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Prophylactic effect of alcohol-free red wine consumption on the development of induced apical periodontitis in rats

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Tese apresentada à Universidade Estadual Paulista (UNESP), Faculdade de Odontologia de Araçatuba, para a obtenção do título de Doutor.

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RESUMO

Ricci R. **Efeito profilático do consumo de vinho tinto sem álcool no desenvolvimento da periodontite apical induzida em ratos.** 2026. Tese (Doutorado) – Faculdade de Odontologia, Universidade Estadual Paulista (UNESP), Araçatuba 2026.

Este estudo investigou os efeitos dos polifenóis do vinho tinto sem álcool (VTSA) na modulação da inflamação e da reabsorção óssea associadas à periodontite apical (PA), por meio de análises *in vivo* e *in vitro*. Trinta e dois ratos Wistar receberam diariamente, por gavagem, VTSA, vinho tinto (VT), solução alcoólica (ALC) ou água (controle). A PA foi induzida no dia 15 e desenvolveu-se por 30 dias, totalizando 45 dias de experimento. Após esse período, amostras de sangue, maxilas e mandíbulas foram coletadas e submetidas à contagem total e diferencial de leucócitos, análise multiplex de marcadores inflamatórios e ósseos no plasma, histologia, imunohistoquímica, qRT-PCR, histometria, microtomografia computadorizada e radiografia. O VTSA reduziu a inflamação periapical, diminuiu a contagem de células TRAP-positivas e reduziu a expressão gênica de IL-1β quando comparado ao controle, aumentou a expressão gênica de IL-10 quando comparado ao VT, e aumentou a imunomarcação de OPG quando comparado ao ALC. Além disso, foi associado à redução de monócitos circulantes em comparação ao controle, sem alterações relevantes nos demais marcadores plasmáticos ou na perda óssea periapical. Já o ALC resultou em maior volume de lesão e em parâmetros inflamatórios desfavoráveis. Complementarmente, em co-culturas de macrófagos (MQ) e fibroblastos do ligamento periodontal (PDLFs), o VTSA induziu um perfil anti-inflamatório, caracterizado por menores níveis de IL-6 e TNF-α e maior IL-10 em relação ao controle. Em conjunto, os achados indicam que o VTSA exerce um efeito imunomodulador com um perfil predominantemente anti-inflamatório, tanto nos modelos animal quanto celular de PA, caracterizado pela redução de mediadores pró-inflamatórios e aumento de mediadores anti-inflamatórios, contribuindo para menor atividade osteoclástica, embora sem impacto significativo no tamanho da lesão ou na perda óssea nas condições experimentais avaliadas.

Palavras-chave: Inflamação; Periodontite Apical; Polifenóis; Remodelação Óssea; Vinho Tinto.

ABSTRACT

Ricci R. **Prophylactic effect of alcohol-free red wine consumption on the development of induced apical periodontitis in rats.** 2026. Doctoral Thesis – School of Dentistry, São Paulo State University (UNESP), Araçatuba, 2026.

This study investigated the effects of dealcoholized red wine (DRW) polyphenols on inflammation and bone resorption associated with apical periodontitis (AP) through *in vivo* and *in vitro* analyses. Thirty-two Wistar rats received daily gavage administration of DRW, red wine (W), alcohol solution (ALC), or water (control). AP was induced on day 15 and developed for 30 days, totaling 45 days of experiment. After this period, blood, maxillae, and mandibles were collected and submitted to total and differential leukocyte counts, multiplex analysis of plasma inflammatory and bone markers, histology, immunohistochemistry, qRT-PCR, histometry, micro-computed tomography, and radiography. The DRW showed reduced periapical inflammation, decreased counts of TRAP-positive cells, and lower IL-1β gene expression compared to the control; increased IL-10 gene expression compared to W; and enhanced OPG immunolabeling compared to ALC. In addition, it was associated with a reduction in circulating monocytes compared to control, without relevant changes in other plasma markers or periapical bone loss. Conversely, ALC resulted in larger lesion volume and unfavorable inflammatory parameters. Complementary in co-cultures of macrophages (MQ) and periodontal ligament fibroblasts (PDLFs), DRW induced an anti-inflammatory profile characterized by lower IL-6 and TNF-α levels and higher IL-10 compared to control. Altogether, the findings indicate that DRW exerts an immunomodulatory effect with an overall anti-inflammatory profile in both animal and cell culture models of AP, characterized by downregulation of pro-inflammatory mediators and upregulation of anti-inflammatory ones, contributing to lower osteoclastic activity, although without significant impact on lesion size or bone loss under the experimental conditions evaluated.

Keywords: Inflammation; Apical Periodontitis; Polyphenols; Bone Remodeling; Red Wine.

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LIST OF ACRONYMS AND ABBREVIATIONS

ALC	Alcohol
AP	Apical periodontitis
ANOVA	Analysis of variance
C	Control
DMEM	Dulbecco's Modified Eagle's Medium
DRW	Dealcoholized red wine
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
HPLC	High-performance liquid chromatography
IL-1β	Interleukin 1 beta
IL-6	Interleukin 6
IL-8	Interleukin 8
IL-10	Interleukin 10
IL-17	Interleukin 17
IL-17A	Interleukin 17A
IRF3	Interferon regulatory factor 3
MAPK	Mitogen-activated protein kinase
MQ	Macrophages
NF-κB	Nuclear factor kappa B
OPG	Osteoprotegerin
PDLFs	Periodontal ligament fibroblasts
RANK	Receptor activator of nuclear factor kappa B
RANKL	Receptor activator of nuclear factor kappa-B ligand
RPMI	Roswell Park Memorial Institute
SPARC	Secreted protein acidic and rich in cysteine
THP-1	Human monocytic leukemia cell line
TLR4	Toll-like receptor 4
TNF-α	Tumor necrosis factor alpha
TRAP	Tartrate-resistant acid phosphatase
VEGF-A	Vascular endothelial growth factor A
W	Red Wine

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1 GENERAL INTRODUCTION

Apical periodontitis (AP) is an inflammatory disease of microbial etiology, primarily caused by dental pulp necrosis and contamination of the root canals (Siqueira & Rôças, 2007). Pulpal necrosis allows microbial invasion to reach the periodontal ligament and alveolar bone, triggering an immune response. This response involves innate and adaptive mechanisms, characterized by the recruitment of inflammatory cells to the lesion site, where essential cytokines are released to drive inflammation and osteoclastic activity, resulting in bone resorption (Siqueira & Rôças, 2007; Jakovljevic et al., 2015).

In AP, bone resorption constitutes a critical factor for lesion expansion, initiated by the proliferation of osteoclast precursor cells and their differentiation into mature osteoclasts, typically identified by the enzyme tartrate-resistant acid phosphatase (TRAP). Receptor activator of nuclear factor kappa-B ligand (RANKL) is a key molecule in osteoclast activation through its binding to the receptor activator of nuclear factor-κB (RANK), a surface protein present on precursor cells. Conversely, osteoprotegerin (OPG) acts as a decoy receptor for RANKL, preventing its interaction with RANK. Thus, an increased RANKL/OPG ratio favors bone resorption through osteoclastogenesis and osteoclastic activation (Kajiya et al., 2010; Cavalla et al., 2021).

The progression of AP reflects an imbalance between inflammation and tissue repair, resulting in persistent periapical damage and continuous immune activation (Márton & Kiss, 2000). Multiple AP foci have been associated with significant systemic repercussions, including alterations in blood parameters and impaired immune homeostasis, with elevated plasma levels of pro-inflammatory cytokines and reduced anti-inflammatory cytokines (Cintra et al., 2014; Cintra et al., 2016; Samuel et al., 2018; Tavares et al., 2021; Azuma et al., 2021). Notably, pulpal-exposure-induced AP has demonstrated the capacity to alter systemic parameters in Wistar rats, increasing lymphocyte, leukocyte, monocyte, and eosinophil counts, as well as enhancing TNF-α and IL-6 expression compared to non-diseased groups (Azuma et al., 2021).

Red wine is a widely consumed beverage worldwide (Kutlesa & Budimir Mrsic, 2016) and has been associated with various health benefits (Pavlidou et al., 2018). Its composition includes water, ethanol, glycerol, polysaccharides, acids, and a complex mixture of predominantly phenolic bioactive compounds, making it one of the main dietary sources of

polyphenols. In general, the beneficial effects of regular and moderate red wine consumption are observed at approximately 150 mL/day for women and 300 mL/day for men (Rotondo, Di Castelnuovo & de Gaetano, 2001; Walzem, 2008).

On the other hand, reducing alcoholic beverages consumption is associated with general health benefits (Fernández-Solà, 2015). Moreover, the beneficial effects of light to moderate alcohol consumption cannot be generalized due to genetic differences among ethnic groups (Hu et al., 2022) and lifestyle variations (smoking, sedentary behavior, dietary habits, etc.), which must always be considered. It should also be noted that initiating alcohol consumption with the aim of obtaining health benefits is not recommended, as various diseases are associated with the harmful effects of excessive alcohol intake (Fernández-Solà, 2015).

Deleterious effects of alcohol consumption have been described in bone tissue, even at low concentrations (8.1% v/v), including reduced cortical area, bone formation rates, and mineral apposition rates (Hogan et al., 1997). Furthermore, chronic alcohol consumption exacerbated the progression of periodontal disease, increasing local inflammatory responses and bone resorption in a dose-dependent manner (de Almeida et al., 2020). In the context of AP, studies have shown that alcohol consumption at 20% v/v, 15% v/v, or at the same dose and concentration present in red wine (12.5% v/v) intensified bone resorption, osteoclastic markers, and inflammation (Dal-Fabbro et al., 2019; Dal-Fabbro et al., 2021). Systemically, alcohol consumption impairs leukocyte migration to sites of injury and infection and causes dysfunctions in T and B lymphocytes, natural killer cells, and monocytes/macrophages, also compromising cytokine production (Deaciuc, 1997; Szabo, 1999; Bautista, 2001).

Conversely, polyphenols appear to play a central role in the health benefits attributed to red wine. Various techniques have been developed to reduce the alcohol content of wine without compromising its phenolic fraction (Belisário-Sanchez et al., 2009). Dealcoholized red wine (DRW) thus emerges as an alternative to obtain the beneficial effects of polyphenols without the adverse effects of alcohol (Belisário-Sanchez et al., 2009). Over the past two decades, winemakers and researchers have developed techniques to reduce the alcohol content of wines and other beverages to meet the growing demand for low- or non-alcoholic products while maintaining good sensory quality. These techniques can be applied during pre-fermentation, fermentation, or post-fermentation stages (Longo et al., 2017; Varela-Varela, 2019; Sam et al., 2021).

Alcohol reduction at these stages involves decreasing fermentable sugars, reducing alcohol production, or removal through membrane and non-membrane processes. Post-fermentation techniques, such as reverse osmosis and osmotic distillation, have been employed to reduce alcohol content to values below 5% v/v without significantly altering the main quality parameters of the wine (Corona et al., 2019). Additionally, the dealcoholization process causes minimal changes to phenolic components and may even increase their concentrations in the final product (Belisário-Sánchez et al., 2009; Corona et al., 2019; Pham et al., 2019).

Consumption of DRW has already demonstrated promising benefits, including cardioprotective effects (Chiva-Blanch et al., 2012), reduction of oxidative stress associated with inflammation (López et al., 2007), modulation of enzymatic activity (Noguer et al., 2012), inhibition of hepatocellular carcinoma (Lan et al., 2023), and reduction of microbial biofilm viability in periodontics (Sánchez et al., 2019). Thus, DRW constitutes an excellent antioxidant source for individuals who must avoid alcohol consumption (Noguer et al., 2012). Taken together, these findings reinforce the hypothesis that the beneficial effects of red wine are related to polyphenols, whereas alcohol exerts an antagonistic effect, promoting bone resorption and inflammation during the development of AP.

Therefore, DRW appears promising not only due to the absence of alcohol but also because of its diverse array of phenolic compounds. To date, no studies have investigated the relationship between DRW consumption and endodontics, particularly its potential action on the development of AP. The null hypothesis of this study is that DRW administration will allow the occurrence of inflammation and bone resorption during the development of induced AP in rats, similarly to water supplementation.

2 OBJECTIVES

2.1 General objective

To evaluate the effects of prophylactic supplementation with DRW on the development of induced AP in Wistar rats, investigating local inflammation and the modulation of bone resorption at the periapical site, the impact on hematological and systemic inflammatory profiles, as well as the cellular mechanisms and the potential immunomodulatory effect of DRW in macrophage (MQ) and periodontal ligament fibroblast (PDLF) co-cultures.

2.2 Specific objectives

- To analyze and quantify the phenolic compounds in wine using high-performance liquid chromatography (HPLC);
- To analyze the inflammatory profile of AP through H&E histology and immunolabeling for the cytokines IL-1β, IL-10, and TNF-α;
- To analyze the bone remodeling process in AP through immunolabeling of RANKL, OPG, and TRAP;
- To analyze and quantify the volume of the periapical lesion associated with AP using micro-computed tomography;
- To analyze and compare the lesion area of AP through histometric and radiographic analyses;
- To analyze and quantify the gene expression of inflammatory and bone metabolism-related markers (IL-1β, IL-10, TNF-α, RANKL, OPG, and TRAP) in AP using qRT-PCR;
- To evaluate the percentage of body weight gain in the animals at the end of the experiment;
- To analyze the hematological profile of the supplemented rats through total and differential leukocyte counts (neutrophils, lymphocytes, monocytes, and eosinophils) using an automated analyzer;
- To analyze the presence of inflammatory and bone markers (IL-10, IL-1β, IL-17A, TNF-α, osteocrin, and SPARC) in blood plasma using multiplex ELISA;

- To investigate the cell viability of MQs and PDLFs exposed to different concentrations of DRW;
- To investigate the immunomodulatory potential of DRW through multiplex analysis of the cytokine profile in MQ and PDLF co-cultures.

3 CHAPTER 1

Impact of Wine Polyphenols on the Inflammatory Profile of Induced Apical Periodontitis in Rats

ABSTRACT

Introduction: This study evaluated the impact of dealcoholized red wine polyphenols on the inflammation and lesion volume associated with apical periodontitis (AP) in rats.

Methods: Thirty-two Wistar rats receiving AP induction were arranged as: Control Group (C), Dealcoholized Red Wine Group (DRW), Red Wine Group (W), and Alcohol Group (ALC). Solutions were administered daily in a volume of 4.28 mL/kg via gavage for 45 days. Mandibles and maxillae were removed for histological, immunohistochemical (RANKL, OPG, TRAP, IL-1β, IL-10, TNF-α) and micro-computed tomography analyses of the AP site. A statistical analysis was performed with a significance level of 5%.

Results: Inflammation and TRAP-positive cell count were similar for DRW and W, but lower when compared to C and ALC ($p<0.001$). The immunohistochemical expression of OPG was higher for DRW than for ALC ($P < .05$). A larger lesion volume was observed in ALC compared to other groups ($p<0.001$).

Conclusions: Prophylactic administration of dealcoholized red wine significantly reduced inflammation, decreased the number of TRAP-positive cells, enhanced OPG expression, and reduced lesion volume compared to water and alcohol solutions.

Keywords Apical periodontitis; bone remodeling; dealcoholized red wine; polyphenols; wine

INTRODUCTION

During apical periodontitis (AP) development, inflammatory cytokines play a significant role in initiating and coordinating cellular events and regulating the host's response to endotoxins (1). The AP induction in Wistar rats has been correlated with increased levels of pro-inflammatory cytokines at the AP site, such as tumor necrosis factor-alpha (TNF-α), interleukin (IL) 6, and IL-1β (2). Bone resorption also occurs as a determining factor in lesion expansion during AP, initiated by the proliferation of immature osteoclast precursor cells and their differentiation into mature osteoclastic cells that degrade organic and inorganic bone components (1).

Red wine is globally consumed, and its light to moderate consumption is associated with various health benefits, including a reduced risk of bone mass loss and fractures, and milder inflammatory response due to its anti-inflammatory and antioxidant effects (3-5). The prophylactic administration of red wine reduced the tartrate-resistant acid phosphatase (TRAP) labeling in rats with induced AP. Additionally, its isolated polyphenols attenuated the inflammation, lesion volume, and TRAP labeling, and increased osteoprotegerin (OPG) and IL-10 expression compared to water supplementation (5).

Moderate red wine consumption, typically ranging from 150 to 300 mL per day (6), has been associated with health benefits due to its polyphenols and alcohol content (7). However, reducing alcohol intake generally improves health, since its excessive intake is linked to many diseases (8). Moreover, genetic differences and lifestyle factors also affect alcohol's impact, limiting its promotion for health benefits (9). Furthermore, studies have shown that when alcohol was given at the same dose and concentration found in wine, bone resorption and inflammation increased in animal experiments (5, 10-12).

Conversely, polyphenols appear to play a significant role in the health benefits associated with red wine. Various techniques have been developed to reduce the alcohol content of wine while maintaining its phenolic compounds (13). The use of dealcoholized red wine provides a means to obtain the benefits of polyphenols without the adverse effects of alcohol (13). Dealcoholized red wine consumption offers promising health benefits, including cardioprotective effects (14), decreased oxidative stress associated with inflammation (15), enzyme activity modulation (16), inhibition of hepatocellular carcinoma (17), and reduced microbial biofilm viability in periodontics (18). Thus, it is an excellent antioxidant source for individuals who should avoid alcohol (16).

To date, no study linked dealcoholized red wine rich in polyphenols with the development of induced AP in terms of inflammation and lesion volume. The hypothesis is that dealcoholized red wine administration results in milder inflammation and lower lesion volume than water administration during the development of induced AP in rats.

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MATERIAL AND METHODS

Quantification of the phenolic compounds present in the wines

The total phenolic compounds in red wine and dealcoholized red wine (Vinoh, Bento Gonçalves, RS, Brazil) were determined by High-Performance Liquid Chromatography (HPLC) as stated in a previous study (19).

Animals

This study was approved by the ethics committee of Araçatuba School of Dentistry, São Paulo State University, Brazil (nº 0221-2022). Thirty-two male Wistar rats (Rattus norvegicus), each weighing 300-350g, were housed in a temperature-controlled environment (22°C±1°C, 70% humidity) with a 12-hour light-dark cycle, having *ad libitum* access to food and water.

Animals were randomly assigned as (n=8): Control Group (C), Dealcoholized Red Wine Group (DRW), Red Wine Group (W), and Alcohol Group (ALC). They were weighed every two days to calculate diet dosage, with weight gain recorded as a percentage of initial and final weights.

Diet administration

The rats from C received potable water as a sham. The rats from DRW and W received dealcoholized red wine and red wine (Vinoh, Bento Gonçalves, RS, Brazil), respectively. The rats from ALC received an alcoholic solution containing 12.5% alcohol by volume (the same amount of alcohol present in the red wine given to W) (5). The administration occurred daily in the morning at 4.28 mL/kg body weight via oral gavage for 45 days, starting 15 days before AP induction and continuing for 30 days (5).

Apical periodontitis induction

On day 15, all animals were anesthetized with ketamine (80 mg/kg) and xylazine (4 mg/kg) for AP induction. The coronal openings of the first and second upper and lower right molars (4 teeth) were performed using a 0.5 mm carbon steel bur (Long Neck Ln Bur - Maillefer, Dentsply), standardizing all pulp exposures. Lesions were analyzed 30 days postinduction (20-22).

Sample collection

On day 45, the animals were euthanized with an anesthetic overdose (20) to collect the right mandibles and maxillae. The hemimandibles were fixed in 4% formaldehyde for histological and immunohistochemical analyses, while the hemimaxillae were frozen for micro-computed tomography analysis.

Histologic/immunohistochemical

Fixed mandibles were decalcified in 10% ethylenediaminetetraacetic acid, embedded in paraffin, and sectioned at 5 μm. For histological analysis, hematoxylin and eosin staining were used to evaluate inflammation and bone loss in the periapical area of the first molar's distal root. Inflammation was analyzed at 400x magnification and scored as follows: 1 - absent/few inflammatory cells; 2 - up to 25 cells, mild reaction; 3 - 25-125 cells, moderate reaction; 4 - more than 125 cells, severe reaction (22).

Immunohistochemical analysis was performed using the immunoperoxidase technique as previously described (5). IL-1β, IL-10, TNF-α, receptor activator of nuclear factor κB ligand (RANKL), and OPG, were analyzed in the periapical lesion of the first molar's distal root at 400x magnification. Immunostaining was defined as brown staining in the cytoplasm of cells and the extracellular matrix and scored as follows: 0 - complete absence of immunoreactive (IR) cells; 1 - low IR cells and weak extracellular matrix staining; 2 - moderate IR cells and moderate extracellular matrix staining; and 3 - high number of IR cells and intense extracellular matrix staining. TRAP-positive cells were quantified at the perimeter and expressed as cells per millimeter. Analyses were conducted by a blinded, calibrated investigator using an optical microscope (Optiphot-2, Nikon).

Micro-computed tomography

The first upper right molars were scanned using the SkyScan 1272 imaging system (Kontich, Belgium). Reconstructed three-dimensional images were generated using Data Viewer (SkyScan, Version 1.4.4 64-bit, Burker, Kontich, Belgium) and NRecon (SkyScan, Burker, Kontich, Belgium) software, and analyzed using CTAnalyser software (2003-11SkyScan, 2012 Bruker MicroCT Version 1.12.4.0, Burker, Kontich, Belgium). The volume of interest included the periradicular bone resorption involving the distal root's periapex. Thus, the space of the resorption area was measured and expressed in mm^3 (5).

Statistical analysis

The data were analyzed using SigmaPlot 12.0™ software (Chicago, IL, USA). The Kruskal-Wallis and Dunn's multiple comparison tests were applied to non-parametric data. One-way ANOVA and Tukey's tests were used for parametric data. The significance level was set at 5% ($P < .05$).

RESULTS

Quantification of the phenolic compounds present in the wines

No differences were observed in the amounts of total phenolic acids, flavanols, proanthocyanidins, stilbenes, and flavanones detected in the dealcoholized red wine and red wine samples ($P > .05$). For flavonols, dealcoholized red wine had higher amounts of myricetin and quercetin 3-glucoside, while red wine had higher amounts of rutin ($P < .05$). For anthocyanins, red wine showed higher amounts of delphinidin 3-glucoside, pelargonidin 3-glucoside, and malvidin 3-glucoside ($P < .05$) (Table 1).

Table 1. Mean and standard deviation for total phenolic compounds detected in dealcoholized red wine (DRW) and red wine (W) samples.

	Sample code	
Phenolic Compounds (mg/L)	**DRW**	**W**
Phenolic acids		
Gallic acid	56.92 ± 1.38[a]	56.40 ± 1.17[a]
Caftaric acid	105.80 ± 2.40[a]	104.92 ± 2.24[a]
Caffeic acid	3.10 ± 0.05[a]	2.91 ± 0.01[a]
p_Coumaric acid	0.77 ± 0.01[a]	0.78 ± 0.04[a]
Flavanols		
Catechin	6.73 ± 0.15[a]	6.77 ± 0.12[a]
Epigallocatechin gallate	0.05 ± 0.00[a]	0.05 ± 0.00[a]
Epicatechin	11.60 ± 0.18[a]	11.93 ± 0.27[a]
Epicatechin gallate	0.96 ± 0.01[a]	0.98 ± 0.02[a]
Proanthocyanidins		
Procyanidin B1	14.65 ± 0.40[a]	14.10 ± 0.19[a]
Procyanidin B2	29.25 ± 0.71[a]	29.60 ± 0.73[a]
Stilbenes		
Cis-Resveratrol	0.91 ± 0.02[a]	1.48 ± 0.20[a]
Trans-Resveratrol	0.43 ± 0.00[a]	0.42 ± 0.01[a]
Flavanones		
Naringenin	4.56 ± 0.37[a]	5.47 ± 0.46[a]

Flavonols		
Myricetin	19.82 ± 0.39[a]	17.15 ± 0.51[b]
Quercetin 3-Glucoside	3.69 ± 0.02[a]	3.57 ± 0.02[b]
Rutin	0.68 ± 0.01[b]	0.84 ± 0.01[a]
Kaempferol 3-glucoside	1.42 ± 0.29[a]	1.48 ± 0.29[a]
Anthocyanins		
Delphinidin 3-glucoside	4.54 ± 0.14[b]	5.13 ± 0.13[a]
Cyanidin 3-glucoside	2.93 ± 0.03[a]	3.11 ± 0.07[a]
Pelargonidin 3-glucoside	17.80 ± 0.30[b]	20.03 ± 0.28[a]
Peonidin 3-glucoside	3.19 ± 0.05[a]	3.42 ± 0.05[a]
Malvidin 3-glucoside	76.94 ± 0.85[b]	85.74 ± 0.92[a]

Different superscript letters indicate significant statistical differences in rows ($P < .05$).

Weight gain average

The mean and standard deviation values for the percentage of weight gain are shown in Figure 3 (A). All groups gained weight during the experiment. Similar weight gains ($P > .05$) were observed for C (38.4 ± 4.654) and DRW (36.178 ± 6.543). Lower weight gains were observed for ALC (27.40 ± 4.933) and W (29.078 ± 3.670) compared to C and DRW ($P < .001$).

Histological analysis

The histologic sections and the inflammatory scores are shown in Figures 1 and 3 (B). All the animals had inflammatory infiltrates in the periapex with bone destruction. Moderate to severe inflammatory reactions were observed for C (score 3) and ALC (score 4) ($P > .05$). While mild to moderate inflammatory responses were observed for DRW (score 2) and W (score 2.5), lower than C ($P < .05$) and ALC ($P < .001$). There was no difference between DRW and W ($P > .05$).

Immunohistochemical analysis

The immunoreactivity patterns for IL-1β, IL-10, TNF-α, RANKL, OPG, and TRAP, are shown in Figures 2 and 3 (D, E, F, G, H, I). A higher OPG expression was observed for DRW (score 2.5) than ALC (score 1) ($P < .05$). A lower number of TRAP-positive cells were found for DRW and W than C ($P < .05$) and ALC ($P < .001$). Additionally, ALC was more TRAP-inductive than all other groups ($P < .001$). No differences were observed for IL-1β, IL-10, TNF-α, and RANKL ($P > .05$) immunolabeling among the groups.

Micro-computed tomography analysis

The data and the representative micro-computed tomography slices are shown in Figures 3 (C) and 4 (A, B, C, D), respectively. By day 45, all groups exhibited periapical hypodense regions, indicating AP onset and development. The lowest lesion volume (0.659 mm^3 ± 0.124 mm^3) was observed for DRW, similar to C (0.824 mm^3 ± 0.169 mm^3) and W (0.859 mm^3 ± 0.305 mm^3) ($P > .05$). The greatest lesion volume was observed for ALC (1.608 mm^3 ± 0.302 mm^3) compared to the other groups ($P < .001$).

DISCUSSION

This study is the first to assess dealcoholized red wine impact on inflammation and lesion volume in rats with induced AP. Despite claims that alcohol contributes to red wine's benefits (7), dealcoholized red wine reduced inflammation compared to water, but had no impact on lesion volume, rejecting the hypothesis of this study. Fewer TRAP-positive cells were also observed for DRW compared to C and ALC, with higher OPG immunolabeling and reduced lesion volume than ALC. This suggests that the benefits attributed to red wine may primarily be due to polyphenols rather than the alcoholic content (5, 15, 16).

Wistar rats are a well-established model for studying induced AP (10, 11, 20-24). While results cannot be directly applied to clinical settings, using Wistar rats allows for controlled conditions that could influence the development of AP, such as weight, diet, and stress levels (23, 24). The intraoral gavage method ensured precise solution administration, unlike free access through water bottles. The 4.28 mL/kg dose was equivalent to a 70 kg male consuming 300 mL (2 glasses) of red wine daily (6).

Weight variation was monitored as an indirect index of overall animal health while ensuring accurate diet doses. All animals gained weight during the experiment, with no significant difference between C and DRW, while the least weight gain was observed for W and ALC. Equivalent results were found comparing AP development in rats on a chronic alcoholic diet versus a regular diet (10, 11). Despite being considered hypercaloric, alcohol is ineffective as a nutritional source and hampers vitamin and nutrient absorption (25).

Histological analysis confirmed effective AP induction, with all groups showing inflammatory infiltrate in the periapical region with bone resorption. A milder inflammatory process was noted for DRW and W. In contrast, ALC exhibited predominantly severe inflammatory reactions. These results align with studies showing increased AP inflammation with alcoholic diets at 12.5%, 15%, and 20% concentrations (5, 10, 11) and exacerbation of periodontitis with chronic alcohol consumption at 14%, 25%, and 36% concentrations in a dose-dependent manner (12). Additionally, resveratrol and quercetin

are found in both dealcoholized and regular red wine. These polyphenols are known to exert anti-inflammatory and antioxidant biological effects, in addition to their capacity to modulate the host osteoimmune inflammatory response (26-28).

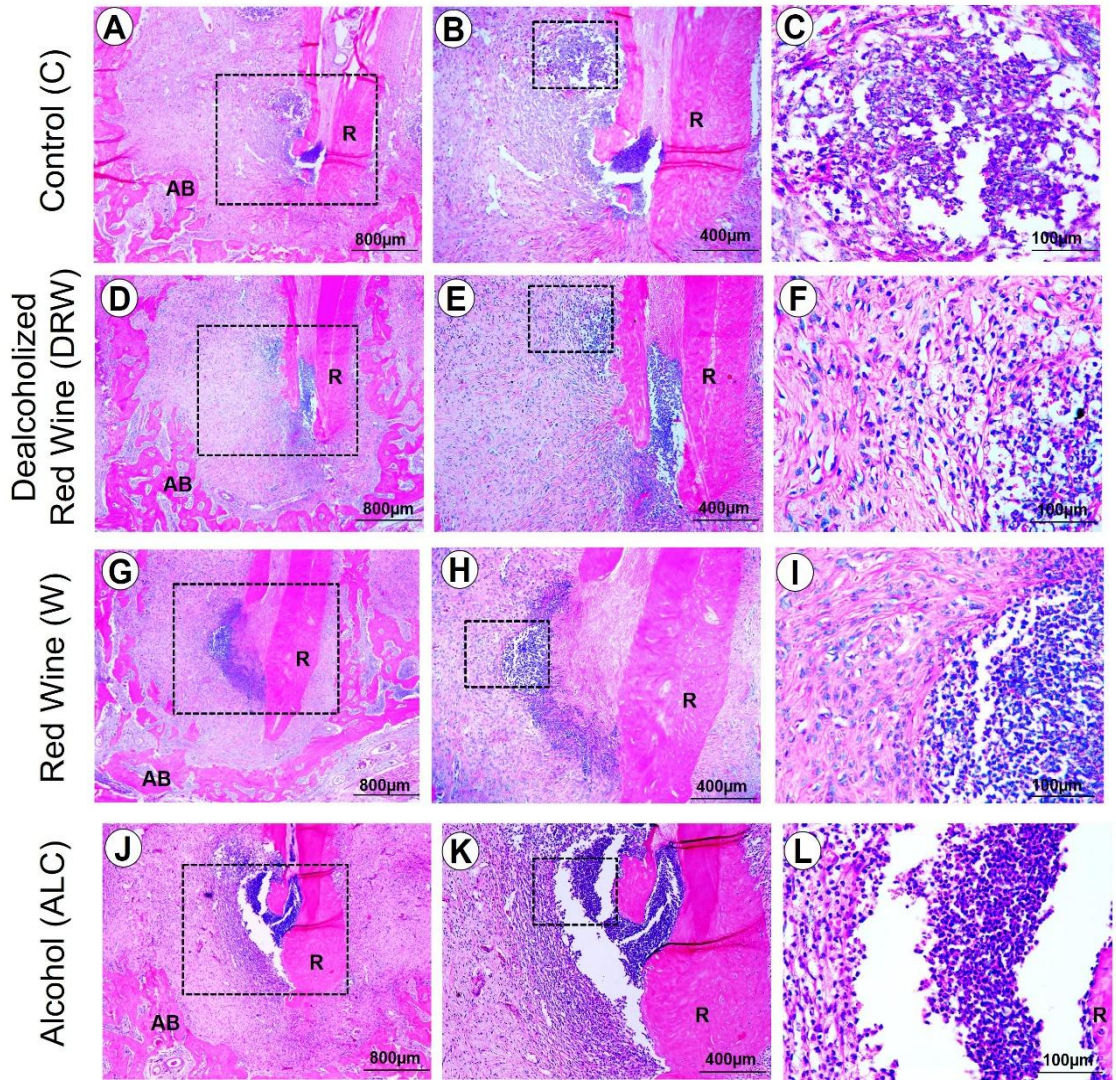

Figure 1. The photomicrographs show the histological aspects and the alveolar bone (AB) loss of the apical regions around the foraminal opening of the distal root (R) of the mandibular first molar. The moderate inflammatory process can be observed for the Control Group (A, B, and C at 50×, 100×, and 400× magnification, respectively). A mild inflammatory process can be observed for the Dealcoholized Red Wine Group (D, E, and F at 50×, 100× and 400× magnification, respectively), and a mild to moderate inflammatory process can be observed for the Red Wine Group (G, H, and I at 50×, 100× and 400× magnification, respectively). The severe inflammatory process can be observed for the Alcohol (J, K, and L at 50×, 100×, and 400× magnification, respectively). Hematoxylin and eosin staining. Rectangles indicate the enlarged area in the next magnification. Scale bars: 800 µm (A, D, G, J), scale bars: 400 µm (B, E, H, K), and scale bars: 100 µm (C, F, I, L).

One mechanism by which polyphenols can exert their immunomodulatory effects is the modulation of inflammatory cytokines (29). In a previous study, red wine showed a tendency to decrease IL-1β and TNF-α while enhancing IL-10 release in the AP site of rats. However, only its polyphenols statistically increased IL-10 levels compared to the control group (5). This study found similar IL-1β and TNF-α levels across groups. These cytokines act simultaneously in inflammation and bone resorption and are frequently found in inflamed periodontal tissues (30). IL-1β, the predominant IL-1 type in human periapical lesions, can increase osteoclastic cells *in vivo* and inhibit osteogenesis (31). TNF-α can be present immediately after pulp exposure with levels potentially increasing for up to 30 days in periapex (32).

All groups had similar IL-10 and RANKL expressions. IL-10 is a potent anti-inflammatory cytokine that can regulate cell differentiation and prevent bone loss by upregulating OPG and downregulating RANKL (33, 34). Under physiological conditions, the RANK/RANKL/OPG ratio guarantees a delicate balance between bone formation and resorption. The RANKL is a critical molecule in osteoclast activation. In turn, OPG binds to RANKL, preventing it from binding to RANK and leading to osteoclast differentiation (1). Higher OPG immunolabeling was observed for DRW compared to ALC, as noted when resveratrol and quercetin were administered to rats with induced AP (5).

Since dealcoholized red wine has multiple bioactive components beyond polyphenols (such as glycerol, polysaccharides, and acids) (13), interactions may occur between them. These interactions can be advantageous or synergistic, as well as negative or neutralizing (37). Additionally, most phenolics in wine are not entirely absorbed by the digestive system, resulting in insignificant blood or tissue concentrations (38). Consequently, the impact of phenolics as a group might differ from that of individual phenolics, which are typically the focus of research. Therefore, studying whole foods versus isolated compounds reveals differences, as demonstrated by resveratrol and quercetin (5).

This study questions whether alcohol is essential for obtaining red wine’s benefits. Our findings suggest further research on dealcoholized red wine polyphenols on AP and other oral diseases. It's important to note that dealcoholized red wine contains several distinct polyphenols that should be individually studied in future research.

CONCLUSION

Prophylactic administration of dealcoholized red wine reduced inflammation and TRAP-positive cells in rats with induced apical periodontitis compared to water. It also increased OPG expression and reduced lesion volume compared to an alcoholic solution.

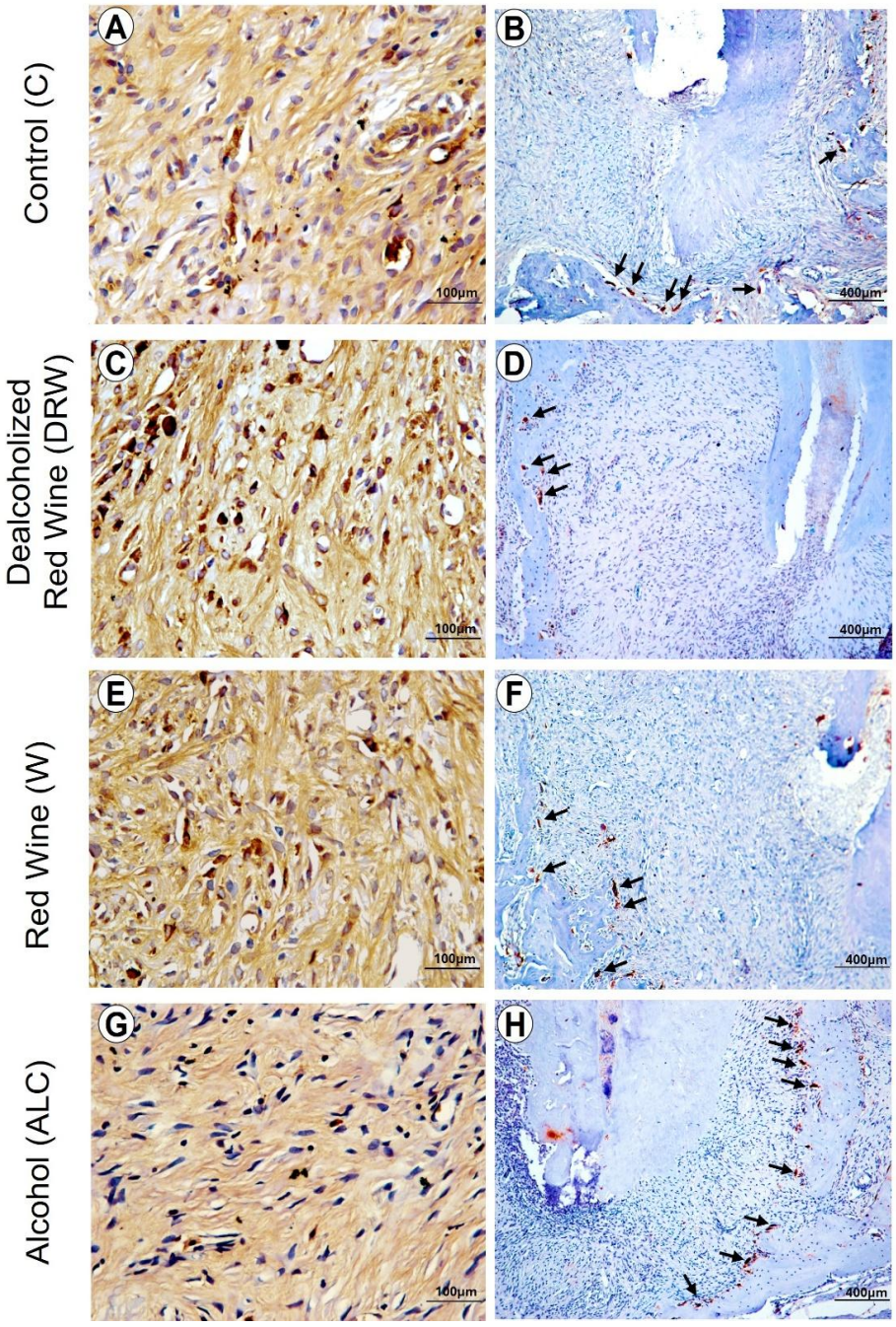

Figure 2. The photomicrographs show the immunostaining for OPG (A, C, E, G) and TRAP (B, D, F, H) in apical periodontitis around the foraminal opening of the distal root of the mandibular first molar for the Control Group (A, B), the Dealcoholized Red Wine Group (C, D), the Red Wine Group (E, F) and the Alcohol Group (G, H). Counterstaining: Harris hematoxylin. Original magnification for OPG: 400× with scale bars: 100 µm, and 100× for TRAP with scale bars: 400 µm.

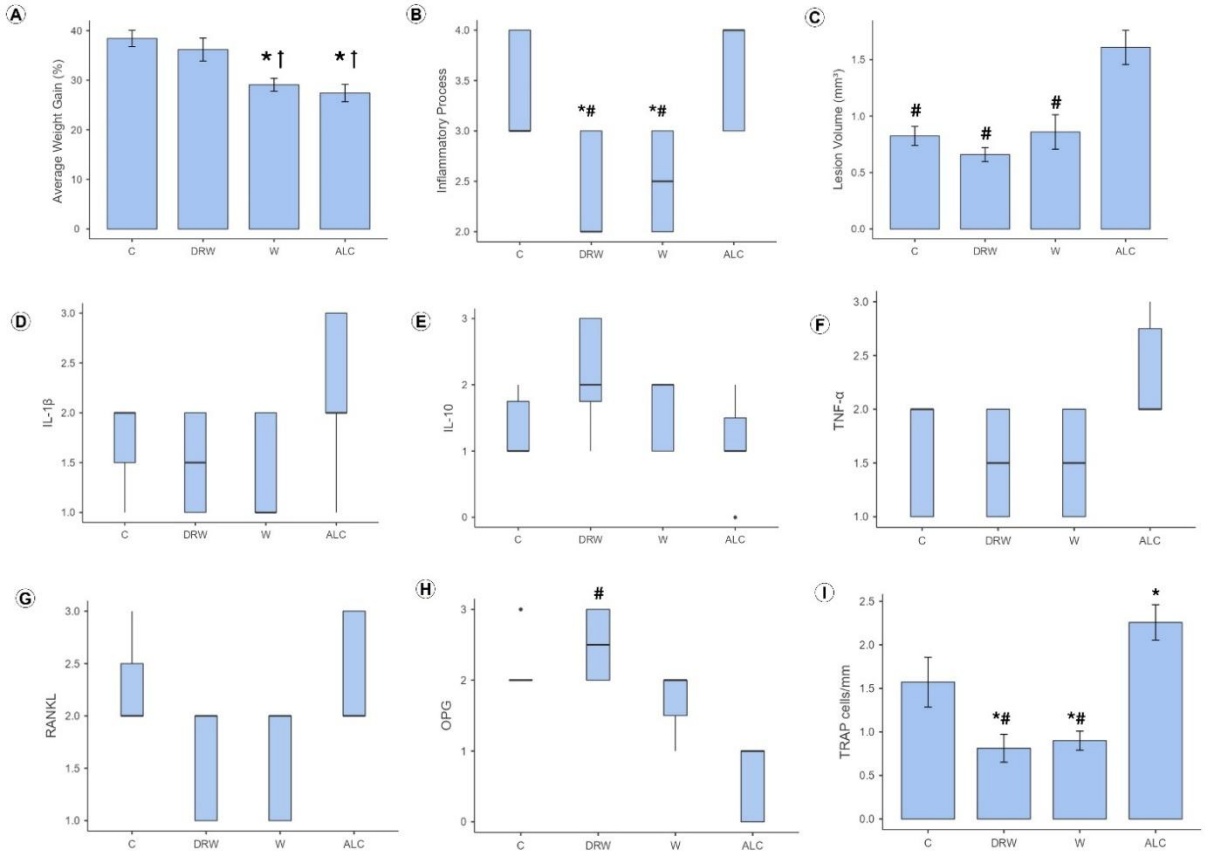

Figure 3. The bar graphs and the charts represent the average weight gain percentage (A), the intensity of the inflammatory process (B), the lesion volume in cubic millimeters (C), the IL-1β (D), IL-10 (E), TNF-α (F), RANKL (G), and OPG (H) immunolabeling, and the positive TRAP cells per millimeter (I). The bar graphs show each group mean and standard deviation. The bold line in the charts represents the median of each group. Symbols: *P < .05 *versus* C; ŧP < .05 *versus* DRW; #P < .05 *versus* ALC.

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CONFLICT OF INTEREST

The authors declare no conflict of interest related to this study.

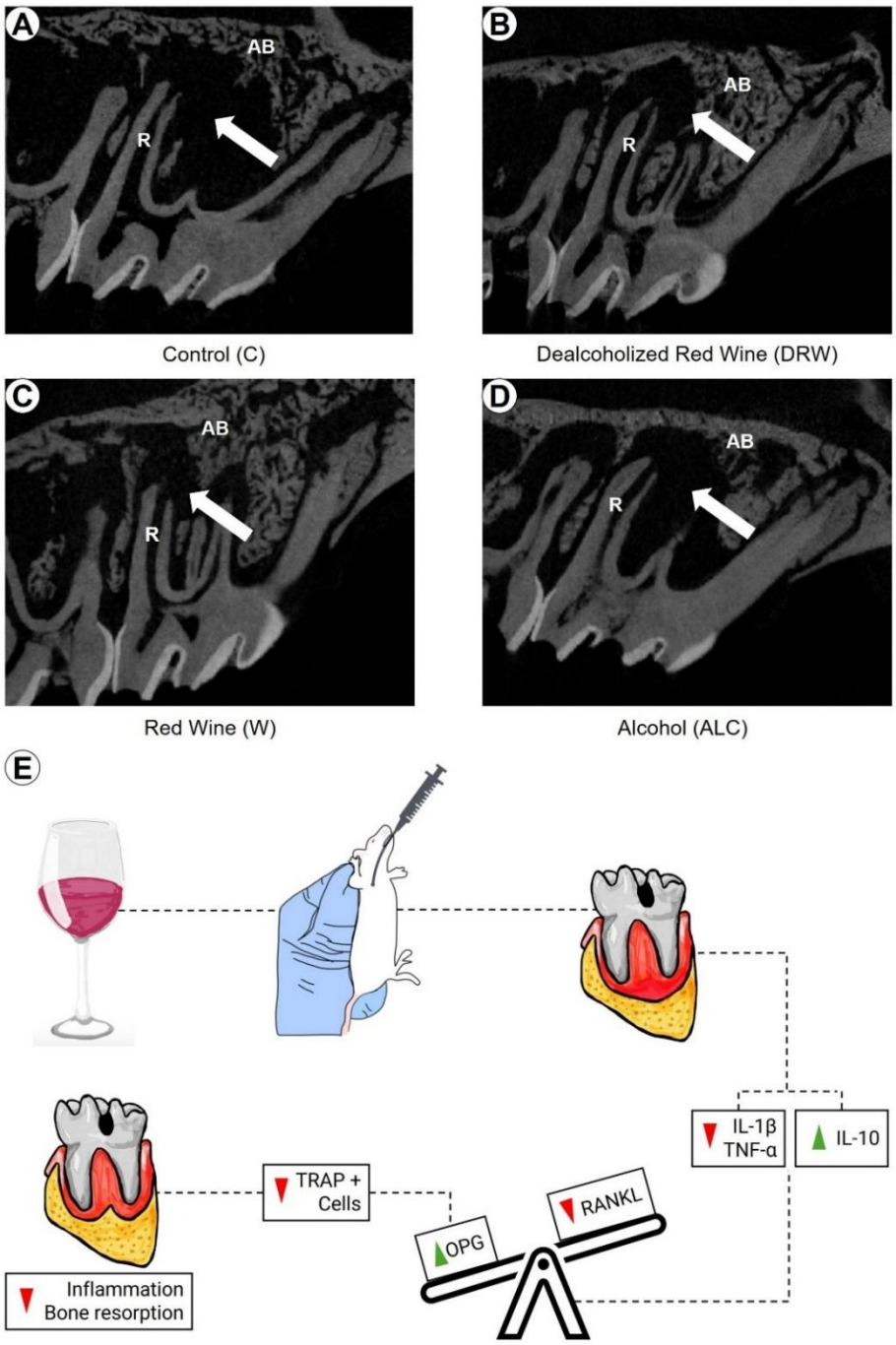

Figure 4. Micro-computed tomography slices of apical periodontitis in the distal root (R) of the upper first molars of each group (A, B, C, and D). Diminished bone resorption on microtomography is noted for the Control Group (A), the Dealcoholized Red Wine Group (B), and the Red Wine Group (C) compared to the Alcohol Group (D) ($p<0.001$). White arrowheads point to the area of the apical periodontitis surrounded by hyperdense alveolar bone (AB). Symbols: #P < .05 versus ALC. Schematic representation of the mechanisms through which dealcoholized red wine influences AP (E).

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4 CHAPTER 2

Cellular and molecular immunomodulatory potential of red wine polyphenols in apical periodontitis

Abstract

This *in vitro* and *in vivo* study assessed dealcoholized red wine (DRW) effects on cytokine profile in macrophage (MQ)–periodontal ligament fibroblast (PDLFs) co-cultures and its impact on blood parameters, inflammatory/bone markers, cytokine expression and periapical bone loss in rat apical periodontitis (AP). A MQ–PDLFs co-culture and Wistar rats with induced AP were exposed to DRW or red wine (W), with DMEM or water as controls (C). Cell cultures were analyzed for cytokine profile using a multiplex immunoassay. Rats underwent blood profiling, radiography, qRT-PCR and histometric analysis of AP. Statistical significance was set at 5%. Multiplex analysis of the co-culture revealed that DRW induced lower interleukin (IL)-6 and tumor necrosis factor (TNF)-α levels compared to C and W, higher IL-10 level than C, and lower IL-1β level only compared to W ($P < .05$). Radiographic images confirmed AP development in rats. DRW showed a reduced monocyte count compared to C ($P < .05$), but the inflammatory/bone markers in plasma were similar ($P > .05$). Additionally, DRW showed lower IL-1β expression than C, and higher IL-10 expression only compared to W in AP ($P < .05$). Periapical bone loss was similar among groups ($P > .05$). In conclusion, DRW promoted an anti-inflammatory profile in co-cultures and *in vivo*. However, these effects did not translate into differences in lesion size or bone loss within the experimental model evaluated.

Keywords: Apical periodontitis; Bone remodeling; Dealcoholized red wine; Inflammation; Red wine

Running title: Immunomodulatory Role of Wine Polyphenols in Inflammation

Introduction

Apical periodontitis (AP) is an inflammatory condition affecting the periapical tissues of teeth with necrotic pulps, primarily triggered by bacterial infection and their by-products (1). Pulpal necrosis allows microbial invasion to extend into the periodontal ligament and alveolar bone, initiating an immune response. This response involves both innate and adaptive mechanisms, characterized by the recruitment of inflammatory cells to the lesion site, where they release key cytokines that drive inflammation and osteoclastic activity, ultimately leading to bone resorption (2,3).

The progression of AP reflects a disruption in the balance between inflammation and tissue repair, resulting in sustained periapical damage and continued immune activation (4). Multiple foci of AP have been associated to significant systemic repercussions, including altered blood parameters and disrupted immune homeostasis, with elevated plasma levels of pro-inflammatory cytokines and reduced levels of anti-inflammatory cytokine (5-9).

A diet rich in polyphenols has been linked to anti-inflammatory and antioxidant responses in various pathological conditions (10,11). Although red wine (W) is widely studied for these benefits, its health-promoting effects are primarily attributed to its polyphenolic rather than the alcoholic content (12-14). In this context, dealcoholized red wine (DRW) emerges as a promising alternative, offering the polyphenols without the health risks and inflammation associated with alcohol consumption (15-17).

Studies have demonstrated that DRW can exert anti-inflammatory effects in various inflammatory conditions (18,19). Previous findings revealed that supplementation with DRW reduced inflammation and bone loss in rats with induced AP (13,14). All together, these results suggest that DRW may modulate the inflammatory response in AP through mechanisms associated with polyphenol activity. However, the cellular and molecular pathways underlying DRW effects remain to be elucidated.

Therefore, the present study aimed to clarify some of these pathways by investigating the immunomodulatory potential of DRW and W in the context of AP. The cytokine and chemokine profile of macrophages (MQ) and periodontal ligament fibroblasts (PDLFs) in direct co-culture with DRW or W exposure was first examined *in vitro*, allowing juxtacrine interactions. In parallel, an *in vivo* model was used to evaluate both systemic and local effects of DRW or W supplementation, including changes in blood profile, inflammatory and bone metabolism markers in plasma, cytokine expression at the lesion site, and histometric analysis of periapical bone loss. We hypothesized that DRW and W differentially influence the inflammatory profile in AP by modulating cytokine production and immune cell recruitment both locally and systemically.

Article formatted according to the *Brazilian Journal of Medical and Biological Research* guidelines, available at https://www.bjournal.org/instructions/, accessed on 09/22/2025.

Material and Methods

Cell cultures

THP-1 monocytes (ATCC TIB-202; American Type Culture Collection, USA) were cultured in complete RPMI 1640 medium (#A104950; Gibco, USA), supplemented with 10% heat-inactivated fetal bovine serum (#19C448; Sigma-Aldrich, USA), 1% antibiotic/antimycotic (# 15240-062, Gibco), and 0.1% beta-mercaptoethanol (# 21985023, Gibco). The cells were incubated at 37 ºC in a humidified atmosphere containing 5% CO_2. Following this, THP-1 cells (2.5 x 10^5 cells/mL) were differentiated into MQ by exposure to 100 nmol/L phorbol 12-myristate-13-acetate (Sigma-Aldrich) for 24 h. This was followed by an overnight resting period in fresh RPMI medium. Differentiation was confirmed by observing cellular adhesion and spreading.

Human PDLFs were provided as a kind gift from Dr. Douglas Hamilton's lab, Schulich's School of Medicine & Dentistry, London, ON, Canada. Cells were isolated from clinical samples of healthy periodontium. PDLFs were cultured in complete Dulbecco's Modified Eagle's Medium (DMEM) (Sigma-Aldrich), supplemented with 10% heat-inactivated fetal bovine serum and 1% antibiotic/antimycotic. The cells were maintained in a 37ºC incubator with 5% CO_2 in a humidified atmosphere. Experiments were conducted using cells from the third to fifth passage.

Cell Viability Assay

To determine the optimal concentration of wine, both DRW and W (Vinoh, Brazil) were opened under sterile conditions within a biosafety cabinet and immediately filtered using a vacuum filtration membrane. The wines were then diluted in complete DMEM to concentrations of 15, 1, and 0.1%.

PDLFs monocultures were exposed to the test media for 24 h. Cells cultured in complete DMEM alone served as the negative control. The media was removed, and triplicate samples of cells were incubated with 200 µL of calcein-AM (Invitrogen/Molecular Probes) for 20 min. Fluorescence was observed at 10× magnification using a fluorescent inverted microscope (Vert.A1; Carl Zeiss, Germany). Images were acquired and analyzed using ImageJ software (National Institutes of Health, USA). Cell viability was calculated as a percentage relative to the negative control, which was considered to be 100% viable (20).

Based on the PDLFs viability results, the most suitable wine concentration was tested in MQ monocultures following the same viability assay protocol. The cytokine and chemokine analysis were performed only after confirming the viability of MQ at the selected concentration.

Assessment of Cytokine and Chemokine Profile

After the determination of optimal concentration, cell culture supernatants of MQ and PDLFs co-culture treated with DMEM (C), 0.1% concentration of DRW (0.1% DRW) or W (0.1% W), were collected at 24 h. The samples were then centrifuged at 10,000 g and 4ºC for 5 min to remove residual cells, aliquoted, and stored at -80ºC until analysis. A multiplex immunoassay was conducted to simultaneously analyze the inflammatory cytokines interleukin (IL)-1β, tumor necrosis factor (TNF)-α, IL-8 (a chemokine), IL-10, IL-6 and vascular growth factor A (VEGF-A) using the HCYTOMAG-60K Milliplex MAP Human cytokine magnetic bead panel (Millipore, USA). The assessment was performed in duplicate. Data are reported in picograms per milliliter (pg/mL).

Animal Experiment

Once approved by the ethics committee (Araçatuba School of Dentistry, São Paulo State University, nº 0221-2022), twenty-four male rats (*Rattus norvegicus, Wistar*), aged 3 months and weighing 300-350 g each, were housed in a temperature-controlled environment (22°C±1°C, 70% humidity) with a 12-h light-dark cycle. The animals were fed a solid diet and water *ad libitum* throughout the experimental period. Solid diet was not provided during the 12 h leading up to the intervention. The sample size was based on previous studies recommending at least seven animals per group (12–14). To account for potential complications, one extra animal was added per group, totaling 24 animals (n=8 per group).

The animals were randomly assigned into three groups as follows: Control (C), rats receiving 4.28 mL/kg body weight of potable water to mimic the same procedure endured by the other animals; DRW, rats receiving 4.28 mL/kg body weight of DRW containing 0.3% ethanol by volume; and W, rats receiving 4.28 mL/kg body weight of W containing 12.5% ethanol by volume (12-14). This amount of the test solutions is considered moderate consumption for a male individual (300 mL per day) (21). The solutions were administered daily through oral gavage for 45 days supplementing the conventional diet. This regimen began 15 days before the induction of AP and continued for an additional 30 days (12,13). All animal experiment protocols were performed in accordance with the ARRIVE guidelines for reporting *in vivo* experiments.

Induction of AP

On the 15th day, all animals were anesthetized with 80 mg/kg ketamine (Avenco Inc., USA) and 4 mg/kg xylazine (Mobay Corp., USA) and received AP induction. The first and second maxillary and mandibular molars on the right side had the pulp exposed using a 0.5 mm diameter carbon steel bur (Long Neck Ln Bur, Maillefer, Dentsply, Switzerland), the coronal openings were standardized and the

teeth remained open until the end of the experiment (6,9,13). After 30 days from induction, the periapical lesion development was confirmed through digital radiography.

Blood profile and assessment of inflammatory/bone markers

Thirty days after inducing AP, the animals were anesthetized following the previously described protocol. A cardiac puncture was performed to collect 5 mL of blood. The blood samples were then placed in EDTA and homogenized for further processing. An aliquot was separated for the blood profile analysis. Afterward, the blood was centrifuged (3,000 rpm, 5 min) to separate the plasma. The plasma samples were transferred to Eppendorf tubes for enzyme-linked immunosorbent assay (ELISA) multiplex analysis.

The blood count parameters of leukocytes and absolute differential count of neutrophils, lymphocytes, monocytes and eosinophils (7) were determined using an automated hematology analyzer (Mindray - BC 2800 Vet; Shenzhen Mindray Animal Medical Technology Co., LTD., China).

For the multiplex analysis, the MILLIPLEX® Rat Cytokine/Chemokine Magnetic Bead Panel - Immunology Multiplex Assay (RECYTMAG-65K, Merck Life Science, LLC, Germany) was used for the quantification of IL-10, IL-1β, IL-17A and TNF-α, and the MILLIPLEX® Rat Myokine Magnetic Bead Panel (RMYOMAG-88K, Merck Life Science, LLC, Darmstadt, Germany) was used for the detection of osteocrin and secreted protein acidic and rich in cysteine (SPARC). All analyses were performed in duplicate. Data are reported in pg/mL (22).

Radiographic, qRT-PCR and histometric analysis of AP

Therefore, the animals were euthanized using an anesthetic solution overdose to collect the right-side maxillae and jaws for radiographic, quantitative reverse transcription polymerase chain reaction (qRT-PCR), and histometric analysis of AP.

The maxillae (n=4) were processed for digital radiography using an X-ray equipment Spectro 70X (Dabi Atlante, Brazil), with a 70 kVp nominal voltage and 7.0 mA tube current, and a digital sensor (Acuity direct X-ray photon detection model 005-000124) to confirm AP development. Radiographic images were acquired with an exposure time of 0.8 seconds and a total focal distance of 20 centimeters. The lesion size was measured using ImageJ Software (National Institutes of Health, USA). The periapical lesion borders and size around the mesial roots of the first molars were marked, and area was calculated in square millimeters and compared between groups (23).

For the qRT-PCR analysis, a bone block was removed from the AP site of the maxillae (n=4) using a 2-mm trephine bur (Trephine Bur 2.0 mm, Harte Surgical Instruments, Brazil). The collected bone blocks were immediately pulverized in liquid nitrogen and homogenized in TRIzol® using a mortar and pestle to ensure complete tissue dissociation for RNA extraction. Total RNA was then extracted (PureLink™ RNA Mini Kit, Thermo Fisher Scientific, USA), DNase I-treated, and purity confirmed by 260/280 (1.8–2.0) and 260/230 (2.0–2.2) ratios. RNA concentration was measured by NanoDrop (Thermo Fisher Scientific, USA), and 2 µg were reverse-transcribed to cDNA (High-Capacity RNA-to-cDNA™ Kit, Applied Biosystems, USA). The gene expression analysis of IL-1β, IL-10 and TNF-α was performed in duplicate using the StepOne Plus™ Real-Time PCR System and TaqMan Gene Expression Assays (Applied Biosystems and Thermo Fisher Scientific). Target gene expression was normalized to Actb and the relative abundance of transcripts determined using the $2^{-\Delta\Delta Ct}$ method (24), with data reported as relative expression.

For the histometric analysis, the jaws (n=8) were demineralized in a 10% EDTA solution, embedded in paraffin and sectioned at 5 µm thick following a general histology protocol. Hematoxylin-eosin staining was used to assess differences in periapical bone loss among the different groups. The periapical area associated with the distal root of the first molar, defined by tracing the lesion boundary, was measured and expressed in square millimeters using Leica Microsystems Software (Leica, Germany) (25).

Statistical Analysis

Statistical analyses were performed using GraphPad Prism 8.0.1 (GraphPad Software, USA). For parametric data, one-way analysis of variance followed by Tukey's *post hoc* test was applied. For non-parametric data, the Kruskal-Wallis test was used, followed by Dunn's *post hoc* test. Comparisons between two groups were conducted using the Student's *t*-test. Results were considered statistically significant when $P < .05$.

Results

Cell Viability Assay

Figure 1 shows the cell viability results for PDLFs after 24 h of exposure. Higher concentrations of DRW and W (15 and 1%) significantly reduced cell viability compared to the negative control ($P < .05$). A dose-dependent improvement in viability was observed as concentrations decreased, with the 0.1% concentration maintaining viability at levels comparable to the control group. Based on these

results, 0.1% was selected for subsequent experiments. Both MQ and PDLFs exposed to 0.1% DRW and 0.1% W remained viable, with no significant differences relative to the control group (P > .05).

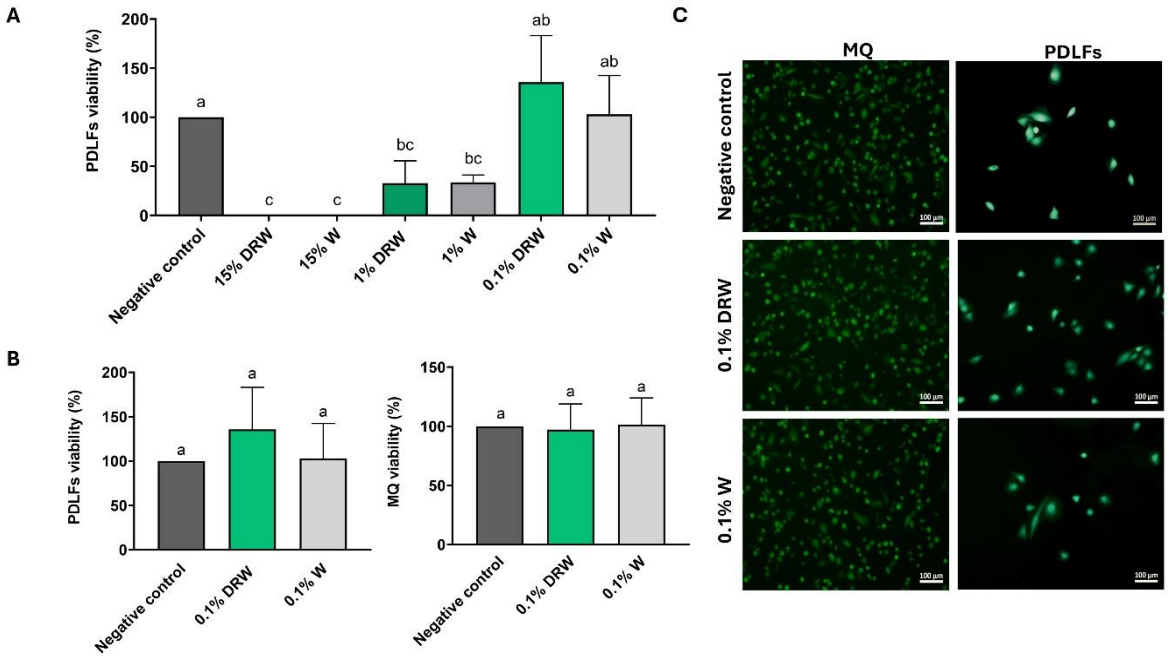

Figure 1. **A**, Cell viability of periodontal ligament fibroblasts (PDLFs) under different concentrations of dealcoholized red wine (DRW) and red wine (W) relative to the negative control; **B**, the cell viability of macrophages (MQ) and PDLFs monocultures under 0.1% of dealcoholized red wine (0.1% DRW) or red wine (0.1% W) relative to the negative control. Data are reported as means and SD. One-way ANOVA with Tukey post hoc test was applied. Different lowercase letters indicate significant difference among the groups (P<0.05). **C**, Calcein AM-stained images (10× magnification, scale bar=100 μm) at 24 h of MQ and PdLFs monocultures under 0.1% DRW or 0.1% W.

Cytokine and Chemokine Profile

Figure 2 presents the cytokine and chemokine profile from the co-culture of MQ and PDLFs. DRW exhibited significantly reduced IL-6 and TNF-α levels compared to C and W, higher IL-10 level compared to C, and lower IL-1β level only compared to W (P < .05).

Blood profile analysis

The results of the blood profile analysis are presented in Table 1. No significant difference was observed in leukocyte count among the groups. However, the absolute differential count revealed a significantly lower monocyte count for DRW compared to C (P < .05), but similar to W (P > .05). The

counts of neutrophils, lymphocytes, and eosinophils showed no significant difference among the groups (P > .05).

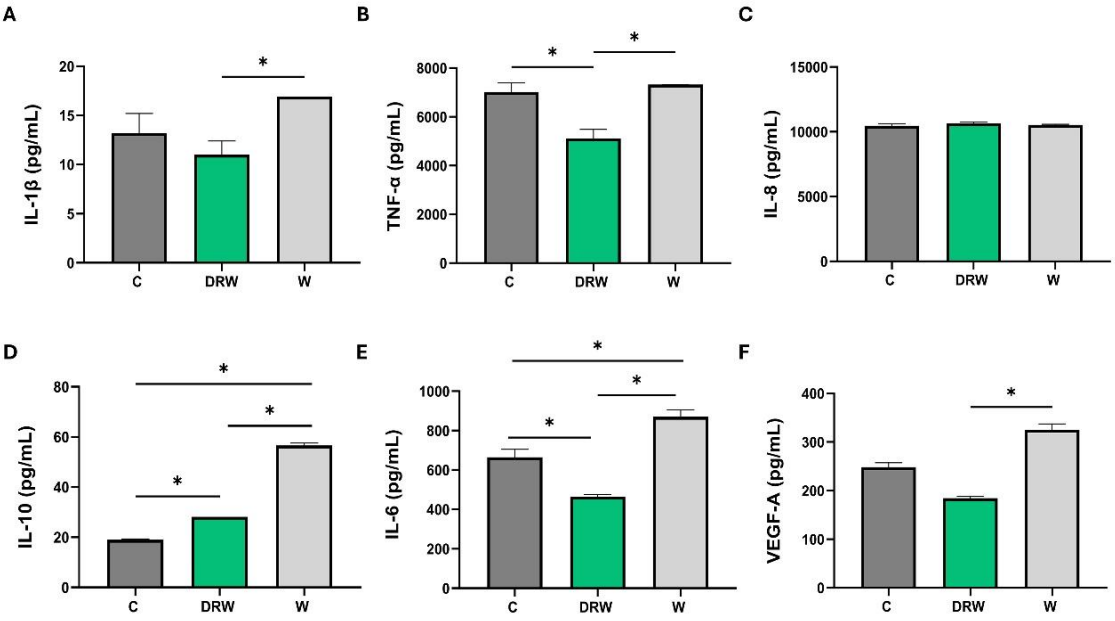

Figure 2. The bar graphs show the mean and standard deviation for the assessment of **A**, IL-1β; **B**,TNF-α; **C**, IL-8; **D**, IL-10; **E**, IL-6; and **F**, VEGF-A for the co-culture of macrophages (MQ) and periodontal ligament fibroblast (PDLFs). Data are reported as means and SD. *P<0.05; one-way ANOVA with Tukey post hoc test was applied.

Assessment of inflammatory/bone markers

The systemic analysis of plasma cytokines showed comparable levels of IL-10, IL-1β, IL-17A, TNF-α, osteocrin, and SPARC among the groups (P > .05) (Figure 3A), indicating a stable systemic cytokine profile under the experimental conditions.

Radiographic analysis of AP

Radiographic imaging of the maxillae confirmed the development of AP in all groups (Figure 4). The periapical lesion areas were measured to assess lesion size, and no statistically significant differences were detected among the groups (P > .05), indicating that the tested supplementations did not alter radiographic lesion size under the present experimental conditions.

Table 1. Blood profile of study groups

Blood parameters	Groups Mean ± SD		
	C	DRW	W
Leukocytes (x10^3/μL)	9.131 ± 1.871[a]	8.925 ± 1.480 [a]	8.593 ± 1.132 [a]
Neutrophils (cells/μL)	1.868 ± 0.748 [a]	1.884 ± 0.683 [a]	1.814 ± 0.432 [a]
Lymphocytes (cells/μL)	6.711 ± 1.278 [a]	6.770 ± 0.988 [a]	6.307 ± 0.951 [a]
Monocytes (cells/μL)	404.4 ± 160.5 [b]	209.8 ± 157.6 [a]	356.3 ± 135.2 [a, b]
Eosinophils (cells/μL)	109.9 ± 75.69 [a]	146.3 ± 54.77 [a]	103.2 ± 119.7 [a]

Data are reported as mean ± SD. Different superscript lower-case letters in rows indicate a significant difference among the groups (P<0.05, one-way ANOVA followed by Tukey's test for multiple comparisons). C: control; DRW: dealcoholized red wine; W: red wine.

qRT-PCR analysis of AP

The graphs for the qRT-PCR analysis are shown in Figure 3B. The AP sites in the maxillae of DRW and W showed significantly lower IL-1β expression compared to C (P < .05). Additionally, IL-10 expression was higher for DRW only compared to W (P < .05). No significant difference was observed in TNF-α expression among the groups (P > .05).

Histometric analysis of AP

Table 2 presents the histometric analysis of AP for all groups. In all groups, histological sections showed evident periapical lesions characterized by bone resorption surrounding the distal root of the first molar. The lesion boundaries were clearly delimited and measured to calculate the periapical area. The periapical bone loss area was similar among groups (P > .05).

Table 2. Area of apical periodontitis in each study group

Group	Histometric analysis (mm^2)
C	2283.976 ± 310.672 [a]
DRW	2099.349 ± 671.320 [a]
W	2094.260 ± 563.611 [a]

Data are reported as mean ±SD. P>0.05, one-way ANOVA followed by Tukey's test for multiple comparisons. C: control; DRW: dealcoholized red wine; W: red wine.

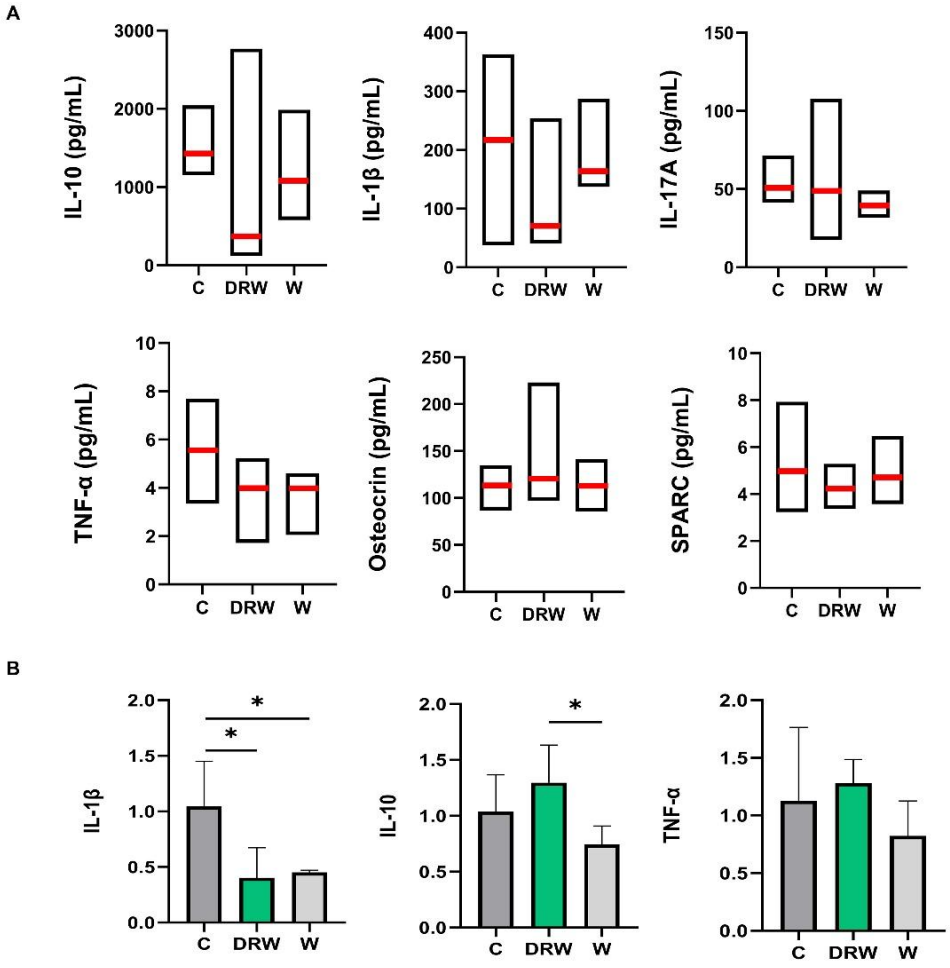

Figure 3. A, Median (red line), minimum, and maximum values for interleukin (IL)-10, IL-1β, IL-17A, tumor necrosis factor (TNF)-α, osteocrin, and secreted protein acidic and rich in cysteine (SPARC) in plasma samples for each group. Kruskal-Wallis test followed by Dunn's post hoc test was applied ($P>0.05$). **B**, Mean and standard deviation of relative gene expression ($2^{-\Delta\Delta Ct}$) from qRT-PCR analysis of apical periodontitis samples for IL-1β, IL-10, and TNF-α. *$P<0.05$; one-way ANOVA with Tukey post hoc test was applied.

Discussion

The findings of this study support the hypothesis that DRW and W differentially modulate the inflammatory profile by influencing key cytokines and immune cell responses. In co-cultures of MQ and PDLFs, DRW promoted a more anti-inflammatory profile than W, characterized by decreased TNF-α and IL-6, with increased IL-10 levels. *In vivo*, DRW intake led to a lower blood monocyte count and reduced IL-1β expression at the AP site, contributing to an anti-inflammatory environment.

After determining the optimal concentration, both DRW and W at 0.1% showed no cytotoxic effects on MQ and PDLFs. These cells play key roles in the development and progression of AP (26). MQ are innate immune cells that participate in inflammation by balancing the production of pro- and anti-inflammatory cytokines, such as IL-6, TNF-α, and IL-10, which in turn influence bone resorption and repair (27,28). PDLFs, resident cells of the periodontal ligament, contribute to tissue homeostasis by maintaining the extracellular matrix and regulating cytokine production through crosstalk with MQ, modulating the immune response (29,30). In addition, they influence bone remodeling by stimulating osteoclast precursors and regulating the expression of receptor activator of nuclear factor-κB ligand and osteoprotegerin, key mediators of osteoclastogenesis and bone turnover (31).

The use of a direct co-culture system allowed to model cell-to-cell interactions occurring in the periapical environment. In this setting, DRW promoted an anti-inflammatory response by suppressing TNF-α and IL-6, while increasing IL-10 levels. Interestingly, IL-1β, TNF-α, IL-6, IL-10 and VEGF-A levels were increased for W compared to DRW.

The anti-inflammatory effects of DRW are likely mediated by polyphenols such as resveratrol, quercetin, and other flavonoids. These compounds modulate signaling pathways in MQ, including nuclear factor-kappaB (NF-κB) and p38 mitogen-activated protein kinase (p38 MAPK), which regulate cytokines such as IL-1β, TNF-α, and IL-6 (32). In lipopolysaccharide(LPS)-stimulated PDLFs, resveratrol suppresses TNF-α, IL-6, and IL-1β in a concentration- and time-dependent manner (33). Inhibition of TLR4-NF-κB/MAPK/IRF3 signaling by resveratrol has also been shown to reduce TNF-α and IL-6 while increasing IL-10 (34). Moreover, polyphenols modulate MAPK and oxidative stress pathways, contributing to the attenuation of pro-inflammatory cascades (35). Since VEGF is often induced by these cytokines (36), its upregulation may enhance vascular permeability and leukocyte infiltration. Differences in responses between DRW and W may be partly due to ethanol in W, which can promote pro-inflammatory signaling (15-17).

As a key mediator of periodontal inflammation, IL-1β promotes immune cell recruitment and bone resorption in AP (3). Therefore, its downregulation observed *in vivo* aligns with reduced circulating monocytes, that migrate to site of injury and differentiate into MQ, regulating both the initiation and maintenance of chronic inflammation (37). Additionally, the higher expression of IL-10 in DRW-treated animals compared to W reinforces its potential regulatory role. These results agree with previous studies where a combination of resveratrol with quercetin or DRW supplementation led to higher IL-10 and reduced IL-1β immunolabeling in developing and established AP models (12,14).

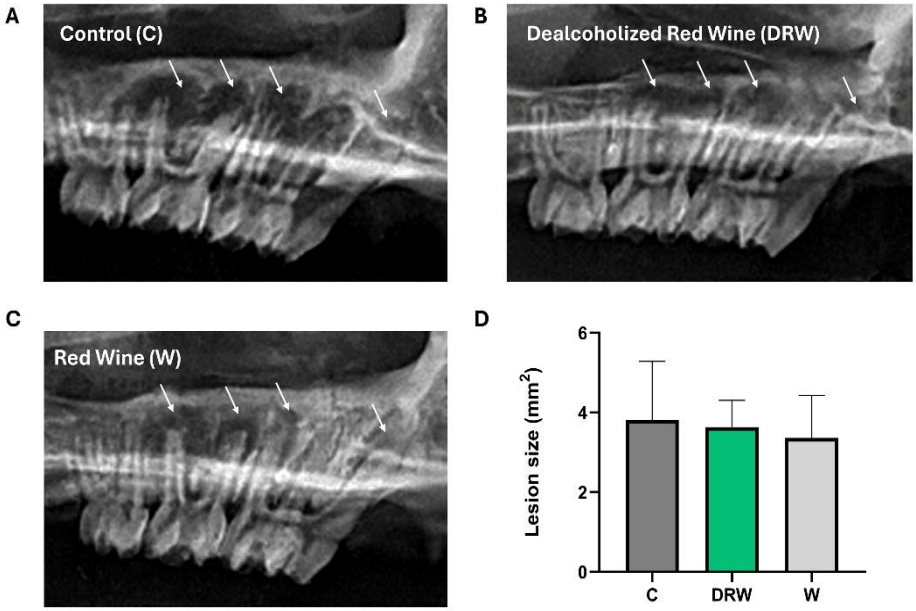

Figure 4. Representative radiographic images from the specimens in the **A**, Control; **B**, dealcoholized red wine (DRW); and **C**, red wine (W) groups. The white arrows indicate the periapical lesions in the first and second maxillary molars. **D**, The bar graph for lesion size shows the mean and standard deviation in mm2 of each group. There were no significant differences among groups (P>0.05). One-way ANOVA with Tukey post hoc test was applied.

Despite the observed molecular changes, ELISA revealed no significant differences among groups in blood levels of IL-10, IL-1β, IL-17A, TNF-α, osteocrin, and SPARC. As previously reported, the inflammatory effects in blood cytokine levels tend to be more evident in animals with multiple foci than those without AP or with a single lesion (6–8), potentially masking anti-inflammatory systemic effects of the tested supplementations. Furthermore, plasma cytokine levels likely reflect a cumulative, time-dependent systemic response, indicating that systemic changes in blood samples of rats with AP can be subtle, transient, and vary with disease progression (38).

Moreover, DRW supplementation did not result in statistical differences in lesion size/periapical bone loss among groups assessed through radiographic and histometric analyses. This may reflect the complexity of bone remodeling, which depends not only on local cytokine levels but also on systemic factors, mechanical influences, and lesion stage, and is tightly regulated by a network of mediators such as IL-1β, TNF-α, IL-6, IL-17, IL-10, and the RANKL/OPG axis (39,40). In the present model, the AP stage may have been too early to allow detectable changes in lesion size or bone loss, since the inflammatory process was still under development. Importantly, evaluating more advanced stages of AP could be key to uncovering the modulatory effects of DRW polyphenols on bone resorption, particularly since differences in lesion size have been documented after 60 days of disease progression (14). These

findings suggest that while DRW polyphenols can influence the inflammatory environment, additional or more sustained interventions may be required to translate these cellular effects into reductions in bone pathology.

Some limitations of this study should be noted. *In vitro*, assessing MQ polarization markers and including bacterial stimuli could clarify cellular pathways and inflammatory responses. *In vivo*, evaluating mediators such as the RANKL/OPG ratio may explain why IL-1β suppression and IL-10 upregulation did not reduce bone loss. Additionally, early peaks in cytokine expression may have been diminished at the 45-day assessment (40). Finally, isolating specific polyphenols could identify the main active components, guiding targeted therapies.

Considering the recent nature of research on the anti-inflammatory effects of DRW in AP, and the complex interplay of local and systemic immune responses involved in the disease, these findings should be interpreted with caution and are not directly translatable to clinical settings. Nonetheless, this study offers valuable insights into the immunomodulatory potential of DRW in both *in vitro* and *in vivo* models, highlighting the need for further investigations to better elucidate its mechanisms of action.

In conclusion, DRW and W exerted distinct effects in both co-culture and *in vivo* AP models. DRW promoted a pronounced anti-inflammatory profile by reducing TNF-α and IL-6 while increasing IL-10 in co-cultures, and by lowering blood monocyte counts and decreasing IL-1β expression *in vivo*. However, these anti-inflammatory effects were not sufficient to alter bone disease progression within the experimental model evaluated.

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The authors declare that there are no conflicts of interest related to this study.

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5 CONCLUDING REMARKS

Although no significant differences were observed in lesion size or bone loss, the administration of dealcoholized red wine modulated the inflammatory response in apical periodontitis, reducing the intensity of the inflammatory process, the gene expression of the pro-inflammatory cytokine IL-1β, circulating monocyte counts, and osteoclastic activity, as evidenced by a decrease in TRAP-positive cells in the animal model. In cell co-cultures, lower levels of IL-6 and TNF-α were observed, accompanied by an increase in IL-10. Thus, dealcoholized red wine contributed to an anti-inflammatory profile in different biological contexts, suggesting a potential modulation of the inflammatory and bone-metabolic responses associated with apical periodontitis, which could ultimately favor post-treatment healing in affected teeth.

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APPENDIX A – Certificate of approval from the animal ethics committee

UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"

CAMPUS ARAÇATUBA
FACULDADE DE ODONTOLOGIA
FACULDADE DE MEDICINA VETERINÁRIA

CEUA - Comissão de Ética no Uso de Animais
CEUA - Ethics Committee on the Use of Animals

CERTIFICADO

Certificamos que o Projeto de Pesquisa intitulado **"Efeito do consumo de vinho tinto sem álcool no desenvolvimento da periodontite apical induzida em ratos"**, Processo FOA nº 0221-2022, sob responsabilidade de João Eduardo Gomes Filho apresenta um protocolo experimental de acordo com os Princípios Éticos da Experimentação Animal e sua execução foi aprovada pela CEUA em 25 de Abril de 2022.

VALIDADE DESTE CERTIFICADO: 30 de Maio de 2025.
DATA DA SUBMISSÃO DO RELATÓRIO FINAL: até 30 de Junho de 2025.

CERTIFICATE

We certify that the study entitled **"Effect of alcohol-free red wine consumption on the developmente of induced apical periodontitis in rats"**, Protocol FOA nº 0221-2022, under the supervision of João Eduardo Gomes Filho presents an experimental protocol in accordance with the Ethical Principles of Animal Experimentation and its implementation was approved by CEUA on April 25, 2022.

VALIDITY OF THIS CERTIFICATE: May 30, 2025.
DATE OF SUBMISSION OF THE FINAL REPORT: June 30, 2025.

Prof. Dr. João Carlos Callera
Coordenador da CEUA
CEUA Coordinator

CEUA - Comissão de Ética no Uso de Animais
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APPENDIX B – "Impact of Wine Polyphenols on the Inflammatory Profile of Induced Apical Periodontitis in Rats" published in the *Journal of Endodontics*

BASIC RESEARCH – BIOLOGY

Impact of Wine Polyphenols on the Inflammatory Profile of Induced Apical Periodontitis in Rats

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ABSTRACT

Introduction: This study evaluated the impact of dealcoholized red wine polyphenols on the inflammation and lesion volume associated with apical periodontitis (AP) in rats. **Methods:** Thirty-two Wistar rats receiving AP induction were arranged as follows: Control Group, Dealcoholized Red Wine Group (DRW), Red Wine Group, and Alcohol Group (ALC). Solutions were administered daily in a volume of 4.28 mL/kg via gavage for 45 days. Mandibles and maxillae were removed for histologic, immunohistochemical (IL-1β, IL-10, tumor necrosis factor-alpha, receptor activator of nuclear factor κB ligand, osteoprotegerin [OPG], and tartrate-resistant acid phosphatase), and micro-computed tomography analyses of the AP site. A statistical analysis was performed with a significance level of 5%. **Results:** Inflammation and TRAP-positive cell count were similar for DRW and Red Wine Group, but lower when compared to Control Group and ALC ($P < .001$). The immunohistochemical expression of OPG was higher for DRW than for ALC ($P < .05$). A larger lesion volume was observed in ALC compared to other groups ($P < .001$). **Conclusions:** Prophylactic administration of dealcoholized red wine significantly reduced inflammation, decreased the number of TRAP-positive cells, enhanced OPG expression, and reduced lesion volume compared to water and alcohol solutions. *(J Endod 2025;51:594–601.)*

KEY WORDS

Apical periodontitis; bone remodeling; dealcoholized red wine; polyphenols; wine

SIGNIFICANCE

Resveratrol and quercetin administration reduced inflammation and bone resorption in rats with induced apical periodontitis. Dealcoholized red wine provides a viable model to investigate the potential anti-inflammatory effects of polyphenols while avoiding the inflammatory risks posed by alcohol consumption.

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During apical periodontitis (AP) development, inflammatory cytokines play a significant role in initiating and coordinating cellular events and regulating the host's response to endotoxins[1]. The AP induction in Wistar rats has been correlated with increased levels of pro-inflammatory cytokines at the AP site, such as tumor necrosis factor-alpha (TNF-α), interleukin (IL) 6, and IL-1β[2]. Bone resorption also occurs as a determining factor in lesion expansion during AP, initiated by the proliferation of immature osteoclast precursor cells and their differentiation into mature osteoclastic cells that degrade organic and inorganic bone components[1].

Red wine is globally consumed, and its light to moderate consumption is associated with various health benefits, including a reduced risk of bone mass loss and fractures, and milder inflammatory response due to its anti-inflammatory and antioxidant effects[3–5]. The prophylactic administration of red wine reduced the tartrate-resistant acid phosphatase (TRAP) labeling in rats with induced AP. Additionally, its isolated polyphenols attenuated the inflammation, lesion volume, and TRAP labeling, and increased osteoprotegerin (OPG) and IL-10 expression compared to water supplementation[5].

Moderate red wine consumption, typically ranging from 150 to 300 mL per day[6], has been associated with health benefits due to its polyphenols and alcohol content[7]. However, reducing alcohol intake generally improves health, since its excessive intake is linked to many diseases[8]. Moreover, genetic differences and lifestyle factors also affect alcohol's impact, limiting its promotion for health benefits[9]. Furthermore, studies have shown that when alcohol was given at the same dose and concentration found in wine, bone resorption and inflammation increased in animal experiments[5,10–12].

Conversely, polyphenols appear to play a significant role in the health benefits associated with red wine. Various techniques have been developed to reduce the alcohol content of wine while maintaining its phenolic compounds[13]. The use of dealcoholized red wine provides a means to obtain the benefits of polyphenols without the adverse effects of alcohol[13]. Dealcoholized red wine consumption offers promising health benefits, including cardioprotective effects[14], decreased oxidative stress associated with inflammation[15], enzyme activity modulation[16], inhibition of hepatocellular carcinoma[17], and reduced microbial biofilm viability in periodontics[18]. Thus, it is an excellent antioxidant source for individuals who should avoid alcohol[16].

To date, no study linked dealcoholized red wine rich in polyphenols with the development of induced AP in terms of inflammation and lesion volume. The hypothesis is that dealcoholized red wine administration results in milder inflammation and lower lesion volume than water administration during the development of induced AP in rats.

MATERIAL AND METHODS

Quantification of the Phenolic Compounds Present in the Wines

The total phenolic compounds in red wine and dealcoholized red wine (Vinoh, Bento Gonçalves, RS, Brazil) were determined by High-Performance Liquid Chromatography as stated in a previous study[19].

Animals

This study was approved by the ethics committee of Araçatuba School of Dentistry, São Paulo State University, Brazil (n° 0221-2022). Thirty-two male Wistar rats (Rattus norvegicus), each weighing 300–350g, were housed in a temperature-controlled environment (22°C ± 1°C, 70% humidity) with a 12-hour light-dark cycle, having *ad libitum* access to food and water.

Animals were randomly assigned as (n = 8): Control Group (C), Dealcoholized Red Wine Group (DRW), Red Wine Group (W), and Alcohol Group (ALC). They were weighed every 2 days to calculate diet dosage, with weight gain recorded as a percentage of initial and final weights.

Diet Administration

The rats from C received potable water as a sham. The rats from DRW and W received dealcoholized red wine and red wine (Vinoh, Bento Gonçalves, RS, Brazil), respectively. The rats from ALC received an alcoholic solution containing 12.5% alcohol by volume (the same amount of alcohol present in the red wine given to W)[5]. The administration occurred daily in the morning at 4.28 mL/kg body weight via oral gavage for 45 days, starting 15 days before AP induction and continuing for 30 days[5].

Apical Periodontitis Induction

On day 15, all animals were anesthetized with ketamine (80 mg/kg) and xylazine (4 mg/kg) for AP induction. The coronal openings of the first and second upper and lower right molars (4 teeth) were performed using a 0.5 mm carbon steel bur (Long Neck Ln Bur - Maillefer, Dentsply), standardizing all pulp exposures. Lesions were analyzed 30 days postinduction[20–22].

Sample Collection

On day 45, the animals were euthanized with an anesthetic overdose[20] to collect the right mandibles and maxillae. The hemimandibles were fixed in 10% formaldehyde for histologic and immunohistochemical analyses, while the hemimaxillae were frozen for micro-computed tomography analysis.

Histologic/Immunohistochemical

Fixed mandibles were decalcified in 10% ethylenediaminetetraacetic acid, embedded in paraffin, and sectioned at 5 μm. For histological analysis, hematoxylin and eosin staining were used to evaluate inflammation and bone loss in the periapical area of the first molar's distal root. Inflammation was analyzed at 400x magnification and scored as follows: 1 - absent/few inflammatory cells; 2 - up to 25 cells, mild reaction; 3 - 25-125 cells, moderate reaction; 4 - more than 125 cells, severe reaction[22].

Immunohistochemical analysis was performed using the immunoperoxidase technique as previously described[5]. IL-1β, IL-10, TNF-α, receptor activator of nuclear factor κB ligand (RANKL), and OPG, were analyzed in the periapical lesion of the first molar's distal root at 400x magnification. Immunostaining was defined as brown staining in the cytoplasm of cells and the extracellular matrix, and scored as follows: 0 - complete absence of immunoreactive (IR) cells; 1 - low IR cells and weak extracellular matrix staining; 2 - moderate IR cells and moderate extracellular matrix staining; and 3 - high number of IR cells and intense extracellular matrix staining. TRAP-positive cells were quantified at the perimeter and expressed as cells per millimeter. Analyses were conducted by a blinded, calibrated investigator using an optical microscope (Optiphot-2, Nikon).

Micro-computed Tomography

The first upper right molars were scanned using the SkyScan 1272 imaging system (Kontich, Belgium). Reconstructed three-dimensional images were generated using Data Viewer (SkyScan, Version 1.4.4 64 bit, Burker, Kontich, Belgium) and NRecon (SkyScan, Burker, Kontich, Belgium) software, and analyzed using CTAnalyser software (2003-11SkyScan, 2012 Bruker MicroCT Version 1.12.4.0, Burker, Kontich, Belgium). The volume of interest included the periradicular bone resorption involving the distal root's periapex. Thus, the space of the resorption area was measured and expressed in mm^3[35].

Statistical Analysis

The data were analyzed using SigmaPlot 12.0 software (Chicago, IL, USA). The Kruskal–Wallis and Dunn's multiple comparison tests were applied to nonparametric data. One-way ANOVA and Tukey's tests were used for parametric data. The significance level was set at 5% ($P < .05$).

RESULTS

Quantification of the Phenolic Compounds Present in the Wines

No differences were observed in the amounts of total phenolic acids, flavanols, proanthocyanidins, stilbenes, and flavanones detected in the dealcoholized red wine and red wine samples ($P > .05$). For flavonols, dealcoholized red wine had higher amounts of myricetin and quercetin 3-glucoside, while red wine had higher amounts of rutin ($P < .05$). For anthocyanins, red wine showed higher amounts of delphinidin 3-glucoside, pelargonidin 3-glucoside, and malvidin 3-glucoside ($P < .05$).

Weight Gain Average

The mean and standard deviation values for the percentage of weight gain are shown in Figure 1*A*. All groups gained weight during the experiment. Similar weight gains ($P > .05$) were observed for C (38.4 ± 4.654) and DRW (36.178 ± 6.543). Lower weight gains were observed for ALC (27.40 ± 4.933) and W (29.078 ± 3.670) compared to C and DRW ($P < .001$).

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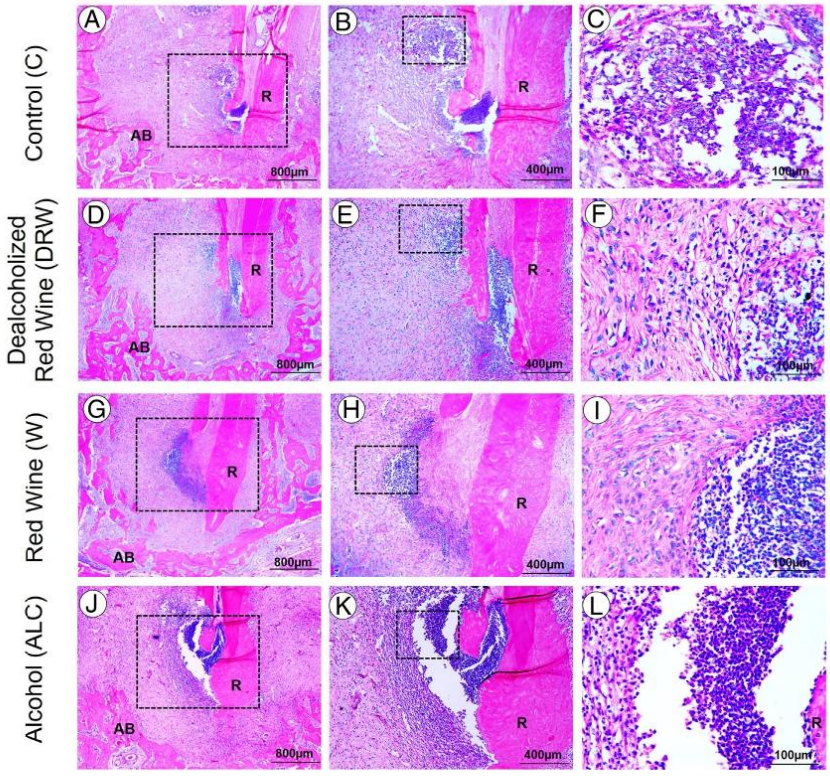

FIGURE 1 – The bar graphs and the charts represent the average weight gain percentage (*A*), the intensity of the inflammatory process (*B*), the lesion volume in cubic millimeters (*C*), the IL-1β (*D*), IL-10 (*E*), TNF-α (*F*), RANKL (*G*), and OPG (*H*) immunolabeling, and the positive TRAP cells per millimeter (*I*). The bar graphs show each group mean and standard deviation. The bold line in the charts represents the median of each group. Symbols: **P* < .05 vs C; †*P*<.05 vs DRW; #*P* < .05 vs ALC. ALC, Alcohol Group; DRW, Dealcoholized Red Wine Group; OPG, osteoprotegerin, TRAP, tartrate-resistant acid phosphatase; RANKL, receptor activator of nuclear factor κB ligand; TNF-α, tumor necrosis factor-alpha; IL, interleukin.

Histologic Analysis

The histologic sections and the inflammatory scores are shown in Figures 1*B* and 2. All the animals had inflammatory infiltrates in the periapex with bone destruction. Moderate to severe inflammatory reactions were observed for C (score 3) and ALC (score 4) ($P > .05$). While mild to moderate inflammatory responses were observed for DRW (score 2) and W (score 2.5), lower than C ($P < .05$) and ALC ($P < .001$). There was no difference between DRW and W ($P > .05$).

Immunohistochemical Analysis

The immunoreactivity patterns for IL-1β, IL-10, TNF-α, RANKL, OPG, and TRAP are shown in Figures 1*D*, *E*, *F*, *G*, *H*, *I* and 3. A higher OPG expression was observed for DRW (score 2.5) than ALC (score 1) ($P < .05$). A lower number of TRAP-positive cells were found for DRW and W than C ($P < .05$) and ALC ($P < .001$). Additionally, ALC was more TRAP-inductive than all other groups ($P < .001$). No differences were observed for IL-1β, IL-10, TNF-α, and RANKL ($P > .05$) immunolabeling among the groups.

Micro-computed Tomography Analysis

The data and the representative micro-computed tomography slices are shown in Figures 1*C* and 4*A*, *B*, *C*, *D*, respectively. By day 45, all groups exhibited periapical hypodense regions, indicating AP onset and development. The lowest lesion volume (0.659 $mm^3 \pm 0.124$ mm^3) was observed for DRW, similar to C (0.824 $mm^3 \pm 0.169$ mm^3) and W (0.859 $mm^3 \pm 0.305$ mm^3) ($P > .05$). The greatest lesion volume was observed for ALC (1.608 $mm^3 \pm 0.302$ mm^3) compared to the other groups ($P < .001$).

DISCUSSION

This study is the first to assess dealcoholized red wine impact on inflammation and lesion volume in rats with induced AP. Despite claims that alcohol contributes to red wine's benefits[7], dealcoholized red wine reduced inflammation compared to water, but had no impact on lesion volume, rejecting the

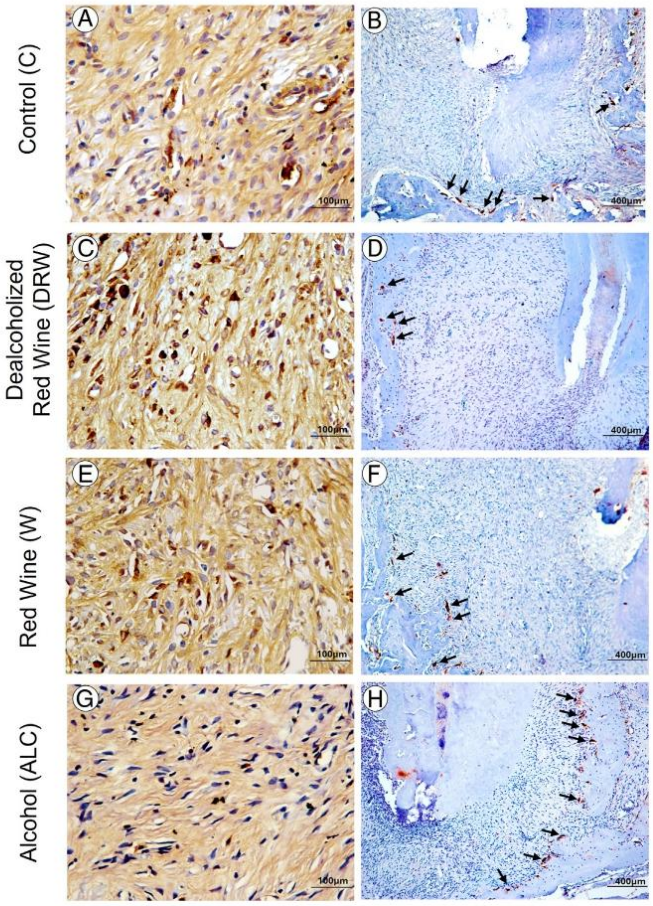

FIGURE 2 – The photomicrographs show the histologic aspects and the alveolar bone (AB) loss of the apical regions around the foraminal opening of the distal root (*R*) of the mandibular first molar. The moderate inflammatory process can be observed for the Control Group (*A*, *B*, and *C* at 50 ×, 100 ×, and 400 × magnification, respectively). A mild inflammatory process can be observed for the Dealcoholized Red Wine Group (*D*, *E*, and *F* at 50 ×, 100 × and 400 × magnification, respectively), and a mild to moderate inflammatory process can be observed for the Red Wine Group (*G*, *H*, and *I* at 50 ×, 100 × and 400 × magnification, respectively). The severe inflammatory process can be observed for the Alcohol (*J*, *K*, and *L* at 50 ×, 100 ×, and 400 × magnification, respectively). Hematoxylin and eosin staining. Rectangles indicate the enlarged area in the next magnification. Scale bars: 800 µm (*A*, *D*, *G*, *J*), scale bars: 400 µm (*B*, *E*, *H*, *K*), and scale bars: 100 µm (*C*, *F*, *I*, *L*).

hypothesis of this study. Fewer TRAP-positive cells were also observed for DRW compared to C and ALC, with higher OPG immunolabeling and reduced lesion volume than ALC. This suggests that the benefits attributed to red wine may primarily be due to polyphenols rather than the alcoholic content[5,15,16].

Wistar rats are a well-established model for studying induced AP[10,11,20–24]. While results cannot be directly applied to clinical settings, using Wistar rats allows for controlled conditions that could influence the development of AP, such as weight, diet, and stress levels[23,24]. The intraoral gavage method ensured precise solution administration, unlike free access through water bottles. The 4.28 mL/kg dose was equivalent to a 70 kg male consuming 300 mL (2 glasses) of red wine daily[6].

Weight variation was monitored as an indirect index of overall animal health while ensuring accurate diet doses. All animals gained weight during the experiment, with no significant difference between C and DRW, while the least weight gain was observed for W and ALC. Equivalent results were found comparing AP development in rats on a chronic alcoholic diet vs a regular diet[10,11]. Despite being considered hypercaloric, alcohol is ineffective as a nutritional source and hampers vitamin and nutrient absorption[25].

Histologic analysis confirmed effective AP induction, with all groups showing inflammatory infiltrate in the periapical region with bone resorption. A milder inflammatory process was noted for DRW and W. In contrast, ALC exhibited predominantly severe inflammatory reactions. These results align with studies showing increased AP inflammation with alcoholic diets at 12.5%, 15%, and 20% concentrations[5,10,11] and exacerbation of periodontitis with chronic alcohol consumption at 14%, 25%, and 36% concentrations in a dose-dependent manner[12]. Additionally, resveratrol and quercetin are found in both dealcoholized and regular red wine. These polyphenols are known to exert anti-inflammatory and antioxidant biological effects, in addition to their capacity to modulate the host osteoimmune inflammatory response[26–28].

One mechanism by which polyphenols can exert their immunomodulatory effects is the modulation of inflammatory cytokines[29]. In a previous study, red wine showed a tendency to decrease IL-1β and TNF-α while enhancing IL-10 release in the AP site of rats. However, only its polyphenols statistically increased IL-10 levels compared to the control group[5]. This study found similar IL-1β and TNF-α levels across groups. These cytokines act simultaneously in inflammation and bone resorption and are frequently found in inflamed periodontal tissues[30]. IL-1β, the predominant IL-1 type in human periapical lesions, can increase osteoclastic cells *in vivo* and inhibit osteogenesis[31]. TNF-α can be present immediately after pulp exposure with levels potentially increasing for up to 30 days in periapex[32].

All groups had similar IL-10 and RANKL expressions. IL-10 is a potent anti-inflammatory cytokine that can regulate cell differentiation and prevent bone loss by upregulating OPG and downregulating RANKL[33,34]. Under physiological conditions,

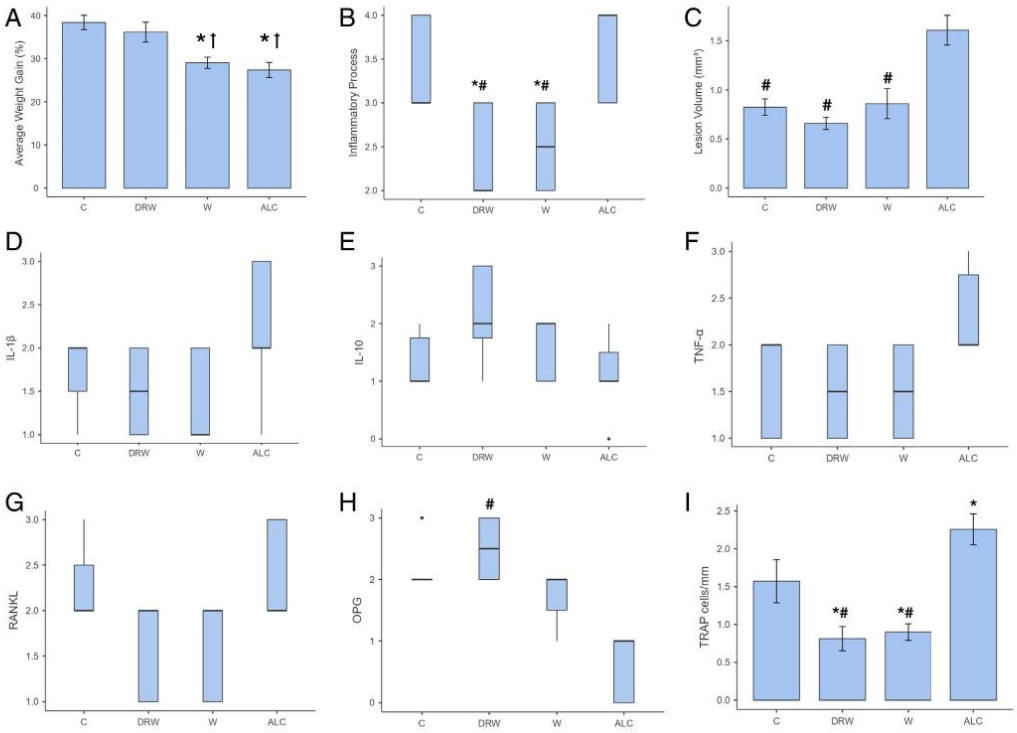

FIGURE 3 – The photomicrographs show the immunostaining for OPG (*A*, *C*, *E*, *G*) and TRAP (*B*, *D*, *F*, *H*) in apical periodontitis around the foraminal opening of the distal root of the mandibular first molar for the Control Group (*A*, *B*), the Dealcoholized Red Wine Group (*C*, *D*), the Red Wine Group (*E*, *F*) and the Alcohol Group (*G*, *H*). Counterstaining: Harris hematoxylin. Original magnification for OPG: 400 × with scale bars: 100 μm, and 100 × for TRAP with scale bars: 400 μm. OPG, osteoprotegerin, TRAP, tartrate-resistant acid phosphatase.

the RANK/RANKL/OPG ratio guarantees a delicate balance between bone formation and resorption. The RANKL is a critical molecule in osteoclast activation. In turn, OPG binds to RANKL, preventing it from binding to RANK and leading to osteoclast differentiation[1]. Higher OPG immunolabeling was observed for DRW compared to ALC, as noted when resveratrol and quercetin were administered to rats with induced AP[5].

A lower load of TRAP-positive cells was observed for DRW and W compared to C. Conversely, ALC was more TRAP inductive than all other groups, reinforcing alcohol's potential to increase bone resorption[5,10–12]. TRAP, a protein in osteoclasts, is upregulated by RANKL and downregulated by OPG, and its secretion can be enhanced during bone resorption[35]. Alcohol has been shown to negatively affect bone metabolism, reducing cortical bone area, bone formation rates, and mineral apposition rates[36]. Micro-computed tomography revealed a reduced lesion volume in DRW and W compared to ALC, consistent with previous research[5] and supports the findings on inflammatory profile and cytokine expression.

Since dealcoholized red wine has multiple bioactive components beyond polyphenols (such as glycerol, polysaccharides, and acids)[13], interactions may occur between them. These interactions can be advantageous or synergistic, as well as negative or neutralizing[37]. Additionally, most phenolics in wine are not entirely absorbed by the digestive system, resulting in insignificant blood or tissue concentrations[38]. Consequently, the impact of phenolics as a group might differ from that of individual phenolics, which are typically the focus of research. Therefore, studying whole foods vs isolated compounds reveals differences, as demonstrated by resveratrol and quercetin[5].

This study questions whether alcohol is essential for obtaining red wine's benefits. Our findings suggest further research on dealcoholized red wine polyphenols on AP and other oral diseases. It's important to note that dealcoholized red wine contains several distinct polyphenols that should be individually studied in future research.

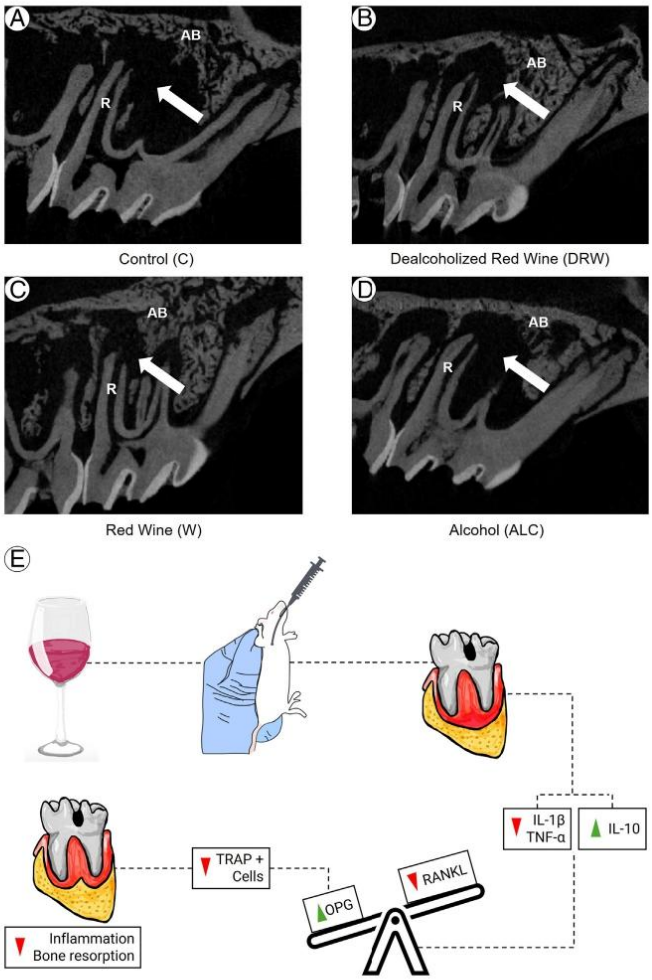

FIGURE 4 – Micro-computed tomography slices of apical periodontitis in the distal root (*R*) of the upper first molars of each group (*A*, *B*, *C*, and *D*). Diminished bone resorption on microtomography is noted for the Control Group (*A*), the Dealcoholized Red Wine Group (*B*), and the Red Wine Group (*C*) compared to the Alcohol Group (*D*) ($P < .001$). White arrowheads point to the area of the apical periodontitis surrounded by hyperdense alveolar bone (AB). Symbols: #$P < .05$ vs ALC. Schematic representation of the mechanisms through which dealcoholized red wine influences AP (*E*). AP, apical periodontitis; ALC, ALC, Alcohol Group.

CONCLUSION

Prophylactic administration of dealcoholized red wine reduced inflammation and TRAP-positive cells in rats with induced apical periodontitis compared to water. It also increased OPG expression and reduced lesion volume compared to an alcoholic solution.

CREDIT AUTHORSHIP CONTRIBUTION STATEMENT

Rafaela Ricci: Conceptualization, Methodology, Data curation, Formal analysis, Resources, Writing – original draft, Writing – review & editing, Visualization, Funding acquisition. **Bharbara de Moura Pereira:** Conceptualization, Methodology, Resources, Visualization. **Julissa Denisse Arguello Alvarado:** Conceptualization, Methodology, Resources, Visualization. **Romulo de Oliveira Sales-Junior:** Conceptualization, Methodology, Resources, Visualization. **Nathália Evelyn da Silva Machado:** Conceptualization, Methodology, Resources, Visualization. **Doany Cevada dos Santos:** Resources, Visualization. **Felipe Haddad Martim Pederro:** Resources, Visualization. **Marciane Magnani:** Formal analysis, Investigation. **Marcos dos Santos Lima:** Formal analysis, Investigation. **Edilson Ervolino:** Conceptualization, Methodology, Validation, Formal analysis, Investigation, Resources. **Luciano Tavares Ângelo Cintra:** Conceptualization, Methodology, Validation, Formal analysis, Investigation, Resources, Writing – review & editing. **Anil Kishen:** Writing – review & editing. **João Eduardo Gomes-Filho:** Conceptualization, Methodology, Formal analysis, Investigation, Resources, Writing – original draft, Writing – review & editing, Visualization, Funding acquisition, Supervision, Project administration.

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The authors deny any conflicts of interest related to this study.

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APPENDIX C – "Cellular and Molecular Immunomodulatory Potential of Red Wine Polyphenols in Apical Periodontitis" published in the *Brazilian Journal of Medical and Biological Research*

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Cellular and molecular immunomodulatory potential of red wine polyphenols in apical periodontitis

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Abstract

This *in vitro* and *in vivo* study assessed dealcoholized red wine (DRW) effects on cytokine profile in macrophage (MQ)-periodontal ligament fibroblast (PDLFs) co-cultures and its impact on blood parameters, inflammatory/bone markers, cytokine expression, and periapical bone loss in rat apical periodontitis (AP). A MQ-PDLFs co-culture and Wistar rats with induced AP were exposed to DRW or red wine (W), with DMEM or water as controls (C). Cell cultures were analyzed for cytokine profile using a multiplex immunoassay. Rats underwent blood profiling, radiography, qRT-PCR, and histometric analysis of AP. Statistical significance was set at 5%. Multiplex analysis of the co-culture revealed that DRW induced lower interleukin (IL)-6 and tumor necrosis factor (TNF)-α levels compared to C and W, higher IL-10 level than C, and lower IL-1β level only compared to W ($P<0.05$). Radiographic images confirmed AP development in rats. DRW showed a reduced monocyte count compared to C ($P<0.05$), but the inflammatory/bone markers in plasma were similar ($P>0.05$). Additionally, DRW showed lower IL-1β expression than C, and higher IL-10 expression only compared to W in AP ($P<0.05$). Periapical bone loss was similar among groups ($P>0.05$). In conclusion, DRW promoted an anti-inflammatory profile in co-cultures and *in vivo*. However, these effects did not translate into differences in lesion size or bone loss within the experimental model evaluated.

Key words: Apical periodontitis; Bone remodeling; Dealcoholized red wine; Inflammation; Red wine

Introduction

Apical periodontitis (AP) is an inflammatory condition affecting the periapical tissues of teeth with necrotic pulps, primarily triggered by bacterial infection and their by-products (1). Pulpal necrosis allows microbial invasion to extend into the periodontal ligament and alveolar bone, initiating an immune response. This response involves both innate and adaptive mechanisms, characterized by the recruitment of inflammatory cells to the lesion site, where they release key cytokines that drive inflammation and osteoclastic activity, ultimately leading to bone resorption (2,3).

The progression of AP reflects a disruption in the balance between inflammation and tissue repair, resulting in sustained periapical damage and continued immune activation (4). Multiple foci of AP have been associated to significant systemic repercussions, including altered blood parameters and disrupted immune homeostasis, with elevated plasma levels of pro-inflammatory cytokines and reduced levels of anti-inflammatory cytokines (5–9).

A diet rich in polyphenols has been linked to anti-inflammatory and antioxidant responses in various pathological conditions (10,11). Although red wine (W) is widely studied for these benefits, its health-promoting effects are primarily attributed to its polyphenolic rather than the alcoholic content (12–14). In this context, dealcoholized red wine (DRW) emerges as a promising alternative, offering the polyphenols without the health risks and inflammation associated with alcohol consumption (15–17).

Studies have demonstrated that DRW can exert anti-inflammatory effects in various inflammatory conditions (18,19). Previous findings revealed that supplementation with DRW reduced inflammation and bone loss in rats with

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induced AP (13,14). All together, these results suggest that DRW may modulate the inflammatory response in AP through mechanisms associated with polyphenol activity. However, the cellular and molecular pathways underlying DRW effects remain to be elucidated.

Therefore, the present study aimed to clarify some of these pathways by investigating the immunomodulatory potential of DRW and W in the context of AP. The cytokine and chemokine profile of macrophages (MQ) and periodontal ligament fibroblasts (PDLFs) in direct co-culture with DRW or W exposure was first examined *in vitro*, allowing juxtacrine interactions. In parallel, an *in vivo* model was used to evaluate both systemic and local effects of DRW or W supplementation, including changes in blood profile, inflammatory and bone metabolism markers in plasma, cytokine expression at the lesion site, and histometric analysis of periapical bone loss. We hypothesized that DRW and W differentially influence the inflammatory profile in AP by modulating cytokine production and immune cell recruitment both locally and systemically.

Material and Methods

Cell cultures

THP-1 monocytes (ATCC TIB-202; American Type Culture Collection, USA) were cultured in complete RPMI 1640 medium (#A104950; Gibco, USA), supplemented with 10% heat-inactivated fetal bovine serum (#19C448; Sigma-Aldrich, USA), 1% antibiotic/antimycotic (#15240-062, Gibco), and 0.1% beta-mercaptoethanol (#2198 5023, Gibco). The cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2. Following this, THP-1 cells (2.5×10^5 cells/mL) were differentiated into MQ by exposure to 100 nmol/L phorbol 12-myristate-13-acetate (Sigma-Aldrich) for 24 h. This was followed by an overnight resting period in fresh RPMI medium. Differentiation was confirmed by observing cellular adhesion and spreading.

Human PDLFs were provided as a kind gift from Dr. Douglas Hamilton's lab, Schulich's School of Medicine & Dentistry, London, ON, Canada. Cells were isolated from clinical samples of healthy periodontium. PDLFs were cultured in complete Dulbecco's Modified Eagle's Medium (DMEM) (Sigma-Aldrich), supplemented with 10% heat-inactivated fetal bovine serum and 1% antibiotic/antimycotic. The cells were maintained in a 37°C incubator with 5% CO_2 in a humidified atmosphere. Experiments were conducted using cells from the third to fifth passage.

Cell viability assay

To determine the optimal concentration of wine, both DRW and W (Vinoh, Brazil) were opened under sterile conditions within a biosafety cabinet and immediately filtered using a vacuum filtration membrane. The wines were then diluted in complete DMEM to concentrations of 15, 1, and 0.1%.

PDLFs monocultures were exposed to the test media for 24 h. Cells cultured in complete DMEM alone served as the negative control. The media was removed, and triplicate samples of cells were incubated with 200 μL of calcein-AM (Invitrogen/Molecular Probes, USA) for 20 min. Fluorescence was observed at 10 × magnification using a fluorescent inverted microscope (Vert.A1; Carl Zeiss, Germany). Images were acquired and analyzed using ImageJ software (National Institutes of Health, USA). Cell viability was calculated as a percentage relative to the negative control, which was considered to be 100% viable (20).

Based on the PDLFs viability results, the most suitable wine concentration was tested in MQ monocultures following the same viability assay protocol. The cytokine and chemokine analysis were performed only after confirming the viability of MQ at the selected concentration.

Assessment of cytokine and chemokine profile

After the determination of optimal concentration, cell culture supernatants of MQ and PDLFs co-culture treated with DMEM (C), 0.1% concentration of DRW (0.1% DRW), or W (0.1% W) were collected at 24 h. The samples were then centrifuged at 10,000 *g* and 4°C for 5 min to remove residual cells, aliquoted, and stored at –80°C until analysis. A multiplex immunoassay was conducted to simultaneously analyze the inflammatory cytokines interleukin (IL)-1β, tumor necrosis factor (TNF)-α, IL-8 (a chemokine), IL-10, IL-6, and vascular growth factor A (VEGF-A) using the HCYTOMAG-60K Milliplex MAP Human cytokine magnetic bead panel (Millipore, USA). The assessment was performed in duplicate. Data are reported in picograms per milliliter (pg/mL).

Animal experiment

Once approved by the Ethics Committee (Araçatuba School of Dentistry, São Paulo State University, No. 0221-2022), twenty-four male rats (*Rattus norvegicus*, *Wistar*), aged 3 months and weighing 300–350 g each, were housed in a temperature-controlled environment (22°C ± 1°C, 70% humidity) with a 12-h light-dark cycle. The animals were fed a solid diet and water *ad libitum* throughout the experimental period. Solid diet was not provided during the 12 h leading up to the intervention. The sample size was based on previous studies recommending at least seven animals per group (12–14). To account for potential complications, one extra animal was added per group, totaling 24 animals (n=8 per group).

The animals were randomly assigned to three groups as follows: Control (C), rats receiving 4.28 mL/kg body weight of potable water to mimic the same procedure endured by the other animals; DRW, rats receiving 4.28 mL/kg body weight of DRW containing 0.3% ethanol by volume; and W, rats receiving 4.28 mL/kg body weight of W containing 12.5% ethanol by volume (12–14).

This amount of the test solutions is considered moderate consumption for a male individual (300 mL per day) (21). The solutions were administered daily through oral gavage for 45 days supplementing the conventional diet. This regimen began 15 days before the induction of AP and continued for an additional 30 days (12,13). All animal experiment protocols were performed in accordance with the ARRIVE guidelines for reporting *in vivo* experiments.

Induction of AP

On the 15th day, all animals were anesthetized with 80 mg/kg ketamine (Avenco Inc., USA) and 4 mg/kg xylazine (Mobay Corp., USA) and received AP induction. The first and second maxillary and mandibular molars on the right side had the pulp exposed using a 0.5 mm diameter carbon steel bur (Long Neck Ln Bur, Maillefer, Dentsply, Switzerland), the coronal openings were standardized and the teeth remained open until the end of the experiment (6,9,13). After 30 days from induction, the periapical lesion development was confirmed through digital radiography.

Blood profile and assessment of inflammatory/bone markers

Thirty days after inducing AP, the animals were anesthetized following the previously described protocol. A cardiac puncture was performed to collect 5 mL of blood. The blood samples were then placed in EDTA and homogenized for further processing. An aliquot was separated for the blood profile analysis. Afterward, the blood was centrifuged (1,710 *g*, 4°C, 5 min) to separate the plasma. The plasma samples were transferred to Eppendorf tubes for enzyme-linked immunosorbent assay (ELISA) multiplex analysis.

The blood count parameter of leukocytes and absolute differential count of neutrophils, lymphocytes, monocytes, and eosinophils (7) were determined using an automated hematology analyzer (Mindray - BC 2800 Vet; Shenzhen Mindray Animal Medical Technology Co., LTD., China).

For the multiplex analysis, the MLLIPLEX® Rat Cytokine/Chemokine Magnetic Bead Panel - Immunology Multiplex Assay (RECYTMAG-65K, Merck Life Science, LLC, Germany) was used for the quantification of IL-10, IL-1β, IL-17A, and TNF-α, and the MILLIPLEX® Rat Myokine Magnetic Bead Panel (RMYOMAG-88K, Merck Life Science, LLC) was used for the detection of osteocrin and secreted protein acidic and rich in cysteine (SPARC). All analyses were performed in duplicate. Data are reported in pg/mL (22).

Radiographic, qRT-PCR, and histometric analyses of AP

The animals were euthanized using an anesthetic solution overdose to collect the right-side maxillae and jaws for radiographic, quantitative reverse transcription polymerase chain reaction (qRT-PCR), and histometric analysis of AP.

The maxillae (n=4) were processed for digital radiography using an X-ray equipment Spectro 70X (Dabi Atlante, Brazil), with a 70 kVp nominal voltage and 7.0 mA tube current, and a digital sensor (Acuity direct X-ray photon detection model 005-000124) to confirm AP development. Radiographic images were acquired with an exposure time of 0.8 s and a total focal distance of 20 cm. The lesion size was measured using ImageJ Software (National Institutes of Health). The periapical lesion borders and size around the mesial roots of the first molars were marked, and the area was calculated in square millimeters and compared between groups (23).

For the qRT-PCR analysis, a bone block was removed from the AP site of the maxillae (n=4) using a 2-mm trephine bur (Trephine Bur 2.0 mm, Harte Surgical Instruments, Brazil). The collected bone blocks were immediately pulverized in liquid nitrogen and homogenized in TRIzol® using a mortar and pestle to ensure complete tissue dissociation for RNA extraction. Total RNA was then extracted (PureLink™ RNA Mini Kit, Thermo Fisher Scientific, USA), DNase I-treated, and purity confirmed by 260/280 (1.8–2.0) and 260/230 (2.0–2.2) ratios. RNA concentration was measured by NanoDrop (Thermo Fisher Scientific), and 2 μg were reverse-transcribed to cDNA (High-Capacity RNA-to-cDNA™ Kit, Applied Biosystems, USA). The gene expression analysis of IL-1β, IL-10, and TNF-α was performed in duplicate using the StepOne Plus™ Real-Time PCR System and TaqMan Gene Expression Assays (Applied Biosystems and Thermo Fisher Scientific). Target gene expression was normalized to Actb and the relative abundance of transcripts determined using the $2^{-\Delta\Delta Ct}$ method (24), with data reported as relative expression.

For the histometric analysis, the jaws (n=8) were demineralized in a 10% EDTA solution, embedded in paraffin and sectioned at 5 μm thick following a general histology protocol. Hematoxylin-eosin staining was used to assess differences in periapical bone loss among the different groups. The periapical area associated with the distal root of the first molar, defined by tracing the lesion boundary, was measured and reported in square millimeters using Leica Microsystems Software (Leica, Germany) (25).

Statistical analysis

Statistical analyses were performed using GraphPad Prism 8.0.1 (GraphPad Software, USA). For parametric data, one-way analysis of variance followed by Tukey's *post hoc* test was applied. For non-parametric data, the Kruskal-Wallis test was used, followed by Dunn's *post hoc* test. Comparisons between two groups were conducted using Student's *t*-test. Results were considered statistically significant when $P < 0.05$.

Results

Cell viability assay

Figure 1 shows the cell viability results for PDLFs after 24 h of exposure. Higher concentrations of DRW and W (15 and 1%) significantly reduced cell viability compared to the negative control ($P<0.05$). A dose-dependent improvement in viability was observed as concentrations decreased, with the 0.1% concentration maintaining viability at levels comparable to the control group. Based on these results, 0.1% was selected for subsequent experiments. Both MQ and PDLFs exposed to 0.1% DRW and 0.1% W remained viable, with no significant differences relative to the control group ($P>0.05$).

Cytokine and chemokine profile

Figure 2 presents the cytokine and chemokine profile from the co-culture of MQ and PDLFs. DRW exhibited significantly reduced IL-6 and TNF-α levels compared to C and W, higher IL-10 level compared to C, and lower IL-1β level only compared to W ($P<0.05$).

Blood profile analysis

The results of the blood profile analysis are presented in Table 1. No significant difference was observed in leukocyte count among the groups. However, the absolute differential count revealed a significantly lower monocyte count for DRW compared to C ($P<0.05$), but similar to W ($P>0.05$). The counts of neutrophils, lymphocytes, and eosinophils showed no significant difference among the groups ($P>0.05$).

Assessment of inflammatory/bone markers

The systemic analysis of plasma cytokines showed comparable levels of IL-10, IL-1β, IL-17A, TNF-α, osteocrin, and SPARC among the groups ($P>0.05$) (Figure 3A), indicating a stable systemic cytokine profile under the experimental conditions.

Radiographic analysis of AP

Radiographic imaging of the maxillae confirmed the development of AP in all groups (Figure 4). The periapical lesion areas were measured to assess lesion size, and no statistically significant differences

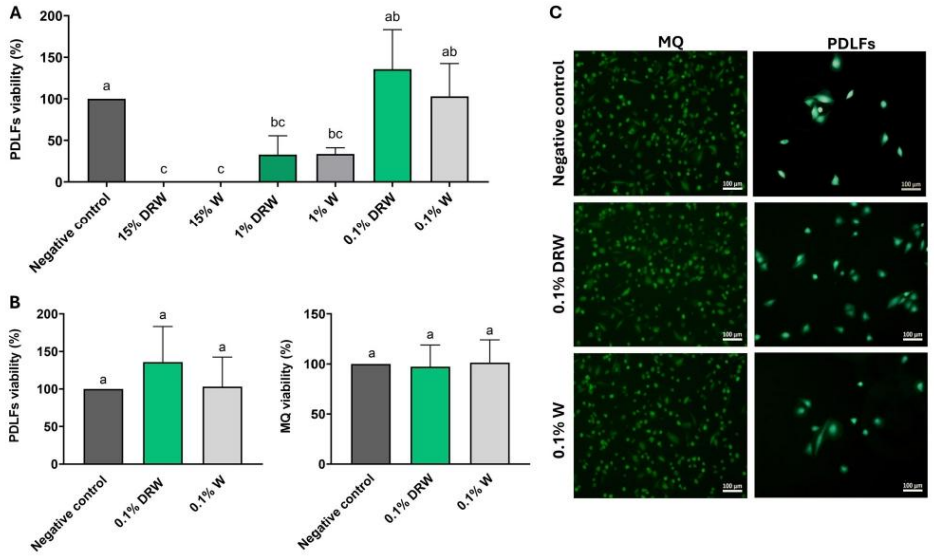

Figure 1. A, Cell viability of periodontal ligament fibroblasts (PDLFs) under different concentrations of dealcoholized red wine (DRW) and red wine (W) relative to the negative control. **B,** Cell viability of macrophages (MQ) and PDLFs monocultures under 0.1% of dealcoholized red wine (0.1% DRW) or red wine (0.1% W) relative to the negative control. Data are reported as means and SD. One-way ANOVA with Tukey *post hoc* test was applied. Different lowercase letters indicate significant difference among the groups ($P<0.05$). **C,** Calcein AM-stained images (10× magnification, scale bar=100 μm) at 24 h of MQ and PdLFs monocultures under 0.1% DRW or 0.1% W.

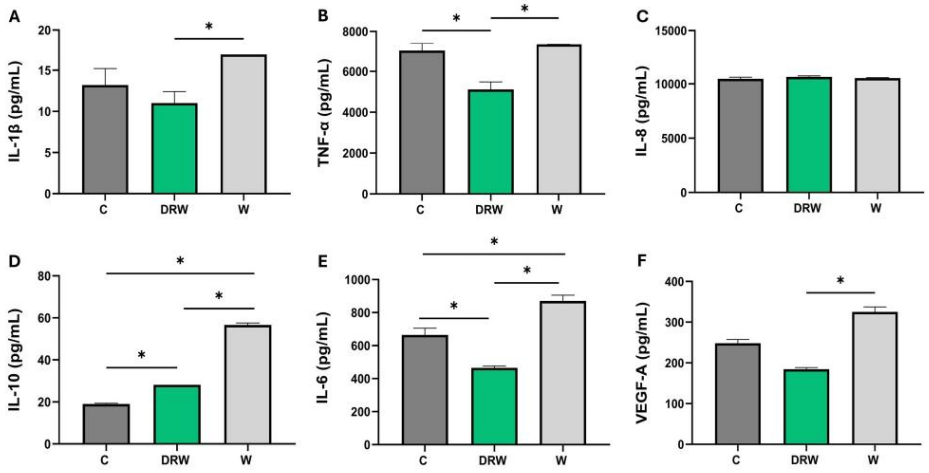

Figure 2. The bar graphs show the mean and standard deviation for the assessment of **A,** IL-1β; **B,** TNF-α; **C,** IL-8; **D,** IL-10; **E,** IL-6; and **F,** VEGF-A for the co-culture of macrophages (MQ) and periodontal ligament fibroblast (PDLFs). Data are reported as means and SD. *$P<0.05$; one-way ANOVA with Tukey *post hoc* test was applied.

Table 1. Blood profile of the study groups.

Blood parameters	Groups		
	C	DRW	W
Leukocytes ($\times 10^3/\mu L$)	9.131 ± 1.871^a	8.925 ± 1.480^a	8.593 ± 1.132^a
Neutrophils (cells/μL)	1.868 ± 0.748^a	1.884 ± 0.683^a	1.814 ± 0.432^a
Lymphocytes (cells/μL)	6.711 ± 1.278^a	6.770 ± 0.988^a	6.307 ± 0.951^a
Monocytes (cells/μL)	404.4 ± 160.5^b	209.8 ± 157.6^a	$356.3 \pm 135.2^{a,b}$
Eosinophils (cells/μL)	109.9 ± 75.69^a	146.3 ± 54.77^a	103.2 ± 119.7^a

Data are reported as means ± SD. Different superscript lower-case letters in rows indicate a significant difference among the groups ($P<0.05$, one-way ANOVA followed by Tukey's test for multiple comparisons). C: control; DRW: dealcoholized red wine; W: red wine.

were detected among the groups ($P>0.05$), indicating that the tested supplementations did not alter radiographic lesion size under the present experimental conditions.

qRT-PCR analysis of AP

The graphs for the qRT-PCR analysis are shown in Figure 3B. The AP sites in the jaws of DRW and W showed significantly lower IL-1β expression compared to C ($P<0.05$). Additionally, IL-10 expression was higher for DRW only compared to W ($P<0.05$). No significant difference was observed in TNF-α expression among the groups ($P>0.05$).

Histometric analysis of AP

Table 2 presents the histometric analysis of AP for all groups. In all groups, histological sections showed evident periapical lesions characterized by bone resorption surrounding the distal root of the first molar. The lesion boundaries were clearly delimited and measured to calculate the periapical area. The periapical bone loss area was similar among groups ($P>0.05$).

Discussion

The findings of this study support the hypothesis that DRW and W differentially modulate the inflammatory

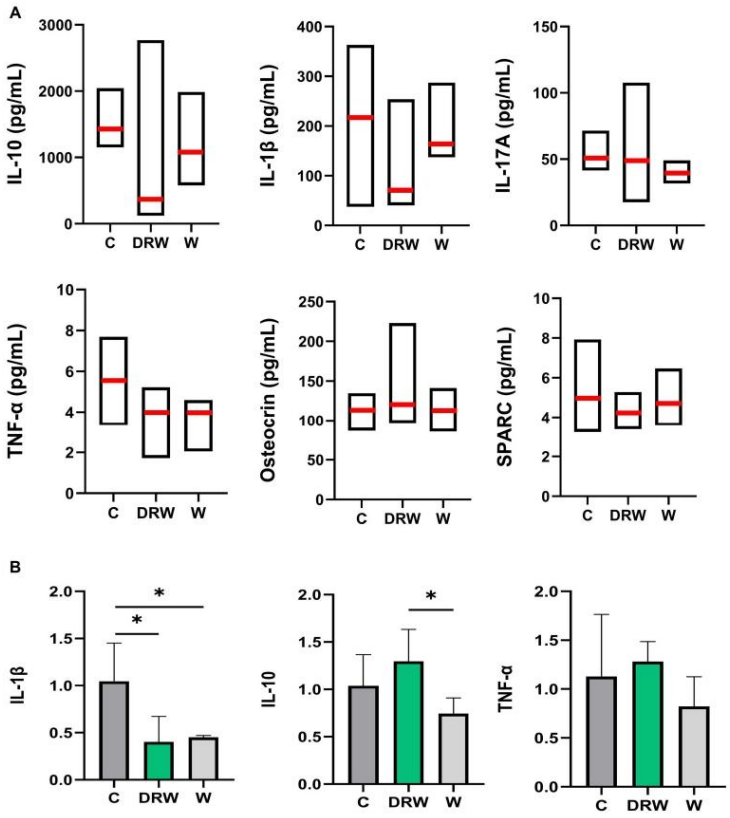

Figure 3. A, Median (red line), minimum, and maximum values for interleukin (IL)-10, IL-1β, IL-17A, tumor necrosis factor (TNF)-α, osteocrin, and secreted protein acidic and rich in cysteine (SPARC) in plasma samples for each group. Kruskal-Wallis test followed by Dunn's *post hoc* test was applied ($P>0.05$). **B**, Mean and standard deviation of relative gene expression ($2^{-\Delta\Delta Ct}$) from qRT-PCR analysis of apical periodontitis samples for IL-1β, IL-10, and TNF-α. *$P<0.05$; one-way ANOVA with Tukey *post hoc* test was applied.

profile by influencing key cytokines and immune cell responses. In co-cultures of MQ and PDLFs, DRW promoted a more anti-inflammatory profile than W, characterized by decreased TNF-α and IL-6, with increased IL-10 levels. *In vivo*, DRW intake led to a lower blood monocyte count and reduced IL-1β expression at the AP site, contributing to an anti-inflammatory environment.

After determining the optimal concentration, both DRW and W at 0.1% showed no cytotoxic effects on MQ and PDLFs. These cells play key roles in the development and progression of AP (26). MQ are innate immune cells that participate in inflammation by balancing the production of pro- and anti-inflammatory cytokines, such as IL-6, TNF-α, and IL-10, which in turn influence bone resorption and repair (27,28). PDLFs, resident cells of the periodontal ligament, contribute to tissue homeostasis by maintaining the extracellular matrix and regulating cytokine production through crosstalk with MQ, modulating the immune response (29,30). In addition, they influence bone remodeling by stimulating osteoclast precursors and regulating the expression of receptor activator of nuclear factor-κB ligand and osteoprotegerin, key mediators of osteoclastogenesis and bone turnover (31).

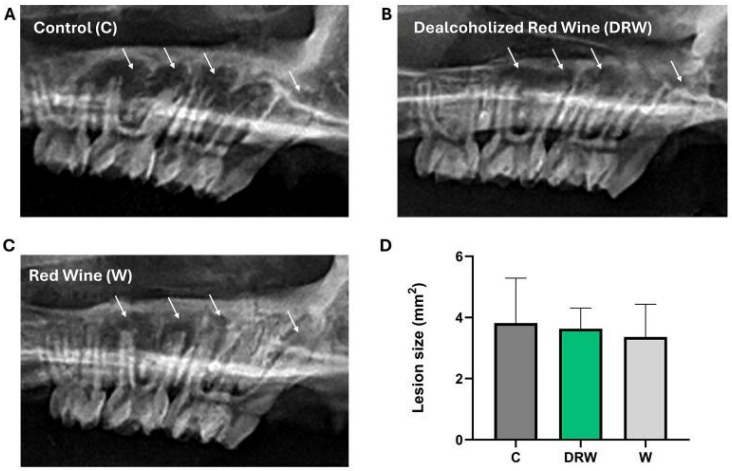

Figure 4. Representative radiographic images from the specimens in the **A,** Control; **B,** dealcoholized red wine (DRW); and **C,** red wine (W) groups. The white arrows indicate the periapical lesions in the first and second maxillary molars. **D,** The bar graph for lesion size shows the mean and standard deviation in mm^2 of each group. There were no significant differences among groups ($P > 0.05$). One-way ANOVA with Tukey *post hoc* test was applied.

Table 2. Area of apical periodontitis in each study group.

Group	Histometric analysis (mm^2)
C	2283.976 ± 310.672
DRW	2099.349 ± 671.320
W	2094.260 ± 563.611

Data are reported as means ± SD. $P > 0.05$, one-way ANOVA followed by Tukey's test for multiple comparisons. C: control; DRW: dealcoholized red wine; W: red wine.

The use of a direct co-culture system allowed to model cell-to-cell interactions occurring in the periapical environment. In this setting, DRW promoted an anti-inflammatory response by suppressing TNF-α and IL-6, while increasing IL-10 levels. Interestingly, IL-1β, TNF-α, IL-6, IL-10, and VEGF-A levels were increased in W compared to DRW.

The anti-inflammatory effects of DRW are likely mediated by polyphenols such as resveratrol, quercetin, and other flavonoids. These compounds modulate signaling pathways in MQ, including nuclear factor-kappaB (NF-κB) and p38 mitogen-activated protein kinase (p38 MAPK), which regulate cytokines such as IL-1β, TNF-α, and IL-6 (32). In lipopolysaccharide (LPS)-stimulated PDLFs, resveratrol suppresses TNF-α, IL-6, and IL-1β in a concentration- and time-dependent manner (33). Inhibition of TLR4-NF-κB/MAPK/IRF3 signaling by resveratrol has also been shown to reduce TNF-α and IL-6 while increasing IL-10 (34). Moreover, polyphenols modulate MAPK and oxidative stress pathways, contributing to the attenuation of pro-inflammatory cascades (35). Since VEGF is often induced by these cytokines (36), its upregulation may enhance vascular permeability and leukocyte infiltration. Differences in responses between DRW and W may be partly due to ethanol in W, which can promote pro-inflammatory signaling (15–17).

As a key mediator of periodontal inflammation, IL-1β promotes immune cell recruitment and bone resorption in AP (3). Therefore, its downregulation observed *in vivo* aligns with reduced circulating monocytes, that migrate to site of injury and differentiate into MQ, regulating both the initiation and maintenance of chronic inflammation (37). Additionally, the higher expression of IL-10 in DRW-treated animals compared to W reinforces its potential regulatory role. These results agree with previous studies where a combination of resveratrol with quercetin or DRW supplementation led to higher IL-10 and reduced IL-1β immunolabeling in developing and established AP models (12,14).

Despite the observed molecular changes, ELISA revealed no significant differences among groups in blood levels of IL-10, IL-1β, IL-17A, TNF-α, osteocrin, and SPARC. As previously reported, the inflammatory effects

in blood cytokine levels tend to be more evident in animals with multiple foci than those without AP or with a single lesion (6–8), potentially masking anti-inflammatory systemic effects of the tested supplementations. Furthermore, plasma cytokine levels likely reflect a cumulative, time-dependent systemic response, indicating that systemic changes in blood samples of rats with AP can be subtle, transient, and vary with disease progression (38).

Moreover, DRW supplementation did not result in statistical differences in lesion size/periapical bone loss among groups assessed through radiographic and histometric analyses. This may reflect the complexity of bone remodeling, which depends not only on local cytokine levels but also on systemic factors, mechanical influences, and lesion stage, and is tightly regulated by a network of mediators such as IL-1β, TNF-α, IL-6, IL-17, IL-10, and the RANKL/OPG axis (39,40). In the present model, the AP stage may have been too early to allow detectable changes in lesion size or bone loss, since the inflammatory process was still under development. Importantly, evaluating more advanced stages of AP could be key to uncovering the modulatory effects of DRW polyphenols on bone resorption, particularly since differences in lesion size have been documented after 60 days of disease progression (14). These findings suggest that while DRW polyphenols can influence the inflammatory environment, additional or more sustained interventions may be required to translate these cellular effects into reductions in bone pathology.

Some limitations of this study should be noted. *In vitro*, assessing MQ polarization markers and including bacterial stimuli could clarify cellular pathways and inflammatory responses. *In vivo*, evaluating mediators such as the RANKL/OPG ratio may explain why IL-1β suppression and IL-10 upregulation did not reduce bone loss. Additionally, early peaks in cytokine expression may have been diminished at the 45-day assessment (40). Finally, isolating specific polyphenols could identify the main active components, guiding targeted therapies.

Considering the recent nature of research on the anti-inflammatory effects of DRW in AP, and the complex interplay of local and systemic immune responses involved in the disease, these findings should be interpreted with caution and are not directly translatable to clinical settings. Nonetheless, this study offers valuable insights into the immunomodulatory potential of DRW in both *in vitro* and *in vivo* models, highlighting the need for further investigations to better elucidate its mechanisms of action.

In conclusion, DRW and W exerted distinct effects in both co-culture and *in vivo* AP models. DRW promoted a pronounced anti-inflammatory profile by reducing TNF-α and IL-6 while increasing IL-10 in co-cultures, and by lowering blood monocyte counts and decreasing IL-1β expression *in vivo*. However, these anti-inflammatory effects were not sufficient to alter bone disease progression within the experimental model evaluated.

Conflicts of Interest: The authors declare that there are no conflicts of interest related to this study.

Data availability statement: All data generated or analyzed during this study are included in this published article.

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