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Original Article

Antibacterial and laxative activities of strictinin isolated from Pu'er tea (*Camellia sinensis*)

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ABSTRACT

Strictinin, the major phenolic compound in Pu'er teas produced from young leaves and buds of wild trees, was isolated to evaluate its antibacterial and laxative activities. The minimum inhibitory concentrations of strictinin against *Propionibacterium acnes* and *Staphylococcus epidermidis* were determined as 250 μM and 2000 μM, respectively, apparently higher than those of several antibiotics commonly used for bacterial infections. The additive and synergistic effects on the inhibitory activities of strictinin combined with other commercial antibiotics were observed in two bacteria tested in this study via the analysis of fractional inhibitory concentrations. Laxative activity was observed on defecation of the rats fed with strictinin. Further analysis showed that the laxative effect of strictinin was presumably caused by accelerating small intestinal transit, instead of enhancing gastric emptying, increasing food intake, or inducing diarrhea in the rats. Taken together with the antiviral activities demonstrated previously, it is suggested that strictinin is one of the active ingredients responsible for the antiviral, antibacterial, and laxative effects of wild Pu'er tea, and has the potential to be developed as a mild natural substitute for antibiotics and laxatives.

1. Introduction

Tea is one of the most widely consumed beverages around the world, and its major components, polyphenols, have been demonstrated to provide a variety of beneficial functions to human health [1–6]. Pu'er tea is produced from young leaves of *Camellia sinensis* var. *assamica* in certain areas of Yunnan Province, China, via a time-consuming process called post-fermentation, in which microorganisms are believed to play

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an important role during the conversion [7]. The production and consumption of Pu'er tea in Yunnan can be traced back as early as the Three Kingdoms era in China's history (A.D. 220–280) [8]. Among diverse teas all over the world, Pu'er tea is unique for its special aroma and taste. Moreover, a wide range of biological effects have been documented for the consumption of Pu'er tea, such as antiobesity, antimutagenic, antiviral, and antimicrobial activities as well as hyperlipidemia, hypoglycemic, and free radical scavenging effects [9–15].

Strictinin, first discovered in green tea by Nonaka and colleagues [16] in 1983, is a hydrolyzable tannin belonging to the family of ellagitannins [17,18]. It has been demonstrated to possess some biological activities, such as antiviral, antiallergic, and immunostimulator effects in the past decades [12,19,20]. Having various beneficial functions to human health, strictinin was regarded as an adequate ingredient to be supplemented in food, cosmetics, and beverages [21]. Strictinin was a relatively minor phenolic compound (approximately 0.2–0.75%) in comparison with the abundant catechins in various teas, and the low yield of this valuable compound in natural sources apparently restricted its potential applications [22]. Recently, strictinin was identified as the major phenolic compound, instead of catechins, in Pu'er teas produced from leaves and buds of wild trees, and thus plenty of wild Pu'er tea trees distributed in several mountain areas of Yunnan were assumed to be an abundant natural source for strictinin [23]. Moreover, the content of strictinin in leaves or buds of wild trees was found to be significantly higher than that in leaves of cultivated shrubs. In other words, massive accumulation of strictinin is tentatively regarded as a major characteristic of wild Pu'er tea plants that grow naturally as large trees in comparison with the dwarf shrubs domestically cultivated in a highly compact manner mostly in the terraced farms of Yunnan.

Empirically, Pu'er teas produced from leaves and buds of wild trees are perceived to possess better biological effects than those produced from leaves of cultivated shrubs, particularly for the healing effects against viral and microbial infections as well as the laxative effect on defecation [23–27]. We wondered if these superior biological effects are a result of the unique and abundant accumulation of strictinin in wild Pu'er teas. Recently, we demonstrated that strictinin and its thermally degraded products, ellagic acid and gallic acid, possessed antiviral activity [23]. In this study, our aim was to evaluate the antibacterial activities of strictinin as well as its laxative activity of enhancing gastrointestinal motility in an animal model.

2. Methods

2.1. Chemicals and materials

All chemicals were purchased from E. Merck Co. (Merck KGaA, Darmstadt, Germany) unless stated otherwise. Erythromycin, neomycin, tetracycline, and amoxicillin were purchased from Bio Basic Inc. (Markham, Ontario, Canada); gentamicin, cefepime, cefmetazole, ceftazidime, and piperacillin were bought from Sigma-Aldrich; flomoxef was purchased from Shionogi (Osaka, Japan). High-performance liquid chromatography (HPLC)-grade acetonitrile and methanol were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Acetic acid (99.7%) was obtained from J.T. Baker (Mallinckrodt Baker, Inc., Phillipsburg, NJ, USA), and double-distilled water was supplied by a Millipore clear water purification system (Direct-Q; Millipore, Billerica, MA, USA). Pu'er tea prepared from the leaves of Yunnan wild trees (*Camellia sinensis* var. *assamica*) in 2008 was purchased from a local store in Taiwan, and used to purify strictinin.

2.2. Purification of strictinin

Strictinin was extracted from wild Pu'er tea according to a previously described method [23]. In each preparation, Pu'er tea leaves of 50 g were ground into fine powder and extracted with 3 L of water at 70°C for 30 minutes. The infusion was partitioned with dichloromethane, ethyl acetate, and butanol. The butanol layer was concentrated, and the powder was redissolved in water and loaded into an HP-20 open column. Fractions containing strictinin were pooled and further purified by a T3 reverse phase column (Waters Corporation, Milford, MA, USA; 4.6 mm × 250 mm, 5 μm). The purified strictinin dissolved in water was adjusted to a concentration of 5mM.

2.3. Preparation of tea infusion and HPLC analysis

Tea infusion was prepared by adding 10 mL of water to 0.5 g of Pu'er tea at 70°C for 15 minutes. After extraction, the brew was filtered through a 0.22-μm polyvinylidene difluoride membrane filter (PALL Corporation, Glen Cove, NY, USA), and used for the following analysis. Chemical constituents in the tea infusion were analyzed on a HPLC system coupled to a Model 600E photodiode array detector (Waters) and performed using a Syncronis T3 column of 250 mm × 4.6 mm (inner diameter), 5 μm (Waters) as described previously [28]. The mobile phase consisted of (A) water and (B) acetonitrile containing 0.5% acetic acid. The gradient was as follows: 0–100 minutes, linearly gradient from 5% to 30% B; and 100–105 minutes, linear gradient from 30% to 5% B. The column was maintained at room temperature and the injection volume was 20 μL at a flow rate of 1 mL/min. The UV absorbance detection wavelength was set at 254 nm. Caffeine, strictinin, and (−)-epigallocatechin gallate shown in the HPLC profile were identified according to a previously described method [23].

2.4. Determination of antibacterial activities

Propionibacterium acnes (BCRC No. 10724), which causes the pathogenesis of acne, and *Staphylococcus epidermidis* (BCRC No. 11030), commonly found in hospital-acquired infections, were purchased from the Bioresource Collection and Research Center (Hsinchu, Taiwan). *P. acnes* was cultured in the brain heart infusion broth (BD Difco, Sparks, MD, USA) with 1% glucose in an anaerobic jar with Anaero pack (Oxoid, Thermo Fisher Scientific, Basingstoke, UK) at 37°C under anaerobic atmosphere. *S. epidermidis* was cultured in the tryptic soy broth (BD Difco) at 37°C. The antibacterial activities of strictinin were determined according a previously described method, with minor modifications [29]. Briefly, *P. acnes* and *S.*

epidermidis were incubated for 72 hours and 24 hours, respectively until logarithmic growth was reached. After adjusting to a concentration of 1×10^7 colony-forming unit (CFU)/mL, bacterial inoculums were added with test compounds of 2-fold serial dilutions. All tests were carried out in sterile flat bottom 96-well microplates. After incubation, 20 μL of freshly prepared Alamar Blue solution (Thermo Fisher Scientific) was added to each sample. A color change from blue to pink in wells of the microplates was monitored and recorded by fluorescence with excitation and emission at wavelengths of 530–560 nm and 580–610 nm, respectively. The samples were assayed in triplicate. The minimum inhibitory concentration (MIC) was defined as the lowest concentration of a test compound to inhibit the growth of *P. acnes* or *S. epidermidis*.

2.5. Combined antibacterial activity

The combination effects of strictinin with antibiotics were evaluated according to a previously described method [30]. In this assay, an inoculum of *P. acnes* or *S. epidermidis* was adjusted to 1×10^7 CFU/mL in 96-well microplates, and then added with strictinin in company with one of the antibiotics ranging from 1/32 to 4 MIC. Similarly, the wells were added with Alamar Blue solution to monitor the color change from blue to pink after incubation. The fractional inhibitory concentration (FIC) index was calculated as (MIC of antibiotic in combination/MIC of antibiotic alone) + (MIC of strictinin in combination/MIC of strictinin alone). For the combination effects, FIC indexes lower than 0.5, between 0.5 and 4, and higher than 4 were defined as synergism, addition, and antagonism, respectively.

2.6. Animals

Male Sprague–Dawley rats weighting 200–250 g were purchased from BioLasco, Taiwan Co. Ltd (Taipei, Taiwan) and adapted for 1 week prior to use. Animals were kept in a controlled environment of 23 ± 2°C, 60 ± 10% humidity, and 12-hour light/dark cycle. The rats were fed with a standard chow diet (calories provided by 28.5% protein, 13.5% fat, and 58% carbohydrate, 5001 Rodent LabDiet; LabDiet, St. Louis, MO, USA) and purified water *ad libitum*. The animal experiments were approved by the Institutional Animal Care and Use Committee of the National Chung-Hsing University (IACUC Approval Number: 103-74). The animals were sacrificed by CO_2 asphyxiation.

2.7. Effect of strictinin on defecation

To examine if strictinin has a laxative effect on defecation, the prokinetic activity of strictinin was evaluated according to the methods modified from previous studies [31,32]. Six rats were randomly selected from each group and fasted for 12 hours in a metabolic cage. The animals were orally administered with vehicle (normal saline) or aqueous solutions containing strictinin (0.25 g/kg or 0.5 g/kg). After the oral administration, each animal was allowed free access to rat chow and water *ad libitum*. The net consumption (food intake) of each rat after feeding for 24 hours was recorded. Feces of the rats were collected every 2 hours for the first 12 hours and finally at 24 hours after feeding. The collected feces were weighted prior to and after drying at 50°C in a dryer. The water content of feces was calculated as (wet weight of feces − dry weight of feces)/wet weight of feces × 100%.

2.8. Effect on gastric emptying

Six rats were randomly selected from each group and then fasted overnight with free access to water. The animals were loaded with vehicle or strictinin (0.5 g/kg) via gastric intubation. After loading for 45 minutes, the rats were fed with 1.5 mL of the gastric emptying indicator containing 1.5% hydroxypropyl methylcellulose solution with 0.05% phenol red. The rats were sacrificed after feeding with the indicator for 0 and 20 minutes. Next, their stomachs were removed, cut into pieces, and homogenized with 25 mL of 0.1M NaOH solution. The mixture was left to settle at room temperature for 1 hour, then 5 mL of the supernatant was taken and added with 0.5 mL of 20% (w/v) trichloroacetic acid. To separate the particulates, the extract samples were centrifuged at 3000*g* for 20 minutes. After centrifugation, 1 mL of the supernatant was taken and mixed with 4 mL of 0.5M NaOH. The absorbance value of the sample mixture was detected at 560 nm in a spectrophotometer. The gastric emptying percentage was calculated as $(1 - x/y) \times 100\%$, where x and y denote the absorbance values after feeding with the indicator for 20 minutes and 0 minute, respectively [33].

2.9. Effect on small intestinal transit

Six rats were randomly selected from each group and then fasted overnight with free access to water. The animals were loaded with vehicle or strictinin (0.5 g/kg) via gastric intubation. After loading for 45 minutes, the rats were fed with the small intestinal transit indicator containing 10 % charcoal and 10% Acacia gum in water. The rats were then sacrificed after feeding with the indicator for 30 minutes. Their small intestines were removed, and the length of the small intestines and the distance of charcoal transit were measured [32]. The percentage of small intestinal transit was calculated as follows: (distance of the charcoal smear traveled)/(length of the small intestine) × 100%

2.10. Statistical analysis

The data were presented as mean values ± standard error of the mean. The differences were analyzed with one-way analysis of variance followed by Duncan's *post hoc* testing. Statistical calculations were performed with SigmaStat (version 3.5). A p value of <0.05 was considered statistically significant.

3. Results

3.1. Isolation of strictinin from wild Pu'er tea

Infusion of Pu'er tea produced from young leaves of wild trees was analyzed in an HPLC system and was found to contain abundant strictinin (Figure 1A). Strictinin, instead of

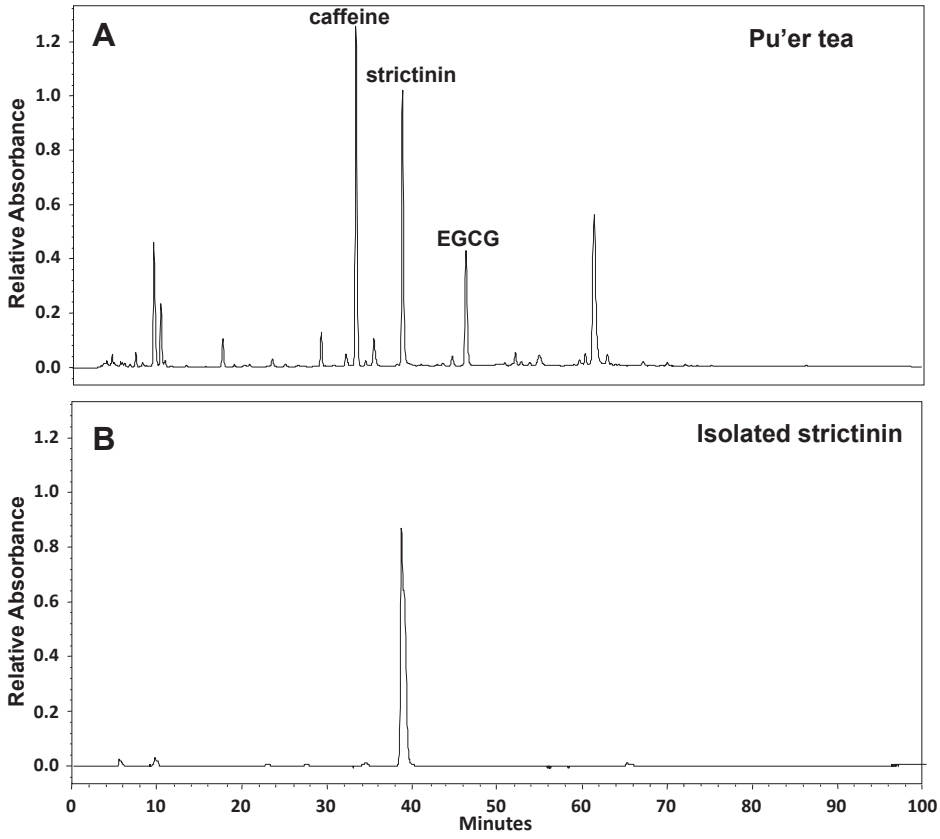

Figure 1 – High-performance liquid chromatography (HPLC) profiles of (A) Pu'er tea infusion and (B) isolated strictinin at 254 nm. Chemical constituents in the infusion of Pu'er tea were separated by HPLC (0–100 minutes). The peaks of caffeine, strictinin, and epigallocatechin-3-gallate (EGCG) are indicated.

catechins, represented the major phenolic compound in the wild Pu'er tea as reported previously [22,23]. Highly purified strictinin was obtained as examined in the same HPLC system (Figure 1B). The isolated strictinin was further confirmed by its complete decomposition to ellagic acid and gallic acid after being autoclaved for 7 minutes (data not shown) according to our previous study [23].

3.2. *Antibacterial activities of strictinin*

The antibacterial activities of strictinin and 10 antibiotics commonly used for the treatments of bacterial infections were evaluated by their inhibitory effects on the growth of *P. acnes* (Table 1) and *S. epidermidis* (Table 2). Among the 10 antibiotics, ceftazidime, tetracycline, piperacillin, and amoxicillin were found incapable of inhibiting *S. epidermidis*. The MIC values of strictinin, apparently higher than those of the antibiotics examined, were determined as 250 μM and 2000 μM for *P. acnes* and *S. epidermidis*, respectively. For the combined antibacterial activity with strictinin against *P. acnes* according to the calculation of FIC indexes, gentamicin and cefmetazole exerted a synergistic effect, whereas the other eight antibiotics displayed an additive effect. For the combined antibacterial activity with strictinin against *S. epidermidis*, all the six antibiotics examined—erythromycin, gentamicin, neomycin, flomoxef, cefepime, and cefmetazole—displayed an additive effect. In our detection, no antagonistic effect was observed for the combined antibacterial activity of strictinin with all the antibiotics examined.

Table 1 – Inhibitory activities of strictinin and 10 antibiotics against *Propionibacterium acnes*.

	Inhibition on *P. acnes*		
	MIC (μM)	FIC_{index}	Combined activity
Strictinin	250	—	—
Erythromycin	0.085	0.75	Additive
Gentamicin	20.938	0.375	Synergistic
Neomycin	16.269	0.75	Additive
Flomoxef	0.065	1	Additive
Cefepime	0.82	0.75	Additive
Cefmetazole	0.162	0.5	Synergistic
Ceftazidime	39.268	0.625	Additive
Tetracycline	0.900	0.75	Additive
Piperacillin	0.123	0.75	Additive
Amoxicillin	0.068	0.75	Additive

FIC = fractional inhibitory concentration; MIC = minimum inhibitory concentration.

3.3. *Laxative effect of strictinin*

To evaluate its laxative effect on defecation, two dosages (0.25 g/kg and 0.5 g/kg) of strictinin were fed to rats, and the feces were analyzed at 6 hours, 12 hours, and 24 hours after

Table 2 – Inhibitory activities of strictinin and 10 antibiotics against *Staphylococcus epidermidis*.

	Inhibition on *S. epidermidis*		
	MIC (μM)	FIC $_{index}$	Combined activity
Strictinin	2000	—	—
Erythromycin	0.681	0.75	Additive
Gentamicin	2.617	0.75	Additive
Neomycin	4.067	0.75	Additive
Flomoxef	1.289	1.25	Additive
Cefepime	1.664	075	Additive
Cefmetazole	5.5065	1.25	Additive
Ceftazidime	ND	—	—
Tetracycline	ND	—	—
Piperacillin	ND	—	—
Amoxicillin	ND	—	—

FIC = fractional inhibitory concentration; MIC = minimum inhibitory concentration; ND = not detectable.

feeding. The results showed that feces of the rats fed with strictinin were evidently more than those of the rats in the control group, and the higher dosage (0.5 g/kg) of strictinin was found to possess a better laxative effect than the lower dosage (0.25 g/kg) of strictinin (Figure 2A). To examine if this laxative effect is a result of the increase in food intake by rats fed with strictinin, the total food intake of rats after feeding for 24 hours was recorded for the three groups. The results showed that there was no statistical significance in terms of food intake among the rats fed with or without strictinin (Figure 2B). Putatively, strictinin had no effect on food intake of the rats, and the laxative effect induced by strictinin was not caused by the enhancement of food intake. To further examine if the laxative effect of rats fed with strictinin resulted from a diarrheal effect, the water content of rat feces in the three groups were recorded after feeding for 24 hours. The results showed that there was no statistical significance in terms of fecal water content in the rats fed with or without strictinin (Figure 2C). Presumably, the laxative activity was not caused by an induction of diarrhea in the rats fed with strictinin.

3.4. *Effect of strictinin on gastrointestinal motility*

To see if enhancement of gastrointestinal motility was responsible for the laxative activity, effects of the rats fed with strictinin of 0.5 g/kg on gastric emptying and small intestinal transit were evaluated. No significant difference was observed for the gastric emptying percentage of the rats fed with or without strictinin (Figure 3A). Apparently, the laxative effect induced by strictinin was an unlikely result of the enhancement of gastric emptying. In contrast, small intestinal transit was significantly accelerated when the rats were fed with strictinin in comparison with those fed without strictinin (Figure 3B). It is probable that the laxative activity resulted from the acceleration of small intestinal transit in rats fed with strictinin.

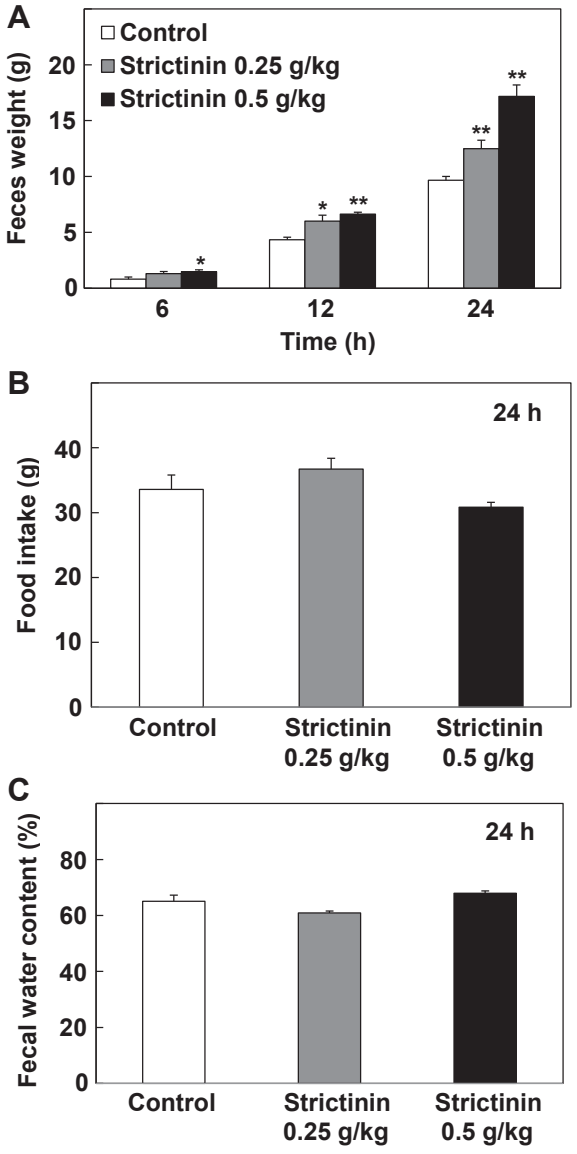

Figure 2 – Effects of strictinin on defecation. Rats were orally administrated with vehicle (control) or aqueous solutions containing strictinin (0.25 g/kg or 0.5 g/kg). (A) Feces weight at 6 hours, 12 hours, and 24 hours. (B) Total food intake at 24 hours. (C) Fecal water content at 24 hours after oral administration. Data represent mean ± SEM of six replicates. Significance levels seen by one-way ANOVA were $^{*}p < 0.05$ and $^{**}p < 0.01$ versus the control. ANOVA = analysis of variance; SEM = standard error of the mean.

4. Discussion

Since the discovery of penicillin in 1928, many antibiotics with different action modes have been identified and used to cure bacterial infectious diseases [34]. However, the abusive use of antibiotics has led a severe consequence—the gradual emergence of many antibiotic-resistant bacterial strains, such as vancomycin-resistant *Enterococcus faecium* [35]. To overcome this urgent problem, many approaches have been attempted to screen or develop new antibiotics as well as supplementary ingredients that are able to reinforce the antibacterial effects of known antibiotics in combinational utilization [36]. Herbal medicine rich in plant secondary metabolites, such as tannins, flavonoids, alkaloids, and terpenoids has been regarded

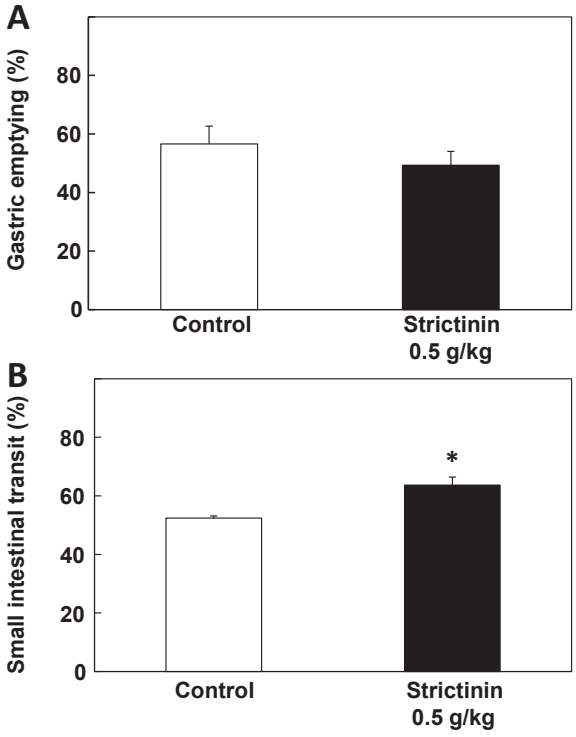

Figure 3 – Effects of strictinin on gastrointestinal motility. Rats were orally administrated with vehicle (control) or aqueous solutions containing strictinin of 0.5 g/kg. (A) Percentage of gastric emptying. (B) Percentage of small intestinal transit. Data represent mean ± SEM of six replicates. A significance level seen by one-way ANOVA was $^{*}p < 0.01$ versus the control. ANOVA = analysis of variance; SEM = standard error of the mean.

as an adequate source fulfilling the above screening purpose. In this study, we demonstrated that strictinin isolated from wild Pu'er tea possessed antibacterial activities. Although the antibacterial activities of strictinin against *P. acnes* and *S. epidermidis* were approximately 10–1000 times weaker than the antibiotics commonly used for the treatments of bacterial infections, an additive or synergistic effect was observed for the combined antibacterial activity of strictinin and the antibiotics examined (Tables 1 and 2). Therefore, it is expectable that combinational utilization with strictinin may be an adequate recipe to reduce the dosages of antibiotics used for the treatments of bacterial infections.

Moreover, we also demonstrated that strictinin possess a laxative activity on rat defecation. According to our animal studies, the laxative activity of strictinin was presumably caused by accelerating small intestinal transit, instead of enhancing gastric emptying, increasing food intake, or inducing diarrhea in the rats. Taken together with the previous studies showing antiviral activities of strictinin [12,23], it is suggested that strictinin is an active ingredient responsible for the antiviral, antibacterial, and laxative effects of wild Pu'er tea, and possesses great potential to be developed as a mild natural substitute for antibiotic and laxative.

In this study, laxative effect was evidentially induced when rats were fed with strictinin (0.25 g/kg). According to the calculation of human dose equivalent (HED) using the formula [37]: HED (mg/kg) = Animal dose (g/kg) × Animal *Km*/Human *Km* (HED = 0.25 g/kg × 6/37 = 0.04 g/kg) where *Km* = Body Weight (kg)/Body Surface Area (m^2), the equivalent dosage of strictinin is approximately 2.4 g for an adult of 60 kg weight (60 × 0.04 = 2.4 g). The tea infusion prepared from 1 g of Pu'er tea contained approximately 0.1 g of strictinin (around 10%). Thus, the tea infusion prepared from 24 g (2.4 ÷ 0.1 = 24 g) of Pu'er tea is assumed to induce a laxative effect in humans. Commonly, 10 g or more of Pu'er tea is used in one preparation of tea infusion, and several tea preparations are consumed by tea drinkers daily. Thus, the results observed in this study are in good agreement with the empirical sensation of enhancing gastrointestinal motility after consumption of wild Pu'er tea.

Medicinal plants such as *Rheum rhabarbarum*, *Aloe vera*, and *Senna alexandrina* have been conventionally used for the preparations of laxatives, and chrysophanol, rhein, aloe-emodin, and sennoside are regarded as the active ingredients in these plants [38]. However, taking these natural laxatives is frequently accompanied with side effects, such as dehydration, electrolyte disturbance, and severe diarrhea via increasing the paracellular permeability across the colonic mucosa, which significantly promotes intestinal peristalsis and accelerates colonic transit [39–41]. Moreover, long-term use of these laxatives may cause relaxation of colon accompanied with upper gastrointestinal bleeding occasionally, and drug addiction subsequently leading to the elevation of effective dosage may potentially raise the risk of liver and renal toxicity [42,43]. In this study, strictinin was demonstrated to possess a laxative activity by accelerating small intestinal transit without induction of diarrhea in the rats. Lacking the severe side effects found in other natural laxatives, strictinin seems to be a promising substitute, provided it undergoes necessary clinical trials. Nevertheless, understanding the detailed molecular mechanism for the laxative effect of strictinin is crucial for the further applications of this valuable natural compound.

Conflicts of interest

All authors declare no conflicts of interest.

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