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Research Article

ANTIMOTOLITY EFFECT OF ORGANIC EXTRACTS OF *ANTHEMIS MAURITIANA* MAIRE AND SENNEN FLOWERS ON THE RODENT ISOLATED JEJUNUM

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ABSTRACT

Objective: The aim of the present study was to investigate the effect of the organic extracts of *Anthemis mauritiana* (AM) Maire and Sennen (Asteraceae) flowers on the contractile responses of the rabbit and rat jejunums and its possible mechanisms.

Methods: Soxhlet extraction with different successive solvents (petroleum ether, dichloromethane, ethyl acetate, and methanol) were prepared and evaluated by preliminary phytochemical test. Antispasmodic effects of the extracts were evaluated against spontaneous contractions of rabbit and rat jejunum contracted by carbachol and high K^+ concentration.

Results: The phytochemical screening analysis revealed that the extracts of AM flowers contain flavonoids and tannins with variable quantities. Petroleum ether and dichloromethane extracts showed the most relaxant effects in the rabbit jejunum at concentrations with IC_{50} value of 9.31±2.65 µg/ml and 25.04±5.87 µg/ml respectively. Ethyl acetate showed a total inhibition at 1 mg/ml, but the methanol extract at 5 mg/ml. In contrast, the residual aqueous extract was a reverse activity. And also all extracts caused an inhibitory effect on both K^+ (75 mM) and CCh (10^{-6} M)induced contractions in the rat jejunum except aqueous fraction. The extracts that have antispasmodic activity induced a marked depression on cumulative concentration-response curve for carbachol and $CaCl_2$.

Conclusions: The results indicate that the antispasmodic effect decreased with the polarity of the organic extracts, but the aqueous fraction had a reverse effect. These results explain and confirm the popular use of genus of this plant for the treatment of gastrointestinal disturbances, and encourage studies on new compounds, in order to obtain new antispasmodic agents.

Keywords: *Anthemis mauritiana*, Organic extract, Antispasmodic, Smooth muscle.

INTRODUCTION

Many plants are used in folk medicine to treat gastrointestinal disorders, such as spasms or indigestion. The genus *Anthemis* (Asteraceae) is distributed by more than 210 species in the Mediterranean region, eastern Africa and southwest Asia [1,2]. Among these species, we are interested in *Anthemis mauritiana* (AM) Maire and Sennen, which is an endemic plant distributed in Morocco and Algeria. The species of this genus are widely used in the pharmaceutics, cosmetics, and food industry. Many compounds have been isolated from these species, including sesquiterpene lactones [3-7], flavonoids [8-11], acetylenes [12] and essential oils [13-16]. Furthermore, this genus, widely used in Mediterranean region in traditional medicine, have been reported to have anti-inflammatory, antioxidant, antibacterial, and spasmolytic effects [17,18]. We have shown that the crude extract and essential of AM flowers had antispasmodic activity [19,20]. Thus, in this report and in continuation of our previous studies we analyze the antispasmodic action of fractions (petroleum ether, dichloromethane, ethyl acetate, methanol, and water) of this latest flowers plant to evaluate in more detail its antispasmodic effects.

METHODS

Plant material

The AM Maire and Sennen flowers were collected during the flowering period in May 2012, from North eastern area of Morocco; the plant was identified by Professor B. Haloui at the department of Biology, Faculty of Science University Mohammed I Oujda, Morocco. A voucher specimen (N° 64666) was previously deposited in Scientific Institute of Rabat.

Preparation of extracts

Soxhlet extraction

AM extract flowers were dried at room temperature and ground to a fine powder; samples (100 g) of powder were heated to reflux temperature (Soxhlet extractor) with 1.5 L solvents of increasing polarity (petroleum ether, dichloromethane, ethyl acetate, methanol, and water). The solvents were removed under reduced pressure using a rotatory evaporator to constant weight. The percentage yields obtained for the extracts were 5.85%, 5.64%, 2.03%, 9.75%, and 3.65%, respectively. All extracts were solubilized in dimethylsulfoxide (DMSO) (1%) for use in experiments except methanol and water extracts that were solubilized in distilled water.

Phytochemical analysis

Phytochemical screening

Preliminary phytochemical analysis was carried out for the presence of flavonoids, and tannins. Extracts plant were tested as positive for flavonoids when they gave yellow color when treated with $AlCl_3$ reagent 1% and for tannins when green or black color was produced with $FeCl_3$.

Flavonoids

A volume of 200 µl of diluted organic or aqueous extract were introduced into a test tube then pure ethanol till 2 ml were added, then 500 ml of $AlCl_3$ 1% were added. In the presence of flavonoids the solution gave yellow.

Tannins

Same procedure for flavonoids revelation, except that here we have added $FeCl_3$ (1%).

Quantification of total phenols contents

To measure the total amount of polyphenolic compounds, 0.25 ml of Folin–Ciocalteu Reagent was added to the extracts solution 0.5 ml and 1.25 ml of Na_2CO_3 (20%) was added [21]. Subsequently, the mixture was vortexed and taked in dark for 40 min at room temperature and then absorbance was noted at 725 nm against a blank containing 0.5 ml of water or 4% DMSO in water. Polyphenolic content was expressed as mg of catechin equivalent/g of the dry plant extracts. All measurements were done in triplicate.

Dosage of tannins

Total tannins content was determined by the procedure of Folin–Ciocalteu previously described, after their adsorption onto bovine serum albumin (BSA/fraction V, ACROS, New Jersey, USA) [22]. 20 ml of each sample (20 mg/ml) were homogenized with 250 mg of BSA and the mixture was stirred for 30 minutes; the preparation obtained was stored for 2 hrs at +4°C. Then the pH was adjusted to 4.6 (pHi of BSA) by 1N HCl solution. After centrifugation at 4000 rpm/15 minutes, no adsorbed phenolics in the supernatant were determined by the Folin–Ciocalteu procedure, as described above. Calculated values were subtracted from total polyphenol contents, and the amount of total tannins expressed.

Dosage of flavonoids

Total flavonoids content was determined by the method described by Jay *et al.* [23]. Briefly, 2.5 ml of the extracts solution were added to 1.25 ml of $AlCl_3$ reagent (133 mg crystalline aluminum chloride and 400 mg crystalline sodium acetate were dissolved in 100 ml of extracting solvent). The mixture was vortexed and absorbance was read at 430 nm after 40 min of incubation in the dark against a blank (2.5 ml of the analyzed solution plus 1.25 ml of water). Flavonoids content was expressed as mg of rutin equivalent/g of the dry plant extracts. All measurements were done in triplicate and measured by using a spectrophotometer.

Spasmolytic study

The contractile motility effect of the extracts was studied using isolated Wistar rat and rabbit jejunum preparations. A portion of jejunum (2 cm) was removed and mounted in 10 ml organ baths containing Krebs's solution (KHB) with the following composition (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. The bath solution was maintained at 37°C, pH 7.4 and gassed continuously with air bubbling. The organic extracts were dissolved in DMSO (1%).

Rabbit jejunum exhibits spontaneous rhythmic contractions and allows tests, by adding the cumulative doses of extracts, for spasmolytic of the basal tonus activity directly.

In order to quantify the effect of organic extracts on KCl and carbachol induced rat jejunum contractions, the tissue preparations were exposed to these extracts cumulatively after they were contracted with the sub-maximal concentration of KCl (75 mM) and carbachol (CCh, 10^{-6} M).

To assess whether the spasmolytic activity of extracts was through calcium channel blockade (CCB), the jejunum was allowed to stabilize in normal KHB solution, which was then replaced with Ca^{2+}-free KHB's solution containing ethylenediaminetetraacetic acid (EDTA) (0.1 mM), used as a chelating agent, for 10 minutes to remove calcium from the rat jejunum and replaced with Ca^{2+} free rich K^+ (75 mM) KHB containing EDTA (0.1 mM), Ca^{2+} was added in a cumulative fashion to obtain dose-response curves to Ca^{2+} after the addition of the extracts as the test substance, or 1 µM verapamil as a positive control.

Also, CCh (10^{-8}-10^{-5} M) was added to the organ bath, and different doses of the extracts were added to the bath 5 minutes before commencing the dose-response curve of the agonist.

Statistical analysis

Data obtained were analyzed using the Student's *t*-test and a $p<0.05$ was considered to be statistically significant for all tests analysis and the 50% maximal inhibitory concentration value (IC_{50} value) was calculated with linear regression method. Our results were expressed as means ± standard error of the mean.

RESULTS

The phytochemical screening analysis revealed that the extracts of AM flowers contain flavonoids and tannins with variable quantities. It appears clearly that the methanolic extract contain more total phenolics compounds than the other extracts with 165.67±4.72 mg catechin equivalent/g dry extract. All organic extract are rich in flavonoids except the residual aqueous extract. The methanolic extract contains at least two times of tannins than ethyl acetate and residual aqueous extracts whereas petroleum ether, and dichloromethane extracts contain very few tannins (Table 1).

All organic extracts, petroleum ether, dichloromethane, ethyl acetate, and methanol, of AM flowers showed a tone inhibition of jejunum rabbit spontaneous contraction on a dose-dependent way with IC_{50} value 9.31±2.65 µg/ml, 25.04±5.87 µg/ml, 180±12.65 µg/ml, 1.64±0.31 mg/ml respectively, except residual aqueous extract that produced a dose-dependent spasmogenic effect. When tested against CCh (10^{-6}) and K^+ (75 mM) induced rat jejunum contractions, petroleum ether extract showed an inhibition with IC_{50} values of 4.60±0.52 and 3.91±0.37 µg/ml respectively (Fig. 1), dichloromethane extract with 3.16±0.27 and 2,12±0.17 µg/ml respectively (Fig. 2), ethyl acetate extract with 40.94±5.86, 49,59±3.78 µg/ml, respectively (Fig. 3), and methanolic extract with 200.54±1.47 and 99±0.41 µg/ml, respectively (Fig. 4). Organic extracts also caused a dose-dependent rightward shift in the $CaCl_2$ or CCh dose-response curves. Verapamil hydrochloride and papaverine hydrochloride also reduced the maximal response in curves induced by $CaCl_2$ and CCh, respectively (Table 2).

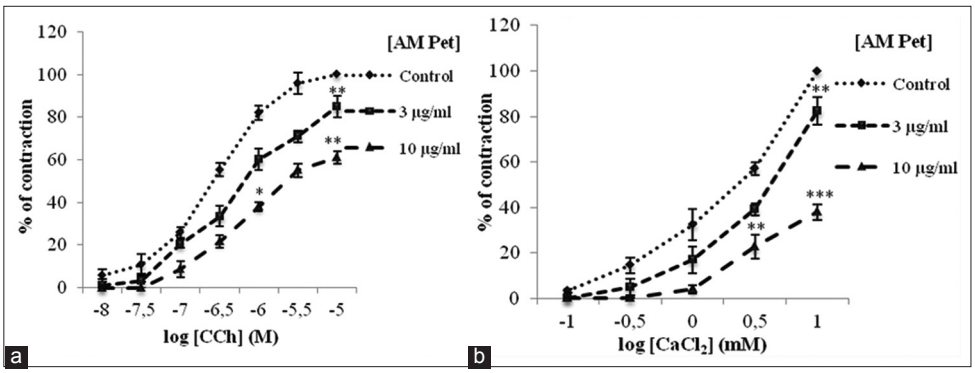

Fig. 1: Effect of *Anthemis mauritiana* petroleum ether fraction (AM Pet), on Curves cumulative dose-response of carbachol-induced contractions (CCh) (a), and $CaCl_2$ (b), in isolated rat jejunum preparations. *p<0.05, **p<0.01 and ***p<0.001 statistically significant difference from control (mean±standard error of mean; n=6)

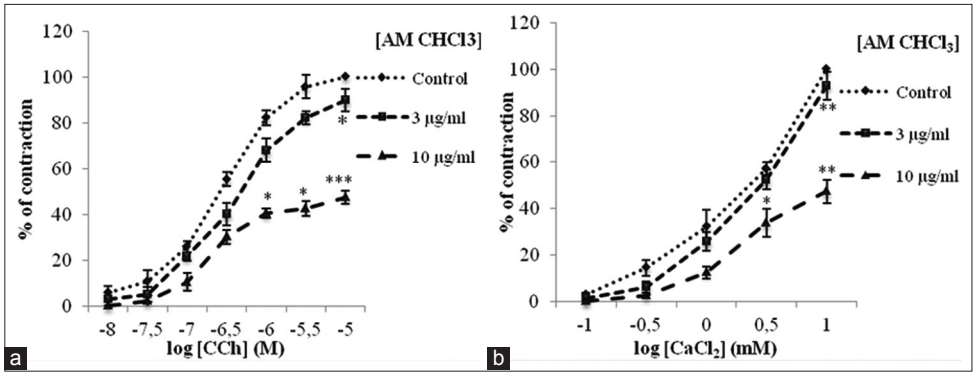

Fig. 2: Effect of *Anthemis mauritiana* dichloromethane fraction (AM $CHCl_3$), on Curves cumulative dose -response of carbachol-induced contractions (CCh) (a), and $CaCl_2$ (b), in isolated rat jejunum preparations. *p<0.05, **p<0.01 and ***p<0.001 statistically significant difference from control (mean±standard error of mean; n=6).

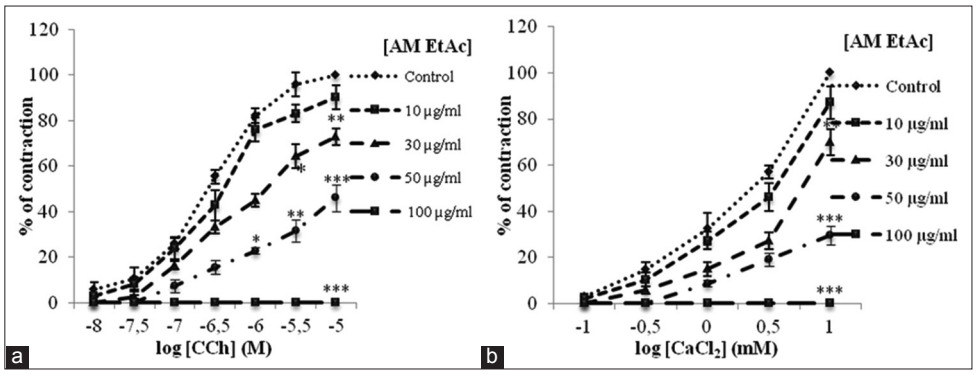

Fig. 3: Effect of *Anthemis mauritiana* ethyl acetate fraction (AM EtAc), on Curves cumulative dose -response of carbachol-induced contractions (CCh) (a), and $CaCl_2$ (b), in isolated rat jejunum preparations. *p<0.05, **p<0.01 and ***p<0.001 statistically significant difference from control (mean±SEM; n=6)

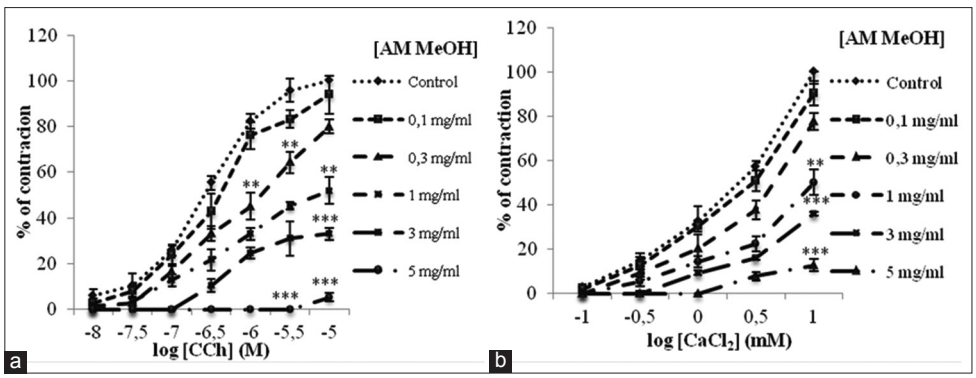

Fig. 4: Effect of *Anthemis mauritiana* methanol fraction (AM MeOH), on Curves cumulative dose-response of carbachol-induced contractions (CCh) (a), and $CaCl_2$ (b), in isolated rat jejunum preparations. * p< 0.05, ** p<0.01 and *** p<0.001 statistically significant difference from control (mean±standard error of mean; n=6).

DISCUSSION

The preliminary phytochemical analysis reveals that all extracts of AM flowers contain high phenol content. Petroleum ether and dichloromethane extracts contain a very low quantity of tannins, whereas ethyl acetate, methanolic and residual aqueous extract contain abundant tanins. All extracts are rich in flavonoids.

The present study demonstrated that all organic extracts prepared from the plant of AM flowers inhibited the spontaneous contractions of the rabbit jejunum when tested *in vitro*. These effects decreased with the polarity of the organic extracts, but the aqueous fraction had a reverse effect. The extracts also inhibited rat contractile stimuli of carbachol and K^+ rich medium. The contraction of smooth muscle preparations including rat and rabbit jejunums is dependent upon an increase in the cytoplasmic free [Ca^{2+}], which activates the contractile elements [24]. There is evidence that there is a significant variation in the degree of participation of extra- and intracellular Ca^{2+} in smooth muscle contraction. This increase is due to either influx *via* voltage dependent Ca^{2+} channels (VDCs) or the release from intracellular stores in the sarcoplasmic reticulum [24].

Relaxation of K^+ induced contraction had suggested a possible calcium channel blockade by the extracts. It was shown that a substance, which inhibits this contraction, is considered to act through blockade of Ca^{2+} VDCs [25,26]. The presence of calcium antagonist constituent(s)

Table 1: Polyphenol contents of AM Maire and Sennen extracts

Extracts	Total phenols[a]	Tanins[a]	Flavonoids[b]
Petroleum ether extract	61.04±0.42	0.085±0.01	55.79±2.10
Dichloromethane extract	64.27±0.33	0.27±0.09	59.47±3.68
Ethyl acetate extract	109.46±5.7	32.52±1.38	75.12±2.13
Methanolic extract	165.67±4.72	90.93±2,1	67.38±1.61
Residuel aqueous extract	66.47±2.18	41.13±0.83	16.75±5.91

Values are expressed as means±SEM of triplicates assays, [a]Expressed as mg catechin equivalent/g dry extract, [b]Expressed as mg rutin equivalent/g dry extract, SEM: Standard error of mean, AM: *Anthemis mauritiana*

Table 2: EC_{50} and maximum effect values obtained from the cumulative dose-response curves to CCh and $CaCl_2$ in rat jejunum

Antagonist (mg/ml)	CCh		$CaCl_2$	
	EC_{50} (M)	Emax±SEM	EC_{50} (M)	Emax±SEM
Control	4.92×10^{-6}	100	3.22×10^{-3}	100
Petroleum ether extract				
0.003	1.25×10^{-6}	85±4.87	2.91×10^{-3}	82,58±7.54
0.01	6.75×10^{-5}	61±2.9	6.65×10^{-2}	37.95±6.04
Dichloromethane extract				
0.003	4.11×10^{-6}	90.7±3.62	4.36×10^{-3}	92,87±3.21
0.01	2.81×10^{-5}	47.51±5.17	0.84×10^{-3}	47.32±2.86
Ethyl acetate extract				
0.01	5.28×10^{-6}	90.23±7.98	2.43×10^{-3}	87.10±5.12
0.03	8.11×10^{-5}	72.98±2.1	1.32×10^{-2}	69.98±3.54
0.05	1.22×10^{-5}	45.98±6.32	1.54×10^{-1}	29.54±8.66
0.1	0	0	0	0
Methanolic extract				
0.1	2.95×10^{-6}	93,91±1.32	5.52×10^{-3}	90,31±4.28
0.3	1.37×10^{-6}	80,02±2.87	2.14×10^{-3}	77,63±4.13
1	5.79×10^{-5}	51,98±6.19	0.83×10^{-3}	50,1±6.43
2	0.2×10^{-5}	33,1±3.4	4.86×10^{-2}	35,98±1.8
3	0.41×10^{-4}	5,36±2.7	7.13×10^{-1}	12,65±5.4
Papaverine (10^{-5} M)	2.63×10^{-5}	55.9±2.06**	-	-
Verapamil (10^{-6} M)	-	-	1.13×10^{-2}	46.6±4,2**

Number of experiments n=6, *p<0.05, **p<0.01 statistically significant difference from control

was confirmed when our organic extracts caused a rightward shift in the Ca^{2+} dose-response curves, similar to that caused by verapamil, a standard calcium-channel blocker [25,26].

Carbachol, a cholinomemetic drug, interacts with muscarinic receptors on intestinal smooth muscle cell membranes [27]. The advantage of using this drug is that it is not hydrolyzed by cholinesterase like acetylcholine. On the rodent intestine, Ach induced contractions involve two different mechanisms coupled to muscarinic receptors (M2 and M3). One is the activation of the release of calcium from the sarcoplasmic reticulum. The other mechanisms are activation of non-selective cation channels in the plasma membrane, which results in membrane depolarization [25,26]. This last event stimulates Ca^{2+} influx through voltage-gated Ca^{2+}-channels [25,26]. The Current study demonstrates that the organic extracts of AM had a relaxant effect on rabbit jejunum contractions and antagonized the spasmogen effect of Carbachol on rat jejunum. The muscarinic receptors blocking activity was confirmed when pre-treatment of the tissue with extracts produced a dose-dependent shift in the carbachol dose-response curves to the right, similar to that produced by papaverine that was used as a positive control to the study [28].

The flavonoids are well-known for their antispasmodic activity [29] and the tannins are known to have a beneficial role in diarrhea [30]. The presence of flavonoids and tannins in the organic extracts of AM organic extracts may take part in relaxant effect of the rodent jejunum via the muscarinic receptors or the VDCs. Since these effects decreased with the polarity of the organic extracts, there are probably apolar and polar substances, which act in the same or in the different ways.

CONCLUSIONS

As a conclusion, our results support that the extracts exhibit antispasmodic activity on rabbit and rat jejunums probably by inhibiting calcium influx. Therefore, it is possible that the extracts contain some apolar and polar compounds, in particular, flavonoids and tannins, which, interfere with the calcium channels activity. Further studies must be conducted in order to clarify, which constituent of the extract is responsible for this activity. Phytochemical studies are currently in progress, to elucidate the active principles of AM and its relevant mechanism of action.

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