

Original Research

# The Antispasmodic Effect of *Warionia saharae* Essential Oil in Experimental Models and its Mechanism of Action

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## Abstract

With several medicinal and aromatic species, the Asteraceae family is one of the largest angiosperm families. The genus *Warionia* is represented in this family by only one species, *Warionia saharae*. In Moroccan traditional medicine, this species is widely used to treat gastrointestinal problems. Essential oil of this plant (EoWs) was studied for possible myorelaxant and antispasmodic activities to rationalize some of the traditional uses. In this investigation, hydrodistillation was used to obtain the essential oil from the aerial part of the dry plant extract (EoWs), which was then analyzed using gas chromatography coupled to mass spectrometry (GC/MS). The major compounds identified in the EoWs are nerolidyl acetate (21.44%),  $\beta$ -Eudesmol (19.47%), linalool (16.48%), 1-terpinene-4-ol (10.93%), and cineole (5.34%). EoWs is relatively safe in the case of acute intake up to 2 g/kg body weight of albino mice. The effect of EoWs on intestinal relaxation was investigated using rabbit and rat jejunal smooth muscle. We have noticed that EoWs produce a myorelaxation on basal rabbit jejunum's contractions in a concentration-dependent manner with a maximal effect at 30  $\mu$ g/mL. This myorelaxation was not dependent on adrenergic receptors. When the rat jejunums were pre-contracted with 25 mM KCl or 10  $\mu$ M Carbachol (CCh), EoWs had an antispasmodic action with an IC<sub>50</sub> values of 15.76  $\pm$  0.37 and 12.04  $\pm$  0.30  $\mu$ g/mL, respectively. Preliminary results showed that it is probable that our plant might act directly through the NO and guanylate cyclase signaling pathway and on muscarinic but not nicotinic receptors. The results reveal that the Essential oil of *W. saharae* appears to have an impact on intestinal relaxation *in vitro* conditions. This finding lends credence to the traditional usage of this plant to treat intestinal disorders.

**Keywords:** *Warionia saharae*; essential oil; rabbit; rat; jejunum; myorelaxation; antispasmodic effects

## 1. Introduction

*Warionia saharae* Ben-them ex Benth. & Coss. is an endemic plant of North Africa regions. This plant is native to Morocco, and it is the sole species in the genus *Warionia* (Asteraceae) [1]. The plant may reach a height of one to three meters. The shrub has a strong trunk covered in a gray peel, a structure of highly wavy terminal leaf bouquets, and a profusion of yellow flowers. The latex that pours out of peel injuries glues to hands in a very tenacious way; picking the leafed stems of this bush clears a very strong and spicy odor [2]. Afessas, abessas, or tazart nifiss are some of the native Berber names for this plant [3]. *W. saharae* leaves are used in traditional medicine to treat inflammatory disorders, gastrointestinal problems, and epileptic seizures [4]. Until recently, *W. saharae* had not sparked the curiosity of scientists, and just a few articles on this species had been published. The hydromethanolic extract of *W. saharae* exhibits significant glucosidase inhibitory activities, accord-

ing to Rechek *et al.* [4]. The essential oil and crude extracts of this plant were found to have antibacterial, cytotoxic, antioxidant, and anti-inflammatory properties against the cancer cell line "KB cells" [4,5].

Essential oils and their constituents have been utilized to treat a wide range of human ailments since ancient times. Essential oils are used in aromatherapy, medicine, fragrances, and cosmetics. Their use is limited to their varied biological functions [6]. Heghes *et al.* [7] 2019 identified 39 plant species containing essential oils with antispasmodic activities. They found that the main mechanisms of antispasmodic action were achieved via inhibiting voltage-dependent calcium channels, modifying potassium channels, and intracellularly controlling AMPc. Several investigations have identified various biological activities of essential oils [8], but the antispasmodic effect, while mentioned in traditional medicine literature, has received far less experimental attention.



The objectives of this study were to investigate at the chemical composition of essential oils extracted from aerial parts of *W. saharae* in the southern region of Morocco and to study their antispasmodic activities.

## 2. Material and Methods

### 2.1 Plant Material

*W. Saharae* aerial parts were collected from southeastern region of Morocco (Errachidia: 31°55'53 N", 4°25'35" W) on April 2018. The plant was identified, and a specimen was registered in the herbarium of the Faculty of Sciences, University Mohamed first Oujda, Morocco, with the number UHPOM 450. Before essential oil extraction, the dried plant material is stored in a laboratory at room temperature (25 °C) in a dry and dark environment.

### 2.2 Extraction of Essential Oil

One hundred grams of air-dried *W. saharae* aerial part were cut into small pieces and placed in a round-bottom flask with 0.1 L distilled water, and the essential oil were extracted by hydro-distillation after 2 hours using a Dean-Stark apparatus. The obtained oil was kept in a dark sealed container at 4 °C for further analysis. The extraction yield was 0.5%.

### 2.3 Gas Chromatography Mass Spectrometry (GC/MS) Analysis

The volatile components of *W. saharae* essential oils were characterized using a gas chromatography coupled with mass spectrometry GC/MS (Shimadzu model QP2010, Kyoto, Japan). Helium (3 mL/min) was employed as the carrier gas in a BPX 25 capillary column (30 m × 0.25 mm, film thickness 0.25 μm). The column temperature program was 50 °C for 5 min, with 10 °C increases per min to 250 °C; which was maintained for 10 min. The temperatures of the detector and injector were maintained at 250 °C and 225 °C, respectively. The split flow method was utilized. Electron impact (EI) mass spectra were collected at 70 eV ionization voltages and the scan rate was 5 scan/s. The scan mass range of the mass spectrometer was 20 to 450 amu. The ionization source temperature was 280 °C, while the interface temperature was 300 °C. The components were identified by comparing their mass spectra to the NIST 147 Mass Spectral Database (National Institute for Standard Technology –NIST-147, 198 compounds LabSolutions version 2.5. USA). The amount of all detected components was evaluated using a percent relative peak area.

### 2.4 Chemicals

All chemicals used were of analytical grade. Calcium chloride (CaCl<sub>2</sub>), carbamylcholine chloride (CCh, Carbachol), verapamil hydrochloride, prazosin, propranolol, dimethyl sulfoxide (DMSO), Methylene blue, and yohimbine were purchased from Sigma Chemical Co. Papaverine hydrochloride and atropine were obtained from Fluka, hex-

amethonium, and L-NAME from Calbiochem. Except for nifedipine, which was dissolved in DMSO, and papaverine, which was dissolved in 120 parts ethanol, all pharmacological agents used are water soluble.

Essential oil dissolved in DMSO was prepared by mixing 10 μL of essential oil with 100 μL DMSO then complete to 1 mL with double distilled water addition.

The Krebs-Hemseleit Buffer (KHB) utilized in this investigation had a pH of 7.4 and was held at 37 °C throughout the experiments.

### 2.5 Animals

New Zealand rabbits (1.5–2 kg), Wistar rats (200–350 g) and Swiss albino mice (20–25 g) of both sexes were utilized. They were housed in standard laboratory animal housing conditions. Animals fast for 18 hours before each experiment and have unrestricted access to water. The experiments were carried out ethically in conformity with the principles outlined in the Declaration of Helsinki, and the study was authorized by the Faculty of Sciences institutional review board in Oujda, Morocco (01/20-LBBEH-04 and 09/01/2020).

Under the antispasmodic experiments, when the animal was anesthetized by light ethyl ether inhalation and euthanized, the abdominal cavity was opened and most of the internal organs were visualized in place, including the digestive mass. It is necessary to notice the state of all organs and precisely that of the intestine, of which sometimes the presence of intestinal alterations can distort the results, and before any experiments it is checked that the organ contracted well in a KHB medium rich in potassium.

The basic spontaneous contractions of the rabbit jejunum are larger and easier to evaluate than those of the rat jejunum. Therefore, it is a good model for studying the spontaneous myorelaxant activity. However, if you want to study the antispasmodic activity and their mechanism of action, rat jejunum contraction induced by CCh and KCl is more practical and cheaper. For each experience 6 animals were used.

We used the mice in the toxicity test because they are very sensitive compared to the other animals, so they are more adapted for the toxicity tests of Natural products.

### 2.6 Acute Toxicity Study

Acute toxicity of EoWs was evaluated following the recommendations by OECD-Guidelines [9]. For this study, 30 mice were divided into five groups of six mice each. After fasting for 18 hours with unrestricted access to water, EoWs treatment was started by giving each mouse a single dose of:

-0.5, 1, 1.5 and 2 g EoWs/kg body weight solubilized in gelatin (5%)

-gelatin (5%) solubilized in distilled water for the control group

The animals were fed after treatment, followed by weight registration and 14-day observation of general indicators of toxicity symptoms, behavior, and mortality. At the end of the investigation, the mice were euthanized by cervical dislocation, and the weights of organs such as the right and left kidney, liver, and heart were collected while the intestines and stomach were inspected.

### 2.7 Isolated Jejunum Preparations

Antispasmodic effect of *W. saharae* L. essential oil (EoWs) was evaluated utilizing isolated jejunum preparations from rabbit and rat: 2 cm long animal small intestine segments were suspended in 10 mL tissue baths using KHB solution with regular oxygenation (bubbling) and kept at 37 °C. During the 30-minute equilibration period, the KHB was changed every 15 minutes before adding any drug or essential oil.

The vehicle DMSO was used to dissolve EoWs, which was then added to the organ bath. At the maximum dose we used (50 µg/mL), the DMSO in the culture medium represents an amount of 0.5%. No toxic effect was observed using this DMOS concentration.

The amplitude of the jejunum contraction is measured using an isotonic transducer (B. Braun Melsungen AG Type 362722 # 203, Germany) related to the intestine mounted in organ isolated bath (10 mL). The graph tracing related to the contractile response of the intestine was recorded using recording cylinder (B. Braun Melsungen AG 861 062 Type, No. 1696, Germany). To calibrate the system: a weight was used to calibrate the signal transducer (1 g equivalent to 100% of contraction for rat jejunum, and 2 g equivalent to 100% of contraction for rabbit jejunum).

### 2.8 Myorelaxant Effect of EoWs on Rabbit Jejunum

In isolated spontaneously contracting rabbit jejunum, the action of the EoWs (10, 30, and 50 µg/mL) was evaluated alone or in the presence of three different kinds of adrenergic receptors inhibitors antagonists:  $\alpha 1$  by prazosin,  $\alpha 2$  by yohimbine, and  $\beta$  by propranolol at 50 µM each). Contraction inhibition was calculated as a percentage of baseline spontaneous contractions in the rabbit intestine.

### 2.9 Antispasmodic Activity of EoWs on Rat Small Intestine

The following experiments were performed:

-The antispasmodic action of EoWs (5, 10, 30 µg/mL) on rat jejunums precontracted by KCl (25 mM) or carbachol (1 µM) was evaluated.

-In the absence and presence of the EoWs, concentration response curves for carbachol (CCh) were produced. Following a 1-hour stabilization period in KHB, the jejunum section was treated with cumulative doses of CCh (0.03–30 µM) (control). The same procedure as the control was done by pre-incubating each time for 5 minutes with a specific concentration of EoWs (5, 10, 30 µg/mL) before adding carbachol.

-To evaluate whether calcium channel was involved in the effect of EoWs in jejunum, the tissue first stabilized in normal KHB solution, then replaced with a calcium-free KHB (NaCl, 121.7; KCl, 4.7; CaCl<sub>2</sub>, 0; MgSO<sub>4</sub>, 1.2; NaHCO<sub>3</sub>, 25; KH<sub>2</sub>PO<sub>4</sub>, 1.2 and glucose 10) and EDTA (0.1 mM) in order to remove calcium from the tissues. This solution was replaced with Calcium free high K<sup>+</sup> KHB ([K<sup>+</sup>] = 76.2 mM) (NaCl, 48; KCl, 75; CaCl<sub>2</sub>, 0; MgSO<sub>4</sub>, 1.2; NaHCO<sub>3</sub>, 25; KH<sub>2</sub>PO<sub>4</sub>, 1.2 and glucose 10). Following an incubation period of 10 min and after the confirmation of no spontaneous contractions of jejunum, CaCl<sub>2</sub> was added in a cumulative concentration (0.3 to 10 mM) to obtain control concentration-response curves of CaCl<sub>2</sub>. The concentration-response curves were repeated in the presence of different concentration of EoWs (5, 10, 30 µg/mL). The verapamil (1 µM) and papaverine (10 µM) were used as a positive control.

In the last experiment, the impact of EoWs (30 µg/mL) in the presence of drug inhibitors (L-NAME (100 µM), blue of methylene (10 µM), atropine (1 µM), hexamethonium (100 µM), and nifedipine (1 µM)) was evaluated. Tissues were incubated for 20 minutes with the various inhibitors before being stimulated with KCl 25 mM (with the exception of nifedipine, which was contracted with CCh (1 µM)).

### 2.10 Statistics

The results were expressed as mean ± S.E.M. Moreover, the difference between the groups was calculated by one-way analysis of variance (ANOVA) using GraphPad Prism 5 for windows (San Diego, CA, USA). The difference was considered significant when *p* is less than 5%. The linear regression approach was used to get the 50% inhibition concentration (IC<sub>50</sub>).

## 3. Results

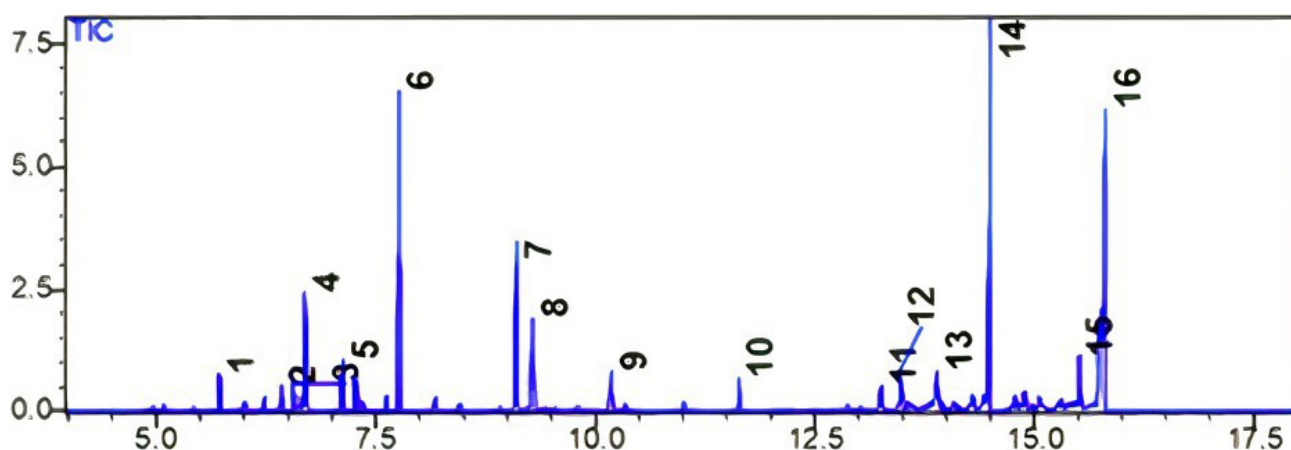
### 3.1 Analysis of *W. Saharae* Essential Oil by GC/MS

The essential oil EoWs was extracted directly from the dried aerial section of *W. saharae* from the Errachidia region (Morocco) and characterized using GC-MS. On a dry weight basis, the obtained essential oil yields were around 0.5% (w/w). Fig. 1 and Table 1 illustrate the chromatogram and chemical content and composition of this essential oil, respectively. These findings indicate that the plant is very rich in a wide range of chemical components.

The chromatographic profile of this essential oil allowed for the identification of 99.99% of the aromatic compounds present in this plant, the most important are, in increasing order, nerolidyl acetate (21.44%),  $\beta$ -eudesmol (19.47%), and linalool (16.48%), which account for 57.39% of the total composition (Fig. 1, Table 1).

### 3.2 Acute Toxicity Test

The acute toxicity study with EoWs revealed that there was no toxicity or mortality at the tested levels (1, 1.5, and 2 g EoWs/kg body weight). All of the animals behaved nor-



**Fig. 1. Typical GC-MS chromatogram of the essential oil of *W. saharae*.** The peaks obtained correspond to 16 compounds representing 99.9% of the aromatic compounds present in this plant.

**Table 1. Chemical composition of essential oil of *W. saharae* (%).**

Pic number	Chemical compound	Retention Time (min)	Abundance (%)
1	4(10)-Thujene	5.73	1.90
2	(+)-4-Carene	6.43	1.19
3	<i>p</i> -Cymene	6.57	1.57
4	Cineole	6.70	5.34
5	$\gamma$ -Terpinene	7.13	1.95
6	Linalool	7.78	16.48
7	1-Terpinen-4-ol	9.10	10.93
8	<i>p</i> -menth-1-en-8-ol	9.30	5.37
9	Geraniol	10.19	1.97
10	$\alpha$ -Terpineol acetate	11.64	2.01
11	E-7-Tetradecen-1-ol	13.26	1.56
12	7-Heptadecene, 1-chloro-	13.48	2.67
13	9-Cedranone	13.90	2.42
14	Nerolidyl acetate	14.51	21.44
15	Agarospirol	15.52	3.72
16	$\beta$ -Eudesmol	15.81	19.47
Total		16.00	99.99

mally when it came to eating, moving, and drinking water. The weights of the mice during the experience period did not change between the control and EoWs-treated groups.

### 3.3 Study of the Muscle Relaxant Effect EoWs on Spontaneous Contractions in the Rabbit Jejunum

The results of this study (Fig. 2A) showed that EoWs at concentrations ranging from 10  $\mu$ g/mL to 50  $\mu$ g/mL exhibited a dose-dependent inhibition of baseline jejunal contractions in rabbits with an  $IC_{50} = 16.80 \pm 0.63$   $\mu$ g/mL. This inhibition is reversible, the spontaneous contractions of the rabbit jejunum reappear after washing with fresh KHB.

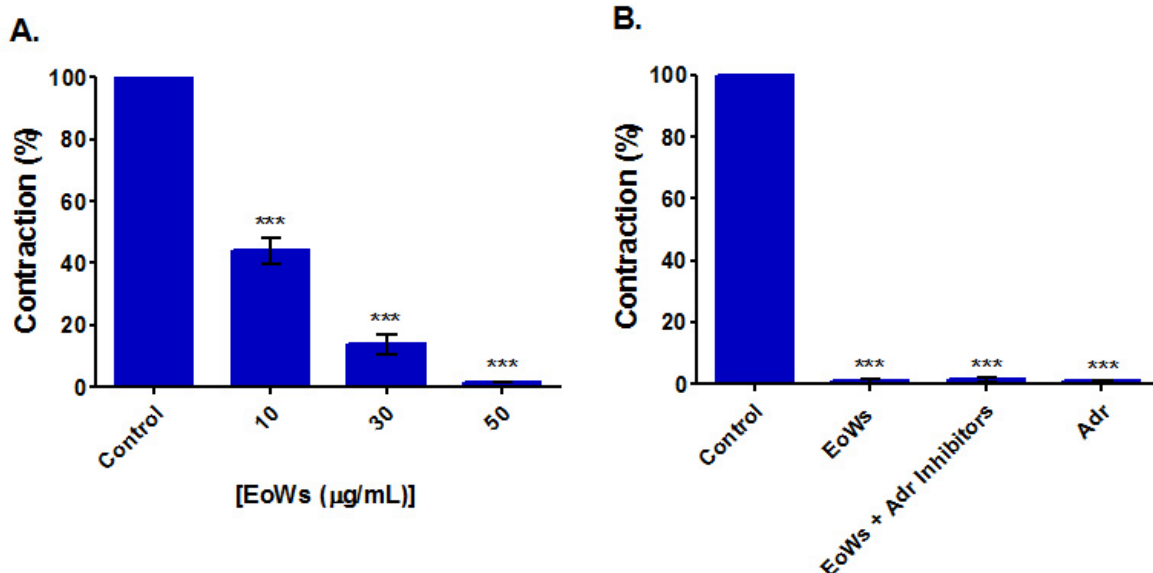
Rabbit basal jejunal contractions are fully suppressed in the presence of 50 g/mL EoWs, even in the presence of three adrenergic inhibitors (propranolol, prazosin, and yohimbine, all used at 0.5  $\mu$ M each) (Fig. 2B).

### 3.3 Antispasmodic Activity of EoWs on Rat Jejunal Tone Provoked by KCl or CCh

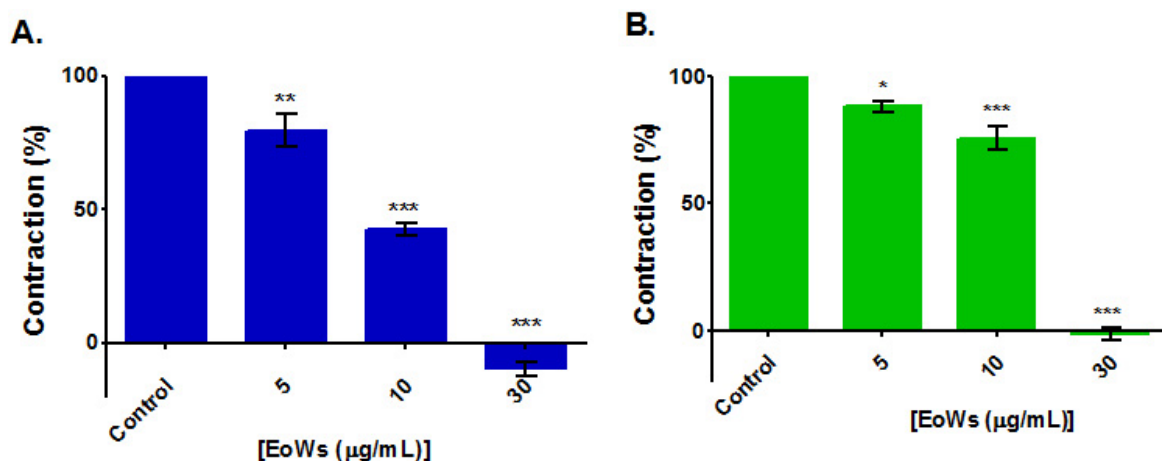
According to the results of this study (Fig. 3), EoWs at doses ranging from 5 to 30 g/mL suppressed rat jejunal tone elicited by 25 mM KCl with an  $IC_{50}$  of  $15.76 \pm 0.37$   $\mu$ g/mL and  $10^{-6}$  M CCh with an  $IC_{50}$  of  $12.04 \pm 0.30$   $\mu$ g/mL.

### 3.4 Study of the Dose-Response Effect of Calcium Chloride ( $CaCl_2$ ) and Carbachol (CCh) in the Presence and Absence of EoWs on Rat Jejunal Contractions

The concentration-response effects of CCh and  $CaCl_2$  in the presence and absence of EoWs are depicted in Figs. 4,5. At doses of 5, 10, or 30  $\mu$ g/mL, EoWs fully suppressed CCh. These EoW effects were equivalent to those observed with a positive control (papaverine 10  $\mu$ M) (Fig. 4).



**Fig. 2.** Myorelaxant effect on spontaneous contractions of the rabbit jejunum of the *W. saharae* essential oil (EoWs). (A) Influence of EoWs applied at different concentrations. (B) Influence of 50 µg/mL EoWs applied in the presence and the absence of Adrenergic inhibitors (Adr Inhibitors) (Prazosin (0.5 µM) + yohimbine (0.5 µM) + propranolol (0.5 µM)). (Adr: Adrenaline). \*\*\* $p \leq 0.001$ : the difference was statistically significant when compared to the control.



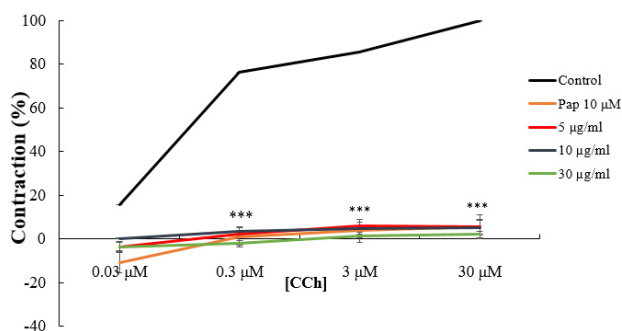
**Fig. 3.** Effect of *W. saharae* essential oil (EoWs). (A) Influence of EoWs on rat jejunum precontracted with carbachol 1 µM. (B) Influence of EoWs on rat jejunum precontracted with KCl 25 mM (B). \*:  $p \leq 0.05$ ; \*\*:  $p \leq 0.01$ ; \*\*\* $p \leq 0.001$ : the difference was statistically significant when compared to the usual control.

When the tissue was pre-contracted with  $\text{CaCl}_2$ , 5 µg/mL of EoWs had a substantial effect, but 30 µg/mL had the highest effect (Fig. 5). In this study, we discovered that 10 µg/mL EoWs had a comparable effect as the positive control (verapamil  $10^{-6}$  M).

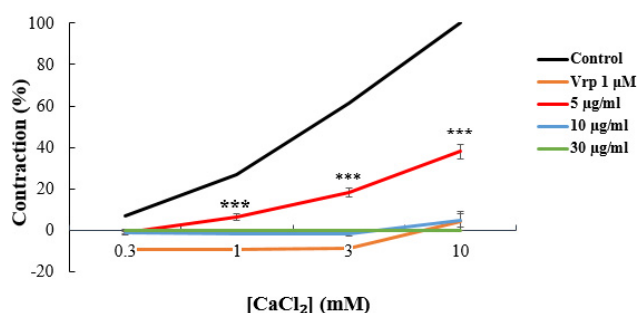
### 3.5 The Study of the Effect of EoWs on Rat Jejunal Contractions Pre-Incubated with Pharmacological Inhibitors and Subsequently Pre-Contracted with KCl

We tested the effectiveness of 30 g/mL EoWs with pharmacological inhibitors that are often used to reduce KCl-induced contraction (atropine is used to inhibit muscarinic receptors, L-Name is used to inhibit NOS, methylene

blue is used to inhibit GC pathway, and hexamethonium is used to inhibit nicotinic receptors). The results show (Fig. 6) that after 20 minutes of pre-treatment with pharmaceutical drugs, the influence of EoWs alone was more effective in lowering KCl-induced contraction than the presence of 1 µM atropine (62.78%), 100 µM L-NAME (32%), and 10 µM methylene blue (56.38%). EoWs, on the other hand, was able to elicit an inhibitory effect in the presence of hexamethonium ( $10^{-4}$  M) equivalent to what occurs in its absence.



**Fig. 4.** Concentration-response curves of CCh in the absence and presence of increasing concentrations of *W. saharae* essential oil (EoWs) and Papaverine (Pap). \*\*\* $p \leq 0.001$ . The difference from the normal control (mean S.E.M,  $n = 6$ ) was statistically significant. In compared to the control, all of the groups showed statistically significant differences.

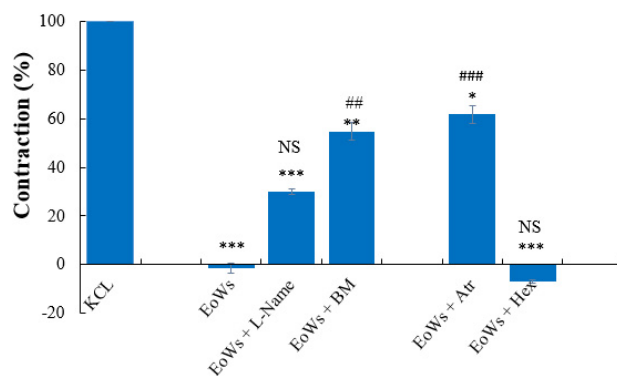


**Fig. 5.**  $\text{CaCl}_2$  dose-response curves with and without different concentration of *W. saharae* essential oil (EoWs). \*\*\* $p \leq 0.001$ . The difference from the normal control (mean S.E.M,  $n = 6$ ) was statistically significant. In compared to the control, all of the groups showed statistically significant differences. Vrp, Verapamil.

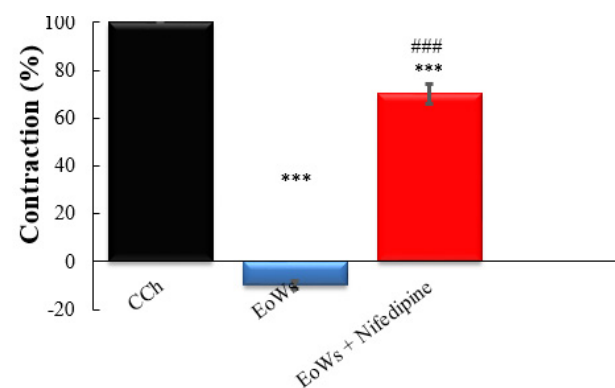
The same result was obtained when KCl was replaced with CCh (Fig. 7). In the absence of the positive control, EoWs (30 g/mL) completely suppressed the contraction caused by CCh; however, the contraction caused by carbachol (1  $\mu\text{M}$ ) was only reduced to 70.15% in the presence of the positive control (1  $\mu\text{M}$  nifedipine this compound is used as antagonist of L-type calcium channel).

#### 4. Discussion

The essential oil was extracted and analyzed using GC-MS from the dried areal parts of *W. saharae*. The essential oil yields were about 0.5% (w/w) on a dry weight basis. Sellam *et al.* [10] found a 1.1% essential oil extraction yield from *W. saharae*. For Mezhoud, the extraction yield was around 0.85% (plant grown in Algeria) [11]. These yield variations might be attributed to extraction techniques, ecotype, or aerial component employed (stem, leaf or flower). The composition of the EoWs was presented as a ratio of numerous molecules from the



**Fig. 6.** Effect of *W. saharae* essential oil (EoWs, 30  $\mu\text{g/mL}$ ) on contractions of rat jejunum preincubated for 20 minutes with L-NAME 100  $\mu\text{M}$ , methylene blue (BM) 10  $\mu\text{M}$ , atropine (Atr) 1  $\mu\text{M}$ , and hexamethonium (Hex) 100  $\mu\text{M}$ , and then precontracted with KCl 25 mM. \*\*\* $p \leq 0.001$  when compared to the control (KCl). NS, no significant. ## $p \leq 0.01$ ; ### $p \leq 0.001$  when compared with the EoWs without drugs.



**Fig. 7.** Effect of *W. saharae* essential oil (EoWs, 30  $\mu\text{g/mL}$ ) on contractions of rat jejunum preincubated for 20 minutes with nifedipine (1  $\mu\text{M}$ ), and then precontracted with CCh (1  $\mu\text{M}$ ). \*\*\* $p \leq 0.001$  when compared to the control (CCh, 1  $\mu\text{M}$ ). ### $p \leq 0.001$  when compared with the EoWs without Nifedipine.

monoterpene, sesquiterpene, terpene esters, and monoterpenol groups. The oil's distinctive and aromatic odor may be explained by its high concentration of identified oxygenated compounds (Table 1). Several investigations have found nerolidyl acetate,  $\beta$ -eudesmol, and linalool to be among the primary compounds found in EoWs, although with varied chemical profiles and quantities in comparison to our data [10–13]. Ramaut *et al.* [12] published the first study on the chemical composition of *W. saharae* essential oils extracted from the leaves. Only three compounds have been identified by the authors:  $\beta$ -eudesmol (42.25%), nerolidol (8.63%), and linalool (17.26%). On the other hand, according to Essaqui *et al.* [13],  $\beta$ -eudesmol (52.7%), *trans*-nerolidol (17.4%), linalool (5.1%), were the main components of the essential oil. Other compounds were identified so far: terpinen-4-ol and 1, 8-cineole, and

guaiol [14]. These variations were explained by the plant genotype, ecology, and technique of extraction.

To assess a substance's potential risk to humans, toxicity studies in animal models are commonly conducted. The study of effect EoWs at concentrations of 0.5, 1.0, 1.5, and 2.0 g/kg body weight showed no signs of toxicity or mortality over a 14-day observation period, nor did the mice's weight change, and even after the animal was euthanized, the weight of vital organs such as the kidneys, liver, and heart, as well as the aspect of the stomach and intestine, did not change. After investigating these factors, we detected no evidence of toxicity. Based on these results, we may infer that our plant is relatively safe in the case of acute intake of up to 2 g/kg body weight of albino mice.

The myorelaxant activity of *W. saharae* essential oil has been examined in the rabbit jejunum, which is characterized by contractions whose amplitude and frequency are rhythmic (do not change over time), owing to the slow waves generated by the Cajal interstitial cells [15]. Furthermore, the waves are clearer and larger than the rat's, making them easier to examine and comprehend. As a result, we chose to concentrate our studies on the myorelaxant impact of EoWs, specifically on the rabbit jejunum. With an  $IC_{50}$  of  $16.80 \pm 0.63 \mu\text{g/mL}$ , EoWs at doses of 10, 30, and 50  $\mu\text{g/L}$  suppressed basal jejunal contractions in rabbits in a dose-dependent and reversible manner. This action is more effective than the myorelaxant effect found for extracts of *Origanum majorana*, with an  $IC_{50}$  of  $59.22 \pm 2.42 \mu\text{g/mL}$  [16] and for *Psidium guajava*, with an  $IC_{50}$  of  $0.84 \pm 1.08 \mu\text{g/mL}$  [17].

Several investigations have indicated that intestine smooth muscle contraction occurs as a result of an increase in free calcium in the cytoplasm, which subsequently activates the contractile elements [18]. The  $Ca^{2+}$  ion accumulation in the cytoplasm is caused by either inflow through voltage-gated calcium (VGC) channels or release from the cytoplasmic reticulum. Membrane depolarization facilitates the control of spontaneous intestine movements, and when it is intensified, it generates an action potential of the cell, which manifests as a fast influx of  $Ca^{2+}$  ions through VGC channels [19]. The myorelaxant action of the *W. saharae* extract, as demonstrated in our investigation, is most likely due to EoWs interfering with  $Ca^{2+}$  ion influx through VGC channels or release from the cytoplasmic reticulum. On the other hand, we established that the muscle relaxant action generated by this plant is reversible, meaning that the essential oil of this plant extract did not injure smooth muscle cells or alter their functioning in an irreversible way. Several studies have reached similar conclusions. Essential oil isolated from the flowers of *Anthemis mauritiana* (Maire & Sennen) displayed an inhibitory impact on the dose-response curves caused by  $CaCl_2$ , but at greater concentrations (100 g/mL), most likely attributable to  $\alpha$ -pinene [20]. The same effect was achieved using an essential oil extracted from the aerial portion of *Artemisia herba alba*

(Asso.), albeit at a lower concentration (200 ng/mL). This latter spasmolytic effect might be related to the presence of camphor, terpinene, 1,8-cineol,  $\alpha$ - and  $\beta$ -pinene, all of which have been reported to be smooth muscle relaxants [21]. Bergapten, a hydrophobic substance isolated from the fruits of *Heracleum leskovii* (Grossch.) (Apiaceae), produced myorelaxation of intestinal preparations at concentrations ranging from 0.0001 to 1 mM. Bergapten induced smooth muscular relaxation or contraction at higher doses [22]. As a result, in order to avoid the opposite effect, the dosages must be carefully established.

Previously, it was established that catecholamines and adrenergic drugs reduced spontaneous contractions of the rabbit jejunum [23]. For this reason, we selected three adrenergic inhibitors: prazosin, yohimbine, and propranolol, which are recognized for their competitive action on  $\alpha_1$ ,  $\alpha_2$  and  $\beta$  adrenergic receptors, respectively. Adrenaline (epinephrine) is a catecholamine neurotransmitter that acts on receptors coupled with G proteins [24]. This compound is added to ensure that all adrenergic receptors have been blocked by the adrenergic inhibitors listed above. Treatment with EoWs (30  $\mu\text{g/mL}$ ) reduced the amplitude of rabbit jejunal contractions, indicating that EoWs do not operate via adrenergic receptors.

Carbachol, a structural analogue of acetylcholine, was employed to evaluate the cholinergic receptor pathway, since it has the advantage of not being degraded in the physiological medium by the action of acetylcholinesterases. In the presence of EoWs, we examined two different cholinergic inhibitors: atropine, a muscarinic antagonist, and hexametonium, a nicotinic inhibitor. EoWs appear to operate via muscarinic receptors but not nicotinic receptors. Then this oil may therefore induce the activation of M2 and M3 muscarinic receptors found in the intestine [25]. Furthermore, muscarinic receptor activation can stimulate non-selective calcium (NSC) channels in the plasma membrane, resulting in a membrane potential and  $Ca^{2+}$  entry via VGC channels [26]. EoWs significantly reduced the tone of the rat jejunum caused by 1  $\mu\text{M}$  CCh in a dose-dependent manner, with an  $IC_{50}$  of  $12.04 \pm 0.30 \mu\text{g/mL}$ . As a consequence, we can postulate that one or more active plant compounds in EoWs inhibit M2 and M3 muscarinic receptors. We examined the dose response effect of Carbachol in the presence or absence of EoWs to corroborate this hypothesis (Fig. 4). The EoWs has a significant inhibitory action on CCh-induced maximal contraction. Papaverine (10  $\mu\text{M}$ ) used as a positive control, relaxes intestinal muscle by inhibiting adenosine monophosphate phosphodiesterase (AMPC), resulting in an increase of this molecule in the intracellular medium, which negatively influences the phosphorylation of the myosin light chains and, as a result, contraction [27].

We obtained the same results, when we progressively added calcium in the presence of EoWs or verapamil. These results, thus, suggest that EoWs operated as an agonist

through one of the two muscarinic receptor contraction pathways. Similar observations were done with plant extracts from *Nepeta cataria* L. [28], *Satureja hortensis* [29], and *Satureja obavata* [30].

Previous research has found that the antispasmodic activity of medicinal plants is generally mediated by blocking calcium channels [31,32]. Similarly, we aimed to determine if our essential oil's antispasmodic activity is driven by similar pathways. The effect of EoWs on rat jejunal contractions generated by 25 mM KCl was studied for this purpose, and the results showed that this essential oil significantly reduced the maximum tone of the jejunum created by the latter in a dose-dependent manner, with an  $IC_{50} = 15.76 \pm 0.37 \mu\text{g/mL}$  (Fig. 3B). To determine if our essential oil acted through calcium channels, we investigated the dose-response impact of  $\text{CaCl}_2$  in the presence and absence of EoWs.  $\text{CaCl}_2$ , like KCl, can induce plasma membrane depolarization by activating L-type calcium channels, which causes muscle contraction [33,34]. Furthermore, a voltage-gated calcium channel inhibitor is defined as a chemical that prevents these contractions. For this, verapamil (1  $\mu\text{M}$ ) was employed as a positive control. This chemical has been identified as a calcium channel blocker for L-type dependent VGC channels [35]. Our results indicate that EoWs prevented calcium entry through the VGC channels and, as a result, calcium release from the endoplasmic reticulum. In general, the chemical composition of aromatic and medicinal plants can fluctuate from year to year depending on climate, soil, altitude, and other factors. The antispasmodic activity of these essential oils can be attributed to one or more components, which can operate alone or in combination. For instance, other essential oils, such as *Origanum majorana* essential oil [16] containing terpenenes, terpineols, limonene, linalool and carvacrol, *Artemisia herba-alba* essential oil [21] containing camphor, terpinene, 1,8-cineol,  $\alpha$ - and  $\beta$ -pinene, or crude extract of *Lavandula stoechas* [36] are described for their spasmolytic activities.

We also explored whether the activity of our plant essential oil is mediated by nitric oxide (NO) or guanylate cyclase to better understand the mechanism of action. L-NAME (NG-nitro-L-arginine methyl ester) [37,38] and methylene blue [39] were used as selective inhibitors of these pathways, respectively. The administration of both inhibitors had a significant effect on EoWs-induced relaxation. As a result of these observations, our extract is likely to function directly through the NO and guanylate cyclase signaling pathways.

This antispasmodic activity can be linked to the chemical components found in this essential oil that have spasmolytic properties, such as Cineole [40], 1-terpinen-4-ol [41], terpineols [42], and linalool [43]. It should also be mentioned that  $\beta$ -eudesmol, which is particularly abundant (15.8%) in our essential oil, stimulated small intestine motility and gastric emptying by inhibiting both the 5-HT<sub>3</sub> receptor and the dopamine D<sub>2</sub> receptor [44]. To our knowl-

edge, except our previous work conducted with the total aqueous extract [45], no study on the antispasmodic action of *W. saharae* essential oil has been done. Although the results of this study were comparable, far higher dosages (in mg of extract) were required to achieve comparable action, demonstrating that the essential oil fraction is certainly the most bioactive and allowing for a better understanding of the phytochemicals that may be responsible for this activity. Similarly, in another study, we observed that the essential oil fraction was more active than the aqueous extract of *Origanum majorana* [16]. The myorelaxing effect might be attributed to a variety of EoWs compounds that need to be identified. This action was most likely caused by different EoWs components acting independently or synergistically. Nonetheless, we cannot rule out the possibility that compounds derived from the aqueous extract of *W. saharae* might possibly explain its antispasmodic effect.

## 5. Conclusions

The antispasmodic activity of *W. saharae* essential oil (EoWs) on isolated rabbit and rat small intestine was here studied. The EoWs demonstrated a significant spasmolytic effect by acting on several pharmacological pathways such as NO, guanylate cyclase, VGC channels, and muscarinic receptors. These results support the traditional usage of this plant to treat intestinal disorders and will allow us to explore future prospects for the treatment of intestinal spasms using natural chemicals derived from this medicinal plant.

## Author Contributions

MA (Mohammed Aziz) conceived and designed research. OA, MM, HM conducted the experiments. OA, MA (Mohamed Addi), MA (Mohammed Aziz) wrote the manuscript. MA (Mohamed Addi), MM contributed to software analysis. CA, AK analyzed the data. CH, JTC critical analysis of the data, review and editing manuscript. All authors contributed to editorial changes in the manuscript.

## Ethics Approval and Consent to Participate

The experiments were carried out ethically in conformity with the principles outlined in the Declaration of Helsinki, and the study was authorized by the Faculty of Sciences institutional review board in Oujda, Morocco (01/20-LBBEH-04 and 09/01/2020).

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## Conflict of Interest

The authors declare no conflict of interest. JTC is serving as one of the Guest Editor of this journal. We declare that JTC had no involvement in the peer review of this article and has no access to information regarding its peer review. Full responsibility for the editorial process for this article was delegated to GA.

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