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Physiological Effects of Condensed Tannins from Black Currant (*Ribes nigrum* L.) on Isolated Rat Duodenal Contraction

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Abstract

Condensed tannins (CTs) extracted from various plants have been shown to possess antioxidant, antidiabetic, anthelmintic, anti-palatable and anti-diarrhea activity. Black currant (*Ribes nigrum* L.), a native plant of northern Europe and Asia, is rich in phenolic compounds, including CTs. Among the biological activities of CTs, their astringent property is likely to affect gastrointestinal motility. This study aimed to investigate the physiological effect of CTs from black currant (*R. nigrum* L.) leaves on isolated rat duodenal contraction. Duodenal segments were fixed in organ baths containing carbogen aerated Krebs solution at the resting tension of 0.7 - 0.8 g. The frequency, amplitude, and tone of duodenal contraction were recorded. Either CTs or acetylcholine (ACh) were cumulatively added into the bath at the concentration of between 0.001 - 10 µg/mL and 10^{-8} - 10^{-4} M, respectively. The mechanisms of CTs and ACh actions were studied using muscarinic receptor antagonist (atropine, 1.55×10^{-5} M) and calcium channel blocker (verapamil, 10^{-6} M). It is found that CTs at the concentration between 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, was suppressed by atropine. Interestingly, in the presence of atropine and verapamil, CTs showed a further significant decrease in the amplitude of duodenal contraction compared to the effect of these 2 blockers alone. It is concluded that CTs would synergize the activity of the muscarinic receptor antagonist and the calcium channel blocker at duodenal enteric neurons or smooth muscle membrane. However, the use of CTs from black currant (*R. nigrum* L.) leaves to treat gastro-intestinal disorders while having muscarinic receptor antagonist or calcium channel blocker need cautions.

Keywords: Condensed tannins, Black currant (*Ribes nigrum* L.), Duodenal contraction

Introduction

Tannins are complex organic plant products that can precipitate proteins, peptides, and other alkaloid compounds and generally have an astringent taste. They comprise a large group of compounds with a vast structural diversity and are distributed throughout the plant kingdom. Naturally, parts of plant that contain tannin concentrations are the bark, leaf, root, fruit, and seed [1]. Tannins are usually classified into 3 main groups including phlorotannins, hydrolyzable tannins (HT) and proanthocyanidins (PA) or condensed tannins (CT) [2].

Condensed tannins (CTs) or proanthocyanidins (PAs) are oligomers and polymers of flavanols and are widely distributed in various vegetables [3]. Their basic structure consists of linked flavan-3-ol units with extender and terminal flavanol sub-units [4], as shown in **Figure 1**. Typically, the structures of condensed tannins are thought to be important for understanding their biological activities. There are many different analytical methods to quantify CTs in plant materials or extracts. However, thiolysis with benzyl mercaptan (BM) is the only method recently used to provide quantitative and qualitative data [5]. Black currant (*R. nigrum* L.), a native plant of northern Europe and Asia, is one of the tannin-containing plants and well represent prodelphinidin (PD) type tannins [6].

Recently, various biological activities of CTs, for instance, antioxidant, antidiabetic, anthelmintic, anti-palatable, anticancer, antithrombotic and anti-diarrhea activity, have been investigated [7-12]. The percentage of PD within CT are crucial in exerting antiparasitic activity *in vitro* even though the mean degree of polymerization (mDP) was gastrointestinal nematode species-dependent [13]. PD is mostly more

potent than PC tannins when it acts as an antiparasitic agent, although the precise mechanism of action(s) have not been demonstrated [14-16].

The interactions between dietary proanthocyanidins (PAs) or CTs and gastrointestinal tract (mouth, stomach, gastric mucosa, small intestine, colon, and colon mucosa) have been reviewed [17]. In the intestine, the effects of CTs are helpful in the treatment and prevention of diarrhea in rodent models [18,19], and the action may be via the stimulation of intestinal opioid receptors without affecting intestinal motility [19]. CTs can also reduce intestinal transit in charcoal-administered rats [20].

It is likely that the astringent property of CTs or their subunits, as shown in **Figure 1**, may exert diverse effects on gastrointestinal movement. The previous experiment showed a CTs subunit, epigallocatechin gallate (EGCG), which was reported to be the most significant component of catechins [21], at the concentration of 10^{-4} M, was able to induce a decrease in both frequency and amplitude of isolated mouse jejunum spontaneous contraction by direct action on smooth muscle cells via guanylate cyclase-dependent pathway [22]. EGCG (50 - 200 µM) has been shown to inhibit pacemaker activity of the cultured interstitial cells of Cajal from mouse small intestine and reduce intracellular calcium oscillations by cAMP-, cGMP- and ATP-sensitive K^+ channel-independent manner [23]. In contrast, EGCG (1 - 20 µM) has been reported to depolarize the myenteric neurons in the guinea-pig small intestine *in vitro* [24] and facilitate cholinergic ganglion enteric neuron transmission [25,26], which brought to the motility contradiction to the previously mentioned experiments. Furthermore, while many flavonoids and phenolic compounds have been shown to delay the intestinal transit in a dose-dependent manner in mice, catechins (up to 200 mg/kg, i.p.) show no significant effect [27].

Thus, it is interesting to investigate the direct effect(s) and mechanism(s) of action of CTs, particularly PDs, which are isolated from black currant (*R. nigrum* L.) leaves, on mammalian gastrointestinal motility, which have not been widely studied both *in vivo* and *in vitro*. This study, then, aimed to investigate the physiological effect of CTs isolated from black currant (*R. nigrum* L.) leaves on isolated rat duodenal contraction. The CTs (PD- type tannins) used was extracted from a native black currant which grown chiefly in northern Europe and Asia and is found rich in phenolic compounds [6], and the effect on rat duodenal contraction was determined by the alterations in frequency, amplitude, and tone of contraction compared to ACh. Up to date, there is no report regarding the direct effect of CTs (prodelphinidin or PD- type tannins) on the intestinal motility besides an inhibitory effect of a monomeric flavan-3-ols, epigallocatechin in gallate form. We then designed to use acetylcholine (ACh)-induced contraction in duodenal smooth muscle as comparable parameter to test the possibility of CTs either on the stimulation or inhibition of duodenal contraction. The mechanism(s) of CTs (PD- type tannins) actions were investigated by using a nonselective muscarinic receptor antagonist (atropine) and a calcium channel blocker (verapamil).

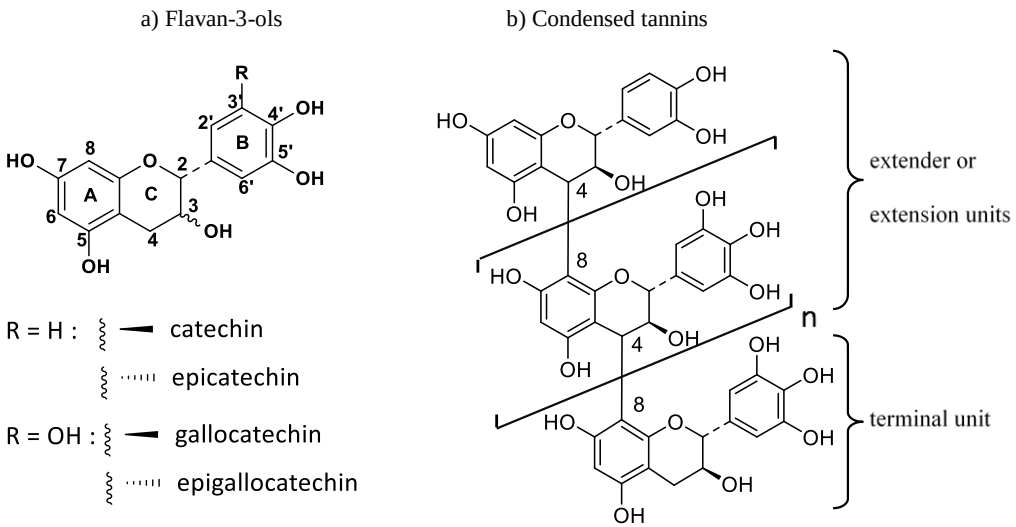

Figure 1 Structures of monomeric and polymeric flavan-3-ols. a) examples of monomeric subunits (Flavan-3-ols) and b) condensed tannins illustrating the position of extension and terminal units. The number (n) in the structure shows the mean degree of polymerization (mDP). After thiolysis, the tannin composition can be calculated according to the structure of monomeric flavan-3-ols as shown in a). If the number of OH group in ring B is either 2 or 3, the structure is called procyanidin (PC) and prodelphinidin (PD), respectively.

Materials and methods

Chemical 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 for acetylcholine chloride were prepared 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.

Preparation of plant extracts and tannin fractions from black currant (*Ribes nigrum* L.)

The preparation of the freeze-dried, fraction 2 (F2-fraction) of the black currant (*R. nigrum* L.) leaves was done as described earlier [6] and was chosen in this study due to the dominated tannin type of high percentages of CT-content, mean degree of polymerization (mDP), prodelphinidins (PD) and *trans*- flavan-3-ols. In brief, the black currant (*R. nigrum* L.) leaves were collected, and freeze-dried powdered (25 g) was extracted with an acetone:water mixture (7:3, v/v; 300 mL) and filtered. The filtrate was further extracted 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 then lyophilized. Then, the freeze-dried extract was re-dissolved in distilled water, filtered under vacuum to remove insoluble particles, and applied to a Sephadex LH-20 column. Fraction 1 (F1) was eluted with acetone/water (3:7, v/v) and fraction 2 (F2) with acetone/water (1:1, v/v). Acetone was removed in a rotary evaporator, and the aqueous residue was freeze-dried. Tannin fractions (F1 and F2) were quantified and characterized by thiolysis with benzylmercaptan [4]. Tannin composition was determined based on the percentage of PCs and PDs, *cis*- and *trans*-flavan-3-ol subunits, and mean degree of mDP [5].

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. All experiments were approved by the Animal Ethics Committee, based on the code of Practice for the Care and Use of Animals for Scientific Purposes, National Committee for Research Animal Development, National Research Council of Thailand (Reference No. WU-AICUC-63-037).

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

The preparation of rat duodenal segment and duodenal contraction study in an organ bath was modified from Basel *et al.* [28]. On the day of the experiment, the 24-h fasted animals were euthanized by cervical dislocation. The duodenum (8 cm) was quickly removed just distal to the pylorus and cut into 6 segments of 1 - 1.5 cm in length each. Each segment was ligated the 2 ends with silk thread. One end was fixed by a glass rod at the bottom of a 25 mL organ bath (6 chambers at once) while the other end was vertically attached to a force transducer (model MLT 1030/D, ADInstruments, Australia) connected to a PowerLab System (ADInstruments, Australia) displayed the isometric contraction recording on the computer. Each organ bath contained Krebs buffer solution with the composition (in mM): KH_2PO_4 1.2, $MgSO_4.7H_2O$ 1.2, KCl 4.7, $NaHCO_3$ 25, NaCl 118.4, ascorbic acid 0.1, Na_2EDTA 0.03, glucose 11.1 and $CaCl_2.2H_2O$ 2.5 and constantly gassed with 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 firstly as the control period and then throughout the experiment.

Experimental design

The experiment was divided into 6 groups (n = 6 - 8, each) namely; Group 1 or vehicle control group, the 25 µL of vehicle solvent was added cumulatively for 5 doses, Group 2 or acetylcholine (ACh) group, the 25 µL of 5 ACh stock solutions were added to make the final concentrations in organ bath at 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5} and 10^{-4} M, respectively, Group 3 or condensed tannins (CTs) group, the 25 µL of CTs stock solutions were added to make final concentration of CTs in organ bath at 0.001, 0.01, 0.1, 1.0, and 10.0 µg/mL respectively, Group 4 or ACh+Atropine group, atropine was prior added in the organ bath at the concentration 4.5 µg/mL (1.55×10^{-5} M) followed by the similar concentrations of ACh as in Group 2, Group 5 or CTs +Atropine, atropine was prior added in the organ bath at the concentration (1.55×10^{-5} M) followed by the

similar concentrations of CTs as in Group 3 and Group 6 or CTs+Verapamil group, verapamil was prior added in the organ bath at the concentration 10^{-6} M followed by CTs at the similar concentrations as in Group 3.

Experimental protocol

After fixing the duodenal segments at 1 g of resting tension, wait until the contraction is stable or in an equilibration period of 20 - 30 min. Each experimental group was exposed to a similar contraction protocol, including frequency, amplitude, and tonus recording over 5 min, which was defined as the control period. After that, either vehicle, ACh, CTs, ACh in the presence of atropine or CTs in either atropine or verapamil was cumulatively added. The duodenal contraction was recorded for 5 min, after adding each dose of either vehicle, ACh, or CTs. In Groups 4, 5 and 6, the duodenal segments were exposed to either atropine or verapamil for 5 min each before adding ACh or CTs (**Figure 2**).

The frequency, amplitude and tone of contraction were averaged from the 5 min recording contraction in each period of experiments and expressed as mean±S.E.M. Each contraction amplitude (g) was measured from the beginning to maximal contraction while the tone was measured from the resting tension (g) to the baseline of each contraction. The frequency was counted and expressed in cycle per min or cpm. The recording data or experimental tracing are shown supplementary figure. The following equation determined the percentage change from the control value:

$$\% \text{ Change from control} = \frac{(\text{mean value after treatment} - \text{mean value of control period})}{\text{mean value of control period}} \times 100$$

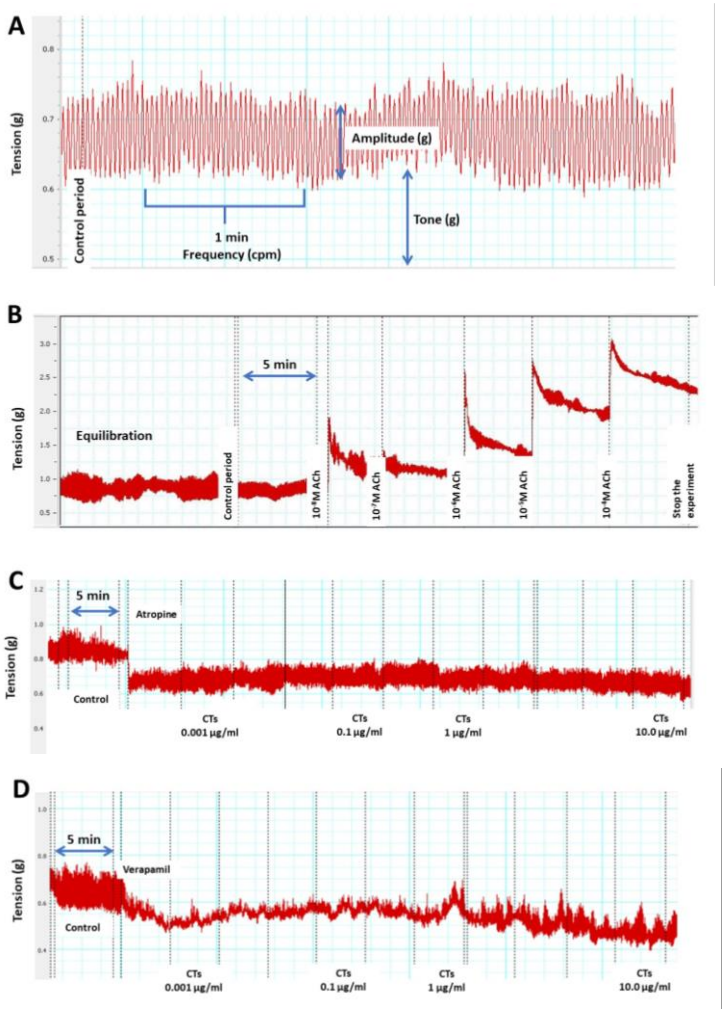

Figure 2 Example of representative tracings: A), the spontaneous contraction of isolated rat duodenal contraction showing the determination of frequency, amplitude, and tone. B), the effects of acetylcholine (ACh) (10^{-9} - 10^{-4} M) on the contraction. The effects of CTs (0.001 - 10.0 µg/mL) in the presence of either 1.55×10^{-5} M atropine C) or 10^{-6} M verapamil D) on the contraction.

Statistical analyses

All data are expressed as mean±S.E.M. Statistical analysis was accomplished using the Sigma plot 14.5 program (Systat Software, Inc., San Jose, CA, USA). Multiple comparisons were performed using one-way repeated ANOVA or one-way ANOVA followed by Student Newman-Keuls post hoc test and t-test. Statistical significance of the mean difference was considered at $p < 0.05$.

Results and discussion

The spontaneous contraction of isolated rat duodenal occurred in the organ bath and was observed and recorded effectively in this experiment as shown in the supplementary figure. This contraction is due to the electrical discharge from the enteric neurons lying in the duodenum. M_2 and M_3 muscarinic receptors (M_2R and M_3R) differentially regulate the intestinal motor activity in which M_2 muscarinic receptors play an essential role in the generation of rhythmic motor activity, and M_3 receptors have a modulatory role in controlling the periodicity of the rhythmic activity together with the myenteric plexus [28]. As shown in **Figure 3**, all mean frequency, amplitude, and tone of duodenal contraction in the control period of Group 1 (Vehicle), 2 (Acetylcholine), and 3 (Condensed tannins) were between 28 - 31 cpm, 0.17 - 0.23 g and 0.71 - 0.87 g, respectively and these 3 parameters of each control period were not significantly different. The frequency of spontaneous isolated rat duodenal contraction in this study was slightly lower than isolated rat phasic longitudinal contractions earlier reported at 36±2 cpm [29] or 38.7±3.65, 35.3±2.45, 35.6±2.54 and 36.5±2.17 cpm [30] which may depend on the set-up techniques. The addition of CTs (0.001-10.0 µg/ml) in this study did not significantly alter these 3 parameters of duodenal contraction, unlike those of ACh (10^{-8} - 10^{-4} M), which significantly increased the total force of duodenal contraction regardless of a significant minor decrease in amplitude of contraction at the doses of 10^{-5} and 10^{-4}M. When released, a parasympathomimetic ACh binds to muscarinic receptors (mainly M_3R) at smooth muscle membrane and signaling an increased intracellular Ca^{2+} level via activation of inositol phosphate pathway and then causes smooth muscle contraction via G protein-coupled receptors (Gq) [31]. ACh can also depolarize membrane potential and causes spike (action) potential generation upon the slow-wave potential increasing the force of contraction. However, the inhibitory actions of ACh via the muscarinic receptor on mouse ileal pacemaker potential was also reported [32].

The doses of CTs designed in this study are considered low or in the physiological range compared to other medicinal plant extracts that exert pharmacological effects. However, these CTs doses are comparable to ACh doses. It is likely that the effect of the CTs can be observed when its pharmacological doses are used, for instance, in the study of an *in vitro* larval exsheathment inhibition assay on gastrointestinal nematode third-stage larvae in which the EC_{50} value of CTs fraction 2 were between 60 to 277 µg/mL [6]

The physiological action of CTs on duodenal contraction was confirmed by comparing its effect with ACh in the presence of a nonselective muscarinic receptor antagonist, atropine. As shown in **Figure 4**, when compared among groups, the addition of ACh (10^{-8} to 10^{-4}M) caused an increase in tonic contraction in a dose-dependent manner, whereas the 2 higher doses significantly decreased the amplitude of contraction. However, in the presence of atropine (1.55×10^{-5} M), either ACh's stimulatory or inhibitory effect on tone and contraction amplitude was suppressed entirely. The addition of the physiological doses of CTs alone in this study showed no alterations in the intestinal contraction compared to the vehicle control group. However, in the presence of atropine, the CTs' effects on the decrease in amplitude, and tonic contraction were pronounced in all doses of CTs used despite a significant increase in amplitude at 1.0 µg/mL dose.

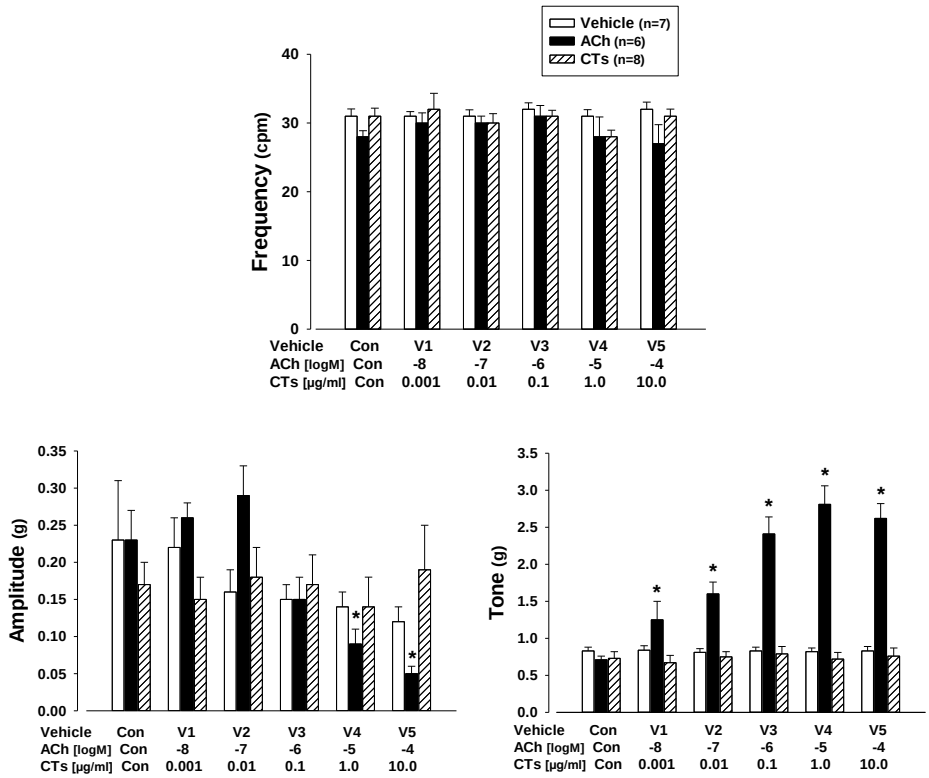

Figure 3 The effects of acetylcholine (ACh) and condensed tannins (CTs) (fraction 2) from black currant (*R. nigrum* L.) leaves on isolated rat duodenal frequency (cpm), amplitude (g), and tone (g) of contraction. Data are mean±S.E.M., * $p < 0.05$ compared with respective control period (one-way repeated ANOVA with multiple comparisons using Student-Newman-Keuls post hoc test).

Figure 5 shows the possible additive effect of CTs with atropine (1.55×10^{-5} M). The addition of atropine alone showed a significant decrease in amplitude (–29.5±8.1 %), and contraction tone (–11.3±4.2 %) but a significant increase in the frequency of contraction (12.6±4.8 %) when compared to their respective control period. The addition of atropine alone in this study showed a contradicting effect on the contraction frequency compared to the addition of 0.1 µmol/L atropine which has no apparent effect on spontaneous contractions in isolated rabbit's small intestine [33]. However, the addition of CTs did not significantly further alter the frequency and tonic contraction caused by atropine. By contrast, the highest dose of CTs (10.0 µg/mL) would be able to synergize the effect of atropine in the decrease in amplitude of contraction significantly (from –29.5±8.1 to –46.0±11.0 %). It is concluded that CTs can synergize the effect of atropine on the amplitude of duodenal contraction, particularly at the dose of 10.0 µg/mL or perhaps higher. Since atropine is a nonselective muscarinic receptor at intestinal smooth muscle membrane and the amplitude of contraction is determined by the number of spike potentials that occur at the smooth muscle membrane, thus, CTs may likely cause either hyperpolarization of the membrane or decrease calcium influx and hence decreases the number of spike potential and then, the magnitude of amplitude of contraction.

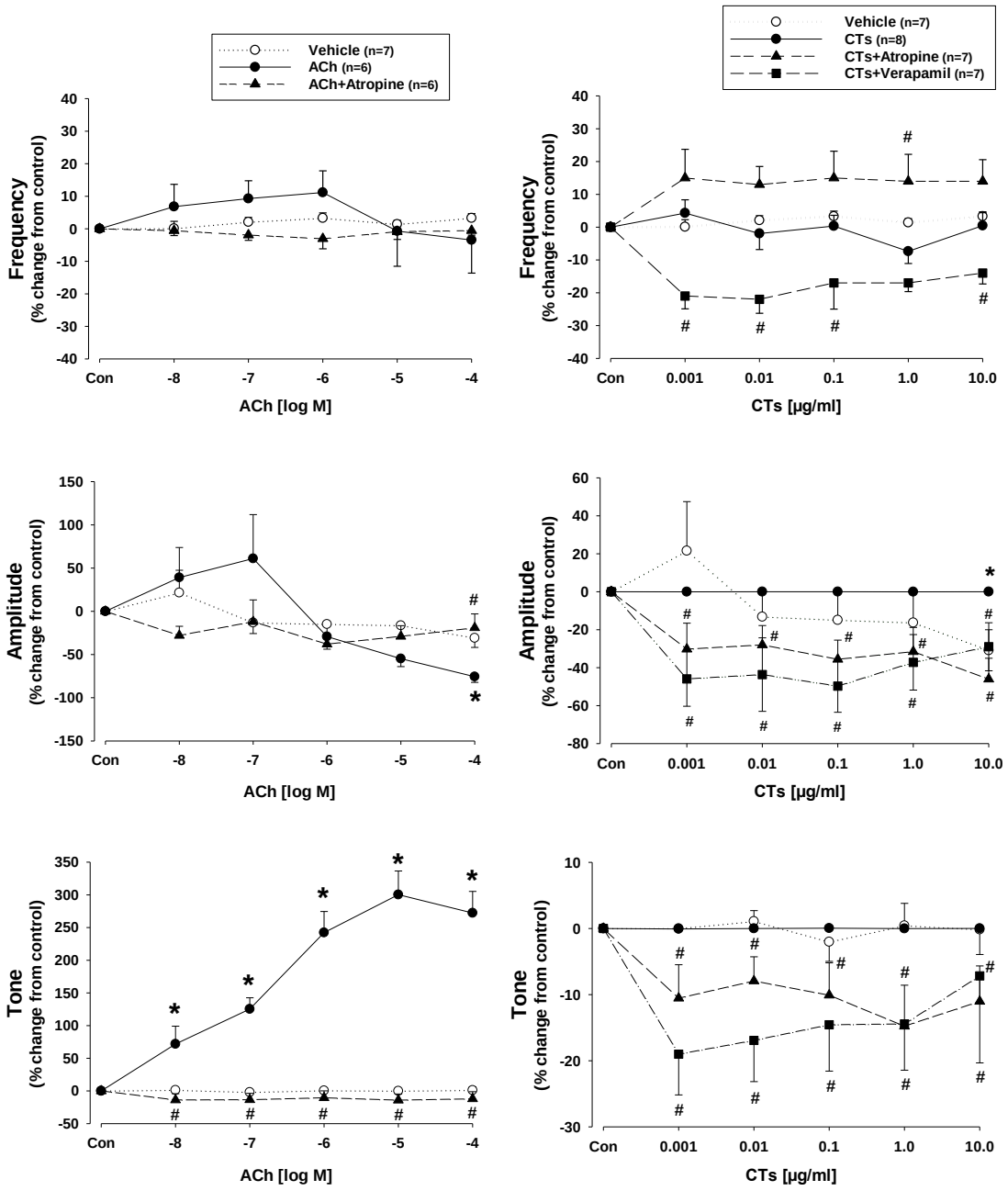

Figure 4 Effects of acetylcholine (ACh) and condensed tannins (fraction 2) (CTs) from black currant (*R. nigrum* L.) leaves alone and in the presence of either atropine or verapamil on the percentage change of the contraction frequency, amplitude, and tone of the isolated rat duodenum. Data are mean±S.E.M. * $p < 0.05$ compared with the vehicle group. # $p < 0.05$ compared with either ACh or CTs group (one way ANOVA with multiple comparisons using Student Newman-Keuls post hoc test or t-test). The raw data of each parameter control period are not shown and set as 0.

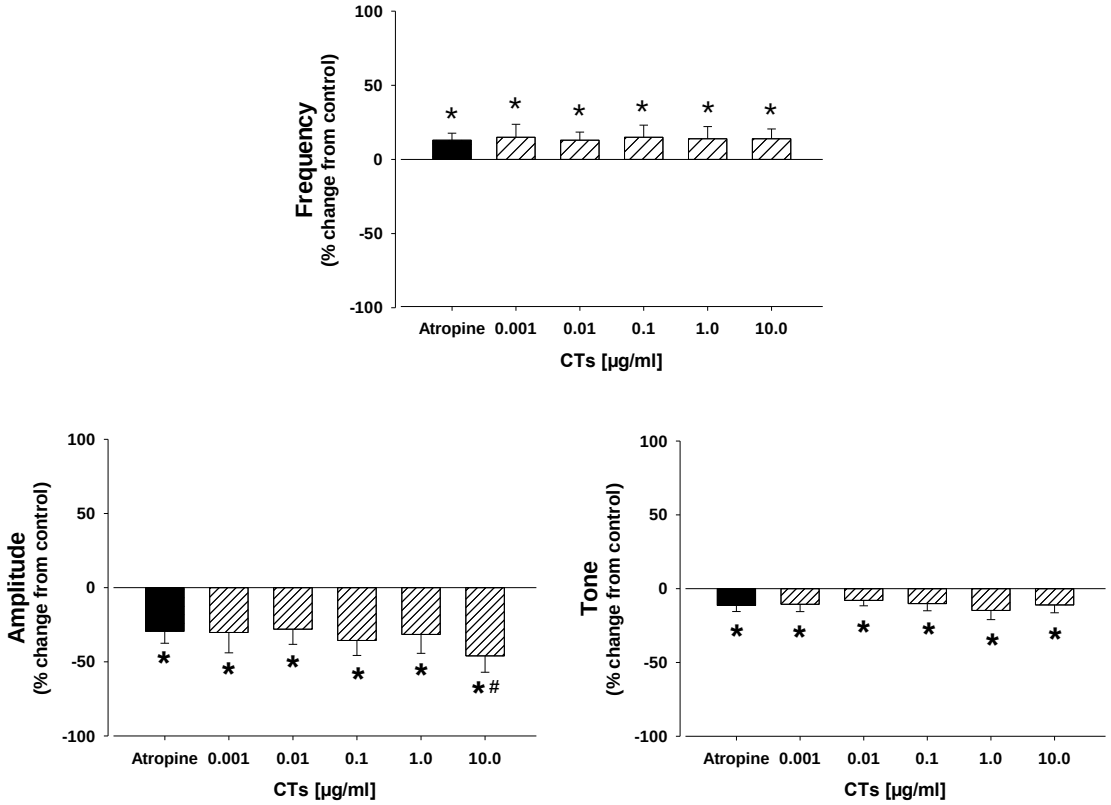

Figure 5 The percentage change of the contraction frequency, amplitude, and tone, from control period after cumulative addition of condensed tannins (fraction 2) (CTs) from black currant (*R. nigrum* L.) leaves in the presence of atropine (n = 7). Data are mean±S.E.M. $*p < 0.05$ compared with control period (one-way repeated ANOVA with multiple comparisons using Student Newman-Keuls post hoc test). $^{\#}p < 0.05$, compared with atropine (paired t-test). The raw data of each parameter control period are not shown and set as 0.

As shown in **Figure 4**, the CTs and 10^{-6} M verapamil's synergistic or additive effect, a calcium channel blocker at the intestinal membrane is possible particularly in the frequency, amplitude, and contraction tone. Almost all doses of CTs in the presence of verapamil showed a significant decrease in these 3 parameters of duodenal contraction when compared to the addition of CTs alone. **Figure 6** shows the results of further elucidation of the synergistic or additive effect of CTs with this calcium channel blocker. The addition of 10^{-6} M verapamil alone significantly decreased the frequency by 16.2±3.1 %, tone by 27.4±5.2 %, and insignificantly decreased the amplitude by 3.8±25.7 % compared to their respective control periods. These results suggest the involvement of Ca^{2+} in the generation of the frequency and tone of duodenal contraction at rest. The 2 types of verapamil-sensitive and less sensitive contraction in the intestinal smooth muscle of guinea-pig caecum have been reported [34], and these may be responsible for a nonsignificant change in amplitude of our finding. The addition of CTs (0.001, 0.1 and 1.0 µg/mL) further significantly decreased the amplitude of contraction from 3.8±25.7 to 45.9±14.1, 43.7±19.4 and 37.2±14.6 %, respectively when compared to the effect of verapamil alone. This result suggested that CTs' synergistic or additive effect with this calcium channel blocker is likely and the mechanism of CTs action at duodenal smooth muscle may differ from that of verapamil.

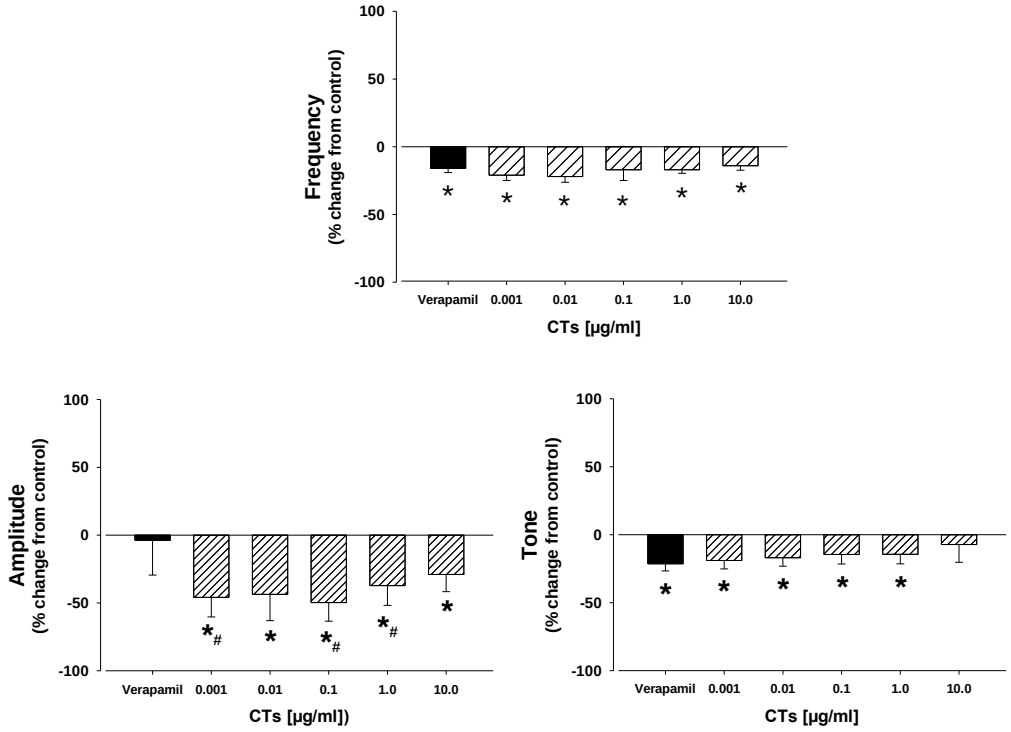

Figure 6 The percentage change of the contraction frequency, amplitude, and tone from control period after cumulative addition of condensed tannins (fraction 2) (CTs) from black currant (*R. nigrum* L.) leaves (n = 9) and CTs in the presence of verapamil (n = 7). Data are mean±S.E.M. $^{*}p < 0.05$ compared with control period (one-way repeated ANOVA with multiple comparisons using Student Newman-Keuls post hoc test). $^{\#}p < 0.05$, compared with verapamil (paired t-test). The raw data of each parameter control period are not shown and set as 0.

The tannin analysis from black currant leaves (F2-fraction) used in this study has been reported in the previous study as a PD-type plant [6]. In brief, The CT contents contained 99.8 (±2.4) g CT/100 g fraction; the mean degree of polymerisation **(**mDP**)** 9.67 (0.1); the tannin PC/PD ratios 5.3 (0)/94.7 and the *cis/trans* flavan-3-ol ratios from 17.8 (0.1)/82.2. The finding of CTs action in this study would support the effect of epigallocatechin gallate, a monomeric subunit of CTs presented in tea, on intestinal motility in mice by decreasing amplitude of contraction [22]. Both CTs and their monomeric subunit could likely cause the alteration in structural protein(s) of smooth muscle membrane and eventually limited the motility. However, the synergistic effects of the physiological doses of CTs on the reduction of rat duodenal amplitude of contraction are likely to occur via smooth muscle muscarinic receptor and calcium channel. However, to further clarify the mechanisms of CTs' action on intestinal contraction, the use of other selective or nonselective receptor blockers such as opioid, oligopeptide, histamine, alpha, beta, serotonin, prostaglandin or oxytocin receptors can be employed when the pharmacological doses of CTs on intestinal contraction have been achieved.

Conclusions

CTs isolated from leaves of black currant (*R. nigrum L.*) at the physiological doses (0.001 - 10.0 µg/mL) have no direct effect on isolated rat duodenal contraction. CTs may possess the synergistic or additive effect with either atropine, a nonselective muscarinic receptor, or verapamil, a calcium channel blocker on duodenal amplitude of contraction. Even though the pharmacological doses and the certain mechanism(s) of CTs action on the intestinal motility have not been investigated in this study. However, the higher doses of CTs are likely to possess either inhibitory or stimulatory effects on intestinal motility. Thus, in the patients who has been treated with calcium channel blockers should be aware of consuming medicinal plants containing high CTs content as alternative medicine since the herb-drug interaction might occur.

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