Periodontal disease has multifactorial etiology. The immune response to the microbial challenge leads to osteoclast activation and resorption of the alveolar bone, resulting in tooth loss. Compounds isolated from Ocimum americanum and Ocimum basilicum were analyzed for apoptotic activity towards osteoclast in this study. Steam distillation was used for the extraction of essential oils (EOs) from dry leaves and flowers. The assessment of apoptosis in osteoclasts was carried out through the TUNEL assay and immunocytochemistry for the Fas receptor. The chemical profile of EOs, characterized through gas chromatography-mass spectrometry analysis, revealed methyl cinnamate (O. americanum), linalool, caryophyllene, 1,8-cineole (O. basilicum) as major components. The results showed that essential oils were not able to induce apoptosis in osteoclast; however, linalool (50 to 300 μg.ml⁻¹) induced 75% of apoptotic osteoclasts at non-toxic concentrations and the apoptotic activity was confirmed by the increasing levels of Fas receptor on osteoclasts treated with this compound. This study suggests that linalool could be used to control osteoclast activity.

**Key words:** Essential oil, linalool, Ocimum, osteoclast, periodontal disease.

**INTRODUCTION**

Periodontal disease is considered a common pathological condition in oral cavity, with possible systemic repercussions, particularly in adult individuals. They are of a multifactorial nature, including a broad spectrum of inflammatory and destructive responses to dental biofilm and bacterial components in a susceptible host. Proinflammatory mediators (IL-1, PGE₂, TNFα) produced by T lymphocytes present in the periodontal pocket promote osteoclast differentiation and activation, culminating in alveolar bone resorption and tooth loss (Ishikawa, 2007).

Osteoclasts (OCs) are specialized motile bone resorptive cells, derived from haematopoietic stem cells and they are the effector cells of alveolar bone loss in periodontal disease. In response to key factors, such as vitamin D₃, parathyroid hormone (PTH) and proinflammatory cytokines (IL-1, IL-6, TNFα, PGE₂, IL-17)
osteoblasts express receptor activator of nuclear factor κB ligand (RANKL), which in combination with CSF-1/M-CSF stimulates osteoclast development from peripheral blood cell precursors by binding to its receptor. Osteoprotegerin (OPG) is a decoy RANKL receptor that prevents RANKL-RANK interaction to inhibit osteoclastogenesis (Emery et al., 1998; Nakashima et al., 2011; Balvers et al., 2015).

Periodontal treatment consists of two sessions at one-week intervals of supragingival scaling and oral hygiene instructions, followed by subgingival scaling and root planing under local anesthesia performed within a period of 14 days, with or without use of antibiotics; however, systemic antibiotics do not act on osteoclasts directly to prevent or suppress bone resorption (Eguchi et al., 2008). Recently, many plants and their extracts have been recognized as useful sources for the prevention and treatment of bone-related disorders, including periodontal disease. In this context, the influence of Stewartia koreana extract on differentiation of osteoclasts was evaluated by Park et al. (2012). According to these authors, the extract (20 μg.mL⁻¹) of this plant was able to inhibit the differentiation of osteoclasts.

The activity of magnolol present in Magnolia officinalis was described by Lu et al. (2015). The administration of magnolol in mice with induced periodontal disease, was able to inhibit alveolar bone resorption and the number of osteoclasts on the bone surface, decreased expression of RANKL, MMP-1, MMP-9, NFKB and showed antimicrobial activity against Porphyromonas gingivalis, Aggregatibacter actinomycetemcomitans and inhibited differentiation of RAW 264.7 macrophages into osteoclasts.

In this study, the in vitro apoptotic activity of essential oils obtained from *O. americanum* and *O. basilicum* was assessed towards osteoclasts. The genus Ocimum, family Lamiaceae, comprises more than 64 herbaceous and subshrub species. This genus exhibits great diversity of species, popularly known in Brazil as “alfavacas” and “manjerícios” (Blank et al., 2002; Lorenzi and Matos, 2008) and are found in tropical and subtropical regions (Paton, 1992). Due to their economic importance, the most cultivated species in the world are Ocimum citriderorum Vis., Ocimum americanum L., Ocimum basilicum L., O. gratissimum L., Ocimum minimo L., and O. tenuiflorum L. (Carović-Stanko et al., 2010).

*Ocimum* species have several biological properties, including anti-inflammatory, antileishmanial (Rabelo et al., 2003; Ueda-Nakamura et al., 2006), antibacterial and antifungal activities (Suppakul et al., 2003) and could be a promising medicinal species to control osteoclast activity.

**MATERIALS AND METHODS**

**Study species**

*O. americanum* L. of the Lamiaceae family originating from Asia and Africa has adapted to the tropical Americas, where it is found spontaneously germinating. Propagation can be done by cuttings or seeds and can be planted all year round and has adapted well to Brazilian climatic conditions. Unlike other species of basil, it is not frequently used for culinary purposes and is more often applied as a medicinal plant (Vieira et al., 2003) to treat asthma, fever, coughs, colds, bronchitis and indigestion (Agra et al., 2008; Chowdhury et al., 2008). The essential oils and extracts of this species present several biological activities, such as antioxidant (Hakkim et al., 2008), repellent (Seyoum et al., 2002), insecticide (Shadidja et al., 2007) and antibacterial against *Propionibacterium acnes* (Vlyoch et al., 2006).

*Ocimum basilicum* L. is an annual aromatic subshrub. The plant is very branched, ranging from 30 to 50 cm, aromatic underbrush, annual or perennial, depending on growing region. Propagation can be done by cuttings or seeds and can be planted all year round. It has simple leaves, membranous, opposite in shape and vary in size depending on the species, wavy edges and protruding ribs, from 4 to 7 cm long, its inflorescence is the espiciporium summit type, and its white flowers, pink or purplish, fruit achene type with small seeds, black and oblong (Couto, 2006; Lorenzi and Matos, 2008) and has adapted well to Brazilian climatic conditions and can be grown year round.

In traditional Indian medicine, *O. basilicum* is used as a sedative and for asthma and diabetes, as well as for cosmetic purposes (Lin and Kan, 1990). The Uyghurs use the species as a cardiotonic and antidiarrheal, and to relieve abdominal pain (Upur et al., 2004). *O. basilicum* is used by the pharmaceutical industry because of its spasmylocatic, carminative, hepatoprotective and diuretic properties (Baritaux et al., 1992).

Scientific studies have shown that *O. basilicum* has antioxidant, antimicrobial, antifungal (Bozin et al., 2006), anticancer (Manosroi et al., 2006) and hypoglycemic activity (Vats et al., 2002). It also has the capacity to reduce platelet aggregation and thrombi in mice (Tothi et al., 2006).

**Plant samples**

Plants were cultivated from March to June 2010, in the Medicinal Plants Garden at the Federal University of Reconquista of Bahia, Santo Antônio de Jesus, Bahia, Brazil, Latitude 12° 57′ 59.2″, Longitude 039° 15′ 49.4″. The herbization treatment was carried out according to Mori et al. (1989). The botanical material collected was deposited in the Herbarium of the State University of Feira de Santana, and it was identified by the taxonomist Teonildes Sacramento Nunes as *O. americanum* L. (Lamiaceae) - HUEFS 167947 and *O. basilicum* L.(Lamiaceae)- HUEFS 167950, according to the Cronquist system (1981).

**Extraction of essential oils**

Steam distillation was performed using a Clevenger-type apparatus for the extraction of essential oils from dry leaves and flowers. The chemical composition of the essential oil was determined by gas chromatography-mass spectrometry (GC/MS) in a Shimadzu GC-2010 gas chromatograph coupled to a GC/MS-QP 2010 Shimadzu mass spectrometer. The extraction process was carried out for 3 h and the components were identified by comparing the obtained mass spectra with the library of the equipment used, and by comparing the calculated Kovats indices with those found in the literature (Adams, 1995) using a homologous series of hydrocarbons. Linalool, E-methyl cinnamate and Caryophyllene were purchased from Sigma-Aldrich Chemical Co., (St. Louis, MO).

Yield of the essential oil (%) was calculated based on fresh and dry biomass in the moisture free basis (MFB) (Santos et al., 2004) and the result was expressed in percentage.
Cell culture

Human primary osteoclasts (OCs) were obtained as described by Matsuzaki et al. (1999), with minor modifications. Briefly, peripheral blood (PB) was collected from healthy volunteers after informed consent was obtained. PB mononuclear cells (PBMC) were obtained from diluted peripheral blood (1:2 in Hanks solution), separated by Histopaque®-1077 (Sigma, St. Louis, MO, USA) and subsequently grown in DMEM High glucose (Euroclone SpA, Milan, Italy), in the presence of M-CSF (25 ng.ml⁻¹), RANKL (30 ng.ml⁻¹) at 37°C in a 5% CO₂ atmosphere for 14 days. To evaluate osteoclastogenesis, TRAP staining was carried out with Acid Phosphatase Leukocyte (TRAP) Kit no. 386 (Sigma, St. Louis, MO, USA), according to the manufacturer's protocol.

Cytotoxicity assay

Mature OCs were plated in 96-well plates and incubated for 3 days in the presence of essential oils (5, 50, and 500 µg.ml⁻¹), and the purified compounds linalool, methyl cinnamate and caryophyllene (Sigma, St. Louis, MO, USA), at 5, 50 and 300 µg.ml⁻¹. A 3% methanol/DMSO solution was used as a negative control, and to solubilize the compounds. Determinations of viable cells were performed after colorimetric assay with MTT (thiazolyl blue). The assay, based on the conversion of the yellow tetrazolium salt MTT (Sigma, St. Louis, MO, USA) to purple formazan crystals by metabolically active cells, provides a quantitative determination of viable cells. After 72 h of treatments in triplicate, 25 µl of MTT was added to each well of cells, and the plate was incubated for 2 h at 37°C. The medium was removed, and the MTT crystals were solubilized with 50% dimethylformamide. Spectrophotometric absorbance of each sample was then measured at 570 nm (Sunrise absorbance reader, Tecan Group Ltd, Männedorf, Switzerland).

Apoptosis assay

At the end of appropriate days of treatment, cells were rinsed two times with PBS solution and fixed for 20 min in 4% paraformaldehyde at room temperature. Apoptotic cells were detected by the DeadEnd Colorimetric Apoptosis Detection System (Promega, Madison, USA) according to the manufacturer's instructions. Moreover, all cells were subjected to hematoxylin solution, showing blue stained nuclei. Cells were mounted in glycerol/PBS 9:1 and observed using a Leica microscope (Leica Microsystems GmbH, Wetzlar, Germany). Measurement of apoptosis was calculated as a percentage of apoptotic nuclei (dark brown nuclei) versus total nuclei of multinucleated TRAP positive cells, evaluated for three different experiments.

Immunocytochemistry assay

Immunocytochemistry analysis was performed using an ImPRESS Universal Reagent Kit (Vector Laboratories, Inc. Burlingame, CA, USA). OCs were seeded in 4-well chamber slides, fixed in cold 100% methanol and permeabilised with 0.2% (v/v) Triton X-100 (Sigma, St. Louis, MO, USA), in TBS (Tris-buffered saline). Cells were incubated in 0.3% H₂O₂ and the endogenous peroxidase was blocked with ready-to-use (2.5%) normal horse blocking serum (ImPRESS Reagent Kit, Vector Laboratories). After reaction with the primary antibodies (1:500 dilution), specific for the Fas receptor (C-20, rabbit anti-human), (Santa Cruz Biotechnology, Inc, Dallas, Texas, U.S.A) the material was incubated for 16 h at 4°C. After rinsing in TBS substrate–chromogen mix (ImmPACT DAB, Vector Laboratories), cells were then incubated for 30 min at room temperature with ImmPRESS reagent (ImPRESS Reagent Kit, Vector Laboratories). After washing, cells were mounted in glycerol/PBS 9:1 and observed using a Leica microscope (Leica Microsystems GmbH, Wetzlar, Germany).

Statistical analysis

The sample test for equal proportions without continuity correction data was carried out, using the program R version 2.10.1 (Copyright Foundation for Statistical Computing ISBN 3-900051-07-0). A value of p < 0.05 was considered statistically significant.

RESULTS

Chromatographic profile of essential oils

The chromatographic profile of essential oils studied showed E-methyl cinnamate as the most concentrated component in O. americanum essential oils, constituting 45.5% of the leaf essential oil and 54.4% of the flower essential oil. The essential oil from leaves of O. basilicum contained 18% methyl eugenol, 12% caryophyllene, 11.2% 1,8-cineole, whereas the flower essential oil had 15.9% linalool, 13.4% 1,8-cineole and 12.5% caryophyllene (Tables 1 and 2).

The essential oil yield of O. americanum essential oils was 1.46% for leaves and 2.95% for flowers, and for O. basilicum, it was 1.81% for leaves and 1.77% for flowers.

Cytotoxicity

Compounds that allowed cell viability greater than or equal to 90% were considered non-toxic. Therefore, the following were found to be non-toxic:

1. Essential oil extracted from leaves of O. americanum, when lower than or equal to 50 µg.ml⁻¹;
2. Essential oil extracted from leaves of O. basilicum, when lower than or equal to 50 µg.ml⁻¹;
3. Essential oil extracted from flowers of O. basilicum at concentrations lower than or equal to 5 µg.ml⁻¹;
4. Linalool at concentrations lower than or equal to 300 µg.ml⁻¹;
5. Methyl cinnamate and caryophyllene, both with non-toxic concentrations lower than or equal to 5 µg.ml⁻¹.

Apoptotic activity in osteoclasts

Only non-toxic concentrations were used to assess the apoptotic activity. The results showed that linalool (50 to 300 µg.ml⁻¹) was the most active component (p < 0.05) and was able to induce 75% of apoptotic osteoclasts (Figure 1). The essential oils of O. americanum (50 µg.ml⁻¹) and O. basilicum (50 µg.ml⁻¹), as well as caryophyllene (5 µg.ml⁻¹) and methyl cinnamate (5 µg.ml⁻¹) were not able to induce apoptosis. Linalool was able to induce
Figure 1. Linalool apoptotic activity at 50 and 300 μg.ml⁻¹ on a human osteoclast culture. Percentage of apoptotic osteoclast in culture after 72 h of treatment.

Table 1. Chemical composition of essential oil extracted from *O. americanum* leaves and flowers.

<table>
<thead>
<tr>
<th>Compound</th>
<th><em>K</em>&lt;sub&gt;lit&lt;/sub&gt;</th>
<th><em>K</em>&lt;sub&gt;calc&lt;/sub&gt;</th>
<th>Leaves (%)</th>
<th>Flowers (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-Pinene</td>
<td>939</td>
<td>939</td>
<td>1.0</td>
<td>0.7</td>
</tr>
<tr>
<td>Sabinene</td>
<td>975</td>
<td>977</td>
<td>0.5</td>
<td>0.3</td>
</tr>
<tr>
<td>β-pinene</td>
<td>979</td>
<td>982</td>
<td>1.1</td>
<td>0.7</td>
</tr>
<tr>
<td>Myrcene</td>
<td>990</td>
<td>992</td>
<td>0.6</td>
<td>0.3</td>
</tr>
<tr>
<td>Limonene</td>
<td>1029</td>
<td>1033</td>
<td>1.2</td>
<td>0.6</td>
</tr>
<tr>
<td>1,8-Cineole</td>
<td>1031</td>
<td>1036</td>
<td>10.4</td>
<td>6.7</td>
</tr>
<tr>
<td>E-β-ocimene</td>
<td>1050</td>
<td>1051</td>
<td>1.6</td>
<td>1.3</td>
</tr>
<tr>
<td>Fenchone</td>
<td>1092</td>
<td>1086</td>
<td>1.2</td>
<td>0.5</td>
</tr>
<tr>
<td>Linalool</td>
<td>1096</td>
<td>1100</td>
<td>3.0</td>
<td>5.6</td>
</tr>
<tr>
<td>Camphor</td>
<td>1146</td>
<td>1150</td>
<td>2.1</td>
<td>2.0</td>
</tr>
<tr>
<td>Terpinen-4-ol</td>
<td>1177</td>
<td>1182</td>
<td>2.3</td>
<td>1.8</td>
</tr>
<tr>
<td>Methyl chavicol</td>
<td>1196</td>
<td>1200</td>
<td>9.7</td>
<td>6.8</td>
</tr>
<tr>
<td>Z-Methyl cinnamate</td>
<td>1299</td>
<td>1305</td>
<td>6.5</td>
<td>4.6</td>
</tr>
<tr>
<td>E-Methyl cinnamate</td>
<td>1378</td>
<td>1388</td>
<td>45.5</td>
<td>54.4</td>
</tr>
<tr>
<td>E-caryophyllene</td>
<td>1419</td>
<td>1427</td>
<td>0.9</td>
<td>1.1</td>
</tr>
<tr>
<td>Germacrene D</td>
<td>1485</td>
<td>1489</td>
<td>1.0</td>
<td>1.1</td>
</tr>
<tr>
<td>Total identified</td>
<td></td>
<td></td>
<td>88.6</td>
<td>88.5</td>
</tr>
</tbody>
</table>

*K*<sub>L</sub>, Kovats index; *K*<sub>lit</sub>, Kovats index in literature; *K*<sub>calc</sub>, calculated Kovats retention index.
either the expression of Fas receptor on osteoclasts.

**DISCUSSION**

In the present study, the effects of essential oils extracted from *O. americanum* and *O. basilicum* on osteoclast activity was investigated. By considering the lack of effects of essential oils of Ocimum species studied on OCs, the effects of purified components (linalool, caryophyllene and methyl cinnamate) on the induction of OCs apoptosis were included in the experimental plan.

Essential oils are complex mixtures of volatile constituents biosynthesized by plants and the interactions between these components may lead to antagonistic, additive or synergistic effects. Some studies have demonstrated that whole essential oils usually have higher activity than the mixtures of their major components, suggesting that the minor components are critical to the synergistic activity, though antagonistic effects have also been observed and this could explain the lack of effects of whole essential oils of Ocimum species towards OCs, in the present study. Antagonistic effect has been attributed to the interaction between non-oxygenated and oxygenated monoterpene hydrocarbons (Goñi et al., 2009). Linalool was considered the most active compound. The chromatographic profile of essential oils studied showed that linalool was presented in both, *O. americanum* and *O. basilicum* essential oils. According to Pandey et al. (2014), the major constituents which have been isolated from different *O. basilicum* oils include linalool, methyl chavicol, eugenol, methyl cinnamate, 1,8-cineole, bergamotene, limonene, camphor, geraniol. Oliveira et al. (2009) reported that in the Brazilian basil leaf essential oils, linalool, geraniol and 1,8-cineole are the major compounds corroborating with this study.

Linalool was able to induce apoptosis in osteoclasts and the apoptotic activity was confirmed by the increasing levels of Fas receptor on osteoclasts treated with this compound. Fas, also called APO-1 (CD95 molecules), plays a role in signal transduction in cellular apoptosis. Fas combines with Fas ligand (FasL), and then interacts with Fas-related death domain structure protein (FADD), to form the FasL-Fas-FADD death-inducing signaling complex (DISC), leading to procaspase-8 activation in the cytoplasm, resulting in eventual apoptosis (Zhang et al., 2016).

Understanding the mechanism of apoptosis can be applied in the development of drugs to control bone resorption by osteoclasts. Bone remodeling is a necessary process to maintain homeostasis of bone tissue; it is the result of a balanced activity between bone resorption by osteoclasts and bone apposition mediated...
by osteoblasts. When there is an imbalance in favor of osteoclastic activity, the pathological resorption compromises the individual's health and problems, such as rheumatoid arthritis, osteoporosis and periodontal disease, can arise.

Moreover, it has been demonstrated that linalool can induce the cell cycle of U937 cells to arrest at the G0/G1 phase, while HeLa cells arrest at the G2/M phase, and its function facilitates the expression of p53, p21, p27, p16 and p18 (CDKIs) and the non-expression of CDK activity (Chang et al., 2015). Therefore, linalool can inhibit the cell cycle of leukemia cells and cervical cancer cells, and it could thus be used to arrest cell cycle of osteoclast and to develop novel therapeutic agents for periodontal disease.

Linalool-incorporated nanoparticles (LIN-NP) were used as novel anticancer agent against epithelial ovarian cancer (Han et al., 2016). The authors reported that LIN NPs had significant cytotoxicity and apoptotic activity against cancer cells and the treatment increased apoptosis through reactive oxygen species (ROS) generation and a subsequent decrease in mitochondrial membrane potential and increase in caspase-3 levels.

The apoptotic activity of linalool, against Candida albicans, was also reported by Khan et al. (2014). Linalool was able to induce chromatin condensation and margination, nuclear envelope separation, nuclear fragmentation, cytoplasmic shrinkage and plasma membrane blebbing in exposed cells. Although, the apoptotic activity of linalool against osteoclast was not found in literature, these findings reinforce the hypothesis that linalool has an apoptotic activity.

Another compound studied in this work was caryophyllene, which was considered toxic to human cells at concentrations above 5 μg.ml⁻¹. However, according to Molina-Jasso et al. (2009), caryophyllene is considered safe for use in industries and therapeutic purposes. Based on metabolism of sesquiterpenes, hydroperoxides are the product of caryophyllene metabolism, which are readily converted to caryophyllene oxide, a second compound that is little reactive and more stable (Sköld et al., 2006). This metabolic process, associated with the DNA repair system and detoxification in mice, could explain the absence of genotoxic effects in vivo studies by Molina-Jasso et al. (2009) and the divergence with the results found in this study, where tests were performed in vitro.

Regarding apoptotic activity, caryophyllene was not able to induce apoptosis in OCs. It is noteworthy that β-caryophyllene exhibited strong antibacterial effect and also displayed strong antioxidant effects (Dahham et al., 2015). Because there is a growing body of evidence suggesting oxidative stress playing a pivotal role in periodontal disease initiation and progression (Ramesh et al., 2016), antioxidant properties of caryophyllene may contribute to the development of novel preventive or therapeutic strategies for oral health.

In this study, the E-methyl cinnamate activity on osteoclasts was not considered effective, as it was toxic above 5 μg.ml⁻¹ and it was not able to induce apoptosis in osteoclasts. Schepetkin et al. (2015) reported that E-methyl cinnamate was not effective in modulating some innate responses like neutrophil migration and ROS production. O. americanum essential oil contained about 55% of methyl cinnamate in its composition; however neither the essential oil extracted from O. americanum nor methyl cinnamate alone were able to induce apoptosis in OCs. Taken together, these data do not support the candidacy of E-methyl cinnamate for the development of new drugs to control osteoclast activity. However, methyl cinnamate was found to show antifungal and antiaflatoxigenic efficacy at a low concentration (0.6 μl/ml) and the nature of its toxicity was fungicidal (Prakash et al., 2012).

There is little information in the literature on the biological activities of the studied plants in the treatment of periodontal disease, one of the most common pathological conditions of the oral cavity. This study demonstrated that Ocimum species studied have a potential biotechnological application in drug formulation for the treatment of periodontal disease, since linalool (50 to 300 μg.ml⁻¹) induced 75% of apoptotic osteoclasts. So, this study suggests that linalool could be used to control osteoclast activity and paves way for future research on the use of O. americanum and O. basilicum compounds for the control of osteoclast activity.

Conflicts of interests

The authors declare that they have no conflict of interest.

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