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Original Article

Antispasmodic activity of *Warionia saharae* Benthem ex Benth. & Coss. on the rabbit and rat jejunums

[Actividad antiespasmódica de *Warionia saharae* Benthem ex Benth. & Coss. sobre yeyunos de conejo y rata]

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Abstract

Context: *Warionia saharae* Benthem ex Benth. & Coss has been widely used for gastrointestinal problems in Morocco and Algeria.

Aims: To explore the possible mechanism for its use to such ailments in order to rationalize some of these folkloric uses. We investigated the aqueous extract of this plant (AqWs) for antispasmodic activity on isolated rat and rabbit jejunums.

Methods: To investigate the contractile activity of isolated jejunum preparations of rat and rabbit we had used *in vitro* techniques. Jejunums were suspended in tissue baths filled with KHB culture medium (37°C) and connected to force transducers. AqWs (0.1–3 mg/mL) was used with or without carbachol (10^{-6} M), KCl (25 mM), yohimbine (10^{-5} M), prazosin (10^{-5} M), propranolol (10^{-5} M), hexamethonium (10^{-4} M), atropine (10^{-6} M), methylene blue (10^{-5} M), L-NAME (10^{-4} M) and nifedipine (10^{-6} M).

Results: AqWs at 0.1–3 mg/mL produced a relaxation on basal rabbit contractions with an IC_{50} = 1.55 ± 0.06 mg/mL. This effect was reversible and was not affected by pretreatment with the inhibitors of α and β adrenergic receptors yohimbine, prazosin and propranolol. The extract had an antispasmodic activity with an IC_{50} = 1.25 ± 0.02 mg/mL on rat jejunum precontracted with KCl rich medium (25 mM). This result suggests that the extract had a Ca^{2+} antagonist effect. This was fortified when pretreatment of the intestine with the extract induced a rightward shift in the Ca^{2+} concentration-response curves. AqWs exhibited also antispasmodic effect on carbachol (10^{-6} M)-induced contractions of rat jejunums with an IC_{50} = 1.53 ± 0.04 mg/mL. The extract at 3 mg/mL on rat jejunums pre-incubated with hexamethonium (10^{-4} M), atropine (10^{-6} M), and methylene blue (10^{-5} M), then contracted by KCl, decreased the maximum contraction but not totally like KCL alone without these inhibitors. For the jejunum pre-incubated with L-NAME (10^{-4} M), the spasmolytic effect of AqWs was comparable with KCL.

Conclusions: These results suggest that the myorelaxant and antispasmodic effects are mediated possibly via Ca^{2+} antagonist, anticholinergic, and guanylate cyclase mechanisms but not by adrenergic and nitric oxide pathway.

Keywords: anticholinergic; antispasmodic; calcium antagonist; guanylate cyclase; jejunum; *Warionia saharae*.

Resumen

Contexto: *Warionia saharae* Benthem ex Benth. & Coss se ha utilizado ampliamente para problemas gastrointestinales en Marruecos y Argelia.

Objetivos: Explorar el posible mecanismo de su utilización ante tales dolencias con el fin de racionalizar algunos de estos usos folclóricos. Investigamos el extracto acuoso de esta planta (AqWs) para determinar la actividad antiespasmódica en yeyunos aislados de ratas y conejos.

Métodos: Para investigar la actividad contráctil de preparaciones aisladas de yeyuno de rata y conejo utilizamos técnicas in vitro. Los yeyunos se suspendieron en baños de tejido llenos de medio de cultivo KHB (37°C) y se conectaron a transductores de fuerza. Se usó AqWs (0,1-3 mg/mL) con o sin carbacol (10^{-6} M), KCl (25 mM), yohimbina (10^{-5} M), prazosina (10^{-5} M), propranolol (10^{-5} M), hexametonio (10^{-4} M), atropina (10^{-6} M), azul de metileno (10^{-5} M), L-NAME (10^{-4} M) y nifedipina (10^{-6} M).

Resultados: AqWs a 0,1-3 mg/mL produjeron una relajación en las contracciones basales del conejo con un IC_{50} = 1,55 ± 0,06 mg/mL. Este efecto fue reversible y no se vio afectado por el pretratamiento con los inhibidores de los receptores adrenérgicos α y β yohimbina, prazosina y propranolol. El extracto tuvo una actividad antiespasmódica con una CI_{50} = 1,25 ± 0,02 mg/mL en yeyuno de rata precontraído con medio rico en KCl (25 mM). Este resultado sugiere que el extracto tenía un efecto antagonista de Ca^{2+}. Esto se reforzó cuando el pretratamiento del intestino con el extracto indujo un desplazamiento hacia la derecha en las curvas de concentración-respuesta de Ca^{2+}. Los AqW también mostraron un efecto antiespasmódico sobre las contracciones del yeyuno de rata inducidas por carbacol (10^{-6} M) con una CI_{50} = 1,53 ± 0,04 mg/mL. El extracto a 3 mg/mL en yeyuno de rata preincubado con hexametonio (10^{-4} M), atropina (10^{-6} M) y azul de metileno (10^{-5} M), luego contraído por KCl, disminuyó la contracción máxima pero no totalmente como KCL solo sin estos inhibidores. Para el yeyuno preincubado con L-NAME (10^{-4} M), el efecto espasmolítico de AqWs fue comparable con KCL.

Conclusiones: Estos resultados sugieren que los efectos miorrelajantes y antiespasmódicos están mediados posiblemente por mecanismos antagonistas de Ca^{2+}, anticolinérgicos y guanilato ciclasa, pero no por la vía adrenérgica y del óxido nítrico.

Palabras Clave: anticolinérgico; antiespasmódico; calcio antagonista; guanilato ciclasa; *Warionia saharae*; yeyuno.

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INTRODUCTION

The genus *Warionia* (*Compositae*) is represented by only one species named *Warionia saharae* Benthem ex Benth. & Coss. For Katinas et al. (2008), the palynological and morphological proof places this plant between the tribes *Cichorieae* and *Cardueae*, and it a shrub endemic in Morocco and Algeria. In Morocco, it is known locally by the vernacular name ''Afessas''. The leaves are used in the traditional remedy of Morocco to treat gastrointestinal disorders, inflammatory diseases and against epileptic crisis (Bellakhdar, 1997). In previous studies, researchers wrought on different extracts of W. *saharae* have shown that it has antioxidant (Sekkoum et al., 2011), anti-inflammatory, antibacterial, and cytotoxic activities against a cancer cell line (Hilmi, 2002a; Znini et al., 2011).

Despite its wide use in the treatment of digestive tract diseases in the southeastern of Morocco, to our best knowledge, no study on *W. saharae* antispasmodic activity was conducted before. Therefore, and like in developing country, this disease is a major health care problem. This study aimed to obtain for the first-time information on the antispasmodic effect of this plant on rodent isolated jejunum.

MATERIAL AND METHODS

Chemicals

Carbamylcholine chloride (carbachol, CCh), calcium chloride ($CaCl_2$), propranolol, prazosin, yohimbine, L-NAME and hexamethonium were purchased from Sigma Chemical Co (Sigma-Aldrich, USA). Atropine from Research Biochemical Incorporated, USA; papaverine hydrochloride (Pap) from Fluka, India; and verapamil hydrochloride (Vrp) from Tocris, USA were also used. All chemical products used were solubilized in distilled water.

Incubation media

The solutions used in this study were normal Krebs-Henseleit buffer (KHB) solution (in mM): NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, $NaHCO_3$ 25, KH_2PO_4 1.2 and glucose 10; calcium free high K^+ KHB ($[K^+]$ = 75 mM): NaCl 48, KCl 75, $CaCl_2$ 0, $MgSO_4$ 1.2, $NaHCO_3$ 25, KH_2PO_4 1.2 and glucose 10; and calcium free KHB (in mM): NaCl 121.7, KCl 4.7, $CaCl_2$ 0, $MgSO_4$ 1.2, $NaHCO_3$ 25, KH_2PO_4 1.2 and glucose 10. All components of these solutions were purchased from Sigma-Aldrich, USA, made up in distilled water. These solutions were employed for incubation jejunum strip and maintained at pH 7.4. Throughout the long-term experiments, while being heated to 37°C and bubbled with 95% O_2 + 5% CO_2.

Plant material

The crude extract of *W. saharae* (AqWs) was obtained by following the traditional use of Moroccan population. Therefore, air-dried aerial part of this plant was infused in boiling distilled water for 30 min, after filtration, the filtrate was concentrated by rotary evaporator, and then dried at 37°C in an oven. Finally, the resulting crude aqueous extract was recovered and preserved at -20°C until use. The specie was collected in Errachidia region (31°55′53″ N, 4°25′35″ W) identified by a Prof. Mostafa Elachouri and a specimen was deposited in the herbarium of Faculty of Sciences, University Mohamed first, Oujda, Morocco, under number HUPOM 450.

Animals

New-Zeeland rabbits (1.5-2 kg) and Wistar rats (200-350 g) of both sexes were used in these experiments. They were placed under standard conditions in the animal house of Faculty of Sciences, Oujda, Morocco, with free access to drinking water *ad libitum*. Maintained at controlled lighting (12 h–12 h light-darkness cycle), humidity, and temperature. All animals were starved 18 h prior the experiments. The experiments were conducted ethically in accordance with the internationally accepted guidelines for the care and use of laboratory animals published by the United States National Institutes of Health (NIH, 1985).

Collection and preparation of animal isolated jejunum strips

Under the experiments, when the animal was anesthetized by light ethyl ether inhalation and sacrificed, 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 medium rich in potassium.

As in previous works (Karim et al., 2010; Aziz et al., 2012), the jejunum segment was isolated from the small intestine and quickly pounded in the normal Krebs-Henseleit buffer (KHB) solution, then each 2 cm fragment was cleared of connective tissue and mounted under tension (1 g for rat jejunum, 1.5 g for rabbit jejunum) in isolated organ bath vessel (10 mL). The time to equilibrate the tissue was 30 min before previously the addition of any extract or drugs. Therefore, KHB was changed with fresh solution every 15 min throughout the time of stabilization. After, the jejunum strip was in direct contact with the extract or drugs for 7 to 8 min cumulative concentration responses.

To describe the pharmacological response to (AqWs), the following protocols were used:

1. The myorelaxant effect of aqueous extract of AqWs (0.3-3 mg/mL) was carried out on rabbit jejunum spontaneous contractions and in the presence or not of three adrenergic inhibitors put together (yohimbine, prazosin, and propranolol at 5×10^{-5} M).
2. The antispasmodic effect of AqWs (0.3-3 mg/mL) was tested on rat jejunums precontracted by KCl (25 mM) or carbachol (10^{-6} M).
3. Concentration response curves for carbachol (CCh) was obtained in the absence and presence of the AqWs. After a stabilization period of 60 min, the jejunum segment was treated with cumulative doses of CCh (10^{-8}-10^{-5} M) only (control). The same thing as the control were done by pre-incubating each time with a precise concentration of AqWs 5 min before adding of carbachol.
3. Concentration-response curves with calcium chloride ($CaCl_2$) was obtained the same way by obtained the concentration-contractile response with carbachol, except the latter was changed by $CaCl_2$ (0.1-10 mM) in the presence of different doses of the extract.

For the last experiment, the jejunum strip was incubated with each pharmacological inhibitor used: L-NAME (10^{-4} M), methylene blue (10^{-5} M), hexamethonium (10^{-4} M), atropine (10^{-6} M), nifedipine (10^{-6} M) for 20 min, and was precontracted with KCl 25 mM (except for the nifedipine, the jejunum strip was precontracted with CCh) and then, the extract was added at concentration of 3 mg/mL.

Statistical analysis

All results are definite as the mean ± SEM (standard error of mean) of six experiments using Graph Pad Prism 5 Software, San Diego, CA, USA. The significance level was determined by Student's test and the $p<0.05$ was considered significant.

RESULTS

Myorelaxant effect of AqWs on spontaneous contraction of rabbit jejunum

This study aimed to check the effect of AqWs on spontaneous contraction of the rabbit jejunum. For that, the amplitude of this latter was measured before and after the addition of the cumulative concentration of the extract (0.3, 1, 3 mg/mL). The amplitude was decreased after addition of the extract in a concentration-dependent mode (Fig. 1) with an IC_{50} = 1.55 ± 0.06 mg/mL. As well as after flushing the fragment with fresh KHB, reversible spontaneous contractions of the rabbit jejunum appeared.

In the presence of three adrenergic inhibitors put together (propranolol, prazosin and yohimbine at 5×10^{-5} M) in which the pharmacologic activity is well known (Michel et al., 2020), the

extract has exhibited a complete inhibition of the spontaneous basal contraction of the rabbit jejunum at 3 mg/mL, this one is identical with the inhibition obtained with the extract alone.

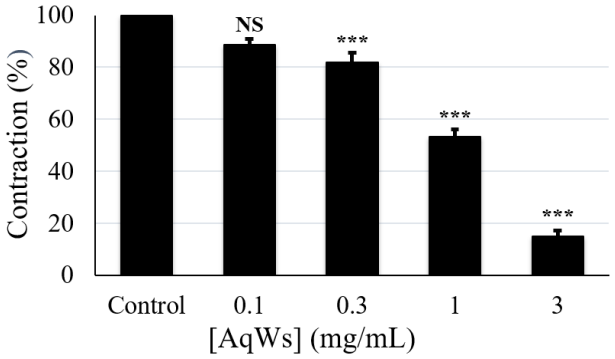

Figure 1. Myorelaxant effect of the crude extract of *W. saharae* (AqWs) on spontaneous contractions of the rabbit jejunum.

NS: no significant; **p≤0.01; ***p≤0.001. The difference was statistically significant from the normal control (using student's t-test with the mean ± SEM, n=6).

Antispasmodic effect of the AqWs on the tone induced by KCl and CCh of the rat jejunum

AqWs reduced in a dose-dependent manner ranging from 0.3 to 3 mg/mL, the maximum tone induced by CCh (10^{-6} M) (an analogue of acetylcholine) with an IC_{50} = 1.53 ± 0.04 mg/mL (Fig. 2), and KCl (25 mM) with an IC_{50} = 1.25 ± 0.02 mg/mL (Fig. 3).

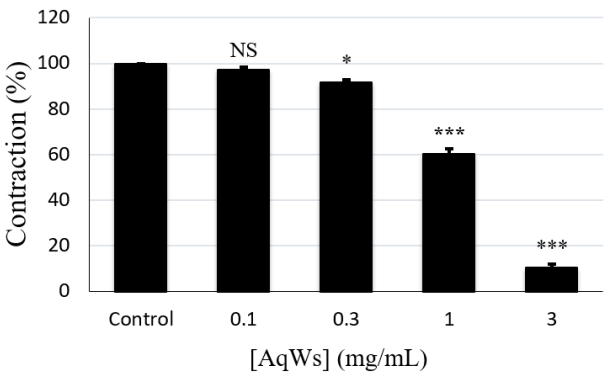

Figure 2. Effect of the crude extract of *W. saharae* (AqWs) on rat jejunum precontracted by carbachol 10^{-6} M.

NS: no significant; *p≤ 0.05; ***p≤0.001 difference statistically significant from the control (using student's t-test with the mean ± SEM, n = 6).

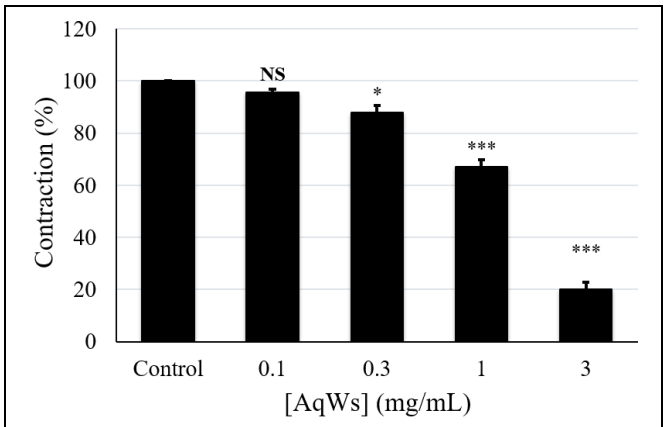

Figure 3. Effect of the crude extract of *W. saharae* (AqWs) on rat jejunum precontracted by KCl 25 mM.

NS: no significant; ***p≤ 0.001. The difference was statistically significant from the normal control (using student's t-test with the mean ± SEM, n=6).

Inhibitory effect of AqWs on CCh and $CaCl_2$ concentration response

In this experiment, the effect of cumulative dose response of CCh and $CaCl_2$ was studied separately in the absence and presence of different concentrations of AqWs. The results have shown that the exposition of tissue with the extract (0.3-3 mg/mL) caused in concentration-dependent fashion, the shifting of CCh or $CaCl_2$ concentration-response curves to the low (Figs. 4 and 5, respectively). For both experiments, the extract has a complete inhibition at 3 mg/mL.

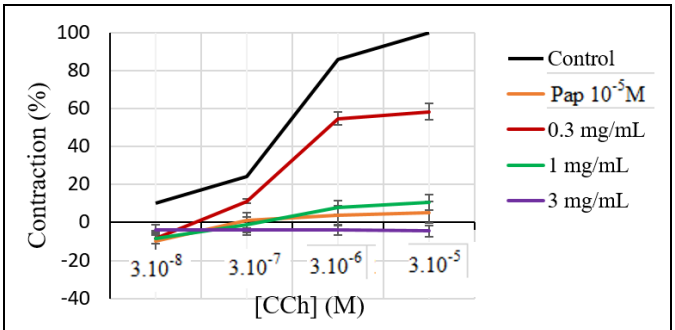

Figure 4. Dose-response curves of carbachol (CCh) in the presence and absence of the crude extract of *W. saharae* (AqWs).

***P≤0.001 difference statistically significant from the normal control (mean values ± SEM, n=6). All the groups represented statistically significant differences with respect to the control. Pap: papaverine.

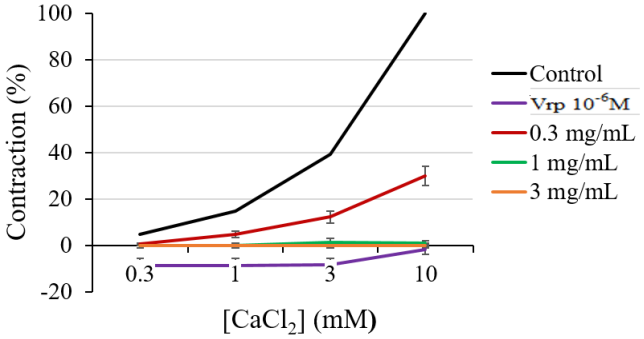

Figure 5. Dose-response curves for $CaCl_2$ in the presence and absence of the crude extract of *W. saharae* (AqWs).

***P≤0.001. The difference was statistically significant from the normal control (mean ± S.E.M, n=6). All the groups represented statistically significant differences with respect to the control. Vrp: verapamil.

The positive control employed for the concentration response of CCh was papaverine at 10^{-5} M and for $CaCl_2$ was verapamil at 10^{-6} M. Papaverine and verapamil produced an identical effect to that of 1 mg/mL of the extract.

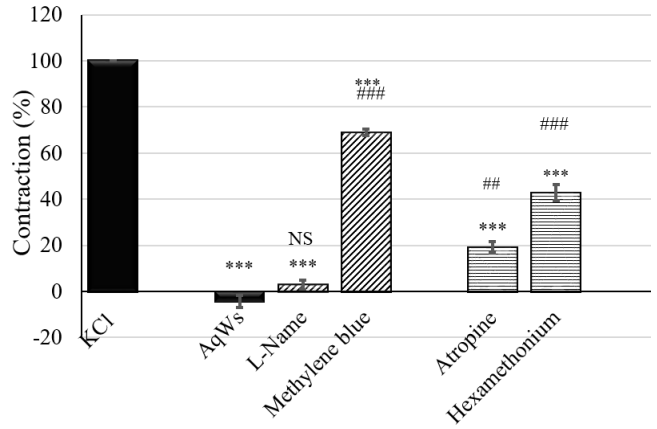

Figure 6. Effect of the crude extract of *W. saharae* (AqWs, 3 mg/mL) on contractions of rat jejunum preincubated with L-NAME 10^{-4} M, methylene blue 10^{-5} M, atropine 10^{-6} M and hexamethonium 10^{-4} M for 20 min and then precontracted with KCl 25 mM.

***P≤ 0.001 in comparison with the normal control. NS: no significant, ##p≤ 0.01; ###p≤ 0.001 in comparison with the extract without drugs (using student's t-test with the mean ± SEM, n = 6).

Effect of different inhibitors (L-NAME, methylene blue, hexamethonium, atropine, and nifedipine) on the provoked jejunum spasm

AqWs at 3 mg/mL on rat jejunums pre-incubated with hexamethonium, atropine, and methylene blue, then contracted by KCl, decreased the maximum contraction but not totally like KCL alone without these inhibitors, the difference was hugely significant (Fig. 6). For the jejunum pre-incubated with L-NAME, the spasmolytic effect of AqWs was comparable with KCL.

In the presence or without of nifedipine, the AqWs at 3 mg/mL induced the same effect on the CCh contracted jejunum by totally relaxing it (Fig. 7).

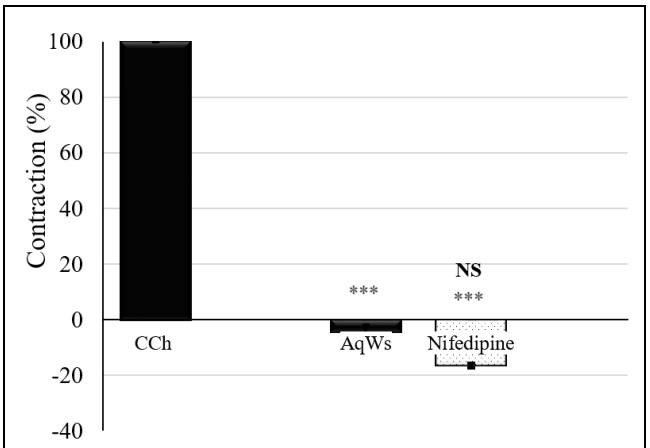

Figure 7. Effect of the crude extract of *W. saharae* (AqWs, 3 mg/mL) on contractions of rat jejunum preincubated with nifedipine 10^{-6} M for 20 min and then precontracted by carbachol (CCh) 10^{-6} M.

***P≤ 0.001 in comparison with the normal control; NS: no significant in comparison with the extract without drugs (using student's t-test with the mean ± SEM, n = 6).

DISCUSSION

The effect of the AqWs on spontaneous contractions of rabbit jejunums have been tested because their amplitude was clear and bigger of that of the rat contractions, so they were easier to be analyzed than the rat jejunum. The present result showed that this aqueous extract exercised in dose-dependent manner a reversible inhibitory effect on the smooth jejunum muscle of the rabbit. In the presence of the blocking adrenergic receptors (β by the propranolol, α_1 by the prazosin, α_2 by the yohimbine), the aqueous extract at the concentration of 3 mg/mL had shown a clear myorelaxant effect on contractions. Then the myorelaxant effect of AqWs did not pass-through adrenergic receptors. Similar results have been established on the other

plant extracts (Aziz et al., 2006; Makrane et al., 2019).

One of the essential cellular events for stimulating contractions in all smooth muscles is the Ca^{2+} flux through the voltage-dependent L-type channels (Gavilánez et al., 2018). When the concentration of K^+ increases in the extracellular medium, a depolarization of the membrane takes place and as a result the voltage-operated Ca^{2+} channels (VOCCs) make Ca^{2+} enter into the cytoplasm (Bolton, 2017) and consequently the contractions make here place. In addition, the substance that inhibited the intracellular contraction mechanism is considered a blocker of this system. The AqWs exercised in dose-dependent manner relaxation of the rat jejunum muscle precontracted by KCl. Thus, in the presence of different doses of this aqueous extract, the maximum response to the increasing and cumulative dose of $CaCl_2$ was diminished by shifting the dose-effect curves to the right. This inhibitory is like a non-competitive antagonist against the VOCCs, which was obtained with other plants (Hajhashemi et al., 2000; Jabeen et al., 2007). These results were comparable with those obtained with the verapamil, which is a specific inhibitor of eukaryotic voltage-gated L-type (Bolton, 2017).

Intestinal problem spasm can be complementary related to the aggravated neuronal release of the neurotransmitter acetylcholine (Souza et al., 2013), furthermore experimental studies *in vitro* clearly demonstrated that acetylcholine acted such as a transmitter for the secreting enterocyte in the small intestine (Tobin et al., 2009). This parasympathetic neurotransmitter can be acted by two functions to regulating the physiological balance between contraction and relaxation in smooth muscle (Español and María, 2000). The contraction can be made by the activation of the M_3 subtype, entraining release of inositol phosphate (IP_3) by the activation of phospholipase C (PLC) on the other hand, the stimulation of the M_2 receptor inhibit the adenylyl cyclase (AC) release entrained relaxation and maintaining of intestine tone (Sales et al., 1997). The AqWs exercised also in a dose-dependent manner relaxation of the rat jejunum muscle precontracted by CCh 10^{-6} M. In the presence of different doses of this aqueous extract, the maximum response to the increasing and cumulative dose of CCh was diminished by shifting the dose-effect curves to the right. Thus, this inhibitory is like to be a non-competitive antagonist against acetylcholine receptors.

To evaluate whether the aqueous extract acted via the cholinergic receptor's pathway, atropine and the hexamethonium were used to antagonize the muscarinic and the nicotinic receptors respectively (Goyal, 1988). Results findings suggested that this extract acted on both nicotinic and muscarinic receptors.

Methylene blue and L-NAME were used in a previous study for their special characters such as blockers of guanylate cyclase or nitric oxide pathways, respectively (Arnold et al., 1977; Mayer et al., 1993; Dib et al., 2017). In the gut NO is synthesized in neural and non-neural tissues by three isoforms of nitric oxide synthase (NOS): neuronal NOS (nNOS), endothelial NOS (eNOS), and inducible NOS (iNOS). It is produced by nNOS in enteric neurons and by eNOS in smooth muscle cells, where it is usually colocalized with vasoactive intestinal polypeptide (VIP) or pituitary adenylate cyclase-activating peptide (PACAP) (Teng et al. 1998; Grider and Murthy 2008). With the preliminary results obtained, it is difficult to specify on which NOS of the intestine AqWs acted. These data supported consequently, that this extract could act via the guanylate cyclase pathway but not by nitric oxide pathway.

The extreme climatic conditions return the Saharan plants resistant to a broad variety of stress conditions. Among these plants, *W. saharae,* which contains phenolic compounds (Bouchouka et al., 2012), different flavonoids (flavonol, flavone, isoflavone) (Cheriti et al., 2013), tannins, saponins, and coumarins (Left et al., 2018). Essential oil of this plant has relieved many compounds such as hedecanoic acid like a major compound in the study realized by Essaqui et al. (2004), and β eudesmol in other studies effected by Amezouar et al. (2012). Hilmi (2002b), isolated twelve new guaianolide type sesquiterpene lactones (SLs) and a new eudesman derivative lß,6a-dihydroxy-costic

acid. The known compounds, dehydroleucodin, reynosin, 1,2-didehydro-3-oxo-costic acid, and the flavonoid hispidulin were reported recently for the first time from this plant. Sesquiterpene and flavonoids have antispasmodic activities (Emendörfer et al., 2005; Mendel et al., 2016). It would be very interesting to check, which is, or which are the *Warionia* compounds responsible for these activities.

CONCLUSIONS

The antispasmodic effect of the aqueous extract of *Warionia saharae* Benthem ex Benth. & Coss. on isolated jejunum of rabbit and rat was tested on jejunum isolated from rabbits and rats. The results obtained suggest that the antispasmodic effect of this plant is like a non-competitive antagonist antagonizing the nicotinic and muscarinic receptors, the VOCCs and the guanylate cyclase pathway. This effect strengthened its use in traditionally medicine as a remedy for intestinal cramps.

CONFLICT OF INTEREST

The authors declare no conflicts of interests.

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AUTHOR CONTRIBUTION:

Contribution	Amrani O	Margich M	Makrane H	Alem C	Aziz M
Concepts or ideas					x
Design					x
Definition of intellectual content	x				x
Literature search	x	x			
Experimental studies	x	x			
Data acquisition			x		
Data analysis				x	
Statistical analysis			x		
Manuscript preparation	x				
Manuscript editing	x				x
Manuscript review	x	x	x	x	x

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