Underlying Mechanisms of Anti-spasmodic, Antidiarrheal, Antioxidant and Acute Toxicity Assessments of Aqueous Extract of *Mentha Suaveolens Ehrh* and its Fourier Transform Infra-red Spectroscopy Analysis

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Authors' contributions

This work was carried out in collaboration between all authors. Author EAJ, performed the entire pharmacological activities experiments and wrote the first draft of the manuscript. Author CA helped in the experiments. Author BA managed the FTIR analyses of the study. Author TA helped in the writing of the manuscript. Author BR supervised the whole work, supported the study, corrected and submitted the paper. All authors read and approved the final manuscript.

Article Information

Original Research Article

ABSTRACT

Objective: The aim of this present study is to investigate the antidiarrheal, spasmolytic and antioxidant activities of aqueous extract of *Mentha suaveolens Ehrh* (AEMS), to study their underlying mechanisms in animal models and to reveal its main functional groups using Fourier Transform Infra-Red Spectroscopy (FTIR).
Methods: *Mentha suaveolens Ehrh* was studied for antidiarrheal activity on Wistar rats of both sexes at the doses of 200 and 800 mg/kg body weight using castor oil-induced diarrhea, castor oil-induced enteropooling and small intestinal transit models.

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The extract was studied for antispasmodic property in isolated rabbit jejunum using various spasmogenic agents including Ach (10^{-5}M), KCl (100 mM) and in the absence and in the presence of L-NAME (10^{-4} M) and the methylene blue (10^{-5} M).The antioxidant capacity of AEMS was carried out using DPPH radical scavenging activity and the ferric reducing antioxidant potential (FRAP). Ascorbic acid and Butylated HydroxyToluene (BHT) were used as references. The functional chemical groups were determined by FTIR.

Results: The great antidiarrheal potential of AEMS seems to be mediated through calcium antagonism. The marked and concentration-dependent induced spasmolytic effect of AEMS appears to involve Ca^{2+} voltage channel blockade and the NO/cGMP pathway activation. AEMS possessed strong and concentration-dependent antioxidant potency using DPPH and FRAP. Polyphenols, carboxyl and carbohydrates were found to be the main functional groups in the AEMS analyzed by FTIR.

Conclusion: Overall, our current findings provide scientific proves in animal models for the traditional use of AEMS in folk medicine for the prevention or the treatment of gastrointestinal diseases in Morocco.

1. INTRODUCTION

Diarrhoeal disease has long been recognized to be the second leading cause of death after pneumonia in low and middle-income countries [1-2]. According to the World Health Organization (WHO), 6 millions of children under 5 years old died of diarrhoea in 2015 [3]. Diarrhoea can last several days, and most people who die from diarrhoea actually die from severe dehydration and fluid loss.

Many people still rely on traditional healing practices and still have a strong belief on medicinal plants despite developing revolutionary technology and huge improvements in healthcare [4]. The WHO estimated that about 80% of the population, especially in developing countries is referred to traditional herbal medicines for their primary health care needs [5]. However, the inappropriate use of traditional medicines or practices can have negative and dangerous effects. It becomes a necessity, therefore, to perform further studies to ascertain the efficiency and safety of these herbal remedies and to provide scientific proves for the traditional utilization of these herb remedies in the folk medicine.

Mentha suaveolens Ehrh, which belongs to the family of the lamiacaeae, grows in several regions in Morocco, where the fresh and dried leaves and flowers are frequently used in herbal tea. *Mentha suaveolens Ehrh* is an aromatic herbaceous and perennial herb with a sickly sweet scent that grows up to 100 cm in height. *Mentha suaveolens Ehrh* is known by Moroccan names «Merssita» or «Timijja».

An ethnobotanical investigation carried out by Cadena et al. [6] has shown that *Mentha suaveolens Ehrh* is used to treat several health challenges including stomach ache, intestinal inflammations, intestinal parasites, fevers, menstrual cramps diaper rash. Another ethnobotanical study of medicinal plants performed by TAHRI et al. [7] indicated that *Mentha suaveolens Ehrh* has numerous pharmacological activities including laxative, toning, digestive, diuretic, carminative, stomachic, eupeptic, antispasmodic, antiseptic, analgesic, anti-hemorrhoidal, anti-rheumatic. Concerning the biological activities, it was found that *Mentha suaveolens Ehrh* has antihypertensive effect [8], acetylcholinesterase inhibitory activity [9] and a monoamine oxidase inhibitory activity [10]. Also, the essential oil *of Mentha suaveolens Ehrh* was reported to have a candidacidal activity [11]. Moreover, it has been proved that the essential oil of the aerial parts of *Mentha suaveolens Ehrh* has anti-inflammatory, cytotoxic, hepatoprotective and antifungal activities [12-14]. Moreover, methanol extract of *Mentha suaveolens Ehrh* provokes the induction of a peripheral analgesic response [15].

Although another study has been previously carried out by Moreno et al. [15] and Božović et al. [16] on the spasmolytic activity of *Mentha suaveolens Ehrh,* however, the major differences and strengths of our present study with it; are the use of different antispasmodic evaluations, the study of the underlying mechanisms involved in the spasmolytic effect of *Mentha suaveolens Ehrh*, performing the functional chemical groups by Fourier transform infrared spectroscopy

(FTIR), the different geographical region of the plant which is an important factor affecting phytochemicals and the pharmacological characteristics of the plant extracts ,and the use of aqueous extract rather than the methanolic extract or the essential oil.

It has been reported that the genus of *Mentha* such as *Mentha longifolia* L [17] possesses antidiarrheal potency. On the other hand, it has been documented that most of the antidiarrheal agents have therapeutic potential in abdominal spasm [18] and it has been reported that the stress oxidative could have direct or indirect effects on responses in the gastrointestinal tract and could thus, be the reason of several diseases including the gastrointestinal troubles like spasm and diarrhea [19,20]. Therefore, the present study was undertaken in order to investigate the antidiarrheal, spasmolytic and antioxidant effects and to explore the putative underlying mechanisms of AEMS respectively on Wistar rats and rabbits models, in order to support the use of AEMS in folk medicine for the prevention or the treatment of gastrointestinal diseases in Morocco.

2. MATERIALS AND METHODS

2.1 Preparation of Aqueous Extract of *Mentha Suaveolens*

Aerial part (leaves and flowers) of *Mentha suaveolens Ehrh* was collected locally from northern Morocco, Taounate (Lat: 34.52, Long: -5.06) between March and June (2017).The authentication of *Mentha suaveolens Ehrh* was done by Professor Derraz (university Sidi Mohamed Ben Abdellah, FST, Fez, Morocco), and a voucher specimen was taxonomically identified and deposited in the herbarium of the Faculty of Sciences and Techniques, Fes, Morocco (No. MA-FSTF 21).

The plant material was thoroughly washed individually under running tap water to remove any traces of soil particles and other dirt, dried in shade and then powdered using electric mixer. The plant extract was prepared following the standard traditional method described in Moroccan Pharmacopoeia [21]. 20 g of the air-dried aerial parts of *Mentha suaveolens Ehrh* were boiled in distilled water (200 mL), centrifuged and the supernatant filtered through whatman filter paper. Thereafter, the water was removed under vacuum in a rotary evaporator

until dry. The percentage yields based on the dried starting material was 20% for dried aqueous extract. The extract was stored at -20°C until the pharmacological investigations were performed.

2.2 In Vivo Experiments

Wistar rats (180-250 g) and rabbits (1.8-2.5 kg) of both sexes were kept in a standard environmental condition in terms of humidity, temperature and light. The animals had free access to water and food until the experiment. However, food was withdrawn 24 hours prior to the experiment. The investigation confirms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No.85-23, revised 1996).

2.3 Acute Toxicity

In order to examine the safety of AEMS, we conducted an acute toxicity test according to the Organization for Economic Cooperation and Development (OECD) guideline 423 (OECD, 2001a) [22]. Wistar rats (180-250g) were randomly divided into different experimental groups of six rats each. The animals were fasted overnight prior to the experiment. AEMS was administered orally by gavage (10 ml/kg) to each treatment group at single dose of 800, 2000, 3000, 5000 mg/kg, respectively. A control group was given distilled water (10 ml/kg). The animals were allowed food and water ad libitum and kept under regular observation within and after 24h following oral administration of the tested materiel, in order to observe the general signs and symptoms of toxicity mainly hypo-activity, piloerection, breathing difficulty, tremors, and convulsion or mortality in any of the animals tested.

2.4 Castor Oil–Induced Diarrhea

To assess the antidiarrhoeal activity of AEMS, we followed a method previously described by Borrelli et al. [23]. Rats of either sex (180-250 g) were fasted for 24 h before starting the experiment. The animals were randomized into five groups of five rats each and were treated as outlined below:

Group I   : Distilled water 10 ml/kg body weight
Group II   : AEMS 200 mg/kg
Group III : AEMS 800 mg/kg

Group IV : loperamide 5 mg/kg
Group V   : loperamide 10 mg/kg

One hour after, castor-oil (3 ml/kg) was administered to each rat. The treated rats were then housed in separate clean cages having paper placed below for collection of fecal matter .The severity of diarrhoea was assessed each hour for 4 h by following these parameters: onset of diarrhoea, number of wet faeces, and total number of faecal output.

POF = (Ft/Fc) x 100

Ft   : the mean fecal weight of each treatment
        group
Fc   : the mean fecal weight of the control
        group
POF : percentage of faecal output

POI = [(Mo-M)/Mo] x100

Where Mo is Mean defecation of control, M is Mean defecation of test sample and POI is percentage of inhibition.

2.5 Castor Oil–Induced Enteropooling

The castor oil-induced enteropooling was carried out following the protocol described by the method of Robert et al. [24]. Over- night fasted rats (180-250 g) were divided randomly into five groups of six animals each. Group I was given 10 ml/kg of distilled water and kept as a control; group II and III received 200 and 800 mg/kg of AEMS respectively, Group IV and V served as a vehicle control and received 5 ml/kg and 10 ml/kg of loperamide respectively. One hour later, all rats were given 3 mL of castor oil orally. After one hour, the animals were sacrificed, the abdomen of each rat was opened; the small intestine was then taken from the pylorus to the caecum; ligated at both ends and dissected out carefully. Each small intestine was weighed and its content was then collected by gentle milking into a graduated tube and the volume of intestinal contents was measured. Each intestine was reweighed and the difference between the full and the empty intestines was calculated. The percentage inhibitions of the volume and weight of intestinal contents were determined according to the following formulae:

POI = [(MVICC)-(MVICT)] x 100 / (MVICC)

Where, MVICC = Mean volume of the intestinal content of the control group; MVICT = Mean volume of the intestinal content of the test group.

2.6 Small Intestinal Transit Time

In this test, Wistar albino rats (180-250 g) were randomly allocated to five groups of five rats each. Distilled water 10 ml/kg body weight was given to group I as control. Group II and group III were received 200 mg/kg and 800mg/kg body weight of AEMS respectively. Group IV and V were given standard drug loperamide 5 mg/kg and 10 mg/kg respectively.

3 ml/kg of the marker diet (10% charcoal suspension in 5% cellulose) was administered orally by gavage one hour after castor-oil treatment. The rats were sacrificed by inhalation of chloroform 1h hour after the charcoal meal and the small intestine was immediately isolated. The peristaltic index (PI), which is the distance traveled by the charcoal meal relative to the total length of small intestine expressed in %, was calculated for each rat using the following equation:

% IP = (LM / LSI) x 100

Where

IP   = peristaltic index
LM   = Length of charcoal meal
LSI   = Length of small intestine

2.7 Spasmolytic Effect

Rabbits (1.8-2.5 g) were slightly anesthetized with ether and then exsanguinated. Segments of jejunum of about 2- 3 cm were quickly isolated and mounted in an organ bath containing Tyrode solution (50 ml) between two stainless steel hooks under 1g initial tension. The lower hook was fixed at the bottom of the organ bath and upper one was connected to an isotonic transducer (Harvard transducer, UK) connected to a Harvard Universal Oscillograph (UK). The Tyrode (in mM: 136 NaCl, 2.7 KCl, 1.4 $CaCl_2$ $2H_2O$, 0.5 $MgCl_2$ $6H_2O$, 11.9 $NaHCO_3$, 0.42 NaH_2PO_4 and 5.56 Glucose) was bubbled continuously with 95% O2, 5% CO2, pH 7.4 at 37°C). Each piece of jejunum was allowed to equilibrate and stabilize in normal Tyrode solution for at least 30 min before the addition of any drug with washout every 10 min.

2.8 Effect of AEMS on Spontaneous Contractions

After stabilization of spontaneous contractions of rabbit jejunum (30 min), the cumulative doses of

AEMS were added (0, 3 mg/ml, 1 mg/ml; 3 mg/ml and 10 mg/ml) in order to test the eventual myorelaxant effect of AEMS. Segments of jejunum that did not show spontaneous contraction were discarded from the experimental protocol. No more than two experiments were realized on the same jejunum which always received the same extract.

2.9 Spasmolytic Effect of AEMS on High Potassium and Ach– induced Contractions

The spasmolytic activity of AEMS was assessed using different spasmogens such as Ach (10^{-5}M), Tyrode high K+ (100 mM) to produce sustained contraction. AEMS was then added to the bath in a cumulative way to obtain concentration-dependent inhibitory responses.

2.10 AEMS Effect on Calcium Induced Contractions

In order to assess if the spasmolytic activity of AEMS was mediated through calcium channel blockade, the jejunum was left for 10 minutes in Ca^{2+} free Tyrode's solution with EDTA (2mM). Ca^{2+} free Tyrode's solution was then replaced by Tyrode high K+ (60mM) Ca^{2+} free solution. A control concentration response curve was obtained by adding $CaCl_2$ (0.5 to 5 mM) to the bath. The study was repeated once in the presence of the maximal concentration of AEMS (10 mg/ml) and twice in the presence of verapamil (5.10^{-6} M), which acted as a positive voltage calcium channel inhibitor.

2.11 Effect of AEMS in the Presence of L-Name and the Methylene Blue

To evaluate the possible contribution of the NO /cGMP pathway in the spasmolytic effect of AEMS, the jejunum preparation was incubated in the presence of L-NAME (10^{-4} M) (NG-nitro-L-arginine methyl ester) as a nitric oxide synthase inhibitor or with the methylene blue (10^{-5} M) a non-specific cGMP inhibitor.

2.12 Antioxidant Activity of AEMS

The antioxidant capacity of AEMS was carried out using antioxidant assays: DPPH (1, 1-diphenyl-2-picrylhydrazyl) radical scavenging activity and the Ferric Reducing Antioxidant Potential (FRAP). Ascorbic acid and Butylated HydroxyToluene (BHT) were used as references.

Plant extract at different concentrations ranging from 0.015 to 1 mg/ml was added to 2 ml of a 0.004% MeOH solution of DPPH•. The mixture was shaken vigorously and kept at room temperature for 30 min in the dark. Absorbance of AEMS and the standard were measured at 517 nm. The capability to scavenge the DPPH• radical was calculated using the following equation from: [(A0-A1)/A0] ×100

Where A0 is the absorbance of the control, and A1 is the absorbance of the extract

The reducing power of AEMS was determined also according to the FRAP method previously described [25]. The extract at different concentrations (0.05, 0.125, 0.25, 0.5,1 mg/ml) was mixed with 2.5 ml of phosphate buffer (0.2 M, pH=6.6) and 2.5 ml potassium ferricyanide [$K_3Fe (CN)_6$] (1%). The mixture was then incubated at 50°C for 30 min. Afterwards, 2.5 ml of trichloroacetic acid (10%) was added to the mixture, which was then centrifuged at 3000 rpm for 10 min. Finally, 2.5 ml of upper layer solution was mixed with 2.5 ml distilled water and 0.5 ml $FeCl_3$ (0.1%), and the absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power.

2.13 FTIR Analysis

Fourier Transform Infra-Red (FTIR) spectroscopy method was used to find out the characteristic functional chemical groups present in AEMS components based on the peak value in the region of infrared radiation. FTIR analysis of a small quantity of *Mentha suaveolens Ehrh* extract was performed using Jasco FT/IR FTIR spectrometer series 6800c, the spectra was recorded in the region between 4000 and 400 cm−1 and the peak values of the FTIR were recorded.

2.14 Statistical Analysis

The data are expressed as means ± SEM (Standard error of mean). The data were analyzed using one-way ANOVA followed by Turkish and student test for comparing the control and the various groups, using Graph Pad Prism version 6.1 (Graph-Pad Software, San Diego, California, USA). Statistical significance was assumed at the 0.05 levels.

2.15 Drugs

Loperamide, verapamil, acetylcholine, EDTA, castor oil, charcoal, DPPH , methylene blue were all purchased from Sigma Chemicals Company (St Louis, MO, the United States). All the drugs were dissolved in distilled water. The drugs were stored at -20°C until use in the pharmacological experiment.

3. RESULTS

3.1 In vivo Results

3.1.1 Acute toxicity study

The results of acute toxicity showed that neither visible clinical signs, symptoms of toxicity, nor death was observed when administering our AEMS at the doses of 800, 2000, 3000, 5000 mg/kg. The oral LD50 was therefore greater than 5000 mg/kg in rats, which means that the extract is safe.

3.2 Castor Oil–Induced Diarrhea

Our results indicate that oral administration of AEMS (200 and of 800 mg/kg) reduced significantly (*p < 0.05) the frequency of defecation, fecal dropping, and the mean weight of feces and delayed the onset time when compared with the untreated group. AEMS exhibited dose-dependent antidiarrheal activity since 200 mg/kg and 800 mg/kg showed 38 and 88.83 % of inhibition of defecation respectively. The diarrhea didn't occur in rats treated with loperamide, a standard antidiarrheal agent, in concentrations of 5 mg/kg and 10 mg/kg and offered that way 100% protection.

3.3 Castor Oil–Induced Enteropooling

The castor oil use lead to the accumulation of water and electrolytes in the intestine. As shown in Fig. 2, the administration of AEMS caused a significant (p < 0.001) reduction of the volume and weight of the intestinal contents in castor oil-induced enteropooling. The inhibition of the intestinal content was 41.03 ± 1.37 and 61.87 ± 3.23 using respectively 200 and 800 mg/kg of AEMS while loperamide produced (p < 0.001) 60.65 ± 0.77 and 73.52 ± 1.50 respectively at the doses of 5 mg/kg and 10 mg/kg.

3.4 Small Intestinal Transit

AEMS markedly decreased the distance travelled by charcoal meal through the gastrointestinal tract when compared with the control group (from 84.35 ± 0.60 to 52.67 ± 0.74 and to 25.37 ± 0.60 respectively for 200 and 800 mg/kg). The observed effect was comparable to that of loperamide (Table 3).

3.5 In Vitro Results

3.5.1 Effect of AEMS on spontaneous contractions of rabbit jejunum preparations

When tested on spontaneous contractions of rabbit jejunum segments, AEMS provoked significant myorelaxant effect in a concentration-dependent manner (0. 3 mg/ml, 1 mg/ml; 3 mg /ml and 10 mg/ml) as illustrated in Fig 1. The highest concentration (10 mg/ml) almost abolished spontaneous contractions.

3.6 Effect of AEMS on Ach and KCl Induced Contractions of Rabbit Jejunum

The contraction of the jejunum preparations under spasmogenic agents, like Ach (10^{-5} M) or KCl (100 mM) were reduced in a concentration-dependent fashion when using AEMS. AEMS concentration- dependently inhibited the response to Ach, with IC50 value of 1, 89 ± 0, and 93 mg/ml and to KCl with IC50 value of 2, 92 ± 0, and 87 mg/ml. As shown in Fig 2, the percentage of contraction was decreased to 7.53 ± 0.59 and 13.22 ± 1.61 using respectively Ach and KCl at 10mg/ml of the AEMS.

3.7 AEMS Effect on Extracellular Calcium Induced Spontaneous Contractions

To investigate an eventual interference of AEMS extract with Ca^{2+} influx, the spontaneous contraction of the jejunum was examined in Tyrode Ca^{2+} free in the presence and in the absence of the extract. As shown in typical trace (Fig. 3a), pretreatment of the preparations for 10 minutes with Tyrode Ca^{2+} free/2 mM EDTA drastically reduced the spontaneous contractions of the jejunum, due to the no presence of extracellular calcium. As expected, the addition of the cumulative concentrations of Ca^{2+} restores the normal jejunum activity, demonstrating the importance of Ca^{2+} influx in spontaneous contraction. As clearly shown in Fig. 3b, addition of AEMS blocked the recovery of spontaneous contractions of the segments seen in control group, suggesting that AEMS causes myorelaxant effect via at least inhibition of extracellular calcium influx in rabbit jejunum.

Table 1. Anti-diarrheal activity of AEMS in rats, on castor oil (CO) -induced diarrhea

Group	Treatment	Onset of diarrhea (min)	Rat rats with diarrhea	Mean weight of wet stools (g)	Mean weight of dry stools (g)	%POF	% Inhibition of defecation
I	Control	60.4 ± 0.57	6⁄6	13.63 ± 0.24	4.21 ± 0.061	—	—
II	AEMS (200 mg/kg)	149.5 ± 0.35*	2⁄6	6.62 ± 0.1*	2,57 ± 0.18*	61.04	38
III	AEMS (800 mg/kg)	180 ± 1.95*	1⁄6	0.82 ± 0.15*	0.47 ± 0.35*	11.16	88.83
V	Loperamide (5 mg/kg)	—	0⁄6	0*	0*	—	100
V	Loperamide (10 mg/kg)	—	0⁄6	0*		0*	0* — 100

Values were expressed as mean ± SEM. (n=6), (*P < 0.05), when compared with control group

Table 2. Effect of AEMS on castor oil (CO) induced- enteropooling in rats

Group	Treatment	Mean weight of intestinal content (g)	Mean volume of intestinal content (ml)	% Inhibition
I	Control	2.98 ± 0.28	3.15 ± 0.29	—
II	AEMS (200mg/kg)	2.47 ± 0.29*	2.44 ± 0.28*	41.03 ± 1.37
III	AEMS (800mg/kg)	1.87 ± 0.12*	1.88 ± 0.11*	61.87 ± 3.23
IV	Loperamide (5mg/kg)	1.66 ± 0.16*	1.33 ± 0.16*	60.65 ± 0.77
V	Loperamide(10mg/kg)	1.32 ± 0.21*	0.92 ± 0.22*	73.52 ± 1.50

Values were expressed as mean ± SEM. (n=6), (*P < 0.05), when compared with control group.

Table 3. Antidiarrheal activity of *AEMS* on castor oil on small intestinal transit in rats

Group	Treatment	Mean Length of Intestine (cm)	Mean Distance Travelled by Charcoal (cm)	Peristaltic Index	% Inhibition
I	Control	99.6 ± 0.557	84.35 ± 0.604	81.93 ± 0.58	—
II	200mg/kg(AEMS)	99.16 ± 0.4*	52.67 ± 0.74*	50.47 ± 0.94*	38.40 ± 0.88
III	800mg/kg(AEMS)	99.65 ± 0.56*	25.37 ± 0.60*	25.45 ±0.58*	68.92 ± 0.76
IV	Loperamide (5mg/kg)	101.58 ± 1.31*	36.17 ± 1.138*	35.95 ± 1.31*	56.46 ± 1.59
V	Loperamide (10mg/kg)	101.15 ± 0.95*	23.00 ± 1.02*	22.74 ± 1.00*	72.24 ± 1.13

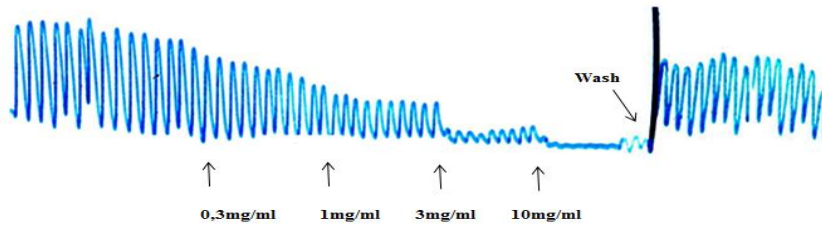*Values were expressed as mean ± SEM. (n=6), (*P < 0.05), when compared with control group*

Fig. 1. Original tracing showing the myorelaxant effect of aqueous extract of *Mentha suaveolens Ehrh* (AEMS) on the spontaneous contractions of isolated rabbit jejunum
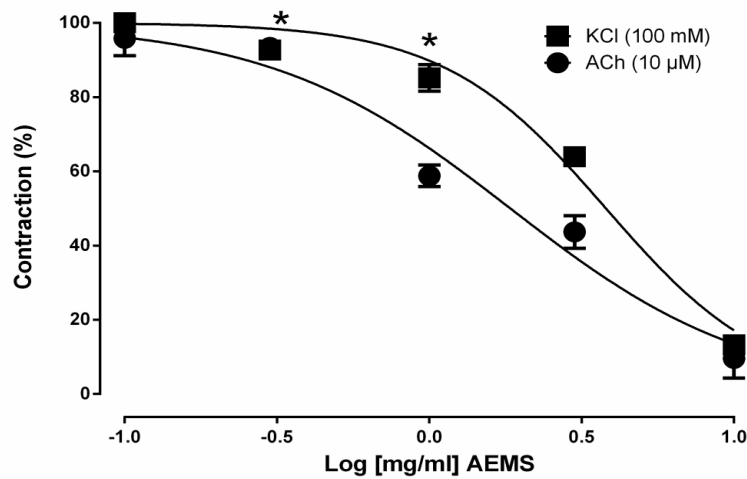

Fig. 2. Spasmolytic effect of cumulative concentrations of AEMS on KCl (100mM) and Ach (10µM) induced contractions in rabbit isolated jejunum (*P<0.05, n=6)
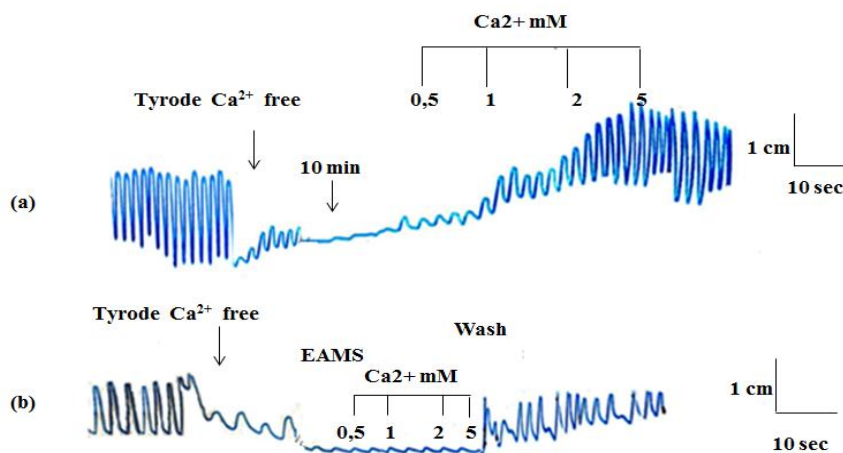

Fig. 3. Typical tracing of contractile activity of rabbit jejunum in Tyrode CA $^{2+}$ free without (a) and with (b) the sub maximal concentration 3 mg/ml of AEMS

3.8 Effect of AEMS on Calcium Channel Voltages Dependents

In order to determine whether the spasmolytic effect of AEMS is due to a blockade of voltage dependent Ca^{2+} channels (VDCC), the extract was assayed on high K^+ (100 mM)-Ca2+-free Tyrode solution.

As shown in Fig. 4, the submaximal concentration of AEMS inhibited the recovery of the high K^+ induced contraction during calcium supplementation and shifted to the right the Ca^{2+} concentration response curves, in a similar way to verapamil (an antagonist of VDCC) used as a positive control, suggesting that the spasmolytic effect of AEMS is through at least voltage-dependent Ca^{2+} channel blockade.

3.9 The Effect of AEMS in the Presence of L-NAME and the Methylene Blue

In order to determine whether the spasmolytic activity of AEMS may involve other molecular pathways such as NO /cGMP activation, the jejunums were incubated with L-NAME a specific NO synthase inhibitor or with methylene blue a non-specific cGMP inhibitor. The results obtained showed that the spasmolytic effect of AEMS was significantly reduced in the presence of L-NAME (100 µM) and methylene blue (10 µM) ($p < 0.05$, n

= 6). The IC50 values of AEMS, L-NAME (100 µM) and methylene blue (10 µM) were 1, 64± 0, 93 mg/ml, 3, 12 ± 0, 86 mg/ml and 2, 99 ± 0, 88 mg/ml respectively. Since the relaxation curves were shifted to the right. Therefore, it seems that the spasmolytic effect of AEMS on the rabbit jejunum preparations may be due also to NO and a cGMP pathways activation.

3.10 Antioxidant Activity of AEMS

In the present investigation, the commonly accepted assays DPPH and FRAP were used for the evaluation of antioxidant activity of AEMS.

3.11 DPPH Radical Scavenging Assay

AEMS exhibited strong antioxidant ability when using DPPH (Fig 6). The results showed that AEMS exhibited a significant dose-dependent inhibition of DPPH• free radical. The percentage of inhibition was 34.32 ± 1.87, 50.11 ± 0.62, 66.44 ± 1.74, 78, 13 ± 0.02 and 79.82% ± 1.64 respectively for 0,062, 0,125, 0, 25, 0, 5 and 1 mg/ml of AEMS with an IC50 of 0.1447± 0.031 mg/ml. The IC50 value of ascorbic acid (0.0525± 0.028 mg/ml) is lower than the IC50 value of EAMS (IC50 0.1447± 0.031 mg/ml) and of BHT (IC50=0.164 ±0.085 mg/ml), but IC50 of AEMS value (0.1447± 0.031 mg/ml) is less than BHT IC50 (0.164±0.085 mg/ml).
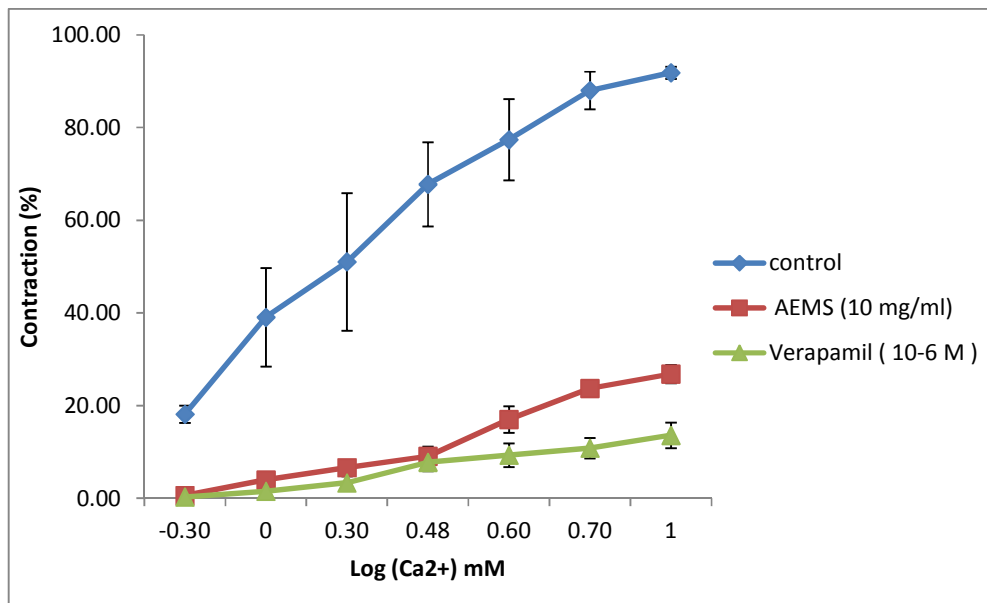

Fig. 4. Typical tracing of effect the aqueous extract of *Mentha suaveolens Ehrh* (AEMS 10 mg/ml) on KCl evoked contraction on rabbit jejunum segments comparing with verapamil
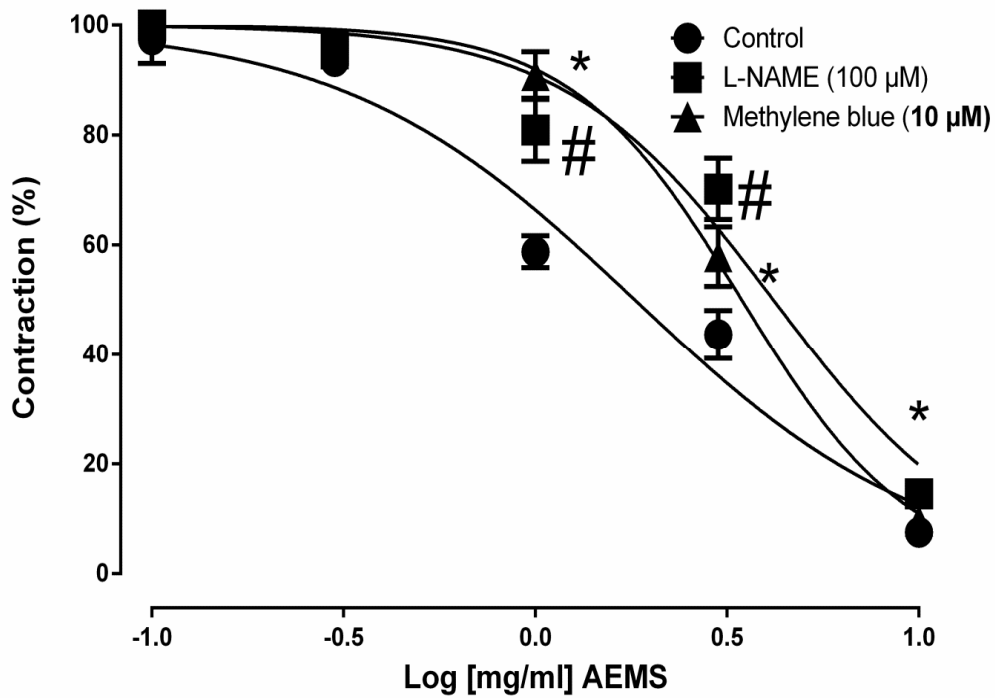
Fig. 5. Typical tracing showing the effect of AEMS on the rabbit jejunum incubated in L-NAME (100 µM) and in methylene blue (10 µM) * p< 0, 05 significantly different from the responses in the absence of L-NAME (n=6), *p< 0, 05 significantly different from the responses in the absence of methylene blue (n=6), by Anova followed by Turkish test
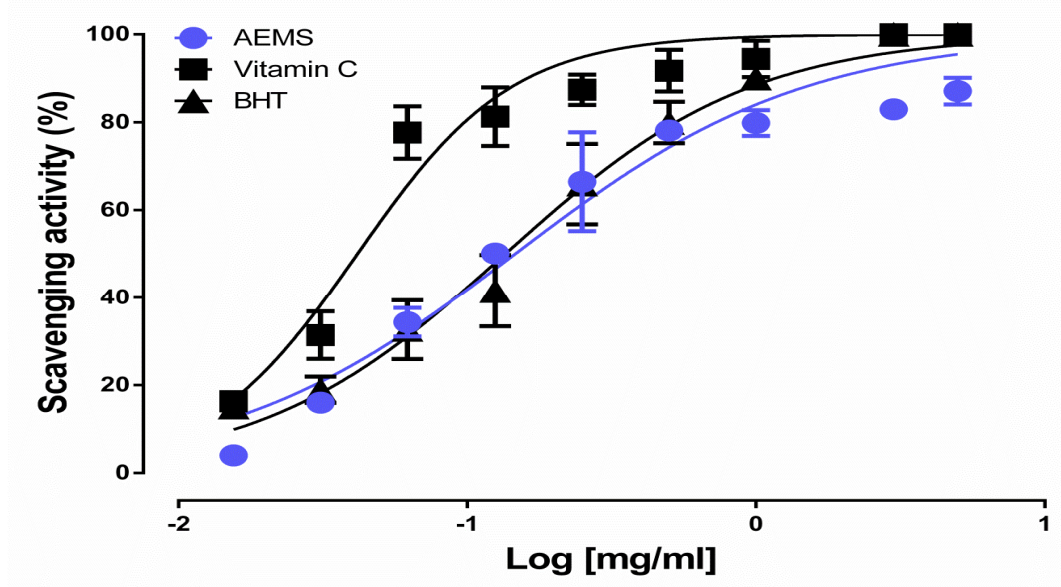

Fig. 6. DPPH radical scavenging assay of standards (Ascorbic acid, and Butylated HydroxyToluene) and aqueous extract of *Mentha suaveolens Ehrh* (AEMS)

3.12 Reducing Power: FRAP

The reductive capability of AEMS compared with ascorbic acid and butylated hydroxy toluene has been illustrated in Fig. 7. The reducing power of AEMS leaves was found to be remarkable, which increased gradually with a rise in the concentration. Considering the maximal concentration ,the reduction power of ascorbic acid, BHT and AEMS (1 mg/ml) was respectively 1.88 ± 0.2, 1.85 ± 0.15 and 1.89 ± 0.28.Thus, these results demonstrated that AEMS is a strong antioxidant.

3.13 Fourier Transform Infrared Spectroscopy (FTIR)

The aim behind performing FTIR analysis is to determine the existence of functional groups that exist in AEMS.

As shown in Fig 8, the FTIR spectrum investigation exhibits the presence of interesting characteristic functional bonds and chemical groups constituting the structural and molecular aspects of AEMS as illustrated in Table 4.

4. DISCUSSION

Although many natural products have been described to possess a number of therapeutic benefits, many others are waiting scientific verification of their efficacy and safety including *Mentha suaveolens Ehrh.* Therefore, the present study was designed in order to validate the medicinal use of AEMS for healing people from gastrointestinal disorder like spasm, diarrhea and oxidative stress in order to provide scientific evidence for its traditional use in folk remedy.

Many mechanisms have been suggested to explain the antidiarrheal effect of loperamide among others, the inhibition of extracellular calcium [26]. However, it is well documented that castor oil produces diarrhea due to its most active metabolite, ricinoleic acid. Ricinoleic acid causes diarrhoea through a series of events which include stimulating the peristaltic activity of the small intestine and reducing and/or inhibiting the activity of Na+-K+ ATPase. These consequently lead to changes in the electrolyte permeability of the intestinal mucosa, hypersecretion of the intestinal contents, and a slowdown of the transport time in the intestine [27-29]. Since in diarrhoea induced by castor oil in Wistar rats, AEMS significantly delayed the onset, decreased the frequency of defecation, fecal dropping, and the mean weight of feces in a concentration- dependent way (Table 1), we think that the extract might have exerted its antidiarrheal action via antisecretory mechanism. Furthermore, AEMS delayed the gastrointestinal transit in rats compared to the control which suggest the increase the absorption of water and electrolyte from the gastrointestinal tract as a probable mechanism of its antidiarrheal effect.
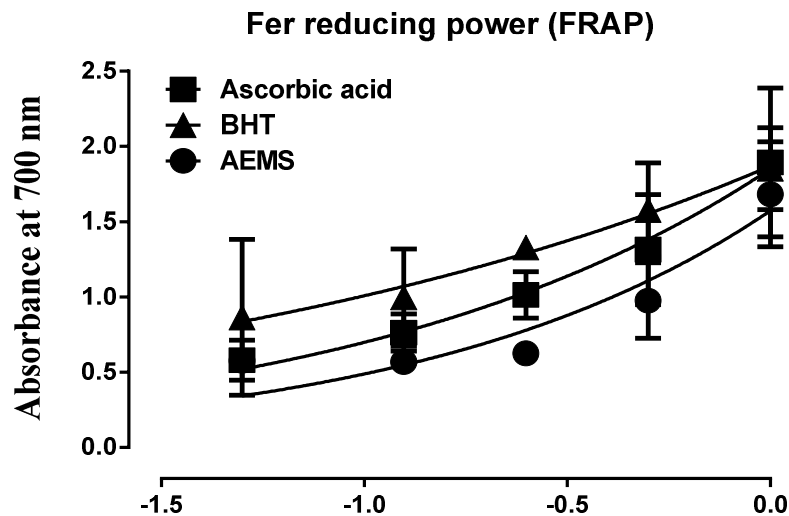

Fig. 7. Total reduction capacity of standards (Ascorbic acid, BHT) and aqueous extract of *Mentha suaveolens Ehrh* (AEMS)
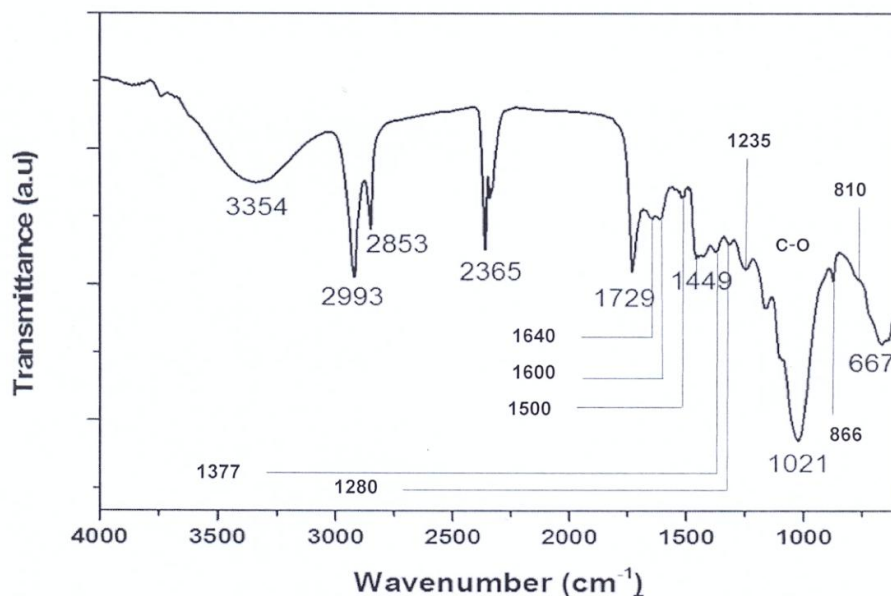
Fig. 8. Fourier transform infrared spectroscopy (FTIR) chromatogram of the aqueous extract of *Mentha suaveolens Ehrh* (AEMS)

Table 4. Main bands in aqueous extract of *Mentha suaveolens Ehrh* FTIR spectra (ν_{as}, ν: asymmetric and symmetric stretching vibration mode; $\delta_{as} + \delta_s$: asymmetric and symmetric bending vibration; δ_{oop}: out of plane bending vibration)

Wavenumber (cm-1)	Band assignments
3750-3100	νOH (phenolic cinnamate derivatives and quinic acid)
3000-2850	νC-H: νas+νs (CH2 + CH3+ CH)
2953; 2853	νasCH2 in pyranose + νas+s CH3 of methoxyl group branched to aromatic
1729	νC=O: conjugate carbonyl ester group, carboxyl groups associated,
1640	νC=C diconjugated
1600, 1500	νCar=Car of aromatic cinnamate derivatives (caffeoyl, rosmarinyl and isoferulyl)
1449	δasCH3 of methoxyl branched to aromatic + δasCH2 for pyranose + δO-H of carbohydrate
1377	δsCH3 of methoxyl; δO-H of carboxyl group
1270	νCar-O of aromatic ferulyl skeleton unit branched to CH3O- + νC-O of carboxyl group
1223-1235	νCar-O of aromatic syringyl skeleton unit branched to alkoxyl groups; δO-H in COOH groups
1156	νasC-O-C: bridge of β-(1-4)-glycosidic linkage or νC-O-C of carbohydrates
1200-920	ν asC-O of secondary and tertiary alcohols glycosidic ring + ν C-O-C of carbohydrates + C-O- ether of CH3-O- ether group in isoferulate ; -CH-O- ester of alkoxyl group of β-O-4 linkages link between cinnamate derivatives and quinic acid
860, 810	δ oopC-Har of aromatic unit 1,2,4-trisubstituted : caffeoylate, rosmarinate, isoferulate
760-400	δoopO-H of quinic acid carbohydrate I_α allomorph in crystalline cellulose I

These results were comparable to those observed in the control groups treating with loperamide, which is one of the most efficient and widely used antidiarrheal drugs [30, 31, 32], at the concentrations of 5mg/kg and 10mg/kg. Loperamide was described to regulate the gastrointestinal tract, to reduce transit in the intestine, and to decrease colon flow rate [33]. It's well reported that the therapeutic effect of loperamide is to be due to its antimotility and antisecretory properties [34]. Since AEMS inhibited the diarrheal effect of castor oil, markedly decreased propulsion of charcoal meal through the gastrointestinal tract and reduce of the volume and weight of the intestinal contents in rats as well, it can be assumed that our plant extract possesses significant antidiarrheal property that may be due to the existence of antidiarrheal components in *Mentha suaveolens Ehrh* (Table 3).

The *in vitro* experiments were undertaken in order to see whether AEMS possesses myorelaxant and spasmolytic effect to confirm the traditional use of the extract against spasm seen in gastrointestinal disorders. As shown, in results section, AEMS inhibited significantly and in a concentration-dependent way, the contractions induced by high KCl (100 mM) and Ach (10^{-5}M) (Fig. 2).

The contractions of smooth muscle preparations are dependent upon an increase in the cytoplasmic Ca^{2+}, which activates the contractile elements [35]. The increase in intracellular Ca^{2+} is due to either influx via voltage dependent Ca^{2+} channels (VDCs) and to release from intracellular stores as the sarcoplasmic reticulum.

It's well documented also, that the substances which inhibit the contractions promoted by k^+ (100mM) are considered to be a calcium voltage channel blocker. Since, AEMS inhibited the contraction of the rabbit jejunum preparation induced by K^+ (100 mM) (Fig. 3); we may suggest that, this plant extract acts via the restriction of Ca^{2+}entry via voltage dependent channels.

In order to test, whether the spasmolytic effect is mediated through the blockade of Ca^{2+} influx, a high dose of K (100 mM) was used to depolarize the tissue. Addition of AEMS caused dependent inhibition of extracellular calcium influx when jejunum was precontracted by high K +, mimicking the action of verapamil, calcium-channel blocker, suggestion that the spasmolytic

effect of AEMS is mediated probably via the restriction of Ca^{2+} influx trough at least blockade of voltage-dependent Ca^{2+} channel (Fig. 3). This effect could explain also the antidiarrheal activity of AEMS since calcium channel antagonism is responsible at least in part for the antidiarrheal actions. These findings are similar with many previous studies [36-41] demonstrating that the spasmolytic effect and antidiarrheal activity of the plant extract were mediated through calcium antagonism. Furthermore, it's known that the nitric oxide plays key role in mediating noradrenergic smooth muscle relaxation through the gastrointestinal tract [42]. It is now widely accepted that NO acts via an increase of cellular concentration of cGMP [43] which activates in its turn a protein kinase G (PKG) responsible for the decrease in the intracellular calcium probably by increasing uptake of Ca^{2+} through activation of sarcoplasmic reticulum Ca^{2+} - ATPase (SERCA) [44]. Considering the fact that the spasmolytic effect produced by the cumulative concentrations of the AEMS on rabbit jejunum segments was affected by L-NAME (100 µM), a nitric oxide synthase inhibitor (Fig. 5), confirms the possible involvement of the activation of the NO pathway in the spasmolytic responses produced by AEMS under the current experimental conditions.

In order to investigate the probable contribution of the cGMP in the potential spasmolytic effect of AEMS, the rabbit jejunum preparations were challenged with Ach (10^{-5}M) in the presence of the methylene blue (10 µM), a non-specific cGMP inhibitor. The response to the lower AEMS concentrations (0, 3 mg /ml, 1mg/ml) was significantly depressed but the curve was similar to that of control when using the highest concentrations and thus we think that cGMP pathway is partially implicated in the spasmolytic effect of AEMS (Fig. 5). Our data are similar with many others studies, confirming the implication of NO/cGMP pathway in spasmolytic effect of plant products reported [45-47].

In this study, the antioxidant activity of the AEMS was also explored using DPPH and FRAP and the results are illustrated in (Fig. 6 and Fig. 7). The obtained results exhibited a significant dose-dependent inhibition of DPPH activity, with IC50 of 0.1447 ± 0.031 mg/ml, 0.0525 ± 0.028 mg/ml mg/ml and 0.164 ± 0.085 mg/ml respectively for AEMS and positive controls ascorbic acid and BHT respectively. It is worth to mention that the antioxidant potential of AEMS is lower than the antioxidant activity of ascorbic acid but higher than that observed with BHT.

When using the FRAP reduction power, AEMS was found to be a strong antioxidant plant extract. EAMS antioxidant potency increased gradually with the rise of the concentration of the extract ranging from 0.05, 0.125, 0.25, 0.5 and 1 mg/ml. Indeed, the reducing power of AEMS is similar to that obtained with the standards antioxidant namely ascorbic acid and BHT tested under the same conditions.

The FTIR analysis of AEMS shows the presence of large and strong absorption band between 3750-3100 cm^{-1} centered at 3354 cm^{-1} which is assigned to stretching vibration of OH (*inter-* and *intra*molecular bonding) related to both phenolic units and carbohydrates (sugars, quinic acid) components [48-50].

The polar fraction of carbohydrate and/or quinic acid is confirmed by the fingerprint absorption region of 1200-920 cm^{-1} centered at 1021 cm^{-1}, illustrating the presence of both C-O and C-O-C stretching vibrations of alcohol (secondary, tertiary) and ether groups contained in sugar structure and quinic acid cycle [51]. The slight inclination signal of the OH acidic stretching vibration in the IR spectral region between 2500-3700 cm^{-1} accompanied by an intense stretching vibration band of associated polar carbonyl acid group (C=O) at 1729 cm^{-1} could be related to quinic acid [52,53,48].

The aromatic fraction containing phenolic groups (genin or heteroside) is confirmed by two weak and polar peaks at 1600 and 1500 cm^{-1} ascribed to $C_{ar}=C_{ar}$ of polar aromatic compounds, such as phenol, hydroxy-phenol or hydroxy-cinnamyl derivatives (*p*-coumaryl, guaiacyl (ferulyl), caffeoyl, rosmarinyl, sinapyl…) [54,55,51]. Moreover, the 1,2,4-trisubstituted aromatic ring is confirmed by the presence of the two weak out of plane bending vibration at 866 and 810 cm^{-1} correlated to $=C-H_{ar}$ and considered as a fingerprint informing on this degree of substitution (case of: coniferyl (guaiacyl), caffeoyl, rosmarinyl [52,53,49].

The two separated signals in the range of 3000-2850 are attributed to primary $v_{as}CH_3 = 2993$ cm^{1} of methoxyl group (CH_3O-) branched to aromatic ring in cinnamate alkyl derivatives, and to secondary $v_sCH_2 = 2853$ cm^{-1} related to quinic acid cycle.

It should be noted that the IR spectrum profile shows the predominance of polar compounds correlated to sugar heterocyclic and/or quinic acid over those derived from polyphenolic nuclei.

The advanced suggestions are justified and consolidated by the presence of intense peaks related to carbohydrates and/or quinic acid (OH: 3700-3100 cm^{-1}, CH_2 and CH: 3000-2800 cm^{-1}, C=O quinic acid: 1729 cm^{-1}, C-O and C-O-C: 1200-920 cm^{-1}) which are more accentuated when compared to hydroxycinnamyle derivatives. The presence of polar phenolic ring is well manifested by the IR large and weak peaks of aromatic $C_{ar}=C_{ar}$ instead of fine ones (caffeoyl, rosmarinyl, isoferulyl).

Many studies have shown that polyphenols and particularly the flavonoids found in medicinal plant extracts are responsible for many biological effects including the antidiarrheal, antispasmodic and antioxidant [55-57]. Our results (data not shown) confirm the presence of polyphenols and flavonoids in AEMS since this later contains respectively 83.46 mg/g of the extract and 78.15 mg/g of the extract. Therefore; we strongly suggest that antidiarrheal, antispasmodic and antioxidant effects of AEMS are due at least to the presence of flavonoids and others polyphenols.

5. CONCLUSION

Overall findings indicated that AEMS possesses a remarkable antidiarrhoeal, spasmolytic, and antioxidant activities. Taking into account the antidiarrheal results in Wistar rats, we think that AEMS exerted its antidiarrheal action via antisecretory mechanism and via increasing the absorption of water and electrolyte from the gastrointestinal tract. Based on the antispasmodic data obtained, we suggest that the spasmolytic effect of AEMS may be mediated through Ca2+ voltage channel blockade, and through the NO/cGMP pathway activation. The antioxidant results indicated clearly that AEMS has a remarkable and dose-dependent antioxidant potency when tested using DPPH radical-scavenging and FRAP. The FTIR analysis revealed that Carbonyl, ester, alcohol and carbohydrates were found to be the main functional groups in *Mentha suaveolens Ehrh*. Our data provided sound pharmacological basis for the traditional use of AEMS in Morocco as a remedy for gastrointestinal diseases and thus demonstrated that AEMS could be possible source for future novel antidiarrheal, antispasmodic and antioxidant agents.

DISCLAIMER

Authors have declared that no competing interests exist. The products used for this

research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

CONSENT

It is not applicable.

ETHICAL APPROVAL

This study was carried out in accordance with the guideline of the Institutional Animal Care and Use Committee at Faculty of Sciences and Technology (FST), USMBA (N°. FST-IACUC-2017-2).

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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Peer-review history:
The peer review history for this paper can be accessed here:
http://www.sdiarticle4.com/review-history/63897