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ARTICLES

Full Length Research Paper

Antioxidant properties of antiepileptic drugs levetiracetam and clonazepam in mice brain after *in vitro*-induced oxidative stress

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This study aimed to investigate the *in vitro* antioxidant activity of the novel anticonvulsant levetiracetam and benzodiazepine clonazepam. To do this, the mice brain homogenates were incubated with levetiracetam (50, 100 or 200 μg/ml) or clonazepam (50, 100 or 200 μg/ml), and then, submitted to heating at 37°C for 1 h. Ascorbic acid (vitamin C, 200 μg/ml) was used as reference antioxidant drug. The markers of oxidative stress, such as lipid peroxidation, nitrite-nitrate content, catalase activity, and reduced glutathione (GSH) levels, were measured in brain homogenates. The group submitted to the heating-induced oxidative stress showed an increase in lipid peroxidation, nitrite-nitrate content, and catalase activity. Previous incubation with levetiracetam and clonazepam, mainly at lower doses (50 and 100 μg/ml), and similarly to vitamin C, prevented these pro-oxidative changes, reducing the lipid peroxidation, nitrite-nitrate contents and catalase activity, and increasing GSH levels. These findings demonstrate antioxidant properties of levetiracetam and clonazepam, and help to elucidate the role of protection against oxidative stress in the neuroprotective mechanism of antiepileptic drugs.

Key words: Levetiracetam, clonazepam, oxidative stress, vitamin C, antiepileptic drugs.

INTRODUCTION

The role of free radical-mediated reactions in human neuropathology continues to attract interest. The production of free radicals is associated with injury to cell structures and the pathogenesis of many neurological disorders, such as Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis and epilepsy (Li et al., 2013; Kong and Lin, 2010). Over-accumulation of reactive oxygen species (ROS) caused by imbalance between the generation and elimination of these entities frequently results in severe harmful effects to cells, a

phenomenon known as oxidative stress (OS) (Gutteridge and Halliwel, 2010; Kong and Li, 2010). Furthermore, in sufficiently high concentrations, nitrogenous compounds, mainly nitric oxide (NO), can also damage cellular structures in a process known as nitrosative stress (NS). This occurs through nitrosation of amines and thiols, which also results in an excess of oxidative species and subsequent molecular damage (Aguiar et al., 2012).

In this context, OS and NS are possible mechanisms implicated in the pathogenesis of epilepsy (Chang and Yu, 2010). Studies have already verified that status epilepticus changes redox potential and decreases the level of ATP, which can lead to a collapse in brain energy production and supply (Aguiar et al., 2012; Wasterlain et al., 1993). Moreover, it was demonstrated that an increase in mitochondrial OS and NS occurs after persistent seizures and promotes subsequent dysfunction of this organelle and cell death (Freitas et al., 2010; Ueda et al., 1997). Therefore, since mitochondrion is the main source of ATP for neurons and has an important role in the homeostasis of intracellular calcium, injury to it may strongly affect neuronal excitability and synaptic transmission (Chang and Yu, 2010). Further support for the role of free radicals in seizures comes from the successful use of exogenously administered antioxidants, as vitamin C and vitamin E, to protect the brain against seizure-induced damage (Barros et al., 2007; Murashima 1998; Santos et al., 2009; Ueda et al., 1997).

The second-generation antiepileptic drug levetiracetam (LEV) is a new molecule that is clearly differentiated from conventional antiepileptic drugs by its pharmacologic properties and mechanisms of action (Gibbs et al., 2006; Lyseng-Williamson 2011; Ueda et al., 2009). Although the exact molecular mechanism of action of LEV remains uncharacterized, it is known that this drug binds to synaptic vesicle protein 2A. By binding to this vesicle, this drug appears to act as a modulator of synaptic vesicle exocytosis, leading to direct inhibition of pre-synaptic neurotransmitter release (Rigo et al., 2002). Moreover, LEV also has been previously demonstrated to protect against oxidative stress-induced neurotoxicity in several models of seizures (Oliveira et al., 2007; Ueda et al., 2009; Zona et al., 2001).

Benzodiazepines, such as clonazepam (CNZ), are established and important agents for the treatment of seizures and epilepsy (Talarek and Fidecka, 2003). Studies have suggested the antioxidant properties of some benzodiazepines. CNZ has been shown to have a protective effect against free radical mediated brain damage in models of diabetic and depressive-like behavior in mice, which involve an oxidative stress process (Haeser et al., 2007; Wayhs et al., 2013[a]; Wayhs et al., 2013[b]). Moreover, nitric oxide synthase (NOS) inhibitors can potentiate the anticonvulsant action of benzodiazepines, which suggests the participation of NO system, and possibly of the NS, in the neuroprotective effect of these drugs (Borowicz et al., 2000; Talarek and

Fidecka, 2003).

However, although the literature suggests that the anticonvulsant effects of antiepileptic drugs (AEDs) could be related to their antioxidant properties, the basis of these properties is still unclear. Therefore, this work aimed to investigate the antioxidant activity of the novel anticonvulsant levetiracetam and benzodiazepine anticonvulsant clonazepam. The study also aimed to investigate how these AEDs could counteract oxidative stress in brain tissues. The investigation of the antioxidant activity of these AEDs and their possible neuroprotective effect may provide stronger evidences for the hypothesis of the involvement of oxidative damage in the pathophysiology of epilepsy.

MATERIALS AND METHODS

Drugs

Levetiracetam (Keppra®) was obtained from UCB Pharmaceutical Sector (Chemin du Foriest, Belgium); clonazepam (Rivotril®) was purchased from A F Hoffmann-La Roche AG (Brazil); ascorbic acid (vitamin C) was obtained from Sigma-Aldrich® (St. Louis, MO, USA). All other chemicals were purchased from Sigma-Aldrich® (St. Louis, MO, USA).

Animals

Experimentally naive, male Swiss mice Mus musculus, from the Animal House of the Federal University of Ceará, weighing 25 to 30 g, were used. The animals were maintained at a controlled temperature (24±2°C) with a 12-h dark/light cycle and food and water *ad libitum*. Mice were caged in groups of 8 in a 41 × 34 × 16 cm cage.

The animals were used according to the NIH Guide for the Care and Use of Laboratory Animals. The experiments were performed after approval of the protocol by the Ethics Committee on Animal Research of the Federal University of Ceará (with protocol number 59/07), in accordance with the Ethical Principles in Animal Research adopted by the Brazilian College of Animal Experimentation (COBEA). Moreover, the experiments were performed to minimize the number of rats and their suffering, following the ethical doctrine of the three "R"s: Reduction, Refinement and Replacement.

Assessment of antioxidant capacity *in vitro*

Antioxidant activity of the compounds was evaluated by measuring the production of thiobarbituric acid (TBARS), as an indicator of lipid peroxidation, reduced glutathione (GSH) levels and catalase activity. In order to assess the effects of these drugs on nitric oxide production, nitrite-nitrate levels were determined. Mice were decapitated and the brains were removed rapidly under standard conditions at 4°C. The whole brain, apart from the cerebellum, was homogenized in 50 mM potassium phosphate buffer (pH 7.4) and the concentration adjusted to 1 g wet weight of brain per 60 ml. Then, 250 µl of the brain homogenates was maintained in the absence or presence of LEV (50, 100 or 200 µg/ml), CNZ (50, 100 or 200 µg/ml) or vitamin C (VIT C) (200 µg/ml) for 24 h at 10°C. Following this, oxidative stress was induced by incubation of the brain homogenates for 1 h at 37°C with samples without oxidative stress acting as controls (Auddy et al., 2003; Mattei et al., 1998; Stocks et al., 1974).

Measurement of TBARS

The homogenates were incubated in a water bath for 1 h at 37°C. After incubation, assessment of the levels of thiobarbituric acid concentration was performed according to the method described by Houng et al. (1998), with absorbance measured at a wavelength of 532 nm and expressed as micromol of malondialdehyde (MDA)/mg of protein. The protein concentration was determined by using Lowry assay (Lowry et al., 1951).

Nitrite-nitrate determination

For the assessment of nitrite-nitrate, derived from nitric oxide (NO), 100 μl of Griess reagent (1% sulfanilamide dissolved in 5% H_3PO_4, 0.1% N-(1-naphthyl)-ethylenediamine dihydrochloride and distilled water, 1:1:1:1) was added to 100 μl of brain homogenate or to 100 μl of $NaNO_2$ at concentrations ranging from 0.75 to 100 μM (standard curve) or for the blanks to 100 μl of the buffer used in the homogenates. The absorbance was measured with a reader plate at 560 nm (Green et al., 1981).

Measurement of catalase activity

This was measured by using hydrogen peroxide to generate H_2O and O_2. The substrate mixture contained 0.3 ml of hydrogen peroxide in 50 ml of 0.05 M phosphate buffer, pH 7.0. The sample aliquot (20 μl) diluted with phosphate buffer was added to 980 μl of the substrate mixture. Absorbances were read after 1 and 6 min at 230 nm. A standard curve was established using purified catalase (Sigma MO, USA) and the results were expressed as μM/min/mg protein (Chance and Maehly, 1955). Protein measurement was performed with the Lowry assay (Lowry et al., 1951).

Determination of glutathione (GSH) levels

GSH levels were evaluated to estimate endogenous defenses against oxidative stress. The method was based on the reaction of Ellman's reagent (DTNB) with free thiol groups. The measurement of this parameter was performed according to the test steps of Sedlak and Lindsay (1998). GSH concentration was determined by the absorbance at 412 nm and was expressed as μg of GSH/μg of protein. The protein concentration was determined with the Lowry assay (Lowry et al., 1951).

Statistical analysis

Data were expressed as mean ± standard error of mean (SEM). Analysis of variance (ANOVA) was used for the statistical analysis followed by Student–Newman–Keuls test to identify differences between experimental groups. Values of p < 0.05 were considered significant. The results of VIT C group was compared with internal groups for each antiepileptic drug, LEV or CNZ, studied in this work.

RESULTS

According to *in vitro* antioxidant assays, brain homogenates exposed to oxidative stress, without pre-incubation with CNZ, LEV or VIT C, (stress control) showed an increase in the lipid peroxidation and nitrite-nitrate contents, when compared with brains not exposed

to oxidative stress (control) (p<0.0001). In comparison, previous incubation with LEV (p<0.001) or CNZ (p<0.05), at all tested concentrations, and with VIT C (p<0.0001) diminished the TBARS levels when compared with stress control. Regarding nitrite-nitrate concentrations, LEV, only at concentrations of 50 and 100 μg/ml (p<0.0001), CNZ, at all tested concentrations (p<0.05), and VIT C (p<0.0001) were able to significantly reduce nitrite-nitrate concentration when compared with stress control. Results of TBARS measurements: LEV (Control: 0.87 + 0.08; Stress control: 5.01 + 0.44; LEV200: 1.21 + 0.11; LEV100: 1.17 + 0.15; LEV50: 0.94 + 0.09; VIT C: 0.89 + 0.6) (Figure 1); CNZ (Control: 0.79 + 0.05; Stress control: 6.57 + 0.15; CNZ200: 2.76 + 0.27; CNZ100: 3.00 + 0.27; CNZ50: 2.15 + 0.23; VIT C: 0.89 + 0.6) (Figure 2), results were expressed in μmol of malondialdehyde (MDA)/mg of protein. Results of nitrite-nitrate levels: LEV (Control: 4.25 + 0.42; Stress control: 57.73 + 5.96; LEV200: 47.53 + 2.59; LEV100: 27.76 + 1.22; LEV50: 34.61 + 1.69; VIT C:12.35 + 0.8) (Figure 3); CNZ (Control: 4.81 + 0.39; Stress control: 58.56 + 5.26; CNZ200: 33.18 + 1.38; CNZ100: 27.76 + 1.22; CNZ50: 27.57 + 1.21; VIT C: 12.35 + 0.8) (Figure 4), results were expressed in μM.

A significant increase in catalase activity was observed in the group of homogenates submitted to oxidative stress (stress control) as compared to the control group (p<0.0001). In the samples pre-incubated with both test drugs, LEV (p<0.0001) and CNZ (p<0.05), at all concentrations, the catalase activity was reduced compared to the stress control, showing that LEV and CNZ pre-incubation was able to keep catalase activity at normal levels. Pre-incubation with VIT C also demonstrated a significant reduction of catalase activity compared to the stress control (p<0.0001). Results of catalase activity: LEV (Control: 8.24 + 0.80; Stress control: 42.37 + 4.05; LEV200: 4.99 + 1.31; LEV100: 7.09 + 1.61; LEV50: 7.34 + 1.35; VIT C: 6.68 + 0.38) (Figure 5); CNZ (Control: 7.40 + 0.60; Stress control: 38.15 + 4.15; CNZ200: 1059 + 1.03; CNZ100: 13.42 + 1.93; CNZ50: 10.06 + 0.96; VIT C: 6.68 + 0.38) (Figure 6), results were expressed in μM/min/mg protein.

No alteration in GSH levels was observed in the samples after the *in vitro* induced oxidative stress. An increase in GSH level was shown at the lowest concentration of CNZ (50 μg/ml) compared to the control group (p < 0.05). An increase in GSH was also demonstrated with the two lowest tested concentrations of LEV (50 and 100 μg/ml) as compared to the control (p < 0.0001) and to the stress control (p<0.001). An increase in GSH levels also occurred in samples with VIT C as compared to the control (p < 0.001) and to the stress control (p<0.001). Results of GSH levels: LEV (Control: 34.23+ 3.14; Stress control: 38.76+ 3.89; LEV200: 50.26 + 5.17; LEV100: 79.59 + 7.63; LEV50: 69.03 + 5.89; VIT C:57.4 + 4.31) (Figure 7); CNZ (Control: 27.96 + 1.12; Stress control: 28.87 + 2.54;
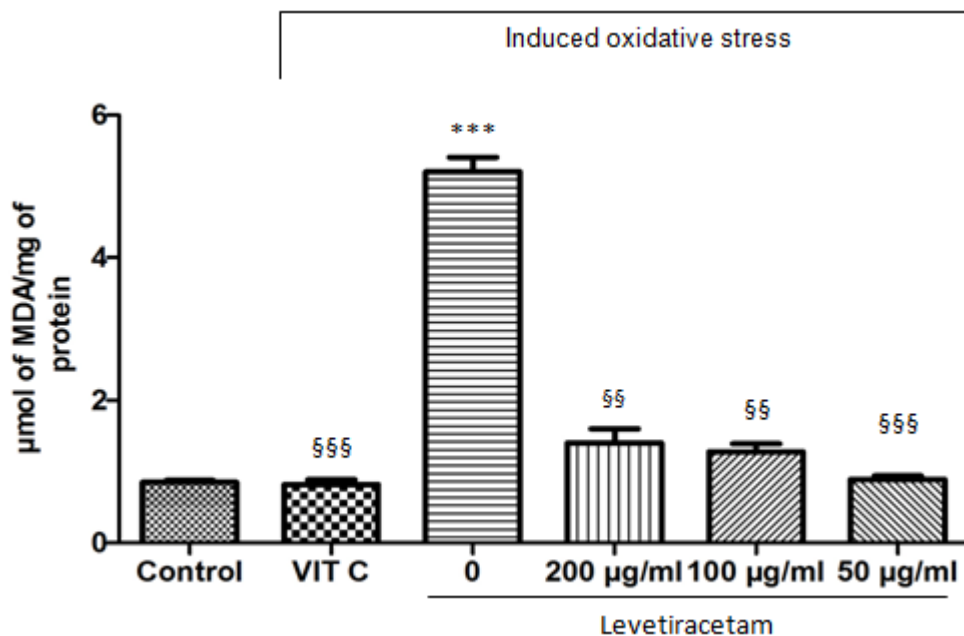
Figure 1. Lipid peroxidation was decreased in samples treated with either LEV as with VIT C. Levels of TBARS (lipid peroxidation level) in mice brain homogenates submitted or not (control) to oxidative stress in the absence (stress control) or presence of different concentrations of levetiracetam (50, 100 or 200 μg/ml) or VIT C (200 μg/ml), after 1 h of incubation of brain homogenate at 37ºC. Each bar represents the mean ± SEM, n=8, ***p<0,0001 vs. control group; §§ = p<0.001, §§§p<0.0001 vs. stress control group (one-way ANOVA followed by post-hoc Student–Newman–Keuls test).
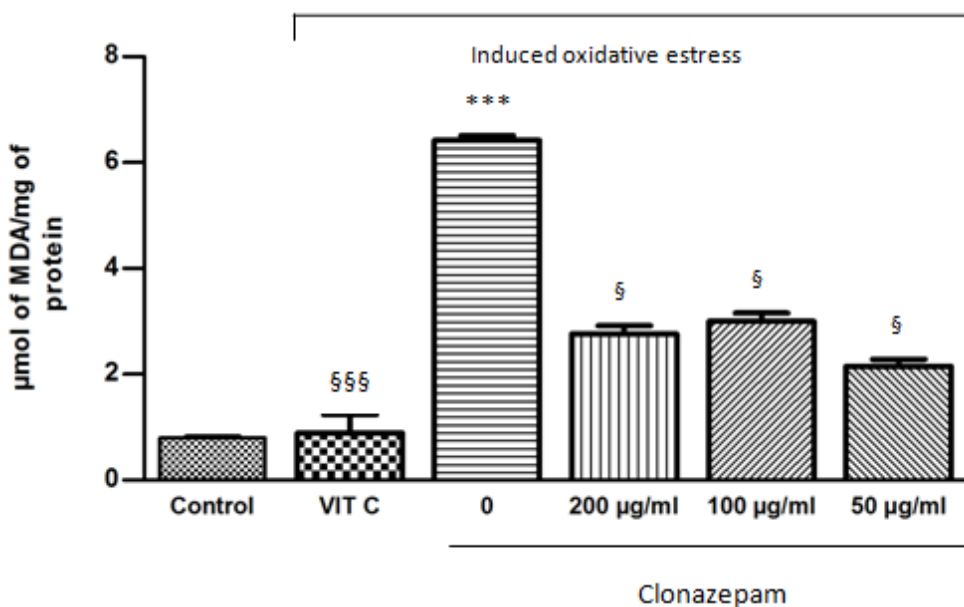
Figure 2. Lipid peroxidation was decreased in samples treated with either CNZ as with VIT C. Levels of TBARS (lipid peroxidation level) in mice brain homogenates submitted or not (control) to oxidative stress in the absence (stress control) or presence of different concentrations of clonazepam (50, 100 or 200 μg/ml) or VIT C (200 μg/ml), after 1 h of incubation of brain homogenate at 37ºC. Each bar represents the mean ± SEM, n=8, ***p<0.0001 vs. control group; § = p<0.05, §§§p<0.0001 vs. stress control group (one-way ANOVA followed by post-hoc Student–Newman–Keuls test).
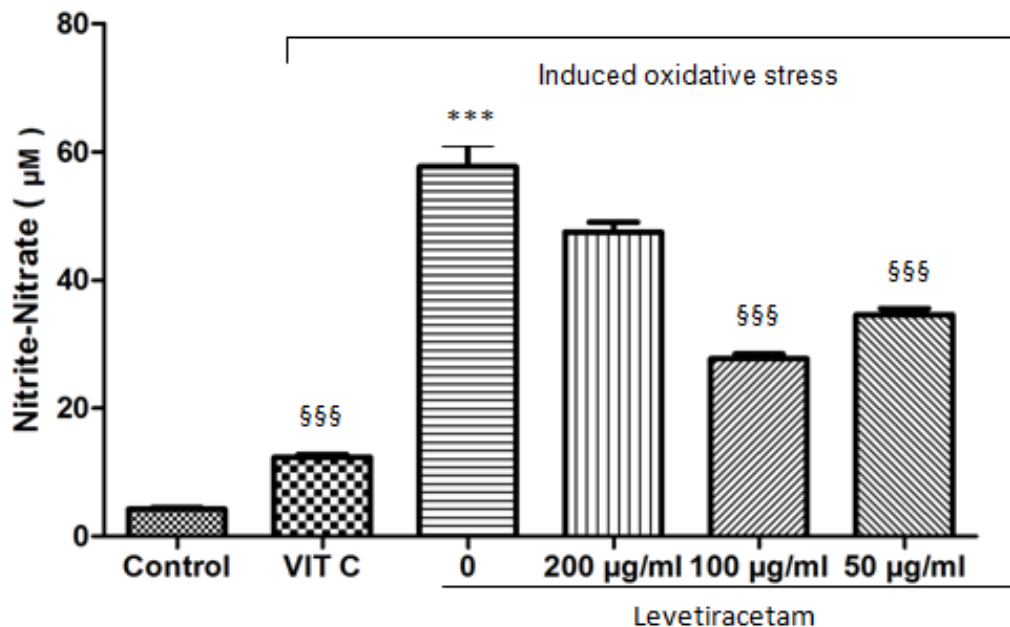
Figure 3. Reduction of nitrite-nitrate content in the samples treated with either LEV as with VIT C. Nitrite-nitrate level in mice brain homogenates submitted or not (control) to oxidative stress in the absence (stress control) or presence of different concentrations of levetiracetam (50, 100 or 200 µg/ml) or VIT C (200 µg/ml), after 1 h of incubation of brain homogenate at 37ºC. Each bar represents the mean ± SEM, n=8, ***$p < 0.0001$ vs. control group; §§§$p < 0.0001$ vs. stress control group (one-way ANOVA followed by post-hoc Student–Newman–Keuls test).
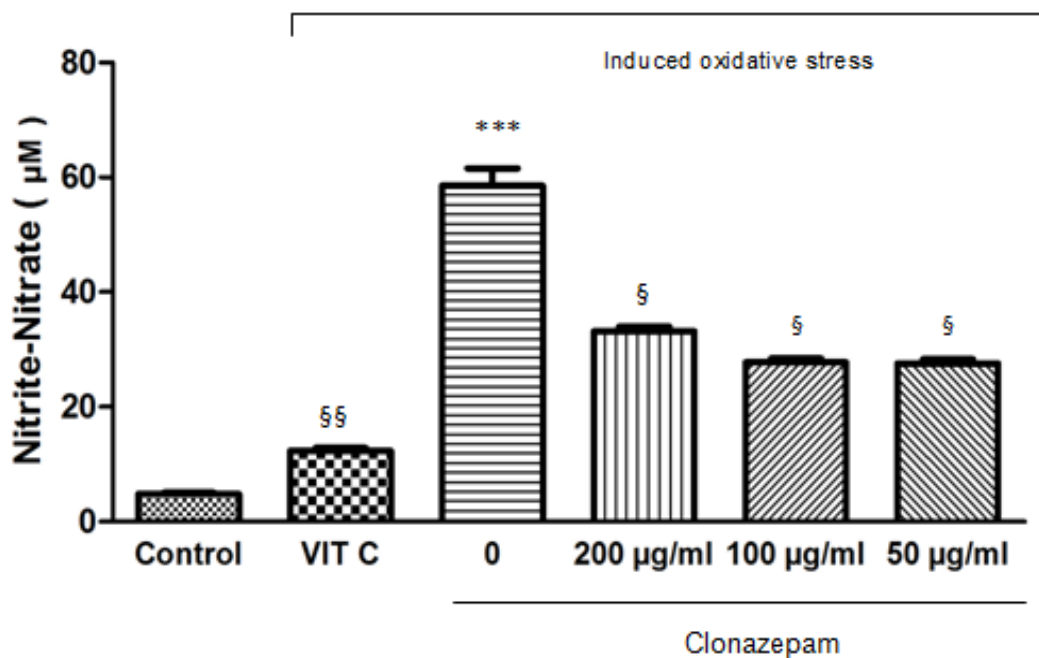
Figure 4. Reduction of nitrite-nitrate content in the samples treated with either CNZ as with VIT C. Nitrite-nitrate level in mice brain homogenates submitted or not (control) to oxidative stress in the absence (stress control) or presence of different concentrations of clonazepam (50, 100 or 200 µg/ml) or VIT C (200 µg/ml), after 1 h of incubation of brain homogenate at 37ºC. Each bar represents the mean ± SEM, n=8, ***$p < 0.0001$ vs. control group; §$p < 0.05$, §§$p < 0.001$ vs. stress control group (one-way ANOVA followed by post-hoc Student–Newman–Keuls test).
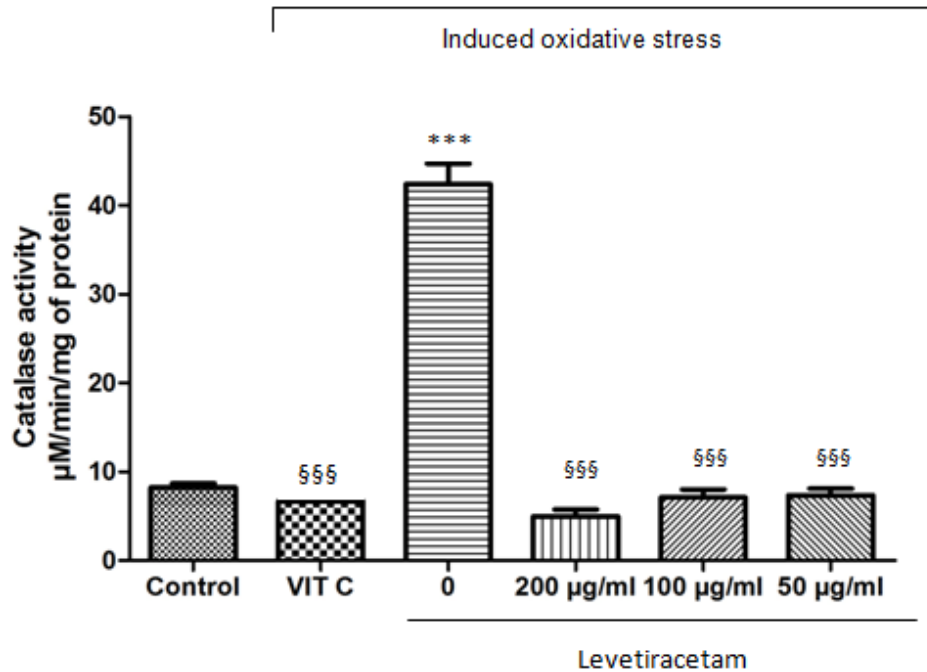
Figure 5. LEV and VIT C were able to approximate the catalase activity of normal levels. Catalase activity in mice brain homogenates submitted or not (control) to oxidative stress in the absence (stress control) or presence of different concentrations of levetiracetam (50, 100 or 200 μg/ml) or VIT C (200 μg/ml), after 1 h of incubation of brain homogenate at 37ºC. Each bar represents the mean ± SEM, n=8, ***$p<0.0001$ vs. control group; §§§$p<0.0001$ vs. stress control group (one-way ANOVA followed by post-hoc Student–Newman–Keuls test).
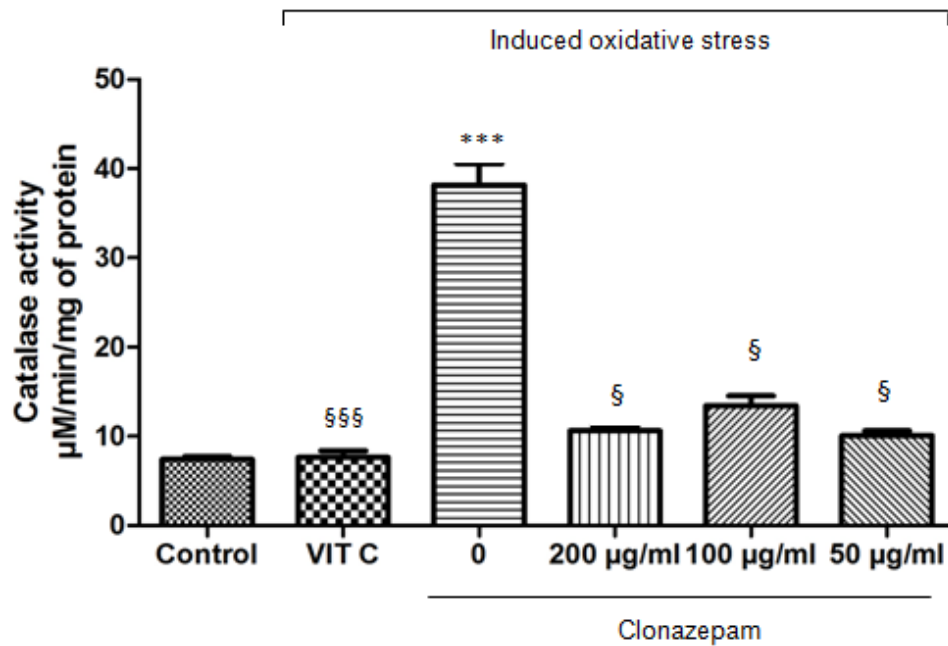
Figure 6. CNZ and VIT C were able to approximate the catalase activity of normal levels. Catalase activity in mice brain homogenates submitted or not (control) to oxidative stress in the absence (stress control) or presence of different concentrations of clonazepam (50, 100 or 200 μg/ml) or VIT C (200 μg/ml), after 1 h of incubation of brain homogenate at 37ºC. Each bar represents the mean ± SEM, n=8, ***$p<0.0001$ vs. control group; §$p<0.05$, §§§$p<0.0001$ vs. stress control group (one-way ANOVA followed by post-hoc Student–Newman–Keuls test).
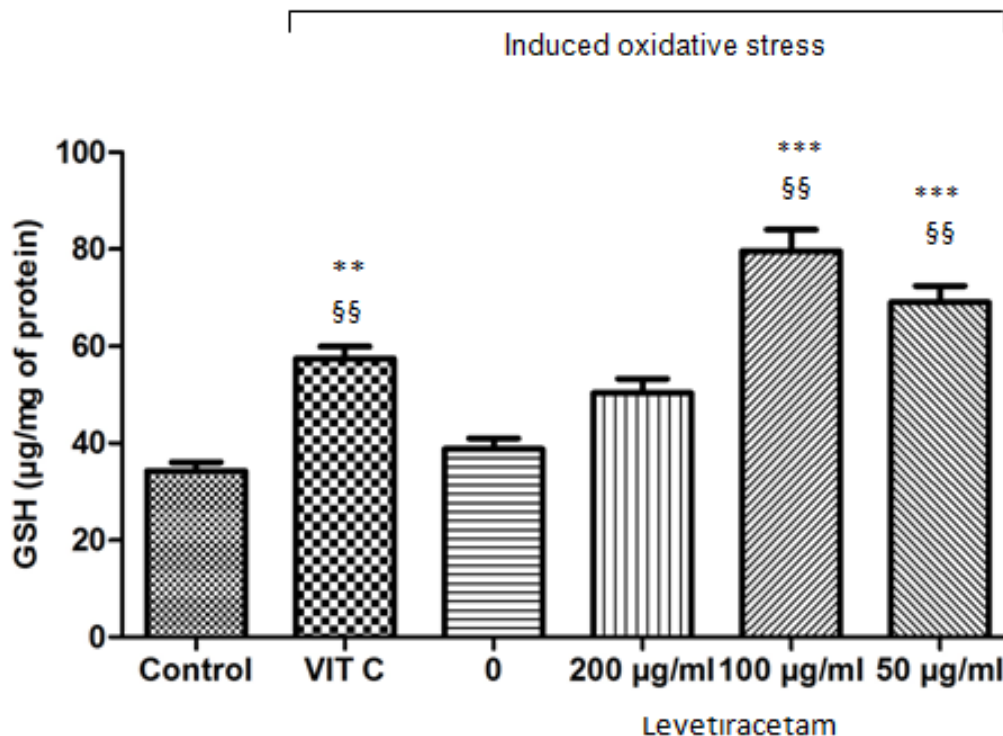
Figure 7. GSH increased especially with the treatment with the two lower concentrations tested of LEV and with VIT C. GSH level in mice brain homogenates submitted or not (control) to oxidative stress in the absence of different concentrations of levetiracetam (50, 100 or 200 µg/ml) or VIT C (200 µg/ml), after 1 h of incubation of brain homogenate at 37ºC. Each bar represents the mean ± SEM, n=8, **p<0.001, ***p<0.0001 vs. control group; §§p<0.001 vs. stress control group (one-way ANOVA followed by post-hoc Student–Newman–Keuls test).

CNZ200: 26.86 + 0.49; CNZ100: 28.44 + 0.06; CNZ50: 33.89+ 1.54; VIT C: 57.4 + 4.31) (Figure 8), results were expressed in µg/µg of protein.

DISCUSSION

In this study, to further investigate the antioxidant properties of levetiracetam and clonazepam, these drugs were tested in an established and specific *in vitro* model of oxidative stress, proposed by Stocks et al. (1974) and modified by Mattei et al. (1998) and Huong et al. (1998). This model is based on the fact that brain homogenates autoxidize spontaneously and reproducibly when heated to 37°C for 1 h, and this preparation may be used to assay the antioxidant effect of several agents in neuronal substrate (Huong et al., 1998; Mattei et al., 1998; Stocks et al., 1974). Moreover, to verify the potential antioxidant capacity of these AEDs in this model, the results obtained were compared with these drugs with the powerful water-soluble antioxidant vitamin C (VIT C). Previous studies have demonstrated not only the well-recognized antioxidant effects of VIT C, but also its anticonvulsant action, suggesting that this drug behaves as a potential

neuronal protective agent, capable of attenuating the behavioral and pro-oxidative changes involved in development of seizures (Santos et al., 2009; Xavier et al., 2007).

Our results showed, as expected, that samples submitted to this *in vitro* stress model showed an increase in MDA and nitrite-nitrate concentrations and higher levels of catalase activity when compared with control samples not submitted to heating. These findings clearly indicate that an oxidative process occurred in brain homogenates submitted to this model. Furthermore, the elevation in free radical formation can be accompanied by a rapid compensatory increase in the activity of free radical scavenging enzymes (Ferreira and Matsubara, 1997; Bellissimo et al., 2001). The high level of catalase activity observed in our results suggests development of an adaptive mechanism against oxidative stress and further supports that this process occurs in this model.

In a similar fashion to VIT C, treatment with LEV and CNZ, at all doses tested, was able to prevent the elevation of MDA levels induced by *in vitro* stress model, with values of MDA similar to those observed in the control group. Regarding nitrite-nitrate concentrations,
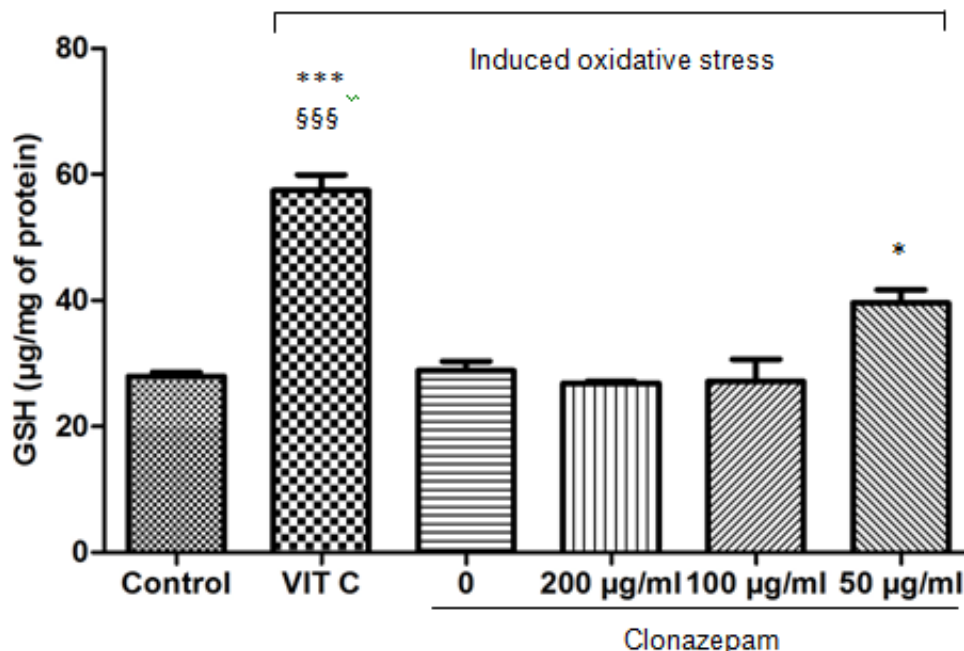
Figure 8. GSH increased especially with treatment with the lower concentration tested of CNZ and with VIT C. GSH level in mice brain homogenates submitted or not (control) to oxidative stress in the absence of different concentrations of clonazepam (50, 100 or 200 µg/ml) or VIT C (200 µg/ml), after 1 h of incubation of brain homogenate at 37ºC. Each bar represents the mean ± SEM, n=8, *$p<0.05$,***$p<0.0001$ vs. control group; [§§§]$p<0.0001$ vs. stress control group (one-way ANOVA followed by post-hoc Student–Newman–Keuls test).

both AEDs demonstrated the capacity to reduce the changes induced by heating, mainly at the lowest doses. VIT C was also able to reduce considerably the nitrite-nitrate concentrations in brain homogenates. Catalase activity was restored to control levels with pre-incubation with LEV and CNZ, and the same effect was observed in samples incubated with VIT C. Finally, the GSH amount was increased by the incubation with both AEDs, especially in lowest doses, and also by incubation with VIT C, when compared with groups submitted to stress procedure.

The degree of lipid peroxidation in tissues is commonly measured by determining the amount of mean product of this process, MDA, and this analysis may relate directly to the level of ROS-mediated injury to a tissue (Kunz et al., 2008; Schihiri, 2014). Free radical-mediated lipid peroxidation proceeds by a chain mechanism, that is, once it was initiated, free radicals can oxidize both lipid molecules in biological membranes and low density lipoproteins. This phenomenon has been shown to induce disturbance to membrane organization and functional loss/modification of proteins and DNA (Frantseva et al., 2000; Schihiri, 2014).

The generation of oxidative damage appears to result from excitotoxicity mechanisms already recognized for participating in several pathological conditions that lead to the development of seizures (Dubinsky et al., 1995;

Duchen, 2000). In this context, overactivation of the glutamate receptors, especially the NMDA receptors, would lead to an increase in intracellular calcium, resulting to mitochondrial dysfunction and generation of ROS and pro-apoptotic factors, fundamental for the damage and cell death (Patel, 2004).

In this context, it has been found that NO is produced in response to N-methyl-D-aspartate (NMDA) receptor stimulation and may be involved in the modulation of neuronal damage (Ferrer et al., 2000; Mülsch et al., 1994). Moreover, the literature has suggested that seizures induce alterations in NO metabolism, increasing the production of its reactive metabolites (nitrite and nitrate). These metabolites, in turn, can interact with NMDA glutamatergic receptors and potentiate its excitotoxicity action on the CNS (Santos et al., 2009). Therefore, elevated nitrite-nitrate levels may be implicated in lipid peroxidation and others oxidative damages on brain tissues submitted to injury situations, as seizures.

Interestingly, according to our findings, LEV and CNZ, similarly to VIT C, was able to reduce the oxidative stress in brain homogenates by decreases of both TBARS and nitrite-nitrate concentrations. Together, these results reinforces the participation of nitrergic pathway, mainly through NO, in oxidative damage in brain tissues and the ability of these antiepileptic drugs, at least in part, through

their antioxidant properties to protect the neurons of oxidative damage.

Furthermore, there is an endogenous ROS scavenging enzyme system, involving the cooperative action of superoxide dismutase (SOD), catalase and glutathione peroxidase (GSH-Px) enzymes. The oxidative stress involved, for example, in status epilepticus and seizures can alter the activity of these enzymes, such catalase, promoting an adaptive cellular response to increased free radicals milieu (Ferreira and Matsubara, 1997; Freitas et al., 2004; Bellissimo et al., 2001). In this context, pre-incubation with LEV and CNZ, preserved the catalase activity in control levels. VIT C was also able to preserve the catalase activity at levels similar to controls samples. These results indicated a possible reduction in toxic reactive substrates in the samples incubated with both AEDs and suggest that these drugs, similarly to a powerful exogenous antioxidant, could help the brain cells to counteract the stress-induced reactive oxygen species overproduction and the oxidative damage.

In the brain, GSH is thought to play a central role in defense against reactive oxygen species. This substance can either directly detoxify reactive oxygen species as can act as a substrate for several peroxidases (Dean et al., 2011). Under conditions of overproduction of free radicals or a deficiency of antioxidant systems, GSH is consumed and its levels fall (Wang and Cynader, 2000). In the present work, no alterations in GSH concentrations were observed in the group of samples submitted to oxidative stress compared to control. However, an increase in this biomarker was induced by pre-incubation with the two lowest tested concentrations of LEV and by the lowest tested concentration of CNZ. An important augment in GSH amount also occurred in samples treated with VIT C. This effect may be probably attributed the capacity of these compounds to avoid the depletion of endogenous antioxidants reserves by the reactive species. Additionally, some of these drugs may potentiate the biosynthesis of brain antioxidants, such as GSH, increasing their amounts. In this context, according to Al-Shorbagy et al. (2013), LEV would be able to up-regulate the action of cystine/glutamate exchanger and to increase the cysteine concentrations in glial cells, the limiting substrate to production of GSH (Al-Shorbagy et al., 2013; Arakawa and Ito, 2007; Kau et al., 2008).

Our findings demonstrate no concentration dependent effects of both LEV and CNZ on the parameters investigated. In fact, in general, the lowest concentrations tested present the highest antioxidant capacity and this property became distinctly smaller with the higher concentration of LEV and CNZ. These results suggest that high concentrations of these drugs could inhibit part of the endogenous antioxidant defense. In accordance with this, previous reports pointed to pro-oxidative effects related to long-term therapy or to administration of high doses of AEDs. In this context, increase in TBARS and decrease in the levels of thiols were detected in blood samples from patients under long-term AED therapy

(Alshafei et al., 2013; Devi et al., 2008; Tan et al., 2009).

Therefore, this study reinforces that the pro-oxidant/antioxidant balance in brain is modulated by antiepileptic drugs. In addition, the ability of these compounds to reduce brain damage caused by seizures and their biochemical changes (that is., markers of oxidative stress) further supports the involvement of free radicals in seizure generation and highlights the possible role for antioxidants as adjuncts to antiepileptic drugs for better seizure control (Devi et al., 2008).

Conclusion

In the present work, it was demonstrated, by an *in vitro* model, the antioxidant ability of two antiepileptic drugs, with different mechanism of action, and that this property is mediated, at least in part, by reduction of lipid peroxidation and nitrite-nitrate contents, preservation of catalase activity at control parameters and increase of GSH levels. These results allow us to infer the participation of this effect in the neuroprotective mechanism of these drugs, and reinforce, even indirectly, the hypothesis of oxidative damage in the pathophysiology of epilepsy.

Additionally, our findings also suggest a potential therapeutic use of antioxidant compounds as alternative or complementary tools to the conventional treatment for this disease. However, more studies need to be performed to elucidate the relation between antioxidant activity and protection against seizure development and epilepsy.

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Conflict of Interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Steady-state bioequivalence study of clozapine psychotic patients in Brazil

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The present work aimed to compare the relative bioavailability of Zolapin with 100 mg tablet Leponex as a reference formulation, through a simple, robust and low-cost bioequivalence assays method. The study design was multiple-dose, randomized, crossover with patients in steady-state, and was performed with 24 schizophrenic male patients. Subjects received 100 mg twice a day of either Leponex or Zolapin for 10 days. At day 10 days of each study phase, blood samples were collected at different times during 12 h after drug administration and the clozapine concentration was determined by high performance liquid chromatography (HPLC). The individual peak plasma concentrations (C_{max}) and the area under the concentration-time curve (AUC_{0-12h}) ratios were calculated. The evaluated pharmacokinetic parameters were quite similar for both formulations. The 90% confidence interval for mean ratio of lnC_{max} (0.9677 to 0.9937) and $lnAUC_{0-12h}$ (0.9811 to 1.0029) were within accepted international guidelines. The results demonstrate that this methodological approach was able to identify Zolapin as bioequivalent to Leponex when orally administered, both in terms of the rate and extent of absorption, and therefore, suitable as a potential low-cost alternative to branded antipsychotic drugs.

Key words: Clozapine, bioavailability, pharmacokinetics, schizophrenia.

INTRODUCTION

The American Psychiatric Association Guidelines for the Treatment of Schizophrenia establish that, after the failure of two or three treatments with atypical antipsychotics, the patient should be considered as bearer of refractory schizophrenia (RS). This means that the patient is a good candidate for treatment with
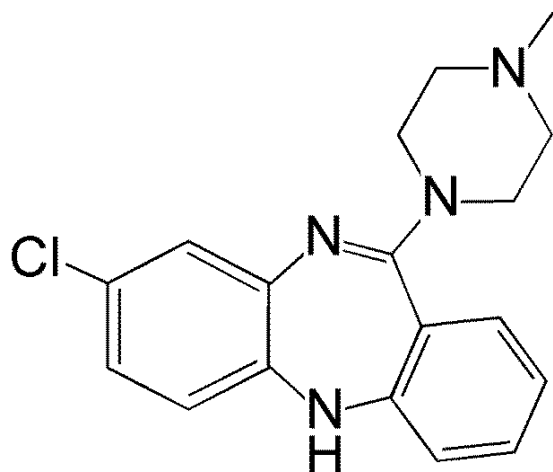
Figure 1. Chemical structure of Clozapine.

bearer of refractory schizophrenia (RS). This means that the patient is a good candidate for treatment with clozapine, the treatment of choice in refractory schizophrenia (third-line agent) in the last two decades (Warnez and Alessi-Severini, 2014). Clozapine is a tricyclic dibenzodiazepine derivative (Figure 1), that was introduced in clinical studies in the United States in the 70s, as the first atypical antipsychotic (Wohlfarth et al., 2011) and has been serving as the best alternative medication for patients with treatment-resistant schizophrenia who failed to respond to other antipsychotics (Suzuki et al., 2011; Kane and Correll, 2010). It is also effective for the treatment of aggressive behavior of schizophrenic and demented patients and in the management of psychosis and aggression in Parkinson's disease (Thomas and Friedman, 2010).

Even though clozapine has been very effective to schizophrenic patients refractory or intolerant to classical antipsychotic therapy, and also induced a good recovery during therapy in some patients with chronic psychosis, a relative risk of serious blood disorders such as agranulocytosis has restricted its use (Asenjo et al., 2010; Meltzer, 2012). Other haematological effects include leucopenia, neutropenia and eosinophilia (Herceg et al., 2010). However, these side effects can be detected, prevented, minimized and treated, and the number of fatalities associated with these effects is actually much reduced (Warnez and Alessi-Severini, 2014; Meltzer, 2012; Herceg et al., 2010; Drew, 2013). The use of clozapine at doses higher than 600 mg daily should follow published recommendations, in order to minimize the risk of seizures; these include anticonvulsant regimens based on blood levels (Cohen et al., 2012).

The success of clozapine treatment is also strongly dependent on the drug´s bioavailability. In this context, 90

to 95% of clozapine is rapidly absorbed following oral administration, and is subject to first-pass metabolism, resulting in an absolute bioavailability of 50 to 60%. The time of onset of maximum plasma clozapine concentration after a single oral dose (75 or 100 mg) is approximately 1.5 h (average, at 2 to 3 h) and its half-life of elimination reaches 4 to 12 h (average of 8 h), in general. Clozapine's plasma concentration may vary widely, depending on several factors, especially at higher doses, above 500 mg/day (Tassaneeyakul et al., 2005). Individual factors may also change the response to this drug, such as cigarette smoking, that induces the metabolism of clozapine (Lucas and Martin, 2013). Clozapine is extensively metabolized by CYO1A2 in the liver and the major metabolites detected in urine and in the plasma are norClozapine (N-desmethylClozapine) and (Clozapine N-oxide) (Wohlfarth et al., 2011). The desmethyl metabolite has only limited pharmacological activity while the hydroxylated and N-oxide derivatives are inactive.

Usually, studies involving the determination of bioequivalence of clozapine employ Leponex (Novartis Pharma AG) as a reference drug. Leponex is an important and widely employed treatment option for many cases of schizophrenia, even though it remains a relatively expensive drug. Therefore, the availability of similar formulations on the market offers an option for patients, especially on developing and low-income countries. In the healthcare scenario of developing countries such as Brazil, in order to deal with high cost of medicaments for the general low-income population, generic drug policies have been implemented since the 90´s, with the aim of encouraging commercial competitiveness, improve the quality of medicines given its interchangeability with the brand drug and increase the population's access to treatment, reducing significantly the cost of drug therapy (Araújo et al., 2010). This legislation recommends that the generic drugs are at least 35% cheaper than the reference brand name drug (Brazil, 1999). Clozapine, despite the availability of its formula in the public domain, has a high cost for Brazilian health programs. Even though the Brazilian government encourages public-private partnerships for the production of generic drugs, there are few options of neuroleptics similar to clozapine, and any novel medicament proposed as a low-cost alternative to branded antipsychotic drugs would have initially pass in criterious assessments of quality and pharmacological performance through parameters such as bioavailability.

Many methods for evaluation of bioequivalence of clozapine formulations in plasma are available in the literature (Wohlfarth et al., 2011; da Fonseca et al., 2013; do Carmo Borges et al., 2012). Amongst the similar generic drugs commercially available at the time this study was started, Zolapin was chosen as being a potential low cost antipsychotic substance under the

scrutiny of the Brazilian Health Authorities. In the present work, we aimed to present and validate an alternative methodology, with simple, reproducible steps for the performance of effective and low cost evaluation of the relative bioavailability of Zolapin (Meizler Biopharma), a formulation of clozapine, employing Leponex, a brand name of clozapine in Brazil, as a reference drug.

MATERIALS AND METHODS

Drugs and chemicals

Clozapine (Figure 1) was purchased from USP reference standards (cat nr.114107). Dothiepin was purchased from British Chemical Reference Substances (cat nr. 134). Both clozapine and Dothiepin were kindly provided by Meizler, São Paulo, Brazil. Other chemicals used in the study were of analytical grade and purchased from Tedia Brazil. Clozapine formulations employed in this study were 100 mg Leponex® tablets (Novartis Pharma AG, Stein, Switzerland, batch no. ZO 007), and 100 mg Zolapin® tablets (Synthon BV - Nijmegen - Holand - batch no. 04H 13 GAC). For disambiguation, it is important to observe that another drug, manufactured by Konark Life Sciences (India), employs the similar name Zolapin MD, but it is a generic formulation containing Olanzapine, and was not used in the present study.

Solutions

Stock solutions of the analytes were prepared by dissolving suitable amounts of each pure substance in methanol (673.300 µ/ml clozapine) and water (0.0236 µ/ml Dothiepin, internal standard). Standard solutions were prepared by diluting stock solutions with the mobile phase.

Subjects

The study protocol was approved by the Ethical Committee of the Bonsucesso General Hospital at Rio de Janeiro, Brazil, under the no. 06/04. Thirty-six male patients regularly admitted and institutionalized in the private EGO Psychiatric Clinic, Itaboraí, Rio de Janeiro, Brazil, were enrolled in the study, 34 of whom completed the clinical steps and 24 were included in the statistical analysis. The test subjects or the person legally responsible for decisions on the subject's behalf were informed, both verbally and in writing, about the experimental procedures and the purposes of the study. A written informed consent was obtained from each participant in the study, following the operational procedures of the Clinical Research Center, nowadays renamed as Clinical Research Unit, at the Fluminense Federal University - UFF - Niterói, Brazil. The personal data were: all male patients (n = 24) aging between 27 to 52 years (mean ± SD, 40.6 ± 7.6); body weight range from of 71.7 to 95.0 kg (mean ± SD, 82.43 ± 12.4), and their body mass indices were in the range of 18 to 34. Exclusion criteria consisted of medical history of allergy to clozapine (or to other anti-psychotic drugs), liver disease, kidney disease, gastrointestinal disorders, cardiovascular diseases, blood disorders, hepatitis, drug abuse, alcoholism, coexisting psychiatric disorders in Parkinson's disease, other psychotic disorders, affective disorders, dyskinesias and related disorders, AIDS or HIV sero-positive (Agência Nacional de Vigilância Sanitária (ANVISA), 2002; Food and Drug Administration (US-FDA), 2014). The blind code was disclosed after all measurements, calculation and plots made as in order to characterize the double-blind study.

Study design

The study was conducted using a multiple-dose, randomized, two-way crossover study design, in three steps:

Step 1: Entry treatment. All the patients received increasing doses of the reference formulation - Leponex (Novartis Pharma AG, Stein, Switzerland) for seven days according to the following schedule: day-1, 12.5 mg after breakfast (8:00 am) and 12.5 mg after dinner (8:00 pm); day-2 and day-3, 25 mg the same time as the first day; day-4, 50 mg after breakfast and 75 mg after dinner; day-5 and day-6, 75 mg twice daily and the day-7, 100 mg twice daily. Drug administration was performed by the nurses of the EGO Psychiatric Clinic.

Step 2: Randomized Exposure. Patients were randomly divided into 2 groups: (i) one group received 100 mg twice daily of the Leponex (Novartis Pharma AG, Stein, Switzerland) and (ii) the other received 100 mg twice daily of the Test formulation, Zolapin (Synthon BV-Nijmegen-Holand), for 10 days, in order to reach the steady-state. At the end of this period, on day-11, the patients were transported to the Clinical Research Center, under medical supervision, and blood samples were taken for analysis by venipuncture in heparinized Vacutainer® blood collectors' tubes. This first sample was collected after 12 h fasting, just before the single morning dose administration of either the test or the reference drug. The subjects were subsequently given one tablet of 100 mg clozapine orally with 120 ml water and blood samples were collected at 0.25, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 5, 6, 8, 10 and 12 h after drug administration (via IV catheter). Blood samples were centrifuged at 1200 × g for 10 min and the supernatant plasma was separated, transferred into polypropylene test tubes and frozen at -20°C until the assay.

Step 3: Crossover. In this phase, a crossover exposure was performed as previously described (Mercolini et al., 2007; Rosland et al., 2007; Golden and Honigfeld, 2008). Patients from each group received a similar treatment, but changing the clozapine formulation from the previous step, that is, patients who received Leponex during Step 2 now received Zolapin during Step 3, and *vice-versa*. There was no period of "washout" between the two treatments (Steps 2 and 3). During the study, patients received close assistance of the medical team. Every meal was standardized by an accredited nutritionist according to the protocol study and the main course was around 2.500 calories. The patients were allowed to drink water freely, except one hour before and one hour after drug administration. After the study, at the discretion of physician, the patients continued to receive 100 mg twice daily of the clozapine reference formulation (Leponex) or had their dose adjusted for other clinically more effective drugs.

Chromatographic apparatus and conditions

The chromatographic system consisted of a HPLC PerkinElmer Series 200, isocratic pump and a detector UV-VIS (Perkin-Elmer, USA). The analytical column used was a Lichrospher RP 18e 5 µm stainless steel column (125 × 3 mm) (MACHEREY-NAGEL GmbH & Co. KG, Germany). The solid phase extraction (SPE) was performed manually, using a Sep-Pak Vac® ColumnStrata C18-E (55 µm, 70 A), 100 mg/3 ml (Waters, USA).

Method validation

Calibration curves

Aliquots of standard solutions at six different concentrations, as

follows: 3.905, 1.953; 0.976; 0.488; 0.0976 and 0.0195 µg/ml, were added to blank plasma. Three calibration curves were obtained with two aliquots of each concentration in each curve. Blank plasma was obtained from the blood of healthy volunteers and subjected to the same procedures reported above for patients' plasma.

Determination of Clozapine in plasma

Plasma concentration of clozapine was assayed by an isocratic HPLC method with UV absorbance detection - HPLC PerkinElmer, Series 200 (PerkinElmer, USA). Initially the plasma was heated at 37°C in water-bath, being placed in polypropylene tubes by shaking for 10 min in vortex, followed by centrifugation (1 min at 1200 g). Subsequently, 0.5 ml of each plasma sample were transferred into Eppendorf tubes and 100 µl of the internal standard (Dothiepin 23.6 mg/ml) were added and mixed briefly. After that, 1 ml 50 mM phosphate buffer solution adjusted to pH 10.0 with NaOH (4 M) was added and vortexed briefly.

Plasma samples were pre-treated by means of solid phase extraction (SPE) with Sep-Pak® Vac 100 mg tC18 cartridges (Waters, USA). The cartridges were equilibrated with 1 ml of methanol and 0.3 ml of phosphate buffer under gentle vacuum. The prepared plasma samples were loaded into the cartridges (2 × 0.7 ml). Washing was carried out with 0.5 ml of phosphate buffer and a 1:1 mixture of methanol and water (2 × 0.75 ml), under gentle vacuum. The analytes were eluted with 0.3 ml of a mixture of mobile phase and acetonitrile (6:4) also on gentle vacuum and 20 µl were injected into the HPLC system. The mobile phase was composed of 7:3 10 mM o-phosphoric acid and acetonitrile, 4 ml/L triethylamine and 2 mg/L N,N,N',N'- tetramethylethylenediamine, the pH being adjusted to 3.55 using 85% H_3PO_4.

The analytical column was a Lichrospher RP 18e (5 µm) stainless steel (125 × 3 mm) (Macherey-Nagel, Germany). Flow rate of the mobile phase was set at 1.0 ml/min and absorbance was monitored at 245 nm. Standard curves were constructed in the clozapine concentration range of 19 to 4000 ng/ml. The standard curve samples were treated in the same manner as the plasma samples collected from the volunteers.

Data analysis

Pharmacokinetic parameters of bioequivalence were set from drug concentration-time curves set by the study protocol. The outstanding measures were: $AUC_{(0-12h)}$, Maximum stabilized plasma concentration (C_{max}), Minimum stabilized plasma concentration (C_{min}) and time of maximum plasma concentration (T_{max}). The area under the clozapine concentration-time curves from 0 to 12 h (AUC_{0-12h}) were calculated using the trapezoidal method and the elimination rate constant (K_e) was estimated using straight regression adjusted in 4 to 6 last log-transformed concentration values.

The relationship between pharmacokinetic variables such as C_{max}, C_{min}, T_{max} and (AUC_{0-12h}) were used to compare the reference and test, determined using analysis of variance (ANOVA) after log-transformed data. The points were estimated at a 90% confidence interval. According to the standard criteria of the Brazilian National Health Surveillance Agency (ANVISA) and the United State Food and Drug Administration (US-FDA), bioequivalence of the two formulations was established when formulation or treatment effect of AUC_{0-12h} and C_{max} should not be different at alpha level of 0.05 and the 90% confidence interval of the mean ratio of AUC_{0-12h} and C_{max} between the tested product and the reference product should stand within the range of 0.80 to 1.25 for log-transformed data. (ANVISA, 2002; US-FDA, 2014).

RESULTS

Under described chromatographic conditions, the retention times for clozapine and internal standard (Dothiepin) were 1.75 at 2.29 and 4.17 at 5.69 min, respectively (Figure 2). Selectivity was evaluated by injecting samples drawn from six different healthy volunteers and none of them produced peaks which could interfere with the analysis. The calibration curves were linear over the range of 20 to 4000 ng/ml. No other peak appeared at the retention times of the compounds of interest (Figure 2B). Within-day coefficients of variation (determined in 5 replicated samples) were 8.45, 6.80 and 7.24% at clozapine concentrations of 40, 1800 and 3200 ng/ml, respectively.

Within-day accuracies were 108.10 ± 8.45, 104.99 ± 6.80 and 100.23 ± 7.24% at 40, 1800 and 3200 ng/ml, respectively, while between-day coefficients of variation for clozapine (determined in 5 replicated samples) were 3.70, 6.68 and 11.50% at 40, 1800 and 3200 ng/ml, respectively. Between-day accuracies were 106.05 ± 3.70, 102.21 ± 6.68 and 97.40 ± 11.50% at 40, 1800 and 3200 ng/ml, respectively.

Patients and steady-state determination

All subjects well tolerated both clozapine formulations. No serious adverse effects were reported during the study. Physical examination, laboratory tests and electrocardiograms revealed that none of the subjects suffered from any serious side effect from the clozapine products used in the study and all were tolerant to both formulations. It was allowed concomitant therapies that had been used during the stabilization of serum concentrations of clozapine (step 1). The steady-state drug concentration was determined in each subject at day-5 and day-6 (Step 1) and day-12 and day-13 (Step 2) prior to the morning dose of the drug. The mean of clozapine concentration-time profiles after administration of the test and reference formulations in 24 subjects are showed in Figure 3.

All of the pharmacokinetic parameters calculated for the Test formulation were close to those of the Reference formulation and there were no statistically significant differences between the two products (Table 1). The mean ratios for lnC_{max} and $lnAUC_{0-12h}$ between the Zolapin and Leponex formulations were 0.9807 ± 0.0391 and 0.9920 ± 0.0327, respectively.

Analysis of variance (ANOVA) of the $lnAUC_{0-12h}$ and lnC_{max} obtained from the Test and Reference formulations revealed that the sequence, period or formulation was not significantly different at p<0.05. The 90% confidence interval for $lnAUC_{0-12h}$ ranged from 0.9811 to 1.0029 and for lnC_{max} ranged from 0.9677 to 0.9937. It should also be noted that none of these values
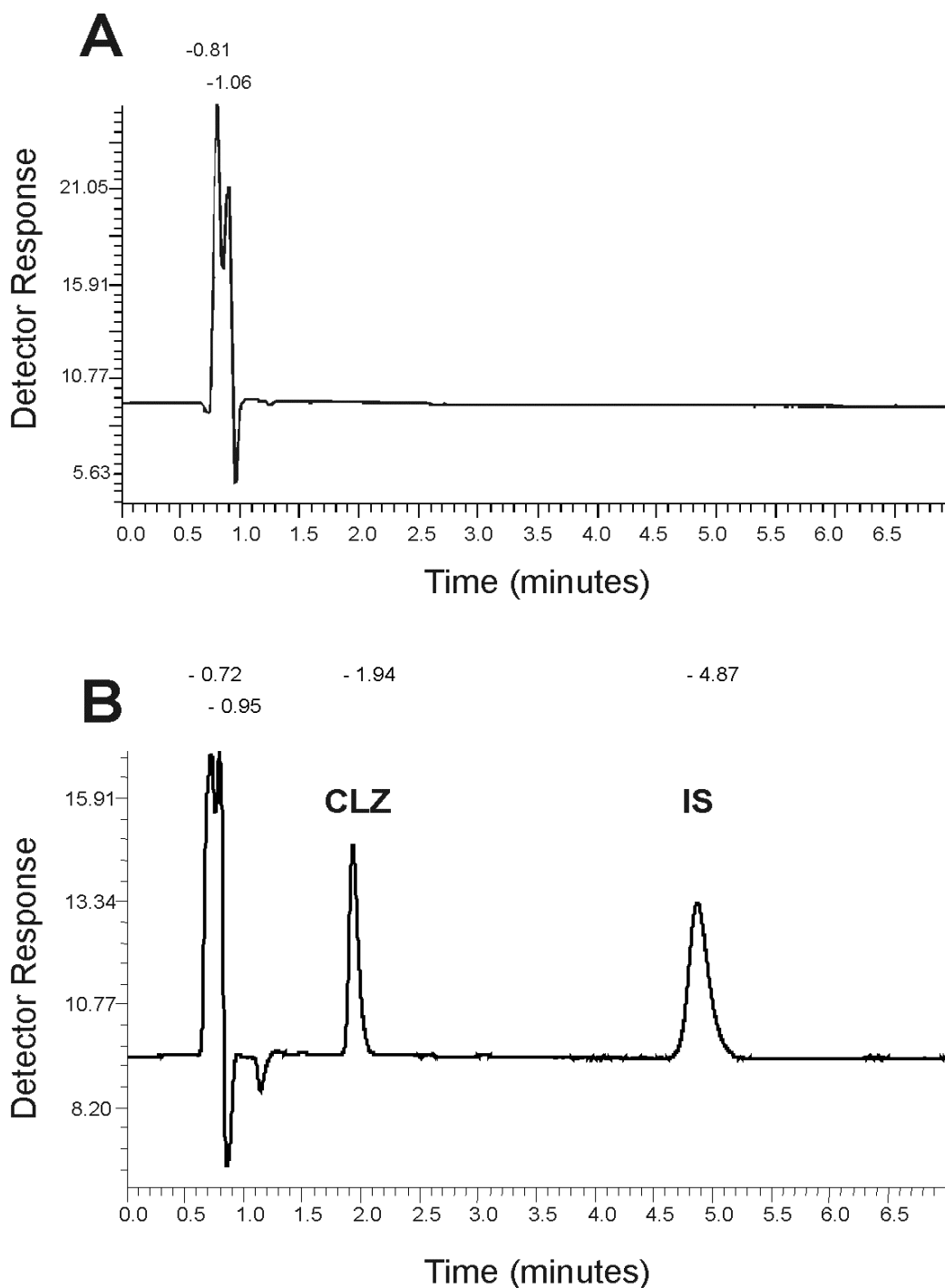
Figure 2. Determination of retention times of Clozapine. A. Chromatogram of a blank plasma sample. B. Plasma sample containing clozapine and Internal Standard.   CLZ= Clozapine   IS= Internal Standard (Dothiepin).

obtained from 24 subjects were outside the range of 0.8 to 1.25. In addition, ANOVA of $\ln T_{max}$ revealed that these two Clozapine products were not significantly different from each other (Table 2).

DISCUSSION

Recent reviews and meta-analysis on clinical practice guidelines and prescribing trends on schizophrenia
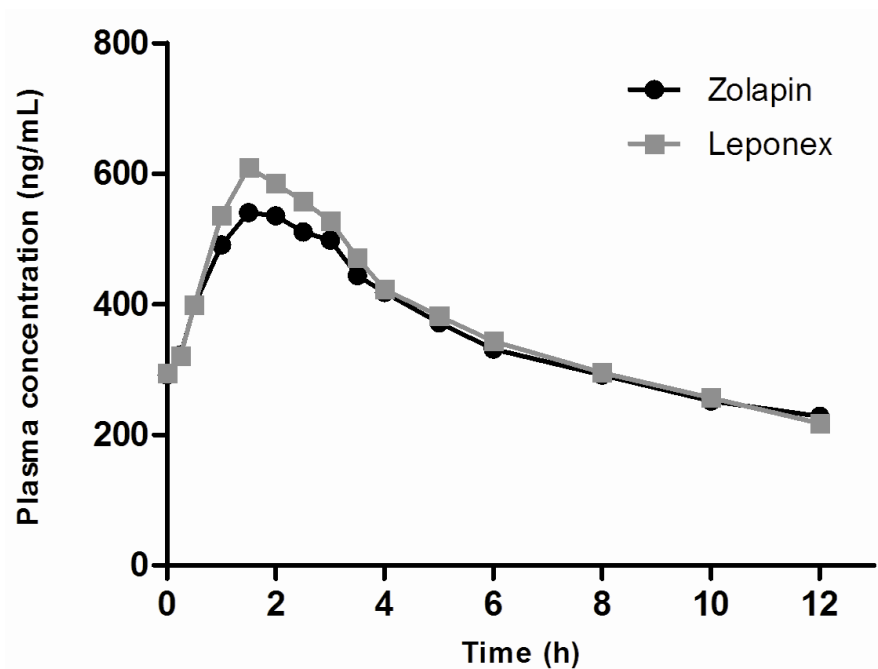
Figure 3. Mean plasma concentration of Zolapin and Leponex after administration. n=24 subjects.

Table 1. Mean pharmacokinetic parameters of Clozapine obtained from 24 subjects after administration of either test or reference formulation.

Parameter	Mean	Median	SD	Minimum	Maximum
Tmax (h)					
Zolapin	1.83	1.50	0.65	1.0	3.0
Leponex	1.81	1.50	0.59	1.0	3.0
Cmax (ng/ml)					
Zolapin	627.66	554.48	304.63	246.50	1284.82
Leponex	690.07	582.74	276.63	280.61	1301.36
Ke (h^{-1})					
Zolapin	0.08	0.08	0.03	0.04	0.14
Leponex	0.08	0.08	0.03	0.03	0.14
t1/2 (h)					
Zolapin	4.15	3.69	1.60	2.14	7.54
Leponex	4.07	3.73	1.68	2.19	8.96
AUCtk (ng/h ml)					
Zolapin	4273.50	3500.57	2549.19	1312.04	11040.16
Leponex	4430.25	2740.11	2379.69	1392.72	10146.08
AUC(∞) (ng/h ml)					
Zolapin	7865.94	5331.03	6009.99	2520.50	24081.06
Leponex	7412.84	4168.23	4471.64	2922.75	19118.47

Table 2. Comparative mean ratio of ln- transformed C_{max} and ln- transformed AUC_{0-12h} of Clozapine in 24 subjects after administration of Zolapin and Leponex.

Subjects nº	Ln (Cmax) Zolapin	Ln (Cmax) Leponex	Zolapin/Leponex	Ln AUC (0-12) Zolapin	Ln AUC (0-12) Leponex	Zolapin/Leponex
1	6.7361	6.287	1.0714	8.6842	8.307	1.0454
2	6.1433	6.3505	0.9673	8.402	8.4919	0.9894
3	5.662	5.9731	0.9479	7.6826	7.8919	0.9735
4	6.6835	6.8039	0.9823	8.662	8.8548	0.9782
5	5.6591	5.637	1.0039	7.3603	7.239	1.0167
6	5.5074	6.1046	0.9022	7.1793	7.7071	0.9315
7	6.3105	6.8021	0.9277	8.355	8.484	0.9848
8	6.351	6.1631	1.0305	8.4407	8.0381	1.0501
9	7.1145	7.009	1.0152	8.9092	9.0728	0.9819
10	5.7592	6.1062	0.9432	7.4685	7.9236	0.9426
11	7.126	7.1712	0.9937	9.186	9.2248	0.9958
12	6.0521	6.286	0.9628	7.7022	7.7963	0.9879
13	6.2313	6.6004	0.9441	8.0203	8.5199	0.9413
14	6.1026	6.5372	0.9335	7.633	8.022	0.9515
15	6.3537	6.3734	0.9969	8.2075	7.757	1.058
16	6.4035	6.362	1.0065	8.1116	8.1118	1
17	6.3255	6.3524	0.9958	8.0663	8.0943	0.9965
18	6.2113	6.0348	1.0292	8.0244	8.0217	1.0002
19	6.909	7.0059	0.9862	8.8481	8.8253	1.0026
20	6.6696	6.8342	0.9759	8.5884	8.5252	1.0074
21	7.1584	6.984	1.025	9.3093	9.0888	1.0242
22	5.9428	6.0109	0.9887	7.5994	7.6023	0.9996
23	6.5527	6.8544	0.956	8.5373	8.704	0.9808
24	6.1079	6.414	0.9523	7.8402	8.1096	0.9667
Mean	6.3364	6.4607	0.9807	8.2007	8.2672	0.992
SD	0.467	0.3998	0.0391	0.5771	0.516	0.0327
90%IC	(6.1796-6.4932)	(6.3265-6.5949)	(0.9677-0.9937)	(8.0069-8.3945)	(8.094-8.4404)	(0.9811-1.0029)

(Warnez and Alessi-Severini, 2014; Sarkar and Grover, 2013) showed the superiority of clozapine for the treatment of refractory schizophrenia compared to other drugs, including for treatment in children and adolescents. In Brazil, the Ministry of Health has Leponex included within Pharmaceutical Healthcare Assistance programs. However, there is still the need for the options of generic clozapine formulations, generating savings in Brazilian health programs, and increasing for patients the right of choice for drugs based on more attractive prices and product quality. In this context, validated and reliable analytical methods allow regulatory agencies to perform bioequivalence studies to evaluate the adequacy of novel generic formulations of clozapine-based drugs for psychiatric patients. The present work involves the determination of the average bioequivalence of a potential generic formulation of clozapine, as compared with the brand formulation, employing statistical analysis of all parameters in compliance with the of both the

Handbook of Good Practices in Bioavailability/Bioequivalence Brazilian Health Surveillance Agency (ANVISA), and the requirements of the US-FDA.

The HPLC method used in this study is simple and straightforward, also providing appropriate sensitivity, specificity for the pharmacokinetic study of clozapine. Previous bioequivalence studies of clozapine (Pokorny at al., 1994) made with healthy patients caused serious adverse effects such as hypotension, bradycardia, and syncope asystole. For this reason, the US-FDA recommends that any bioequivalence study of clozapine should be conducted only in psychiatric patients. Therefore, the present study was carried out following the recent US-FDA Guidance and ANVISA - Brazil Guidance (ANVISA, 2002; US-FDA, 2014), using a multiple-dose, steady-state bioequivalence study in schizophrenic patients. The validation method is a simple, fast and robust HPLC assay for the determination of clozapine

and can be applicable to pharmacokinetic studies and therapeutic drug monitoring, which may be useful in order to determine the optimal dosing for each individual (Cohen et al., 2012).

In the present results, comparing the mean transformed C_{max} and AUC of the 24 volunteers in Table 2, lnC_{max} (0.9677 to 0.9937) and $lnAUC_{0-12h}$ (0.9811 to 1.0029), we verify that they are within the 90% CI, satisfying the condition of bioequivalence between products within the parameters recommended by ANVISA.

Employing the proposed method, a wide interpatient variability in pharmacokinetic parameters was also observed for both clozapine formulations. The large variability in plasma concentrations of clozapine is both due to individual differences in bioavailability and to the fact that clozapine is metabolized by the highly variable enzyme CYP1A2. This enzyme varies among individuals due to induction, inhibition and probably genetic factors (Cohen et al., 2012). The sources of these variabilities also could be explained by age, smoking and simultaneous use of others drugs, which are known for their influence on plasma Clozapine concentrations (Lucas and Martin, 2013). Bioequivalence assays enable the manufacture and distribution of generic drugs for the Brazilian health system at a price much lower than the brand name drug. As clozapine´s reference formulation (Leponex) is an expensive drug and therefore finds limited use in the Brazilian public health system, it is very important to assess possible new formulations of generic clozapine for reducing the cost of treatments (Lewis et al., 2006).

In this context, the present work has shown that candidate generic drugs can have validated analytic methods, following the preconized standards of international studies, providing safe and interchangeable alternatives for patients. The validation of simple methods which can be performed at university and public laboratories must also be an incentive to more initiatives on the production and testing of novel low-cost generic drugs for important clinical conditions. Since the inclusion and exclusion criteria for studies with clozapine are already well stablished, this incentive becomes more straightforward to the generic drug policies in countries like in Brazil, where healthcare services related to the use of this neuroleptic cannot reach the majority of the affected population due to high-cost of medicaments (Araújo et al., 2010; Rumel et al., 2006). Therefore, we believe that simpler and low-cost bioequivalence assays may facilitate the registration of novel products, with their safety incurred by laboratory analyses in governmental laboratories, for widespread distribution to low-income patients.

It is also important to note that not only patients with refractory schizophrenia, but also with Parkinson disease-related psychosis could benefit for generic dlozapine formulations.

Conclusion

The bioavailability of Zolapin is equivalent to that of the reference drug Leponex, when orally administered, both in terms of the rate and extent of absorption, as measured on a simplified and low-cost methodology.

Conflicts of interests

The authors have not declared any conflict of interest.

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Full Length Research Paper

In- *vivo* and *vitro* bioactivities of the essential oil of *Duguetia lanceolata* branches

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The present study investigated the *in- vivo* and *vitro* bioactivities of the essential oil of *Duguetia lanceolata* branches (EODL) and caryophyllene oxide (CO). The chemical composition of EODL was analyzed by GC and GC/MS. Acetic acid-induced writhing, formalin, hot plate and tail-immersion methods were used to evaluate the antinociceptive effect in mice. Anti-inflammatory activity was tested by carrageenan-induced paw edema and pleurisy in rats, and in mouse models of acute ear inflammation induced by croton oil and arachidonic acid. 2,2-Diphenyl-1-picrylhydrazyl (DPPH), reducing power and β-carotene bleaching assays were used to determine the antioxidant bioactivity. β-Elemene, caryophyllene oxide and β-eudesmol were the major components found in EODL. When tested, EODL and CO reduced the number of writhes and both phases of the time of paw licking, while the reaction time on a hot plate and the pain latency duration by the tail-immersion assay were increased. EODL and CO were effective against the paw edema, exudate volume, leucocyte migration and ear edema. EODL and CO also showed *in vitro* antioxidant activity. These results suggest that caryophyllene oxide is one of the active compounds found in EODL and could partially justify the bioactivity of this oil.

Key words: *Duguetia lanceolata*, essential oil, caryophyllene oxide, antinociceptive activity, anti-inflammatory activity, antioxidant activity.

INTRODUCTION

Nociceptive pain derived from a tissue injury occurs due to activation of nociceptors that send signals to the central nervous system in response to a potentially harmful stimulus (Dubin and Patapoutian, 2010; Rodriguez, 2015). The nociceptors are then sensitized by the action of mediators such as acetylcholine, bradykinin, histamine, serotonin, leukotriene, substance P, platelet activating factor, acid radicals, potassium ions, prostaglandins, thromboxane, interleukins, tumor necrosis factor (TNF), nerve growth factor (NGF) and cyclic

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adenosine monophosphate (cAMP), which are present in the tissue environment (Amaya et al., 2013). These mediators are also associated with the development of inflammation. Inflammation is characterized by vasodilatation, increase permeability of the capillaries, migration of leucocytes into the tissue and swelling of the tissue cells (Amaya et al., 2013). In addition, pain and inflammation, as well as the generation of free radicals, have been related to various pathological conditions, including cardiovascular and metabolic complications, diabetes, peptic ulcer, cancer, neurodegenerative diseases (Sen et al., 2010; Carbone and Montecucco, 2015; Fakhoury, 2015). Under these conditions involving pain and inflammation, opioid analgesics, corticosteroids and non-steroidal anti-inflammatory drugs have been widely used (Coutinho and Chapman, 2011; Bacchi et al., 2012; Sousa et al., 2013; Ren et al., 2015). However, the adverse effects of these drugs, such as sedation, dizziness, nausea, vomiting, constipation, physical dependence, tolerance, and respiratory depression, irritation of gastric mucosa and ulcer, water retention and nephrotoxicity, have compromised their therapeutic applications (Benyamin et al., 2008; Slater et al., 2010; Teslim et al., 2014). On the other hand, alternative therapies, highlighting acupuncture, homeopathy, anthroposophy and, especially, herbal medicine have received great attention in recent decades for the treatment of different disorders (Pak et al., 2015). The use of natural products, as essential oils and their components, has been shown large pharmaceutical and pharmacological potentials for the treatment of pain and inflammation associated with oxidative damage (Edris, 2007; Miguel, 2010).

Essential oils belong to the terpenes (mono- and sesquiterpenes), phenylpropanoids and polyketides (Bakkali et al., 2008; Miguel, 2010). These compounds have been studied for their spasmolytic, anxiolytic, anticonvulsant, antinociceptive, anti-inflammatory and antioxidant properties (Bakkali et al., 2008; Miguel, 2010; Wei and Shibamoto, 2010; Sousa, 2011). Regarding the biological activities and the action of oil constituents, the anti-inflammatory activity, for example, has been revealed for limonene, linalyl acetate, β-trans-caryophyllene, 1,8-cineole, p-cymene, thymol, eugenol, chamazulene and α-bisabolol that have inhibitory action on lipoxygenase, an important enzyme involved in the leukotriene pathway (Wei and Shibamoto, 2010; Kamatou and Viljoen, 2010). Another mechanism is based on the action of 1,8-cineole, santoline, spathulenol and caryophyllene oxide, α-pinene, camphor and p-cymene that have a capacity to inhibit the pro-inflammatory interleukins expression (IL-1β and IL-6) (Chao et al., 2005; Juhás et al., 2009). In addition, α-humulene and trans-caryophyllene are able to inhibit the NF-kB activation and neutrophil migration (Passos et al., 2007). These actions can also be related to the antinociceptive effects of components of essential oils since caryophyllene oxide is active in nociception and

inflammation models (Chavan et al., 2010). However, compounds as (-)-linalool and (-)-menthol possess analgesic action by their interaction with several receptors, together with opioids, adenosine A1 and A2, cholinergic M2, and produces changes in K^+ channels (Sousa, 2011).

Duguetia lanceolata St. Hil. (Family: Annonaceae) known as "pindaíba", can be mainly found at the Brazilian Cerrado and Atlantic Forest in Minas Gerais, Mato Grosso and São Paulo States (Sousa et al., 2008). This plant has been used as traditional medicine for various purposes such as anti-inflammatory, cicatrizing, analgesic, sedative and antimicrobial (Sousa et al., 2004, 2008). From the chemical point of view, β-elemene, caryophyllene oxide, β-selinene, β-eudesmol and humulene epoxide II are the most concentrated components in the essential oil from barks and this oil has antimicrobial and cytotoxic properties (Sousa et al., 2012). In addition, the alkaloidal fraction produced a yield of 0.34% (Fischer et al., 2004). Pharmacological studies have proven the antinociceptive and anti-inflammatory (Sousa et al., 2004, 2008) and antiplasmodial (Fischer et al., 2004) activities.

Chemically and pharmacologically, the essential oil from *D. lanceolata* branches (EODL) has not been previously studied, despite few reports published by our research group using other parts (barks and leaves) of this plant species. Based on this fact, in order to establish scientific evidence for the therapeutic uses, the present study evaluated the chemical composition and the antinociceptive, anti-inflammatory and antioxidant activities of EODL using *in- vivo* and *vitro* tests. For a better understanding of these activities, caryophyllene oxide (CO), a reference component, was also assessed.

MATERIALS AND METHODS

Plant material collection, identification and preparation

Plant material from *D. lanceolata* St. Hil. was collected from the city of Juiz de Fora, Minas Gerais State, Brazil (43° 21' 01" W longitude, 21° 45' 51" S latitude) in April 2010. A voucher specimen (CESJ no 29750) was identified, authenticated and deposited in the Herbarium Leopoldo Krieger of the Federal University of Juiz de Fora, Juiz de Fora, MG, Brazil. After collection of plant, the branches were dried at room temperature and triturated in an electric grinder for the extraction of the essential oil.

Extraction of the essential oil

Dried and powdered branches (100 g) of *D. lanceolata* were subjected to extraction by hydrodistillation using Clevenger-type apparatus for 2 h at 100°C. After extraction, the essential oil was dried over anhydrous sodium sulphate and stored in sealed vials at low temperature (-18°C) (Lucchesi et al., 2004).

GC-FID and GC/MS analyses

EODL was subjected to the analysis by gas chromatography

coupled with flame ionization detector (GC-FID) and gas chromatography coupled with mass spectrometry (GC-MS). Quantitative and qualitative analysis of EODL were carried out by using a Shimadzu GC 2010 machine equipped with a ZB-1MS fused silica capillary column (30 m × 0.25 mm × 0.25 μm film thickness). Analysis conditions by GC-FID were as follows: injector temperature, 260°C, detector temperature, 290°C and programmed temperature from 60 to 290°C (10°C/min); hydrogen at 1.0 mL/min was used as carrier gas. Using these conditions, the percentages of the compounds were obtained.

The GC/MS qualitative analysis of EODL was performed by using a GC-QP2010 PLUS Shimadzu machine with a ZB-5MS fused silica capillary column (30 m × 0.25 mm × 0.25 μm film thickness). The parameters used in this analysis were as follows: injector temperature, 270°C, detector temperature, 290°C and programmed temperature from 60 to 290°C (3°C/min); helium at 1.0 mL/min was used as carrier gas. EODL components were identified by comparison of their retention indices and mass spectra with published data (Adams, 2007) and computer matching with WILEY 275 and National Institute of Standards and Technology (NIST 3.0) libraries provided with the computer controlling the GC-MS system. The retention indices were calculated for all volatile constituents using the retention data of linear C8–C24 n-alkanes.

Chemicals and reagents

Chemical and reagents used in this study were as follows: Croton oil, arachidonic acid, capsaicin, dexamethasone, indomethacin, phosphoric acid, rutin and (-)-caryophyllene oxide (Sigma-Aldrich, St. Louis, MO, USA), acetic acid (Vetec Química Farm. Ltda, Rio de Janeiro, RJ, Brazil), potassium ferrocyanide (Labsynth, Diadema, SP, Brazil), formaldehyde and acetylsalicylic acid (Reagen Quimibrás Ind. Química S.A., Rio de Janeiro, Brazil), ascorbic acid (Cromoline Química Fina, Diadema, SP, Brazil), DPPH, morphine hydrochloride (Merck Inc., Whitehouse Station, NJ, USA), naloxone, carrageenan (Sigma Chemical Co, St. Louis, MO, USA), ketamine chloride and xylazine chloride (Syntec, Hortolândia, SP, Brazil).

Animals

Male Wistar rats (90–110 days, weighing 180–220 g) and male Swiss albino mice (50–70 days, weighing 25–30 g) were used for the experiments. All animals were obtained from Central Biotery of the Federal University of Juiz de Fora and the experimental procedures were performed in the Laboratory of Pharmacology of Natural Products of this institution. The animals were grouped and housed in plastic cages (47 × 34 × 18 cm^3) under a 12 h light/12 h dark cycle at room temperature (22±2°C), fed with the balanced feed (Nuvilab Rodents - Nuvital Nutrients, Colombo, Brazil) and received water. After each procedure, the animals were euthanized with an overdose of ketamine and xylazine. Animal care and the experimental protocol were in accordance with the principles and guidelines recommended by the Brazilian College of Animal Experimentation (COBEA) and the rules of the Council for International Organizations of Medical Sciences. Experiments were approved by the local Ethical Committee (protocol number 049/2012).

Acute toxicity

In this procedure, mice were divided into groups of ten animals and received oral doses of 0.5, 1, 1.5, 2 and 3 g/kg of EODL dissolved in sterile saline containing 1% DMSO, and the control group was administered with the vehicle (sterile saline in 1% DMSO). The groups were observed for 48 h and at the end of this period, the

mortality was recorded for each group. General signs of toxicity, motor activity and mortality parameters were recorded for 48 h. The 50% lethal dose (LD$_{50}$) was determined by probit test using a percentage of death *versus* doses' log (Litchfield and Wilcoxon, 1949).

Writhing test

The writhing test was performed according to the method described by Collier et al. (1986) using 0.6% acetic acid as inducer of nociception. Initially, groups of mice (n = 8) were treated with EODL (50, 100 and 200 mg/kg) and CO (10, 25 and 50 mg/kg) orally. The negative control group received, sterile saline in 1% DMSO (control group, 10 mL/kg, p.o.) orally, while the positive control group was administrated acetylsalicylic acid (200 mg/kg, p.o.) and indomethacin (10 mg/kg, p.o.). After 1 h of treatment, intraperitoneal injections (0.25 mL) of 0.6% acetic acid were administered. The total number of writhes was quantified between 10 and 30 min after injection of acetic acid. At this time interval, the acetic acid produced less variation in the number of writhes in each experimental group.

Formalin test

In this experiment, the methodology described by Hunskaar and Hole (1987) was used. The animals were pre-treated with EODL (50, 100 and 200 mg/kg, p.o.), CO (10, 25 and 50 mg/kg, p.o.) and morphine (5 mg/kg, subcutaneous, s.c.). Control animals were treated with similar volume of sterile saline in 1% DMSO (10 mL/kg, p.o.). After 1 h of treatment, 20 μL of 2.5% formalin (in 0.9% saline) were injected under the skin of the dorsal surface of the right hindpaw. The duration of paw licking was determined between 0 and 5 min (first phase) and 15 and 30 min (second phase) after formalin injection. These intervals were established to dissociate nociceptive and inflammatory pain, respectively.

Hot plate test

Following the methodology described by Eddy and Leimbach (1953), the mice were grouped (n = 8) and treated with EODL (50, 100 and 200 mg/kg, p.o.), CO (10, 25 and 50 mg/kg, p.o.), morphine (5 mg/kg, s.c.) and sterile saline in 1% DMSO (10 mL/kg, p.o.). In addition, the effect of EODL (200 mg/kg, p.o.), CO (50 mg/kg, p.o.) and morphine (5 mg/kg, s.c.) in the presence of naloxone (2 mg/kg, s.c.) were also evaluated in separate groups of animals (n = 8). After drug administration, the animals were placed on a hot-plate (Model LE 7406, Letica Scientific Instruments, Barcelona, Spain) at 55 ± 1°C and the reaction times were measured at 0, 30, 60 and 90 min observing two behavioral components, namely paw licking and jumping. To avoid animal paw lesion, the cut-off time was set at 30 s.

Tail immersion test

Based on the description of Ramabadran et al. (1989), the animals were divided into groups of eight animals (n = 8) to perform the tail immersion test. The negative control received sterile saline in 1% DMSO (10 mL/kg, p.o.) orally, while morphine (5 mg/kg, s.c.) was administrated in positive control subcutaneously. Three groups of mice were treated with EODL (50, 100 and 200 mg/kg, p.o.) and three others with CO (10, 25 and 50 mg/kg, p.o.). Then, the reaction times of animals were recorded at 0, 30, 60, 90 and 120 min after the tail immersion in water maintained at a constant temperature (55±1°C). To avoid tissue damage, the cut-off was set

at 10 s. In this test, immersion of an animal's tail in hot water provokes an abrupt movement of the tail and sometimes the recoiling of the whole body.

Carrageenan-induced paw edema method

Carrageenan-induced paw edema in Wistar rats was performed according to the method described previously by Winter et al. (1962). The animals were divided into groups of six, which received oral doses of EODL (50, 100 and 200 mg/kg, p.o.), CO (10, 25 and 50 mg/kg, p.o.), sterile saline in 1% DMSO (10 mL/kg, p.o.) or indomethacin (10 mg/kg, p.o.).

After 60 min of treatment, the paw edema was induced by the injection of 0.1 mL 1% carrageenan (an edematogenic agent) into the subplantar region of the right hind paw of the rat, while in the left paw (used as control), 0.1 mL of sterile saline was injected. The measurement of paw edema was calculated by the difference between the volume displaced by the right and the left paw using a plethysmometer (model LE 7500, Letica Scientific Instruments, Barcelona, Spain) and evaluated at 1, 2, 3 and 4 h postcarrageenan response.

Carrageenan-induced pleurisy method

Using the methodology described by Vinegar et al. (1973) with some modifications, eight groups of animals were separated with six Wistar rats each (n = 6). The groups were treated with EODL (50, 100, and 200 mg/kg, p.o.), CO (10, 25, and 50 mg/kg, p.o.), sterile saline in 1% DMSO (p.o.) or indomethacin (10 mg/kg, p.o.). After 1 h of treatment, the pleurisy was induced by intrapleural administration of 0.4 mL 1% carrageenan suspension in sterile saline between the third and fifth ribs on the right side of the mediastinum.

Animals were euthanized with an overdose of ketamine and xylazine after 4 h of carrageenan injection, and the skin and pectoral muscles were retracted. A longitudinal incision was made between the third and fifth ribs on each side of the mediastinum. Then, the exudate was collected and transferred to a 15 mL conical centrifuge tube and the total volume was determined. A 20 µL aliquot of the exudate was used to determine the total leucocytes using Neubauer haemocytometer chamber under microscopic analysis.

Croton oil-induced ear edema

Croton oil-induced ear edema in mice was performed as described by Schiantarelli et al. (1982). Eight groups with eight mice each (n = 8) were separated and 20 µL of 2.5% croton oil (v/v, diluted in acetone) were administrated topically on the inner surface of the right ear. To accompany the baseline in the groups, 20 µL of acetone (vehicle) were applied in the left ear. After 15 min, the right ears were treated with EODL (0.1, 0.5 and 1.0 mg/ear), CO (0.1, 0.5 and 1.0 mg/ear) or dexamethasone (0.1 mg/ear, positive control), while the negative control (untreated) received 20 µL of acetone. The weight of the ear edema (mg) was evaluated after 6 h of croton oil application by the difference between the masses of the right and left ears.

Arachidonic acid-induced ear edema

Edema was induced in mice (n = 8/group) by topical administration on the inner surface of the right ear using arachidonic acid (AA) (2.0 mg/ear in 20 µL of acetone) according to Young et al. (1984). The left ear received 20 µL of acetone as vehicle. After 15 min, the right ear was topically treated with EODL (0.1, 0.5 and 1.0 mg/ear in 20 µL of acetone), CO (0.1, 0.5 and 1.0 mg/ear in 20 µL of acetone), and indomethacin (2.0 mg/ear in 20 µL of acetone, positive control). The negative control (untreated) received topically 20 µL of acetone on the right ear. As in croton oil test, the weight (mg) of the ear edema was evaluated after 1 h of AA application by the difference between the masses of the right and left ears.

DPPH radical scavenging activity

Using the stable DPPH free radical, the free radical-scavenging activity of EODL and CO was determined by the method of Mensor et al. (2001). For this reaction, EODL (60 to 280 µg/mL) and CO (60 to 280 µg/mL) were prepared and added to an equal volume (0.03 mM) of DPPH in test tubes in triplicate. After 60 min, the absorbance was recorded at 518 nm. Rutin was used as standard control. EC_{50} values denote the concentration (µg/mL) of sample, which is required to scavenge 50% of DPPH free radicals.

Test of iron reducing power

The iron reducing power of EODL and CO was determined according to the method described by Oyaizu (1986). EODL (80 to 320 µg/mL) and CO (80 to 320 µg/mL) were prepared with 0.2 mM phosphate buffer pH 6.6 and 1% potassium ferrocyanide incubation at 50°C for 20 min. This mixture was added to 10% trichloroacetic acid followed by centrifugation at 3000 g for 10 min. Then, the supernatant was mixed with 1% ferric chloride and the absorbance measured at 700 nm in triplicate. Ascorbic acid (0.5 to 10µg/mL) was used as reference substance. EC_{50} (50% effective concentration) values (µg/mL) were calculated and indicated the effective concentration at which the absorbance was 0.5 for reducing power.

β-Carotene/linoleic acid bleaching assay

β-Carotene bleaching assay was carried out according to the method developed by Miller (1971). For this, 0.2 mg/mL β-carotene (1 mL) diluted in chloroform was mixed with linoleic acid (0.02 mL) and Tween 20 (0.2 mL). Using the rotary evaporator (BUCHI, Germany), the chloroform was removed and the mixture was diluted with 100 mL of distilled water to form an emulsion. The emulsion (5 mL) was transferred into different test tubes containing EODL (38.46 to 1.20 µg/mL) and CO (38.46 to 1.20 µg/mL) and placed in a water bath at 50°C for 2 h. Absorbance of the samples was measured at every 15 min for 105 min at 470 nm using a spectrophotometer (UV-VIS Spectrophotometer, Shimadzu). Rutin and BHT were used as positive control (at the same concentration as samples).

All samples were assayed in triplicate and the results were expressed in percentage of inhibition of lipid peroxidation (%) which is the sample concentration providing 50% inhibition of linoleic acid oxidation.

Statistical analysis

Data are expressed as mean ± S.E.M. Statistical significance was analyzed by the one-way analysis of variance (ANOVA) followed by the Student Newman-Keuls or Tukey test. P values below 0.05 were considered significant. The percentage of inhibition was calculated by using: $100 - T \times 100/C$ (%) or $T \times 100/C - 100$ (%); where C and T indicate non-treated (vehicle) and drug-treated, respectively (Jaiswal and Sontakke, 2012). The Graph Pad® Prism 5.0. Software was used for statistical analyses.

RESULTS

Chemical characterization by GC/MS

A total of thirty-seven compounds were identified in EODL and classified as monoterpene hydrocarbons, oxygenated monoterpenes, sesquiterpene hydrocarbons, oxygenated sesquiterpenes and hydrocarbons (Table 1). Among the terpenes identified, thirteen were monoterpenes and twenty-two were sesquiterpenes. β-Pinene (1.1%) and *trans*-pinocarveol (1.0%) were revealed as the most abundant monoterpenes. Considering the sesquiterpenes, β-elemene (8.3%), caryophyllene oxide (7.7%), β-eudesmol (7.2%), β-selinene (7.1%), humulene epoxide II (6.6%) and β-caryophyllene (6.2%) were the most concentrated components.

Acute toxicity

After 48 h of treatment, EODL was toxic to mice with LD_{50} of 2.9 g/kg (95% confidence intervals 1.7-4.9 g/kg). General signs of toxicity (as cyanosis, piloerection, writhing, ptosis, tremors, convulsions, ataxia, hypnosis, red urine and diarrhea) were not detected. The parameters motor activity, such as respiration, corneal reflex, righting and withdrawal, body tone and amount of pats, were also unaffected.

Effects of EODL and CO on acetic acid-induced writhing

The treatment with EODL (100 and 200 mg/kg, p.o.) produced a significant inhibition of the abdominal writhes when compared with the control (Figure 1). After administration of CO, an interesting antinociceptive effect was also observed at doses of 10, 25 and 50 mg/kg with reduction of the number of writhes in 7.81, 11.43 and 13.23%, respectively (Figure 1). As drug references, ASA and indomethacin were effectives against acetic acid-induced nociception.

Effects of EODL and CO on the formalin test

The intraplantar injection of formalin promoted a biphasic characteristic response (first and second phases) (Figure 2). In the first phase (neurogenic), EODL caused a significant and dose-dependent inhibition in the licking time at the doses of 50 (14.34%), 100 (31.3%) and 200 mg/kg (44.4%), while CO reduced the paw licking time by 9.6 and 21.1% at the doses of 25 and 50 mg/kg, respectively. After treatment, doses of 50, 100 and 200 mg/kg of EODL also inhibited the second phase (inflammatory). CO was active at the second phase,

producing a significant response of 10.8 (25 mg/kg) and 13.9% (50 mg/kg). Morphine (5 mg/kg, s.c.), an opioid analgesic, significantly reduced the formalin response in both phases, while indomethacin had no effect in the early phase.

Effects of EODL and CO on the hot plate test

The central effect observed in the first phase of the formalin test was confirmed through the hot plate assay. The effect of EODL and CO in the hot plate test varied according to the doses and the time of observation (Table 2). At 0 and 30 min times, no significant antinociceptive effect was detected. The reaction time in the hot plate significantly increased after 60 and 90 min of treatment with EODL and CO. The treatment was also performed in the presence of naloxone, an opioid antagonist. It was observed that the naloxone was able to inhibit the response of the morphine, but it was not able to completely block the effect of EODL and CO.

Effects of EODL and CO on the tail immersion test

The antinociceptive effect of EODL and CO on the tail immersion latencies is shown in Table 3. After 90 and 120 min of treatment, doses of 100 and 200 mg/kg of EODL and 25 and 50 mg/kg of CO significantly increased the pain latency duration. Morphine was active after 30 min of treatment.

Effects of EODL and CO on the carrageenan-induced paw edema

In this study, EODL and CO showed a significant inhibitory effect on the edema formation at the 3 and 4 h (Figure 3). At 4 h, EODL produced reduction of the paw edema in 18.3 (50 mg/kg), 32.3 (100 mg/kg) and 44.1% (200 mg/kg). In this time, doses of 10, 25 and 50 mg/kg of CO also reduced the paw edema in 11.8, 18.3 and 31.2%, respectively. Indomethacin reduced the paw edema by 25.9% after 4 h of carrageenan application.

Effects of EODL and CO on the carrageenan-induced pleurisy

There was an intense formation of exudate volume and leucocyte migration in the pleurisy of the control group after 4 h of intrapleural application of carrageenan. EODL at the highest dose of 200 mg/kg significantly reduced the carrageenan induced exudate volume and number of leucocytes (50.0 and 32.2%, respectively) as compared to the indomethacin (10 mg/kg) (60.0 and 37.8%, respectively). Decrease of exudate volume and number

Table 1. Constituents of the essential oil of *D. lanceolata* branches.

Compound	RI	Concentration (%)	Identification
α-pinene	936	0.6	RI, GC-MS
verbenene	984	0.5	RI, GC-MS
β-pinene	1018	1.1	RI, GC-MS
p-cymene	1075	1.0	RI, GC-MS
limonene	1033	0.3	RI, GC-MS
p-cymenene	1153	0.5	RI, GC-MS
α-canfolenal	1202	0.2	RI, GC-MS
trans-pinocarveol	1215	1.0	RI, GC-MS
trans-p-2-menthene-1-ol	1225	0.3	RI, GC-MS
terpinen-4-ol	1174	0.3	RI, GC-MS
α-terpineol	1188	0.4	RI, GC-MS
myrtenol	1278	0.7	RI, GC-MS
trans-carveol	1302	0.9	RI, GC-MS
bornyl acetate	1374	0.7	RI, GC-MS
δ-elemene	1336	4.1	RI, GC-MS
α-cubebene	1348	0.1	RI, GC-MS
cyclosativene	1367	1.7	RI, GC-MS
α-copaene	1377	0.4	RI, GC-MS
β-elemene	1390	8.3	RI, GC-MS
β-patchulene	1380	2.1	RI, GC-MS
β-caryophyllene	1420	6.2	RI, GC-MS
aromadendrene	1439	0.3	RI, GC-MS
β-selinene	1489	7.1	RI, GC-MS
α-salinene	1494	1.8	RI, GC-MS
γ-cadinene	1513	1.7	RI, GC-MS
δ-cadinene	1522	5.5	RI, GC-MS
α-calocorene	1548	2.9	RI, GC-MS
palustrol	1557	3.4	RI, GC-MS
caryophyllene oxide	1581	7.7	RI, GC-MS
humulene epoxide II	1642	6.6	RI, GC-MS
β-eudesmol	1654	7.2	RI, GC-MS
cadina-1,4-dien-3-ol	1658	5.2	RI, GC-MS
patchouli alcohol	1659	3.7	RI, GC-MS
cadalene	1674	4.8	RI, GC-MS
caryophyllene acetate	1704	3.4	RI, GC-MS
n-nonadecane	2022	0.1	RI, GC-MS
n-eicosane	2129	0.1	RI, GC-MS
Total (%)		92.9	
Monoterpene hydrocarbons		4.0	
Oxygenated monoterpenes		3.8	
Sesquiterpene hydrocarbons		40.0	
Oxygenated sesquiterpenes		44.9	
Hydrocarbons		0.2	
Unidentified		7.1	
Yield (%)		0.4	

RI: Retention index; MS, NIST and Wiley library spectra and the literature; GC-MS: gas chromatography-mass spectrometry analysis. Data are the means of experiments performed in duplicate.

of leucocytes was also observed at 50 and 100 mg/kg (Table 4). In addition, these biological parameters were significantly inhibited after treatment with CO (10, 25 and 50 mg/kg).
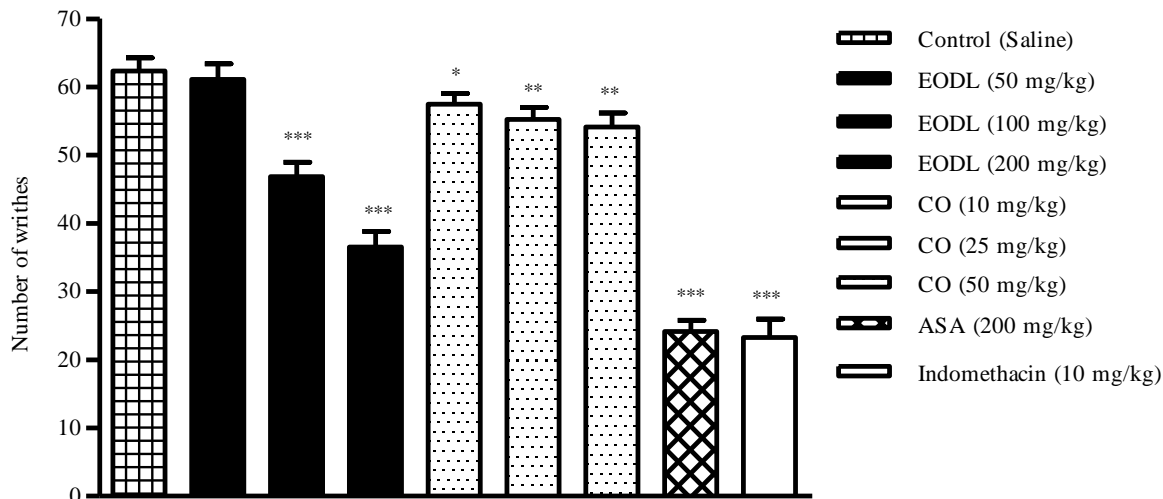
Figure 1. Effects of EODL and CO on acetic acid induced writhing in mice. The data represent the mean ± S.E.M. (n = 8). *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ *vs.* control group after ANOVA followed by Student Newman-Keuls' test. EODL: essential oil from *Duguetia lanceolata* branches; CO: caryophyllene oxide; ASA: acetylsalicylic acid.
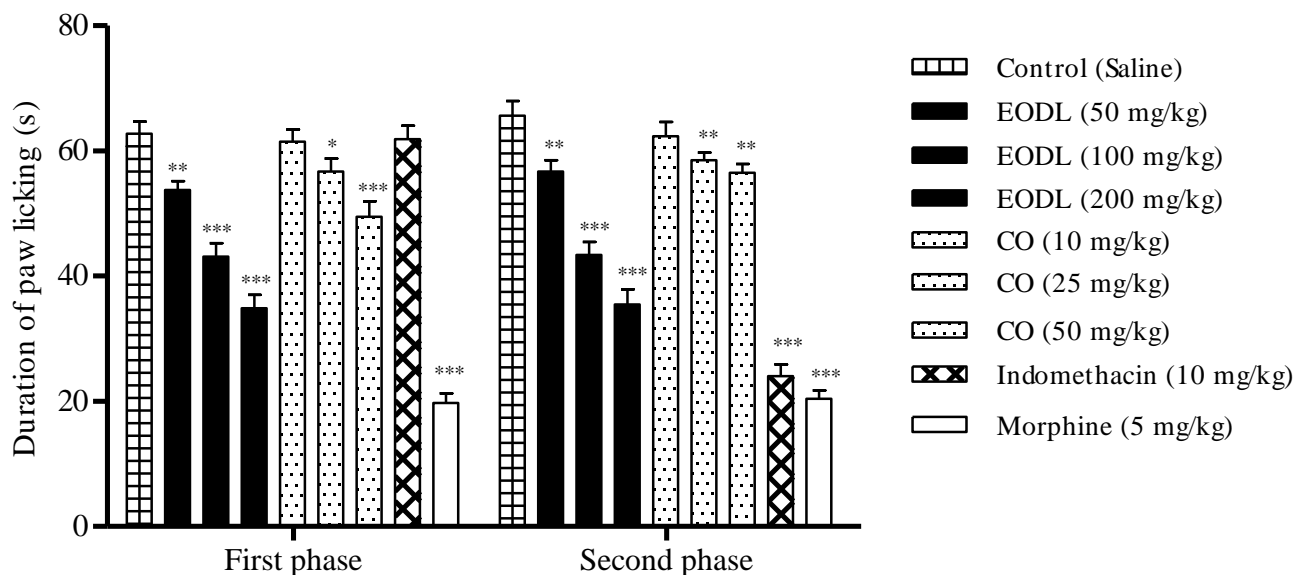

Figure 2. Effects of EODL and CO on formalin-induced nociception in mice. First phase = 0–5 min after formalin injection; second phase = 15–30 min. The data represent the mean ± S.E.M. (n = 8). *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ *vs.* control group after ANOVA followed by Student Newman-Keuls' test. EODL: essential oil from *Duguetia lanceolata* branches; CO: caryophyllene oxide.

Effect of EODL and CO on the croton oil-induced ear edema

The negative control group demonstrated the greatest degree of edema, while the mice group pretreated with EODL and CO revealed significant reduction of the edema in a concentration dependent manner (Figure 4). The positive control (dexamethasone, 0.1 mg/ear) caused a reduction of the edema by 64.6%.

Effect of EODL and CO on the AA-induced mice ear edema

EODL significantly decreased the ear edema induced by arachidonic acid in a concentration-response manner by 21.9, 34.1 and 46.3%, at 0.1, 0.5 and 1.0 mg/ear, respectively. On the other hand, only 1.0 mg/ear of CO was able to inhibit ear edema by 19.5%. The level of inhibition induced by indomethacin (2.0 mg/ear) was

Table 2. Effects of EODL and CO on the latency time of mice exposed to the hot plate test.

Group	Dose (mg/kg)	Time after drug administration (s)			
		0 min	30 min	60 min	90 min
Control	Saline	5.3±0.3	5.8±0.3	6.2±0.3	6.4±0.3
EODL	50	5.5±0.4	6.0±0.3	7.4±0.2*	8.1±0.1*
	100	5.5±0.3	6.2±0.3	9.6±0.3***	10.5±0.4***
	200	5.7±0.3	6.4±0.3	11.2±0.5***	13.6±0.4***
CO	10	5.5±0.2	5.6±0.2	6.4±0.2	6.7±0.2
	25	5.5±0.2	5.6±0.2	7.0±0.3*	7.7±0.3**
	50	5.4±0.3	6.5±0.3	8.5±0.2***	8.9±0.3***
Morphine	5	5.5±0.2	8.4±0.3***	12.1±0.5***	15.5±0.4***
Naloxone+morphine	2+5	5.7±0.4	7.8±0.2***	6.4±0.3	6.6±0.3
Naloxone+EODL	2+200	5.6±0.3	6.2±0.3	8.9±0.3***	10.1±0.5***
Naloxone+CO	2+50	5.5±0.2	6.5±0.3	7.5±0.3**	7.7±0.3**

The data represent the mean ± S.E.M. (n = 8). *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ *vs.* control group after ANOVA followed by Student Newman-Keuls' test. EODL: essential oil from *D. lanceolata* branches; CO: caryophyllene oxide.

Table 3. Effects of EODL and CO on tail-immersion test in mice.

Group	Dose (mg/kg)	Reaction time (s)				
		0 min	30 min	60 min	90 min	120 min
Control	Saline	2.7±0.4	2.8±0.4	2.8±0.4	3.0±0.4	3.0±0.4
EODL	50	2.9±0.5	2.8±0.4	3.3±0.4	3.6±0.5	3.8±0.4
	100	2.9±0.4	3.0±0.4	3.5±0.4***	4.9±0.4**	5.9±0.3***
	200	3.0±0.4	3.2±0.5	4.8±0.4***	7.0±0.3***	7.8±0.5***
CO	10	2.8±0.5	2.9±0.4	3.0±0.3	3.3±0.0.4	3.4±0.4
	25	2.8±0.5	2.9±0.5	3.5±0.3	4.3±0.5*	5.0±0.4**
	50	2.9±0.4	3.0±0.4	4.3±0.3**	6.4±0.4***	6.9±0.4***
Morphine	5	2.9±0.4	4.7±0.3**	6.1±0.4***	8.1±0.3***	8.6±0.3***

The data represent the mean ± S.E.M. (n = 8). *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ *vs.* control group after ANOVA followed by Student Newman-Keuls' test. EODL: essential oil from *D. lanceolata* branches; CO: caryophyllene oxide.

51.2% (Figure 5).

DPPH radical scavenging, Fe^{+3} reducing power and β-carotene bleaching antioxidant activity assessment

The DPPH activity of EODL was higher than that of Fe^{+3} reducing power (Table 5). Using DPPH radical, CO produced EC$_{50}$ greater than 280 μg/mL. In addition, EODL and CO inhibited the lipid peroxidation in 41.5 and 24.1%, respectively. As expected, rutin, ascorbic acid and BHT exhibited potent antioxidant activity (Table 5).

DISCUSSION

One previous chemical study on the essential oil obtained from *D. lanceolata* barks performed by our research group revealed that the major components were sesquiterpenes, β-elemene, caryophyllene oxide, β-selinene, β-eudesmol and humulene epoxide II using GC/MS (Sousa et al., 2012). In the present study, 37 compounds were identified, which accounts for 92.7% of the total oil content with the presence of mono- and sesquiterpenes. Despite the difference in relation to the total oil content and number of components, the chemical composition was similar to barks' oil (Sousa et al., 2012). Interestingly, although some compounds have been found in other species of *Duguetia*, the divergent data regarding the previous component characterization might be related to the constitution of the plant material (Maia et al., 2006; Almeida et al., 2010). It is possible that the similarity of compounds into genus has a chemotaxonomic significance and the major compounds can represent the chemical markers of EODL. In addition, essential oils and their components have been associated
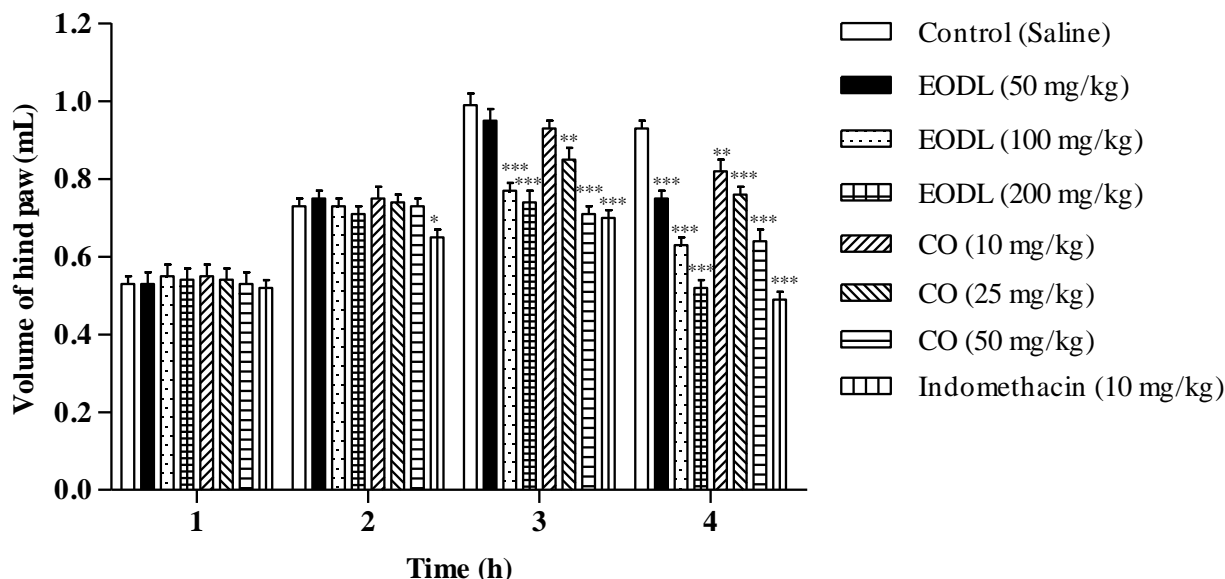
Figure 3. Effects of EODL and CO on the rat paw edema induced by carrageenan. The data represent the mean $\pm$ S.E.M. (n = 6). $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ *vs.* control group after ANOVA followed by Student Newman-Keuls' test. EODL: essential oil from *Duguetia lanceolata* branches; CO: caryophyllene oxide.

Table 4. Effects of EODL and CO on pleural exudation and number of leucocytes in carrageenan-induced pleurisy in rats.

Group	Dose (mg/kg)	Exudate volume (mL)	Inhibition (%)	Nº leucocytes (x 10^3cells/mm^3)	Inhibition (%)
Control	Saline	1.0±0.1	-	14.3±0.2	-
EODL	50	0.8±0.1*	20.0	12.8±0.3***	10.5
	100	0.6±0.1***	40.0	10.5±0.2***	26.6
	200	0.5±0.1***	50.0	9.7±0.2***	32.2
CO	10	0.7±0.1**	30.0	13.0±0.3**	9.1
	25	0.7±0.1**	30.0	12.1±0.2***	15.4
	50	0.5±0.1***	50.0	11.0±0.3***	23.1
Indomethacin	10	0.4±0.1***	60.0	8.9±0.2***	37.8

The data represent the mean $\pm$ S.E.M. (n = 6). $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ *vs.* control group after ANOVA followed by Student Newman-Keuls' test. EODL: essential oil from *D. lanceolata* branches; CO: caryophyllene oxide.

with several biological properties, antinociceptive, anti-inflammatory and antioxidant activities (Bakkali et al., 2008; Miguel, 2010).

The acute toxicity test revealed that EODL (LD_{50} of 2.9 g/kg) can be toxic to mice and this data appeared as crucial to define the doses in the pharmacological experiments (50, 100 and 200 mg/kg). In this test, the major signs of toxicity and parameters motor activity were not affected. However, considering the components found in EODL, β-elemene showed low toxicity when administered to cancer patients (Chen et al., 2012; Li et al., 2013), while CO inhibited the mitochondrial electron transport chain having high toxicity (Monzote et al., 2009). Therefore, our findings are relevant as regard the safety of the use of EODL in the medicinal applications.

The present results show that EODL and CO have antinociceptive effect since they reduced the abdominal writhes in mice (Figure 1). The mechanism might be associated with the blocking of the cyclooxygenase and/or lipoxygenase pathways preventing formation of inflammatory mediators, as prostaglandins (PGE2 and PGF2α) (Deraedt et al., 1980; Schmidt et al., 2010). After application of acetic acid, these mediators were generated in the abdominal cavity and contributed to the increased sensitivity to nociceptors activating the ascending pathways of pain (Deraedt et al., 1980; Schmidt et al., 2010). However, this finding confirms the analgesic effect of CO described by Chavan et al. (2010) and is similar to that reported for the barks' oil (Sousa et al., 2004) and ethanol extract from leaves of *D. lanceolata*
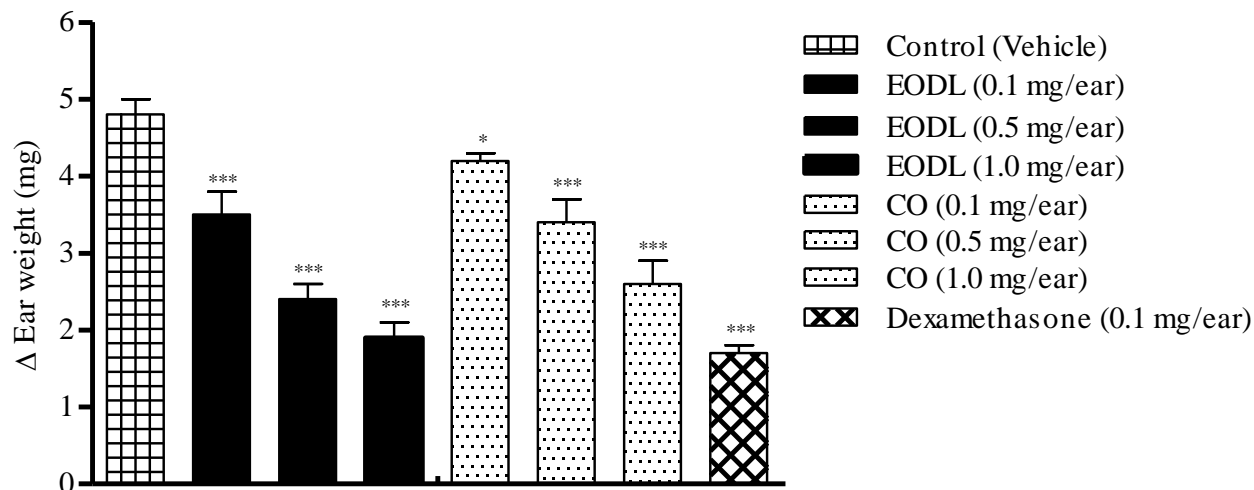
Figure 4. Topical effect of EODL and CO on mice ear edema induced by croton oil. The data represent the mean ± S.E.M (n=8). *$P < 0.05$; ***$P < 0.001$ *vs.* control group after ANOVA followed by Student Newman-Keuls' test. EODL: essential oil from *Duguetia lanceolata* branches; CO: caryophyllene oxide.

Figure 5. Topical effect of EODL and CO on mice ear edema induced by arachidonic acid. The data represent the mean ± S.E.M. (n=8). *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ *vs.* control group after ANOVA followed by Student Newman-Keuls' test. EODL: essential oil from *Duguetia lanceolata* branches; CO: caryophyllene oxide.
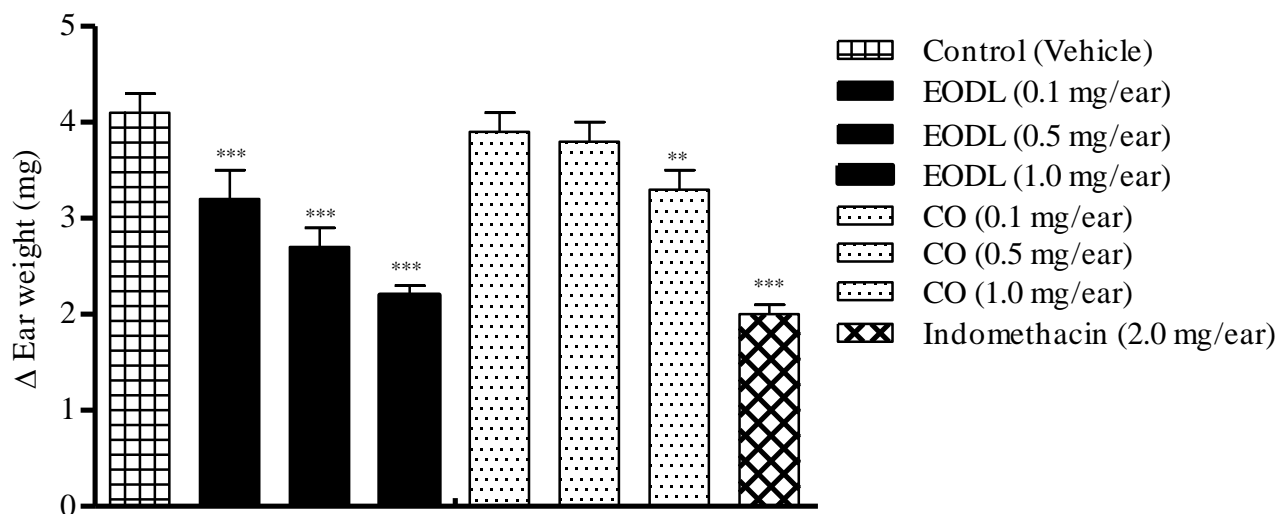
(Sousa et al., 2008).

According to Shibata et al. (1989), the formalin-induced pain produces mediators such as substance P and bradykinin which participate in the manifestation of the first phase, while histamine, serotonin, prostaglandin and bradykinin are involved in the second phase causing central and peripheral responses, respectively. The present study results (Figure 2) show that EODL and CO are able to promote a significant inhibition in both phases by blocking the direct stimulation of nociceptors (central effect; 0 to 5 min) and inflammatory response (peripheral effect; 15 to 30 min) (Hunskaar and Hole, 1987; Shibata

et al., 1989; Tjolsen et al., 1992). These effects are similar to the actions of opioid and nonsteroidal anti-inflammatory drugs. It is worth mentioning that this data has not been previously described using the formalin test, but research conducted by our group demonstrated these effects with the barks' oil (Sousa et al., 2004) and ethanol extract of the leaves (Sousa et al., 2008).

The hypothesis of central effect revealed in the formalin test was investigated using two animal models (hot plate and tail immersion methods) to check the action on opioid receptors in the spinal and supraspinatus levels (Le Bars et al., 2001). This research results demonstrated that the

Table 5. Antioxidant activity of EODL and CO by DPPH assay, Fe^{+3} reducing power and β-carotene/acid linoleic methods. EODL: essential oil from *D. lanceolata* branches; CO: caryophyllene oxide.

Oil/chemical	EC50 (µg/mL)		Inhibition of lipid peroxidation (%)
	DPPH	Fe^{+3} Reducing Power	
EODL	159.4±1.2	187.8±0.6	41.5±2.4
CO	>280.0	>320.0	24.1±1.5
Rutin	9.11±0.1	-	31.7±1.2
Ascorbic acid	-	5.0±0.1	-
BHT	-	-	65.1±0.9

The data represent the mean ± S.E.M. (n = 3). In the same column, there was significant difference between the means considering $P < 0.05$ after ANOVA followed by Tukey's test.

oral administration of EODL and CO exerted significant prolongation in the response latency time to the heat stimulus (Tables 2 and 3) showing a central analgesic effect, particularly on µ opioid receptors (Le Bars et al., 2001). In this test, the central effect of EODL and CO might also be associated with non-opioid system as pretreatment with naloxone did not completely changed the latency time (Table 2), which was also observed in a previous study using the ethanol extract of *D. lanceolata* leaves (Sousa et al., 2008). Although CO has revealed an antinociceptive effect using hot plate test in a study by Chavan et al. (2010), the data showed that this effect may involve additional mechanisms on non-opioid systems. Considering the tail immersion assay, the lack of response at the dose of 50 mg/kg may be due to the greater sensitivity of this model, since it is a spinal integration (reflex) (Carstens and Wilson, 1993).

Initially, the anti-inflammatory effect of EODL and CO (indicated in the formalin test) was investigated using carrageenan-induced paw edema method. These results showed a significant reduction in paw edema which may be associated with the inhibition of prostaglandins biosynthesis that appears in the second phase (3 to 5 h) of the postcarrageenan response (Necas and Bartosikova, 2013) and were similar to the studies reported by Sousa et al. (2004, 2008). However, for a better understanding of this effect, the inflammatory response against carrageenan-induced pleurisy was evaluated. As described by Ammendola et al. (1975), cyclooxygenase inhibitors (indomethacin, phenylbutazone, aspirin and flufenamic acid) are able to reduce the exudate volume and leucocytes migration between 3 and 6 h after application of carrageenan. Thus, as shownin Table 4, EODL and CO inhibited these inflammatory parameters after 4 h of carrageenan application, producing a similar action to the anti-inflammatory agents. Therefore, EODL and CO played a crucial role as protective factors against the carrageenan-induced acute inflammation.

The ear edema models have often been used to corroborate the findings obtained from other methods of acute inflammation through a better understanding of the mechanisms involved in the pharmacological responses. However, although the mechanisms are similar, the topical route has a great advantage in establishing products that can be applied in inflammatory skin diseases (such as psoriasis and allergic contact dermatitis) (Nestle et al., 2009). Based on this viewpoint, the croton oil and arachidonic acid were used as skin inflammation inducers and evaluated in the presence of EODL and CO. According to Gábor (2000), the topical application of croton oil promotes an inflammatory response with oxidative stress that increases vascular permeability, edema formation and leucocyte migration with release of prostaglandin E2, leukotrienes, histamine, serotonin and IL-1. These mediators are generated through different activation pathways and are blocked by cyclooxygenase and 5-lipoxygenase inhibitors, corticosteroids (for example dexamethasone), and leukotriene B4 receptor antagonists. The results showed that EODL and CO were effective on mice ear edema induced by croton oil in a concentration dependent manner. Based on this evidence and in the results of the nociception (abdominal writhing and formalin) and inflammation (paw edema and pleurisy) tests described above, at least, one of the mechanisms involved is associated with the inhibition of prostaglandin synthesis. In this sense, the results of the inhibitory action of EODL and CO on the ear edema induced by arachidonic acid confirmed this hypothesis (Figure 5).

In recent decades, essential oils have been targeted for investigation due to its ability to scavenge free radicals and reduce cellular damages that are related to different pathologies, particularly those associated with inflammatory processes (Miguel, 2010; Adorjan and Buchbauer, 2010). The present study findings revealed that EODL possesses antioxidant effect using DPPH, reducing power and β-carotene assays, while CO inhibited the lipid peroxidation. Nevertheless, considering the antinociceptive and anti-inflammatory properties supported in this study, the antioxidant action of EODL and CO may partially clarify such properties, since reactive oxygen species (ROS) have been implicated in inflammatory response with generation of inflammatory

mediators, edema formation, leucocyte migration and pain (Miguel, 2010). It is important to note that the antinociceptive activity of EODL and CO is also related to the reduction of the liberation of these mediators which are induced by ROS.

Finally, the effects described in this work can be assigned to other components of EODL. Limonene, β-trans-caryophyllene, *p*-cymene and α-pinene are able to inhibit enzyme involved in the leukotriene pathway or the blocking of the production of pro-inflammatory interleukins (IL-1β and IL-6) (Chao et al., 2005; Juhás et al., 2009; Wei and Shibamoto, 2010). Analgesic and anti-inflammatory effects of the essential oil of *Ugni myricoides* are associated with its main components (caryophyllene oxide, humulene epoxide II and β-caryophyllene), especially α-pinene (52.1%) (Quintão et al., 2010).

Conclusion

The results were obtained through the *in- vivo* and *vitro* well-established experiments performed in the present study by adding more subsidies for the use of essential oil from *D. lanceolata* branches due to their antinociceptive, anti-inflammatory and antioxidant properties. Based on the data, caryophyllene oxide is one of the active constituents of this essential oil and represents a promising target for the treatment of pain, inflammation and oxidative stress damage. However, further studies should be conducted to ensure the safety, feasibility and sustainability of usage.

Conflict of Interests

The authors have not declared any conflict of interest.

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