SPASMOLYTIC EFFECTS OF EXTRACTS FROM *KALANCHOE CRENATA* ANDREWS (CRASSULACEAE) LEAVES

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Summary

Organic extracts from the leaves of *Kalanchoe crenata* have been shown to possess inhibitory effects against abdominal pain that can be induced by visceral spasm. The present work examined the spasmolytic effects of extracts from the leaves of *K. crenata* on the rat and guinea pig isolated ileum. Methanol/methylene chloride extract obtained from dry leaves of *K. crenata* was suspended in distilled water and successively extracted in hexane, methylene chloride, ethyl acetate and n-butanol. The spasmolytic effects of the resulting extracts were tested on the spontaneous contractions and contractions induced by KCl, carbachol and histamine. The effects of two possible antagonists, propranolol (3 µM) and prazosin (1 µM), on the relaxant effects of the n-butanol extract were investigated. The extracts reduced in concentration-dependant manner rat ileum spontaneous contraction and contraction induced by KCl, the n-butanol extract been the most active. The relaxant effect of the n-butanol extract was significantly antagonised by prazosin (40%). This extract at the concentration of 300 µg/ml significantly inhibited response to carbachol by 66.8%. At the same concentration, the extract totally inhibited histamine induced contraction. Both inhibitions were non-competitive. When essayed on the phasic contraction induced by KCl, the extract at the concentration of 300µg/ml induced an inhibition of 75.56 %. These data suggest that *K. crenata* extracts possess spasmolytic effects on the intestinal smooth muscle, which may account for their analgesic activities. The n-butanol extract may interfere with the calcium metabolism in the smooth muscle cells.

Key words: *Kalanchoe crenata*, ileum, spasmolytic, rat, guinea pig

Introduction

Kalanchoe crenata (Adrews) Haworth is an ornamental plant belonging to the family Crassulaceae. Commonly known as "never die" or "Dog's liver", this plant is widely used in traditional medicine in the treatment of inflammation, earache, headache, asthma, palpitation, abdominal pain, convulsion and general debility (1, 2). Phytochemical investigations have reported the presence of alkaloids and saponins in the aqueous and alcohol extracts of *K. crenata* leaves (1) and lectins in the juice from fresh leaves (3). Recent works carried out in our laboratory have shown the analgesic properties of the aqueous and ethanolic extracts (4) as well as analgesic and anticonvulsant properties of organic extracts from the leaves of *K. crenata* (5).

Considering the fact that some drug that inhibited pain such as opioids are spasmolytic (6) and that abdominal pain can be induced by visceral spasm, in consistence with the intensive use of this plant against abdominal pain, it can be thought that *K. crenata* possesses spasmolytic properties.

The present work was undertaken to evaluate the spasmolytic properties of the hexane, ethyl acetate, methylene chloride and n-butanol fractions of the methanol/methylene chloride extract of the leaves of *Kalanchoe crenata* on the rat and guinea pig intestinal muscle.

Materials and methods

Animals

Rats and guinea pigs of either sex, aged between 10 and 16 weeks obtained from the animal house of the Institute of Pharmacology and Toxicology of the Technical University of Munich were used in this study. The animals were housed in colony cages and have free access to food and water.

Preparation of the plant extracts

Fresh leaves of *K. crenata* were harvested in Dschang (West Cameroon) in September 2001 and authenticated at the National Herbarium Yaounde in Cameroon where a voucher specimen has been deposited under number 50103/YA. The powder (780 g) obtained from the sun dried and grounded leaves was macerated in a mixture of methanol/methylene chloride (1:1) for 72 h with occasional stir. The mixture was filtrated and the filtrate was concentrated to dry to give 110.8 g of extract. 104.8 g of this extract were suspended in distilled water and successively extracted with hexane, methylene chloride, ethyl acetate and n-butanol. Organic extracts were concentrated using rotary evaporator while aqueous residue was evaporated in an oven at 55° C. The following fractions were obtained: hexane (32.8 g); methylene chloride (2.8 g); ethyl acetate (10.2 g); n-butanol (22.38 g) and aqueous residue (11.8 g). The solutions of the ethyl acetate and hexane fractions were prepared in DMSO. The n-butanol and methylene chloride fractions were dissolved in the mixture containing DMSO and Tween 20 while the aqueous residue was prepared in the distilled water.

Phytochemical screening

The total extract as well as its fractions were used to perform a comparative thin layer chromatography (TLC). These TLC were observed under UV (254 nm and 365 nm) and after sprayed with anisaldehyde sulphuric acid reagent in order to determine the presence of bufadienolides (Wagner and Bladt, 1996).

All the extracts were also submitted to the Liberman Buchard, ferric chloride, copo of magnesium and Vanillin-sulphuric acid tests in the goal to determine the presence of sterols, phenolic compounds, flavonoids and saponins respectively.

Ileum Preparation

Rats were killed by over dose of ether while guinea pigs were killed by cervical dislocation. Ileum was isolated, cleaned of connective and cut in strips of about 1.5 cm long. Strips were suspended vertically in 50 ml organ bath chamber containing a modified Krebs-Henseleit solution of the following composition (mM): NaCl 114.9; KCl 1.22; $CaCl_2$ 3.2; $NaHCO_3$ 24.9; KH_2PO_4 1.18; $MgCl_2$ 1.18; glucose 11. The physiological solution was maintained at 36° C and continuous gassed with 95% O_2 and 5% CO_2. Before starting the experiments, the ileum strips were equilibrated for 45 min under a resting tension of 1g. During this period, the solution was changed every 15 min. Muscle contraction were measured using isometric transducer (Fa. Hottiger Baldwin Messtechnik, Darmstadt, Germany)

Pharmacological studies

Relaxing effects

This experiment was performed on rat ileum strips. After the equilibration period, contraction were recorded for 10 min and used as 100% initial spontaneous contractions. Vehicle or cumulative concentrations of *K. crenata* extracts (10 – 300 µg/ml) were then added in the incubation medium. In another serie of experiment, the ileum was precontrated with KCl (60 mM). After the contraction has been steady for at least 3 min, the amplitude was consider as 100% initial contration. Extracts (10 – 1000 µg/ml) or nifedipine (10 – 1000 nM) concentrations were added cumulatively in the organ bath.

The effect of each extract or nifedipine concentration was observed for 5 min. The amplitude of contraction at the end of the effect of each concentration was expressed as the percentage of initial amplitude.

After these series of experiments, the n-butanol extract was chosen for further studies. The relaxing effects of the n-butanol extract on KCl – contracted ileum were evaluated in the absence and in the presence of propranolol (0.3 µM, a non selective β bloker) and prazosin (1µM, an α_1 antagonist). After the contraction has reached the steady state, the antagonist substance was added in the medium and observed for 10 min before the extract was injected. Contractions that relaxed during this period for more than 10% were discarded.

Inhibitory effects

Rat's ileum was equilibrated and contracted with carbachol (10 µM). The organ was washed two times in interval of 10 min. Cumulative concentration-effect curve of carbachol (0.1 – 10 µM) were constructed after 5 min incubation with n-butanol extract (30 and 300 µg/ml) or vehicle. The same experiment was performed on guinea pig ileum with histamine (5 nM – 50 µM) after a reference contraction induced by 1µM of histamine. Pyrilamine maleate (10 and 100 nM) was used as reference drug. The effect of each agonist concentration was expressed as a percentage of a reference contraction.

The effect of the n-butanol extract was also evaluated on the phasic contraction induced by KCl. The organ was contracted with 60 mM KCl, washed and equilibrated for 15 min. After this period, the organ was incubated for 5 min in n-butanol extract (30 and 300 µg/ml) or nifedipine (0.3 µM) and further contracted with KCl (60 mM). The amplitude of this second contraction was expressed as a percentage of initial contraction.

Statistical analysis

Data are expressed as mean ± SEM. Significant differences among the mean values of multiple groups were evaluated by ANOVA repeated-measures followed by Bonferroni as post hoc test using Graph Pad Instant Biostatistic version 3.0. EC_{50} was calculated using Graph Pad Prism version 3.0.

Results

Phytochemical screening

From the different TLC, it was observed that none of the compounds present in the *K. crenata* extracts was common to all the fractions. The phytochemical screening revealed the presence of sterols in the total extract and in the hexane fraction. A bufadienolide compound was detected in the total extract and in the hexane and methylene chloride fractions, with a higher concentration in methylene chloride fraction. Sterols, flavonoids and saponins were found in the methylene chloride and ethyl acetate fractions. Phenolic compounds, flavonoids and saponins have been detected in total extract and in the n-butanol fraction.

Effects of *K. crenata* extracts on the spontaneous contraction

The *K. crenata* extracts showed different ranges of concentration-dependent inhibition of the amplitude of spontaneous contractions of the rat ileum. The aqueous residue presented a biphasic response, with contracting effect at lower concentrations (10 and 30 µg/ml) and relaxing effect (EC_{50} = 394.8 µg/ml) at higher concentrations. The n-butanol extract proved to be the most active fraction with an EC_{50} value of 5.1 µg/ml (figure 1).

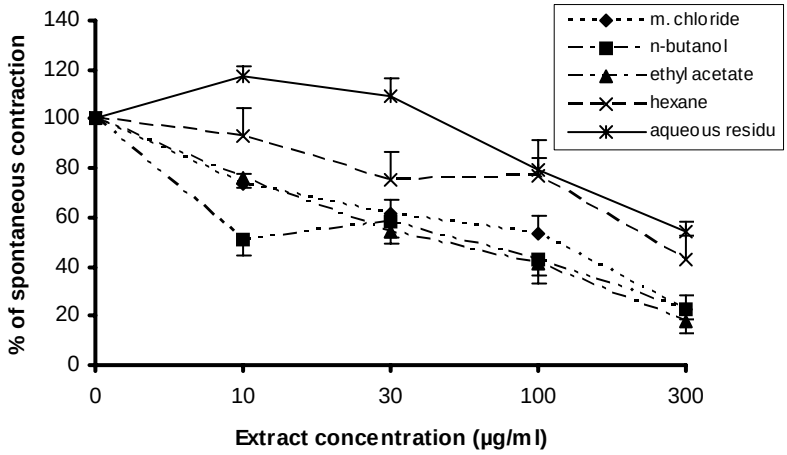

Figure 1: Inhibitory effects of the *Kalanchoe crenata* extracts on the spontaneous contractions of rat ileum (n=6)

Effect of extracts on evoked contractions of the isolated ileum

The relaxing effects of methylene chloride, hexane, n-butanol and ethyl acetate fractions were tested on KCl-contracted ileum. All the extracts relaxed this contraction in concentration-dependent manner. The n-butanol fraction exhibited the most important activity with an EC_{50} value of 30.15 µg/ml while nifedipine, used as reference drug, showed an EC_{50} of 8.05 nM (table 1 and figure 2).

The relaxing effect of n-butanol fraction was significantly inhibited by prazosin. In the presence of 1µM of prasozin, the relaxant activities induced by 30 and 1000µg/ml of extract were reduced by 40%. Propranolol did not significantly affect the extract activity (figure 3).

Table 1: Relaxing effects of extracts from *Kalanchoe crenata* leaves on the rat ileum contracted with KCl (60 mM).

Extract	Emax (% relaxation)	EC_{50} (µg/mL)
Methylene chloride	125.93 ± 6.10	34.27 (11.02 – 106.60)
n-butanol	124.95 ± 3.91	30.15 (19.64 – 46.60)
Hexane	101.88 ± 5.98	40.87 (9.92 – 168.4)
Ethyl acetate	136.04 ± 8.17	260.21 (15.98 – 458.30)

n=6; Values in brackets represent the 95% confident intervals.

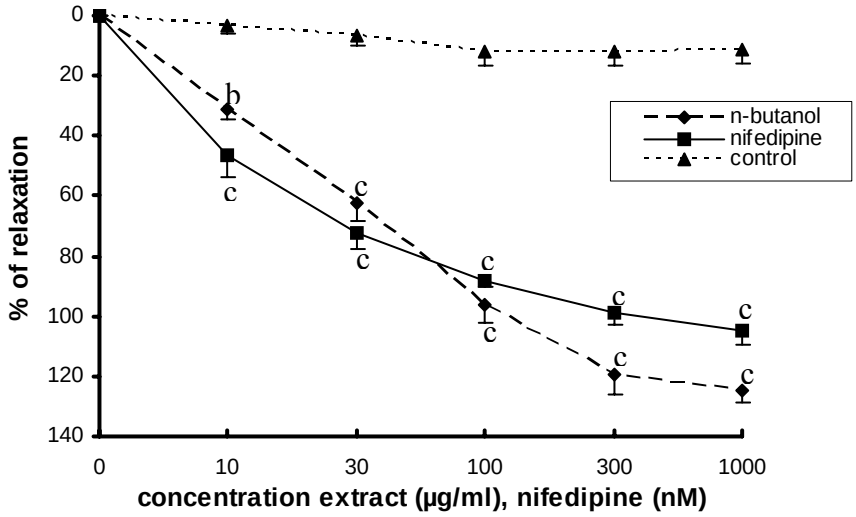

Figure 2: Relaxant effects of *kalanchoe crenata* n-butanol extract and nifedipine on the rat ileum contracted with KCl (60 mM) (n=6)
[b]p<0.01; [c]p<0.001 significantly different compared to control

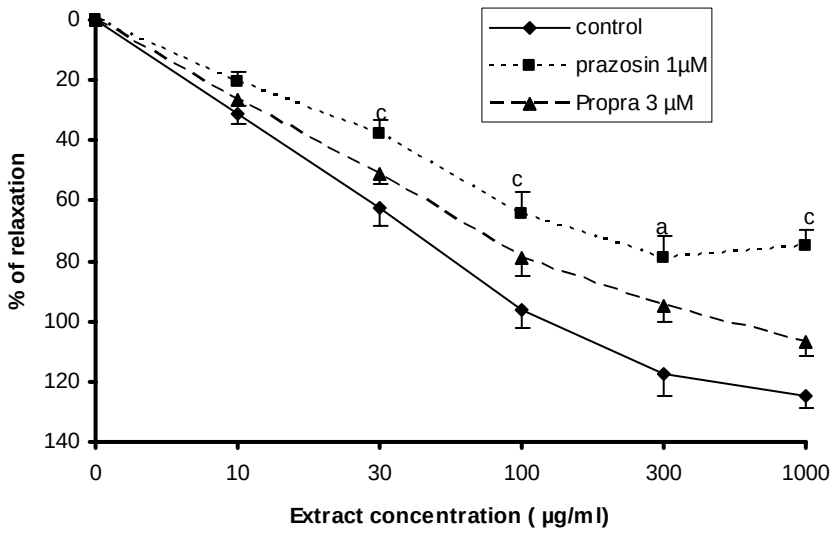

Figure 3: Effects of prazosin and propranalol on the relaxant activity of *Kalanchoe crenata* n-butanol extract on the KCl (60 mM) precontracted ileum (n=6)
[a]p< 0.05; [b]p< 0.01; [c]p< 0.001 significant different compared to control

In further studies, the inhibitory activities of the n-butanol extract were essayed on the cumulative contraction-response curve of carbachol on the rat ileum and histamine on guinea pig ileum. The extract significantly inhibited contractions induced by all the agonists. The extract, at the concentration of 300 µg/ml, inhibited the maximal carbachol-induced contraction by 66.8% (figure 4) and completely inhibited contraction induced by histamine (figure 5A). Pyrilamine maleate used as reference antagonist in the histamine-induced contraction, shifted the concentration-response curve of histamine to the right. The histamine EC_{50} increased from 217.1 nM to 2881 and 32816 in the presence of 10 nM and 100 nM of pyrilamine respectively (figure 5B).

n-butanol extract at 300 µg/ml reduced the phasic contraction induced by KCl from 127.5% to 31.16%, while nifedipine at the concentration of 300 nM reduced it till 19.8% (figure 6).

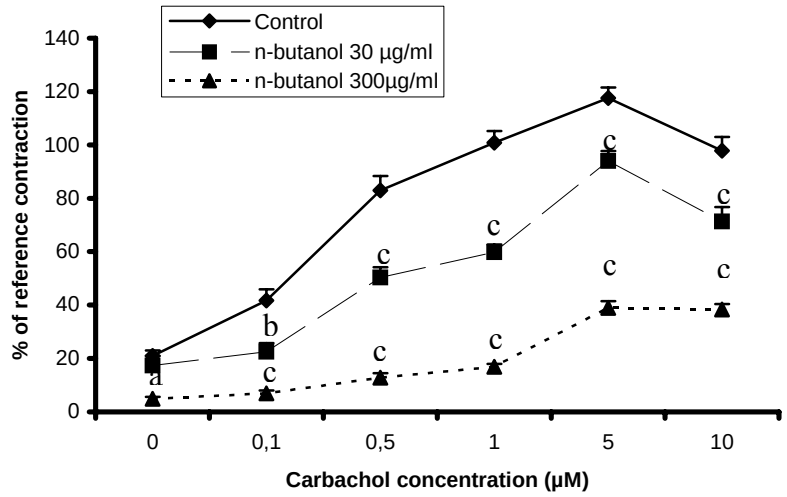

Figure 4: Effects of *Kalanchoe crenata* n-butanol extract on the cumulative concentration-effect curve of cabachol (n=6)
[a]p< 0.05; [b]p< 0.01; [c]p<0.001 significant different compared to control

Discussion

The distinctive finding in this study is that extracts from the leaves of *K. crenata* have a myorelaxant effects on isolated preparations of rat and guinea pig intestinal ileum.

It has been shown that activation of adrenergic receptors in ileal smooth muscle leads to relaxation (7). In order to assess if the extract relaxed intestine by binding on beta or alpha receptors, the relaxing effect of the extract was examined in the presence of propranolol or prazosin. Propranolol did not significantly affect the activity of the extract, suggesting that extract does not have any effect on beta adrenergic receptors. Prazosin significantly reduced the maximal relaxant effect of the extract, inferring that the extract may have an effect either on alpha adrenergic receptors or on the biochemical pathway of relaxation induced by the stimulation of these receptors. The stimulation of alpha adrenergic receptor results to an increase in cAMP intracellular concentration that induced relaxation (8). The fact that at the concentration 1µM prazosin exhibited only 40% of inhibition suggested that this may not be the main mechanism of action of the extract.

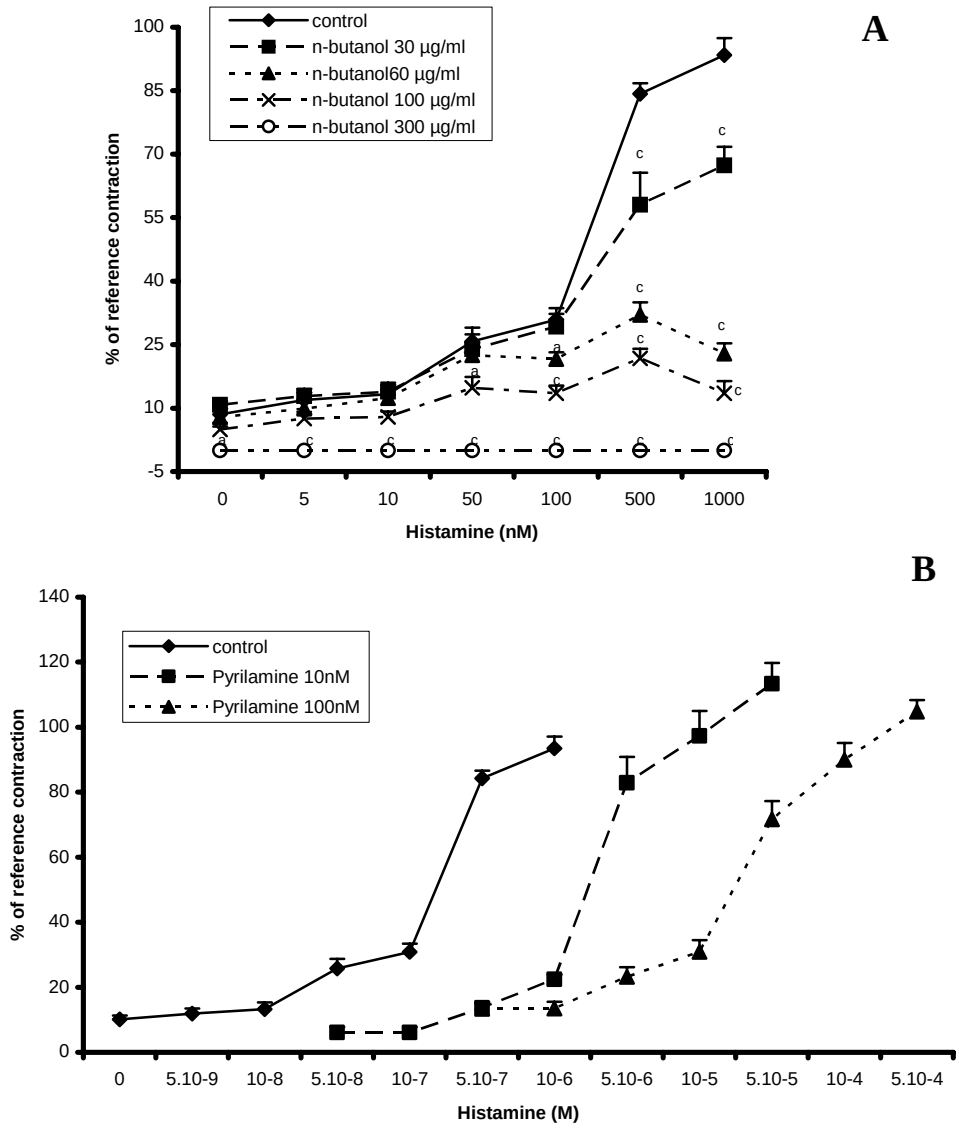

Figure 5: Effects of *Kalanchoe crenata* n-butanol extract (A) and pyrilamine maleate (B) on the histamine concentration-effect curve on the guinea pig ileum (n=6)
[a]$p<0.05$; [c]$p<0.001$ significantly different compared to control

Extracts significantly relaxed rat ileal spontaneous contractions which depend predominantly to acetylcholine (9). Acetylcholine activates ROCs largely by binding to muscarinic receptors. There are two well known mechanisms related to the muscarinic receptor stimulated smooth muscle contraction. One is the IP_3-induced Ca^{2+} release (10) and the other is the activation of non-selective cation channels (11), which would depolarise the membrane potential to activate the voltage-dependent Ca^{2+} channels. It could be possible that *K. crenata* extracts bind on muscarinic receptors or affect at least one of these mechanisms. To assess this hypothesis, the n-butanol extract which was the most active, was examined on the cumulative concentration-response curve of carbachol, a specific agonist of muscarinic receptors. This extract decreased the maximal response of contractions induced by carbachol and could not shift in parallel manner the concentration response curve of this

agonist. This suggests that the extract effect is not mediated directly trough muscarinic receptors binding.

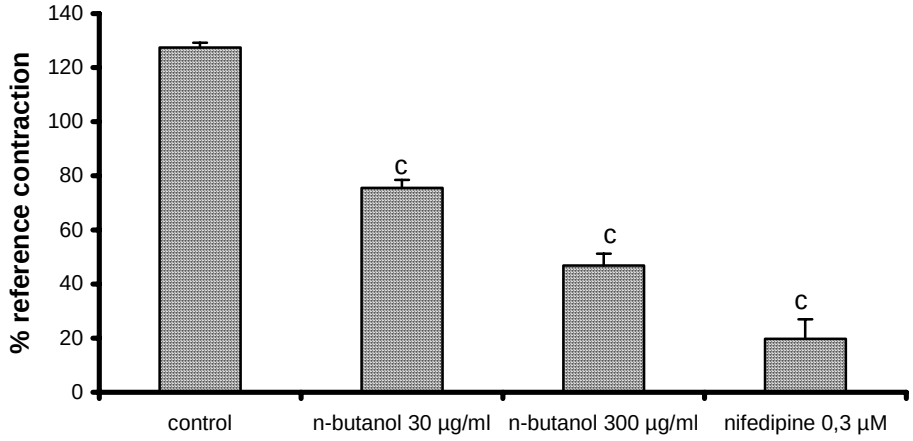

Figure 6: Effects of *Kalanchoe crenata* n-butanol extract and nifedipine on the phasic contraction induced by KCl (60 mM).(n=6)
[c]p< 0.001 significantly different compared to control

The effect of the extract was also examined on cumulative concentration-response curve of histamine on guinea pig ileum. This tissue was chose in regard to his high sensitivity to histamine. H_1 histamine receptors are known to mediate the contractile effects of histamine on the isolated guinea pig ileum (12). This receptor subtype signals through Gq to trigger phosphoinositide hydrolysis (13). Unlike pirylamine maleate, the extract exhibited a significant non-competitive inhibition. It can then be thought that the n-butanol extract from the leaves of *K. crenata* interfere with the IP_3 pathway. n-butanol extract from the leaves of *Bryophyllum calycinum* which belongs to the family of Crassulaceae have been shown to possess antihistanminic activity (14). This may be likely the case of the n-butanol extract from *K. crenata*, though the *Bryuphyllum calycinum* extract effect was showed to be competitive on H_1 receptors. Although it appears that the *K. crenata* extract does not bind to histamine receptors, the potent antagonist activity of the extract against histamine induced contraction may support the traditional use of *K. crenata* in the treatment of asthma.

Considering the fact that even acting trough receptors operated channels, the contraction induced by carbachol and histamine depend on the calcium influx and release from internal reserves, it is possible that calcium antagonists or calcium released inhibitors relaxed spontaneous contractions and contraction induced by receptors agonists.

The extract was then essayed on the phasic component of contraction induced by KCl. The phasic contraction is due to direct calcium influx trough L-type voltage dependant channels (15, 16). The n-butanol extract significantly inhibited the phasic contractions induced by KCl. These results suggested that the extract may have inhibitory effect on L-type voltage dependant calcium channels or reduces the sensitivity of contractile system to calcium.

The presence of flavonoids in the n-butanol extract of *K. crenata* may account for its anti-spasmodic activity. This group of secondary plant metabolites widely occurring in the vegetable kingdom have been shown to display a remarkable array of biochemical and pharmacological actions, including relaxing effects on intestinal smooth muscle (17, 18, 19).

In conclusion, extracts from *K. crenata* possess antispasmodic and spasmolytic activities on the rat and guinea pig ileum. The n-butanol extract showed the most important relaxing effect. This extract may interfere with calcium metabolism in intestinal smooth muscle cells. These antispasmodic and spasmolytic activities of *K. crenata* extracts may account for their analgesic activities.

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