

## Research Article

# The Vasorelaxant Effect of *p*-Cymene in Rat Aorta Involves Potassium Channels

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Received 25 July 2014; Revised 15 December 2014; Accepted 29 December 2014

Academic Editor: Xavier Norel

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The monoterpenes are the main constituents of most essential oils and *p*-cymene is a monoterpene commonly found in various species of aromatic herbs, which has been reported for anti-inflammatory, antinociceptive, and antimicrobial activities. However, there is no report concerning its pharmacological activity on the vascular smooth muscle. The aim of current work was to investigate the effects of *p*-cymene in isolated rat aorta and also study its mechanism of action. In this work, we show that *p*-cymene has a relaxant effect, in a dose-dependent way, on the vascular smooth muscle, regardless of the presence of the endothelium. Using a nonselective potassium channel blocker, the CsCl, the relaxant effect of *p*-cymene was attenuated. In the presence of more selective potassium channels blockers, such as TEA or 4-AP, no change in the relaxant effect of *p*-cymene was evidenced, indicating that BK<sub>Ca</sub> and K<sub>V</sub> channels are not involved in that relaxant effect. However, in the presence of glibenclamide or BaCl<sub>2</sub>, K<sub>ATP</sub> and K<sub>ir</sub> blockers, respectively, the relaxant effect of *p*-cymene was attenuated. The data presented indicate that *p*-cymene has a relaxing effect on rat aorta, regardless of the endothelium, but with the participation of the K<sub>ATP</sub> and K<sub>ir</sub> channels.

## 1. Introduction

Essential oils are volatile, have strong smell, present a complex composition, and are formed by the secondary metabolism of aromatic plants [1]. Due to their characteristic odor, essential oils are widely used in fragrances, cosmetics, and sanitary products. Besides, they are used in holistic medicine therapies such as aromatherapy. Furthermore, due to the complexity of their constituents, these oils are used in medicines, in dentistry, in pest control, and in canned food preservatives [2].

Some studies indicate that many of these essential oils have biological activities, such as cardiovascular effects [3],

inhibition of the oxidation of LDL cholesterol, and blood pressure lowering effect [4, 5].

It is known that the major chemical components of the essential oils are monoterpenes and sesquiterpenes [6]. A monoterpene that is commonly found in various species of herbs is the *p*-cymene. Indeed, this monoterpene is present in the volatile oils of over 100 plant species and occurs naturally in more than 200 kinds of foods, such as orange juice, grapefruit, tangerine fruit, carrots, raspberries, butter, nutmeg, oregano, and most spices [7].

It has been demonstrated that the *p*-cymene has anti-inflammatory, analgesic, and antinociceptive activities [8–10]; immunomodulatory effect [11]; antibacterial activity

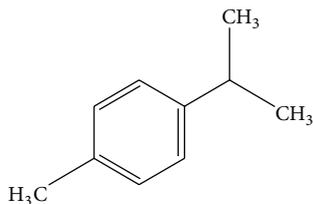


FIGURE 1: Chemical structure of *p*-cymene.

against *Escherichia coli* [12]; hypotension and bradycardia effects in urethane anaesthetized rats [13]; and antioxidant activity and relaxant effect in rat aorta [14]. Although there are reports on the relaxing activity in rat aorta, little is known about the mechanism of action of this monoterpene regarding its relaxing effect.

Concerning the aforementioned, the objective of this work was to access the role of endothelium and potassium channels in the relaxing effect of *p*-cymene in isolated rat aorta.

## 2. Materials and Methods

**2.1. Animals and Ethics Considerations.** In all experiments, male Wistar rats were used, weighing 250–350 g, fed on standard rat chow with free access to food (PURINA, Brazil) and tap water *ad libitum*. The animals were maintained in a 12 h light–dark cycle (lights on: 06:00–18:00 h) under controlled temperature conditions ( $21 \pm 1^\circ\text{C}$ ). All experimental procedures were performed in accordance with the guidelines proofed by the Ethics Committee on Animal Experimentation of UNIVASF (CEUA/UNIVASF number 0002/131211) and follow the recommendations of the National Council for Control of Animal Experimentation of Brazil (CONCEA).

**2.2. Chemicals and Krebs Solution.** For chemicals, acetylcholine chloride (ACh), cesium chloride (CsCl), *p*-cymene (Figure 1), glibenclamide, dimethyl sulphoxide (DMSO), phenylephrine hydrochloride (PE), tetraethylammonium chloride (TEA), 4-aminopyridine (4-AP), and barium chloride ( $\text{BaCl}_2$ ) were purchased from Sigma-Aldrich (St. Louis, MO, USA). All salts used in Krebs solution were purchased from Vetec QuímicaFina Ltda. (Duque de Caxias, RJ, Brazil). *p*-Cymene was dissolved in DMSO (100%) and diluted in distilled water according to the requirements of each experiment.

For all experiments with isolated rat aorta, Krebs salt solution was used, with the composition and concentration (in mM) as follows: NaCl (118), KCl (4.6),  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  (5.7),  $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$  (1.1),  $\text{CaCl}_2$  (2.5),  $\text{NaHCO}_3$  (25), and glucose (11) with pH adjusted to 7.4 with 1 N HCl solution.

**2.3. Preparation and Protocols of Isolated Rat Aorta.** In order to record the isotonic contractions, isolated rat aorta rings (4–5 mm) were suspended in organ chambers (10 mL) of an isolated organ bath system, model EFF 321 from Insight Instruments (São Paulo, SP, Brazil), and attached to isometric

transducers, model TRO015 from Panlab S.L.U. (Barcelona, Spain), coupled to a bridge system amplifier 321 from Insight Instruments (São Paulo, SP, Brazil) and connected to a computer.

The rats were euthanized following the principles of Laboratory Animal Care in accordance with the guidelines of bioethics committee. The thoracic aorta was removed from the animals, immediately immersed in Krebs solution bubbled with carbogen mixture (95%  $\text{O}_2$  plus 5%  $\text{CO}_2$ ), and cleaned up from fat and connective tissues. The aortas were cut into 5 mm long rings and transferred to an organ chamber with Krebs solution at  $37^\circ\text{C}$ . The aortic rings were allowed to stabilize for 60 min at preload tension of 1 g (baseline). During the resting time, the Krebs solution was changed every 15 min to avoid accumulation of metabolites. After that elapsed time, the aortic rings were contracted by the addition of PE ( $1\ \mu\text{M}$ ) and the isometric tension was recorded. When a stable contraction was attained (plateau reached in 15–20 min), ACh ( $1\ \mu\text{M}$ ) was added to the organ bath to confirm the presence or absence of functional endothelium, as described earlier by Furchgott and Zawadzki [15]. Aortic rings without endothelium were obtained by means of the mechanical removal of the endothelial layer. After 30 min, a second PE-induced stable contraction was induced and cumulative-concentration curves by *p*-cymene were performed in absence or in the presence of functional endothelium, CsCl (5 mM), TEA (1 mM or 10 mM), 4-AP (3 mM),  $\text{BaCl}_2$  (0.1 mM), or glibenclamide ( $3\ \mu\text{M}$ ) for the assessment of the participation of potassium channels in the relaxant effect of *p*-cymene. In experiments involving the use of potassium channel blockers, the absence of the endothelium was verified by the lack of relaxation induced by Ach. All potassium channels blockers were added 30 min before a second contraction induced by phenylephrine.

**2.4. Statistics.** All the data are expressed as the mean  $\pm$  S.E.M. and *n* refers to the number of animals used in each set of experiments. The  $\text{EC}_{50}$  values (half-maximal effective concentration) were calculated through nonlinear regression of the concentration-response curves of *p*-cymene in each protocol. The  $E_{\text{max}}$  value refers to a maximal effect induced by a substance in percentage, being equal to 0% in maximum contraction tension elicited by phenylephrine and 100% when initial preload tension level was reached (baseline). Differences between the means were statistically compared using nonpaired Student's *t*-test, where such differences were considered significant when *P* values were less than 0.05. Statistical analyses were performed using the software GraphPad Prism<sup>®</sup> 5.1 (GraphPad Software Inc., San Diego, USA).

## 3. Results

**3.1. Effect of *p*-Cymene in Aortic Rings with and without Endothelium.** The *p*-cymene relaxed the isolated rat aorta rings, precontracted by phenylephrine, in a concentration-dependent manner, in the presence and absence of the endothelium (Figure 2), reaching an  $E_{\text{max}}$  value of 100% of relaxation at the concentration of  $10^{-3}$  M (Figure 2). For rings with endothelium, the *p*-cymene presented an  $\text{EC}_{50}$  value

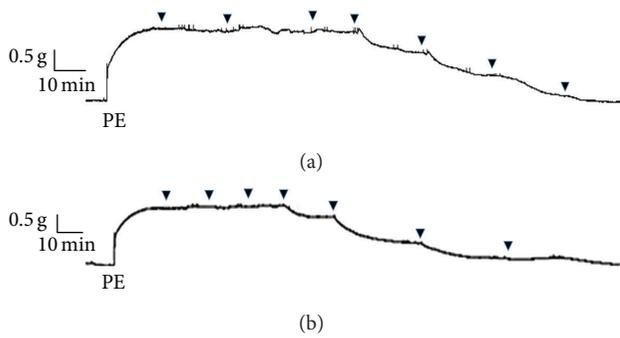


FIGURE 2: Representative original record of the effect of *p*-cymene on the isolated rat aortic rings contracted by phenylephrine with (a) and without (b) endothelium. The arrows represent the time-course of the *p*-cymene administration ( $10^{-6}$  to  $10^{-3}$  M).

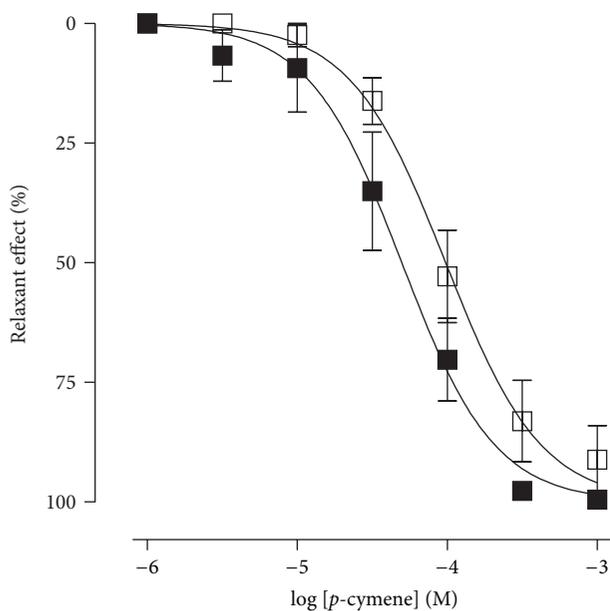


FIGURE 3: Relaxant effect of *p*-cymene on the isolated rat aortic rings contracted by phenylephrine with (■) and without (□) endothelium.

of  $5.8 \pm 1.6 \times 10^{-5}$  M and for rings without endothelium, an  $EC_{50}$  value of  $1.9 \pm 0.9 \times 10^{-4}$  M (Figure 3). There was no statistical difference between values of  $E_{max}$  and  $EC_{50}$  presented by *p*-cymene in the presence and absence of the endothelium. The contractile effect of phenylephrine was completely reestablished after 30 min of washout of the organ with a fresh new Krebs solution, to remove the *p*-cymene of the organ chambers (data not shown). Concerning a possible direct effect of the solvent, the addition of DMSO (1% v/v), which was the maximum concentration of the solvent in the organ bath in our experiments, caused no significant effect on the tonus of the aortic rings constricted (data not shown).

**3.2. Effect of *p*-Cymene in the Presence or Absence of CsCl.** In the presence of CsCl, *p*-cymene also relaxed the aortic rings without endothelium in a concentration-dependent manner. However, the dose-response curve was shifted to the right

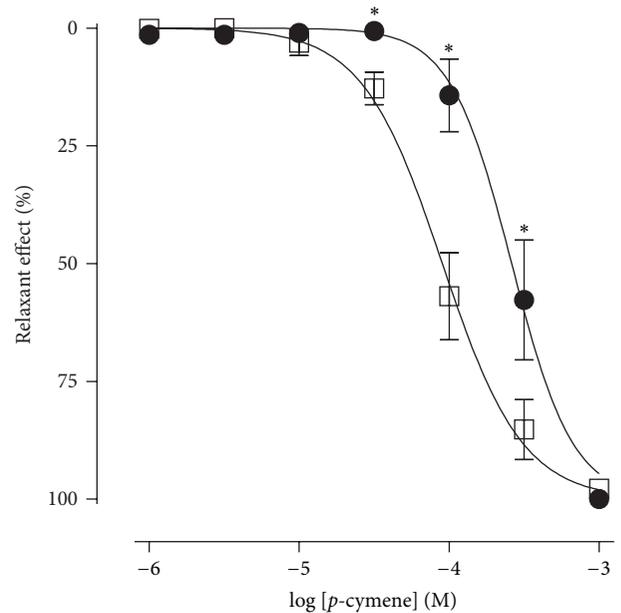


FIGURE 4: Relaxant effect of *p*-cymene on the isolated rat aortic rings without endothelium, contracted by phenylephrine, in the absence (□) and presence of CsCl 5 mM (●). \* $P < 0.05$  (unpaired *t*-test: absence  $\times$  presence of CsCl).

when compared to the control curve (Figure 4). Nevertheless, no change was observed in  $E_{max}$ , which was reached at a concentration of  $10^{-3}$  M, similarly to the control. The  $EC_{50}$  values were  $1.1 \pm 0.2 \times 10^{-4}$  M and  $2.4 \pm 0.4 \times 10^{-4}$  M for the control condition and in the presence of CsCl, respectively, showing statistical difference between them.

**3.3. Effect of *p*-Cymene in the Presence or Absence of TEA or 4-AP.** The *p*-cymene relaxed the aortic rings without endothelium, in a concentration-dependent manner, in both the absence (control) and the presence of TEA (1 mM), TEA (10 mM), or 4-AP (3 mM), with  $EC_{50}$  values of  $1.1 \pm 0.3 \times 10^{-4}$  M,  $1.6 \pm 0.5 \times 10^{-4}$  M,  $1.3 \pm 0.2 \times 10^{-4}$  M, and  $2.2 \pm 0.1 \times 10^{-4}$  M, respectively, showing no statistical differences between them ( $P < 0.05$ ). In all experimental conditions, the  $E_{max}$  value was 100% and reached  $10^{-3}$  M (Figure 5).

**3.4. Effect of *p*-Cymene in the Presence or Absence of BaCl<sub>2</sub> or Glibenclamide.** The *p*-cymene relaxed the aortic rings without endothelium, in a concentration-dependent manner, in both the absence (control) and the presence of BaCl<sub>2</sub>. However, in the presence of BaCl<sub>2</sub>, the dose-response curve was significantly shifted to the right with a change in  $E_{max}$  when compared to the control curve (Figure 6). The presence of BaCl<sub>2</sub> reduced the  $E_{max}$  from  $97.8 \pm 1.7\%$  to  $56.4 \pm 7.4\%$  and increased the  $EC_{50}$  value from  $1.1 \pm 0.2 \times 10^{-4}$  M to  $8.8 \pm 1.5 \times 10^{-4}$  M. Both values present a statistical difference between control values.

In the same way, *p*-cymene relaxed the aortic rings without endothelium in both the absence (control) and the presence of glibenclamide, but in the presence of that

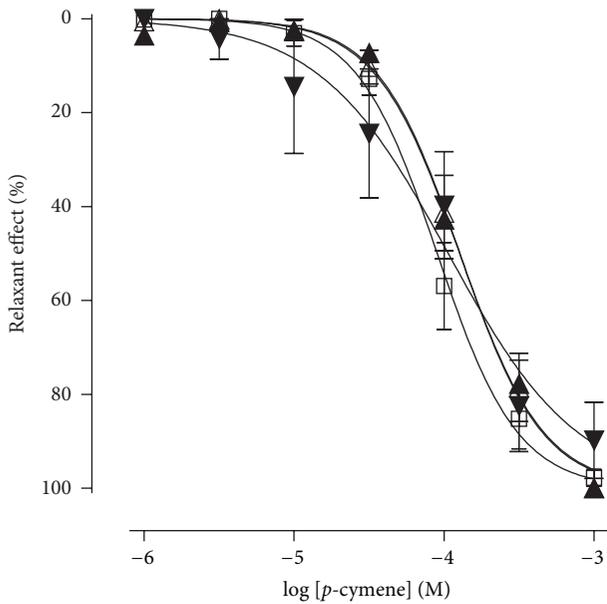


FIGURE 5: Relaxant effect of *p*-cymene on the isolated rat aortic rings without endothelium, contracted by phenylephrine, in the absence (□) and presence of TEA 1 mM (▲), TEA 10 mM (△), or 4-AP 3 mM (▼).

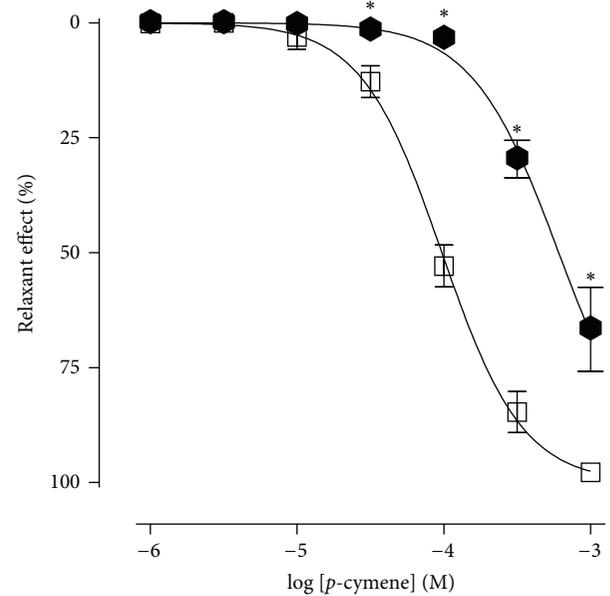


FIGURE 7: Relaxant effect of *p*-cymene on the isolated rat aortic rings without endothelium, contracted by phenylephrine, in the absence (□) and presence of glibenclamide 3  $\mu$ M (●). \* $P < 0.05$  (unpaired *t*-test: absence  $\times$  presence of glibenclamide).

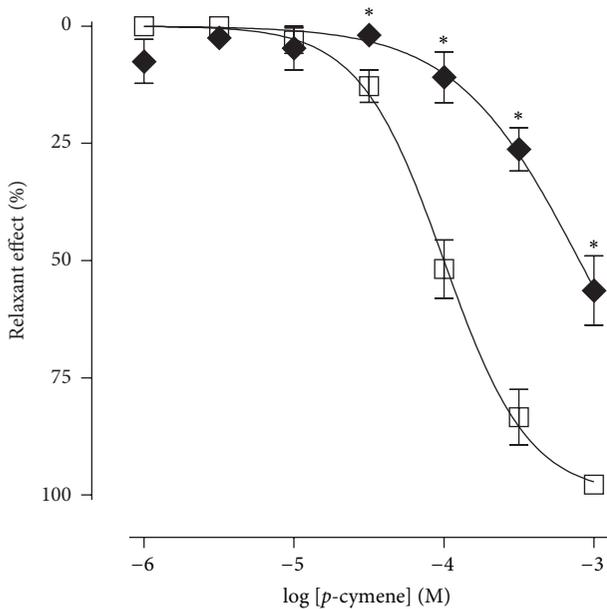


FIGURE 6: Relaxant effect of *p*-cymene on the isolated rat aortic rings without endothelium, contracted by phenylephrine, in the absence (□) and presence of BaCl<sub>2</sub> 0.1 mM (◆). \* $P < 0.05$  (unpaired *t*-test: absence  $\times$  presence of BaCl<sub>2</sub>).

potassium channel blocker, the dose-response curve was significantly shifted to the right with a decrease in  $E_{max}$  value when compared to the control curve (Figure 7). The presence of glibenclamide reduced the  $E_{max}$  from  $98.0 \pm 1.1\%$  to  $67.4 \pm 9.1\%$  and increased the  $EC_{50}$  value from  $1.1 \pm 0.3 \times 10^{-4}$  M to

$6.8 \pm 1.2 \times 10^{-4}$  M. Both values present a statistical difference between control values.

#### 4. Discussion

In this work, we demonstrated that *p*-cymene has a dose-dependent vasorelaxant effect, which was independent of the vascular endothelium with a marked involvement of potassium channels, especially the  $K_{IR}$  and  $K_{ATP}$  channels.

Smooth muscle contraction is ultimately determined by phosphorylation of the 20 kDa myosin light chain subunits (MLC<sub>20</sub>), which can occur through the  $Ca^{2+}$ /calmodulin-dependent actions of myosin light chain kinase (MLCK) or through the  $Ca^{2+}$ -independent actions of many additional kinases. The main event that determines the activation of that  $Ca^{2+}$ /calmodulin pathway with a subsequent activation of MLCK, which leads to the contraction of the smooth muscle, is increased intracellular  $Ca^{2+}$  [16]. Two classical ways to increase the  $Ca^{2+}$  driving to muscle contraction are pharmacomechanical and electromechanical coupling mechanisms. The first mechanism involves  $Ca^{2+}$  mobilization of sarcoplasmic reticulum via G-protein-coupled receptors as well as  $Ca^{2+}$  entry by  $Ca_{V1,2}$  calcium channels; the second mechanism involves change in membrane potential and activation of  $Ca_{V1,2}$  channels, with a subsequent  $Ca^{2+}$  influx from extracellular medium and increase in its intracellular levels [17].

On the other hand, the mechanisms that lead to relaxation may involve multiple signaling pathways. In the vascular smooth muscle cells, both endothelium-derived factors and the membrane potential are important in the regulation of the vascular tone [18]. Thus, a possible role for the endothelium

and  $K^+$  channels in the relaxation induced by *p*-cymene was investigated in this work.

The endothelium produces and releases at least three important relaxant factors: nitric oxide (NO), prostacyclin ( $PGI_2$ ), and the endothelium-derived hyperpolarizing factor (EDHF) [15, 19, 20]. Currently, it is generally accepted that endothelium-derived factors, such as NO, have an important role in modulating relaxation in the rat aorta. However, our results showed that *p*-cymene relaxes aortic rings in an endothelium-independent manner, with similar  $E_{max}$  and  $EC_{50}$  values in the presence or the absence of endothelium. That suggests that the effect of *p*-cymene does not depend on the release of these endothelium-derived relaxant factors.

Since the  $Ca_{V1.2}$  is the main type of voltage-opened calcium channel present in smooth muscle cells [21], which is important for the development and maintenance of contraction in smooth muscle, it is known that the opening and closing of  $Ca_{V1.2}$  are closely related variations in membrane potential. Therefore, potassium channels play an important role in controlling this membrane potential and consequently control the function of  $Ca_{V1.2}$  channels [22]. Once determined that the relaxing effect of *p*-cymene is not dependent on the presence of the vascular endothelium, denoting a likely direct effect on the smooth muscle, investigation of the role of potassium channels in that effect was carried out in the absence of endothelium. In this work it was shown that, in the presence of CsCl, a well-known blocker of potassium channels [23], the vasorelaxant effect of *p*-cymene was attenuated, indicating that some types of potassium channels may be involved in its relaxant effect.

Several different types of potassium channels are present in the vascular smooth muscle, including the calcium-activated potassium ( $K_{Ca1.1}$  or  $BK_{Ca}$ ), blocked by TEA 1–10 mM [22], voltage-gated potassium channels ( $K_V$ ), blocked by 4-AP [23], inward rectifying potassium channel ( $K_{ir}$ ), blocked by  $Ba^{2+}$  [24], and ATP-sensitive potassium channels ( $K_{ATP}$ ), blocked by glibenclamide [25].

In this work, it was demonstrated that *p*-cymene relaxed aortic rings in the presence of TEA 1 or 10 mM, which in the lowest concentration is a selective  $BK_{Ca}$  blocker and in higher concentration is a nonselective blocker for some types of potassium channels, as well as in the presence of  $K_V$  blocker, the 4-AP, without significant differences in its  $E_{max}$  and  $EC_{50}$  values. These data indicate that neither  $BK_{Ca}$  nor  $K_V$  would be involved in the relaxing effect of *p*-cymene. However, in the presence of both  $Ba^{2+}$  and glibenclamide, the vasorelaxant response to *p*-cymene was significantly reduced, with changes in the  $E_{max}$  and  $EC_{50}$ , indicating that  $K_{ir}$  and  $K_{ATP}$  could be involved in its relaxing effect on aortic rings.

*p*-Cymene is a known monoterpene, which has some applications in the odorant industry and also many biological effects, such as the modulation of MAPK and NF- $\kappa$ B activation [11], anti-inflammatory and antinociceptive effects [9], and antimicrobial effects in food [26]. Here we demonstrate, for the first time in literature, the *p*-cymene effect on the vascular smooth muscle, trying to determine its relaxing mechanism of action. Despite our best efforts, further studies

are necessary for a better comprehension of its effect on the smooth muscle.

## Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

## Acknowledgments

The authors would like to thank UNIVASF and CNPq for the financial support to conduct this work.

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