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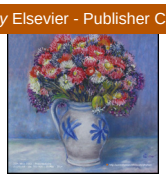

A comparative study of two clerodane diterpenes from *Baccharis trimera* (Less.) DC. on the influx and mobilization of intracellular calcium in rat cardiomyocytes

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ABSTRACT

Baccharis trimera (Less.) D.C. (*Asteraceae*) is a medicinal species native to South America and used in Brazilian folk medicine to treat gastrointestinal and liver diseases, kidney disorders and diabetes. The aqueous extract (AE) of the aerial parts of this species presented two mainly constituents: the *ent*-clerodane diterpene (Fig. 1) and the *neo*-clerodane diterpene (Fig. 2). The objective of this work was to study their activities on the blockade of Ca^{2+}-induced contractions in KCL-depolarized rat portal vein preparations, and on the influx and mobilization of cytosolic calcium in rat cardiomyocytes by fluorescence measurements. The results showed that both the *neo*- and the *ent*-clerodane diterpenes reduced the maximal contractions induced by $CaCl_2$, in KCl depolarized rat portal vein preparations, without modifying the EC_{50}. The data on the concentration of cytosolic calcium ($[Ca^{2+}]_c$) showed that, while the *neo*-clerodane diterpene stimulates the mobilization of $[Ca^{2+}]_c$ in rat cardiomyocytes, this effect was not observed with the *ent*-clerodane diterpene. On the other hand, the influx of calcium was not altered by the *neo*-clerodane diterpene, but was reduced in the presence of the *ent*-clerodane diterpene, indicating that this compound induces a blockade of the voltage-dependent calcium channels.

Introduction

Baccharis trimera (Less.) DC. (*Asteraceae*), known as "Carqueja", is a medicinal species from South America and widely distributed in South and East of Brazil. The plant is commonly used in folk medicine for the treatment of gastrointestinal and liver diseases, inflammatory processes and diabetes (Balbachas 1959; Correa 1984; Sousa et al. 1991; Biondo et al. 2011). Although the gastrointestinal and cardiovascular activities of this species are already described in the literature, there is no clear description of the involved action mechanisms.

Many phytochemical studies of the aerial parts of this plant were done, describing the presence of flavonoids and terpenes (Herz et al. 1977; Bohlmann and Zdero 1970). Pharmacological studies of *B. trimera*, in rats and mice, revealed anti-hepatotoxic (Soicke and Leng-Peschlow 1987), anti-inflammatory and analgesic actions (Gené et al. 1996). Toxicological studies of the tea prepared with the aerial parts the plant and the carquejol, its predominant chemical constituent, were also carried out and did not reveal any toxicity in rodents (Pavan 1952; Caujolle et al. 1960). Previous studies from our laboratory showed that the aqueous extract (AE) and an aqueous fraction (AF) of *Baccharis trimera* presented anti-secretory gastric acid activity, anti-ulcerogenic effect and inhibition of intestinal motility (Gamberini et al. 1991; Lapa et al. 1992). The aqueous extract (AE) also reduced the systolic blood pressure in unanesthetized rats, after 10 days of treatment (Gamberini 1996), while the organic fraction (OF), resultant of the partition of the AE in chloroform, did not show any acid anti-secretory activity, but inhibited the Ca^{2+}-induced contractions in KCl-depolarized rat portal vein preparations (Gamberini 1996). The FA_1, a subfraction of OF, mainly consisting of a dilactonic *ent*-clerodane diterpene and the flavonoid eupatorin (Torres et al. 2000), also reduced the maximal contraction induced by $CaCl_2$ in uterus and rat portal vein preparations, as well as blocked the uptake of ^{45}Ca in rat uterus cell cultures (Gamberini 1996).

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On the mean period a second diterpene (*neo*-clerodane diterpene) was isolated from OF by prep HPLC (Tanae et al. 2008). This new compound was shown to inhibit the gastric acid secretion induced either by histamine or carbachol in rabbit gastric glands (Biondo et al. 2011).

The present work aimed at analyzing both diterpene activities on the blockade of Ca^{2+}-induced contractions of KCl-depolarized rat portal vein preparations and on the influx and mobilization of cytosolic calcium in cultures of cardiomyocytes from ventricles of new born rats, by fluorescence measurements.

Materials and methods

Drugs: Acetoximetilester of Fura-2 (Fura-2/AM), type 1A collagenase, isoproterenol hydrochloride, verapamil hydrochloride, N-2-hydroxyethylpiperazine-N′-2-etanosulfonic acid (HEPES) and trypsin were purchased from Sigma-Aldrich (USA). Horse serum and bovine fetal serum were obtained from Gibco Life Technologies (USA). Ethyl acetate, ethyl alcohol, methyl alcohol, hexane and sulfur vaniline were purchased from Synth (USA). Silica gel (200–325 mesh) and Sephadex LH-20 were from Pharmacia (Sweden). All other reagents were of analytical grade.

Animals: Adult Wistar rats were housed under standard environmental conditions (23 ± 1 °C, humidity 60 ± 5% and a 12 h/12 h dark/light cycle), with food and water available *ad libitum*, in accordance to the Guide for the Care and Use of Laboratory Animals, US Department of Health and Human Services, 1985. The protocol was approved by the Ethics Committee from the Federal University of São Paulo.

Plant extraction and isolation of the diterpenes

The powdered dried aerial parts of *B. trimera* (50 g) were extracted with 1 L of distilled water (73 °C, 30 min), yielding the aqueous extract (AE). After concentration in vacuum, the aqueous extract (AE) was lyophilized (12% yield). The purification of the aqueous extract was accomplished in HPLC, column Shim-Pack, ODS (2500 × 4.6 mm id). The AE was dissolved in deionized water under agitation and filtrated in 0.22 μm filter (Millipore – USA). Samples (1 mL/200 mg) were then injected into the HPLC column. The mobile phase was made of a water/acetonitrile gradient (3–70%) with a 30 min run and a flow rate of 18 mL per minute. The AE purification resulted into 10 fractions (F1–F10). These fractions were submitted to a system of analytical chromatography (Shimadzu-Japan). The same mobile phase was used then with a constant flow of 1.0 mL/min. The samples were dissolved in 5% H_2O/ACN, filtered in 0.22 μm filter (Millipore, USA) and 20 μL samples were injected in the HPLC column. In the F8 and F10 fractions, the *ent*-clerodane (Fig. 1) and the *neo*-clerodane diterpenes (Fig. 2), respectively, were identified.

Pharmacological studies

Portal vein preparations

Adult Wistar rats (150–250 g) were killed by concussion and exsanguination. The portal vein was excised, cleaned of the connective tissue and cut into rings (1.5 cm wide). These preparations were suspended in 2 mL organ baths containing Krebs solution (mM: NaCl 119, KCl 4.6, $MgCl_2$ 1.2, $NaHCO_3$ 15, $CaCl_2$, 1.5, NaH_2PO_4 1.2 and glucose 11). The solution was kept at 37 °C and gassed continuously with 95% O_2 and 5% CO_2. The preparation was equilibrated for 1 h with exchanges in individual bath, every 15 min. The resting tension was adjusted to 1 g. After stabilization, the nutritive solution was changed by a Ca-depleted high KCl (80 mM) solution. The organs were kept equilibrated for 1–1.5 h with exchange every 15 min. Then, cumulative concentration-effects curves of $CaCl_2$ (10 μM to 100 mM) were obtained, in absence or presence of the *ent*-clerodane and *neo*-clerodane diterpenes.

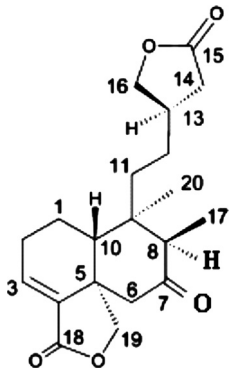

Fig. 1. Structure of *ent*-Clerodane.

Rat cell culture

Briefly, muscles from ventricles of 1–3 day old rat pups were dissected and cut into small pieces. The tissue was then transferred to an ice-cold Hank' balanced salt solution (HBSS), containing 200 UI/mL collagenase (Type IA, Sigma), albumin (0.5 mg/mL) and 2.7 mM $CaCl_2$, for 60–120 min at 37 °C, in a water-saturated atmosphere of 95% air and 5% CO_2 under mild agitation. The cells were washed with HBSS for the collagenase withdrawal. They were then re-suspended in Dulbecco' modified Eagle' medium (DMEM), with 10% (v/v) heat-inactivated horse serum (HS), 10% fetal calf serum (FCS) and gentamicin (40 μg/mL). After 45 min pre-plating to purify the myocyte population, the medium supernatant containing primarily unattached myocytes (>95%) was removed and filtered in Nytex membranes (100 μm). The densities of 0.8×10^6 cells/dish (35 mm) were used for cytosolic Ca^{2+} measurements. The cells normally grew in DMEM with 10% (v/v) heat-inactivated horse serum (HS), 10% fetal calf serum (FCS) and gentamicin (40 μg/mL), in an atmosphere of 95% air and 5% CO_2 at 37 °C. After 120 h (5 days *in vitro*), the confluence was reached and the serum-rich medium was changed to a serum-poor medium (DMEM supplemented with 10% HS and 2% FCS). Cardiomyocytes initiated spontaneous contractions, visible after the 3rd day in culture. The cells were used after 6–7 days in culture.

$[Ca^{2+}]_i$ measurements with the fluorescent dye, fura-2/AM

Intracellular free calcium was measured with the fluorescent dye fura-2/AM. The loading buffer was Krebs/Hepes solution (mM: 143.3 Na^+; 4.7 K^+; 2.5 Ca^{2+}; 1.3 Mg^{2+}; 125.6 Cl^-; 25 HCO_3^-; 1.2 $H_2PO_4^-$; 1.2 SO_4^{2-}; 11.7 glucose and 10 HEPES; pH 7.4). The cells were loaded with 10 μM acetoxymethylester fura-2 (AM form,

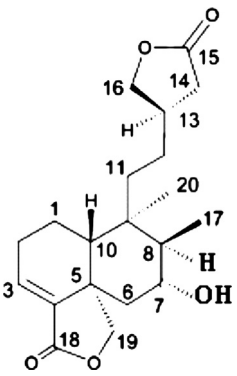

Fig. 2. Structure of *neo*-Clerodane.

dissolved in dimethylsulfoxide) (Molecular Probes, USA) and 5 μl (10% in DMSO) pluronic acid, and incubated for 40 min in the dark at 37 °C, with constant agitation.

After the incorporation, the cells were washed with Krebs/Hepes and incubated 15 min in the dark, at room temperature, to attain a complete deesterification of the probe. After this period, the cells were washed again and the fluorescence was recorded at 37 °C. The cell number was adjusted to 0.8 million cells/mL. The alteration in the fluorescence intensity was monitored by a PTI (MD-5020) dual wavelength spectrofluorometer, by alternative excitation at 340/380 nm (PhoCal, Life Science Resources Ltd., Cambridge, UK). The emission was estimated at 510 nm and the ratio of the emitted light from the 2 wavelengths (R) was used as a measurement of $[Ca^{2+}]_i$ (Grynkiewicz et al. 1985). The intensity of fluorescence was calculated automatically. The $R_{máx}$ and $R_{mín}$ values were determined by addition of digitonin (50 μM) and Mn^+ (2 mM) + EGTA (5 mM), respectively. The $[Ca^{2+}]_i$ levels at rest, as well as maximal increases evoked by agonists were calculated by computer, according to the formula: $[Ca^{2+}]_i = 224 \times [(R - R_{mín})/(R_{máx} - R)]$ (Grynkiewicz et al. 1985).

Measurements of the influx of calcium by the Mn^{2+}-induced quenching of fura-2-Ca^{2+} complex in cardiomyocytes

The Mn^{2+}-induced quenching of fura-2 fluorescence was estimated at 360 nm excitation wavelength (F_{360}), which represents the isosbestic wavelength and is not sensitive to the changes in the $[Ca^{2+}]_i$ and emission at 510 nm. These measurements indirectly reflect the influx of Ca^{2+}, independently of the $[Ca^{2+}]_i$ (Merritt et al. 1989).

Samples of a cardiomyocyte suspension (0.8–1.5 × 10^6 cells/mL) were submitted to the incorporation with fura-2/AM, as described above. After the period of deesterification, the samples were washed and re-suspended in Krebs/HEPES solution (2.5 mL) containing EGTA (0.1 mM), without calcium.

After the registration of the basal fluorescence, the cells were submitted to the alternate excitation at 340 and 360 nm, and the fluorescence emission at 510 nm monitored in the spectrofluorometer model PTI (Photon Technology International – MD-5020, USA). The quenching registrations were accomplished using $MnCl_2$ (0.1 mM) and $CaCl_2$ (2 mM). The results were expressed as the decline of the fluorescence, as a function of time, obtained by excitation at 360 nm (F_{360}) induced by the addition of Mn^{2+}.

Results and discussion

Effects of the B. trimera fractions in the rat portal vein preparations

The smooth muscle contraction is proportional to both the intracellular free calcium concentration released from sarcoplasmic reticulum (Tribe et al. 1994) and the Ca^{2+} influx through calcium channels in the cell membrane (Hughes 1995). In this study, we monitored the effect of the two diterpenes tested, on vascular preparations, depolarized by raising the K^+ concentration in the standard bath solution. The depolarization of the vascular smooth muscle cells in a high-KCl medium allows the influx of Ca^{2+} through the L-type Ca^{2+} channels, triggering a contractile response (Magnon et al. 1995). Under these conditions, the concentration-response curves of $CaCl_2$ were obtained. The cumulative addition of $CaCl_2$ (10 μM to 100 mM) caused concentration-related contractions of the smooth musculature, with a geometric mean effective concentration (EC_{50}) of 2.31 mM (95% confidence limits: 1.94–2.75 mM).

The incubation of *ent*-clerodane diterpene (30, 100 and 300 μM) reduced the maximum responses to $CaCl_2$ proportionally to its concentrations (30 μM: 84.86 ± 7.15%; 100 μM: 69.91 ± 4.70% and 300 μM: 70.90± 2.19%, as related to the control) (Fig. 3). The EC_{50} values in these response curves were not different from that of the control. The wash out of the vascular preparations with a nutritious solution reverted totally the effect.

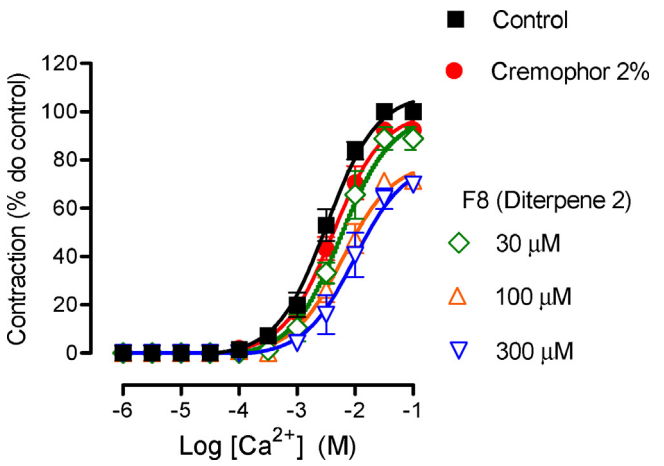

Fig. 3. Effects of *ent*-clerodane diterpene (30, 100 and 300 μM), isolated from the EA of *Baccharis trimera*, on the calcium-induced isometric contraction of rat portal vein segments depolarized in 80 mM KCl Ca^{2+}-free Krebs solution. The *ent*-clerodane diterpene was dissolved in 2% Cremophor EL. Control concentration-response curves to calcium, obtained in the absence and presence of the vehicle (Cremophor EL), were not significantly different. The symbols and vertical bars are means ± SEM (n = 4).

The incubation of the *neo*-clerodane diterpene (30, 100 and 300 μM) reduced the preparation maximum responses to $CaCl_2$ proportionally to its concentrations: 93.2 ± 1.1; 72.7 ± 0.8 and 66.1 ± 2.1% of the control, respectively (Fig. 4). The EC_{50} values in these curves were not different from the control. The wash out of the preparation with a nutritious solution also reverted totally the effect of the *neo*-clerodane diterpene. Our results showed that both the *ent*-clerodane and the *neo*-clerodane diterpenes blocked the vascular smooth muscle contraction induced by the extracellular Ca^{2+} concentration, in KCl-depolarized preparations.

Fluorescence measurements of the mobilization of cytosolic calcium in cultures of neonatal rat cardiomyocytes

In order to verify whether the mechanism of action of the two diterpenes was linked to the blockade of the influx of calcium,

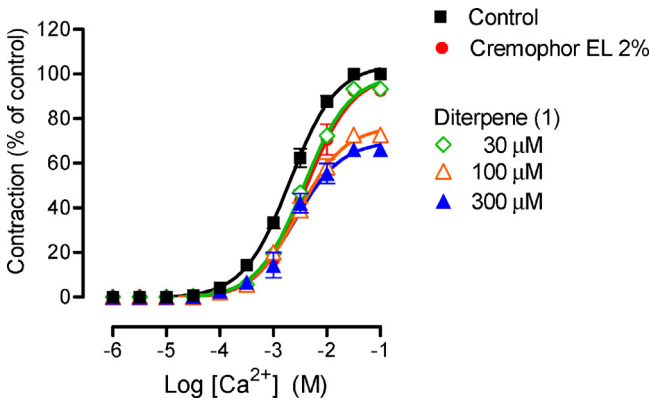

Fig. 4. Effects of the *neo*-clerodane diterpene (30, 100 and 300 μM), isolated from OF (chloroform) of *Baccharis trimera*, on the calcium-induced isometric contraction of rat portal vein segments, depolarized in 80 mM KCl Ca^{2+}-free Krebs solution. The *neo*-clerodane diterpene was dissolved in 2% Cremophor EL. Control concentration-response curves to calcium, obtained in the absence and presence of the vehicle (Cremophor EL), were not significantly different. The symbols and vertical bars are means ± SEM (n = 4).

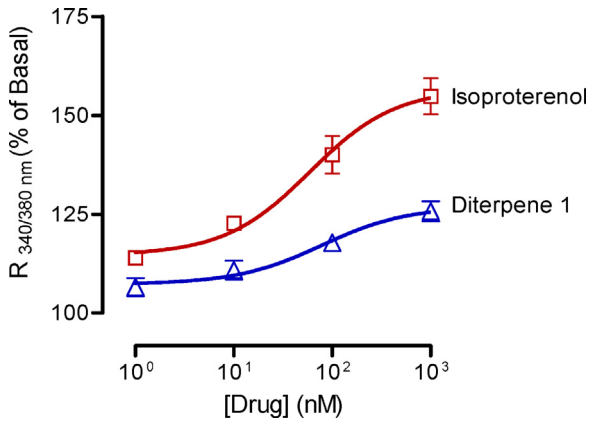

Fig. 5. Effects of isoproterenol ($n = 4$) and *neo*-clerodane diterpene ($n = 5$), 1 nM to 1 μM, on the cytosolic calcium concentration of cardiomyocytes in rat ventricles muscle, after 6 days in culture.

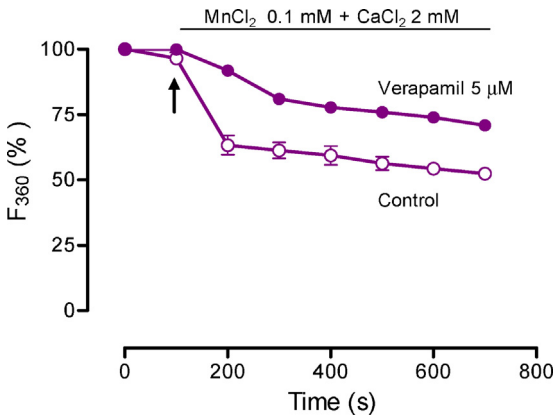

Fig. 6. Quenching of fura-2 fluorescence, induced by Mn^{2+}, obtained in the absence and presence of verapamil (5 μM). Fura-2-loaded cardiomyocytes after 6 days in culture were incubated with verapamil (5 μM), for 10 min. The arrow indicates the incubation of $MnCl_2$. The symbols and vertical bars are means ± SEM ($n = 3$).

cultures of cardiomyocytes from newborn rats were used, since these cells possess the necessary machinery for the measurement of the Ca^{2+} cytosol concentration. In the neonatal period, the formation of the T-tube system starts, the sarcoplasmic reticulum (SR) is developed and a few dihydropyridine (DHP) receptors close to ryanodine receptors (RyRs) can evoke the calcium spark (Tohse et al. 2004). The cultures of ventricular myocytes of newborn rats, used in our study, presented individual spontaneous contractions after 48 h, and areas with rhythmic contractions after 3 days of cultivation were observed. These characteristics indicated that the cultures presented compatible development with the normal calcium homeostasis. In fact, there are evidences that the rhythmic spontaneous contraction of the cardiomyocytes is related to the expression and functionality of proteins of SR, as SERCA 2A and the fosfolambam (Vetter et al. 1998).

During the cardiac action potential, Ca^{2+} enters the cell by depolarization-activated Ca^{2+} channels, as inward Ca^{2+} current (I_{Ca}) which contributes to the action potential plateau. Ca^{2+} entry triggers Ca^{2+} release from the sarcoplasmic reticulum (SR). The combination of Ca^{2+} influx and release raises the free intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), allowing Ca^{2+} to bind to the myofilament protein, troponin C, which then switches on the contractile machinery (Bers 2002).

In samples of rat cardiomyocytes, the incubation of isoproterenol (1 nM to 1 μM) as positive control increased the intracellular concentration of calcium, expressed as $R_{340/380}$, to 10.9 ± 3.2, 17.9 ± 4.9, 32.7 ± 8.0 and 45.0 ± 10.4% of the basal (2.26 ± 0.23; $n = 4$), respectively (Fig. 5).

In another series of samples, the incubation of the *neo*-clerodane diterpene (1 nM to 1 μM) stimulated the mobilization of $[Ca^{2+}]_i$, increasing the values of $R_{340/380}$ to 7.7 ± 2.6, 10.9 ± 2.4 and 17.8 ± 1.9 and 25.7 ± 2.6% of the basal (2.51 ± 0.33; $n = 5$), respectively (Fig. 5). This effect was not observed in the presence of the vehicle (1% Cremophor EL).

Analyses of actions of the *neo*-clerodane diterpene on the concentration of cytosolic Ca^{2+} ($[Ca^{2+}]_c$), accomplished by fluorometer with the fluorescent indicator fura-2/AM, showed that this compound stimulates the mobilization of $[Ca^{2+}]_c$ in rat cardiomyocytes. Although it presented a lower relative potency, as related to that of the positive control (isoproterenol), in the increase of $[Ca^{2+}]_c$, this effect of the *neo*-clerodane diterpene had not been described before. On the other hand, analyses of actions of the *ent*-clerodane diterpene, on the concentration of cytosol Ca^{2+}, showed that this compound does not stimulate the mobilization of $[Ca^{2+}]_i$ in the rat cardiomyocytes (not shown).

Effects of the ent-clerodane and neo-clerodane diterpenes on the Mn^{2+} influx in fura-2-loaded rat cardiomyocytes

The influx of Ca^{2+} can be estimated by measuring the quenching rate of fura-2 by Mn^{2+}. Indeed, Mn^{2+} ions can traverse Ca^{2+} channels, where they are not taken up by Ca^{2+} pumps or by exchangers of the sarcoplasmic reticulum or plasma membrane (Missiaen et al. 1990). In the cytosol, Ca^{2+} rapidly binds to fura-2 and quenches the fluorescence (Chen and Rembold 1992).

Fig. 6 shows the fluorescence quenching of the fura-2 (F_{360}), induced by Mn^{2+} in controls and in the presence of verapamil (5 μM), a blocker of calcium channels (Hockerman et al. 1997). The speed of decline of F_{360} in the presence of verapamil (5 μM) was 0.53 ± 0.03 s^{-1} ($n = 3$), which is significantly smaller than that under control conditions (0.80 ± 0.04 s^{-1}, $n = 3$).

The study of the actions of the *neo*-clerodane diterpene on the influx of Ca^{2+} in cardiomyocytes, by measuring the speed of extinction of the fluorescence of the fura-2 for Mn^{2+} (quenching), showed that this compound does not influence the influx of Ca^{2+} in concentrations that increase the mobilization of $[Ca^{2+}]_i$, differently from that observed with verapamil (5 μM) (Fig. 7).

In the presence of 1, 3 and 10 μM concentrations of the *neo*-clerodane diterpene, the quenching speed of the fluorescence induced by $MnCl_2$ (0.1 mM), in the presence of the highest tested concentration (0.78 ± 0.03 s^{-1}, $n = 3$), did not differ from that of the control.

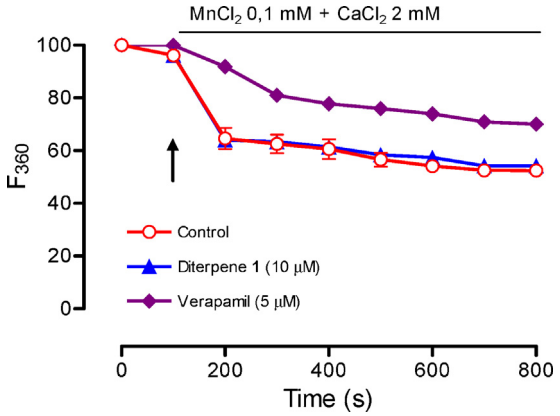

Fig. 7. Quenching of fura-2 fluorescence, induced by Mn^{2+}, obtained in the absence and presence of the *neo*-clerodane diterpene (10 μM) or verapamil (5 μM). Fura-2-loaded cardiomyocytes after 6 days in culture were incubated with verapamil and *neo*-clerodane diterpene, for 10 min. The arrow indicates the incubation of $MnCl_2$. The symbols and vertical bars are means ± SEM ($n = 3$).

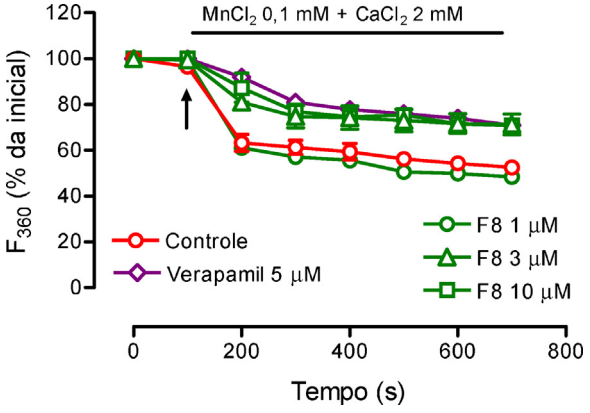

Fig. 8. Quenching of fura-2 fluorescence, induced by Mn^{2+}, obtained in the absence and presence of *ent*-clerodane diterpene (1, 3 and 10 μM, $n = 3$) or verapamil (5 μM). Fura-2-loaded cardiomyocytes after 6 days in culture were incubated with verapamil and the *ent*-clerodane diterpene, for 10 min. The arrow indicates the incubation of $MnCl_2$. The symbols and vertical bars are means ± SEM ($n = 3$).

Fig. 8 shows the quenching of the fura-2 fluorescence F_{360}, in fura-2-loaded cardiomyocytes. In the presence of *ent*-clerodane diterpene (3 and 10 μM) or verapamil (5 μM), the quenching rate was significantly decreased. The rates of fluorescence quenching by $MnCl_2$ were $0.803 \pm 0.042\ s^{-1}$ ($n = 3$), $0.800 \pm 0.089\ s^{-1}$ ($n = 3$), $0.509 \pm 0.089\ s^{-1}$ ($n = 3$), $0.495 \pm 0.041\ s^{-1}$ ($n = 3$) in the presence of neo-clerodane diterpene (1, 3 and 10 μM), respectively.

The influx of calcium, obtained indirectly by quenching measures of the complex fura-2-Ca^{2+} by Mn^{2+}, was not altered by the *neo*-clerodane diterpene, but was reduced in the presence of *ent*-clerodane diterpene. These results indicate that the blockade of the voltage-dependent calcium channels produced by this compound may be involved in the hypotensive effect of the *B. trimera* extract, observed in experiments *in vivo*.

In conclusion, the main results obtained were as follows: the EA presented two mainly constituents, the *neo*-clerodane diterpene, already described in *B. trimera*, and the *ent*-clerodane diterpene. Both the *neo*-clerodane and the *ent*-clerodane diterpenes reduced the maximal contractions, induced by $CaCl_2$, in KCl-depolarized rat portal vein preparations without modifying the EC_{50}. The effects of the *neo*-clerodane diterpene on the concentration of cytosolic calcium ($[Ca^{2+}]_c$), analyzed by fluorescence measurements, showed that this compound stimulates the mobilization of $[Ca^{2+}]_c$ in rat cardiomyocytes, an effect that was not observed with the *ent*-clerodane diterpene. The influx of calcium, obtained indirectly by quenching measures of the complex fura-2-Ca $^{2+}$ by Mn^{2+}, was not altered by the *neo*-clerodane diterpene but was reduced in the presence of *ent*-clerodane diterpene, indicating a blockade of the voltage-dependent calcium channels. Similar results were described for marrubenol, a diterpene isolated from another species, *Marrubium vulgare*, used in folk medicine as an antihypertensive, effect attributed to the blockade of L-type calcium channels (El Bardai et al. 2003).

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