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Table of Contents: Volume 10 Number 28 25 July, 2016

## ARTICLES

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*Full Length Research Paper*

# The impact of compounds isolated from *Ocimum* sp. on apoptotic activity of osteoclast

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**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. Pro-inflammatory mediators (IL-1, $PGE_2$, 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 proinflamatory cytokines (IL-1, IL-6, TNFα, $PGE_2$, IL-17)

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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 $\mu g.mL^{-1}$) 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, NFκB 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 "manjericões" (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 citriodorum* 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 (Shadia et al., 2007) and antibacterial against *Propionibacterium acnes* (Viyoch 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 espiciforme 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 spasmolytic, 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 (Tohti et al., 2006).

### Plant samples

Plants were cultivated from March to June 2010, in the Medicinal Plants Garden at the Federal University of Reconcavo of Bahia, Santo Antônio de Jesus, Bahia, Brazil, Latitude 12° 57′ 59.2”, Longitude 039° 15′ 49.4” LO. The herborization 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. Loius, 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% $CO_2$ 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 cinnammate and caryophylenne (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 25 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% $H_2O_2$ 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 and flowers 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

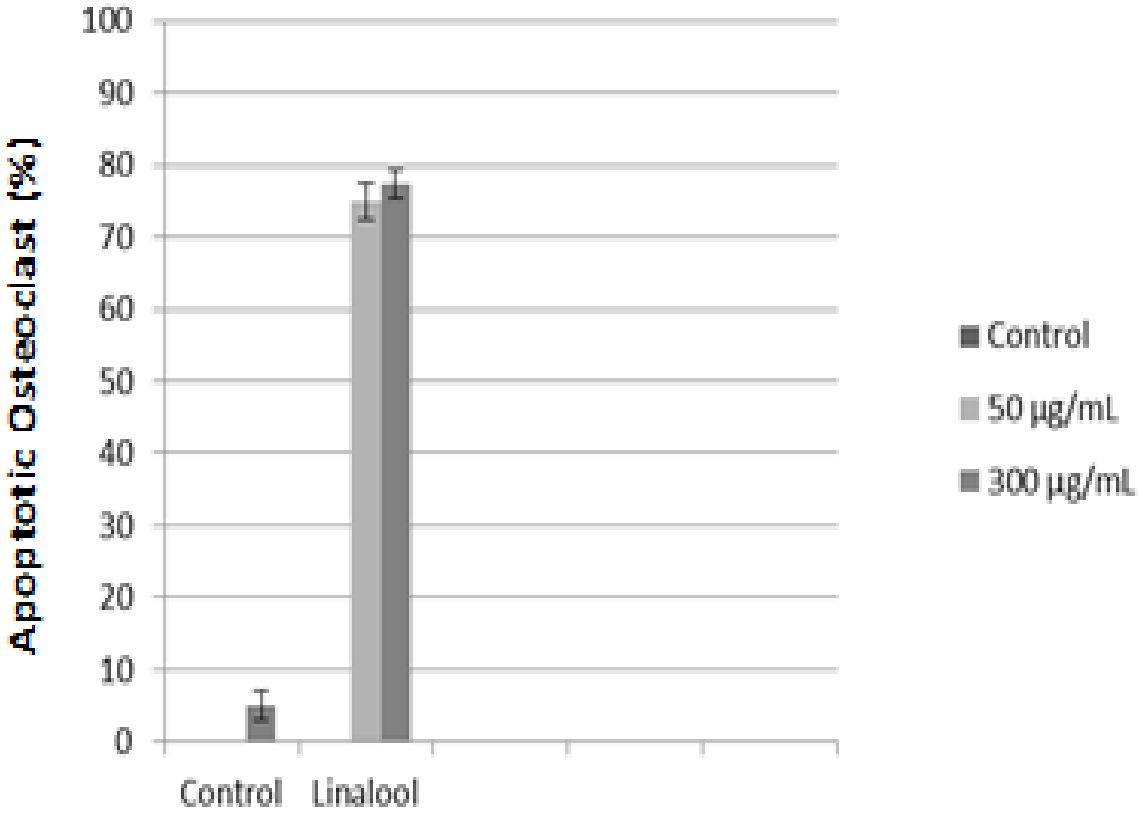


**Figure 1.** Linalool apoptotic activity at 50 and 300 µg.ml$^{-1}$ on a human osteoclast culture. Percentage of apoptotic osteoclast in culture after 72 h of treatment.

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

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

KI, Kovats index; $Ki_{lit}$, Kovats index in literature; $Ki_{calc}$, calculated Kovats retention index.

**Table 2.** Chemical composition of essential oil extracted from *O. basilicum* leaves and flowers.

| Compound | $KI_{lit}$ | $KI_{calc}$ | Flowers (%) | Leaves (%) |
|---|---|---|---|---|
| α-Pinene | 939 | 939 | 0,9 | 0,5 |
| Sabinene | 975 | 977 | 0,5 | 0,4 |
| β-pinene | 979 | 982 | 1,8 | 1,3 |
| Myrcene | 990 | 992 | 0,6 | 0,5 |
| Limonene | 1029 | 1033 | 0,4 | 0,5 |
| 1,8-Cineole | 1031 | 1036 | 13,4 | 11,2 |
| Z-β-ocimene | 1037 | 1044 | 3,1 | 5,1 |
| Linalool | 1096 | 1100 | 15,9 | 2,2 |
| Eugenol | 1359 | 1359 | 2,9 | 9,3 |
| β-Elemeno | 1390 | 1396 | 6,4 | 2,7 |
| Methyleugenol | 1403 | 1403 | 2,1 | 18,0 |
| caryophyllene | 1419 | 1427 | 12,5 | 12,0 |
| α-humulene | 1454 | 1461 | 3,5 | 2,6 |
| β-selinene | 1490 | 1492 | 1,7 | 2,2 |
| Bicyclogermacrene | 1500 | 1502 | 14,6 | 4,7 |
| α-Bulneseno | 1500 | 1502 | 2,8 | 4,1 |
| Elimicina | 1557 | 1558 | 2,1 | 14,6 |
| Spathulenol | 1578 | 1584 | 1,8 | 1,8 |
| Total identified | | | 87,0 | 93,7 |

KI, Kovats index; $Ki_{lit}$, Kovats index in literature; $Ki_{calc}$, 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 pro-caspase-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 $\mu g.ml^{-1}$. 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 $\mu g.ml^{-1}$ 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 neuthophil 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 $\mu g.ml^{-1}$) 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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*Full Length Research Paper*

# The effects of an aqueous leaf extract of *Clausena anisata* (Willd.) Hook.f.ex Benth. on blood pressure, urine output, angiotensin II levels and cardiac parameters in spontaneously hypertensive rats

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***Clausena anisata* (Willd.) Hook.f. ex Benth (Rutaceae) is a medicinal plant indigenous to Southern Africa and scientific studies report on its biomedical activities and possible antihypertensive property by demonstrating *in vitro* angiotensin converting enzyme inhibition. This study investigated the antihypertensive effects of an aqueous leaf extract of *Clausena anisata* in a spontaneously hypertensive rat model, and determined whether these blood pressure lowering effects could be attributed to diuresis, the inhibition of the renin-angiotensin-aldosterone blood pressure control system and/or possible negative inotropic or chronotropic cardiac effects. Aqueous extracts were prepared from ground leaves of *Clausena anisata*. Four groups of ten rats each received 50, 100, 200 or 400 mg/kg.*bw* of aqueous extracts intra-arterially respectively to obtain a dose response relationship. Another two groups of fifteen rats each received either plain water (control group) or the plant extract added to their drinking water (experimental group) for 40 days. Urine output was measured at 10 day intervals. All cardiovascular data was recorded with a Powerlab 200 recording system in anaesthetised rats that were catheterised via the carotid artery. Plasma angiotensin II levels were determined using a commercially available enzyme immunosorbent assay kit. The results showed that *Clausena anisata* was effective in reducing aortic blood pressure at the highest dose tested (400 mg/kg.*bw*). Furthermore, *Clausena anisata* significantly reduced the blood pressure over 40 days. This appears to have occurred via a mechanism that causes a reduction in plasma angiotensin II levels and not via diuresis or negative chronotropic effects.**

**Key words:** angiotensin-converting-enzyme inhibitor (ACE inhibitor), negative inotropic effect, diuresis, antihypertensive agents, traditional medicinal plants, lethal dose 50 cardiac contractility, South Africa.

## INTRODUCTION

In 2008, almost one billion individuals globally had hypertension, with the vast majority residing in developing countries. The highest number of reported cases came from the Sub-Saharan African region (WHO, 2011). Because of the high cost involved in treating and managing hypertension with conventional pharmaceutical

drugs and the inadequate supply of these drugs to many rural areas with limited resources (Patwardhan, 2005), these populations have to rely on treatment by traditional healers who use plant remedies that have not been adequately tested for their effectiveness. . Approximately 80% of the world's population, primarily those in developing countries, relies on plants and plant-derived medicines for primary health care requirements (Gurib-Fakim, 2006; Talha et al., 2011). South Africa has a very large biodiversity that contains more than 30,000 species of higher plants (Van Wyk et al., 2002). However, only a limited number of plant species have been investigated for their possible antihypertensive effects (Duncan et al., 1999; Mackraj and Ramesar, 2007).

*Clausena anisata* (*C. anisata)* belongs to the plant family Rutaceae and is indigenous to Southern Africa where it is commonly known as 'isifudu' in Zulu. It usually grows in areas with high rainfall, such as evergreen forests and woodlands, and along streams and rivers (Coates Palgrave et al., 2000). In traditional medicine, *C. anisata* is used as a remedy against worm infections, respiratory ailments, heart disorders, and hypertension (Hutchings et al., 1996). In addition, traditional healers claim that the steam arising from boiling leaves of *C. anisata* strengthens the heart, cures rheumatism and decreases fever (Coates Palgrave et al., 2000). A number of studies have also shown the therapeutic potential of the plant as an antimalarial (Clarkson et al., 2004), hypoglycaemic (Ojewole, 2002) and antiparasitic (Makanju, 1983) remedy. Phytochemical studies reported that the plant contains large amounts of coumarins, such as chalepin, osthole, coumarins, scopoleptin, xanthoxyletin, and heliettin (Duncan et al., 1999). Also, the presence of carbozole alkaloids, such as clausenol and clausamine, has shown to exhibit antimicrobial properties (Chakraborty et al., 1995). Furthermore, many terpenoid hydrocarbons, sesquiterpenoids and fatty acids have been isolated from the pericarps, roots and leaves of this plant (Reisch et al., 1985). However, very little is known about the antihypertensive properties of the plant as claimed by traditional healers. One study, however, suggested that the plant may possess a possible antihypertensive property by showing angiotensin converting enzyme (ACE) inhibition when an aqueous leaf extract was tested *in vitro* (Duncan et al., 1999). The current study was therefore conducted to investigate whether an aqueous leaf extract of *C. anisata* has blood pressure (BP) lowering effects in the spontaneously hypertensive rat (SHR) and, to further investigate whether the antihypertensive effects could be attributed to diuresis, inhibition of the renin-angiotensin-aldosterone BP control system, possible negative inotropic or chronotropic cardiac effects or a combination of these effects.

## MATERIALS AND METHODS

### Preparation of the plant extract

Fresh leaves of *C. anisata* were collected from the South African National Biodiversity Institute in Pretoria, South Africa. The plant was authenticated by the botanist, Mrs J. Maphuta as *C. anisata* (Willd.) Hook.f. ex Benth (the plant name has been checked on www.theplantlist.org) and a voucher specimen (Genspec 3346-1) was deposited at the institute's herbarium. Directly after collection, the fresh leaves were washed with water and air-dried at room temperature for 48 h. The dried leaves were then ground into a fine powder using a coffee grinder, vacuum packed at -0.8 bar (La.va V300 vacuum packing machine, La.va, South Africa) and stored at 4°C.

The leaf powder was used to prepare a crude aqueous extract using a procedure similar to the method described by Duncan et al. (1999). Briefly, 15 g of the powdered leaves were added to 150 mL of distilled water in a beaker that was surrounded by ice and subjected to ultra-sonication for 60 min (Soniprep 150 ultrasonic disintegrator, MSE Scientific Instruments, England). After sonication, 150 mL of warm distilled water (40°C) was added to the mixture, stirred for 1 h on a magnetic stirrer and then left to stand overnight to allow the plant residues to settle to the bottom. The supernatant was then filtered (Whatman no 1 filter paper), and the plant residue was then repeatedly (2 times) extracted with 50 mL of distilled water (40°C). The solution was then stirred for 5 min and left standing for 2 min; then, the supernatant was filtered, after which the filtrate was centrifuged for 10 min at 3000 x *g* (17RS, Heraeus Sepatech centrifuge, USA). The supernatant was then decanted into an empty pre-weighed Petri dish and was oven dried at 40°C for 24 h. The dried yield was weighed, sealed and then stored at 4°C in a refrigerator until testing.

### Toxicity testing

Toxicity testing of the plant extract was performed by using the brine shrimp toxicity assay as described by Krishnaraju et al. (2006). Briefly, artificial sea water was prepared by adding 38 g of artificial sea salt (Tropic Marin®) to 1 L of distilled water (3.8% artificial sea water), and the pH was adjusted to 8.5 using 1 M NaOH. *Artemia salina* eggs were then incubated in an Erlenmeyer flask containing artificial sea water at room temperature. An aerator was placed into the flask that was exposed to an artificial light source for 48 h. Once the eggs hatched, the completely hatched nauplii were used as the test organism. Varying concentrations of plant extract (0.5 mL) were added into sample vials containing 10 nauplii in 4.5 mL artificial sea water solution to achieve a final concentration of 0 to 10 g plant extract/L. Determinations were done in quadruplicate. After the 24-h incubation period, at room temperature and exposed to a light source, the number of dead nauplii was counted and recorded. The following equation was used to correct for the control:

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$$\% \text{ Mortality} = \frac{x-y}{z} \times 100$$

Where x = the number of dead nauplii in the treated group after 24 h, y = the number of dead nauplii in the control group after 24 h and z = the number of nauplii at 0 h. From these data, the median lethal dose ($LD_{50}$) value was extrapolated from a best fit line of log concentration vs % mortality.

**Animals and experimental design**

The animal design, administration of anaesthesia, recording of aortic and cardiac parameters was performed using a procedure as described by Hay et al. (2008). Seventy six-month-old SHR, weighing between 300 and 350 g, were obtained from the animal unit of the University of Cape Town, South Africa. The rats were housed in individual standard rat cages and were kept in an environmentally controlled room (comfortable room temperature approximately 24°C, relative humidity between 45 and 55%, proper ventilation, 12 h light/dark cycles and a quiet environment). The animals had access to a sufficient amount of tap water (40 mL/day) and a nutritionally balanced rat diet (supplied by Epol Pty Ltd, RSA). The investigation conforms to the South African Guide for the care and use of laboratory animals (South African National Standard, 2008) in accordance with the internationally accepted principles for laboratory animal use and care (Directive 86/606/EEC, 1986). Ethical approval was obtained from the Medical Research and Ethics Committee of the University of Limpopo (Medunsa Campus).

**Methodology for dose response experiments**

Forty SHR were randomly divided into four groups of 10 each to obtain dose-dependent effects. Each animal was anaesthetised with an intra-peritoneal injection of a 3:1 mixture of Anaket-V (ketamine 100 mg/mL, Kyron Laboratories Pty Ltd. Johannesburg, SA) and Chanazine (2%) (xylazine 20 mg/mL, Centaur labs, SA) at a concentration of 1.3 mL/kg of bodyweight (*bw*). Directly before the experiments started, the Hewlett-Packard pre-conditioner (8805D) and quartz pressure transducer (1290A) were calibrated with a mercury manometer according to the manufacturer's instructions.

The right carotid artery was surgically exposed and carefully separated from the surrounding structures in a sterile environment using standard surgical techniques. A catheter (Arrow 20 G x 12 cm) was inserted into the exposed carotid artery, advanced into the aorta and the proximal end connected to a fluid-filled Hewlett-Packard quartz pressure transducer (1290AH-E01) that was interfaced with a Hewlett-Packard multi-channel (3968A) and Powerlab 200 (AD Instruments) recording system. The following parameters were obtained from the aortic blood pressure (AOP) curve: Systolic blood pressure (SBP); diastolic blood pressure (DBP); mean arterial pressure (MAP), calculated as [SBP+DBP]/2; and pulse pressure (PP). The data obtained were captured and analysed with a Powerlab 200 (AD Instruments) application program (Chart 4.2.3 for Windows).

Four different concentrations of *C. anisata* (50, 100, 200, and 400 mg/kg of *bw*) were prepared from the dried yield and brought to 0.5 mL with saline. The four different concentrations were intra-arterially administered over one minute as 0.5 mL bolus injections. Baseline aortic BP values were recorded followed by a bolus injection and recorded for 20 min. Anaesthesia was maintained throughout the procedure by further administration of the anaesthetic as needed. A constant anaesthetic plane was continually assessed by means of the tarsal pinch reflex.

**Methodology for chronic experiments over 40 days**

***Water consumption and urine output measurements***

During the 40-day feeding period, urine output and water consumption was measured at 10-day intervals from the two groups of rats as follows: six rats were randomly selected from each group and placed in individual metabolic cages situated in the same animal room where the other rats were kept. These rats were placed in the metabolic cages a day before the actual measurements were taken. Both water consumption and urine output was measured over a 48-hour period.

***Recording of BP and cardiac parameters***

For these experiments, 30 SHR were randomly divided into two groups. The control group received a known quantity (40 mL/day) of tap water and the standard rat diet *ad libitum* for 40 days, and the experimental group was treated as above, except that the group received the leaf extract of *C. anisata* at 400 mg/kg of *bw* dissolved in 40 mL of drinking water. At the end of the 40-day feeding period, each animal was anaesthetised and catheterised as previously described under the methodology for dose response experiments. However, for chronic experiments the catheter was also advanced into the left ventricle for recording the left ventricular pressure (LVP) to obtain the left ventricular systolic pressure (LVSP), left ventricular end-diastolic pressure (LVEDP), heart rate (HR) and maximum rate of the rise of LV pressure ($dP/dt_{max}$), indicating LV contractility. The pressure recordings were continuously monitored to confirm the position of the catheter either in the aorta for AOP recordings or in the left ventricle for LVP recordings.

***Determination of plasma angiotensin II levels***

At the end of the AOP and LVP recordings, 5 mL of arterial blood was withdrawn via the arterial catheter and collected in ethylenediaminetetraacetic acid (EDTA) coated tubes (Transpharm, Pretoria), mixed thoroughly by gentle tilting and placed immediately on ice. After enough blood was collected, the rats were sacrificed by further exsanguination. The blood samples were then centrifuged at 3000 x *g* for 20 min at 4°C. Plasma was removed by aspiration, placed into Eppendorf tubes and immediately stored at -21°C. Once all of the samples were accumulated, an angiotensin II (AII) enriched fraction was prepared using hydrophobic interaction chromatography with phenyl cartridges (SPI-Bio/Bertin, Part Number PH-S-00/3) according to the manufacturer's instructions. Briefly, this entailed loading the plasma samples on prewashed columns. Unbound material was washed off with water. The AII enriched fraction was eluted with methanol that was dried under a stream of nitrogen at 37°C. The AII levels were thereafter determined with a sandwich enzyme linked immunosorbant assay kit (SPI bio/Bertin, Catalogue Number A05880) according to the manufacturer's instructions. All determinations were performed in triplicate.

**Statistical analysis**

Toxicity data were evaluated using Excel Microsoft 2010. All other data were analysed using an interactive statistical program and were expressed as the mean ± standard error of the mean (SEM) (SigmaStat version 8.0, SPSS Science, USA). Data were also analysed for statistical significance and comparison among groups using Student's t-test and Paired t-test, One Way ANOVA (bonferoni test) as applicable. A *p*-value of <0.05 was considered to

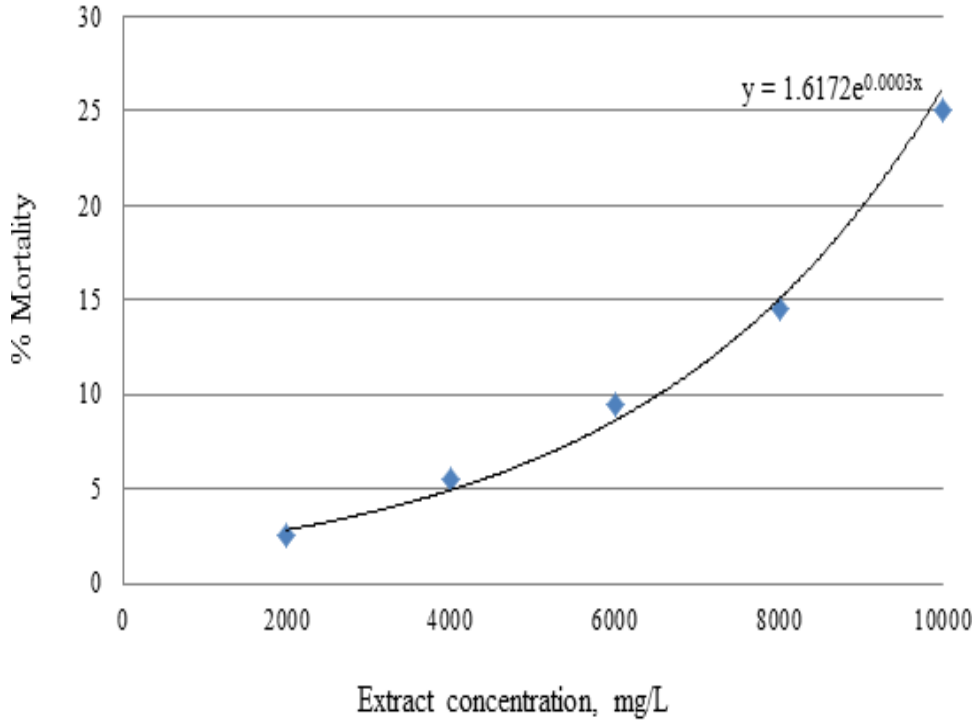


**Figure 1.** The brine shrimp toxicity result showing that a crude extract of *C. anisata* (10 g/L) had a very low toxicity ($LD_{50}$ of 27172.5 mg/L).

be statistically significant, and all graphical presentations were performed using either Excel Microsoft 2010 or SigmaPlot (version 8.0, SPSS Science, USA).

## RESULTS

### Toxicity results

An exponential relationship of increasing doses of aqueous leaf extracts of *C. anisata*, ranging from 0 to 10 000 mg/L, and toxicity was established (Figure 1). The results indicated that the leaf extract had a very low toxicity; at a concentration of 10 g/L, only 30% of nauplii were killed. The $LD_{50}$ was calculated using the formulae of the fitted line to be 27172.5 mg/L. A dosage of 400 mg/kg of *bw*/day should therefore have no substantial toxic effect on rats.

### Dose response relationship of different concentrations of *C. anisata* on aortic BP over 20 min

As indicated in Figure 2; at 50 mg/kg of *bw*, SBP was significantly decreased by 33 mmHg; DBP was decreased by 34 mmHg and MAP was decreased by 25 mmHg. Furthermore, a 100 mg/kg of *bw* dose significantly reduced SBP by 41 mmHg, reduced DBP by 37 mmHg, and reduced MAP by 35 mmHg. In addition, 200 mg/kg of *bw* significantly decreased SBP by 47 mmHg, decreased DBP by 39 mmHg, and decreased MAP by 37 mmHg. However, the 400 mg/kg of *bw* produced the largest significant decrease in SBP by 50 mmHg, decreased DBP by 40 mmHg, and decreased MAP by 40 mmHg. Compared to other concentrations, there is only a slight difference between the 200 mg/kg of *bw* and the 400 mg/kg of *bw*. PP did not show any significant change for all concentrations.

### Chronic effects of *C. anisata* (400 mg/kg of *bw*/day) on water consumption, urine output, BP, cardiac parameters and plasma AII levels

#### *Effects on water consumption and urine output*

Figures 3 and 4 show that administration of *C. anisata* (400 mg/kg of *bw*/day) over a 40-day period did not significantly influence the urine output or water consumption of SHR compared to day 0; control rats receiving plain tap water had significantly larger urine output and water consumption over the same period.

#### *Effects on aortic blood pressure*

Figure 5 shows that administration of *C. anisata* (400

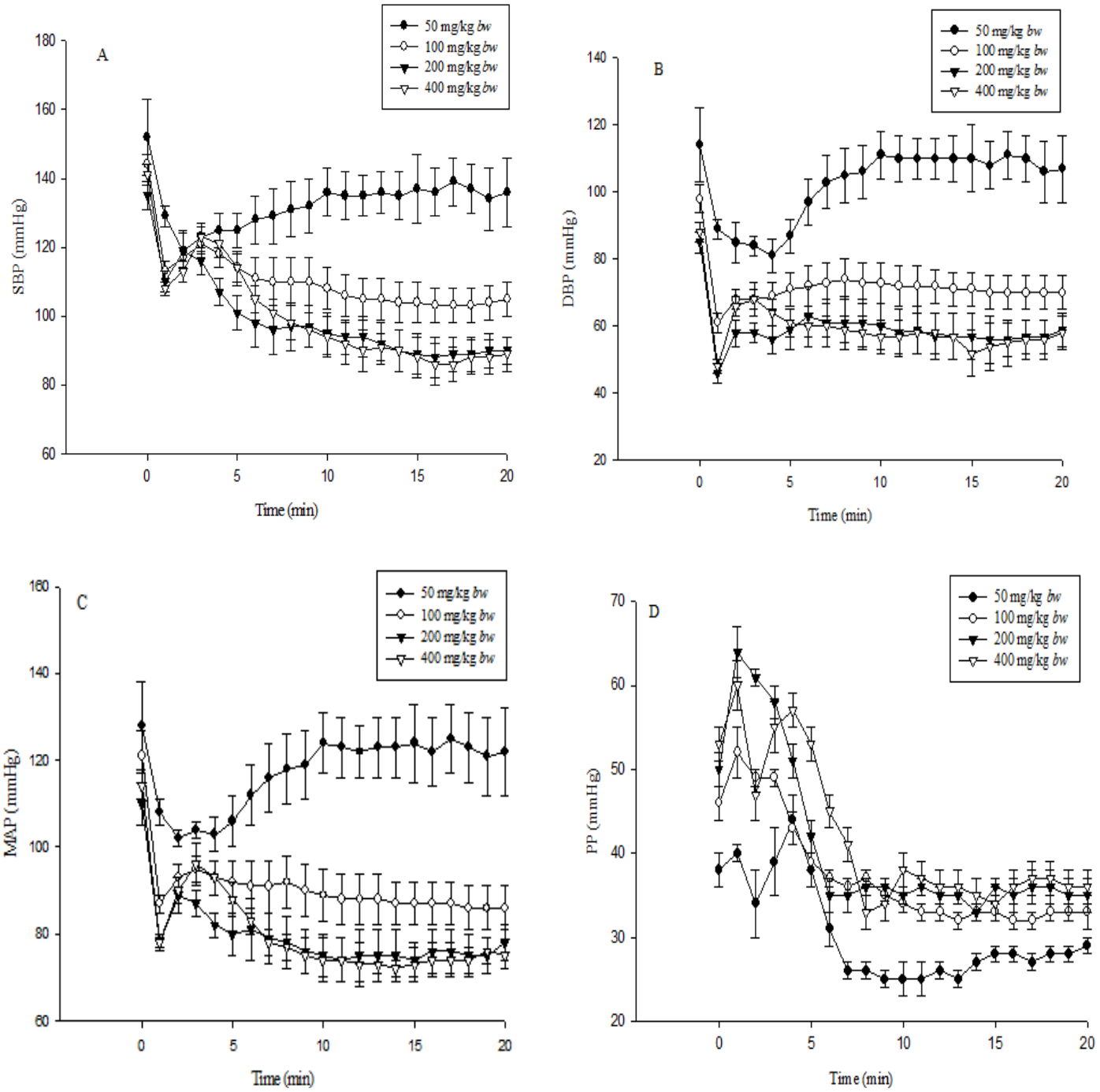


**Figure 2.** Effects of different concentrations of *C. anisata* (50, 100, 200 and 400 mg/kg of *bw*) on aortic BP over 20 minutes. (A) Changes in systolic BP; (B) Changes in diastolic BP; (C) Changes in mean arterial pressure, and (D) Changes in pulse pressure. The 400 mg/kg of *bw* showed the largest decrease in BP. Data was expressed as mean ± SEM. Paired t-test used to compare changes over 20 min with time 0. One Way ANOVA (Bonferoni test) did not show significant changes between the different groups (data not shown) (n = 40).

mg/kg of *bw*/day) significantly ($p < 0.05$) decreased SBP by 17 mmHg (6%), decreased DBP by 13 mmHg (7%) and decreased MAP by 13 mmHg (5%), while PP did not change significantly.

***Effects on the left ventricular pressure***

Figure 6 indicates that *C. anisata* significantly decreased LVEDP by 3.6 mmHg (9%), while LVSP was not

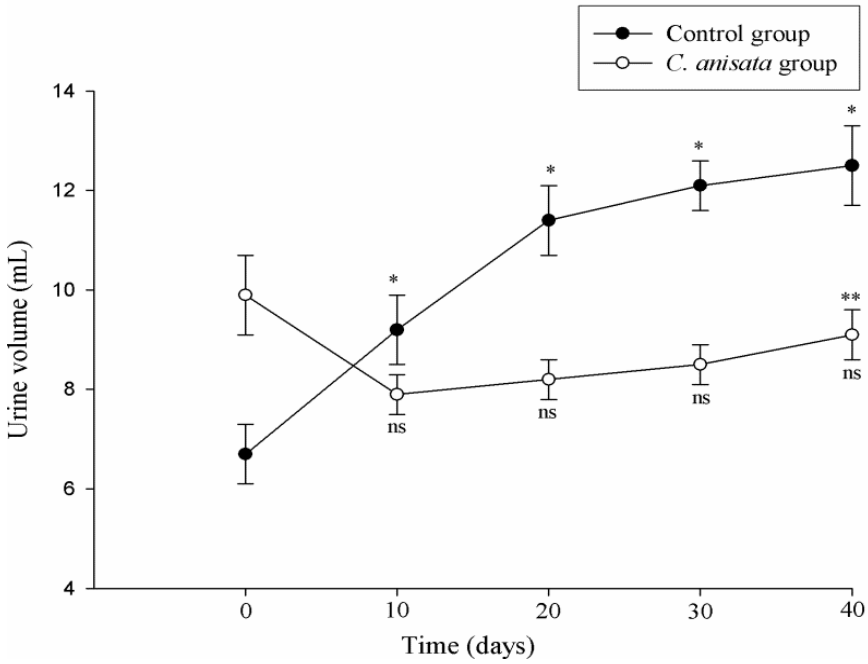


**Figure 3.** Effects of *C. anisata* (400 mg/kg of *bw*/day) on urine output over 40 days. Control rats receiving plain water had a significantly larger urine output compared to day 0, while rats treated with a leaf extract of *C. anisata* did not show significant changes in urine output when compared to day 0, **p* < 0.05 and ns (non-significant) (Paired t-test). ***p* < 0.05 compared to control group (Student's t-test). Data was expressed as mean ± SEM (n = 12).

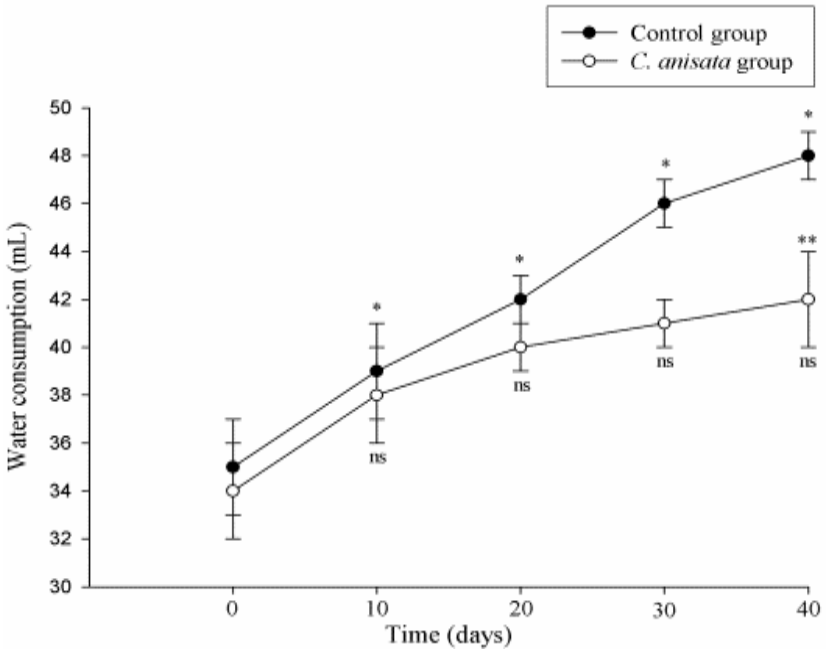


**Figure 4.** Effects of *C. anisata* (400 mg/kg of *bw*/day) on water consumption over 40 days. Control rats receiving plain water had significantly larger water consumption, while rats treated with *C. anisata* did not significantly influence water consumption when compared to day 0, * *p* < 0.05 and ns (non-significant) (Paired t-test). ** *p* < 0.05 compared to control group (Student's t-test). Data was expressed as mean ± SEM (n = 12).

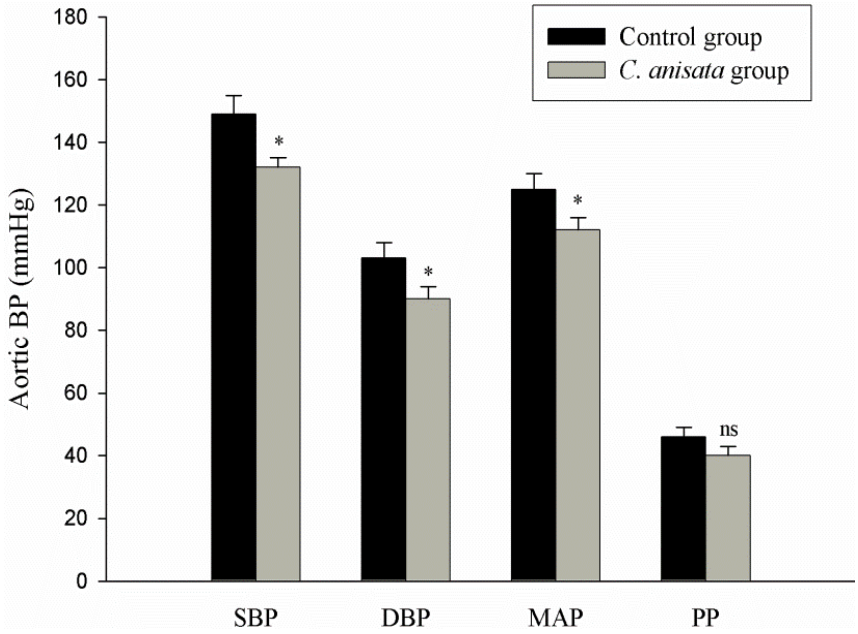


**Figure 5.** Effects of *C. anisata* (400 mg/kg of *bw*/day) on systolic BP, diastolic BP, mean arterial BP and pulse pressure. Administration of *C. anisata* (400 mg/kg of *bw*/day) significantly decreased all the BP parameters after 40 days. *$p < 0.05$ and ns (non-significant) compared to control group (Student's t-test). Data was expressed as mean ± SEM (n = 30).

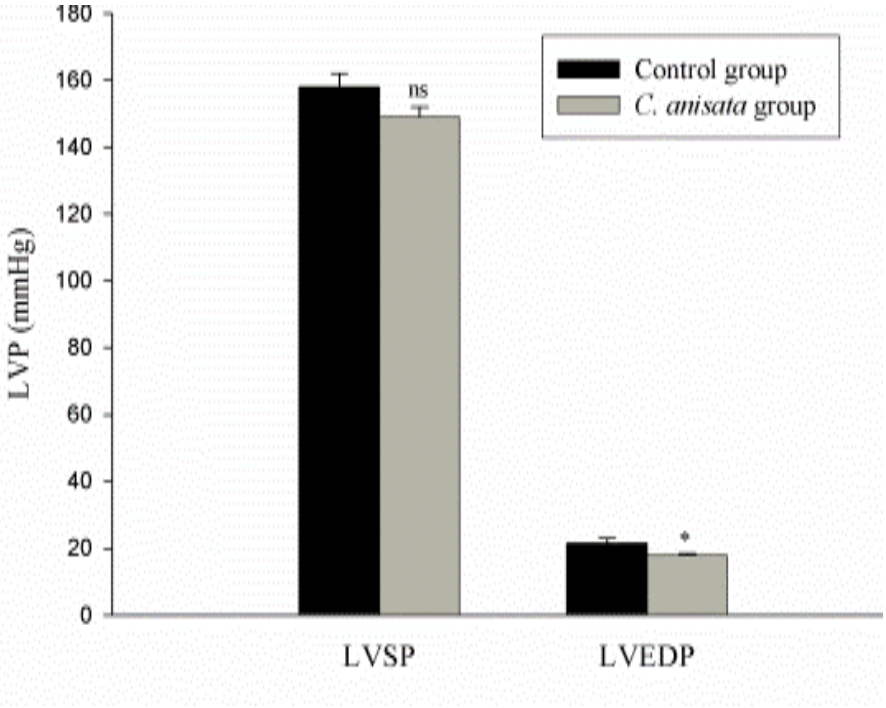


**Figure 6.** Effects of *C. anisata* (400 mg/kg of *bw*/day) on left ventricular systolic and end-diastolic pressures after 40 days. *C. anisata* significantly decreased LVEDP by 9%, while LVSP was not significantly affected. * $p < 0.05$ and ns (non-significant) compared to control group (Student's t-test). Data was expressed as mean ± SEM (n = 30).

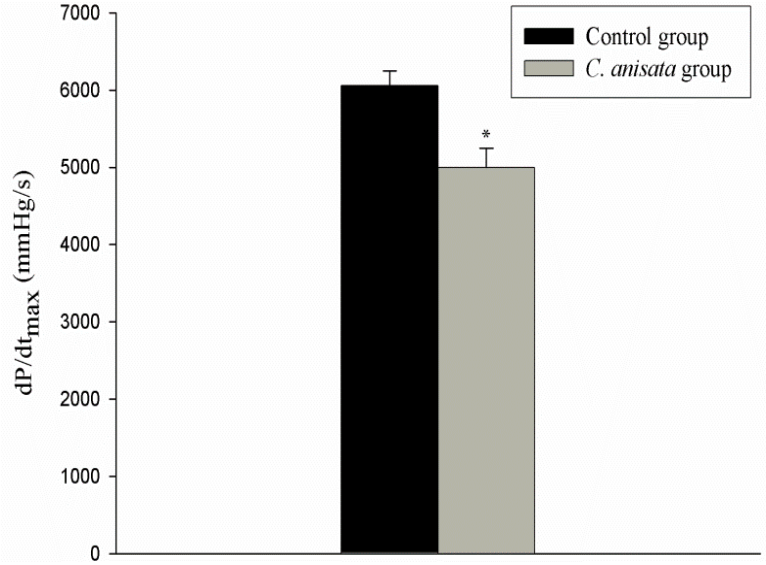


**Figure 7.** Effects of *C. anisata* (400 mg/kg of *bw*/day) on $dP/dt_{max}$ after 40 days suggested that the left ventricular contractility was significantly reduced. * $p < 0.05$ compared to control group (Student's t-test). Data was expressed as mean ± SEM (n = 30).

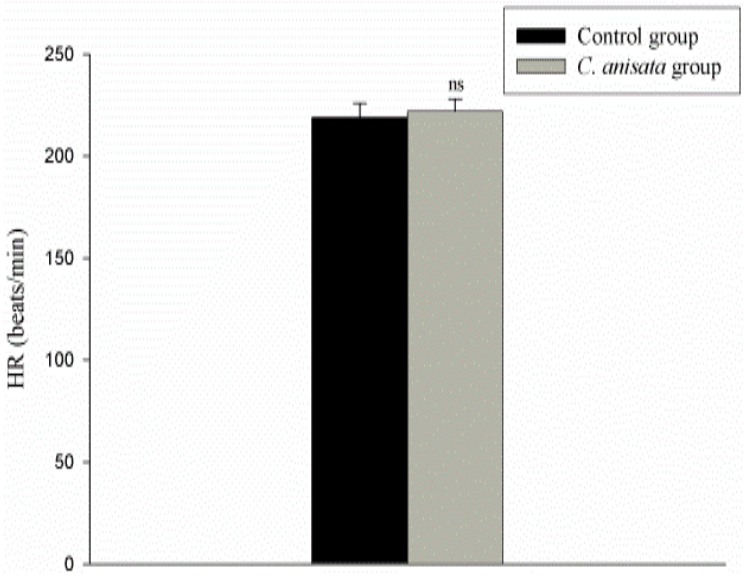


**Figure 8**. *C. anisata* (400 mg/kg of *bw*/day) did not significantly affect heart rate after 40 days. ns (non-significant) compared to control group (Student's t-test). Data was expressed as mean ± SEM (n = 30).

significantly affected.

### *Effects on the left ventricular dP/dt$_{max}$ and heart rate*

Figure 7 shows that *C. anisata* significantly reduced $dP/dt_{max}$ by 1062 mmHg (10%), while HR was not significantly affected (Figure 8).

### *Effects of C. anisata on plasma AII levels*

Figure 9 show that *C. anisata* significantly decreased plasma AII levels by 92.8 ± 25.3 pg/mL (31%) after 40

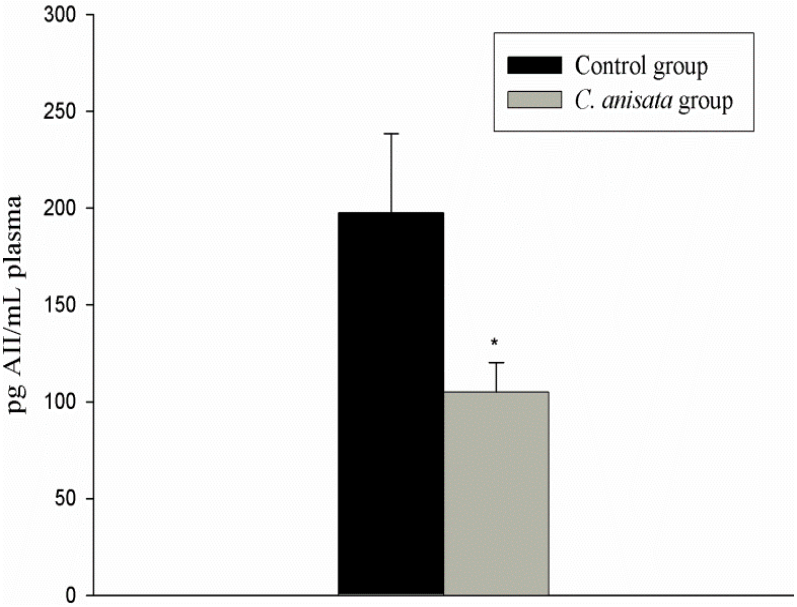


**Figure 9**. Effects of *C. anisata* (400 mg/kg of *bw*/day) on plasma angiotensin II after 40 days. Rats treated with *C. anisata* significantly decreased plasma angiotensin II levels by 31% after 40 days. * $p < 0.05$ compared with control group (Student's t-test). Data was expressed as mean ± SEM (n = 30).

days.

## DISCUSSION

Medicinal plants have been used to treat a wide variety of human ailments (Duncan et al., 1999; Hutchings et al., 1996; Osamaor and Owumi, 2010). In South Africa, Duncan et al. (1999) identified twenty plant species showing some ACE inhibitory potential *in vitro*. *C. anisata* is one of those plants and is used by traditional healers to treat hypertension. However, its antihypertensive potential has not been fully investigated to substantiate the antihypertensive claims that are made by the traditional healers. The current study showed that bolus injections of *C. anisata* (50, 100, 200, and 400 mg/kg of *bw*) to hypertensive rats significantly decreased aortic blood pressures, with 400 mg/kg of *bw* producing the largest effect. The study further showed that a leaf extract of *C. anisata* (400 mg/kg of *bw*) added daily to the drinking water of SHR significantly reduced SBP, DBP and MAP after 40 days. A possible diuretic effect, which is a common occurrence for many plant species to reduce BP (Bhadoriya et al., 2010; Reddy et al., 2011), was, however, ruled out because the results did not show any significant increases in urine output. Similarly, the non-significant changes in heart rate could not be offered as an explanation for the decrease in BP. Although phytochemical screening was not carried out in this study, it has been shown that *C. anisata* is biologically active due to compounds such as the carbozole alkaloids and coumarins (Chakraborty et al., 1995; Duncan et al., 1999). The biological activities reported for coumarins have been shown to possess antihypertensive properties (Gilani et al., 2000). It is therefore possible that the decrease in BP observed in this study could be attributed to these compounds.

In contrast to the parameters discussed above, LV $dP/dt_{max}$, which is an index for LV contractility, decreased significantly. However, its overall contribution to the decrease in BP is weakened by the lack of a matched change in LVSP. A possible explanation for this lies in the role of AII in contractility. It is known that AII has a direct positive inotropic effect by affecting myocardial calcium channels (Mori and Hashimoto, 2006), increasing intracellular calcium (Petroff et al., 2000); because the plant extract used in our study significantly reduced AII levels, this most likely results in the negative inotropic effect of the plant extract. In addition, because AII is also known to be a very powerful vasoconstrictor (Rosenthal, 1992), the significant decrease in the AII levels could therefore explain the significant decreases in BP parameters, especially the lower DBP reported in this study. AII is a vasoconstrictive hormone that increases the systemic blood pressure, renal perfusion pressure and the glomerular filtration rate. Therefore, the decrease in the AII levels could also provide impetus to the *in vitro* observation that *C. anisata* possesses ACE inhibitory

activity, as suggested by Duncan et al. (1999).

## Conclusion

The results suggest that *C. anisata* extracts significantly reduced the BP of hypertensive rats. The mechanism most likely involves a reduction in the AII levels, which might act through an ACE inhibitory mechanism as suggested by Duncan et al. (1999). This study provides an important basis for further investigations into the isolation and characterization of the active compounds that might be responsible for lowering the BP. Further investigations should also be conducted on isolated hearts to investigate a possible direct negative inotropic effect and possible active compounds of the plant extract on ventricular muscle. The findings of this study should then provide traditional healers with a scientific basis for the antihypertensive effects of *C. anisata*. We also acknowledge the fact that more research is needed on human subjects to determine the effectiveness and safety of this plant as an antihypertensive remedy in humans.

## Conflict of Interests

The authors declare that there is no conflict of interests including any financial, personal, or other relationships with people or organizations

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