

Article

Efficacy of Oregano Essential Oil Extract in the Inhibition of Bacterial Lipopolysaccharide (LPS)-Induced Osteoclastogenesis Using RAW 264.7 Murine Macrophage Cell Line—An In-Vitro Study

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Abstract: Gram-negative, anaerobic bacteria are predominant in periapical infections. The bacterial lipopolysaccharide (LPS) initiates the process of inflammation and periapical bone resorption. Usage of various medicaments retards or inactivates the bacterial endotoxin (LPS). However, the results are not highly effective. In recent years, owing to antimicrobial resistance, the shift from conventional agents to herbal agents has been increased tremendously in research. Keeping this in mind, the present study was formulated to evaluate the efficacy of *oregano* essential oil in inhibiting bacterial LPS-induced osteoclastogenesis. Four different concentrations (0 ng/mL, 25 ng/mL, 50 ng/mL, and 100 ng/mL) of *oregano* essential oil extract were added into 96-well culture plate. Under light microscope, quantification of osteoclast cells was performed. One-way ANOVA with post-hoc Tukey test was carried out on SPSS v21. A significant reduction ($p < 0.001$) in the osteoclast was observed in the experimental groups compared to no *oregano* essential oil extract (control). A dose-dependent significant reduction ($p < 0.001$) in osteoclast formation was observed among the experimental groups, with lesser osteoclast seen in group IV with 100 ng/mL of *oregano* essential oil extract. Thus, it can be concluded that *oregano* essential oil extract can be utilized as a therapeutic agent that can target bacterial LPS-induced osteoclastogenesis. However, randomized controlled studies should be conducted to assess the potential use of this extract in the periapical bone resorption of endodontic origin.

Keywords: lipopolysaccharide; monoterpene; *Oregano* essential oil; osteoclastogenesis; bone resorption; periapical infection; *Porphyromonas endodontalis*; carvacrol; RAW 264.7 macrophages; natural products

1. Introduction

In endodontic practice, physiological or pathological resorption of the periapical bone has always been a matter of significant concern [1]. In permanent dentition, pathological periapical bone resorption is usually associated with conditions such as traumatic injuries of the tooth, orthodontic treatment, and periapical inflammatory lesions [2]. Apical periodontitis is an inflammatory condition that results from the chronic insults of oral fluids laden with microorganisms reaching the apical region of the infected root canals [3]. Endodontic infection is primarily a biofilm-associated disease, and the association of microorganisms with the biofilm is an important etiological factor in dental caries, periodontal disease, and endodontic infection [4,5]. Predominantly, gram-negative bacteria such as *Enterococcus faecalis*, *Prevotella*, and *Porphyromonas* are associated with endodontic infections. They release lipopolysaccharide (LPS) to stimulate cytokine secretion responsible for osteoclast activation, thus eventually causing bone resorption [6,7].

Periapical bone resorption is primarily carried out by motile, multinucleated giant cells known as osteoclast. They are formed by the fusion of mononuclear precursor cells derived from the spleen and bone marrow, unlike osteoblast, which is derived from the skeletal muscle [8]. At the site of injury during bone remodeling, the pro-inflammatory cytokines result in active recruitment of osteoclast. However, the initiation of bone resorption primarily requires the attachment of osteoclast to the bone surfaces by the formation of podosomes. These surfaces mainly consist of actin and $\alpha\text{v}\beta\text{3}$ integrin, which interact with bone matrix proteins to form a sealing zone on the bone material. This interaction complex secretes hydrochloric acid via the V-type ATPase pathway present on the ruffled membrane of the osteoclast to create an acidic environment conducive for bone resorption [9,10]. Following the binding of receptor activator of nuclear factor kappa-B ligand (RANKL) to its receptor on osteoclast precursors, trimerization and downstream activation of signaling pathways such as I κ B-a/b kinase (IKK), nuclear factor kappa-B (NF- κ B), mitogen-activated protein kinases (MAPKs)—p38, and extracellular-signal-regulated kinase (ERK) occur [11]. This leads to the activation of an inflammatory process where the bone hemostasis mechanism is wholly disrupted, leading to an initiation of a distinct intrinsic signaling cascade for osteoclast activation [12].

In endodontics, during an event of an injury, the odontoblastic and pre-dentin layers tend to get damaged and thus allow for the inculcation of osteoclast into the area. Over some time, this leads to the loss of dental hard tissue structures [13]. In the event of over activation of osteoclast cells, there can be an imbalance in the bone remodeling process, leading to excessive resorption of periapical bone [13,14]. When periapical bone resorption is left untreated, it can lead to perforation and later fracture the tooth. Therefore, either elimination or reducing the osteoclast activity is of paramount concern.

In current medical practice, oral bisphosphonates have been used to retard the osteoblastic mediated bone loss in various conditions such as post-menopausal women, steroid-induced osteoporosis, and tumors metastasizing to the bone [15,16]. However, their prolonged application has reported adverse effects such as bisphosphonate-induced osteonecrosis of the jaw (BRONJ) [17]. This undesired outcome is known to occur even in individuals without co-morbidities, often requiring surgical management for treatment [18]. Despite consequential side effects, they are known for their retarding effect against bone resorption. Hence, there is a paradigm shift in dispensing bisphosphonates as a local drug to bypass the adverse effects resulting from the oral administration of the drug [13]. Most of the literature on the usage of bisphosphonates as local drug delivery is restricted to periodontics for controlling or treating periodontal defects in patients with chronic periodontitis. Studies have reported the effectiveness of 1% alendronate gel in patients with periodontitis [19–22]. Conversely, in a recent systematic review, systematic bisphosphonate usage in patients undergoing orthodontic treatment showed compromised results, with prolonged treatment time and moderate changes around the root [23]. However, according to our best literature search, the usage of bisphosphonates on controlling endodontic lesions of periapical origin is not available.

About the adverse effects of generic drugs, an upsurge in traditional medications has been observed. Various natural essential oils or gels such as eucalyptus oil, peppermint oil, cinnamon oil, tea tree oil, neem gel, and lavender oil are known for their medicinal property [24,25]. Numerous active compounds of medicinal plants such as flavonoids, terpenoids, glycosides, lignans, coumarins, alkaloids, polyphenols, limonoids, and quinones effectively suppress osteoclastogenesis and bone resorption [26], although the specific mechanism of their inhibitory effects on osteoclast is not entirely understood.

A recent study using macrophage lineage RAW 264.7 has indicated the involvement of intricate signaling pathways [27]. Few in-vitro cell line-based studies have proved the efficiency of various natural agents, including citrus fruits, propolis, and green tea, in controlling osteoclastogenesis and bone resorption [26]. *Oreganum vulgare* (Family-Lamiaceae-, Genera- *Oreganum*-, Species—*Vulgare*) is a natural herb that grows in different regions of Europe and Asia India; it is found in the region of the Himalayas. In the present study, *oreganum* fresh leaves were collected manually from the southern part of Tamil Nadu, India. *Carvacrol* (5-Isopropyl-2-methylphenol), a monoterpene phenol, is a primary compound found in oregano essential oil. Based on the previously published reports, it possesses anti-inflammatory, bactericidal, and antioxidant properties [28]. The principal compound in the oregano essential oil exhibits suitable antibacterial properties against *Enterococcus faecalis* and therefore can likely be used in endodontic infections [29]. In endodontics, the underlying molecular mechanism of oregano essential oil extract, a novel anti-osteoclastogenic compound, has not been studied yet.

The prepared oregano essential oil extract was exposed to LPS to evaluate the anti-osteoclastogenic activity. The research question we intended to answer in the present study was “Is there any effect of oregano essential oil extract on retarding the bone resorption of endodontic lesions in the periapical area?”

The present study aimed to assess the anti-osteoclastogenic activity of oregano essential oil extract in bone resorption induced by endodontic infections. The design of the study was constructed at the in-vitro molecular level. RANKL-treated RAW264.7 macrophages were exposed to LPS-induced *Porphyromonas endodontalis* for TRAP staining to quantify the osteoclastic cells.

2. Materials and Methods

2.1. Preparation of Oregano Essential Oil Extract

In the present study, *oreganum* fresh leaves were collected manually from the southern part of Tamil Nadu, India. The hot air oven was operated at 60 °C for drying the leaves till a constant weight was obtained. To extract oil, leaves were placed in a Soxhlet extractor for 8 h with petroleum ether as solvent [28]. Oregano essential oil was separated from petroleum ether using a rotary evaporator. To identify the individual compounds, gas chromatography and mass spectroscopy were performed (Table 1). For preparing 10 mg/mL of oregano essential oil as a stock solution, 10% dimethyl sulfoxide (DMSO) was added as a vehicle, and the solution was frozen as aliquots in −80 °C. At the beginning of the experiment, the stock solutions were diluted to working concentrations of 0, 25, 50, and 100 ng/mL in a culture medium.

Table 1. Chemical constituent.

S.No.	Compound Name	Retention Time	Peak Area %
1	3-thujene	4.9	0.4
2	α-pinene	7.6	0.6
3	β-Pinene	10.15	0.5
4	α-Terpinene	10.47	1.19

Table 1. Cont.

S.No.	Compound Name	Retention Time	Peak Area %
5	p-cymene	12.04	9.47
6	γ -terpinene	12.8	12.68
7	β -linalool	14.035	0.67
8	Borneol	14.767	0.4
9	Terpinen-4-ol	15.94	0.41
10	Carvacrol	20.672	41.2
11	β -caryophyllene	21.84	0.83
12	β -bisabolene	22.47	0.601
13	myristicin	22.573	0.25
14	spathulenol	25.28	0.4
15	apiol	27.16	0.14

2.2. Study and Sample Characteristics

An in-vitro study was planned after receiving ethical approval from the local ethics committee of the institute. Four study groups were created based on the *oreganum* extract concentration added into the culture plates: Group II with 25 ng/mL, Group III containing 50 ng/mL, and Group IV having 100 ng/mL. The culture plate with DMSO alone (0 ng/mL of oregano essential oil extract) was treated as the control group (Group I). There are four study groups, and we took 96 wells plates per study group. In each plate, three wells were considered as a test run (control). Therefore, we get a sample of 93 wells per group ($96 - 3 = 93$), and hence the sample size in the present study was 372 ($93 \times 4 = 372$).

2.3. Preparation of Culture Medium

Human RANKL was purchased from Insight Biotechnology (Middlesex, UK). RAW264.7 murine macrophages were purchased as American Type Culture Collection (ATCC[®] TIB-71[™], Gaithersburg, MD, USA) and from Sigma-Aldrich (cat. no. 91062702, Gaithersburg, MD, USA). RANKL-treated RAW264.7 macrophages were differentiated into osteoclasts for 5 days [30]. The cells were maintained in Dulbecco's modified eagle's medium (DMEM) with 10% fetal bovine serum (FBS) (Grand Island- GIBCO, NY and Amersham, Little Chalfont, Buckinghamshire, UK). RANKL was used (15 ng/mL) to activate cells. The cell culture media and factors were replenished every third day, and differentiation was stopped on the fifth day (31). The cells were incubated in a humidified atmosphere at 37 °C with 5% CO₂. Lastly, the RAW264.7 cell line was cultured in DMEM supplemented with 10% FBS, 100 U/mL penicillin, 100 µg/mL streptomycin, and 2 mmol/l L-glutamine.

2.4. Staining Procedure

RAW264.7 macrophages (5×10^3 /well) were exposed to 200 ng/mL of LPS derived from *Porphyromonas endodontalis* (American type culture collection 25611, Manassas, Virginia, USA) and were differentiated for seven days [31].

2.5. Quantification of Osteoclast

The cells were plated in 96-well plates for Tartrate-resistant acid phosphatase (TRAP) staining (Sigma, St Louis, MO, USA). Positively stained cells were counted in 5 randomly chosen microscopic fields, and later an average was considered to measure the osteoclast formation. Multinucleated TRAP-positive cells with three or more nuclei were considered osteoclasts when viewed under a 2.5 \times magnification using a light microscope [32]. TRAP staining showing predominantly numerous plump eosinophilic cells, and dispersed, multiple basophilic nuclei is suggestive of osteoclast. The cells that were smaller in size with fewer nuclei were suggestive of immature osteoclast.

2.6. Statistical Analysis

A power analysis was carried out using G power software version 3.10 (Heinrich Heine University, Düsseldorf, Germany) [33] for the current study. Data was gathered and presented in mean with standard deviation. Intergroup comparative analysis was carried out with one-way ANOVA followed Post-hoc Tukey analysis using IBM SPSS (Version 21, IBM, Armonk, NY, USA) (statistical package of social sciences) predictive analytics community, Armonk, New York software v. 23.

3. Results

A power of 0.98 was achieved for the study with four groups, an effect size of 0.25, and α of 0.05. RANKL-treated RAW264.7 cells was induced to undergo osteoclastogenic differentiation by $1\mu\text{g}/\text{mL}$ LPS in the presence or absence of oregano essential oil at different concentrations (0, 25, 50, and $100\text{ ng}/\text{mL}$) (Figure 1A–D).

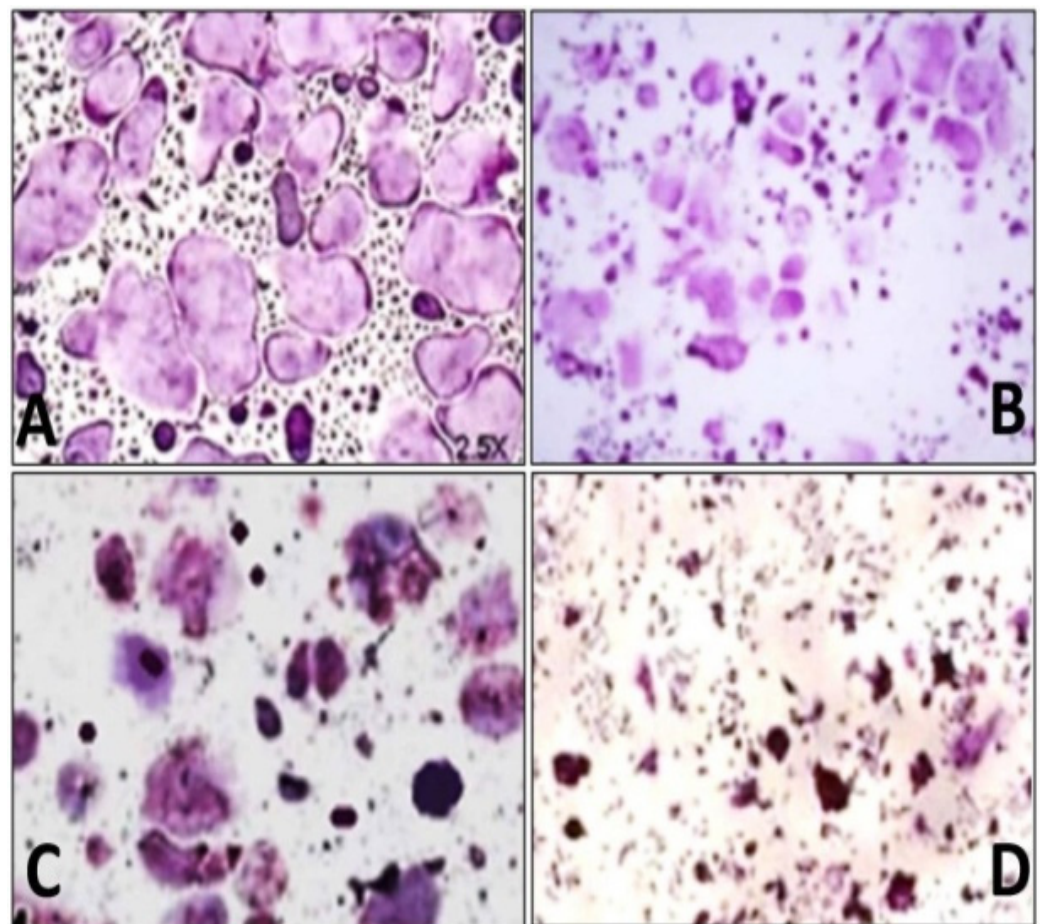


Figure 1. Microscopic image ($2.5\times$) depicting the TRAP staining at different concentrations of oregano essential oil extract: (A) Image shows TRAP staining in control (group I) with numerous plump eosinophilic cells and multiple basophilic nuclei dispersed predominantly along the periphery of the cell, suggestive of osteoclastic differentiation; (B) image shows TRAP staining in group II ($25\text{ ng}/\text{mL}$) with scattered eosinophilic cells and a few basophilic nuclei rimming the periphery of the cells. The cells are smaller with fewer nuclei suggestive of immature osteoclasts; (C) image shows TRAP staining in group III ($50\text{ ng}/\text{mL}$) with very few cells with eosinophilic to deeply basophilic cells cytoplasm and condensed nuclei. Cell lysis is evident in a few areas, suggestive of cells undergoing necrosis; (D) image shows TRAP staining in group IV ($100\text{ ng}/\text{mL}$) predominantly composed of cellular fragments—enucleate, amorphous, eosinophilic cells with ill-defined cellular outline along with cellular debris with no clear cell outline discernible, suggestive of necrotic debris.

At 2.5× magnification under light microscope TRAP staining, different concentration of oregano essential oil extract was visualized. On histological examination, numerous eosinophilic cells with multiple basophilic nuclei were identified as osteoclast. Compared to the control group, a significantly ($p < 0.001$) lower number of TRAP-positive cells were reported in oregano essential oil-treated groups of different concentrations (25 ng/mL, 50 ng/mL, 100 ng/mL). The TRAP positive cells were observed more with 25 ng/mL of oregano essential oil extract (8.0 ± 0.3). The numbers of osteoclast cells were significantly ($p < 0.05$) decreased with the increasing concentration of oregano extract oil (Table 2). At 100 ng/mL, significant reduction of osteoclast cells was evident. Histological image shows necrotic debris when 100 ng/mL of oregano-essential-treated groups was exposed to RANKL-treated RAW 264.7 cells exposed to LPS.

Table 2. Post-Hoc analysis of osteoclastogenesis.

Control Group (without Oregano Essential Oil)	Experimental Group (Different Concentration of Oregano Essential Oil)		
	25 ng/mL	50 ng/mL	100 ng/ml
12.0 ± 0.4 b***c***d***	8.0 ± 0.3 a***c***d***	5.0 ± 0.3 a***b***d***	1.0 ± 0.2 a***b***c***

Note: Results expressed in mean ± SD; *** p value < 0.001; SD—standard deviation; a = compared to control group; b = compared to 25; c = compared to 50; and d = compared to 100.

4. Discussion

In recent years, due to the possible adverse effects of conventional treatment and also due to increased incidence of antimicrobial resistance, the focus has switched from current practice to alternative medicine. In endodontics, over the last decade research has been concentrated towards the use of herbal extracts [34]. Recently, evidence has shown natural agents to be an effective therapeutic option for retarding osteoclastogenesis. For instance, an anthrocylic glycoside aloin derived from *Aleo vera* has been shown as an effective alternative treatment for retarding bone resorption by inhibiting micro ribonucleic acid-21 (miRNA-21) via the NFκB signaling pathway, which is shown to be a primary molecular event in RANKL-RANK signaling [35]. To the best of our knowledge, there are no previous reports on the usage of oregano essential oil extract as an anti-osteoclastogenic agent on aberrant osteoclastogenesis induced by bacterial-LPS. Thus, the current in vitro study at the cellular level was performed as first of its kind to report an osteoclastic inhibitory effect of *Oreganum vulgare*, a plant-based extract.

Macrophages are present in chronic inflammation and exhibit three significant functions: antigen presentation, phagocytosis, and immunomodulation [36]. During inflammation upon activation, they initiate inflammatory cascade through pro-inflammatory cytokines such as IL (Interleukin)-8, TNF (Tumor Necrosis Factor)-α, and IL (Interleukin)-1b [37]. These cytokines influence the production of collagenases, cellular adhesion, and bone adhesion properties and play a role in the recruitment and activation of osteoclast [37]. In addition to the cytokine-mediated pathway, RANKL plays a crucial role in producing giant osteoclasts by activating different signaling pathways such as TGF-β, and TAK1, involving signaling complexes containing RANK, TRAF6, and RAW 264.7 cells [38]. In order to simulate the above-mentioned molecular chain of events, the RANKL-treated RAW 264.7 murine macrophage cell line was used in the present study, which is in corroboration with the previous study [39].

Prior research has reported that the activation of macrophage, release of inflammatory cytokine, and subsequent osteoclast formation are initially triggered by LPS of *Porphyromonas endodontalis*. An oxygen-sensitive microbe is usually seen in infected root canals with acute periapical infections [40]. It is identified as gram-negative anaerobic rods and is described as a black pigmented bacterium (BPB). However, it is a *Porphyromonas* species, but it is seen exclusively in endodontic infections as it lacks adhesins on its surface necessary for adhesions in other regions [41]. Previous authors have reported the high incidence

of *P. endodontalis* in infected root canals, and other members of BPB such as *P. gingivalis* and *Prevotella intermedia* [42] reported the presence of BPB in 76% of the cases, with *P. endodontalis* being the most commonly witnessed species.

Additionally, these species were found to be most commonly associated with sinus tract and abscess formation. On the contrary, the incidence of *P. endodontalis* was reported to be 23% of *P. endodontalis* in the Brazilian population [43]. Furthermore, studies have also evaluated the direct effects of LPS on osteoclast [44,45]. Additionally, Dahlen et al. [46] analyzed monkeys (*Macaca fascicularis*) and root canals after bacterial LPS. The study revealed the inflammatory reactions in the periapical tissues with signs of bone resorption after 3 and 7 months. Reports also revealed the direct action of LPS in stimulating the osteoclast, increasing the expression of RANKL and eventually resulting in the induction of osteoclastogenesis [47]. Thus, in the present study, considering the preponderance of *Porphyromonas endodontalis* in endodontic infections and their potential of inducing bone resorption [44] in periapical lesions, LPS of *P. endodontalis* was used as a model to investigate bacteria-induced osteoclastogenesis.

In endodontics, calcium hydroxide $\text{Ca}(\text{OH})_2$ is a common biomaterial used. It was initially introduced as a pulp capping agent and later found suitable for other treatment procedures. Predominantly, calcium hydroxide has been widely used as intracanal medicament for routine endodontic procedures due to its antimicrobial property. In addition to its antimicrobial property, it has been shown to inhibit osteoclastogenesis due to the hydroxyl group providing an alkaline environment that allows for repair and calcification. This environment effectively acts on the osteoclast by inhibiting lactic acid secretions and activating alkaline phosphatase, which is crucial in hard tissue formation [48]. In cases of periapical infection with large periapical lesion, $\text{Ca}(\text{OH})_2$ acts as an intra appointment intracanal medicament. Several studies have explored the use of calcium hydroxide [$\text{Ca}(\text{OH})_2$] for inhibiting LPS- induced resorptive process during root canal treatment and found a significant reduction in the osteoclast differentiation [3,49,50]. The highly toxic lipid A molecule responsible for the damaging effects of endotoxin was seen to be hydrolyzed by $\text{Ca}(\text{OH})_2$ [51]. However, several other studies have reported on the cytotoxic nature of $\text{Ca}(\text{OH})_2$ while being used as an intracanal medicament [52–54]. Due to its strong alkaline properties, it affects the dentinal matrix structure, in the long run causing dissolution of the collagen and hydroxyapatite crystals, leading to fracture of the tooth. Due to these reasons, long-term use of $\text{Ca}(\text{OH})_2$ is not advocated.

For the reasons mentioned above, it is imperative to explore an alternative agent for inhibiting osteoclastogenesis. The usage of natural agents such as *aloe vera*, cinnamon, and garlic has been explored in endodontics due to their medicinal and biocompatible properties [55]. According to the earlier reports, curcumin, a natural polyphenol compound, reduces osteoclastic formation and maturation by acting on the RANKL site [56]. It was also shown to inhibit c-fos and c-jun (AP-1) expression, responsible for the differentiation into osteoclasts [57].

The present study used *Oreganum vulgare*, which has shown similar antioxidant and antimicrobial properties to curcumin due to the presence of common compounds such as carvacrol and thymol. The effect of carvacrol on the development of osteoclasts was investigated. Carvacrol effectively reduced the production of TRAP+ osteoclasts produced by RANKL and LPS without being cytotoxic. This could be one of the mechanisms responsible for the reduction of osteoclastogenesis. It has been reported to have anti-inflammatory properties as it can reduce pro-inflammatory cytokines such as $\text{TNF-}\alpha$, IL-6, and IL-1 β in a dose-dependent manner [58]. *Oreganum* essential oil has also been shown to have good antimicrobial, antifungal, bactericidal, and antiviral activity. Based on the previous study, results of gas chromatography and mass spectroscopy revealed various available compounds in oregano extract, in which carvacrol has been expressed in higher amounts and thus considered as the principal component responsible for its known bioactivity [28,59]. Earlier reports have shown the potential of carvacrol in inhibiting osteoclastogenesis by activating and downregulating various molecular levels pathways. Hence, the present study

was conducted to investigate the effects of different concentrations of *Oreganum* essential oil on RANKL-treated murine macrophage RAW 264.7 cell line exposed to LPS-induced *Porphyromonas endodontalis* for TRAP staining to explore osteoclastogenesis.

The current in-vitro experimental setup revealed a significant reduction in osteoclastogenesis in the presence of oregano essential oil extract. This could be mainly due to the carvacrol compound, a monoterpene, and the principal constituent in oregano essential oil. Monoterpene has been shown to interfere with isoprenoid synthesis [60], which is essential for cellular functions [61] and osteoclast formation [62]. In addition, it is shown to inhibit different signaling pathways necessary for osteoclastogenesis. The primary mechanism of bone resorption is the interplay within the RANK–RANKL mechanism, in which osteoclastic pathways such as MAPKs, Akt, and NF- κ B are activated. In these pathways, NF- κ B is of significant interest as it induces and separates inhibitor I κ B from further entering the nucleus, thus regulating the activation of NFATc1 transcription factor responsible for osteoclastic precursor cell differentiation [56]. The previous study showed that monoterpenes inhibit bone resorption in the bone marrow and spleen cells [63]. Sapkota et al. investigated the effect of thymol on RANKL-induced osteoclastogenesis in murine macrophages, and the result showed that thymol inhibits RANKL-induced TRAP-positive multinucleated cells and LPS-stimulated inflammatory bone loss [64]. Hence, it can be suggested that monoterpene facilitates retarding osteoclastogenesis. However, long-term clinical studies should be conducted to substantiate the current observations.

In the present study, the reduction in osteoclast formation was significantly reduced with the increasing concentration of oregano essential oil extract. The least osteoclastic cells were formed in group IV (100 ng/mL), whereas the maximum was found in the control group, which was not treated with oregano essential oil extract. Our results follow [31], which showed dose-dependent downregulation of RANKL-induced NF- κ B activation, thereby inhibiting osteoclastogenesis. Regarding cytotoxicity of the *oreganum* extract, our recently published article on the rat model showed 250 ng/mL concentration to be mild cytotoxic [54]. Additionally, a previous study showed that the concentration of 1.25–20 μ g/mL reported no cytotoxicity in corroboration with the present study [65]. Hence, in the present study, we adjusted the concentrations to an optimal level of 100 ng/mL, which was potentially not cytotoxic and, at the same time, was in the therapeutic range.

The authors of this study also keep a standpoint based on our unpublished cell line study report; oregano essential oil extract possesses the most negligible cytotoxic effect on L929 fibroblast cells, showing its biocompatible nature. Based on the observations, it can be suggested that oregano essential oil could be used as a potential intracanal medicament to reduce periapical bone resorption.

5. Strength, Limitations, and Future Directions

Herbal agents are generally shown to have significant advantages compared to synthetic agents, which can cause long-standing complications with their chronic usage. [66]. A preliminary cell line study evaluating the effect of *oregano* essential oil extract on osteoclastogenesis showed an encouraging response. Considering the in-vitro study design of the present research, the results need to be cautiously adopted for future clinical studies. Further experimental research should be carried out to explore the extract's chemical compounds and exact nature to delineate its effectiveness in bone resorption of periapical origin and other possible therapeutic uses. Additionally, clinical trials on human subjects should be conducted to affirm the efficiency of oregano essential oil extract to inhibit osteoclastogenesis or as an anti-bone resorptive agent. Further research has to be focused on the effect of oregano essential oil on cell signaling and cytokines.

The other interesting aspect that needs to be concentrated on in future studies is dispensing the extract as a local drug targeting the lesion, especially in the case of endodontic infections that usually remain localized.

6. Conclusions

A significant reduction of LPS-induced osteoclastic cells was evident when 100 ng/ml of oregano essential oil extract was used. The results of the study infer that, as the concentration of the oregano essential oil increases, reduction of osteoclastic cells occurs. Therefore, it can be concluded that oregano essential oil extract can be utilized as a therapeutic agent targeting LPS-induced osteoclastogenesis. Hence, these preliminary, in vitro study results can be a foundation for conducting randomized controlled trials on the retardation of periapical bone resorption in endodontic infections.

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Informed Consent Statement: Not applicable.

Data Availability Statement: The data set used in the current study will be made available on reasonable request.

Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

LPS	Lipopolysaccharide
NF- κ B	nuclear factor kappa-B
MAPKs	mitogen-activated protein kinases
ERK	extracellular-signal-regulated kinase
BRONJ	bisphosphonate-induced osteonecrosis of the jaw
ATCC	American Type Culture Collection
DMEM	Dulbecco's modified eagle's medium
FBS	Fetal Bovine Serum
TRAP	Tartrate-resistant acid phosphatase
ANOVA	One-way Analysis of Variance
SPSS	statistical package of social sciences
miRNA-21	micro ribonucleic acid-21
RANK	Receptor Activator of Nuclear Factor- κ B-
RANKL	Receptor Activator of Nuclear Factor- κ B-Ligand
TNF	Tumour necrosis factor IL- Interleukin
TGF- β	Transforming growth factor
TAK1	Transforming growth factor beta-activated kinase 1
Ca(OH) ₂	Calcium Hydroxide
MAPKs	Mitogen Activated Protein Kinases
Akt	Protein kinase B
I κ B	inhibitor of nuclear factor kappa B
NFATc1	Nuclear Factor of Activated T cells 1

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