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PHARMACOLOGICAL EVALUATIONS FOR THE RELAXANT EFFECT OF THE HYDROALCOHOLIC EXTRACT OF *TAPINANTHUS DODONEIFOLIUS* ON RAT TRACHEA

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Abstract

The present study was designed to investigate the blocking of calcium by the hydroalcoholic extract of *Tapinanthus dodoneifolius* (Tapidod), "*in vitro*", on rat trachea. To evaluate this effect, the contractile activity of tracheal chains from Wistar Kyoto rats was isometrically recorded. On the isolated tracheal rings the extract produced the following effects: (a) a reduction of the contraction obtained by $BaCl_2$, (b) a bronchorelaxing action, on strips precontracted by KCl, which was not influenced by TEA ($3x10^{-3}$ M), (c) a concentration-dependent decrease of the spasm evoked by calcium chloride ($CaCl_2$) in K^+-rich Ca^{2+}-free physiological salt solution, before and after intracellular calcium depletion (d), an inhibitory effect on contraction induced by acetylcholine in Ca^{2+}-free Krebs-Heinseleit solution supplemented with EDTA ($5x10^{-4}$M). It is concluded that: 1. The activation of the potassium channels does not play a significant role in the relaxant effect of Tapidod. 2. The antispasmodic property of Tapidod seems to be mediated by the blockade of intracellular Ca^{2+} release. 3. Most likely an inhibition of the intracellular Ca^{2+}-regulating proteins is involved.

Keys words: Extract of *Tapinanthus dodoneifolius (Tapidod);* Trachea; Calcium.

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Introduction

Asthma is a worldwide chronic airway inflammatory disease, the etiology of which is not known (Holgate, 1997). The mortality and morbidity associated with this disease have not appreciably declined (Sly and O'Donnell, 1997), and its treatment has not changed over the last two decades (Wenzel, 1998). Therefore, research has not reached its goal, and the treatment of asthma is still seeking for targeted, anti-inflammatory therapy (Vaali, 1999).

Phytotherapy research represents a promising approach to the discovery of biologically active molecules that may be beneficial in the treatment of asthma. *Tapinanthus dodoneifolius* is extensively used as medicinal plant in traditional medicine in Burkina Faso. According to Boussim (2002) *Tapinanthus dodoneifolius* is used in the treatment of cholera, asthma and diabetes. The aqueous extract is taken against gynecologic disturbances, digestives disorders and nervous confusions (Nacoulma/Ouédraogo 1996). Cardiovascular properties of *T. dodoneifolius* have been recently described by Ouédraogo et al. (2005a). Cepeleanu et al. (1994) revealed larvicidal and molluscidal effects of *T. dodoneifolius*. Preliminary pharmacological investigations on rat trachea indicate that *Tapinanthus dodoneifolius* total aqueous extract inhibited contractile activity induced by physiological and non-physiological agonists (Ouédraogo et al., 2005b).

Chemical analyses of different extracts from *T. dodoneifolius* yielded components as triterpenes, sterols, carotenoïdes, saponosides, anthracenosides, anthocyanosides and tannins (Traoré, 2000).

The present study was conducted to analyze comprehensively the relaxant effect and to elucidate the mechanism of action of Tapidod in rat trachea.

Material and Method

Plant and extracts

Tapinanthus dodoneifolius (DC.) Danser (syn: *Agelanthus dodoneifolius* (DC.) R.M. Polhill& D. Wiens) was collected in Loumbila (Burkina Faso) in April 2002 and taxonomically identified by an expert botanist (Prof Boussim I. J., University of Ouagadougou). A voucher specimen is deposited in the Herbarium of the Department of Vegetal Biology, University of Ouagadougou, Burkina Faso, with the reference no. 002. The drying operation was carried out under room temperature with out exposure to sunlight. Air-dried and powdered plants of *T. dodoneifolius* (100g) were soaked in chloroform (1000 ml) for 24 hours. After filtration, the remainder was dried at room temperature and extracted in ethanol (1000 ml) for 24 hours. The extract was centrifuged, concentrated by rotary evaporation, frozen and lyophilized.

Tissue preparation

Tracheal rings were dissected from either male or female Wistar rats (weighing 200-300g) following ethyl carbamate anesthesia and exsanguination by carotid artery transection. Thoracic tracheas were removed rapidly and cleaned of surrounding tissue. The isolated tracheas were cut as spirals according to the method described by Brunelleschi et al. (1987); Hazekamp et al. (2001) and suspended in 20 ml organ bath chambers containing modified Krebs-Henseleit physiological solution at pH of 7.4, continuously oxygenated and maintained at 37° C. The rings were mounted by means of two parallel L-shaped stainless steel holders inserted into the lumen. The upper holder was attached with thread to a transducer while the lower one was tied to a stationary glass rod. The preparations were allowed to equilibrate for at least 90 min while the base-line tension was adjusted progressively to 2g as recommended by Tschirhart et al. (1987). Isometric contraction of the smooth muscle was recorded with chart recorder via a force-displacement transducer (Grass FT 03C).

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Experimental procedures

Acetylcholine and barium chloride are tested by cumulative addition, directly in the tissue bath. The total amount of drugs added did not exceed 5 % of the total volume (1ml) of the bath (Van Rossum, 1963). For the determination of the effects of antagonists on the response to the addition of agonists, tissues were incubated in the presence of antagonists 15 or 30 min prior to the addition of agonists.

To investigate the involvement of voltage-sensitive K^+ channels activation in Tapidod-induced relaxation, two K^+ channel blockers ($BaCl_2$ and TEA) (Emine et al., 1998; Imaizumi and Watanabe, 1981) and the method of depolarization described by Karaki and Weiss (1988) were used.

In order to study the inhibition of the availability of the calcium by Tapidod three different experimental protocols were applied. These protocols derived from method described by (Lagaud et al. (1996)

In the first experimental condition, concentration-response curves to $CaCl_2$ were constructed by cumulative application in a K^+-rich Ca^{2+}-free solution. Trachea was previously incubated in a normal physiological salt solution (equilibration period) and depolarized in the K^+-rich Ca^{2+}- free solution. The K^+-rich Ca^{2+}- free solution was obtained by KCl ($8x10^{-2}$ M) substitution for an equimolar amount of NaCl, omission of calcium and addition of EDTA ($5x10^{-4}$ M) (Guan et al. 1987).

In the second experimental protocol which intended to study the component of the Tapidod-induced relaxation due to the blocking of the Ca^{2+} influx, the first experimental condition was repeated after depletion of intracellular Ca^{2+}. Depletion was obtained by repeated exposure of the trachea to acetycholine maximally active concentration (10^{-3}M), in a Ca^{2+}-free solution containing 2mM EDTA , until there was no response to acetylcholine.

To study the component of the Tapidod-induced relaxation due to the inhibition of internal Ca^{2+} release, an intracellular Ca^{2+} mobilizing agent (acetylcholine) was used on rat trachea in Ca^{2+}-free solution. Acetylcholine was added cumulatively to the bath. A washout period of 45 min was allowed between each experimental protocol.

Expression of results

The bronchodilatory efficacy of the extract was expressed as E_{max}=maximal bronchodilatory response, evaluated as % relaxation of the KCl-induced contraction. The potency of the plant extract was expressed as pEC_{50} = -log EC_{50} (Jenkinson et al. 1995), calculated as concentrations (g of extract per ml of organ bath) required to produce 50% of E_{max} (Computer program: GRAPH PAD PRISM® 2.01).

Drugs

The Krebs-Heinsleit solution had the following composition (millimolar): NaCl, l18; KCl, 4.75; $CaCl_2$, 2.5; KH_2PO_4, 1.2; $MgSO_4$ $7H_2O$, 1.2; $NaHCO_3$, 25 and Glucose, 5.55. Acetylcholine chloride and verapamil were obtained from Sigma. Tetraetylammonium chloride was provided by Fluka. Ethylene Diamine Tetraacetic Acid (EDTA), $BaCl_2$, $CaCl_2$ and KCl were purchased from Prolabo. All the drugs were prepared as concentrated stock solution and diluted to the final concentration in Krebs-Heinseleit solution. In preparing the stock solution, all the drugs were dissolved in distilled water. Before each experiment the lyophilized extract was dissolved and diluted in distilled water.

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Statistical analysis

Results are expressed as means±SEM of number (n) of experiments. The significance of differences was evaluated by means of ANOVA and student's *t* test for unpaired data. P values lower than 0.05 were considered to be significant.

Results

Tapidod effects on the concentration-response curve of $BaCl_2$

$BaCl_2$ (10^{-4}M to 10^{-1} M) produced a concentration dependent contraction on rat trachea (pEC_{50}=2.03±0.06). Tapidod reduced significantly the contraction induced by $BaCl_2$ and caused a rightward shift of the concentration-response curve for $BaCl_2$. In tracheal rings the maximal contractile response to $BaCl_2$ in the presence of Tapidod ($1x10^{-3}$g/ml) was 59.326±2.54 % of the maximal response observed in its absence (Figure 1).

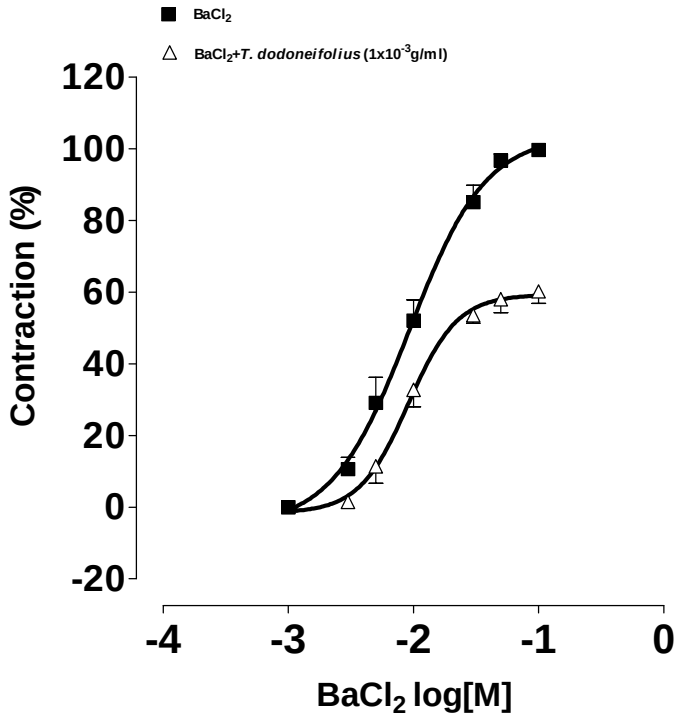

Figure 1: Concentration-response curves in rat trachea contraction induced by $BaCl_2$ in the absence (■) or in the presence (Δ) of *T. dodoneifolius* extract ($1x10^{-3}$ g/ml). In the abscissae, the molar concentrations of $BaCl_2$ are expressed as logarithms. Vertical lines represent the S.E.M. (n =5)

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Effects of Tapidod on the KCl-induced contraction

TEA ($3x10^{-3}$ M) potentiated KCl-induced contraction. Tracheal rings, pre-treated by TEA, maximal response to $3x10^{-2}$ M KCl did not differ significantly from $4x10^{-2}$ M KCl-induced contraction without TEA. This equivalence in developed tension has been determined by comparing KCl-induced contraction concentration-response curves in the absence or in the presence of TEA ($3x10^{-3}$ M). Tapidod elicited a bronchodilatory effect on rat trachea pre-contracted by KCl (pEC_{50}=3.02±0.13, E_{max}=106.2±15.22%, in absence of TEA; pEC_{50}=2.97±0.075, E_{max}=102.9±5.33% in presence of TEA)(Figure 2). In tracheal rings precontracted by KCl $8x10^{-2}$M, the extract produced a concentration-dependant bronchorelaxant response (pEC_{50}=2.61±0.07 and E_{max}=107.8±7.02%).

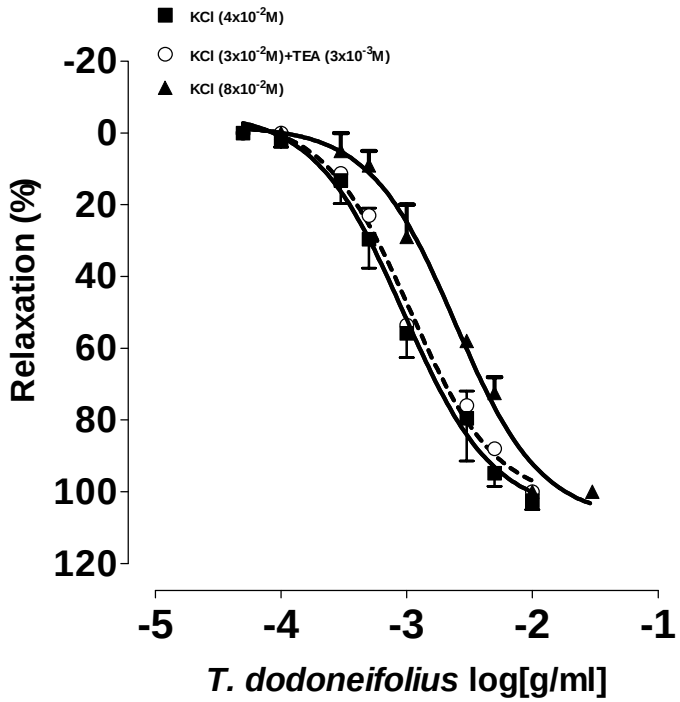

Figure 2: Concentration-resposne curves for the relaxation induced by the *T. dodoneifolius* extract on trachea contracted with KCl ($4x10^{-2}$ M) (■) and ($8x10^{-2}$M) (▲) without TEA, or with KCl ($3x10^{-2}$ M) in the presence of TEA ($3x10^{-3}$ M) (O). The responses are expressed as % of the maximal KCl-induced contraction. Vertical bars indicate the S.E.M. values. In the abscissea, the concentrations g/ml of the extract are expressed as logarithms. (n=5)

Tapidod effects on the concentration-response curve of $CaCl_2$ in k^+ rich Ca^{2+}-free solution

In trachea bathed by K^+-rich Ca^{2+}-free physiological salt solution, $CaCl_2$ (10^{-5}M to 10^{-2} M) evoked concentration-dependent spasm (pEC_{50}=3.68±0.06). When the effect of Tapidod ($5x10^{-4}$, $2x10^{-3}$, $5x10^{-3}$ g/ml) was evaluated on $CaCl_2$ cumulative concentration-effect curve a dose-dependant depression of the curve was detected in the high K^+ Ca^{2+}-free solution (pEC_{50}=2.86±0.07 and E_{max}=103.3±5.09%; pEC_{50}=2.13±0.06 and E_{max}=38.67±2.11%;

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pEC_{50}=1.38±0.16 and E_{max}=33.11±5.14%, respectively) (Fig. 3). Calcium depletion did not influenced Tapidod ($2x10^{-3}$g/ml) antispasmodic effect (pEC_{50}=3.01±0.06, E_{max}=39.96±2.34%) (Figure 4).

Tapidod effects on the concentration-response curve of acetylcholine in Ca^{2+} free solution.

Calcium removal from the bath shifted to the right the concentration-response curve for acetylcholine but did not influenced E_{max}. Tapidod ($1x10^{-3}$g/ml) inhibited the effect of acetylcholine in Ca^{2+}-free solution. (pEC_{50}=5.041±0.04 in the absence of Tapidod; pEC_{50}=4.55±0.05 and E_{max}= 69.40± 2.12% in the presence of Tapidod; Figure 5).

Effect of Verapamil and Tapidod on acetylcholine dose-response curve

The contraction induced by acetylcholine was markedly inhibited by the pre-treatment with Tapidod (pEC_{50}=5.041±0.04 in the absence of Tapidod; pEC_{50}=5.43±0.12 and E_{max}=71.68±2.76% in presence of Tapidod ($1x10^{-3}$ g/ml)) (Figure 6). However, in the presence of verapamil ($1x10^{-6}$M), acetylcholine concentration-effect curve was not significantly influenced (pEC_{50}=4.75±0.06 and E_{max}=106.02±2.33%).

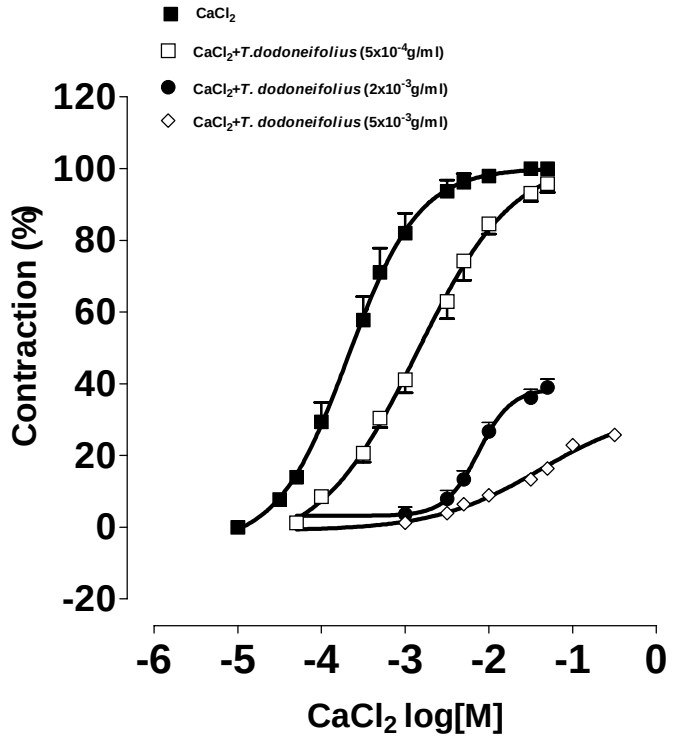

Figure 3: Concentration-response curves with $CaCl_2$, in K^+-rich Ca^{2+}-free solution, in the absence (■) or in the presence of *T. dodoneifolius* extract ($5x10^{-4}$ M) (□) ; ($1x10^{-3}$ g/ml) (●) ; ($2x10^{-3}$ g/ml) (◊). In the abscissae, the molar concentrations of $CaCl_2$ are expressed as logarithms. Vertical lines represent the S.E.M. (n=5-9)

Discussion

The presence of Voltage-dependant K^+ Channels, Ca^{2+}-dependant K^+ channels and ATP-dependant K^+ channels in tracheal smooth muscle has been reported by Kotlikoff et al. (1990); Marthan et al. (1989) and Black et al. (1990). Barium, a K^+ channels blocker

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(Thirstrup et al., 1997), induced concentration dependant contractile activity inhibited by Tapidod. The blocking of potassium conductance increases the transmembrane Ca^{2+} influx as a result of the cell membrane depolarization (Corrompt et al., 1998), eliciting a contractile response. It has been suggested that the activation of K^+ channels reduces the voltage-dependant Ca^{2+} influx and $[Ca^{2+}]i$ through tonic hyperpolarisation of smooth muscle cells (Kannan and Johnshon, 1995). As Tapidod inhibits the tracheal smooth muscle response to the blocking of K^+ conductance , we suggest that this extract may act by activating the K^+ influx or by modulating the Ca^{2+} influx. Moreover, one way to study a possible K^+ channel opener is to increase the K_e to 40 mM level (Vaali, 1999). Tapidod relaxed 80 mM K^+-induced contraction indicating that its bronchorelaxing action is not mediated by a K^+ channels activation.

Tapidod prevented the contraction induced by Ca^{2+}on rat tracheal muscle bathed by K^+-rich Ca^{2+}-free physiological salt solution. Intracellular calcium depletion did not influence Tapidod effect. These inhibitory effects indicate a blocking of the Ca^{2+}-influx. L-type Ca^{2+} channel blocker (Verapamil, $1x10^{-6}$ M) did not reverse acetylcholine-induced contraction. This result is similar to those mentioned by Foster et al. (1984) and Advenier et al. (1984). However, *T. dodoneifolius* antagonized acetylcholine indicating that the pharmacodynamic profile of Tapidod differs from that of the L-type Ca^{2+} channels blockers.

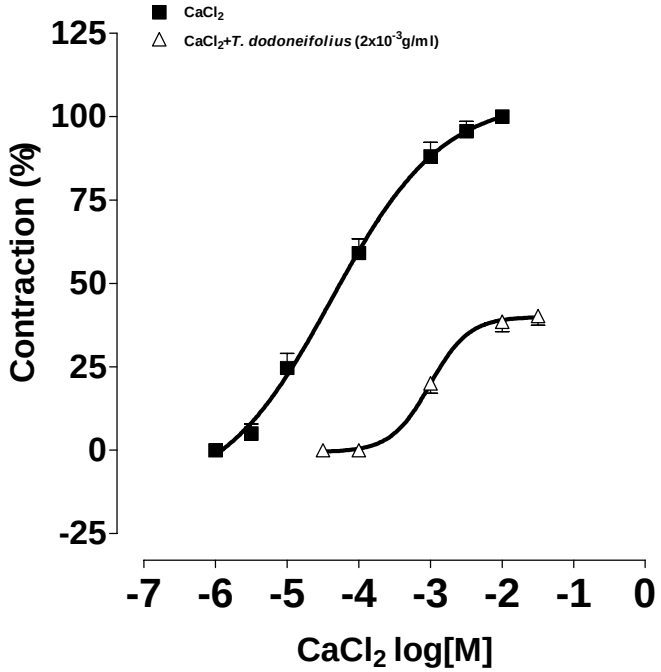

Figure 4 : Concentration-response curves with $CaCl_2$, in K+-rich Ca^{2+}-free solution, on calcium-depleted trachea, in the absence of ($CaCl_2$) (■), and in the presence of *T. dodoneifolius* ($2x10^{-3}$ g/ml) (Δ). In the abscissae, the molar concentrations of $CaCl_2$ are expressed as logarithms. Vertical lines represent the S.E.M. (n= 5)

In Ca^{2+}-free solution Tapidod produced a depression of the contractile response to acetylcholine . Eglen et al. (1994) and Marthan et al. (1987) reported that acetylcholine elicits contractile activity by using calcium from internal stores. One characteristic in smooth muscle is that pharmacomechanical coupling can be seen even when extracellular Ca^{2+} concentration is zero and must therefore depend upon intracellular stores. The depression of the contractile

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response to acetylcholine in Ca^{2+}-free Krebs solution suggests an action on the intracellular mobilization of Ca^{2+} ions.

The two most important factors affecting smooth muscle contraction are $[Ca^{2+}]_i$ and the contractile machinery's sensitivity to calcium (Vaali, 1999). As the precise mechanism of action of Tapidod remains to be elucidated, the present results suggest that its inhibitory action may involve its binding to intracellular Ca^{2+}-regulating proteins (i.e. calmodulin) thereby preventing Ca^{2+} from binding to these proteins. Similar mechanisms have been reported by Hazekamp et al. (2001) to explain the bronchodilatory effect of *Clerodendrum petasites (Verbenaceae)* on guinea-pig trachea.

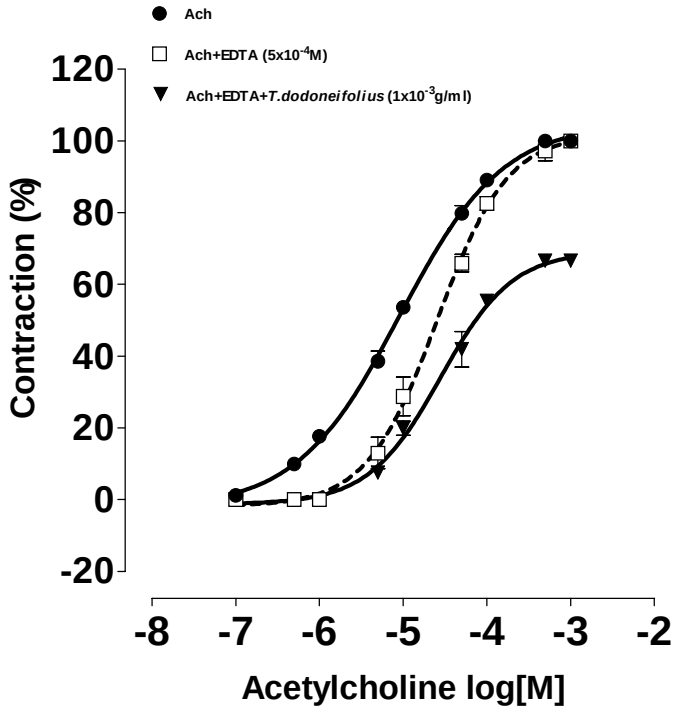

Figure 5: Concentration-response curves with acetycholine, in Ca^{2+}-free solution, in the absence (■) or the presence of the EDTA (5.10^{-4} M) (□); EDTA+*T. dodoneifolius* extract ($1x10^{-3}$ g/ml) (▼). In the abscissae, the molar concentrations of acetylcholine are expressed as logarithms. Vertical lines represent the S.E.M. (n= 7-9)

Conclusion

It is beyond the scope of this paper to speculate further on the structure-activity relationship. Tapidod is a crude extract that would contain several constituents able to present additional effects. The purpose of this paper was to demonstrate the Ca^{2+}-blocking activity of Tapidod, which relaxes KCl-induced contraction on rat trachea and inhibits agonists-induced contraction. It was concluded that Tapidod effect may involves an intracellular calcium sequestration or/and a calcium-regulating proteins inhibition.

Acknowledgements

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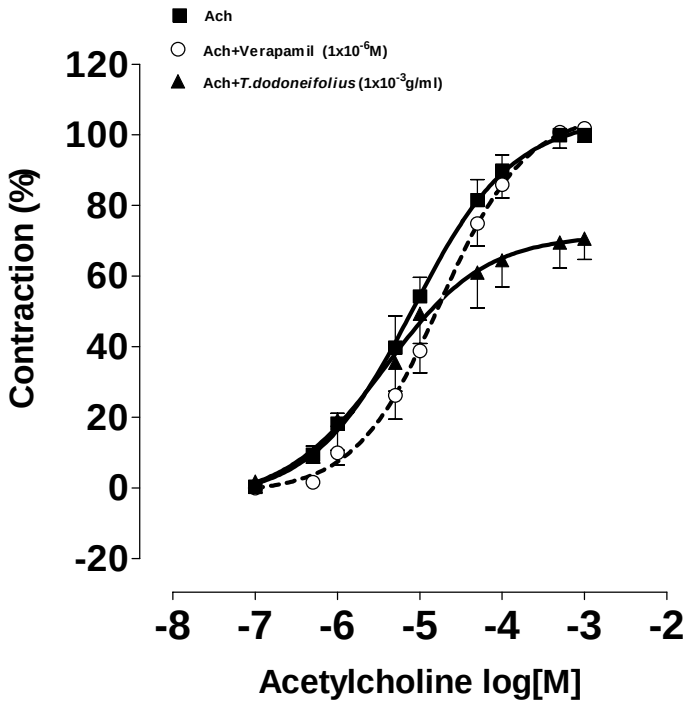

Figure 6: Concentration-response curves in rat trachea contraction induced by acetycholine in the absence (●) or in the presence of verapamil (10^{-6} M) (Δ); and of *T. dodoneifolius* extract ($1x10^{-3}$ g/ml) (▲). In the abscissae, the molar concentrations of acetylcholine are expressed as logarithms. Vertical lines represent the S.E.M. (n= 8-10)

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