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Original Research Article

Antispasmodic and Relaxant Effects of *Thymus algeriensis* Dichloromethane fraction on Intestinal Smooth Muscle Motility of *Wistar rats*

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ABSTRACT

Digestive disorders are common reasons for medical consultations, making natural remedies based on medicinal plants increasingly popular. This study explored the potential benefits of *Thymus algeriensis* on the motility of the smooth intestinal muscle to address digestive tract disorders. The study focused on an *in vitro* test using the rat jejunum to evaluate the antispasmodic and relaxant effects of its dichloromethane fraction. The results showed that the dichloromethane fraction caused a significant relaxing effect on spontaneous contraction and an antispasmodic effect on rat jejunum precontracted with carbachol or KCl in a dose-response manner ($p < 0.001$). The inhibitory activity demonstrated was comparable to those produced by a non-competitive antagonist of voltage-dependent Calcium channel and cholinergic receptors. These findings highlight the potential of *Thymus algeriensis* dichloromethane fraction to address digestive disorders.

***Keywords*:** *Thymus algeriensis*, dichloromethane fraction, antispasmodic, smooth muscle, rat jejunum, digestive disorders

Introduction

Thymus algeriensis is a perennial herbaceous species of the Lamiaceae family. It can be recognized by its small evergreen hairy leaves with a light green to light gray color; It also has small purplish-pink or whitish flowers.[1,2] This plant belongs to the *Hyphodromi* section of the genus Thymus and is one of the 215 species included in this genus.[3] It is well distributed in the Mediterranean region.[1,3] In Morocco particularly, *Thymus algeriensis*, is easily found in mountainous regions such as Middle Atlas, High Atlas, Western Anti-Atlas, Oriental and Rif areas, where it is commonly named 'Zaitra', 'Tazerkahha', 'Azoukni', 'Djertil'.[2,4] Local people harness it in traditional medicine and often recommend it for addressing a wide range of diseases. Like most *Thymus* species that are traditionally used to treat skin, urinary, circulatory, genital, nervous, respiratory diseases, and digestive ailments; such as stomach aches, diarrhea, dysentery, and intestinal ulcers.[5-11] The research on *Thymus algeriensis* chemical composition showed the presence of oxygenated monoterpenes (α-terpineol, carvacrol, thymol, linalool, camphor, and borneol), Monoterpene hydrocarbons (p-cymene, γ-terpinene, α-terpinene, and camphene), and polyphenols (rutin, coumaric acid, luteolin, cinnamic acid, apigenin, and quercetin).[10-13] That might support the several biological activities and the traditional use of this plant.

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In prior research, *Thymus algeriensis* aqueous extract showed antispasmodic and antidiarrheal activities on rodents.[13,14] The focus of this study was to continue the research on this plant by assessing the relaxant and antispasmodic properties of its dichloromethane fraction.

Material and Methods

Plant Material

Thymus algeriensis was harvested from the Jbel Hamra, located to the southeast of Oujda in Morocco. The harvesting occurred between April and May 2019. The plant were identified at Mohammed First University Oujda by Professor Benyounes Haloui, a botanist from the Department of Biology. A designated reference specimen (No. HUMPOM 425) was kept in placed within the university's herbarium collection.

Animal Model

The study of the pharmacological activities of the fraction was conducted on jejunum sections obtained from Male and female *Wistar rats*, weighing between 250 and 300g. The animals were bred and acclimatized under normal environmental conditions in the animal facility of the Department of Biology, Mohammed First University, Morocco. The animals were subjected to an 18-hour food withdrawal period before the experiment. All animal procedures were adhered to the guidelines as outlined by the National Research Council's for the care and utilization of laboratory animals.[15]

Buffers

The Krebs-Henseleit buffer (KHB) was used as a physiological fluid in multiple version (Table 1).

Chemicals and Pharmacological Substances

Dichloromethane, papaverine, verapamil, Atropine and carbamylcholine chloride (CCh) were obtained from Sigma-Aldrich Co. and Acros Organics. All chemicals employed were of analytical grade, and were dissolved in distilled water.

Preparation of dichloromethane Fractions
The aerial parts (stems, leaves, and flowers) of *Thymus algeriensis* were air-dried in the shaded conditions at room temperature for a minimum of ten days. After which they were ground into a powder.
The extraction was carried out on 182 g of plant powder using a Soxhlet apparatus, using a sequential process with solvents of increasing polarity: The obtained dichloromethane fraction was filtered using Whatman No. 3 filter paper, and the filtrate was evaporated to dryness using a rotary evaporator (Buchi B-480, Switzerland). Subsequently, the fraction were subjected to overnight drying at 30°C in an oven.[16,17]
The extraction yield (3.39%) was calculated as a percentage according to equation 1 (eq 1), and the obtained dichloromethane fraction was stored in the freezer (-20°C) until further use. The dichloromethane fraction was dissolved in Dimethyl sulfoxide at a concentration lower than 1%.

(eq1) Extraction Yield % = $\left(\frac{\text{Final weight of dichloromethane fraction}}{\text{Initial weight of the dry plant}}\right)$ x 100.

Preparation of Jejunum Fragments
The animals were fasted for 18 hours before each experiment with free access to water. They were anesthetized using ethyl ether by an inhalation method. The bodies of the animals were fixed on a board, and the abdominal cavity was opened to collect and preserve 2 cm-jejunum section in oxygenated normal KHB physiological fluid for the tests. The jejunum section was incubated in an isolated organ bath containing 10 ml of normal KHB physiological solution. It was then subjected to a tension of 1g and connected to an isotonic transducer. The jejunum section was maintained at a temperature of 37°C and a pH of 7.4 while being continuously oxygenation via an air bubbler for a duration of one hour.

Signals Recording System
After stabilizing the jejunum fragment in the organ bath, the KHB was changed every 15 minutes to equilibrate the organ before adding the fraction or other pharmacological substances. During all tests, were recorded for a minimum duration of 7 to 8 minutes using a recording cylinder rotating at a speed of 13 mm/min. This allowed obtaining tracings that would be analyzed.

Antispasmodic Study
The protocol was adapted from previous works published by our team.[16,18]

Antispasmodic Effect of dichloromethane fraction on Pre-contracted Rat Jejunum with Carbachol (CCh) or Potassium Chloride (KCl)
After stabilizing of the basic contractions of rat jejunum, we pre-contract this later with a high-potassium medium (KCl 75 mM) or CCh 10^{-6} M. When the jejunum's smooth muscle tone was stable, we added cumulative doses of the dichloromethane fraction (TaDF) (0.01, 0.1, 0.3 mg/ml) which gave us the most significant effect in the preliminary tests.

Concentration-Response curves of Carbachol in the Presence of Thymus algeriensis dichloromethane fraction (TaDF) or Atropine
Concentration response curves for CCh were produced. The jejunum section was contracted with cumulative doses of CCh ranging from 10^{-8} to 10^{-5} M (control). The other experiments began by introducing a concentration of TaDF (0.01; 0.1 or 0.3 mg/ml) or atropine (10^{-6} M) into the isolated organ bath. Once stabilization was achieved, the same procedure as the control was followed.

Concentration-Response curves of $CaCl_2$ in the Presence of Thymus algeriensis dichloromethane fraction (TaDF) or Verapamil
In order to validate the impact of TaDF on calcium channels, the normal KHB solution was substituted, without interrupting the recording, with a calcium-free high K^+ solution and EDTA (Ethylenediaminetetraacetic acid) (0.1 mM) to eliminate calcium from the tissues for duration of 10 minutes. Following this, it was substituted with KHB rich in potassium and calcium-free. After stabilization, increasing and cumulative doses of $CaCl_2$ (0.1, 0.3, 1, 3, 10 mM) were introduced. The same protocol was followed for the remaining tests, with the exception that the different doses of TaDF (0.01, 0.1, 0.3 mg/mL) or Verapamil (10^{-6} M) (Positive control) was added prior to $CaCl_2$.

Statistical Analysis
The results are expressed as mean with standard error of the mean (SEM). Statistical analysis was conducted by utilizing the one-way analysis of variance (ANOVA) followed by a post hoc Tukey test in all experiments. The statistical analysis was performed using GraphPad Prism software, version 5.01 from San Diego, California USA. Statistical significance was established when the probability value (p) fell below 5%. Significance levels were denoted as follows: (*): $p < 0.05$ (**): $p < 0.01$ (***): $p < 0.001$.
The 50% inhibitory concentration (IC_{50}) was calculated by linear regression method.

Table 1: Different Krebs-Henseleit buffers used in the experiments

Compounds (mM)	Normal KHB	Calcium free high K^+ KHB	Calcium free KHB
NaCl	118	48	121.7
KCl	4.7	75	4.7
$CaCl_2$	2.5	0	0
$MgSO_4$	1.2	1.2	1.2
$NaHCO_3$	25	25	25
KH_2PO_4	1.2	1.2	1.2
Glucose	10	10	10

Results and discussion

Antispasmodic Effect of Thymus algeriensis dichloromethane fraction (TaDF) on Pre-contracted Rat Jejunum with Carbachol (CCh) or Potassium Chloride (KCl)
Thymus algeriensis dichloromethane fraction *(TaDF)* showed an effective dose-dependent inhibition of CCh-induced contractions (Figure. 1) and KCl-induced contractions (Figure. 2) compared to the control, with a maximum antispasmodic effect at 0.3 mg/ml with IC_{50} values equal to 0.136 ± 0.015 and 0.162 ± 0.012 mg/ml respectively. The difference between the control and each dose is statistically significant (Figure. 1-2).

Concentration-Response curves of $CaCl_2$ and CCh in the Presence of different doses of TaDF
The effect of $CaCl_2$ concentration-responses in the presence and absence of *Thymus algeriensis* dichloromethane fraction was tested on rat intestine. The results demonstrated that in the presence of this fraction (0.01 to 0.3 mg/ml), the concentration-response curves were moved to the right and downward from the control. The effect of Verapamil (10^{-6} M) was similar to that obtained by 0.3 mg/ml of *Thymus algeriensis* dichloromethane fraction (Figure. 3).

Other experiments were performed to determine whether *Thymus algeriensis* dichloromethane fraction suppressed CCh-induced jejunum contractions. Initially, jejunum contractions were recorded with and

without *Thymus algeriensis* dichloromethane fraction treatment. *Thymus algeriensis* dichloromethane fraction at a ranging dose of 0.1 to 0.3 mg/ml significantly reduced ($p < 0.001$) the CCh-induced contraction and the concentration-response curves were moved to the right and downward from the control (Figure. 4). Similarly, in the presence of 10^{-6} M of Atropine, the contractile response effect of increasing and cumulative doses of CCh was suppressed significantly ($p < 0.001$) (Figure. 4).

Herbal remedies have gained prominence as a viable option for treating a range of health conditions, due to their remarkable pharmacological abilities. Among these properties, they have a myorelaxant and antispasmodic effects.[19,20] Therefore, we studied the myorelaxant and antispasmodic effects of *Thymus algeriensis* dichloromethane fraction using rat jejunums in an *in vitro* model. Our preliminary results showed that all organic fractions caused a dose-dependent relaxing effect. This relaxing effect was reversible after washing on spontaneous contractions. This suggested that the observed inhibition was not the result of intestinal damage caused by the organic fractions used. This effect was also observed in previous studies with fractions of other plants.[16,21,22] TaDF exerted the most significant relaxing effect on spontaneous contractions compared to the other fractions of the plant, with a complete inhibition. The findings provide the impetus for further research on the *Thymus algeriensis* dichloromethane fraction, aimed at improving understanding of the mechanism responsible for the observed relaxant effect. For this, we pre-contracted the rat jejunum with a high KCl medium. The elevated potassium concentration induced a biphasic muscle response, comprising a phasic contraction followed by a sustained tonic contraction, with a brief relaxation phase in between. This type of response has also been observed in the ileum, colon, and stomach of rats.[23,24] The increasing of potassium concentration above 30 mM involves the depolarization of the membrane, which triggers the activation of voltage-dependent calcium channels (VDCCs). This activation permits the influx of Ca^{2+} into the cytoplasm.[25] In fact; the jejunum smooth muscle contractions are regulated by depolarization and repolarization cycles that are mediated by an increase in Ca^{2+} influx through voltage-dependent calcium channels. This activates the contractile elements.[26] and mobilizes Ca^{2+} from sarcoplasmic reticulum via inositol triphosphate (IP_3) receptors. Therefore, Ca^{2+} originates from either the sarcoplasmic reticulum or the extracellular matrix through calcium channels. Natural products that induced an inhibitory effect on contractions provoked by KCl inhibit the influx of Ca^{2+} ions.[27] *Thymus algeriensis* dichloromethane fraction exhibited an inhibitory effect on rat jejunum contractions induced by a high KCl medium in a dose-dependent manner, similar to the inhibition of the spontaneous contractions of the *Rabbit* jejunum. Therefore, it can be suggested that the inhibition of contraction in rat jejunum reflects the entry of Ca^{2+} through VDCCs. This was substantiated by the significant inhibitory effect of the *Thymus algeriensis* dichloromethane fraction on the maximum response to $CaCl_2$, causing a shift in the dose-response curves both downwards and to the right. Hence, we can hypothesize that one or more components in the *Thymus algeriensis* dichloromethane fraction exert a non-competitive antagonistic action on VDCCs. On the other hand, verapamil, a membrane calcium channel antagonist also reduced the maximal response in the curves induced by $CaCl_2$. This molecule remarkably inhibits calcium entry via L-type VDCCs. Relaxation of the intestinal smooth muscle is due to a reduction in intracellular calcium, which can be caused by a decrease in extracellular calcium entry through calcium channels or a reduction in calcium released from the sarcoplasmic reticulum (SR). The effect of TaDF was more pronounced than that of other plant extracts mentioned in the literature, such as the aqueous extract of *Thymus algeriensis*,[13] the dichloromethane fraction of *Anthemis mauritiana*,[16] and *Artemisia campestris* hydroethanolic extract.[28] However, it was found to be less effective than *Buddleja scordioides* and *Buddleja perfoliata*.[29]

The pathway involving cholinergic receptors was also tested using carbachol (CCh), a structural analog of acetylcholine (ACh).[30] This latter is a neurotransmitter secreted by postganglionic parasympathetic neurons that innervate the intestine. The response to ACh is mediated by the activation of two specific muscarinic receptors (M_2 and M_3)[31] that can produce changes in tension without necessarily affecting the membrane potential. Activation of these receptors opens ROC-type membrane calcium channels and releases calcium from its storage sites, increasing intracellular Ca^{2+} concentration. This increase is mediated by IP_3, which acts on receptors located on the membranes of the sarcoplasmic reticulum, allowing the release of stored calcium into the cytoplasm,[32] inducing phasic and tonic contractions. Carbachol is not degraded by acetylcholinesterase and is a cholinomimetic drug that interacts with muscarinic receptors on the smooth muscle cell membrane.[31] On the rat jejunum; carbachol caused a contraction involving two different mechanisms coupled to muscarinic receptors. The main subtypes of muscarinic receptors in the gastrointestinal tract are M_2 and M_3, both of which are coupled to G proteins. M_2 primarily acts by inhibiting adenylyl cyclase, thereby decreasing cAMP levels in the cell, while M_3 induces activation of phospholipase C. The latter results in the breakdown of phosphatidylinositol bisphosphate (PIP_2) into IP_3 and diacylglycerol (DAG).[33] IP_3 interacts with RS receptors and releases stored calcium from this organelle into the cytoplasm.

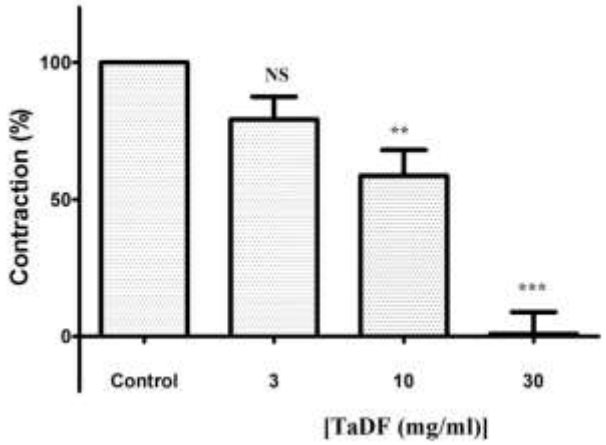

Figure 1: Antispasmodic effect of *Thymus algeriensis* dichloromethane fraction (TaDF) on rat jejunum pre-contracted with (CCh 10^{-6} M). NS, No significant. **, $p < 0.01$; ***, $p < 0.001$. The difference is statistically significant compared to the control (mean ± SEM, n = 6).

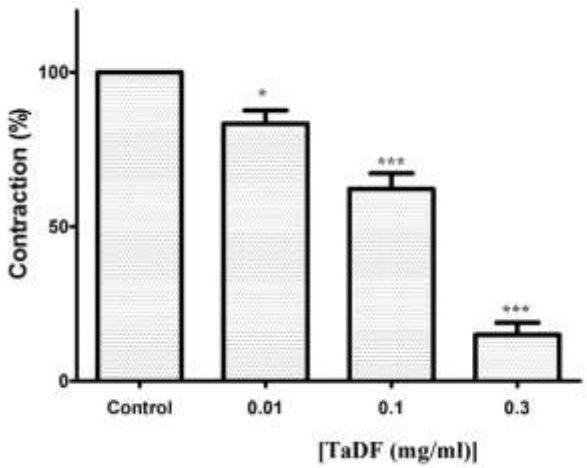

Figure 2: Antispasmodic effect of *Thymus algeriensis* dichloromethane fraction (TaDF) on rat jejunum pre-contracted with (KCl 75 mM). **, $p < 0.01$; ***, $p < 0.001$. The difference is statistically significant compared to the control (mean ± SEM, n = 6).

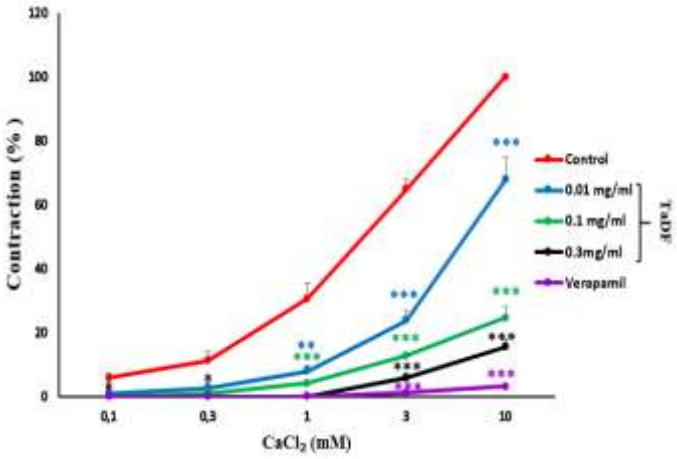

Figure 3: Concentration-response curves of $CaCl_2$ in the presence and absence of the dichloromethane fraction of *Thymus algeriensis*. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. The difference is statistically significant compared to the control (mean ± SEM, n = 6).

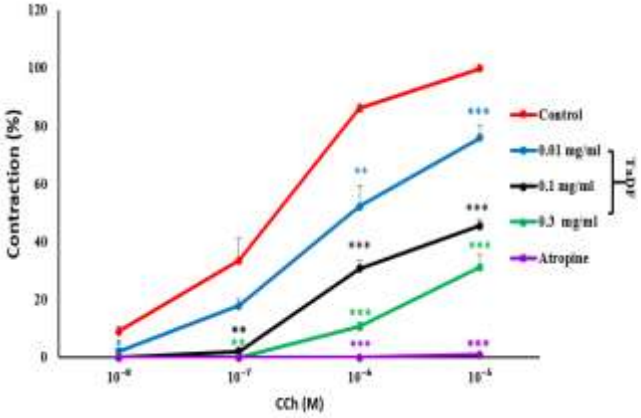

Figure 4: Concentration-response curves of Carbachol in the presence and absence of different doses of the dichloromethane fraction of *Thymus algeriensis*. **, $p < 0.01$; ***, $p < 0.001$. The difference is statistically significant compared to the control (mean ± SEM, n = 6).

TaDF inhibited the tonic contraction of rat jejunum induced by 10^{-6} M of Carbachol in a dose-dependent manner. Cumulative doses of carbachol in the presence or absence of the *Thymus algeriensis* dichloromethane fraction leads to a significant suppressive activity on the maximal contraction provoked by carbachol while shifting the concentration-response curves to the right and down. This effect was similar to that observed with the use of Atropine as a positive control. Based on these findings, it can be suggest that one or more components in the *Thymus algeriensis* dichloromethane fraction could act as non-competitive antagonists via one of the two contraction pathways coupled to muscarinic receptors. These results are comparable to those obtained previously of a medicinal plant that exhibit non-competitive antagonistic activity against CCh in the smooth muscles of ileum.[34] The inhibition of these contractions induced by CCh and KCl by the *Thymus algeriensis* dichloromethane fraction may indicate that one or more spasmolytic compounds act as non-specific antagonists.

In prior study using the aqueous extract of *Thymus algeriensis* we demonstrate that the spasmolytic effects are mediated possibly through Ca^{2+} antagonist mechanism and cholinergic receptors. However, there was no observed impact on adrenergic receptors, nitric oxide, or guanylate cyclase. These effects can be due to the presence of Apeginin, Luteolin and Quercitin in in aqueous extract of *Thymus algeriensis* as these compounds are renowned for their antispasmodic properties. These components could act alone or together with other unidentified compounds.[13] Several studies demonstrate that the dichloromethane fraction of medicinal plants had the most significant antispasmodic effect such as *Origanum majorana* L.[17] *Piliostigma reticulatum*,[35] *Cynara scolymus*.[36] *Persea cordata*.[37] Therefore, the antispasmodic effect of medicinal plants are concentrated in the nonpolar fractions. These fractions contain a substantial amount of lipids and essential oils, comprising a variety of compounds like monoterpenols, sesquiterpenes, and terpene esters. Notably, compounds such as terpinenes, terpineols, limonene, and linalool within these fractions are known for their antispasmodic activities. Moreover, the utilization of medicinal plants in the treatment of digestive ailments across various regions worldwide lends support to our initial hypothesis.[38,39]

Conclusion

The *in vitro* tests on rat jejunums was used to evaluate the antispasmodic and relaxant effects of the *Thymus algeriensis* dihloromethane fraction. *Thymus algeriensis* dichloromethane fraction showed an antispasmodic and myorelaxant effects. These effects are mediated through the antagonistic mechanism of Ca^{2+} and cholinergic receptors. The results support the traditional uses of this plant in treating gastrointestinal disorders.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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