



Effects of dibenzylbutyrolactone lignans arctigenin and trachelogenin on the motility of isolated rat ileum

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ABSTRACT

Dibenzylbutyrolactone-type lignans are phenolic compounds of medical importance. The purpose of the study was to determine the effects of two such lignans, arctigenin and trachelogenin on the motility of isolated rat ileum and obtain indications on their mechanism of action. They were isolated from *Arctium lappa* and *Cirsium arvense*, respectively, which have been used traditionally to treat gastrointestinal disorders. 1–1.5 cm long segments of distal ileum were obtained from adult male Wistar rats. The intestinal segments were suspended vertically in a well-aerated organ-bath according to Magnus mounting method. The intestinal motility was monitored for 30 min before treatment to obtain the baseline, followed by treatment with 1 μ M, 10 μ M, 20 μ M and 40 μ M concentrations of arctigenin and 0.5 μ M, 1 μ M, 10 μ M and 20 μ M of trachelogenin concentrations. The amplitude, tone, and period of spontaneous contractions were measured after 15 and 30 min of treatment. To investigate their mechanism of action, cholinergic, glutamatergic, adrenergic antagonists and compounds inhibiting nitric oxide synthase and L-type calcium channels were also tested. Arctigenin and trachelogenin decreased the frequency of contractions in a dose-dependent manner. At the concentration of 20 μ M and 40 μ M of trachelogenin and arctigenin, respectively, there was a marked alteration in spontaneous contraction pattern with an observable increase in the period time. This activity was comparable to 0.5 μ M nifedipine (L-type calcium channel blocker) treatment. Our results demonstrate relaxant effect of arctigenin and trachelogenin on the ileum motility that may be mediated by L-type calcium ion channel blockade.

1. Introduction

Traditionally used medicinal plants are abundant sources of secondary metabolites expressing various biological activities. *A. lappa* (L.) and *C. arvense* (L.) Scop, Asteraceae have been used traditionally for the treatment of gastrointestinal disorders [1,2]. So far, dibenzylbutyrolactone lignans have been confirmed as the main compounds in their fruits [3–5].

Lignans are phenolic compounds, composed of two phenylpropane units (C6-C3) categorized into various structural groups including butyrolactone ring-containing dibenzylbutyrolactone lignans [DBBLs] [6]. As we confirmed recently, two representatives of

dibenzylbutyrolactone lignans i.e., arctigenin (ATG) and trachelogenin (TGN) can be isolated straightforwardly from the fruits of *A. lappa* and *C. arvense*, respectively [3,4]. ATG and TGN are of increasing importance in medicine: they possess significant antiproliferative [3,7] and antiviral effects [8]. In addition, ATG has also been shown to have neuroprotective effects in the rat brain neocortex via non-NMDA-type glutamate receptors [9,10].

However, the effects of arctigenin and trachelogenin on gastrointestinal motility have not been investigated. Gastrointestinal motility involves the interaction and communication between the autonomic nervous system, the enteric nervous system, the interstitial cells of Cajal and smooth muscle cells [11]. The enteric nervous system is organized

Abbreviations: AP5, DL-2-Amino-5-phosphonopentanoic acid; ATG, arctigenin; CNQX, 6-cyano-7-nitro-quinoxaline-2,3-dione; DMSO, Dimethyl sulfoxide; HPLC, High performance liquid chromatography; L-NAME, N^w-Nitro-L-arginine Methyl Ester, Hydrochloride; NMDA, N-Methyl-d-aspartic acid; TGN, trachelogenin.

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into myenteric and submucosal plexuses, it expresses essentially all CNS neurotransmitters [12,13]. Interstitial cells of Cajal are muscle-like pacemaker cells in the myenteric plexus that produce slow waves, which evoke smooth muscle contraction by calcium ion influx via L-type calcium channels [14]. Smooth muscle relaxation is mediated by activation of adenylate or guanylate cyclase, extracellular calcium release, intracellular calcium uptake and L-type calcium ion channel blockade [15,16]. Thus, exogenous agents affecting cytosolic calcium levels can influence gastrointestinal motility.

Dietary excitotoxins cause enteric nervous system dysfunction, resulting in incurable gastrointestinal motility disorders, such as irritable bowel syndrome (IBS) characterized by impaired intestinal motility affecting approximately 11 % of the world population [17,18]. Irritable bowel syndrome is mainly treated with calcium ion channel blockers whose adverse side effects have been reported [18].

Plant compounds mediate smooth muscle relaxation mainly via anticholinergic, adrenergic, nitrergic mechanisms and calcium ion channel blockade [19]. In isolated organs of laboratory animals, ATG, TGN, schisandrin A and magnolol have been reported as smooth muscle relaxants or L-type calcium ion channel inhibitors [20–22]. ATG also showed airway smooth muscle relaxation, nitric oxide elevation and acetylcholinesterase inhibition in different in vitro systems [23,25,26].

Hence, we aimed to determine the effects of arctigenin and trachelogenin on the spontaneous motility of isolated rat ileum and elucidate their mechanism of action.

2. Materials and methods

2.1. Drugs and chemicals

Atropine, 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) >99 % (HPLC) [Hello Bio], Dimethyl sulfoxide (DMSO) ≥ 99.9 % (HPLC), DL-2-Amino-5-phosphonopentanoic acid (AP5) >99 % (HPLC) [Hello Bio], Nifedipine ≥ 98 % (HPLC), N^w-Nitro-L-arginine Methyl Ester, Hydrochloride (L-NAME) ≥ 98 % (TLC), Phentolamine hydrochloride ≥ 98 % (TLC), Propranolol hydrochloride ≥ 99 % (TLC) were used. Tyrode's solution whose composition was as follows [mM]: [NaCl 136.9, KCl 2.7, KH₂PO₄ 0.32, CaCl₂ 1.8, MgSO₄ 0.98, NaHCO₃ 11.9, glucose 5.5; pH 7–7.4] was prepared in the Department of Physiology and Neurobiology, Eötvös Loránd University. Nifedipine, CNQX and Bay K 8644 (Hello Bio Ltd., Dunshaughlin, Ireland) were dissolved in DMSO while AP5, propranolol hydrochloride, phentolamine hydrochloride, L-NAME and atropine were dissolved in distilled water to obtain stock solutions that were placed in Eppendorf tubes and stored at – 20 °C. Due to photosensitive nature of nifedipine, nifedipine-containing Eppendorf tubes were wrapped in aluminum foil before storage. All compounds were freshly diluted in Tyrode solution just before recordings. Unless stated otherwise, all chemicals were purchased from Sigma-Aldrich (St Louis, Mo, USA).

2.2. Extraction, isolation, and formulation of the test compounds arctigenin and trachelogenin

Ripe fruits of *C. arvensis* and *A. lappa* were collected from different Hungarian habitats. Their voucher specimens were deposited in the herbarium of the Department of Plant Anatomy, Eötvös Loránd University, Budapest, Hungary.

Lyophilized and pulverized fruit samples (2.0 g weighed with analytical precision) were suspended in 5.0 ml of distilled water and were heated at 40 °C for 60 min. Thereafter, the samples were lyophilized and extracted with 25 ml of 80 % (v/v) methanol held under reflux at boiling point for 60 min. Thereafter, the insoluble, centrifuged material was extracted for a second time, as before. The combined supernatants were dried by a rotary vacuum evaporator at 30–40 °C. The dried extracts were dissolved in 2.5 ml of 80 % (v/v) methanol for the isolation of ATG and TGN by preparative HPLC (high performance liquid

chromatography) as described in our previous manuscript [4]. Structures of isolated ATG and TGN were confirmed by high performance liquid chromatography hyphenated with ultraviolet and high-resolution mass spectrometry detections and by nuclear magnetic resonance spectroscopy. Using a HPLC-UV-MS method, purity of isolated ATG and TGN was determined to be 95.4 % and 96.7 %, respectively (Supplementary material, Fig. A.1).

Isolated compounds (ATG and TGN) were dissolved in DMSO to obtain 10 mM stock solutions that were then stored at – 20 °C. The stock solution was diluted in 200 ml Tyrode's solution to obtain the final concentrations of 0.5 μM, 1 μM, 10 μM, 20 μM, and 40 μM.

2.3. Experimental animals

Adult male Wistar rats weighing 200–255 g, were purchased from Toxi-Coop Ltd., Budapest, Hungary. Before the experiment, they were housed under controlled laboratory conditions, 12:12-h light: dark photocycle and temperature of 22 ± 2 °C. They were fed with standard rat pellets and tap water was provided ad libitum. All experiments were conducted in accordance with the Hungarian act of animal care and experimentation. All procedures were reviewed and approved by the Animal Care and Use Committee of Eötvös Loránd University.

2.4. Experimental protocol

The test rats were sacrificed by decapitation using rodent guillotine and immediately followed by an abdominal incision using scissors to expose the intestines. The small intestine was trimmed off the mesentery, pulled off from the abdomen using a pair of forceps and the terminal ileum was excised. The intraluminal contents were flushed by injection of warm Tyrode's solution using a syringe and the distal ileum was then cut into 1–1.5 cm segments using a pair of scissors into a beaker containing Tyrode's solution. The beaker containing the ileum segments was then transferred into a water-bath maintained at 39 °C and the segments were continuously aerated.

The ileum segments were mounted vertically in an organ bath equipped with mechanosensor and connected to an amplifier (Experimetria Ltd., Budapest, Hungary). The organ bath contained 50 ml air-bubbled Tyrode's solution (39 °C and pH 7–7.4) and recording of motility was done using Analyze-wide computer software (written by L. Détári). Fresh Tyrode's solution was added to the organ-bath, contractility of the segments was monitored for 15 min followed by wash-out of 50 ml of the Tyrode's solution. Thereafter, it was monitored for 15 min and then recording was done for 2 min before treatment to obtain the baseline. The test compounds were applied and monitored for another 15 min followed by 2 min recording. This was then followed by treatment with 50 ml of the test compounds. It was then monitored for another 15 min and 2 min recording was done [Fig. 1].

2.5. Data analysis

The intestinal motility was measured using the Analyze-wide software to obtain the amplitude, tone, and period of the spontaneous contractions [Fig. 1B]. The mechanosensor was calibrated with small weights ranging from 0.1 g to 5 g. In this range, the operation of the sensor was linear (correlation coefficient=1). Using the linear equation, amplitude of the intestinal contractions was transformed into force units (mN).

At least 10 waves/recording segments were evaluated obtained from at least 5 animals per treatment/control group. The data were converted into means, standard deviations and standard error of the mean using MS Excel for Windows. Data were transformed as a ratio of the baseline; different treatments were compared using two-way analysis of variance followed by Bonferroni's post hoc tests and presented in the form of graphs using Graphpad Prism version 8.4.3 for Windows. A *p*-value of less than 0.05 was used to indicate statistical significance.

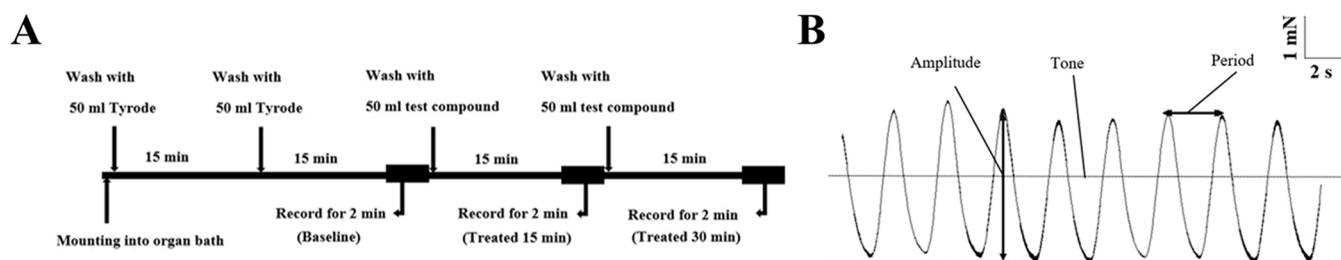


Fig. 1. Protocol of the experiments and analyzed parameters of the spontaneous contractions. (A) Schematic diagram showing the experimental protocol. (B) Original recorded signal showing the analyzed parameters: amplitude, period and tone of spontaneous contractions.

3. Results

Original experimental recordings of spontaneous contractions of the isolated rat ileum segments before and after treatment with control solutions, different concentrations of ATG and TGN, and selected antagonists are shown in Fig. 2.

3.1. Effects of dibenzyl butyrolactone lignans on contraction amplitude

There was no significant difference between the contraction amplitude of ileum segments incubated in DMSO-containing Tyrode (vehicle control) and pure Tyrode. The amplitude of contractions showed a gradual increase after 15 and 30 min, but it was not significantly different from the baseline in these groups ($p > 0.05$) [Fig. 3].

ATG lowered the amplitude of contractions in a dose- and time-dependent manner. 40 μM ATG inhibited the contractions significantly already after 15 min treatment ($p < 0.01$) while 10 μM ATG only after 30 min treatment ($p < 0.05$). In turn, the medium concentration (20 μM) did not have a significant effect on contraction amplitude, similarly to the lowest concentration (1 μM) [Fig. 3].

TGN decreased the amplitude of contractions in a dose-dependent manner. 20 μM TGN had a significant inhibitory effect ($p < 0.05$), which was comparable to the effect of 40 μM ATG ($p > 0.999$). Furthermore, there was no significant difference between the effects of 1 μM ATG, 10 μM ATG, 1 μM TGN and 10 μM TGN ($p > 0.999$) which caused no significant change in the contraction amplitude [Fig. 3].

In addition to the test lignans, various receptor antagonists and mediators were also studied to address the mechanism of action of lignans. The AMPA/KA type glutamate receptor antagonist CNQX (10 μM) had no significant effect on contraction amplitude and doubling this concentration did not result in further change in activity ($p > 0.05$). The NMDA type glutamate receptor antagonist AP5 (20 μM) increased the amplitude, however the change was not significant ($p > 0.05$). The adrenoceptor antagonist propranolol (1 μM) plus phentolamine (1 μM) [Pro+Phe] had no effect on the amplitude of contractions, either ($p > 0.05$) [Fig. 3]. Nifedipine, an antagonist of L-type calcium channels dramatically decreased the amplitude of contractions. Its activity was evaluated after 5 min treatment, as it abolished spontaneous phasic contractions completely at 0.2 μM and above already after 15 min 0.5 μM nifedipine had greater activity than 1 μM ATG and 0.5 μM TGN but caused a significant decrease in contraction amplitude, which was comparable to the effect of 40 μM ATG and 20 μM TGN ($p > 0.05$) [Fig. 3]. In contrast, the nitric oxide synthase inhibitor L-NAME had no significant effect ($p > 0.05$). However, the muscarinic receptor antagonist atropine (0.1 μM) decreased the amplitude of contractions significantly ($p < 0.05$).

3.2. Effects of the dibenzyl butyrolactone lignans on contraction period

There was no significant difference between the contraction period times of the intestinal segments incubated with Tyrode and DMSO-containing Tyrode and their effect did not differ from the baseline after 15- or 30-min incubation ($p > 0.999$) [Fig. 4].

ATG decreased the frequency of contractions in a dose-dependent manner, manifested by the increased period time [Fig. 4]. 20 μM ATG and 40 μM ATG showed a significantly increased period time characterized by alteration in spontaneous contraction [Fig. 2]. Lower concentrations of ATG did not influence contraction frequency ($p > 0.999$) [Fig. 4].

Likewise, TGN increased the contraction period in a dose-dependent manner. After 15 and 30 min, 20 μM TGN increased the period time significantly ($p < 0.05$). 20 μM TGN was also marked by alteration of the spontaneous contraction pattern that became clear after 30 min [Fig. 2]. Though there was an observable increase in period time after treatment with 10 μM TGN, it was not significantly different from 0.5 μM TGN and 1 μM TGN and control groups ($p > 0.999$).

When comparing the effects of the two lignans, there was no difference between the effect of 10 μM ATG and 10 μM TGN ($p > 0.05$). However, 20 μM TGN had a higher effect on contraction frequency than 20 μM ATG ($p < 0.05$) and comparable to 40 μM ATG over the same period ($p > 0.05$) [Fig. 4].

CNQX, AP5, propranolol plus phentolamine, L-NAME and atropine (0.1 μM) had no effect on the frequency of contractions ($p > 0.999$). 0.5 μM nifedipine increased the period time of contractions significantly ($p < 0.05$), to a similar degree as 40 μM ATG and 20 μM TGN ($p > 0.999$) [Fig. 4]. 1 μM nifedipine abolished the phasic contractions totally, this effect reversed after 15 min of washout with Tyrode's solution [Fig. 2].

3.3. Effects of the dibenzyl butyrolactone lignans on contractile tone

Incubation with DMSO-containing Tyrode slightly increased the tonicity of the ileum segments, but this change was insignificant compared to baseline and its effect was the same as the effect of pure Tyrode ($p > 0.05$). ATG had no effect on tone in all the tested concentrations up to 40 μM , but a dose-dependent tendency to decrease the tone was observed. The effect was similar in case of TGN, but here, 20 μM TGN significantly decreased the tonicity ($p < 0.001$), the effect of lower concentrations was nonsignificant. The tone was unaffected by CNQX, AP5, propranolol plus phentolamine and atropine ($p > 0.05$). 0.5 μM nifedipine significantly lowered the tone ($p < 0.05$) and this activity was comparable to that of 20 μM TGN ($p > 0.05$). Conversely, L-NAME significantly increased the tone after 30 min ($p < 0.05$), while at 15 min, the effect appeared only as a tendency [Fig. 5].

3.4. Effects of the dibenzyl butyrolactone lignans may be mediated via calcium channels

As L-NAME, a specific antagonist of L-type calcium channels exerted a similar effect on contraction parameters of the isolated ileum segments than ATG and TGN, additional experiments were performed to study the possible mechanism of action via calcium channels. A specific L-type calcium channel agonist, Bay K 8644 was applied in pretreatment and also in combination with ATG to test their antagonistic effects. Perfusion with Bay K 8644 (0.5 μM) increased the amplitude of spontaneous contractions significantly compared to baseline activity, while the tone was somewhat increased and period time did not change. Subsequent

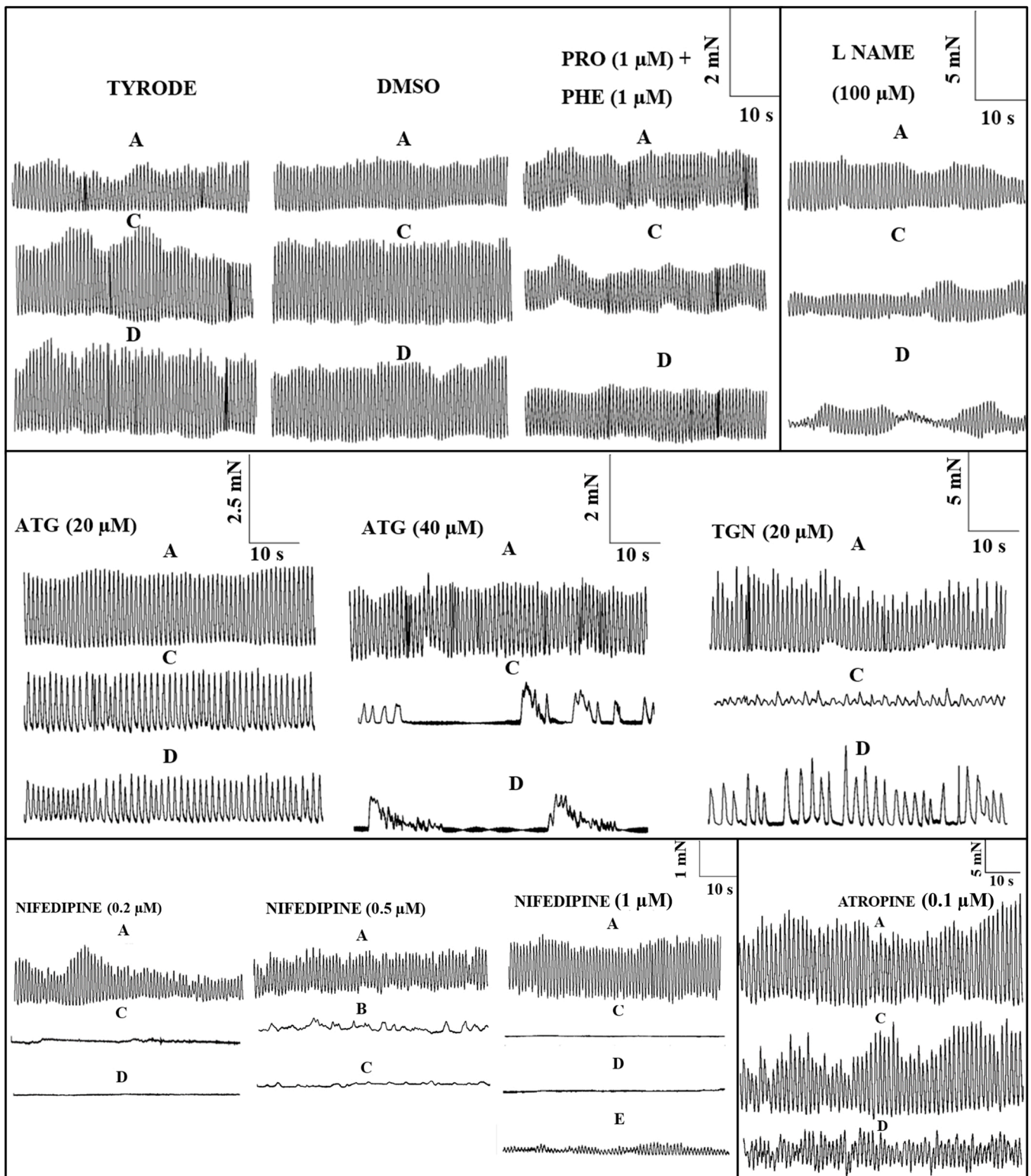


Fig. 2. Representative experimental recordings of spontaneous contractions of isolated rat ileum before and after treatment with Tyrode, DMSO, arctigenin (ATG), trachelogenin (TGN), propranolol plus phentolamine (Pro+Phe), L-NAME, nifedipine and atropine. (A) Baseline (B) Effect after 5 min (C) Effect after 15 min (D) Effect after 30 min and (E) Tyrode solution washout. 40 μM ATG and 20 μM TGN showed an increased period time and decreased amplitude after 15 and 30 min, which was absent in Tyrode, DMSO, propranolol plus phentolamine (Pro+Phe), L-NAME and atropine. 1 μM nifedipine abolished the phasic contractions, which was in turn restored after 15 min of Tyrode's solution washout.

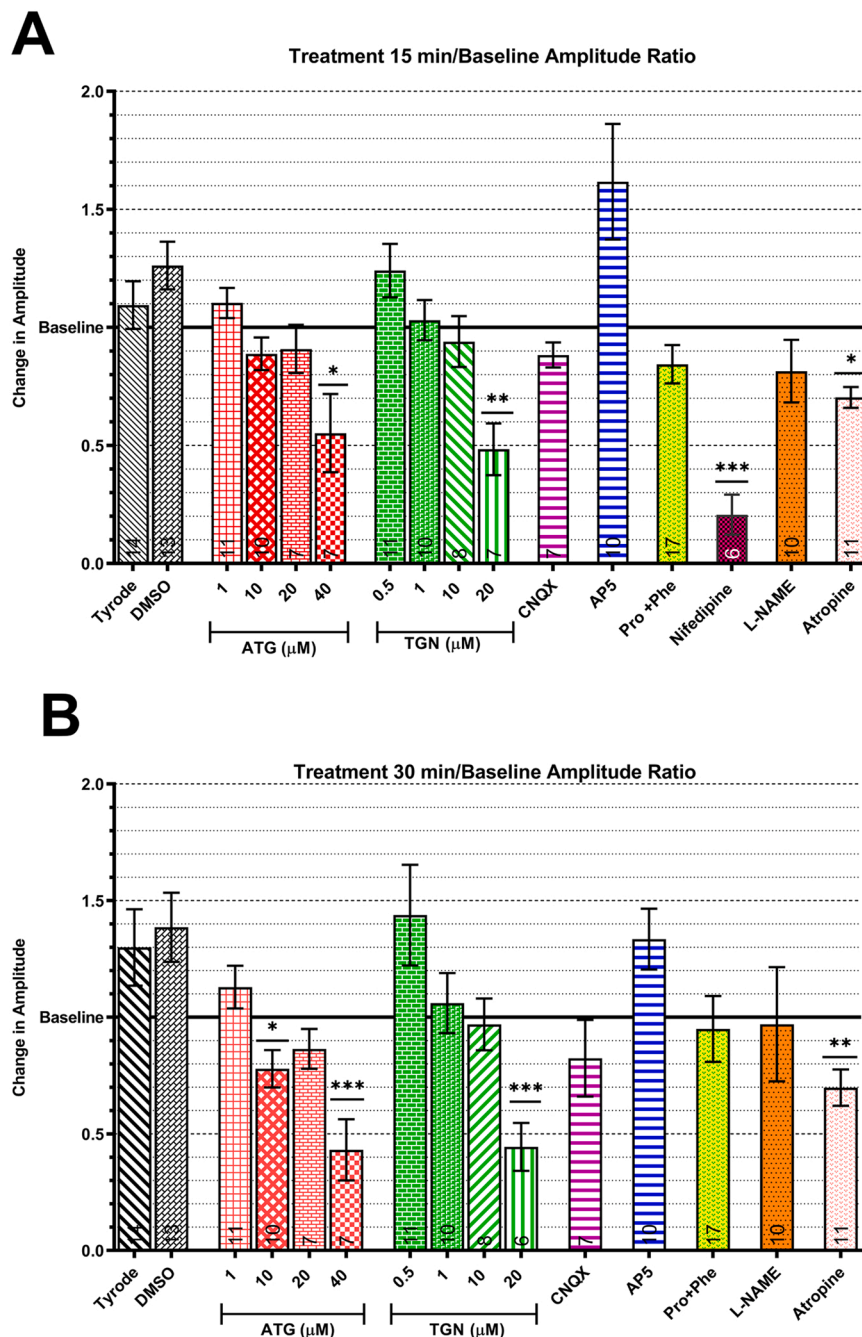


Fig. 3. The change in contractile amplitude of isolated rat ileum after treatment with arctigenin (ATG) and trachelogenin (TGN). Panels A and B show the effect of different concentrations of arctigenin (ATG) and trachelogenin (TGN) on contraction amplitude after 15 and 30 min, respectively. Bars represent mean values \pm SEM, * p < 0.05, ** p < 0.01, and *** p < 0.001, indicates the differences in mean from DMSO control. The numbers in each bar indicate sample numbers. Concentrations of antagonists were: CNQX (10 μ M), AP5 (20 μ M), Propranolol (1 μ M) plus Phentolamine [Pro+Phe] (1 μ M), L-NAME (100 μ M) and atropine (0.1 μ M). The effect of 10 μ M ATG, 40 μ M ATG and 0.5 μ M nifedipine was significantly different from DMSO control. Activity of 0.5 μ M nifedipine however was measured after 5 min as the phasic contractions were abolished after 15 min.

perfusion with ATG (50 μ M) in the absence of Bay K 8644 had an opposite effect, it lead to the decrease in amplitude and tone, and contractions became less frequent, as shown by the significant increase in period time [Fig. 6A]. If perfusion with Bay K 8644 was followed by perfusion with ATG in combination with Bay K 8644, the effects were similar although the presence of Bay K 8644 decreased the inhibitory action of ATG on intestinal motility [Fig. 6B].

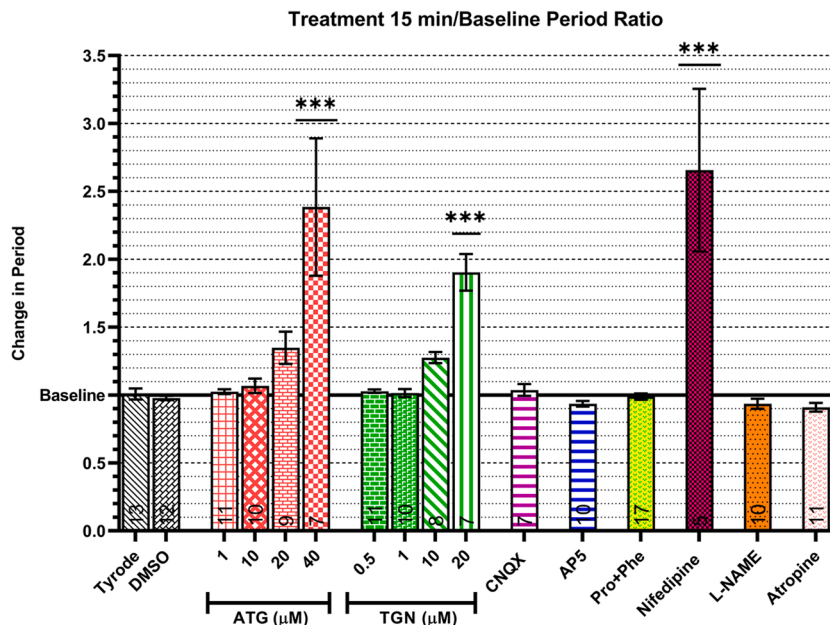
4. Discussion

Arctium lappa and *Cirsium arvense* have been used as traditional medicine in alleviation of constipation and diarrhea, respectively [1,2, 27–29]. The underlying mechanisms of effect are, however, unknown. There are some sporadic data about the effects of their bioactive compounds on different test systems. In isolated organs of laboratory

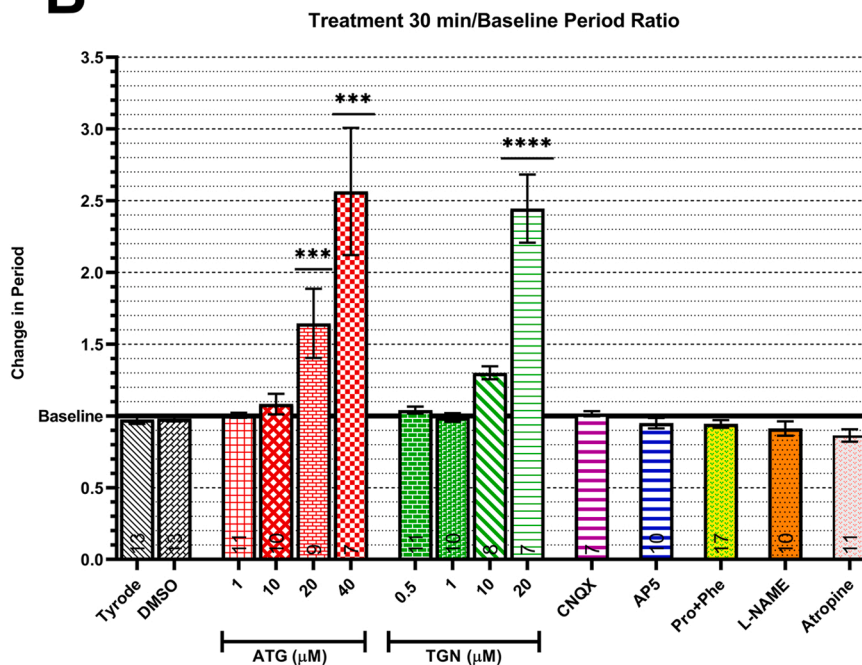
animals, ATG and TGN have been reported as smooth muscle relaxants and calcium antagonists [20–22]. Later, ATG has been shown to be an L-type calcium ion channel antagonist in airway smooth muscle tissue [22]. ATG produced blood pressure decrease via endothelial nitric oxide level elevation [22,23,25]. A memory ameliorating effect of ATG, presumably via acetylcholinesterase inhibition has also been reported in mice [23]. Besides, antigitamatergic effects of ATG on brain tissue have also been demonstrated [10].

We aimed to investigate the effects of ATG and TGN on the motility of isolated rat ileum and to elucidate their probable mechanism of action. Altogether, the two compounds exerted an inhibitory action on ileum motility. The most prominent effect of the lignans was a dose-dependent decrease in the frequency of spontaneous contractions, together with a decrease in contraction amplitude, and in the case of TGN at high concentrations, a decrease in the tonicity. There was a clear

A



B



difference in the potency of the two lignans on this test system, with TGN being about twice as potent as ATG. 40 μM ATG or 20 μM TGN evoked irregular contractions, characterized by prolonged contractile period and decreased amplitude, while at lower concentrations, only the effect on contraction period could be observed, with the preservation of a regular pattern.

The regulation of gastro-intestinal motility depends on the interaction and communication between enteric neurons, ICCs and gastrointestinal smooth muscle cells [30]. Acetylcholine, norepinephrine and non-adrenergic non-cholinergic mediators such as nitric oxide are the main enteric neurotransmitters that control intestinal motility. In the ileum, acetylcholine triggers smooth muscle contraction by binding to

M2 and M3 muscarinic receptors, which increase cytosolic calcium levels via activation of nonselective cation channels in the plasma membrane and release of intracellular calcium [31,32]. M2 receptors act via Gi/o proteins by partly inhibiting cyclic adenosine monophosphate (cAMP) and ion channels in smooth muscle cells, while M3 acts via Gq/11 proteins that activate phospholipase C (PLC), leading to the formation of inositol 1,4,5-trisphosphate (IP3), which in turn causes the release of calcium ions from the sarcoplasmic reticulum [33].

Also present in the rat ileum are α₁-, α_{2D}-, β₁-, β₂-, and β₃-adrenoreceptors [34–37]. Norepinephrine (noradrenaline) acts presynaptically to decrease the activity of cholinergic neurons in the enteric nervous system by activating α₂-adrenoreceptors, which couple to G_i proteins

Fig. 4. The dose-dependent increase in contractile period of isolated rat ileum elicited by arctigenin (ATG) and trachelogenin (TGN). Panels A and B show the effect of different concentrations of arctigenin (ATG) and trachelogenin (TGN) on period of contraction after 15 and 30 min, respectively. Bars represent mean ± SEM, ***p<0.001, ****p<0.0001 indicates significant difference from DMSO control. The numbers in each bar indicate sample numbers. Concentrations for antagonists were: CNQX (10 μM), AP5 (20 μM), Propranolol (1 μM) plus Phentolamine (1 μM) (Pro_Phe), L-NAME (100 μM), and atropine (0.1 μM).

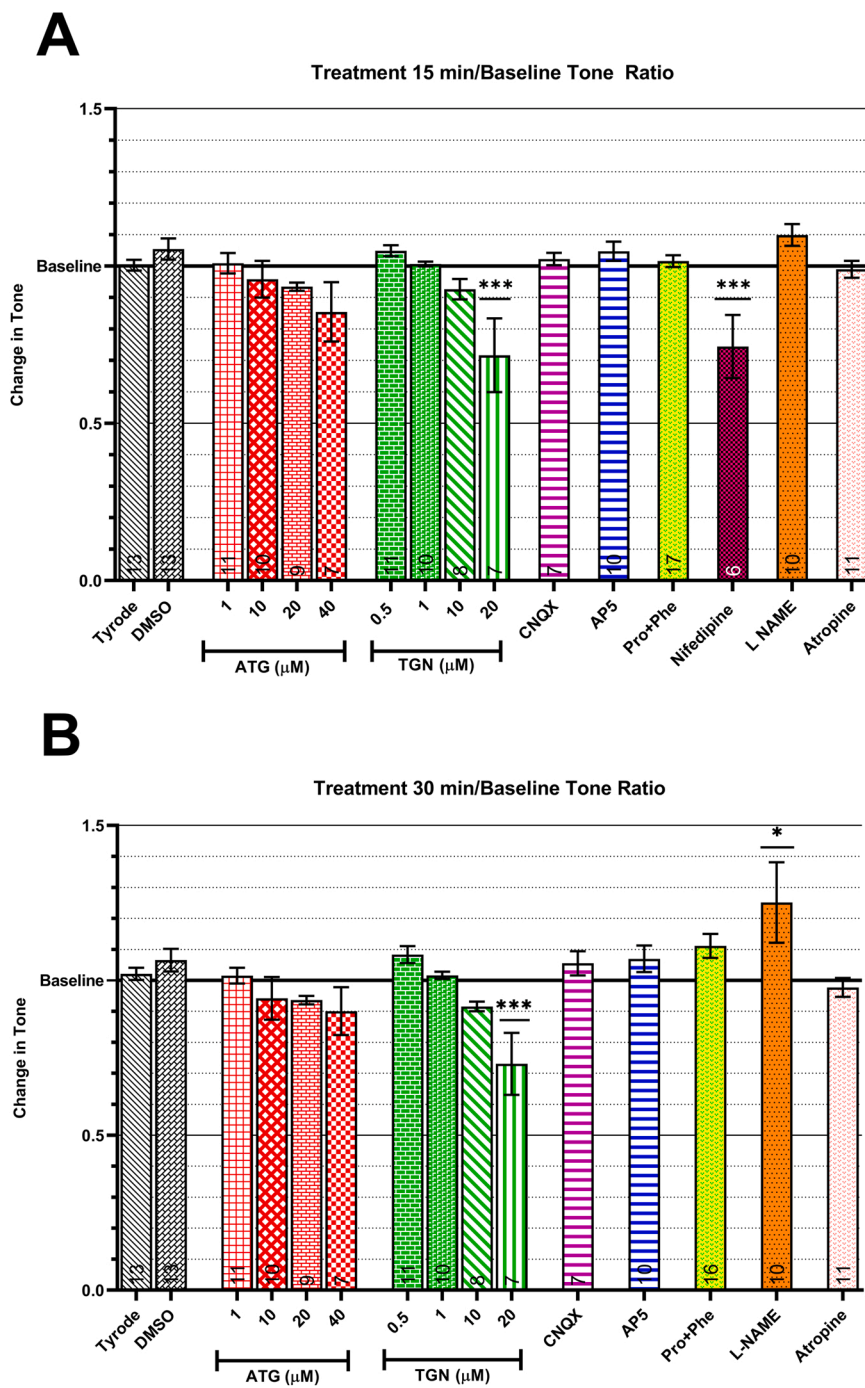


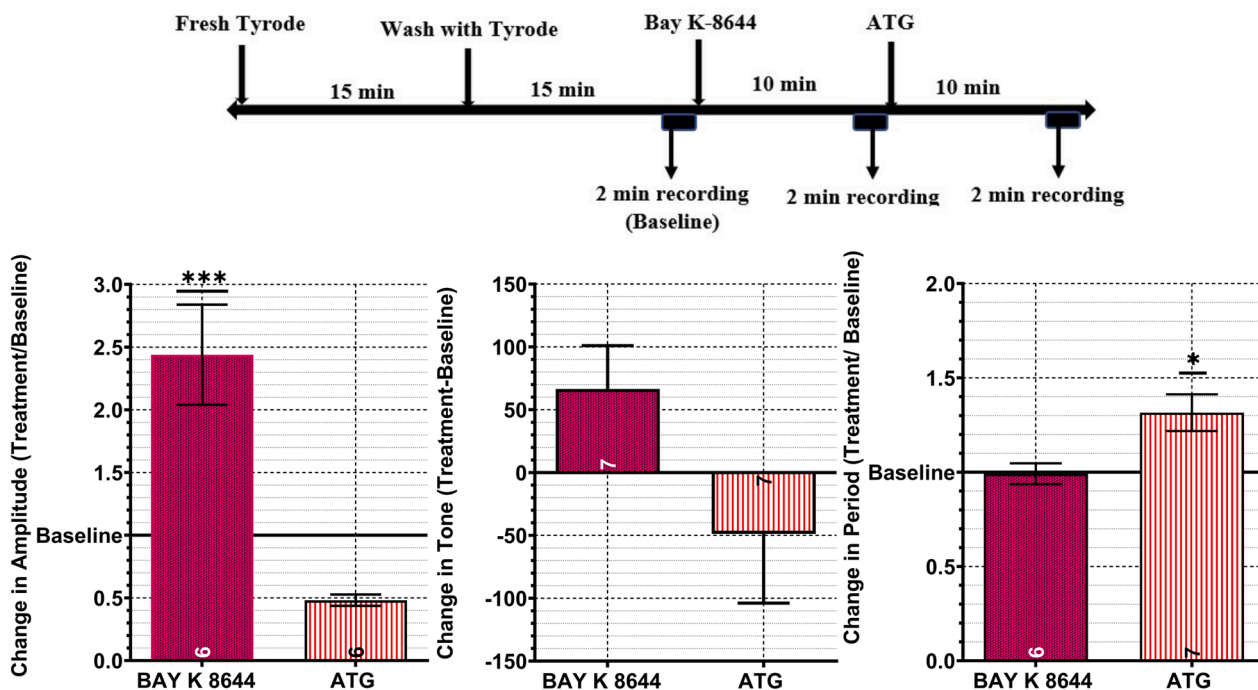
Fig. 5. The dose-dependent decrease in tone of isolated rat ileum elicited by arctigenin (ATG) and trachelogenin (TGN). Panels A and B show the effect of different concentrations of arctigenin (ATG) and trachelogenin (TGN) on contraction tone after 15 and 30 min, respectively. Bars represent mean \pm SEM, * $p < 0.05$, *** $p < 0.001$ indicates significant difference from DMSO control. The numbers in each bar indicate sample numbers. Concentrations of antagonists were: CNQX (10 μ M), AP5 (20 μ M), propranolol (1 μ M) plus phentolamine (1 μ M), L-NAME (100 μ M) and atropine (0.1 μ M).

and inhibit adenylate cyclase, thereby decreasing cAMP levels [38]. Norepinephrine also acts directly on intestinal smooth muscle cells by activating β -adrenoreceptors, which couple to G_s proteins and activate adenylyl cyclase leading to an increase in cAMP, which in turn activates protein kinase A, which phosphorylates myosin light chain kinase, resulting in smooth muscle relaxation [35,38]. Nitric oxide is synthesized from L-arginine by nitric oxide synthase, which exists in three isoforms: neuronal, endothelial and inducible nitric oxide synthase. Neuronal nitric oxide synthase-expressing neurons are located in the myenteric ganglia throughout the gastrointestinal tract [39] and generate nitric oxide, which mediates smooth muscle relaxation by activating soluble guanylate cyclase, thereby increasing cyclic guanosine monophosphate level [40]. Calcium is a fundamental second messenger in smooth muscle cells responsible for smooth muscle

contraction. An influx of extracellular calcium ions via L-type calcium ion channels or the release of calcium ions from the sarcoplasmic reticulum lead to elevation in cytosolic calcium levels, activating a signaling cascade leading to smooth muscle contraction [15]. Therefore, exogenous agents that interfere with cytoplasmic calcium levels affect intestinal motility [41].

First, we speculated that the inhibitory effects of the lignans on ileum motility could be mediated by an adrenergic mechanism. The synergistic effect of arctigenin with β_2 -adrenoreceptor agonists on airway smooth muscle relaxation has been demonstrated [42]. To investigate the involvement of adrenergic receptors, a combination of phentolamine (non-selective α -adrenergic receptor antagonist) and propranolol (non-selective β -adrenergic receptor antagonist) was tested. The results were consistent with previous studies in the rat ileum showing no effect

A



B

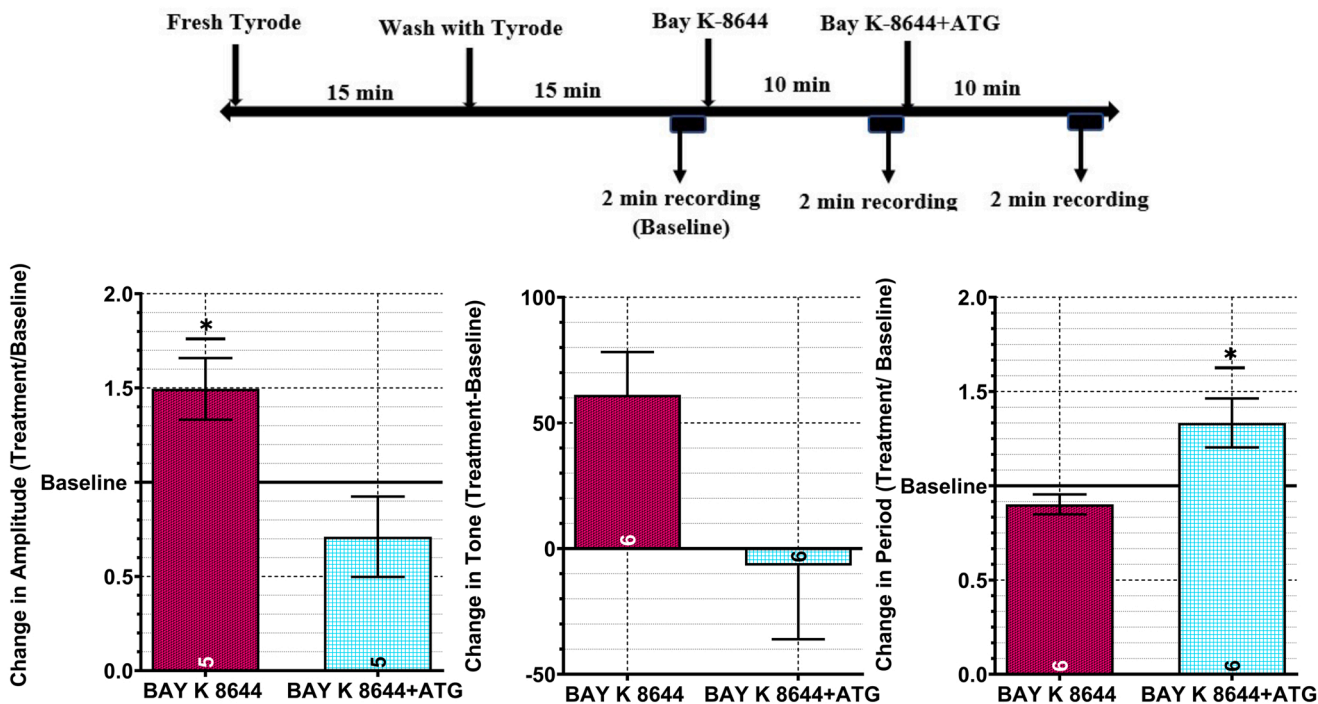


Fig. 6. The effects of arctigenin (ATG) on the amplitude, tone and motility period after the L-type calcium ion channel was activated. Panels A show the effect of arctigenin (ATG) [50 μM] after perfusion of the L-type calcium ion channel agonist (Bay K 8644) at 0.5 μM. Panels B show the effect of arctigenin (ATG) [50 μM] + Bay K 8644 [0.5 μM] in combination with Bay K 8644 (0.5 μM). Bars represent mean ± SEM, ***p<0.001, *p<0.05 indicates significant difference from the baseline. The numbers in each bar indicate sample numbers.

on muscle tone [43] and confirmed the ineffectiveness of norepinephrine on the frequency of contractions [37]. Thus, in our preparation, blockade of adrenergic receptors had no effect on the contraction frequency and tone.

To determine the lignans' effect on the cholinergic pathway, we tested a muscarinic receptor antagonist, atropine, which decreased the amplitude of the contraction but had no effect on period time or tone. The result is consistent with previous studies in rabbit ileum that showed no effect on frequency [44]. Blockade of muscarinic receptors by atropine resulted in decrease in cytosolic calcium level and thus a decrease in amplitude.

However, our results indicate a clear relaxant effect of the test lignans, as evidenced by prolongation of contractile period and decrease in contraction amplitude and tone. The effect of ATG on nitric oxide production has been demonstrated in previous studies [25,45]. Therefore, we investigated whether the relaxant effect was mediated via the nitric oxide pathway. To determine this effect, we tested L-NAME (a nitric oxide synthase inhibitor) that showed an increase in tone, but no change in period or amplitude of the spontaneous ileum contractions. These effects are consistent with those obtained on rabbit ileum at the same concentration after 5 min [46]. The increase in tone may be due to increased acetylcholine release as demonstrated in dog and guinea pig ileum circular muscle [47]. Nitric oxide had the same effects as in the human small intestine [48]. Nitric oxide is a retrograde messenger that increases the release of glutamate, which in turn decreases acetylcholine release; thus, inhibition of the enzyme may have led to elevated acetylcholine level.

As anti-glutamatergic effects of ATG have been previously indicated [10], we investigated whether manipulating glutamatergic transmission would mimic ATG and TGN effects. The presence of glutamate receptors in the enteric nervous system has been demonstrated [49–51]. To determine their possible involvement in the ileal motility, the non-NMDA receptor antagonist CNQX and the NMDA receptor antagonist AP5 were tested. An observable increase in amplitude occurred after administration of AP5, but no effect was observed with CNQX on the period and tone of the spontaneous ileum contractions. The lack of CNQX effects on ileal contraction frequency was consistent with previous results on mouse colon [51]. The effect of AP5 may be attributed to increased release of acetylcholine in intrinsic neurons, as the inhibitory effect of glutamate on acetylcholine release in the rat ileum is mediated by NMDA receptors [52]. Thus, these findings suggest that the glutamatergic effect on ileum motility may be mediated indirectly via the modulation of acetylcholine and noradrenaline release from the myenteric plexus, as previously shown [53–55].

Finally, the prolonged contractile period and decreased contractile amplitude caused by the test lignans may be attributed to decreased cytosolic calcium ion levels, which decreases myosin light chain kinase activity, resulting in smooth muscle relaxation. Regulation of calcium ion influx through L-type calcium channels (dihydropyridine channel) is critical in controlling the contractile state of smooth muscle and a calcium ion action potential is generated when the threshold is reached [41]. To address this issue, we investigated whether the blockade of L-type calcium ion channels could be in the background of the observed lignan effects on ileum motility. Nifedipine, an antagonist of L-type calcium channels produced a similar alteration in contraction pattern as that caused by ATG and TGN. A disruption of the spontaneous contraction pattern occurred, characterized by smaller contractions followed by a delay before another contractile event. The decreased intracellular calcium level may have inhibited the initiation of the action potential in smooth muscle cells. The effects observed by us after treatment with 1 μ M nifedipine are consistent with previous results in rabbit ileum [44]. An agonist of L-type calcium channels, Bay K 8644 had an effect opposite to that of nifedipine: increase in contraction amplitude and tonicity occurred. In some samples, frequency of contractions increased as well. Arctigenin could counteract these actions in the presence of Bay K 8644, suggesting that the two compounds may

have shared targets. Altogether, although our results do not provide information on how lignans block the L-type calcium ion channel in rat ileum on the molecular level, but they represent strong evidence on the involvement of L-type calcium ion channels in the mechanism of action of the blocking effects of lignans on the intestine. The stronger activity of TGN compared to ATG was possibly due to its higher potency as a calcium antagonist with an IC_{50} of 1.1 μ M compared with IC_{50} of 8.8 μ M of ATG [20]. The difference in activity could be due to the variation in molecular structure [56].

5. Conclusion

In conclusion, the present study demonstrates relaxant effects of arctigenin and trachelogenin in isolated rat ileum. The results indicate that the effect is not mediated by nitric oxide, cholinergic, adrenergic or glutamatergic signaling pathways while L-type calcium ion channel blockade is likely to be involved, although further studies on the molecular level are required to ascertain this. Thus, we propose a possible pharmacological basis for traditional uses of *Arctium lappa* and *Cirsium arvense* for treatment of gastrointestinal disorders. Arctigenin and trachelogenin may be applied as an alternative L-type calcium ion channel blocker and used in the management of gastrointestinal disorders. There is also a need to identify and study other medicinal plants of Asteraceae family due to the presence of potential antispasmodic (spasmodic) compounds.

CRedit authorship contribution statement

Peter Kiplang'at Koech: Investigation, Formal analysis, Visualization, Writing – original draft. **Imre Boldizsár:** Investigation, Writing – review & editing. **Árpád Dobolyi:** Conceptualization, Methodology, Supervision, Writing – review & editing. **Petra Varró:** Conceptualization, Methodology, Supervision, Writing – review & editing.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.toxrep.2022.05.019](https://doi.org/10.1016/j.toxrep.2022.05.019).

References

- [1] F. Steven, D. James, *A Field Guide to Medicinal Plants and Herbs of Eastern and Central North America*, second ed., Houghton Mifflin Harcourt., New York, 2000.
- [2] D. Wang, A.S. Bădărau, M.K. Swamy, S. Shaw, F. Maggi, L.E. da Silva, V. López, A. W.K. Yeung, A. Mocan, A.G. Atanasov, Arctium species secondary metabolites chemodiversity and bioactivities, *Front. Plant Sci.* 10 (2019), <https://doi.org/10.3389/fpls.2019.00834>.

- [3] A. Solyomváry, Z. Mervai, G. Tóth, Á.E. Ress, B. Noszál, I. Molnár-Perl, K. Baghy, I. Kovalszky, I. Boldizsár, A simple and effective enrichment process of the antiproliferative lignan arctigenin based on the endogenous enzymatic hydrolysis of *Serratula tinctoria* and *Arctium lappa* fruits, *Process Biochem* (2015), <https://doi.org/10.1016/j.procbio.2015.09.011>.
- [4] I. Boldizsár, Z. Füzfa, F. Tóth, É. Sedlák, L. Borsodi, I. Molnár-Perl, Mass fragmentation study of the trimethylsilyl derivatives of arctiin, matairesinoid, arctigenin, phylligenin, matairesinol, pinoresinol and methylarctigenin: their gas and liquid chromatographic analysis in plant extracts, *J. Chromatogr. A* 1217 (2010) 1674–1682, <https://doi.org/10.1016/j.chroma.2010.01.019>.
- [5] I. Boldizsár, M. Kraszni, F. Tóth, B. Noszál, I. Molnár-Perl, Complementary fragmentation pattern analysis by gas chromatography-mass spectrometry and liquid chromatography tandem mass spectrometry confirmed the precious lignan content of *Cirsium* weeds, *J. Chromatogr. A* 1217 (2010) 6281–6289, <https://doi.org/10.1016/j.chroma.2010.08.018>.
- [6] D. Simpson, S. Amos, Other plant metabolites, *Pharmacogn. Fundam., Appl. Strategy* (2017) 267–280, <https://doi.org/10.1016/B978-0-12-802104-0.00012-3>.
- [7] Z. Mervai, A. Solyomváry, G. Tóth, B. Noszál, I. Molnár-Perl, K. Baghy, I. Kovalszky, I. Boldizsár, Endogenous enzyme-hydrolyzed fruit of *Cirsium brachycephalum*: Optimal source of the antiproliferative lignan trachelogenin regulating the Wnt/ β -Catenin signaling pathway in the SW480 colon adenocarcinoma cell line, *Fitoterapia* 100 (2015) 19–26, <https://doi.org/10.1016/j.fitote.2014.10.017>.
- [8] R.B. Taponno, S. Kusari, M. Spittler, Recent advances in research on lignans and neolignans, *Nat. Prod. Rep.* (2016), <https://doi.org/10.1039/c6np00021e>.
- [9] Y.P. Jang, S.R. Kim, Y.H. Choi, J. Kim, S.G. Kim, G.J. Markelonis, T.H. Oh, Y. C. Kim, Arctigenin protects cultured cortical neurons from glutamate-induced neurodegeneration by binding to kainate receptor, *J. Neurosci. Res.* (2002), <https://doi.org/10.1002/jnr.10204>.
- [10] S. Borbély, G. Jócsák, K. Moldován, É. Sedlák, É. Preininger, I. Boldizsár, A. Tóth, P.T. Atlason, E. Molnár, I. Világi, Arctigenin reduces neuronal responses in the somatosensory cortex via the inhibition of non-NMDA glutamate receptors, *Neurochem. Int.* (2016), <https://doi.org/10.1016/j.neuint.2016.03.006>.
- [11] A. Aggarwal, M. Bhatt, Commonly used gastrointestinal drugs. *Handbook of Clinical Neurology*, first ed., Elsevier B.V., 2014 <https://doi.org/10.1016/B978-0-7020-4087-0.00043-7>.
- [12] J.B. Furness, B.P. Callaghan, L.R. Rivera, H.J. Cho, The enteric nervous system and gastrointestinal innervation: integrated local and central control, *Adv. Exp. Med. Biol.* (2014), https://doi.org/10.1007/978-1-4939-0897-4_3.
- [13] R. Serio, M.G. Zizzo, M. Mastropalo, The enteric nervous system: new developments and emerging concepts, *Malta Med. J.* (2011) 23.
- [14] K.M. Sanders, S.M. Ward, S.D. Koh, Interstitial cells: regulators of smooth muscle function, *Physiol. Rev.* (2014), <https://doi.org/10.1152/physrev.00037.2013>.
- [15] I.Y. Kuo, B.E. Ehrlich, Signaling in muscle contraction, *Cold Spring Harb. Perspect. Biol.* (2015), <https://doi.org/10.1101/cshperspect.a006023>.
- [16] K.M. Sanders, S.D. Koh, S. Ro, S.M. Ward, Regulation of gastrointestinal motility: insights from smooth muscle biology, *Nat. Rev. Gastroenterol. Hepatol.* (2012), <https://doi.org/10.1038/nrgastro.2012.168>.
- [17] M.T. Liu, J.D. Rothstein, M.D. Gershon, A.L. Kirchgessner, Glutamatergic enteric neurons, *J. Neurosci.* (1997), <https://doi.org/10.1523/jneurosci.17-12-04764.1997>.
- [18] V.A.C. Barney, A.F.O. Hernández, The role of antispasmodics in managing irritable bowel syndrome, *Rev. Colomb. Gastroenterol.* (2019), <https://doi.org/10.22516/25007440.309>.
- [19] S.H. Hejazian, S.M. Bagheri, F. Safari, Spasmolytic and anti-spasmodic action of *Trachyspermum ammi* essence on rat's ileum contraction, *New Am. J. Med. Sci.* (2014), <https://doi.org/10.4103/1947-2714.147982>.
- [20] K. Ichikawa, T. Kinoshita, U. Sankawa, S. Nishibe, The Ca²⁺ antagonist activity of lignans, *Chem. Pharm. Bull.* 34 (1986) 3514–3517, <https://doi.org/10.1248/cpb.34.3514>.
- [21] J.M. Yang, P.S.P. Ip, C.T. Che, J.H.K. Yeung, Relaxant effects of *Schisandra chinensis* and its major lignans on agonists-induced contraction in guinea pig ileum, *Phytomedicine* 18 (2011) 1153–1160, <https://doi.org/10.1016/j.phymed.2011.06.003>.
- [22] M. Zhang, K.H. Zang, J.L. Luo, F.P. Leung, Y. Huang, C.Y. Lin, Z.J. Yang, A.P. Lu, X. D. Tang, H.X. Xu, J.J.Y. Sung, Z.X. Bian, Magnolol inhibits colonic motility through down-regulation of voltage-sensitive L-type Ca²⁺ channels of colonic smooth muscle cells in rats, *Phytomedicine* (2013), <https://doi.org/10.1016/j.phymed.2013.07.008>.
- [23] I.-A. Lee, E.-H. Joh, D.-H. Kim, Arctigenin isolated from the seeds of *arctium lappa* ameliorates memory deficits in mice, *Planta Med.* 77 (2011) 1525–1527, <https://doi.org/10.1055/s-0030-1270746>.
- [24] Y. Liu, G. Wang, M. Yang, H. Chen, Y. Zhao, S. Yang, C. Sun, Arctigenin reduces blood pressure by modulation of nitric oxide synthase and NADPH oxidase expression in spontaneously hypertensive rats, *Biochem. Biophys. Res. Commun.* (2015), <https://doi.org/10.1016/j.bbrc.2015.11.041>.
- [25] Z. Zhao, Y. Yin, Z. Wang, R. Fang, H. Wu, M. Jiang, G. Bai, G. Luo, Arctigenin exhibits relaxation effect on bronchus by affecting transmembrane flow of calcium, *Biol. Trace Elem. Res.* 156 (2013) 181–187, <https://doi.org/10.1007/s12011-013-9839-9>.
- [26] P.M. Guarrera, Traditional phytotherapy in central Italy (Marche, Abruzzo, and Latium), *Fitoterapia* 76 (2005) 1–25, <https://doi.org/10.1016/j.fitote.2004.09.006>.
- [27] S. Wetzel, L.C. Duchesne, M.F. Laporte, *Bioproducts from Canada's Forests: New Partnerships in the Bioeconomy*, Springer Science & Business Media, 2006.
- [28] A.B.A. De Almeida, M. Sánchez-Hidalgo, A.R. Martín, A. Luiz-Ferreira, J.R. Trigo, W. Vilegas, L.C. Dos Santos, A.R.M. Souza-Brito, C.A. De La Lastra, Anti-inflammatory intestinal activity of *Arctium lappa* L. (Asteraceae) in TNBS colitis model, *J. Ethnopharmacol.* (2013), <https://doi.org/10.1016/j.jep.2012.12.048>.
- [29] O. Al-Shboul, The importance of interstitial cells of cajal in the gastrointestinal tract, *Saudi J. Gastroenterol.* 19 (2013) 3–15, <https://doi.org/10.4103/1319-3767.105909>.
- [30] G.Y. Sy, A. Sarr, A.M. Dièye, B. Faye, Myorelaxant and antispasmodic effects of the aqueous extract of *Mitragyna inermis* barks on Wistar rat ileum, *Fitoterapia* 75 (2004) 447–450, <https://doi.org/10.1016/j.fitote.2004.03.006>.
- [31] T. Unno, H. Matsuyama, T. Sakamoto, M. Uchiyama, Y. Izumi, H. Okamoto, M. Yamada, J. Wess, S. Komori, M 2 and M 3 muscarinic receptor-mediated contractions in longitudinal smooth muscle of the ileum studied with receptor knockout mice, *Br. J. Pharm.* (2005), <https://doi.org/10.1038/sj.bjp.0706300>.
- [32] Y. Tanahashi, S. Komori, H. Matsuyama, T. Kitazawa, T. Unno, Functions of muscarinic receptor subtypes in gastrointestinal smooth muscle: A review of studies with receptor-knockout mice, *Int. J. Mol. Sci.* 22 (2021) 1–24, <https://doi.org/10.3390/ijms22020926>.
- [33] L. Liu, I.M. Coupar, Evidence for functional α 2D- adrenoceptors in the rat intestine, *Br. J. Pharm.* 117 (1996) 787–792, <https://doi.org/10.1111/j.1476-5381.1996.tb15261.x>.
- [34] N. Mirzaei Damabi, A.A. Moazedi, S.M. Seyyednejad, The role of α - and β - adrenergic receptors in the spasmolytic effects on rat ileum of *Petroselinum crispum* Latifolium (parsley), *Asian Pac. J. Trop. Med* (2010), [https://doi.org/10.1016/S1995-7645\(10\)60208-8](https://doi.org/10.1016/S1995-7645(10)60208-8).
- [35] S.J. Roberts, M. Papaioannou, B.A. Evans, R.J. Summers, Characterization of β -adrenoceptor mediated smooth muscle relaxation and the detection of mRNA for β 1-, β 2- and β 3-adrenoceptors in rat ileum, *Br. J. Pharm.* (1999), <https://doi.org/10.1038/sj.bjp.0702605>.
- [36] R. Seiler, A. Rickenbacher, S. Shaw, B.M. Balsiger, α - and β -adrenergic receptor mechanisms in spontaneous contractile activity of rat ileal longitudinal smooth muscle, *J. Gastrointest. Surg.* (2005), <https://doi.org/10.1016/j.gassur.2004.05.012>.
- [37] L.E.A. Montgomery, E.A. Tansey, C.D. Johnson, S.M. Roe, J.G. Quinn, Autonomic modification of intestinal smooth muscle contractility, *Adv. Physiol. Educ.* 40 (2016) 104–109, <https://doi.org/10.1152/advan.00038.2015>.
- [38] M.K. Jarvinen, W.J. Wollmann, T.A. Powrozek, J.A. Schultz, T.L. Powley, Nitric oxide synthase-containing neurons in the myenteric plexus of the rat gastrointestinal tract: Distribution and regional density, *Anat. Embryol.* 199 (1999), <https://doi.org/10.1007/s004290050213>.
- [39] N.M. Matsuda, S.M. Miller, Non-adrenergic non-cholinergic inhibition of gastrointestinal smooth muscle and its intracellular mechanism(s), *Fundam. Clin. Pharm.* (2010), <https://doi.org/10.1111/j.1472-8206.2009.00761.x>.
- [40] K.M. Sanders, Signal Transduction in Smooth Muscle Invited Review: mechanisms of calcium handling in smooth muscles, *J. Appl. Physiol.* 91 (2001) 1438–1449, <https://doi.org/10.1152/jappl.2001.91.3.1438>.
- [41] Y. Hou, B. Cheng, M. Zhou, R. Fang, M. Jiang, W. Hou, G. Bai, Searching for synergistic bronchodilators and novel therapeutic regimens for chronic lung diseases from a traditional Chinese medicine, *Qingfei Xiaoyan Wan*, *PLoS One* 9 (2014), <https://doi.org/10.1371/journal.pone.0113104>.
- [42] E. Nocerino, A.A. Izzo, F. Borrelli, F. Capasso, R. Capasso, A. Pinto, L. Sautebin, N. Mascolo, Relaxant effect of capsaepine in the isolated rat ileum, *Naunyn. Schmiede Arch. Pharmacol.* 365 (2002) 187–192, <https://doi.org/10.1007/s00210-001-0522-x>.
- [43] L. Grasa, E. Rebollar, M.P. Arruebo, M.A. Plaza, M.D. Murillo, The role of Ca²⁺ in the contractility of rabbit small intestine in vitro, *J. Physiol. Pharmacol.* 55 (2004) 639–650.
- [44] F. Zhao, L. Wang, K. Liu, In vitro anti-inflammatory effects of arctigenin, a lignan from *Arctium lappa* L., through inhibition on iNOS pathway, *J. Ethnopharmacol.* (2009), <https://doi.org/10.1016/j.jep.2009.01.038>.
- [45] L. Grasa, E. Rebollar, M.P. Arruebo, M.A. Plaza, M.D. Murillo, The role of NO in the contractility of rabbit small intestine in vitro: effect of K⁺ channels, *J. Physiol. Pharmacol.* (2005).
- [46] H. Kilbinger, Modulation of acetylcholine release by nitric oxide, *Prog. Brain Res* 109 (1996) 219–224, [https://doi.org/10.1016/S0079-6123\(08\)62105-6](https://doi.org/10.1016/S0079-6123(08)62105-6).
- [47] V. Wittmeyer, T. Merrot, B. Mazet, Tonic inhibition of human small intestinal motility by nitric oxide in children but not in adults, *Neurogastroenterol. Motil.* (2010), <https://doi.org/10.1111/j.1365-2982.2010.01532.x>.
- [48] A.L. Kirchgessner, M.T. Liu, F. Alcantara, Excitotoxicity in the enteric nervous system, *J. Neurosci.* (1997), <https://doi.org/10.1523/jneurosci.17-22-08804.1997>.
- [49] A. Baj, E. Moro, M. Bistoletti, V. Orlandi, F. Crema, C. Giaroni, Glutamatergic signaling along the microbiota-gut-brain axis, *Int. J. Mol. Sci.* (2019) 20, <https://doi.org/10.3390/ijms20061482>.
- [50] M. Seifi, J.D. Swinny, Immunolocalization of AMPA receptor subunits within the enteric nervous system of the mouse colon and the effect of their activation on spontaneous colonic contractions, *Neurogastroenterol. Motil.* 28 (2016) 705–720, <https://doi.org/10.1111/nmo.12768>.
- [51] S.M. Jankovic, S.V. Jankovic, D.R. Milovanovic, Effect of the exogenous glutamate and the NMDA on electric field-stimulated contractions of isolated rat ileum, *Pharmacol. Res.* 50 (2004) 529–532, <https://doi.org/10.1016/j.phrs.2004.02.011>.
- [52] C. Giaroni, E. Zanetti, F. Marino, M. Cosentino, A. Senaldi, L. Somaini, M. Ferrari, R. Bombelli, S. Lecchini, G. Frigo, Glutamate receptors of the AMPA type modulate neurotransmitter release and peristalsis in the guinea-pig isolated colon, *Life Sci.* 67 (2000) 1747–1757, [https://doi.org/10.1016/S0024-3205\(00\)00760-8](https://doi.org/10.1016/S0024-3205(00)00760-8).

- [54] E.A. Milusheva, V.I. Kuneva, D.E. Itzev, N.I. Kortežova, B. Sperlagh, Z. N. Mizhorkova, Glutamate stimulation of acetylcholine release from myenteric plexus is mediated by endogenous nitric oxide, *Brain Res. Bull.* (2005), <https://doi.org/10.1016/j.brainresbull.2005.05.011>.
- [55] V. Filpa, E. Carpanese, S. Marchet, V. Prandoni, E. Moro, S. Lecchini, G. Frigo, C. Giaroni, F. Crema, Interaction between NMDA glutamatergic and nitroergic enteric pathways during in vitro ischemia and reperfusion, *Eur. J. Pharm.* (2015), <https://doi.org/10.1016/j.ejphar.2015.01.021>.
- [56] V. Temml, S. Kuehn, D. Schuster, S. Schwaiger, H. Stuppner, D. Fuchs, Interaction of *Carthamus tinctorius* lignan arctigenin with the binding site of tryptophan-degrading enzyme indoleamine 2,3-dioxygenase, *FEBS Open Bio* 3 (2013) 450–452, <https://doi.org/10.1016/j.fob.2013.08.008>.