

## Relaxant effect of thymol on smooth muscle Efecto relajante del timol en músculo liso

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### Abstract:

Thymol is a phenolic monoterpene in a colorless crystalline form or white crystalline powder with a pungent, acidic, and sweet taste, present in different *Thymus* genus species, especially *T. vulgaris* and *T. zizis* (10% and 60% in the aerial part of both plants). Its chemical structure is characterized by a hydroxyl (-OH) attached to a benzene ring that derives from the p-cymene hydroxylation. Various therapeutic properties have been reported for this molecule, such as anticancer, anti-inflammatory, fungicidal, antibacterial, antioxidant, hepatoprotective, and spasmolytic in different species, notably rats, mice, rabbits, guinea pigs, and human cell lines. Different pathologies arise from the smooth muscle tissues, the most studied gastrointestinal tissue. Thymol concentrations have varied in each study, and decreased contractions induced with substances such as acetylcholine, KCl, and carbachol have been observed. This narrative article describes the processes of smooth muscle contraction and relaxation and thymol's spasmolytic effect in different smooth muscle tissues in rats, guinea pigs, rabbits, and mice. Through this research, it is suggested to the scientific community to study this molecule's possible mechanisms of action in the different types of smooth muscle tissue for further clinical studies.

### Keywords:

Thymol, medicinal plant, spasmolytic, smooth muscle

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### Resumen:

El timol es un monoterpeno fenólico con forma cristalina incolora o en polvo cristalino blanco de sabor picante, ácido y dulce, presente en diferentes especies del género *Thymus* especialmente *T. vulgaris* y *T. zizis* con un porcentaje que fluctúa entre el 10% y 60% encontrado en la parte aérea de ambas plantas. Su estructura química se caracteriza por un grupo hidroxilo (-OH) unido a un anillo de benceno, derivado de la hidroxilación de p-cimeno. Se han registrado propiedades terapéuticas diversas para esta molécula tales como anticancerígeno, antiinflamatorio, fungicida, antibacteriano, antioxidante, hepatoprotector y espasmolíticos realizados en diferentes especies, destacando las ratas, ratones, conejos, cobayos y líneas celulares de humanos. Son diferentes las patologías que surgen del desbalance del proceso fisiológico de la contracción/relajación del músculo liso, por lo que su efecto espasmolítico ha sido probado en diferentes tejidos de músculo liso; siendo el tejido gastrointestinal el más estudiado. Las concentraciones de timol han sido variadas en cada estudio y se observan disminución de las contracciones inducidas con diferentes sustancias como acetilcolina, KCl y carbacol. El objetivo de este artículo narrativo es describir los procesos de contracción y relajación del músculo liso y el efecto espasmolítico del timol en diferentes tejidos de músculo liso de rata, cobayo, conejo y ratón. A través de esta investigación se sugiere a la comunidad científica estudiar los posibles mecanismos de acción de esta molécula en los distintos tipos de tejido de músculo liso para posteriores estudios clínicos.

### Palabras Clave:

Timol, planta medicinal, espasmolítico, músculo liso

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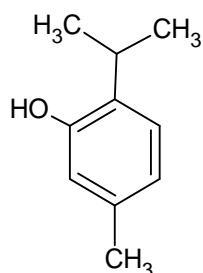
### INTRODUCTION

The research of new compounds that can be used to resolve different health problems in society is becoming a research field of high demand; for this purpose, the study of medicinal plants' main active compounds has generated significant interest. Thymol (C<sub>10</sub>H<sub>14</sub>O; 2-isopropyl-5-methyl phenol) is a

monoterpene phenolic with a hydrophobic characteristic that can form colorless translucent crystals or a white crystalline powder with a "thyme" smell. Its flavor is described as spicy, acidic, and sweet. Its chemical structure is characterized by a hydroxyl group (-OH) attached to a benzene ring (Figure 1).<sup>1</sup>

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**Figure 1.** Thymol chemical structure.<sup>3</sup>

This molecule is derived from the hydroxylation of p-cymene and its carvacrol isomer; the mevalonic acid pathway biosynthesizes it. Concerning its pharmacokinetics, thymol is rapidly absorbed by the oral route and in the small intestine. Thymol is not found freely in plasma or urine; however, it is metabolized as thymol sulfate in plasma and in urine as thymol glucuronide and thymol sulfate. Peak plasma concentration (93.1 ng/mL) was reached after above two h, and the mean terminal elimination half-time is 10.2 h. These metabolites are excreted through the urine, and the total elimination is 24 hours after ingestion. The highest plasma concentration in humans (93.1 ng/mL) was reached in 1.97 h. (Table 1).<sup>3,4</sup>

**Table 1.** Thymol pharmacokinetic data in human plasma.

$C_{max}$ =Maximum plasma concentration,  $t_{max}$  = elimination time,  $t_{1/2}$ = elimination half-time, CL= full clearance,  $V_{dss}$ = distribution volume.<sup>3,4</sup>

Average Time	
Dose (mg)	1.08
$C_{max}$ (ng/ml)	93.11
$t_{max}$ (hr)	1.97
$T_{1/2}$ (hr)	10.2
Cl (L/hr)	1.2
$V_{dss}$ (L)	14.7

This compound is found in plasma as thymol sulfate, undergoing enzymatic hydrolysis and subsequent phase II conjugation by a sulfotransferase. Besides, the conjugate thymol glucuronide is found only at doses higher than 1.08 mg; this could result from lower hepatic UDG glucuronyltransferase activity than sulfotransferase. The excretion of these metabolites is by urine present, where the elimination half-life is 10.2 hours, and total elimination is 24 hours after ingestion. Renal clearance is 1.2 hours. The distribution volume (14.7 l) suggests that thymol sulfate remains in the extracellular space; its bioavailability after administration is 16% (Table 1).<sup>2-4</sup> In other experimental animal studies, thymol metabolites are rapidly excreted by the urine. In rat plasma, thymol's sulfate and

glucuronide have been detected simultaneously. They are also present in rabbits' and dogs' urine.<sup>5,6</sup> The lethal dose 50 (DL<sub>50</sub>) of thymol in rats and guinea pigs has been reported to be 980 mg/kg and 88 mg/kg, respectively; in male mice, its DL<sub>50</sub> is 1200 mg /kg and 1050 mg/kg in female mice while cats and rabbits it is 20 mg/kg and 750 mg/kg. As an alimentary additive, thymol is considered safe with negligible toxicity.<sup>7</sup> However, the results are mixed with other toxicity studies assessing a cell's damage at the DNA level. One study reports that it is not genotoxic in mammalian cells; another study shows that high concentrations of thymol (greater than 0.1 mM) induce structural aberrations and changes in the frequency of human lymphocytes, while at low concentrations (less than 0.05 mM), it does not generate any risk to them. Furthermore, it shows no genotoxic effect on the human colon carcinoma cell line Caco-2.<sup>8</sup>

This monoterpene is found in the *Thymus* genus of the *Lamiaceae* family, especially in *T. vulgaris* (thyme) and *T. zizis* (thyme saffron) in a percentage range between 10% and 60% located in the aerial part of all the constituents of these; however, this compound is not exclusive of *Thymus* genus. *Lagoecia cuminoides* (common wild cumin) has 72.8-94.8% thymol in its essential oil and *Trachyspermum copticum* (bishop's weed) between 37.2 and 72.3% thymol in its fruits and seeds.<sup>3,9-11</sup> This phenolic compound is attributed to different therapeutic properties as an antimicrobial, fungicidal, spasmolytic, hepatoprotective, antioxidant, anticancer, and anti-inflammatory agent; these studies have been carried out in different smooth muscle tissues of different species: rats, guinea pigs, rabbits, mice, chickens, and human cell lines.<sup>7,12-19</sup>

Specifically, its action has been assessed in humans, proving its anti-inflammatory and antioxidant effect in the lungs, its effect antitussive, and the reduction of asthma symptoms.<sup>20-22</sup> This narrative article describes the processes of contraction and relaxation effect of thymol in different smooth muscle tissues.

## SMOOTH MUSCLE

Smooth muscle is present in different body systems performing several essential functions, in the gastrointestinal tract it acts in bolus propulsion, while the cardiovascular system regulates blood flow and pressure through vascular resistance. In the urinary system, the smooth muscle sets urine flow; in the genital system controls sperm propulsion and penile erection and modulates uterine contractions; and in the respiratory tract, it is responsible for tuning bronchioles diameter. Smooth muscle is innervated by the autonomic nervous system, which involuntary manages its contractions and control using hormones, neurotransmitters, and other receptors. Under the microscope, the smooth muscle is observed as elongated fiber lamellae because its filaments are not organized into sarcomeres. Its cellular structure is mononuclear, spindle-shaped, and contains invaginations called caveolae; each cell contains a sarcoplasmic

reticulum which stores the necessary calcium for contractions; a cell's distinctive feature is the dense bodies, responsible for thickening the actin filaments.<sup>23,24</sup>

### PHYSIOLOGICAL MECHANISM OF SMOOTH MUSCLE CONTRACTION

An increase in intracellular  $\text{Ca}^{2+}$  concentration mediates smooth muscle contractions. The cell can increase its ion concentration through the 1) extracellular plasma membrane and 2) sarcoplasmic reticulum (SR). Voltage-operated calcium channels are found in the extracellular plasma membrane; while L-type channels stand out in this type along the entire membrane length; calcium channels are operated by a receptor and channels activated by physical parameters. On the other hand, there is SR; this organelle in smooth muscle cells functions as a calcium reservoir and can release the ion at the cytosol by sarco/endoplasmic reticulum  $\text{Ca}^{2+}$ -ATPase (SERCA), by calcium-induced channels (CLCIC), and by second messenger inositol triphosphate ( $\text{IP}_3$ ) dependent calcium channels.<sup>24</sup> The increase of calcium through extracellular membrane-associated channels occurs by depolarization of the extracellular membrane, causing the opening of these channels. Once intracellular  $\text{Ca}^{2+}$  increases, CLCIC in the SR is activated, further increasing the concentration of this ion in the cytosol. Conversely, when an agonist activates Gq-protein coupled receptor, an intracellular signaling pathway activates phospholipase C (PLC). At the same time, PLC leads to forming two-second messengers:  $\text{IP}_3$  and diacylglycerol (DAG); when  $\text{IP}_3$  concentrations are elevated, this second messenger dependent in the SR opens, resulting in increased intracellular  $\text{Ca}^{2+}$  increase. Subsequently,  $\text{Ca}^{2+}$  binds to the cytosolic protein calmodulin. When this complex has 3 or 4 ions, it binds to a myosin light chain kinase (MLCK: myosin light chain kinase), causing a conformational change on this enzyme allowing phosphorylation of myosin light chain (MLC<sub>20</sub> myosin light chain 20 kDa). Once this process occurs, there is a conformational change in the myosin head, and the ATPase activity of this protein increase, generating the interaction between myosin and actin to produce smooth muscle contraction. (Figure 2).<sup>24,25</sup>

### PHYSIOLOGICAL MECHANISM OF SMOOTH MUSCLE RELAXATION.

Smooth muscle relaxation implies intracellular calcium decrease and myosin light chain of phosphatase activity (MLCP; myosin light chain phosphatase). Once the membrane's exit stimulus ends, ATPases of  $\text{Ca}^{2+}$  and  $\text{Na}^+$  pumps remove calcium from the extracellular space. SERCA ( $\text{Ca}^{2+}$  ATPases) of Sr recharge  $\text{Ca}^{2+}$  reserves from the cytosol into its interior, while channels operated by reserves (COR) introduce calcium from extracellular space. Once intracellular  $\text{Ca}^{2+}$  decreases, MLCK shuts down. Then, a plasmatic membrane of the SR  $\text{Ca}^{2+}$

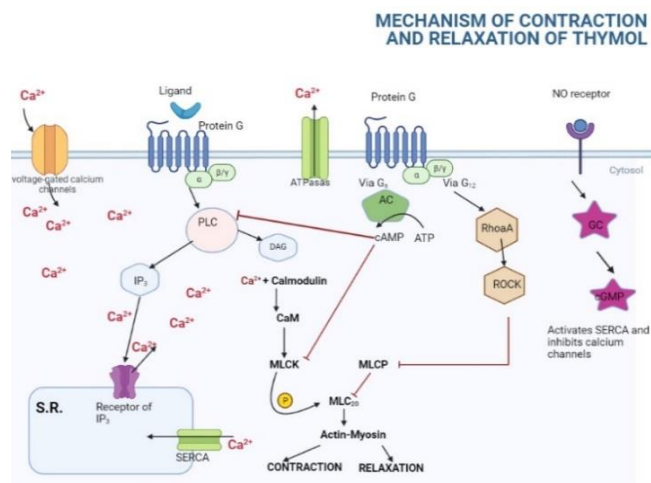
-  $\text{Mg}^+$  ATPases binds to two  $\text{Ca}^{2+}$  ions reducing this ion concentration inside the cell, and Mg binds to the catalytic site of this pump to mediate its reaction. While MLCP participates in MLC dephosphorylation, promoting smooth muscle relaxation.<sup>24</sup> On the other hand, the different G-protein signaling pathways greatly influence smooth muscle relaxation mechanisms; for example, Rho-kinase (ROCK) is essential in smooth muscle relaxation because when G-protein RhoA is activated by a ligand (noradrenaline, endothelin, angiotensin, etc.) ROCK is also activated; this enzyme phosphorylates MLCP and inhibits its action. ROCK inhibitors such as Y-27632 and fasudil compete with this enzyme promoting muscle relaxation.<sup>25</sup> Another critical enzyme in this mechanism is cAMP-dependent protein kinase (PRKA; cAMP-dependent protein kinase), which is activated once the Gs subunit by a receptor causes a stimulation of adenylyl cyclase (AC; adenylyl cyclase); this interaction results in the second messenger cyclic Adenosine monophosphate (cAMP; cyclic adenosine monophosphate), this nucleotide activates PRKA which phosphorylates MLCK and PLC and also activates the  $\text{Na}^+/\text{K}^+$  ATPase pump. cAMP phosphatase and inhibits RhoA in smooth muscle. It is important to mention that the different phosphodiesterases regulate the concentrations of cAMP.<sup>26,27</sup> Currently, the relaxant effect of  $\beta$ -receptors in smooth muscle has been proven (being the cyclic cAMP pathway one of them). Additionally, membrane repolarization plays a critical role in the mechanism of smooth muscle relaxation and may also be a significant event mediated by these receptors. The role of the  $\text{K}^+$  channel in depolarization is crucial; currently, 2-subtypes of these channels have been identified. The evidence shows that PRKA activation implies the phosphorylation of this channel, and the activation of PRKA independent channel is modulated by G-protein. However, this mechanism has not been fully understood.<sup>28</sup> Another studied relaxation mechanism is through the cyclic monophosphate guanosine nucleotide (cGMP cyclic guanosine monophosphate). The activation of this pathway is initiated by nitric oxide (NO, nitric oxide) responsible for guanylyl cyclase activation (GS; guanylyl cyclase), inducing cGMP formation. Cyclic guanosine monophosphate activates G-kinase (PKG; protein kinase G); this protein has three different action points for muscle relaxation: 1) L-type  $\text{Ca}^{2+}$  channel inhibition, 2) Inhibition of  $\text{IP}_3$  calcium-dependent channels, 3) acts in SERCA promoting calcium recapture in the SR (Figure 2).<sup>29,31</sup>

### SPASMOLYTIC EFFECT OF THYMOL IN SMOOTH MUSCLE TISSUE

In recent years there has been an advance in the research on the properties of thymol. One of them has been as a spasmolytic in smooth muscle tissue to apply in solution for the treatment of different health problems such as premature birth, vasoconstriction, abdominal pain derived from spasms, and dysmenorrhea, among others.<sup>2,7</sup> The first record about this

property of *T. vulgaris* dates to 1962 by Jensen and Dyrud; however, the main active compound in this plant that could provide this beneficial effect was not yet known.<sup>32</sup> The following is a synthesis of different research results about thymol as antispasmodic over the last few years, classified by type of smooth muscle tissue.

**Figure 2. Physiological mechanism of smooth muscle relaxation.**<sup>24,25,29-31</sup> Black arrows are interpreted as activators in the indicated direction. The red lines represent inhibitory effects on proteins. PLC: phospholipase C, DAG: Diacylglycerol; IP<sub>3</sub>: inositol triphosphate; CaM: Complex Ca<sup>2+</sup>-calmodulin; MLCK: myosin light chain kinase; MLCP: Myosin light chain phosphatase; MLC<sub>20</sub>: Myosin light chain 20; SR: Sarcoplasmic reticulum; SERCA: Ca<sup>2+</sup> ATPases; ROCK: Rho kinase; GC: guanylyl cyclase; cGMP: cyclic guanosine monophosphate; cAMP: cyclic adenosine monophosphate; AC: Adenylyl cyclase; ATP: Adenosine triphosphate; NO: Oxide nitric.



### ANTISPASMODIC EFFECT ON GASTROINTESTINAL SMOOTH MUSCLE TISSUE

The results obtained from rat-isolated duodenum tissue showed that increasing concentrations of *T. vulgaris* inhibit acetylcholine-induced contractions reversibly.<sup>34</sup> Other study on gastrointestinal smooth muscle tissue (duodenum and ileum) of rats and guinea pigs demonstrated that the extract of *T. vulgaris* had spasmolytic properties and was the first to suggest that the possible mechanism of action was a blockage of Ca<sup>2+</sup> channels by interference between the nerve axon and the muscle membrane.<sup>35</sup> Also, different concentrations of thymol effects (0.02-2 mM) electrical and mechanic activities of guinea pig *Taenia coli* smooth muscle cells found that thymol at 0.05 mM suppresses the membrane's action potential and reduces its resistance and potential; likewise, 1mM concentration inhibits spike generations, hyperpolarizes membrane and its resistance.<sup>36</sup> On the other hand, the antispasmodic activity of

thymol was analyzed in rabbit jejunum in order to know if it is possible to use this compound to relieve irritable bowel discomfort; the results of this experiment showed that it does have an inhibitory effect on contraction (IC<sub>50</sub> 2.85 × 10<sup>-2</sup> ± 1.2 × 10<sup>-2</sup> µg/mL).<sup>37</sup> It is worth mentioning that the result rules out that the mechanism of action of thymol in intestinal tissue is by activation of the β<sub>2</sub>-adrenergic receptors present in this tissue. In addition, the effect of thymol was analyzed in guinea pig trachea to test its traditional use as a bronchodilator, and at high concentrations, thymol has significant results. Subsequently, the spasmodic effect of *T. vulgaris* extract in guinea pig ileum depends on its concentration, obtaining that the maximum concentration evaluated is 1.7 mg/mL. In addition, the results show that this plant extract inhibits acetylcholine-induced contractions, concluding that this effect may come from an anticholinergic activity as a serotonergic blockade activity.<sup>38</sup> Likewise, the authors tested *T. vulgaris* spasmolytic effect at low and normal concentrations as well as its effect separately: isolated thymol (15 µg/ mL) showed inhibition of 43% in contractions induced with 60 K<sup>+</sup> mM and contractions induced by 60 K<sup>+</sup> mM its EC<sub>50</sub> (in the range 70 µg/ mL) in a concentration-dependent manner, was found to be more than carvacrol (200 µg/ mL) in ileum guinea pig tissue. In contrast, thymol alone has no relevant results in the *in vivo* rat trachea model, and the authors suggest that the standardization of thymol should be reconsidered as a spasmolytic tissue. Regarding the comparison of the effect of *T. vulgaris* extract with low thymol (< 0.005%) and the extract with normal amounts of thymol (> 0.038%), the latter is more effective against acetylcholine-induced contractions, thus interpreting that thymol has significant anticholinergic effects in guinea pig ileum.<sup>39</sup> Afterwards, in 2020, researchers studied the effect of *T. vulgaris* extract combined with excipients compared with pure extract for possible treatment of irritable bowel syndrome with diarrhea; the results showed that in colon tissue, the contractions are almost annulled using the extract plus excipients; however, in the ileum, the pure extract was shown to have more effect in reducing tone. In addition, the extract with the excipients showed that it blocks L-type calcium channels, which would reduce gastrointestinal muscle tone.<sup>40</sup>

### RELAXING EFFECT OF THYMOL IN AIRWAY SMOOTH MUSCLE

Concerning airway smooth muscle, some authors compared the spasmolytic effect of *T. vulgaris* extract and ethanol with different spasmogenic agents; interestingly, in pure ethanol, a 2% relaxation of tracheal smooth muscle was observed in contractions provoked with BaCl<sub>2</sub>, carbachol e histamine but 0% in contractions stimulated with PGF<sub>2α</sub>; however, the ethanol extract of *T. vulgaris* had relaxation with PGF<sub>2α</sub> (89% de inhibition), histamine (73% inhibition), BaCl<sub>2</sub> (43% inhibition), this article was decisive in attributing to this herbal extract spasmolytic property.<sup>41</sup> The antispasmodic activity of thymol

was analyzed in rabbit jejunum to prove its traditional use as a bronchodilator. At high concentrations, thymol showed significant results ( $10^{-4}$ – $10^{-2}$  M).<sup>37</sup> In 2016, the safe use of *T. vulgaris* and *Primula officinalis* extracts was confirmed as a natural and medicinal treatment in relieving cough symptoms by acting as a bronchodilator for its antispasmodic properties. Schonknecht and cols. affirm that the extract of both plants causes a comparable synergy with Ambroxol medicine.<sup>12</sup>

### THYMOL AS VASCULAR SMOOTH MUSCLE RELAXANT

One study about the effects of thymol in rat aortic strips found that specific low doses of thymol ( $3 \times 10^{-8}$  y  $3 \times 10^{-7}$  M) produces a contractile activity, reaching 15% contractile activity as a consequence of  $\alpha^1$  y  $\alpha^2$  adrenergic receptor engagement; however, with increasing dose ( $> 10^{-6}$  M) muscle tone and contractile activity is wholly inhibited due to activation of  $\beta$ -adrenergic receptors; the authors suggest 3 action pathways of action of thymol to function as a vasodilator: 1) net spasmolytic effect on smooth muscle cells, 2) inhibition of noradrenaline through activation of  $\alpha_2$ -receptors and 3) activation of A-kinase through activation of  $\beta$ -receptors.<sup>42</sup>

### UTERINE RELAXING EFFECT OF THYMOL

In 1962, being the first record of *T. vulgaris* as a spasmolytic, the authors proved that in isolated Wistar rat uterus tissue, this extract reversibly inhibits acetylcholine-induced contractions.<sup>34</sup> In 2022, the most recent study published aimed to answer the question of whether thymol has an antagonist role in acetylcholine (ACh)-induced contraction in utero; increasing concentrations of thymol inhibited contractions in a concentration-dependent manner, where the  $IC_{50}$  was evaluated 5.35 Mm in utero, based on the results of their study it was hypothesized that thymol competitively antagonizes muscarinic receptors since thymol at low concentration with low concentrations had an inhibitory effect and thymol at low but high concentrations of ACh shown to have a significant inhibitory effect; in turn, high concentrations of thymol decreased all ACh-induced contractions.<sup>43</sup>

### THYMOL MECHANISMS OF ACTION

Thymol mechanisms of action are not completely defined or understood; however, some authors have proposed hypotheses about its influence on smooth muscle relaxation ranging from calcium channel blockade to concentration-dependent inhibition of muscarinic ACh receptors.<sup>34,36,43</sup> Nevertheless, results of the study demonstrated that thymol micro concentrations reduce  $Ca^{2+}$ -ATPase activity and increase  $Ca^{2+}$  permeability in the sarcoplasmic membrane; on the other hand, they show that this compound exerts an agonists effect on  $\alpha_1$ ,

$\alpha_2$ , and  $\beta$  adrenergic receptors in guinea pig portal vein and stomach isolated tissue producing a relaxing effect on them, these results suggest that the molecule's action mechanism differs depending on smooth muscle type.<sup>41</sup> The evidence to understand thymol's action mechanism on smooth tissue is scarce, so these mechanisms have been investigated in different tissues or cell lines. Furthermore, authors proved that skeletal muscle blocks voltage-operated sodium channels, obtaining an  $IC_{50}$  de 104  $\mu$ M; thymol (100  $\mu$ M) has also been reported to block voltage-dependent sodium channels in rat brain HEK 293 cells, attributing contraceptive and anesthetic properties.<sup>44</sup> Also, thymol blocked  $Ca^{2+}$  entrance through  $Ca^{2+}$  channels on canine and human ventricular cardiomyocytes in a similar way that verapamil (calcium channel blocker) drug does, suggesting that thymol binds to channel proteins, modifying them.<sup>45</sup> It is essential to know the mechanism of action as an enzyme inhibitor or activator, as a direct G protein-coupled receptors ligand, or as an activator/blocker of voltage-dependent channels involved in the physiological process of contraction and relaxation in order to propose it as a drug or auxiliary to known drugs.

### CONCLUSION

Thymol has been shown to have relaxation effects on the contractions of different types of smooth muscle; these contraction and relaxation processes are based on MLC's phosphorylation and dephosphorylation; these biochemical processes are regulated by an exchange of different intracellular molecules that can be activated through a direct stimulus on the membrane or utilizing a ligand, because of the insufficient knowledge about thymol mechanisms of action on the smooth muscle; investigating further is suggested in order to use this molecule in future clinical research.

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