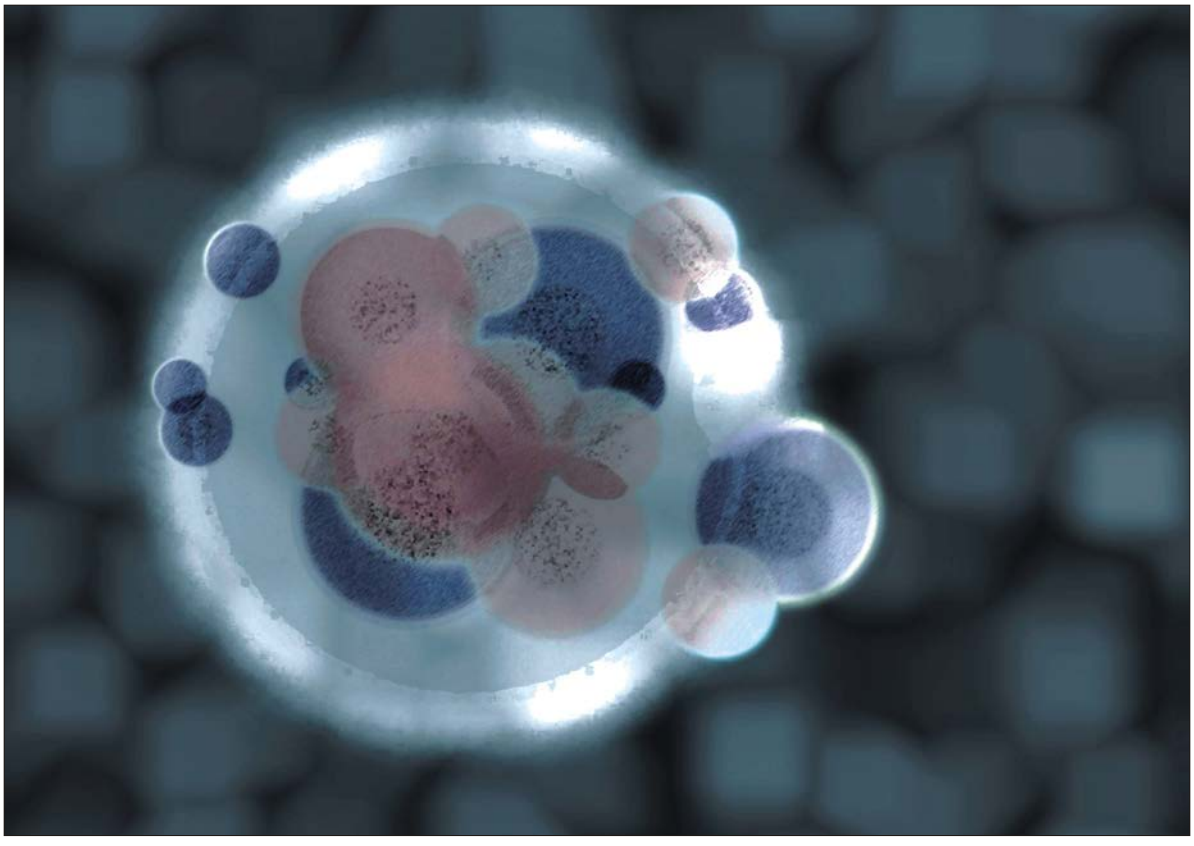

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Investigated Antispasmodic Effect of *Ruta chalepensis* Leaf on Rat's Ileum at Present of KCl and Different Concentrations of Calcium Chloride

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Abstract: In this study, the effect of *Ruta chalepensis* on rat's ileum contraction has been investigated because the hydroalcoholic extract of the leave of *Ruta chalepensis*, grown in Iran were investigated for pharmacological properties. *Ruta chalepensis* (Rue) is annual herb is used in the traditional medicine in Iran for intestinal disorder. In this study the effect of hydroalcoholic extract of Rue on isolated rat's ileum contraction was investigated by KCl (60 mM). A segment of ileum was removed and suspended in 10 mL organ bath containing aerated Tyrode solution (37°C, pH = 7.4).The responses were determined and recorded on isotonic transducer and Harvard Universal Oscillograph. The result demonstrate that the extract significantly reduced the ileum contraction induced by KCl (60 mM) in a dose dependent manner ($n = 7$, $p<0.001$). Also, adding extract before influence of KCl, cause reduce effect of KCl ($n = 7$, $p<0.001$). In another study, in high potassium Ca^{2+}-free tyrode solution, cumulative concentrations of $CaCl_2$, induced ileal contraction, however, the extract reduced these contractions in a dose dependently ($n = 7$, $p<0.001$). Therefore, the results suggest that relaxatory effect of rue on the ileum may be due to blocking of voltage gated calcium channels.

Key words: Antispasmodic, *Ruta chalepensis*, calcium channel, isotonic, ileum

INTRODUCTION

Ruta L. includes eight species pungently aromatic more or less woody perennial herbs (Huxley and Griffiths, 1999). Rue (*Ruta chalepensis*) belongs to the Rutaceae family. It grows in the north of Boshehr (south of Iran) Saudi Arabia and Mediterranean regions (Mozaffarian, 2004). A Subshrub, 20-60 cm, stem glabrous. Lower leaves long petiolate, two or three pinnatisect, ultimate segment 1.5-6 mm wide narrowly oblong-lanceolate or obovate (Huxley and Griffiths, 1999). Phytochemical screening of the aerial parts of the plant showed the presence of alkaloids, flavonoids, coumarins, tannins, volatile oil, sterols and/or triterpenes (Al-Sagair, 2004a). In folk medicine as an antirheumatic, an antispasmodic and a treatment for snakebites and headaches. A few of the coumarins from *R. chalepensis* exhibited antifertility effects and some quinoline alkaloids isolated from *Ruta* species displayed mutagenic, ganglionic-blocking, curare-like and spasmolytic activities (El Sayed *et al*., 2000). Other results indicate that rue inducing abortion (Ciganda and Laborde, 2003). Dried leaf infusions of rue, ruda, were found to cause perinatal changes in mice (Zeichen de Sa and Ray, 2000).The effects of an ethanol extract of the aerial parts of Rue on the Central Nervous System (CNS) induce a depressant activity on the CNS (Gonzalez-Trujano *et al*., 2006). Comparison inhibitory effect of hydroalcoholic extract of *Ruta chalepensis* in absent and presence of propranolol indicate that block of the β-adrenoceptors by propranolol significantly affect the relaxation response of Rue extract (Moazedi *et al*., 2008). Also reported that Rue extract reduced blood levels of nitrite (Iuak *et al*., 2004). *Ruta chalepensis* possesses normoglycemiant properties by raising serum circulating insulin levels through stimulation of insulin release from the pancreas without any counterregulatory factors and metabolic bias. It would be of great interest as a natural source for treatment of diabetes (Al-Sagair, 2004b). Chloride (Cl^-) secretion across intestinal epithelium plays an important role in regulating water secretion into intestinal lumen and is under close regulation of hormonal, neuronal and paracrine mediators. A disturbed regulation of Cl^- secretion can result in a change in the water balance and cause pathophysiological situations like diarrhea, in which excessive water secretion occurs (Oprins *et al*., 2001). In Iranian traditional medicine Rue

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use for the treatment of diarrhea. The aim of this study was to investigate the relaxation effect of leaf hydroalcoholic extract of Rue on isolated male Wistar rat's ileum contraction *in vitro* for examine its probability antispasmodic activity.

MATERIALS AND METHODS

Drugs and solution: The following drugs were used for the experiments: KCl (Merck), $CaCl_2$ (Merck), Rue(hydroalcoholic extract). Chloride potassium (KCl) was made up as 60 mM (Nasu *et al*., 1994) stock solution in Tyrode. Various concentration of $CaCl_2$ were made up as (0.225,0.45, 0.9, 1.8 and 3.6 mM) stock, dilution in Tyrode (without $CaCl_2$ and high concentration of K^+). Tyrode's solution composed of (mM): NaCl(139.9), KCl(2.68), $CaCl_2$ (1.8), $MgCl_2$ (1.05), $NaHCO_3$ (11.9), NaH_2PO_4 (0.42) and glucose (5.55), was made up in distilled water (Sadraie *et al*., 2003). All chemical were purchased from Merck.

Preparation of extract: This research has been done in April 2006 until May 2007. Aerial parts of *Ruta chalepensis* were collected in March from the north of Boshehr (South of Iran). The plants were identified by Boshehr's natural resource center. The leaves were powdered by electric grinder and powder was extracted by maceration using 70% ethanol for 72 h at room temperature. The mixture then filtered (Whatman No. 1) and the solvent evaporated at 30°C by blowing air to the extract. The extract was stored at 4°C until further use. All the concentrations are the final concentrations in the organ bath. The amount of total hydroalcoholic was 30%. From dried hydroalcoholic extract (starting with 10 g).

Experimental procedure: Male Wistar rats (200-250 g) breed were maintained in controlled conditions at 24°C, under a 12 h light/dark cycle and were given a standard diet and free access to water, however, food was withdrawn 24 h prior to experimentation. The terminal portion of the rat's ileum, the 8 cm portion nearest the caecum being discarded, was cleaned and transferred to the Tyrode solution. pH 7.4 and was aerated continuously and the organ bath were maintained a constant temperature of 37°C. Segments of ileum (2 cm) were placed directly in a 10 mL organ chamber. The preparations were given an initial load of 1 g and were equilibrated for 1 h before the experiments were initiated with a 15 min washout interval. The experiments were performed on three or four segments of ileum taken from each of seven animals. Segments that did not show spontaneous activity were discarded. At the beginning of each experiment, the tissue was contracted 2 times by using 60 mM KCl in each muscle bath, until a stable response was observed. The activity of the extracts was tested in isolated spontaneously contracting rat ileum. The inhibition of contractions in the test material was assessed as a percentage of the basal spontaneous contractions in the rat ileum. The spasmolytic effect was studied using potassium depolarized for adding K^+ 60 mM to the tissues. A concentration response was obtained by the cumulative concentration of extract (0.01, 0.02, 0.03, 0.04, 0.05, 0.06 and 0.07 mg mL^{-1}) after adding 60 mM KCl. The relaxation of the rat's ileum was expressed as a percentage of control K^+-induced contractions. In next step, after equilibrium period, the ileum was contracted by 60 mM when the plateau was achieved, the non-cumulatively extract (0.01, 0.02, 0.03, 0.04, 0.05, 0.06 and 0.07 mg mL^{-1}) were added to the organ bath. In next protocols, ileums tissue incubated with various concentration of extract (0.01, 0.02, 0.03, 0.04, 0.05, 0.06 and 0.07 mg mL^{-1}), then KCl 60 mM was added to the bath After incubation tissue with each concentration of extract, washing it for 15 min. To assess whether the spasmolytic activity of rue extracts was via a calcium channels, the rat's was allowed to stabilize, with a K^+ rich (60 mM) Ca^{2+}-free Tyrode solution for 60 min. Following an incubation period of 60 min and after the confirmation of no spontaneous contractions of ileum, Ca^{2+} was added in a cumulative fashion (0.225, 0.45, 0.9, 1.8 and 3.6 mM), to obtain control concentration-response curves of Ca^{2+}. The dose-response curves of Ca^{2+} were repeated following 5 min incubation with the extracts. Extract and spasmogen were added directly to the organ bath in volumes usually not exceeding 5% of bath volume.

Statistical analysis: The results obtained from at least seven different animals are presented as Mean±SEM. Two sample comparisons were made by a two-tail unpaired Student's t-test and one-way analysis of variance (ANOVA). Statistical significance was assumed at $p<0.05$. The plateau of the contraction caused by each spasmogen (KCl and $CaCl_2$) in the absence of the extract was considered as the 100% contraction.

RESULTS AND DISCUSSION

Effect of extract on ileum's contraction induced by KCl: Hydroalcoholic extracts from the leaves of *Ruta chalepensis* caused a dose-dependent antispasmodic effect in the contraction of rat's ileum. For relaxation, precontraction of ileum was induced by 60 mM KCl; the cumulative extracts (0.01, 0.02, 0.03, 0.04, 0.05, 0.06 and 0.07 mg mL^{-1}) of interest were then added to the organ bath. The extracts to the concentration-dependent manner

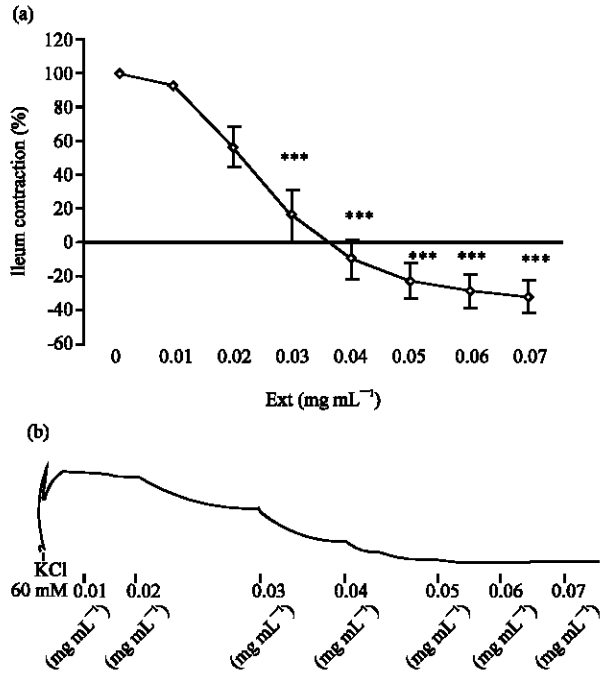

Fig. 1: The effect of cumulative concentration of hydroalcoholic Rue leaf extract on KCl-induced contraction in the adult male rats ileum (n = 7, *** p<0.001). (a) Dose-response curve and (b) One sample of oscillogram

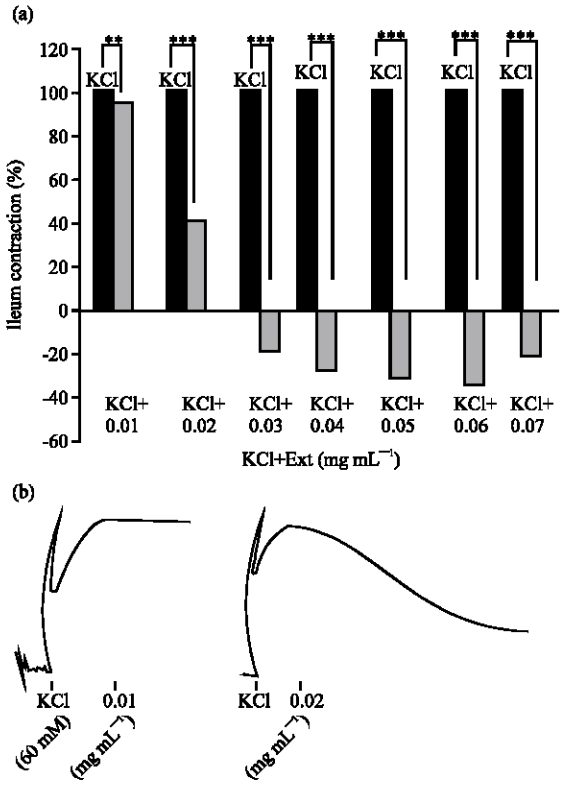

Fig. 2: Non-cumulative concentration of hydroalcoholic rue extract on KCl-induced contraction in the adult male rats ileum (n = 7, ***p<0.001). (a) Histogram and (b) One sample of oscillogram

inhibited the ileum contraction induced by 60 mM KCl (n = 7, p<0.001) and relaxation of this contraction was evaluated as a percentage (Fig. 1a, b). In next step, non-cumulative concentration of extracts (0.01, 0.02, 0.03, 0.04, 0.05, 0.06 and 0.07 mg mL^{-1}) reduced the KCl induced ileum contraction in a dose dependent manner and significantly (n = 7, p<0.001) (Fig. 2a, b).

The effect of extract on ileum before effect of KCl: The ileums were maximally contracted with KCl 60 mM, then tissue was washed and incubated with the various concentration of extract for 5 min. Afterwards, the tissue was either washed or not and then a second contraction elicited by using KCl. The second contraction was expressed as a percentage of the pre-contraction (Fig. 3a, b).

The effect of various contraction of calcium on ileum's contraction in absent and present of extract: A contractile response was obtained by the addition of calcium (0.225, 0.45, 0.9, 1.8 and 3.6 mM), to a previously decalcified rat ileum (Fig. 4a, b). Under the same conditions, but in presence of the extracts (0.01, 0.02 and 0.03 mg mL^{-1}) a decrease in the contractile response was observed, producing a shift in the Ca^{2+} curves (Fig. 5).

The main feature of the small intestine is to absorb and secrete. Diarrhoea results from an imbalance between the absorptive and secretive mechanisms in the intestinal tract, accompanied by intestinal hurry, which results in an excess loss of fluid through the faeces. Many animal based on studies have investigated the bioactivity and effects on intestinal function of plants traditionally used as treatment for diarrhoea. Plant extracts can have antispasmodic effects, delay gastrointestinal transit, suppress gut motility, stimulate water adsorptions or reduce electrolyte secretion. These activities may explain the benefits of using a particular plant in the treatment of diarrhea diseases (Tangpu and Yadav, 2006). Most regions of the gastrointestinal tract generate spontaneous electrical and mechanical activity in the absence of stimulation (Hirst and Edwards, 2004). The results from the present study indicate that hydroalcoholic extracts of *R. chalepensis* had a relaxant effect on rat's ileum, to the dose-dependent manner inhibited the ileum contraction where reversibly blocked spasms induced by 60 mM KCl stimuli. Certainly, the contractions induced by KCl are dependent on the entry of Ca^{2+} into the cells through

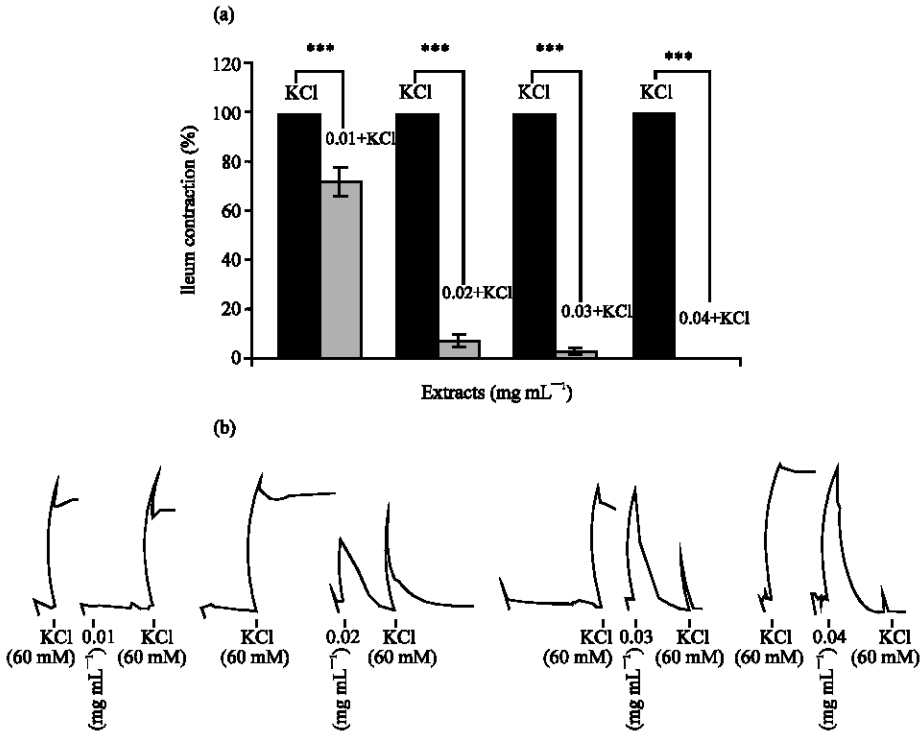

Fig. 3: The effect of various concentration of hydroalcoholic rue leaf extract on adult male rats ileum, before influence of KCl 60 mM (n = 7, *** p<0.001). (a) Histogram and (b) One sample of oscillogram

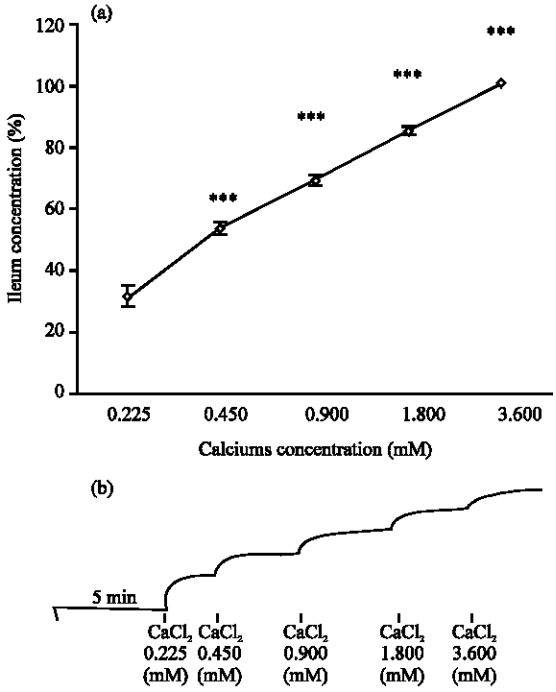

Fig. 4: The dose-response Calcium (a) The effect of cumulative concentration of calcium on contraction of adult male rats ileum (n = 7, *** p<0.001) and (b) One sample of oscillogram

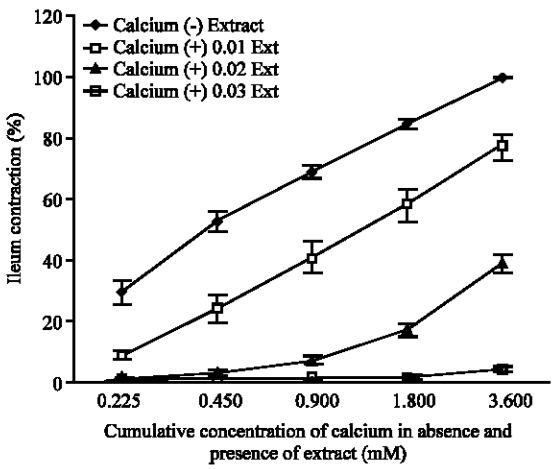

Fig. 5: The effect of various concentration of $CaCl_2$ on contraction of adult male rat's ileum at absent of cumulative *R. chalepensis* extract (n = 7, *** p<0.001)

voltage-dependent calcium channels, therefore a substance which can inhibit high K^+-induced contraction is, considered to be a Ca^{2+} channel blocker(Cortes *et al*., 2006). The inhibitory effect was probably due to different components acting separately or together (Fig. 1a). Only second study showed that the inhibitory effects

observed, was not arising from tired smooth muscle ileum. Non-cumulative concentrations of extracts have reduced the KCl induced ileum contraction in a dose dependent manner (Fig. 2a). On the other hand, the inhibitory effects of all extracts were reversible after washing the tissue with fresh tyrodes solution. Therefore, utilization from KCl could contractions on ileum for several times which were no significant difference between them (Fig. 2b). Exposure of ileum rings incubated with rue leaf extract caused a direct effect on the smooth muscle. These results indicate that *Ruta chalepensis* contains a combination of spasmogenic and spasmolytic constituents. Rue extract in first caused a dose-dependent spasmogenic effect and then followed by relaxation at same doses (Fig. 3b). It seems that rue leaf extracts reduce in a dose dependent KCl contraction (Fig. 3a). Photochemical screening of the aerial parts of this plant showed that the presence of alkaloids, flavonoids, coumarins, tannins, volatile oil, sterols and/or triterpenes (Al-Sagair, 2004a). Many flavonoids show vasorelaxing properties, due to different and often not yet completely clarified mechanisms of action. Among them, the activation of vascular potassium channels has been indicated as a possible pathway (Calderone *et al*., 2004). Sadraei reported that alkaloid extract of *Pycnocycla spinosa* in a concentration-dependent manner inhibited the ileum contraction induced by 80 mM KCl (Sadraei, 2003). Other scientist have shown that in the isolated rabbit jejunum preparation the crude *Acorus calamus* extract, which tested positive for the presence of alkaloid, saponins and tannins, caused inhibition of spontaneous and high K^+ (80 mM)-induced contractions, thus showing spasmolytic activity, mediated possibly through calcium channel blockade (Gilani *et al*., 2006). Ghayur and Gilani (2004) demonstrated that A spasmolytic effect of curve Ginger observed and inhibition of high K^+ (80 mM)-induced contractions indicating a calcium antagonist effect. Phytochemical analysis revealed the presence of saponins, flavonoids and alkaloids in the crude extract. So, the relaxation effect of *Ruta chalepensis* leaf on rat's ileum may be due to presence of alkaloid and flavonoid in the extract. On the other hand KCl-induced contraction has long been known to be due to membrane depolarization causing Ca^{2+} entry through voltage-operated Ca^{2+} channels (VOCCs). KCl-induced Ca^{2+} sensitization in this tissue is largely the result of alpha-adrenergic receptor stimulation by norepinephrine released from sympathetic nerves. Studies done as early as 1984, with the use of intact smooth muscle tissues, showed that G-Protein coupled receptor agonists can produce greater increases in force for a given increase in $[Ca^{2+}]$i than KCl (Ratz *et al*., 2005).

Contraction of smooth muscle is regulated by cytosolic Ca^{2+} levels and the sensitivity to Ca^{2+} of the contractile elements in response to changes in the cell. There are differences in the degree of participation by extra- and intracellular Ca^{2+} in smooth muscle contraction (Grasa *et al*., 2004). Ca^{2+} is the primary signal responsible for activation of smooth muscle contractile proteins. Cytosolic free Ca^{2+} concentration ($[Ca^{2+}]$i) is finely tuned by multiple membrane-based Ca^{2+} channels, pumps, compartments and intracellular buffers to suit the immediate contractile requirement of muscle cells. However, Ca^{2+} does not directly activate smooth muscle motor proteins. Rather, phosphorylation of 20-kDa Regulatory Myosin Light chain (MLC) serves as the switch to turn on smooth muscle actomyosin ATPase activity, increasing cross-bridge cycling rates and muscle contraction. Stimuli that increase $[Ca^{2+}]$i elevate MLC phosphorylation levels by increasing the Ca^{2+}-calmodulin-dependent MLC kinase-to-phosphatase activity ratio (Ratz *et al*., 2005), whereas relaxation is mediated by cAMP-and or cGMP-dependent protein kinase which inhibit the initial [ca]i transient and reduced the sensitivity of MLC kinase to $[Ca]^i$ (Ohya and Horowitz *et al*., 2002). In order to confirm the interaction of contraction smooth muscle with voltage dependent Ca^{2+} channels, in this study, the tissue was pretreated with high potassium and calcium free. Cumulative addition of various concentrations of $CaCl_2$ to the bath tissue that including high potassium caused strong contraction (Fig. 4a). Applying cumulative concentrations of $CaCl_2$ to the Ca^{2+}-free and high potassium (60 mM) Tyrode solution caused ileal contractions dose dependently(Fig. 4b).

Rue extracts in a dose dependent decrease in the contractile response was observed, producing a shift in the Ca^{2+} curves (Fig. 5). Also there is evidence for that in guinea pig ileum and Taenia coli, high K^+ elicits an increase in $[Ca^{2+}]$ i and transient contractions The spontaneous movements in longitudinal muscle of sheep duodenum persist in Ca^{2+}-free solutions with EDTA for 20 min. Verapamil, nifedipine and diltiazem, all of which are Ca^{2+} antagonists, reduce the mechanical activity of rabbit ileum, rat duodenum, sheep duodenum and canine ileum. The amplitude, frequency and tone of spontaneous activity in smooth muscle from rabbit small intestine were eliminated after 10 min of preincubation in Ca^{2+}-free solutions with EGTA. Furthermore, the parameters of motility decreased when the intestinal segments were pre-incubated with verapamil or nifedipine, antagonists of L-type voltage-dependent Ca^{2+} channels. Those results indicate that extracellular Ca^{2+} participates in spontaneous activity and enters cytosol by L-type voltage-dependent Ca^{2+} channels. The ACh and KCl responses were reduced

in Ca^{2+}-free solutions, verapamil and nifedipine. Those results suggest that the ACh- and KCl induced contractions in rabbit small intestine are mediated by extracellular Ca^{2+}, although intracellular Ca^{2+} might also be implicated (Grasa *et al*., 2004). In conclusion it seems, Rue could relax the intestinal smooth muscle by blocking voltage-gated calcium channels.

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