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Effect of condensed tannins from black currant (*Ribes nigrum*) on acetylcholine-induced contractility in isolated rat intestine

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ABSTRACT: The condensed tannins (CTs) from black currant (*R. nigrum*) leaves were isolated as a tannin fraction to investigate their effect on isolated rat duodenal contraction induced by acetylcholine (ACh). The mechanisms of CT action were compared with those of a muscarinic receptor antagonist (atropine) and a calcium channel blocker (verapamil). Duodenal segments were fixed in organ bath, and the frequency, amplitude, and tone of contraction were recorded. The experiments were performed in seven groups, including the vehicle control, CTs (10, 50, 100, and 300 μg/ml), atropine (1.55×10^{-5} M), and verapamil (10^{-6} M), with cumulative additions of ACh (10^{-8}–10^{-4} M). The possibility that CTs might act by altering ion channel activity in intestinal epithelial cells was tested in a human colonic adenocarcinoma cell line by measuring the equivalent short-circuit current, compared with that induced by an adenylyl cyclase activator forskolin (10 μM). It was found that CTs at low concentrations (10 and 50 μg/ml) significantly decreased the frequency of ACh-induced duodenal contractions. Only the CTs at high concentration (300 μg/ml) were able to inhibit ACh-induced amplitude and tone of contraction, similar to those of atropine, but not verapamil. Preliminary data of equivalent short-circuit currents were unaltered after the four doses of CTs, in contrast to forskolin. It is concluded that the mechanism of CT action is more likely mediated via the muscarinic receptor signaling pathway rather than through inhibition of Ca^{2+} channels in intestinal pacemaker and smooth muscle cell membranes or epithelial cAMP-dependent Cl^- secretion.

KEYWORDS: proanthocyanidins, prodelphinidin, duodenal contraction, muscarinic receptors

INTRODUCTION

Condensed tannins (CTs) or proanthocyanidins (Pas) are classified in the tannin group according to their chemical structures. CTs are polymers of flavan-3-ols and are found abundantly in many medicinal plants and vegetable foods that are widely consumed. CTs are complex organic compounds that generally possess astringent properties and would be able to precipitate proteins, peptides, and other compounds such as alkaloids [1, 2]. The structure of CTs is oligomers and polymers of flavanols and comprises the linked flavan-3-ol units with extender and terminal flavanol subunits [3]. The biological activities of CTs have been reported along with the different characteristics in CT composition between extension and terminal units. Thiolysis with benzyl mercaptan (BM) is one of the techniques that has recently been developed to provide the quantitative and qualitative data of CTs [4]. During the thiolysis reaction, the terminal units of CTs are released mainly as free flavan-3-ols, including catechin (C), epicatechin (EC), gallocatechin (GC), and epigallocatechin (EGC), and the extension units are as benzyl mercaptan (BM) derivatives, including C-BM, EC-BM, GC-BM, and EGC-BM (Fig. 1) [4]. Moreover, the structural composition of CTs can be identified after thiolytic degradation by High-Performance Liquid Chromatography (HPLC) or Liquid Chromatography-Mass Spectrometry (LC-MS) chromatograms, such as procyanidin/prodelphinidin ratio (PC/PD), *cis*/*trans* ratio, and mean degree of polymerization (mDP) [4].

The beneficial biological activities of CTs, such as antioxidant, antimicrobial, antiviral, anti-inflammatory, anthelmintic, anti-palatable, anticancer, antithrombotic, and antidiarrheal activity, have been widely investigated [2, 5–8]. The antimicrobial activity of CTs has been shown to interfere with the ulcerogenic pathogen *Helicobacter pylori* in human gastric mucosa. In addition, CT-rich beverages have been reported to reduce *H. pylori* colonization in human gastrointestinal tract [2]. The anti-nutritional effect may be attributed to the protein-binding activity of CTs, which can be beneficial for ruminants because the complexes could bind with essential amino acids, thereby preventing their degradation in the rumen [6]. CTs could lead to a reduction in palatability because of the astringency of the CT-protein complexes formed with the proteins in saliva [7]. Recently, both condensed tannins (CTs) and hydrolyzable tannins (HTs) have been explored

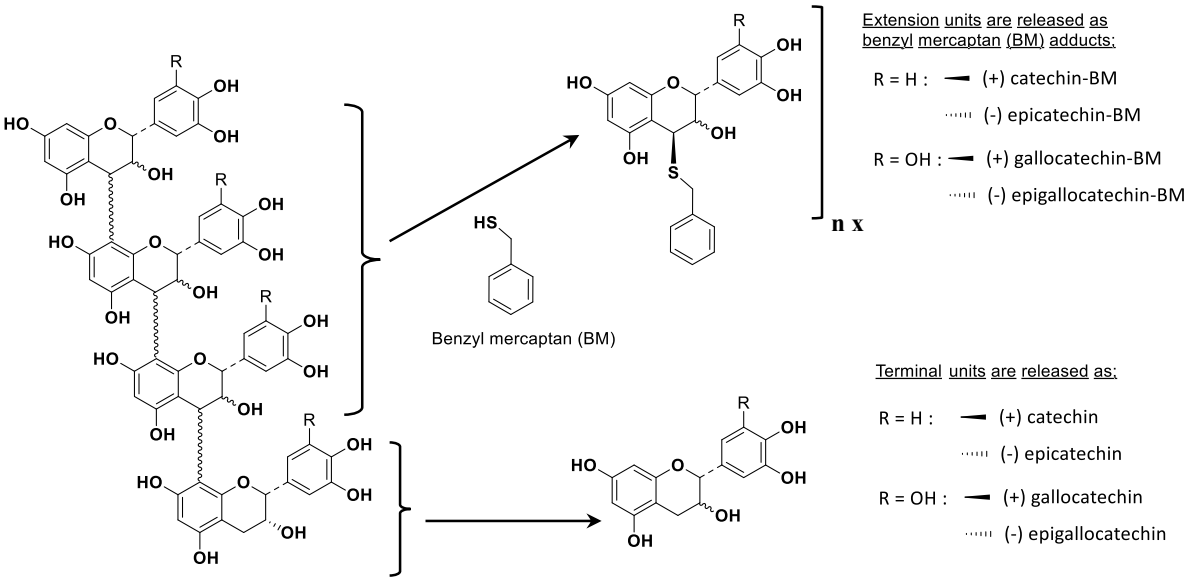

Fig. 1 Degradation of condensed tannin polymers using benzyl mercaptan (BM).

as potential natural products due to their anthelmintic properties. For instance, previous research reported that many CT-rich plants can provide sustainable control of gastrointestinal nematodes (GIN) in livestock [8]. Furthermore, different types of CTs have shown varying anthelmintic (AH) effects [6, 9–14]. In comparison, HT-rich plants, such as *Syzygium aromaticum*, contain a high content of eugenol in both methanolic and hexanic extracts, which exhibit nematicidal activity against egg hatching and larvae exsheathment of *Haemonchus contortus*, as well as antifungal activity [15].

The structure-activity relationships between condensed tannins and their AH effects have been explored by testing the *in vitro* AH properties of different tannin types against GIN [10]. Notably, high proportions of PD showed greater anthelmintic effects than high PC levels in some nematode species; however, in other species, higher PD levels combined with larger condensed tannin (CT) polymer sizes resulted in increased efficacy [10]. Therefore, both the size and the percentage of PD within CTs are vital in influencing antiparasitic activity [11]. These results agreed with other studies, which also supported that PD is more potent than PC tannins in exerting their biological activities, even though the precise mechanism of action(s) still needs to be examined [11–14].

The dietary Pas or CTs with large molecules, a mDP greater than 3, remain unabsorbed in the gastrointestinal (GI) tract and accumulate in the colonic lumen. As a result, this impacts the main physiological processes occurring in the GI tract and exerts protective properties in some pathological conditions. For example, in the mouth, CTs can bind with salivary proteins, whereas in the small intestine, they can prevent and alleviate diarrhea [2]. The prevention of diarrhea was also reported in other rodent models [16]. The mechanism of CT action on the GI tract may occur via the stimulation of intestinal opioid receptors without affecting the intestinal motility [17].

A few experiments have been reported on the pharmacological effects of CT subunits. One of the major tea catechins, epigallocatechin gallate (EGCG), at the concentration of 10^{-4} M, reduced the frequency and amplitude of spontaneous contraction in isolated mouse jejunum by direct action on smooth muscle cells via a guanylate cyclase-dependent pathway [18]. Another experiment revealed that EGCG, at concentrations of 50–200 μM, can inhibit pacemaker activity of the cultured interstitial cells of Cajal from the mouse small intestine and reduce intracellular calcium oscillations by cAMP-, cGMP-, and ATP-sensitive K^+ channel-independent manner [19]. In contrast, EGCG at concentrations of 1–20 μM was able to depolarize the myenteric neurons in guinea-pig small intestine *in vitro* [20] and facilitate cholinergic ganglion enteric neuron transmission [21], which brought about the motility contradiction to the previously mentioned experiments. However, one study revealed that flavonoids and phenolic compounds, including narigenin, silibinin, silymarin, and taxifolin, significantly reduced small- and large-intestinal transit in a dose-dependent manner in mice, whereas catechins (up to 200 mg/kg, i.p.) showed no significant effect [22].

The study of the direct effects and mechanisms of action of CTs, particularly PDs, isolated from black currant leaves on mammalian gastrointestinal motility has not been widely investigated both *in vivo* and *in vitro*. There is only one study that revealed the physiological effect of CTs isolated from black currant leaves

on isolated rat duodenal contractions. This study reported that the PD-type tannins and their effects on rat duodenal contraction were determined by the alterations in frequency, amplitude, and tonic contraction compared to ACh, a predominant neurotransmitter of excitatory motor neuron. The mechanisms of CT (PD-type tannins) actions were investigated using a nonselective muscarinic receptor antagonist, atropine, and a calcium channel blocker, verapamil. It was found that CTs at concentrations of 0.001–10 μg/ml had no direct effect on duodenal frequency, amplitude, and tone of contraction, whereas ACh showed a significant increase in tonic contraction, and this effect was suppressed by atropine. However, in the presence of atropine and verapamil, CTs caused a further significant decrease in the amplitude of duodenal contraction compared with the effect of the two blockers alone. It was concluded that CTs may synergize with muscarinic receptor antagonists and calcium channel blockers at duodenal enteric neurons, smooth muscle cell membranes, or both [23].

Therefore, this study aimed to investigate the pharmacological effects of CTs (10–300 μg/ml) on isolated rat duodenal rhythmic contraction induced by ACh (10^{-8} to 10^{-4} M). The effects of CTs were then compared with a muscarinic receptor antagonist, atropine (1.55×10^{-5} M), and a calcium channel blocker, verapamil (10^{-6} M). The possibility that CTs may exert its action via cAMP-dependent Cl^- secretion was also tested in an intestinal epithelial cell line (human colonic adenocarcinoma) by measuring equivalent short-circuit current compared with an adenylyl cyclase activator, forskolin (10 μM).

MATERIALS AND METHODS

Chemicals and drugs

Acetylcholine chloride ($CH_2CH_2OCOCH_3Cl$, MW 181.66) and atropine ($C_7H_{23}NO_3$, MW 289.375) were purchased from Sigma-Aldrich (Steinheim, Germany); the stock solutions of acetylcholine chloride were dissolved in distilled water and adjusted in 5 respective concentrations at 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5} M, while atropine was prepared at 1.125 mg/ml. Verapamil (MW 454.602) was obtained from T.O. Pharma (Bangkok, Thailand), the stock solution was prepared at 10^{-3} M.

Chemicals for Krebs buffer solution: KH_2PO_4, KCl, NaCl, $C_{10}H_{14}N_2Na_2O_8\cdot 2H_2O$ and D-Glucose were purchased from KemAus (New South Wales, Australia), $MgSO_4\cdot 7H_2O$ and $CaCl_2\cdot 2H_2O$ from RCI Labscan (Bangkok, Thailand), $NaHCO_3$ from CarloErba (VAL DE REUIL Cedex, France), and L-Ascorcic acid from Loba Chemie (Mumbai, India). All chemicals for HEPES buffer solution were purchased from Sigma (St. Louis, Missouri, USA), except NaCl from Merck Millipore (Darmstadt, Germany).

Preparation of plant extracts and tannin fractions from black currant

Black currant (*Ribes nigrum*) leaves were collected from Hildred PYO-Farm (Goring-on-Thames, Reading, UK) in August 2012. The plant sample was cleaned and dried at room temperature, followed by ball-milling and freeze drying. The aqueous fractions (F1 and F2) of the *R. nigrum* leaves were obtained as described in a previous study [9]. This plant was selected for this experiment because it represents a tannin type with high contents of CT, mDP, PD, and *trans*-flavan-3-ols. This tannin type has been reported to show synergistic effects with a muscarinic receptor blocker and a Ca^{2+} calcium channel blocker at the physiological doses on the reduction in isolated rat duodenal contraction [22]. The collected black currant leaves and the freeze-dried powdered plant material (25 g) were further extracted with a 70% acetone/water mixture (7:3, v/v; 300 ml) and filtered under vacuum. Chlorophyll and lipid were removed from the filtrate by liquid-liquid extraction with dichloromethane (250 ml), and the organic phase was discarded. The aqueous phase was rotary evaporated under vacuum at 40 °C to remove residual organic solvents, and the extracts were freeze-dried and stored at −20 °C. The freeze-dried extract was re-dissolved in distilled water and applied to a Sephadex LH-20 column with distilled water. The elution with an acetone/water mixture (3:7, v/v) yielded fraction 1, CTs (F1); a second elution with an acetone/water mixture (1:1, v/v) yielded fraction 2, CTs (F2). Acetone was removed on a rotary evaporator with a water bath at 35 °C, and the aqueous residue was freeze-dried. Tannin fractions (F1 and F2) were quantified and characterized by thiolysis with benzylmercaptan [3]. Tannin composition was determined based on the percentages of PCs and PDs, *cis*- and *trans*-flavan-3-ol subunits, and mDP using LC-MS (Agilent Technologies, Waldbronn, Germany). The F2 tannin fraction stock solutions were prepared in distilled water for the isolated rat intestinal contraction experiments and cell culture studies.

Experimental animals

Male Wistar rats (body weight 220–250 g) were purchased from the National Laboratory Animal Center, Mahidol University, Thailand. All animals were housed under controlled conditions (temperature 23–24 °C, humidity 50–55%, lighting 06:00–18:00 h), fed with a laboratory diet containing 34.2 mmol sodium chloride/kg dry weight food, and allowed for free access to reverse osmosis water.

Preparation of the duodenal segment and the duodenal contraction study in an organ bath

The preparation of the rat duodenal segment and duodenal contraction study in an organ bath was modified from Basel et al [24]. On the day of the experiment,

24-h fasted animals were euthanized by cervical dislocation. The duodenum (8 cm) was quickly excised just distal to the pylorus and cut into seven segments, each 1 cm in length. Each segment was ligated at both ends with silk thread. One end was fixed by a glass rod at the bottom of a 25 ml organ bath (seven chambers at once), while the other end was vertically attached to a force transducer (Model MLT 1030/D, ADInstruments, New South Wales, Australia) connected to a PowerLab System (4/26 PL2604 ADInstruments) for recording isometric contractions on a computer. Each organ bath contained Krebs buffer solution with the following composition (in mM): KH_2PO_4 1.2, $MgSO_4 \cdot 7\,H_2O$ 1.2, KCl 4.7, $NaHCO_3$ 25, NaCl 118.4, ascorbic acid 0.1, Na_2EDTA 0.03, glucose 11.1, and $CaCl_2 \cdot 2\,H_2O$ 2.5. The solution was constantly gassed with a 95% O_2 and 5% CO_2 mixture. The temperature of this buffer solution was maintained at 37 °C. The basal tension of each isolated segment was initially adjusted to 1 g and allowed for 20–30 min equilibration before starting the experiment. The spontaneous frequency, amplitude, and tone of isometric duodenal contraction were recorded first during the control period and then throughout the experiment.

Experimental design for duodenal contraction study

The experiment was divided into seven groups (n = 6–8, each), including Group 1 or vehicle group, in which 25 µl of vehicle solvent was added to the organ bath followed by cumulative addition of five doses of ACh to achieve final concentrations of 10^{-8}–10^{-4} M; Group 2–5 (CT groups), in which CTs were added to achieve final concentrations of 10, 50, 100, and 300 µg/ml in the bath followed by ACh addition at the same concentrations as Group 1; and Group 6 and 7 (blocker groups), in which atropine and verapamil were added to achieve final concentrations of 1.55×10^{-5} and 10^{-6} M, respectively, prior to cumulative addition of ACh at the same concentrations as Group 1.

Experimental protocol for isolated duodenal contraction study

After the duodenal segments were fixed at a resting tension of 1 g, they were allowed to equilibrate until contractions became stable, typically within 20–30 min. Each experimental group was subjected to a similar contraction protocol, including measurements of frequency, amplitude, and tonus over 5 min, which was defined as the control period. After that, vehicle, CTs, atropine, or verapamil was added, and the three contraction parameters were recorded for 5 min prior to the cumulative addition of the five doses of ACh. The effect of ACh in the presence of vehicle, CTs, or blockers was further recorded for 5 min at each concentration.

The frequency, amplitude, and tone of contraction were averaged from the 5-min recordings in each experimental period and expressed as mean ± S.E.M. Each contraction amplitude (g) was measured from the beginning to the maximal contraction, while the tone was measured from the resting tension (g) to the baseline of each contraction. The frequency was determined and expressed in cycles per minute (cpm). The percentage change of each contraction parameter from the control value was calculated using the following equation: % Change from control = [(mean value after treatment − mean value of control period)/mean value of the control period] ×100.

Cell culture

The human intestinal epithelial (colonic adenocarcinoma) cell line, T84 cells, was purchased from the American Type Culture Collection (ATCC). The cells were grown in 1:1 Dulbecco's modified Eagle's medium and Ham's F-12 medium (DMEM-F12) supplemented with 10% fetal bovine serum and 100 U/ml penicillin and 100 mg/ml streptomycin. They were maintained in 75 cm^2 flasks and incubated in a humidified atmosphere of 5% CO_2 at 37 °C until reaching 70% confluence, before being plated on Snapwell inserts (Corning, New York, USA). For Ussing chamber studies, the cells were plated on Snapwell inserts at a density of 5×10^5 cells/insert. Monolayers were incubated for 7–14 days until tight junctions developed.

Short-circuit current measurement (Ussing chamber study)

The Snapwell insert was mounted in a Ussing chamber (Micro-Ussing chamber, Model P2300, Physiologic Instruments, San Diego, USA) connected to a data acquisition system (PowerLab 4/30, ADInstruments). Intact T84 monolayers were bathed in HEPES buffer solution with the following composition (in mM): NaCl 130, KCl 4, H-HEPES 5, Na^+-HEPES 5, $MgCl_2$ 1, $CaCl_2$ 1, and glucose 5; pH 7.4. Transepithelial potential difference (V_{te}) was continuously monitored using Chart V 7 program, with reference to the basolateral side of the epithelium. Transepithelial resistance (R_{te}) was determined by applying short (200 ms) current pulses of 3 µA with a period of 6 s. The equivalent short-circuit current (I_{sc}) was calculated according to Ohm's law ($I = V/R$). After the T84 monolayers were allowed to equilibrate in the Ussing chamber for 10–20 min, CT extract dissolved in distilled water at concentrations of 10, 50, 100, and 300 µg/ml was added to either apical or basolateral bathing solution, and the changes in V_{te} were recorded for at least 10 min.

Statistical analyses

All data are expressed as mean ± S.E.M. Statistical analyses were performed using the Sigma plot software version 14.5 (Systat Software, Inc., San Jose, CA, USA). Multiple comparisons were performed using

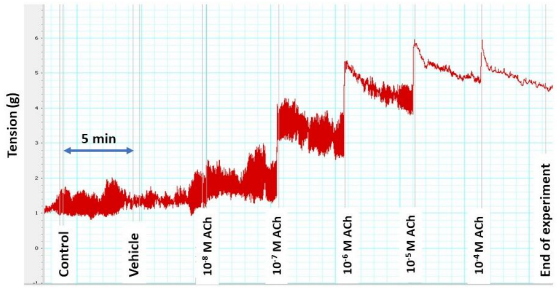

Fig. 2 Representative tracing of isolated rat duodenal rhythmic contractions showing the effect of cumulative addition of 10^{-8} to 10^{-4} M acetylcholine (ACh).

one-way repeated ANOVA or one-way ANOVA, followed by Student–Newman–Keuls post hoc test and *t*-test. Statistical significance of the mean differences was considered at $p < 0.05$.

RESULTS AND DISCUSSION

Results of isolated duodenal contractions

As shown in Table 1, the spontaneous contractions of the isolated rat duodenum observed in the organ bath were well recorded. The frequency, amplitude, and tone of isolated duodenal contractions during the control period of Group 1 (vehicle) in this study were 28.7 ± 0.5 cpm, 0.38 ± 0.06 g, and 1.04 ± 0.09 g, respectively.

Intestinal smooth muscle contraction results from the electrical discharge and propagation from pacemaker cells called interstitial cells of Cajal (ICCs), which are electrically coupled to smooth muscle cells of the gastrointestinal tract and thus control the motility [25–27]. The integrated effects of the slow waves and spike action potentials generated at ICCs determine smooth muscle activity. Slow-wave activity apparently occurs as voltage-gated Ca^{2+} channels depolarize the cell and increase intracellular Ca^{2+} level, followed by the opening of Ca^{2+}-activated K^+ channels, which repolarize the cell [28, 29]. The number of ICC slow waves that reach the membrane threshold and the spike potential generation determine the frequency and amplitude of smooth muscle rhythmic contractions.

Intestinal smooth muscle contraction is considered spontaneous and consists of two major characteristics: rhythmic and tonic contractions. Rhythmic contraction occurs as a result of an alteration between spontaneous contraction and relaxation of the smooth muscle continuously. Tonic contraction (tonic tension, basal tension, or basal tone), in contrast, is the sustained and long-term continuous contraction that results in a certain basal tone generation in the smooth muscle wall. In addition, a smaller rhythmic contraction also occurs simultaneously along with an increase in tonic contraction, particularly under the regulation of ACh, a predominant neurotransmitter of excitatory motor neurons. This can be observed as an effect of ACh at higher doses (10^{-6} to 10^{-4} M) in this study (Table 1 and Fig. 2).

In this study, the addition of ACh (10^{-6} and 10^{-5} M) into the organ bath resulted in a significant increase in frequency, while all doses (10^{-7} to 10^{-4} M) significant increase in tone of duodenal rhythmic contraction. However, ACh at the doses of 10^{-5} and 10^{-4} M resulted in a significant decrease in the amplitude of contraction. The smaller rhythmic contraction also occurs simultaneously along with tonic contraction, as shown in Table 1 and Fig. 2. It is suggested that ACh can depolarize the membrane potential and causes spike potential generation upon the slow-wave potential of ICCs, thereby resulting in an increased frequency of contraction. The acetylcholine receptors at the intestine, namely M2 and M3 muscarinic receptors (M2R and M3R), differentially regulate the intestinal motor activity, in which M2R plays an essential role in the generation of rhythmic motor activity, and M3R has a modulatory role in controlling the periodicity of the rhythmic activity together with the myenteric plexus [30]. The action of parasympathomimetic ACh in this study would result in the opening of voltage-gated Ca^{2+} channels via either M2R or M3R signaling pathway, or both, at the smooth muscle cells, ultimately leading to in an increased force of contraction. The M3R signaling pathway at the smooth muscle cell membrane involves an increase in intracellular Ca^{2+} level via activation of inositol triphosphate pathway, which then causes smooth muscle contraction via G protein-coupled receptors (G_q) [31].

It has been previously reported that the physiological effects of CTs (0.001, 0.01, 0.1, 1, and 10 µg/ml) on isolated rat duodenal contractions differ from those of ACh (10^{-8}–10^{-4} M). However, in the presence of atropine (1.55×10^{-5} M) and verapamil (10^{-6} M), those doses of CTs exhibited a synergistic or additive effect with either atropine, a nonselective muscarinic receptor, or verapamil, a calcium channel blocker, on duodenal amplitude of contractions [23]. This suggests the action of CTs as an inhibitor of ACh-induced duodenal contractions. Thus, this study was designed to compare the pharmacological effects of CTs (10, 50, 100, and 300 µg/ml) on ACh-induced isolated rat duodenal contractions with those of atropine and verapamil at previously reported doses.

The effects of four doses of CTs (10, 50, 100 and 300 µg/ml) on ACh-induced duodenal contractions are shown in Fig. 3. Not only were all doses of CTs able to subside 10^{-5} M ACh-induced increase in contraction frequency, but they also caused a significant decrease in frequency at doses of 10 and 50 µg/ml by 18.2 ± 5.5% and 18.8 ± 5.7%, respectively. However, only the highest concentration of CTs (300 µg/ml)

Table 1 The effects of 10^{-8}–10^{-4} M acetylcholine (ACh) on the frequency (cycle per minute, cpm), amplitude (g), and tone (g) of rhythmic contractions in isolated rat duodenum.

Group 1 (Vehicle, $n = 6$)	Frequency (cpm)	Amplitude (g)	Tone (g)
Control	28.7 ± 0.5	0.38 ± 0.06	1.04 ± 0.09
Vehicle	29.4 ± 0.5	0.47 ± 0.07	1.17 ± 0.72
10^{-8} M ACh	29.4 ± 0.6	0.49 ± 0.11	1.32 ± 0.13
10^{-7} M ACh	28.9 ± 0.5	0.44 ± 0.12	$2.01 \pm 0.23^{\#*}$
10^{-6} M ACh	$30.9 \pm 0.8^{\#}$	0.25 ± 0.05	$2.95 \pm 0.31^{\#*}$
10^{-5} M ACh	$31.5 \pm 0.5^{\#*}$	$0.08 \pm 0.02^{\#*}$	$3.24 \pm 0.39^{\#*}$
10^{-4} M ACh	29.1 ± 0.8	$0.05 \pm 0.01^{\#*}$	$2.98 \pm 0.42^{\#*}$

Data are mean ± S.E.M., n = number of animals. #, * $p < 0.05$ compared with respective control period and vehicle treatment (one-way repeated ANOVA with multiple comparisons using Student–Newman–Keuls post hoc test).

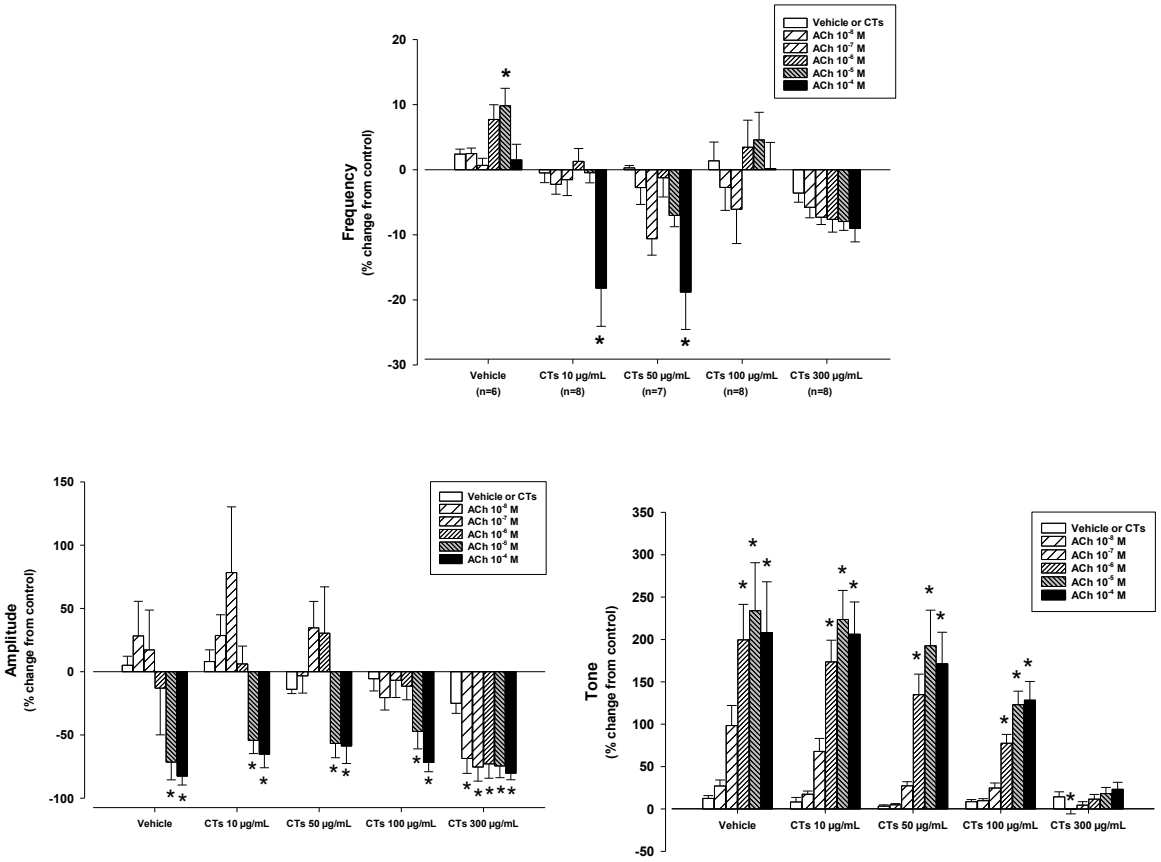

Fig. 3 The percentage change of the contraction frequency, amplitude, and tone from the control period after cumulative addition of acetylcholine (Ach; 10^{-8}–10^{-5} M) in the presence of either vehicle or condensed tannins (fraction 2, CTs) from black currant (*R. nigrum* L.) leaves (10, 50, 100, and 300 µg/ml). Data are mean ± S.E.M., n = number of animals. Legend symbols and the number of animals correspond across all graphs. * $p < 0.05$ compared with either vehicle or each CT treatment (one-way repeated ANOVA with multiple comparisons using Student–Newman–Keuls post hoc test). The raw data of each parameter during control period are not shown and set as zero.

was able to further suppress ACh-induced decrease in amplitude and ACh-induced increase in tone of contraction. The action of CTs is likely to inhibit membrane ion channel activity such as L-type Ca^{2+} channel [32] or Na^{+} channel [33], resulting in a decrease in slow wave generation and propagation from ICCs to adjacent duodenal smooth muscle via gap junctions. These ion channels are transmembrane proteins whose conformation structures can be affected by CTs. The ability of CTs and their fractionations that may possess protein binding ability and enzymatic activity inhibition were reported at least in gastrointestinal

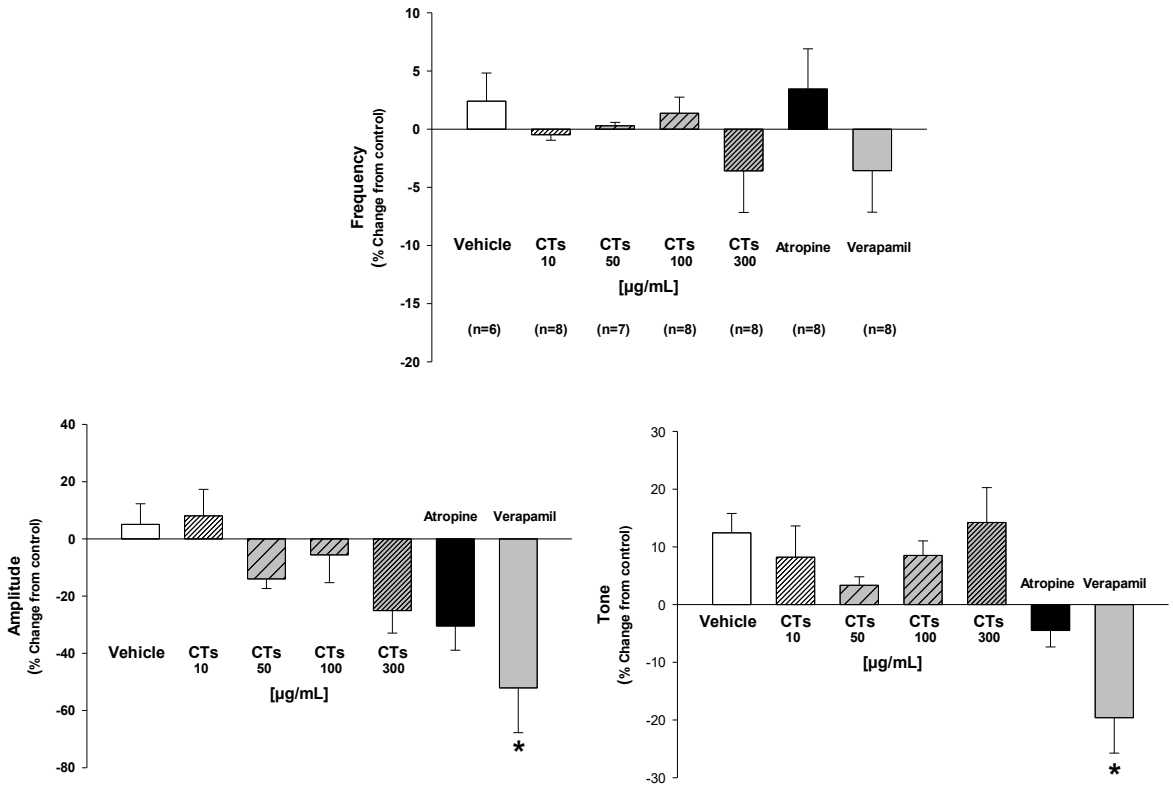

Fig. 4 The effects of either condensed tannins (fraction 2, CTs) from black currant (*R. nigrum* L.) leaves (10, 50, 100, and 300 µg/ml), atropine (1.55×10^{-5} M), or verapamil (10^{-6} M) on the percentage change of the contraction frequency, amplitude, and tone from the control period. Data are mean ± S.E.M., n = number of animals, which are correspondent in all graphs. * $p < 0.05$ compared with vehicle (one-way ANOVA with multiple comparisons using Student–Newman–Keuls post hoc test). The raw data of each parameter during the control period are not shown and set as zero.

nematode experimental study [34, 35]. In addition, a higher proportion of the CT subunit, PD, was able to affect protein aggregation by decreasing the apparent α-helix content and increasing β-sheet content of bovine serum albumin *in vitro* [36]. These mechanisms are likely to responsible for a decrease in frequency, amplitude, and tone of intestinal contraction observed in this study, as the black currant F2 fraction used contains a high percentage of PD (94.7%), high CT content (99.8 g CT/100 g fraction), high mDP (9.67), and a high percentage of *trans*-flavan-3-ols (82.2%) as previously analyzed and reported [9]. Moreover, CTs may interfere with the muscarinic receptor signaling pathway, resulting in a decrease in cytosolic Ca^{2+} level and a lessened force of contraction. However, other CT precise mechanisms of action on intestinal motility that might involve guanylate cyclase or adenylate cyclase-dependent pathway or other mucosal chemical and mechanical receptors should be further investigated.

To investigate whether the mechanism of action of CTs is similar to that of the muscarinic receptor antagonist, atropine, or calcium channel blocker, verapamil, the results are shown in Fig. 4. The addition of atropine (1.55×10^{-5} M) or any of the four doses of CTs (10, 50, 100, and 300 µg/ml) alone had no effect on the duodenal contractions. In contrast, addition of verapamil (10^{-6} M) showed a significant reduction in both amplitude and tone of contraction (by 52.1 ± 15.6% and 19.6 ± 6.1%, respectively). It is likely that this calcium channel blocker inhibits the entry of calcium ions via slow L-type calcium channels, similar to those occurring in myocardium and vascular smooth muscle [24]. This inhibitory effect finally resulted in the reduction of duodenal force of contraction. The lack of effect of verapamil on contraction frequency, but not on amplitude and tone of duodenal contraction, further suggests the involvement of verapamil-sensitive and less-sensitive calcium channel activities at rest, as previously reported in guinea pig caecum [37].

The results of further addition of ACh (10^{-8}–10^{-4} M) after pretreatment of CTs, atropine, or verapamil on duodenal contractions are shown in Fig. 5. The profile effect of cumulative addition of ACh after CTs (300 µg/ml) on the contraction frequency was similar to that of atropine and verapamil in terms of abolition of 10^{-5} M ACh-induced significant increase

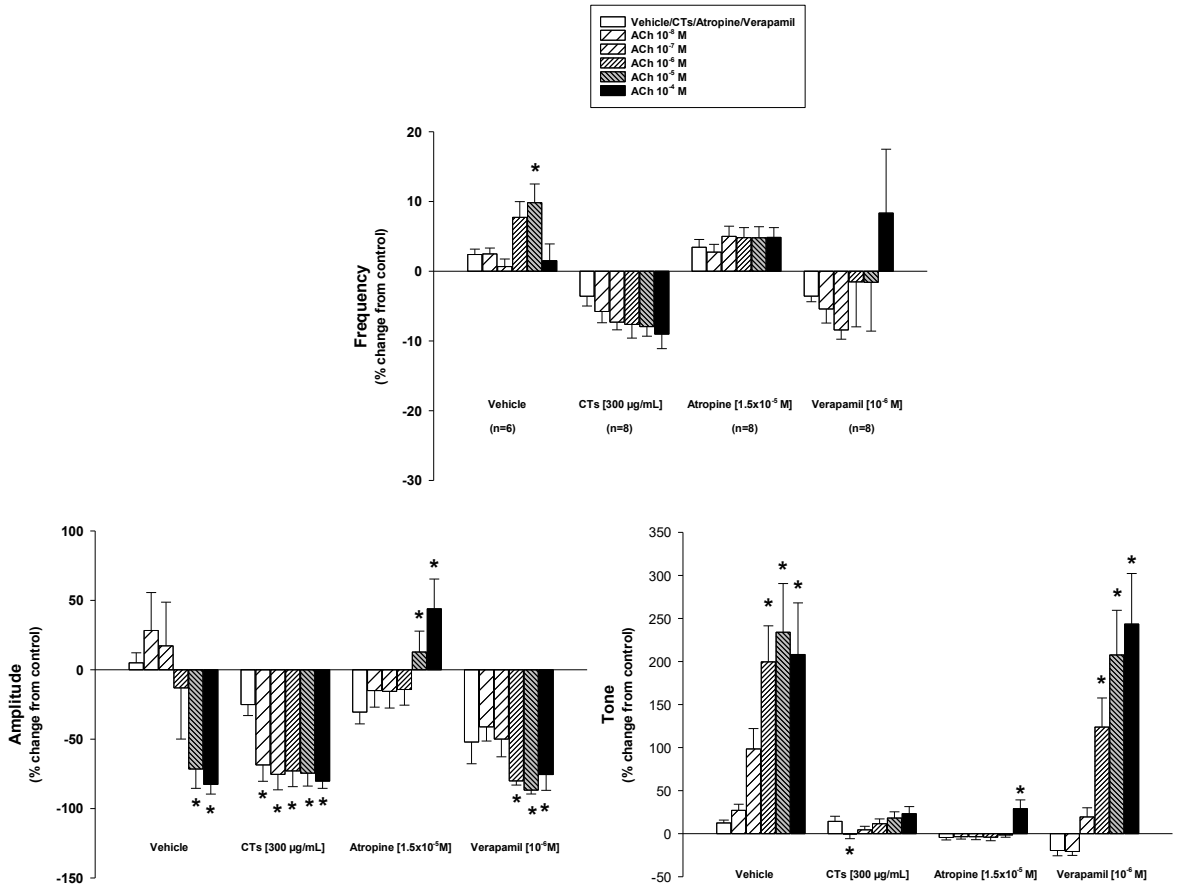

Fig. 5 The percentage change of the contraction frequency, amplitude, and tone from control period after cumulative addition of acetylcholine (ACh; 10^{-8}–10^{-5} M) in the presence of either vehicle, condensed tannins (fraction 2, CTs) from black currant (*R. nigrum* L.) leaves (300 µg/ml), atropine (1.55×10^{-5} M), or verapamil (10^{-6} M). Data are mean ± S.E.M., n = number of animals. Legend symbols and the number of animals are correspondent in all graphs. * $p < 0.05$ compared with either vehicle, CTs, atropine, or verapamil treatment (one-way repeated ANOVA with multiple comparisons using Student–Newman–Keuls post hoc test). The raw data of each parameter during control period are not shown and set as zero.

in this pacemaker potential. This reversal in the stimulatory action of ACh by CTs suggested that the action of CTs might occur via the alteration of duodenal pacemaker potential. The profile effects of the cumulative addition of Ach, which significantly decreased both amplitude and tone of duodenal contractions at the two and three higher doses, are shown in Fig. 5 (vehicle group). Atropine, but not verapamil, was able to significantly counteract the effect of ACh in the reduction of amplitude. In terms of amplitude reduction, the action of CTs was likely similar to that of verapamil (10^{-6} M) and possibly more potent. However, regarding tonic reduction, the action of CTs was more similar to that of atropine than to verapamil, as shown in Fig. 6. It is suggested that CTs may exert a reverse ACh-like effect on duodenal pacemaker cells by inhibiting calcium ion entry, ultimately hyperpolarizing the duodenal pacemaker slow-wave potential and propagation, thereby decreasing contraction frequency and amplitude. The inhibitory effect of CTs likely occurs via muscarinic receptor signaling pathway at duodenal smooth muscle cell membrane, as both CTs and atropine demonstrate a similar pattern in inhibiting tonic contraction. The increase in cytosolic calcium via the internal sarcoplasmic reticulum (SR) store is probably more pronounced in regulating tonic contraction than the opening of calcium channels in the smooth muscle cell membrane. Thus, the mechanism of action of CTs resembles that of a calcium channel blocker (verapamil) in reducing contraction amplitude and that of a muscarinic receptor antagonist (atropine) in reducing tonic contraction.

Results of the cell culture study

The potential involvement of the ion channel activity in response to CTs was further investigated in the human colonic adenocarcinoma (T84) cell line using Ussing chamber study. The equivalent short-circuit current

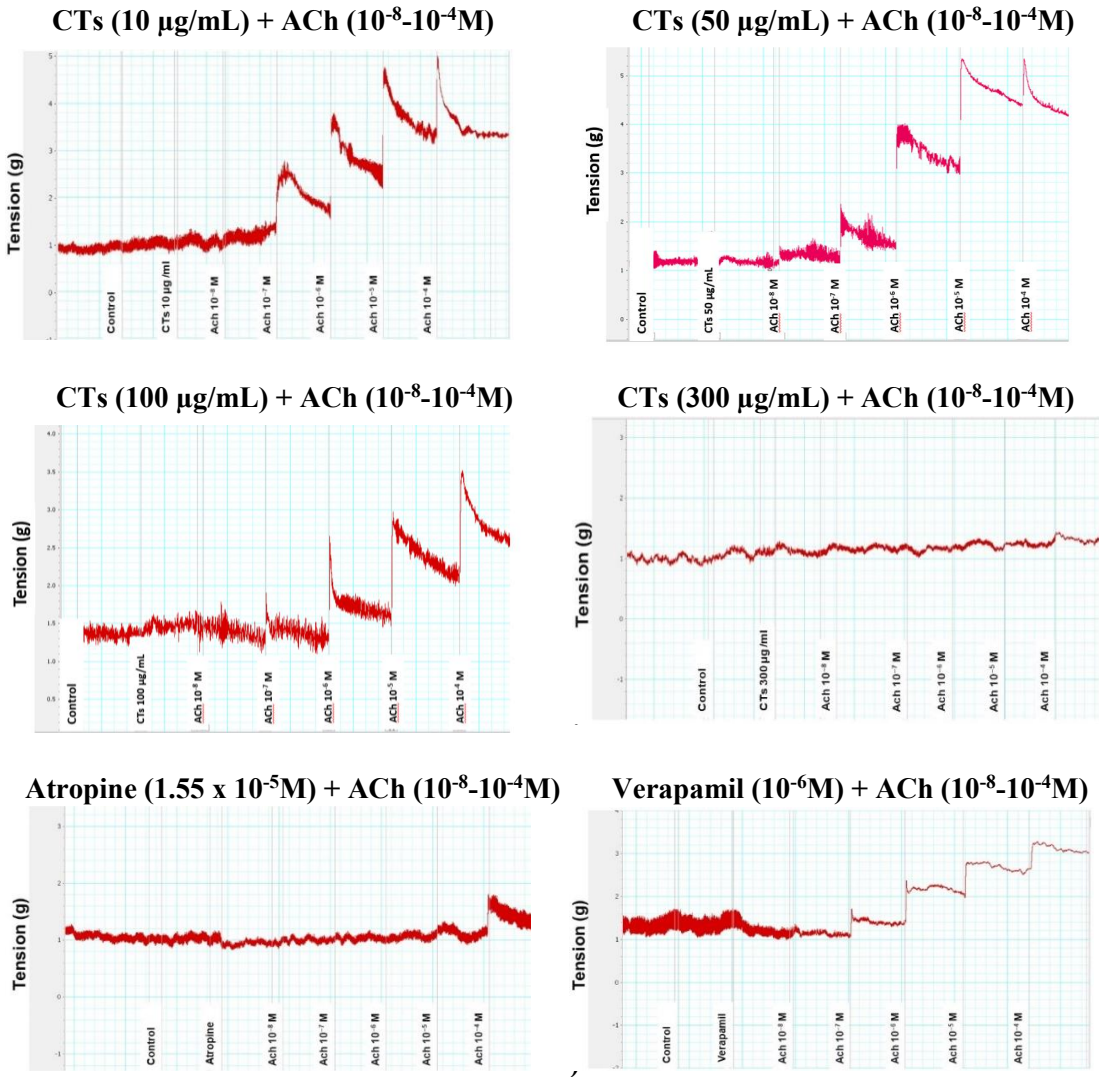

Fig. 6 Representative tracing of isolated rat duodenal rhythmic contractions showing the effect of CTs (10, 50, 100, and 300 µg/ml), atropine (1.55×10^{-5} M), and verapamil (10^{-6} M) pretreatment on acetylcholine (ACh; 10^{-8}–10^{-4} M)-induced isolated intestinal contractions.

(I_{sc}) was calculated according to Ohm's law and shown in Table 2. Application of each of the four doses of CTs to either the apical or basolateral side of the intestinal cell line monolayers did not cause significant changes in the equivalent short-circuit current compared to the vehicle control. When forskolin, a substance that increases the intracellular level of cAMP, was added to the basolateral site of the monolayer, there was a significant change of I_{sc} observed (-5.30 ± 0.66 versus -0.48 µA/cm^2). Thus, the involvement of an ion channel that might alter the membrane potential according to the addition of CTs or an increase in the cAMP-dependent Cl^- secretion signaling pathway is unlikely, at least in the colonic adenocarcinoma cell line. In addition, some smooth muscle cells can contract or relax without any change in membrane potential caused by the opening of membrane ion channels. For example, a neurotransmitter binding to its receptor can activate a G protein, leading to IP_3 generation and Ca^{2+} release from sarcoplasmic reticulum, which subsequently causes the contraction. Contraction can also occur via an increase in Ca^{2+} sensitivity of the contractile apparatus, [38].

CONCLUSION

CTs isolated from leaves of black currant (*R. Nigrum*) at doses of 10–300 µg/ml demonstrate a dose-dependent inhibition of ACh-induced contractions in isolated rat duodenum, affecting frequency, amplitude, and tone. At the pharmacological dose (300 µg/ml), CTs can reverse the ACh-induced increase in contraction fre-

Table 2 Preliminary data of equivalent short-circuit current (I_{sc}) calculated by Ohm's law (I = V/R). Transepithelial potential difference (V_{te}) was monitored with reference to the basolateral side of the epithelium. Transepithelial resistance (R_{te}) was determined by applying short (200 ms) current pulses (3 μA, period 6 s).

Treatment	I_{sc} (μA/cm^2)	I_{sc} (μA/cm^2)
	Apical	*Basolateral*
Control (distilled water)	0.02 ± 0.08 (2)	−0.48 (1)
CTs 10 μg/ml	0.46 ± 1.13 (2)	1.04 (1)
CTs 50 μg/ml	0.20 ± 0.07 (2)	−0.10 (1)
CTs 100 μg/ml	−0.19 ± 0.25 (2)	0.07 (1)
CTs 300 μg/ml	−0.28 ± 0.20 (2)	0.16 (1)
Forskolin 10 μM	−	−5.30 ± 0.66*(3)

Data are mean ± S.E.M. $^*p < 0.05$ compared with control (*t*-test). Numbers in parentheses indicate the number of experiments.

quency and tone and enhance ACh-induced decrease in contraction amplitude. The cellular action of CTs in suppressing ACh-induced tonic contraction mainly occurs via downregulation of the muscarinic receptor signaling pathway, similar to that of atropine. CTs may act on the pacemaker calcium ion channels, which are responsible for the reverse action of ACh in controlling the frequency of contraction and enhancing the effect on the amplitude of contraction, similar to that of verapamil.

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