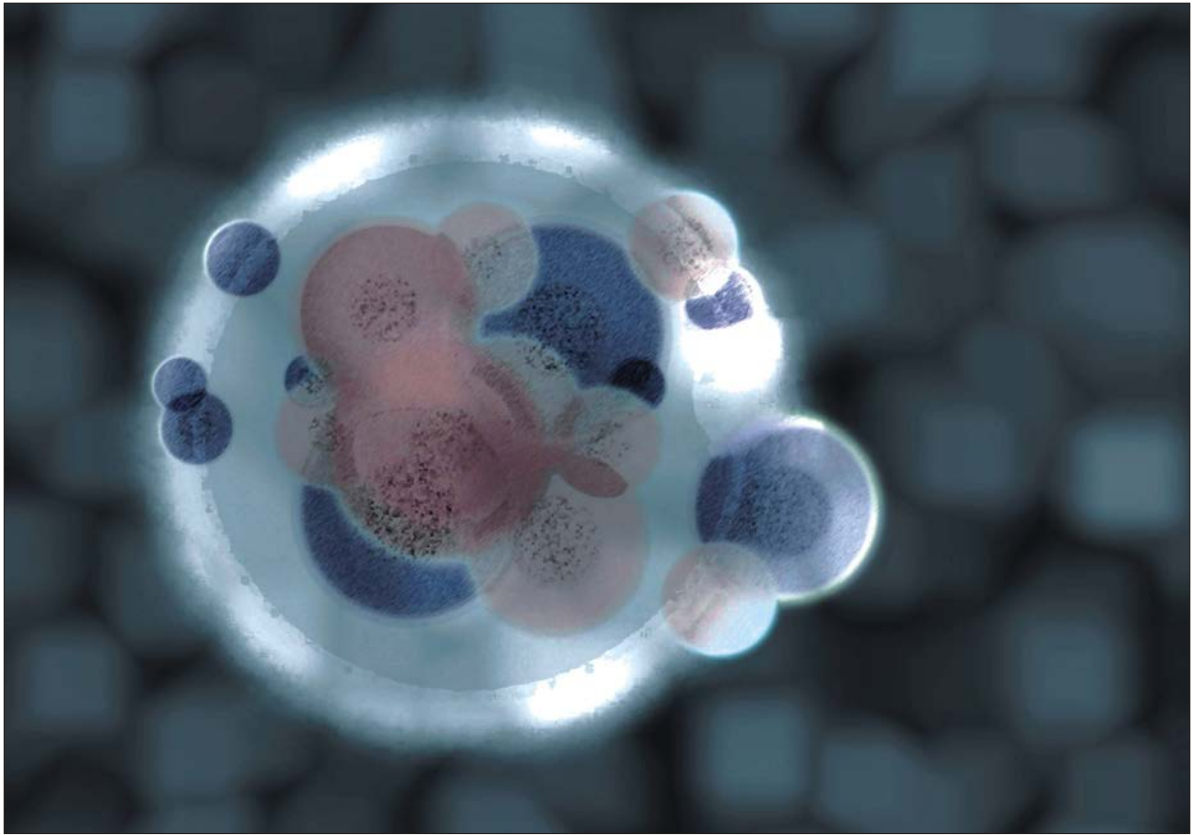




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The Role of β -Adrenoceptors at Inhibitory Effect of Hydroalcoholic Extract of *Ruta chalepensis* Leaf in Male Rat's Ileum

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Abstract: The aim of present study was to investigate the effect of hydroalcoholic extract of *Ruta chalepensis* on the ileum contractions by some known spasmogens and also to study the possible mechanism involved. Rue extract was prepared by macerated with alcohol (70%). A piece of ileum (2 cm) was removed from male Wistar rats and mounted in an organ bath containing air bubbled Tyrode solution with 1 g initial tension and contractions were recorded by an isotonic transducer. The relaxed ileum by non-cumulative concentration of Rue extract (0.01, 0.02, 0.03, 0.04 mg mL⁻¹) were effected on KCl (60 mM) and ACh (1 μ M) spasmogens. The results demonstrate that the Rue leaf extract significantly dose-dependently ($p < 0.001$) reduces the ileum contractions induced by KCl (60 mM). Acetylcholine (1 μ M)-induced ileum contractions were attenuated at the same concentration of the extract ($p < 0.0001$). The Rue leaf extract (1 mg mL⁻¹) induced relaxation on the ACh-induced contraction in ileum was unaffected by beta-adrenoceptor antagonist (propranolol 1 μ M). Cumulative concentrations of extract (0.01, 0.02, 0.03, 0.04, 0.05, 0.06 and 0.07 mg mL⁻¹) have reduced the KCl-induced ileum contractions in a dose dependent manner and significantly ($p < 0.001$). The spasmolytic effect of the cumulative concentration of extracts (0.01, 0.02, 0.03, 0.04, 0.05, 0.06 and 0.07 mg mL⁻¹) was reduced after tissue incubation 30 min with propranolol (1 μ M). Furthermore, it seems the portion relaxatory effect of Rue extract on the rat ileum may be due to β -adrenergic receptors.

Key words: Spasmolytic, *Ruta chalepensis*, β -adrenergic receptors, rat, ileum

INTRODUCTION

Rue or Herb of Grace, is by far the best known of this genus of sixty species native to the Mediterranean and western Asia and typifies the rue family Rutaceae. Common rue, well known and highly regarded since ancient times, is frequently mentioned in the literature, including the writings of Milton and Shakespeare (Al-Sagair, 2004). In folk medicine as an antirheumatic, an antispasmodic and a treatment for snakebites and headaches (El Sayed *et al.*, 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). The gastrointestinal tract receives a dense nervous innervations from the lumbosacral parasympathetic outflow and it is this system that exerts the major influence on activity. A sympathetic innervation also exists and interactions between the systems operate at a prejunctional and postjunctional level. The main neurotransmitter released within the parasympathetic system is Acetylcholine (ACh) (Uchiyama and Chess-

Williams, 2004). Rue extract has an anticholinergic action on the small intestine of male guinea pigs. Furthermore, relaxation and inhibition in the motility of the female small intestine, induced by Rue extract (Molina *et al.*, 1991). KCl has long been used as a convenient stimulus to bypass G protein-coupled receptors (GPCR) and activate smooth muscle by a highly reproducible and relatively simple mechanism involving activation of voltage-operated Ca²⁺ channels that leads to increases in cytosolic free Ca²⁺ (Ratz *et al.*, 2005).

However, there is little information regarding the underlying mechanism that mediated its spasmolytic action. The aim of the present study was, therefore, to investigate the possible mechanism that mediated the effects of Rue leaf hydroalcoholic extract on male Rat's ileum.

MATERIALS AND METHODS

Drugs and solution: Acetylcholine (ACh), propranolol were purchased from Sigma (USA), all chemicals were used of reagent grade, purchased from Merk Germany.

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 (Sadraei *et al.*, 2003). Chloride potassium (KCl) was made up as 60 mM (Nasu *et al.*, 1994) stock solution in Tyrode. ACh and Propranolol were made up as 1 μM (Gharib Naseri and Heidari, 2007) stock, dilution in Tyrode.

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).

Animals and tissue preparation: Male Wistar Rats (200±250 g) were obtained from animal house of Jundishapur Ahwas University of Medical Sciences and kept at 20-24°C under 12/12 h light/dark cycle and had free access to food and water. The Rats were housed in individual cages with wire-mesh bottoms for 24 h before experiment and were deprived of food but had free access to drinking water. Rats were sacrificed by a blow to the skull and cervical dislocation. After laparotomy, a segment (2 cm) of distal part of the ileum was dissected out and rinsed intraluminally with cold oxygenated Tyrode solution. The ileum was suspended in an organ bath (10 mL) containing Tyrode solution (37°C, pH = 7.4) between two stainless steel hooks and solution was bubbled with air continuously. The lower hook was fixed at the bottom of the organ bath and the upper one was connected to an isotonic transducer (Harvard Transducer). Under Tyrode solution 1 g resting tension, the ileal contractions were recorded (Universal Harvard Oscillograph) after 60 min equilibrium period. The bath solution was refreshed every 15 min. The experiments were performed on three or four segments of ileum taken from each of seven animals. Each tissue was only used for one of the spasmogens. Segments that did not show spontaneous activity were discarded. At the beginning of each experiment, the tissue was contracted 2 times by using spasmogens in each muscle bath, until stable responses were observed. After equilibrium period, ileums tissue incubated with various concentration of extract

(0.01, 0.02, 0.03 and 0.04 mg mL^{-1}), then, contracted by KCl 60 mM or by ACh 1 μM that were added to the bath. Then, during 15 min, the bath solution was exchanged three times with fresh Tyrode solution. The same protocol was repeated but with the higher extracts concentration. To investigate the involvement of β -adrenoceptors, first, the inhibitory effect of extract (0.01 mg mL^{-1}) was recorded then ACh was added for contraction. After 15 min and several exchanging the bath solution, the same protocol was repeated in the presence of propranolol 1 μM for 30 min (Oprins *et al.*, 2001).

To assess whether the spasmolytic activity of rue extracts was via a β -adrenoceptors, the spasmolytic effect of cumulative concentration extract (0.01, 0.02, 0.03, 0.04, 0.05, 0.06 and 0.07 mg mL^{-1}) on KCl (60 mM) induced contraction were recorded and then the same procedure was repeated 30 min after incubation of tissue with propranolol, in separate protocols.

Statistical analysis: The results obtained are presented as Mean±SEM. Two sample comparisons were made by Student's t-test. Multiple means were compared by analysis of variance (ANOVA). Statistical significance was assumed at p-levels less than 0.05.

RESULTS

Effect of pre-incubation with hydroalcoholic extract of *Ruta chalepensis* on ileum in responses of KCl and ACh: In this stage, the ileums were maximally contracted with KCl 60 mM or by ACh 1 μM , then tissue was washed and incubated with the non-cumulative concentration of extract for 5 min afterwards, the tissue was either washed

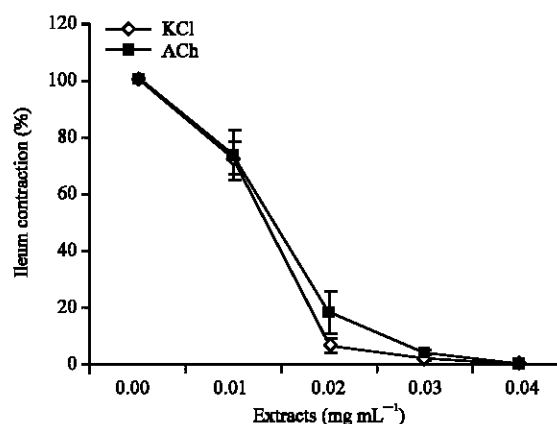


Fig. 1: Contraction in response to KCl or ACh reduced in tissue that before were reversibly inhibited by non-cumulative concentration of hydroalcoholic extract of *Ruta chalepensis* leaf ($p < 0.001$)

or not and then a second contraction elicited by using KCl or by ACh. The second contraction was expressed as a percentage of the pre-contraction. The results indicated that in the presence of hydroalcoholic Rue leaf extract a dose dependently, prevented from contraction-induced by KCl or by ACh (student's-test, $p < 0.001$, $n = 7$) (Fig. 1).

Inhibitory effect of extract on response ACh contraction in absent and presence propranolol: The ileums were contracted with ACh $1 \mu\text{M}$, then tissue was washed and

then after exposure to Rue leaf extract (0.01 mg mL^{-1}) and no washing, ileum contraction with ACh $1 \mu\text{M}$ reduced of its control ACh contraction (student's-test, $p < 0.001$, $n = 7$). The same protocol was repeated but in the presence of propranolol as a β -adrenoceptor antagonist ($1 \mu\text{M}$, for 30 min, $n = 7$). Figure 2 shows that the inhibitory effects of extract (0.01 mg mL^{-1}) on response ACh contraction are not different in the absence and in the presence of propranolol.

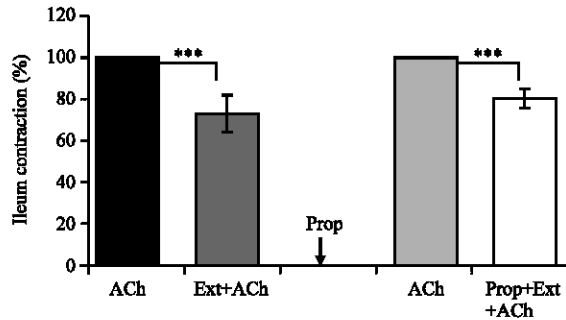


Fig. 2: Spasmolytic effect of Rue leaf hydroalcoholic extract (Ext = 0.01 mg mL^{-1}) in response contraction induced by ACh ($1 \mu\text{M}$) in absence and in the presence (30 min) of β -adrenoceptor antagonist, propranolol, (Prop. = $1 \mu\text{M}$) in rat's ileum (***) $p < 0.001$, $n = 7$)

The effect of extract on ileum's contraction-induced by KCl: Rats ileum suspended in Tyrodes solution shows irregular spontaneous contractile activity, which attenuates by changing of bath fluid. KCl 60 mM (non-receptor stimulation for opening Ca channels) produced a sustained tonic contraction, which was maintained during the course of experiments. Cumulative concentration of Rue leaf extracts (0.01 , 0.02 , 0.03 , 0.04 , 0.05 , 0.06 and 0.07 mg mL^{-1}) reduced the ileum contraction induced by KCl 60 mM in a dose dependent manner (ANOVA, $p < 0.001$, $n = 7$). The relaxation of this contraction was evaluated as a percentage (Fig. 3).

Inhibitory effect of extract on ileum KCl induced contraction in the presence of propranolol: The activity of the extracts was tested in isolated spontaneously contracting Rat ileum, in the presence of agent including

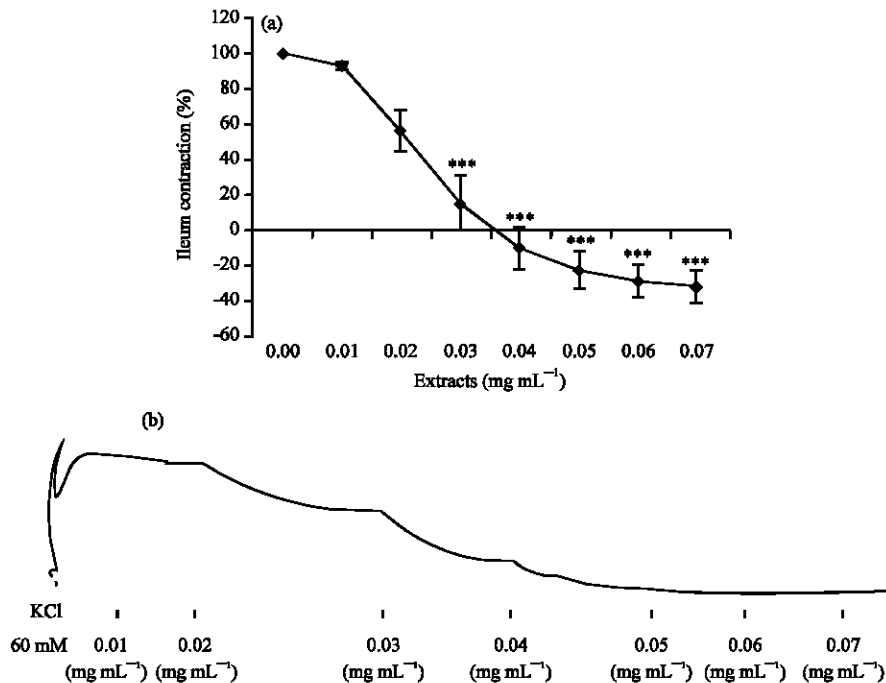


Fig. 3: Effect of cumulative concentrations of the Rue leaf hydroalcoholic extract on rat ileal contraction induced by KCl 60 mM (***) $p < 0.001$, $n = 7$). (a) Dose-response curve (b) One sample of oscillogram

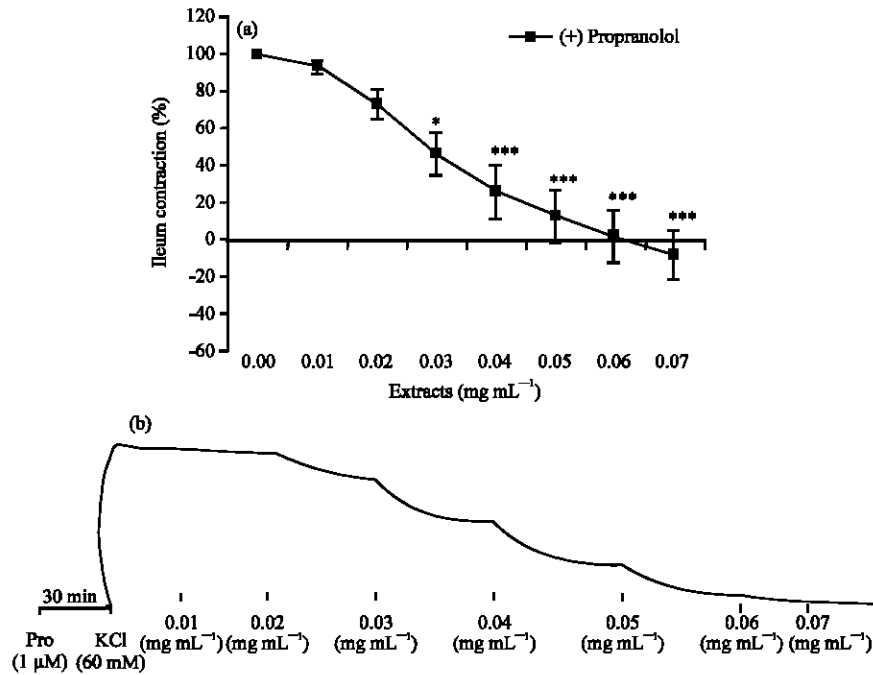


Fig. 4: Effect of exposure of ileum to propranolol on relaxation time in response of the Rue extract after contraction with KCl 60 mM. (a) Dose-response curve (b) One sample of oscillogram

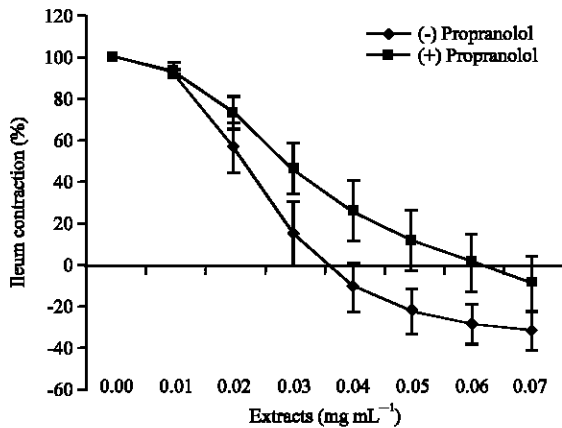


Fig. 5: Comparison effect of pre-incubation (30 min) with propranolol on relaxation of hydroalcoholic extract of *Ruta chalepensis* in response to 60 mM KCl ($p < 0.001$)

non-selective β -adrenergic antagonist: propranolol (1 μ M). KCl (60 mM) was used as a positive control. The inhibition of contractions in the test material was assessed as a percentage of the basal spontaneous contractions in the Rat ileum. In the presence of propranolol, cumulative concentration of extract (0.01, 0.02, 0.03, 0.04, 0.05, 0.06 and 0.07 mg mL⁻¹) reduced KCl-induced contraction in a dose dependent manner (ANOVA, $*p < 0.05$, $***p < 0.001$, $n = 7$) (Fig. 4).

Comparison inhibitory effect of hydroalcoholic extract of *Ruta chalepensis* in absence and presence of propranolol:

Pre-incubation of ileum by propranolol for 30 min caused a reduction in response to cumulative concentration of extract in a dose dependent manner to contraction induced by KCl of the control. The inhibitory effects of extract on the KCl-induced contraction are different in the absence and in the presence of propranolol (1 μ M) as a β -adrenoceptor antagonist (ANOVA, $p < 0.001$, $n = 7$) (Fig. 5).

DISCUSSION

The parasympathetic nervous system is responsible for maintaining normal intestinal function, contracting the smooth muscle by releasing the neurotransmitters acetylcholine (ACh) and ATP. The receptors mediating postganglionic responses are the muscarinic receptors (Uchiyama and Chess-Williams, 2004). The contractile responses obtained by activation of different muscarinic receptor subtypes in the longitudinal muscle of the rat ileum (Elorriaga *et al.*, 1996). ACh is the main transmitter released and smooth muscle contraction is mediated via a mixed M2/M3 receptor population. KCl is often used as a tool to bypass G-protein coupled receptor (GPCR) stimulation and activate smooth muscle by changing the K⁺ equilibrium potential and clamping membrane potential at some value above the resting level (Ratz *et al.*, 2005). The depolarization induced by high potassium

concentration activates the L-type Voltage Dependent Calcium Channels (VDCCs) (Karaki *et al.*, 1997). The present study showed that Rue leaf hydroalcoholic extract is a potent relaxant on response contractions induced by a variety of spasmogens in Rat ileum. It reduced the KCl-induced contraction, a calcium channel mediated spasmogen, as well as those produced by acetylcholine, a receptor mediated agent (Fig. 1). Furthermore, following washing, these inhibitory activities were totally reversible, suggesting that these effects are membrane mediated. On the other hand, these inhibitory effects could not be related to reduction of smooth muscle myofilaments sensitivity to calcium, otherwise, washing the tissue was unable to reverse the inhibitory effect. Some report indicated that Rue has an anticholinergic action on the small intestine of male guinea pig. Administration of Rue extract (20%) before ACh in different segments of small intestine from male guinea pig inhibited the contraction induced by ACh and this effect returned after washing (Molina *et al.*, 1991). The two components of intracellular and extracellular calcium were involved in the contraction of smooth muscle induced by ACh. The first phasic contraction induced by release of intracellular calcium. The second phasic contraction based on the inflow of extracellular calcium through the Receptor-Operated Calcium (ROC) (Zhang *et al.*, 2005). In this study, it seems that Rue extract significantly inhibited the second phasic contractions of smooth muscle induced by ACh because, this effect returned after washing. In ileal, colonic, gastric smooth muscle the density of M2 receptors is far greater than the density of M3 receptors, the M2:M3 ratio being 3:1 in most species including man. M2 receptors couple to Gi proteins to induce responses via an inhibition of adenylate cyclase, a reduction in cAMP levels and smooth muscle relaxation (Uchiyama and Chess-Williams, 2004). As show in Fig. 2, the inhibitory effects of extract (0.01 mg mL^{-1}) on the ACh-induced contraction are not significant in the absence and in the presence of propranolol (β -adrenoceptor antagonist). This suggests that either the components in the 0.01 mg mL^{-1} extract or actions that are unrelated to the β -blocking action of propranolol could be the mediators of this action. Also researchers have shown that the *in vitro* responses of isolated strips of gastrointestinal tract tissue following stimulation with agonists such as carbachol or oxotremorine have been examined in many species including humans. In all species so far examined, the responses of isolated tissue preparations appear to be mediated exclusively via the M3-muscarinic receptor subtype, with M3-receptor selective antagonists such as 4-DAMP having a high affinity for the receptor whilst M2-receptor selective antagonists like methoctramine having a relatively low

affinity. Furthermore, wherever Schild analysis has been performed the plots for these antagonists have slopes of unity indicating that the M3 receptor is the only muscarinic subtype involved in mediating contractile responses to muscarinic agonists *in vitro* (Uchiyama and Chess-Williams, 2004). M3 receptor couple to Gq/11 guanine nucleotide binding proteins and alter cellular activity by stimulating phospholipase C and generating the second messengers inositol trisphosphate (IP3) which induces the release of calcium from intracellular stores and diacylglycerol (DAG) which causes the influx of extracellular calcium to induce responses (Caulfield and Birdsall, 1998). On the other hand, diacylglycerol activates protein kinase C, which opens the Ca^{2+} channels in the plasma membrane (Araujo and Bendhack, 2003). Influx of extracellular calcium, in part is through L-type calcium-channels (Aronsson and Holmgren, 2000). In view of the fact, Rue extract has an anticholinergic action, it seems, the extract influence on M3 receptor signaling of cholinergic pathway. Since, relaxation effect of extract returned after washing tissue, therefore, these effects are membrane mediated. Furthermore, it has suggested that substances, which inhibit the ACh-induced contraction, act by blocking L-type voltage dependent calcium channel (VDCCs). This suggestion is supported by the existence of L-type VDCCs in the rat intestine (El-Bardai *et al.*, 2004).

In the next step, the effects of cumulative concentration hydroalcoholic extract of *Ruta chalepensis* on contraction by KCl and the roles of extract on β -adrenergic system have been investigated. This experiment showed that, hydroalcoholic extract of *Ruta chalepensis* in a concentration-dependent manner inhibits rat ileum contraction induced by 60 mM KCl (Fig. 3). The inhibitory effect was probably due to different components acting separately or together. 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-Said *et al.*, 1990). A high extracellular concentration of KCl and a depolarizing electrical stimulus promote Ca^{2+} influx by voltage-operated channels (Bean *et al.*, 1986). The L-type Ca^{2+} channels require relatively strong depolarization for activation (Beech, 1993). Certainly, the contractions induced by KCl are dependent on the entry of Ca^{2+} into the cells through 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).

β -adrenoceptors are integral membrane proteins mediating a wide variety of the physiological actions of catecholamines, through coupling to guanine nucleotide binding regulatory proteins (G proteins) and activation of

adenylate cyclase. In general, it is accepted that all three β -adrenoceptor subtypes (β_1 , β_2 and β_3 -subtypes) are coupled to the activation of adenylate cyclase and acted through the same effector pathway-cAMP as a primary mechanism for signal transduction (Koike *et al.*, 1995). In rat ileum, β_1 -, β_2 - and β_3 -AR mRNA was detected (Roberts *et al.*, 1999). The contractile response induced by high concentration of KCl was concentration dependent and stable over time. Demonstrating that the time used for incubation with the various antagonists did not change the contractile responses to KCl. Laboratory experiments were performed to investigate the mechanism of relaxation of the rue extract on smooth muscle with the β -adrenergic receptor antagonist propranolol (1 μ M). Under this condition, in presence of propranolol a decrease in the relaxation response was observed (Fig. 4). Comparison inhibitory effect of hydroalcoholic extract of *Ruta chalepensis* in absence and presence of propranolol indicate that block of the β -adrenoceptors by propranolol significantly affect the relaxation response of Rue extract (Fig. 5). The corresponding pathway leading from stimulation of β -adrenergic receptors and increases in cAMP to relaxation of phasic smooth muscle is not fully understood. It involves a reduction in Ca^{2+} levels but also a reduction in the effectiveness of Ca^{2+} in invoking contraction. Uncoupled Protein 1 (UCP1) functions as a regulated transporter for proton equivalents over the mitochondrial membrane, leading to energy being released as heat instead of being captured in ATP.

This may result in mitochondrial uncoupling, a significant fraction of the proton gradient may be diverted from the ATP-syntheses and instead pass through UCP1, resulting in lower ATP levels. In different organs exhibiting peristaltic motion, β -adrenergic receptors also have a role: to induce relaxation/inhibition of contraction via an increase in cAMP levels. Other relaxing agents (VIP, PACAP) have also been suggested to induce relaxation through this cAMP-dependent pathway. As activated UCP1 in the mitochondria of smooth muscle cells may functionally explain the lowered effectiveness of Ca^{2+} in inducing contraction observed under these conditions (Shabalina *et al.*, 2002). Mechanisms of relaxant effects mediated by cyclic AMP may be summarized as follows, (1) inhibition of the receptor-mediated signal transduction resulting in the inhibition of all the effects of agonists including Ca^{2+} release, Ca^{2+} influx and Ca^{2+} sensitization, (2) dissociation of contraction from MLC phosphorylation, (3) increase in SR Ca^{2+} uptake, (4) decrease in the Ca^{2+} sensitivity of MLC phosphorylation possibly by activating MLC phosphatase and (5) increase in noncontractile $[\text{Ca}^{2+}]_i$ which may result in activation of K^+ channels and membrane hyperpolarization. A part of these effects may be mediated by G kinase but not by A kinase

(Karaki *et al.*, 1997). *Ruta chalepensis* is a rich source of important secondary metabolites. Besides, it is a medicinal plant and still used in traditional medicine. So, the effects of chemical components and its medical properties are recommended.

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