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Original Article

Significant elevation of antiviral activity of strictinin from Pu'er tea after thermal degradation to ellagic acid and gallic acid



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ABSTRACT

Compared with abundant catechins, strictinin is a minor constituent in teas and has been demonstrated to possess inhibitory potency on influenza virus. In this study, strictinin was found as the major phenolic compound in Pu'er teas produced from leaves and buds of wild trees. Due to its thermal instability, strictinin, in tea infusion or in an isolated form, was completely decomposed to ellagic acid and gallic acid after being autoclaved for 7 minutes. A plaque reduction assay was employed to compare the relative inhibitory potency between strictinin and its thermally degraded products against human influenza virus A/Puerto Rico/8/34. The results showed that the antiviral activity of ellagic acid regardless of the presence or absence of gallic acid was significantly higher than that of strictinin. Thermal degradation of strictinin to ellagic acid and gallic acid seems to be beneficial for the preparation of Pu'er teas in terms of enhancing antiviral activity.

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1. Introduction

Strictinin is a hydrolysable tannin of the ellagitannin family [1,2]. It is detected as a relatively minor phenolic compound (approximately 0.2–0.75%) in comparison with the abundant catechins in various teas examined so far [3]. In the past decade, some biological activities, such as antiallergic and immunostimulatory effects, have been reported for strictinin [4,5]. Recently, strictinin was also demonstrated to inhibit the

replication of influenza virus *in vitro* [6]. Having various beneficial functions to human health, strictinin is regarded as an adequate ingredient to be supplemented in food, cosmetics, and beverages [7]. However, strictinin has been found to be thermally unstable, such that it might be substantially decomposed into ellagic acid and gallic acid during the heating processes of tea manufacture [8]. Thermal instability of strictinin in company with its low yield in natural sources apparently restricts the promising applications of this valuable compound. Nevertheless, ellagitannins (such as strictinin) and

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their thermally degraded product, ellagic acid, were found to be antiviral and antimicrobial agents [9]. Furthermore, ellagic acid has been shown to inhibit the growth of pathogens in humans, probably coupled with proteins in bacteria walls, such as those of *Bacillus*, *Staphylococcus*, and *Salmonella* [10].

Tea is one of the most widely consumed beverages around the world, and has been found to have beneficial health functions [11–13]. Among diverse teas produced from different varieties of tea plants, the content of strictinin was found to be relatively high in Pu'er tea [3]. Pu'er tea, produced from young leaves of *Camellia sinensis* var. *assamica*, is famous for its special aroma and taste. Moreover, a wide range of biological effects have been documented for Pu'er tea, such as antiobesity, antimicrobial activity, antitumor, and free radical scavenging activity [14–19]. Pu'er tea is generally categorized into two major types: raw/green tea and ripened/black tea. Traditionally, ripened tea is converted from raw tea by a time-consuming process called postfermentation, in which microorganisms are believed to play an important role. Polyphenols, such as catechins and strictinin, in ripened tea were found to be significantly lower than those in raw tea; and it has been observed that the contents of polyphenols were gradually reduced year by year in the long-term process of postfermentation [20].

The contents of phenolic compounds in tea leaves are affected by cultivation conditions, such as fertilization, sunlight, geographic altitude, and growth season [21–23]. In normal growth environments, Pu'er tea plants grow naturally as large trees. For a commercially massive production of Pu'er tea, tea plants are dwarfed to shrubs and domestically cultivated in a highly compact manner mostly in the terraced farms of Yunnan Province of China. The Pu'er tea produced in this shrub cultivation is called Tai-di tea in contrast with Gu-shu tea produced from large tea trees [24]. Large tea trees used to produce Gu-shu tea may either be cultivated with good care as Tai-di tea plants or grow naturally in wild forests. Whether significant variation of phenolic composition is present among Pu'er teas produced from tea plants grown in different environments is basically unknown.

In this study, we first detected and compared the phenolic compounds in raw Pu'er teas produced from cultivated and wild plants. The results show that strictinin, instead of catechins, was present as the major phenolic compound in leaves and buds of wild Pu'er tea trees. Strictinin, in tea infusion or in an isolated form, was found to be thermally unstable and decomposed to ellagic acid and gallic acid. To evaluate the effect of the thermal decomposition on the antiviral activity of strictinin, a plaque reduction assay was employed to compare the relative inhibitory potency between strictinin and its thermally degraded products (ellagic acid and/or gallic acid) against human influenza virus A.

2. Methods

2.1. Chemicals and materials

All chemicals were purchased from E. Merck Co. (Darmstadt, Germany) unless stated otherwise. High-performance liquid chromatography (HPLC) grade acetonitrile was from Fisher Scientific (Fair Lawn, NJ, USA). Acetic acid (99.7%) was

obtained from J. T. Baker (Mallinckrodt Baker, Inc., Phillipsburg, NJ, USA). Ellagic acid, gallic acid, and dimethyl sulfoxide (DMSO) were from Sigma Co. (Sigma-Aldrich, St Louis, MO, USA). Water was purified using a clear water purification system (Direct-Q; Millipore, Billerica, MA, USA). Different Pu'er teas prepared from cultivated or wild tea plants (*Camellia sinensis* var. *assamica*) in 2008 and 2009 were gifts from a local tea producer, Mr. Chao-jie Lee (Taipei, Taiwan). All Pu'er tea samples used for the analyses in this study were raw Pu'er teas prepared by local manufacturers in Yunnan, China, and refined with slight baking by Mr. Lee in Taipei, Taiwan.

2.2. Cell and virus

Madin–Darby canine kidney (MDCK) cells were subcultured in minimal essential medium (MEM; Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum (Invitrogen). The cell culture condition was set at 37°C with 5% CO₂. Human influenza virus A/Puerto Rico/8/34 (PR8, H1N1) was kindly provided by Paul Digard (University of Cambridge, Cambridge, UK) and H.K. Shieh, and was propagated in the MDCK cells.

2.3. Purification of strictinin

Strictinin was extracted from Pu'er tea from wild trees. Briefly, 50 g of Pu'er tea leaves were ground into fine powder and extracted with 3 L water at 70°C for 30 minutes. The infusion was separated from tea leaves by filtration, and partitioned with dichloromethane, ethyl acetate, and butanol. The butanol layer was concentrated using a rotary evaporator. The powder was redissolved in water and loaded into an HP-20 open column. Fractions containing strictinin were pooled and further purified using a T3 reverse phase column (Waters, 4.6 mm × 250 mm, 5 μm). The highly purified strictinin was dissolved in water to a concentration of 5mM.

2.4. Structural identification of strictinin

The isolated strictinin was identified by liquid chromatography/electrospray ionization–mass spectroscopy and nuclear magnetic resonance spectroscopy (NMR). The molecular weight was determined as 634 by the negative [M-H]⁻ ion showing a peak at *m/z* 633. The fragment characterization for MS² ions at *m/z* 463 and *m/z* 301 corresponded to the cleavage of gallic acid and gallic acid plus glucosyl residue. NMR spectrum was acquired on a Varian Mercury plus 400 NMR instrument at a constant temperature controlled and adjusted to around 300K, using the residual solvent resonance as internal shift reference. δ: 3.60 (1H, t, *J* = 10.0 Hz, H-2), 3.72 (1H, t, *J* = 10.0 Hz, H-3), 3.82 (1H, d, *J* = 13.0 Hz, H-6), 4.05 (1H, dd, *J* = 10.0, 6.5 Hz, H-5), 4.86 (1H, t, *J* = 10.0 Hz, H-4), 5.23 (1H, dd, *J* = 13.0, 6.5 Hz, H-6), 5.68 (1H, d, *J* = 8.0 Hz, H-1), 6.56 (1H, s, HHDP-H-3'), 6.70 (1H, s, HHDP-H-3), and 7.15 (2H, s, galloyl-H-2,6).

2.5. Preparation of tea infusions and HPLC analysis

Tea infusions were prepared by adding 15 mL of water to 2 g of various Pu'er teas 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



Fig. 1 – Leaves and buds of different Pu'er teas. Leaves and buds of Pu'er teas produced from cultivated and wild plants are shown after tea preparation.

used for the following analysis. Chemical constituents in the tea infusions were analyzed on a liquid chromatography system coupled to a Model 600E photodiode array detector (Waters Corporation, Milford, MA, USA) and performed using a 250 mm × 4.6 mm i.d., 5 μm, T3 reversed-phase column

(Waters Corporation) as described previously [25]. The mobile phase consisted of (A) acetonitrile and (B) water containing 0.5% acetic acid. The linear gradient started with 5% (A) and increased to 25% (A) in 100 minutes. The column was maintained at room temperature and the injection volume was

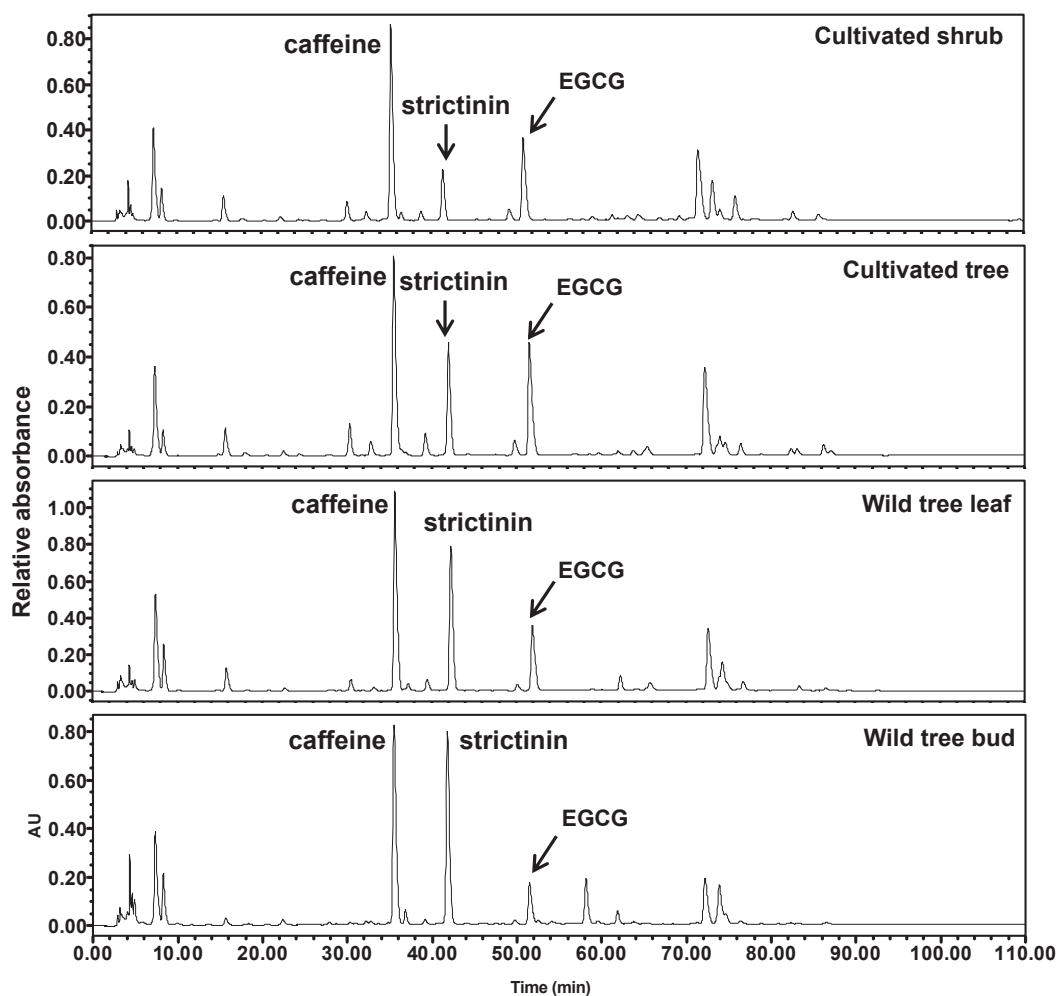


Fig. 2 – High-performance liquid chromatography (HPLC) chromatograms of infusions from different Pu'er teas. Chemical constituents in tea infusions of two cultivated and two wild Pu'er teas were analyzed and compared by HPLC (0–120 min). The peaks of caffeine, strictinin and EGCG in the HPLC profiles are indicated. EGCG = (–)-epigallocatechin gallate.

10 μL at a flow rate of 1 mL/min. The UV absorbance detection wavelength was set at 254 nm. Caffeine, strictinin and (–)-epigallocatechin gallate (EGCG) shown in the HPLC profiles of this study were identified as described previously [26].

2.6. Cytotoxicity test

A cytotoxicity test was performed by using the XTT assay (Biological Industries, Beit-Haemek, Israel). MDCK cells (2×10^4) were preseeded with MEM in 96-well plates Corning Incorporated (Corning, NY, USA) for 12 hours. The seeding medium was removed, and the cells were added with MEM containing various concentrations of tea compounds (0–50 μM) for 24 hours. In the combination of gallic acid and ellagic acid, equal concentrations of gallic acid (0–50 μM) and ellagic acid (0–50 μM) were mixed and then added to the medium. After removal of the medium, the plates were added with the XTT reagent for 2 hours, and then the absorbance wavelength at 475 nm was measured by Tecan infinite 200 PRO spectrophotometer (Tecan, Männedorf, Switzerland). The cell proliferation rate of control treatment was set as 100%.

2.7. Plaque reduction assay

Virus titer was determined by a plaque assay. MDCK cells were cultured with MEM in 12 well plates (2×10^5 /well) for 12 hours prior to viral infection. The cells were washed with phosphate-buffered saline (Invitrogen, Carlsbad, CA, USA) and infected with virus of 10 fold serial dilutions. For a plaque reduction assay, the cells were infected with influenza virus A of 2000 plaque-forming units in the presence of tea compounds at various concentrations for 1 hour (0–50 μM). In the combination of gallic acid and ellagic acid, equal concentrations of gallic acid (0–50 μM) and ellagic acid (0–50 μM) were mixed and then added with influenza virus. After 1 hour of infection, the suspension containing unbound virus was removed and the plates were covered with MEM (1 mL/well) supplemented with 0.1% trypsin (Worthington Biochemical Corp., Lakewood, NJ, USA) and 0.6% agarose (Lonza, Rockland, ME, USA). The plaques were observed after incubation for 48 hours by staining with 1% crystal violet (Sigma–Aldrich). The plaque formation number with mock treatment was regarded as 100%. The data were analyzed with the Student t test using SigmaStat (Version 3.5 integrated with SigmaPlot 10) from Systat Software (San Jose, CA, USA). A p value <0.001 was considered to be statistically significant.

3. Results

3.1. Comparison of major phenolic compounds in different Pu'er teas

Raw Pu'er teas produced from young leaves of cultivated shrubs and trees as well as those produced from young leaves and buds of wild trees were collected. Both leaves and buds of wild trees were stiff while those of cultivated shrubs and trees were soft after tea preparation (Fig. 1). Interestingly, the leaves of wild trees were found to be slightly smaller, but apparently thicker than those of cultivated shrubs and trees. Similar

patterns of phenolic compounds were observed in HPLC profiles for the four tea samples (Fig. 2). However, the content of strictinin in leaves or buds of wild trees was significantly higher than that in leaves of cultivated shrubs or trees. Apparently, strictinin, instead of catechins, represented the major phenolic compound in the Pu'er teas produced from leaves and buds of wild trees.

3.2. Thermal degradation of strictinin

To further confirm the major phenolic compound, strictinin, in Pu'er teas of wild trees, tea infusion was subjected to thermal treatment at 70°C, 80°C, and 90°C for 1 hour, 2 hours,

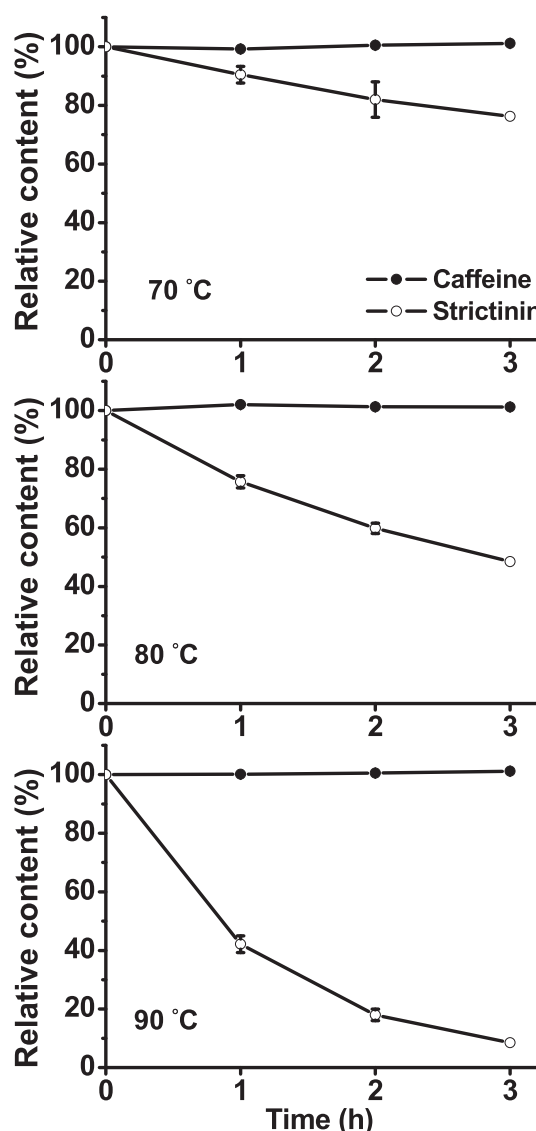


Fig. 3 – Contents of strictinin and caffeine in the infusion of wild Pu'er tea after different thermal treatments. The infusion of wild Pu'er tea was treated at 70°C, 80°C, and 90°C for 0 hours, 1 hour, 2 hours, and 3 hours. The contents of caffeine and strictinin in high-performance liquid chromatography profiles were detected and compared. Data are presented as mean \pm standard error of the mean of three replicates.

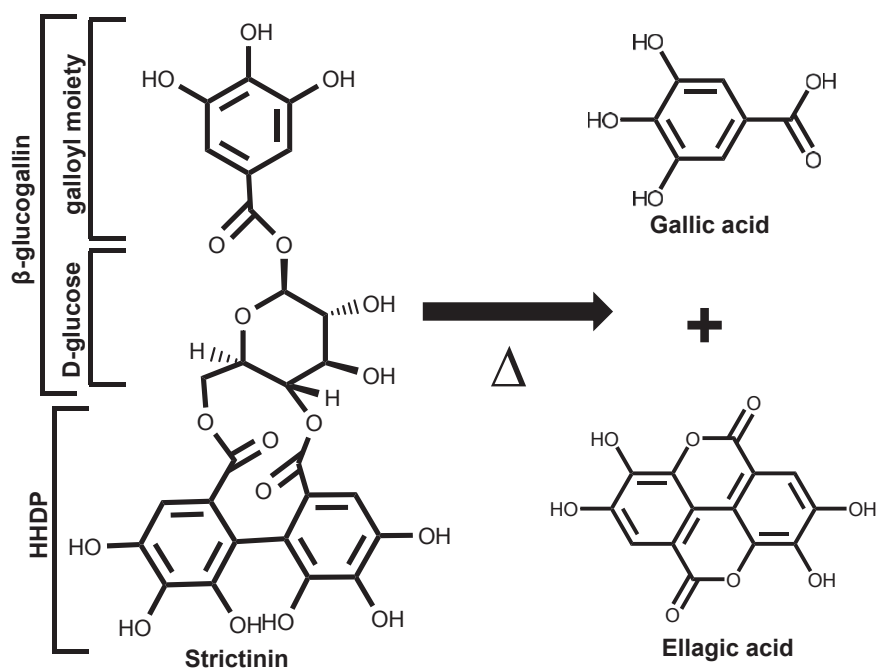


Fig. 4 – Chemical structures of strictinin and its thermally degraded products, gallic acid, and ellagic acid. Strictinin is composed of a β -glucogallin (a D-form glucose linked with a galloyl group) and a hexahydroxydiphenic acid (HHDP). After thermal treatment, strictinin is decomposed to gallic acid and ellagic acid.

and 3 hours. The results showed that strictinin was thermally unstable and degraded rapidly at 90°C, while the content of caffeine remained unchanged in the tea infusion during the thermal treatment (Fig. 3). With a more vigorous heating treatment, strictinin, either in tea infusion or in an isolated form, was completely decomposed to ellagic acid and gallic acid after being autoclaved for 7 minutes (Figs. 4 and 5).

3.3. Antiviral activity of strictinin, ellagic acid and gallic acid

A plaque reduction assay was used to determine the relative inhibitory potency between strictinin and its thermally degraded products against human influenza virus A (Fig. 6). The results showed that ellagic acid and/or gallic acid, but not strictinin, at a concentration of 50 μ M inhibited >50% of plaque formation (Fig. 7). No synergistic or additive effect on the inhibitory potency was observed for the combined supplement of ellagic acid and gallic acid. The antiviral activity of ellagic acid regardless the presence or absence of gallic acid was significantly higher than that of strictinin. Quantitatively, the IC_{50} (50% inhibitory concentration) value of ellagic acid was approximately 6 μ M while strictinin at 50 μ M displayed only 35% inhibition on plaque formation (Fig. 8). It seems that thermal degradation of strictinin to ellagic acid and gallic acid in Pu'er teas may significantly elevate their inhibitory potency on virus infection.

4. Discussion

Strictinin was first discovered in green tea by Nonaka et al [27] and regarded as a minor polyphenol in green tea [3,27]. In

this study, we discovered that strictinin was the major phenolic compound, instead of catechins, in Pu'er teas produced from leaves and buds of wild trees (Fig. 2). The history of Pu'er tea in Yunnan Province, China can be traced back as early as in the Three Kingdoms period (CE 220–280) [28]. Indeed, plenty of wild Pu'er tea trees are distributed in several mountain areas of Yunnan, and a few wild ones are also sporadically scattered in the whole province. These wild trees seem to be an abundant natural source for strictinin. Moreover, it will be interesting to see if the contents of strictinin in cultivated Pu'er trees and shrubs can be significantly elevated when these tea plants are no longer supplied with fertilizers and pesticides.

In accord with the observation in the Benifuki tea cultivar [29], the content of strictinin in buds was higher than that in leaves of wild Pu'er tea trees examined in this study (Fig. 2). By contrast, the content of EGCG, the major catechin in buds was lower than that in leaves of wild Pu'er tea trees. The inverse correlation between the content of strictinin and that of EGCG seems to be reasonable as these two phenolic compounds share a common precursor in their biosynthetic pathways. Structurally, strictinin is composed of a β -glucogallin (a D-form glucose with a galloyl group) linked with a hexahydroxydiphenic acid (Fig. 4). β -glucogallin is also the precursor molecule used for the galloylation of non-gallate-type catechins to form gallate-type catechins, such as EGCG, by an enzyme, epicatechin (1-O-galloyl- β -D-glucose O-galloyltransferase) [30,31]. The active biosynthesis of strictinin may deplete β -glucogallin, and thus the biosynthesis of EGCG is slowed down in buds of wild Pu'er tea trees. The same interpretation seems to be applicable to the inverse correlation between the content of

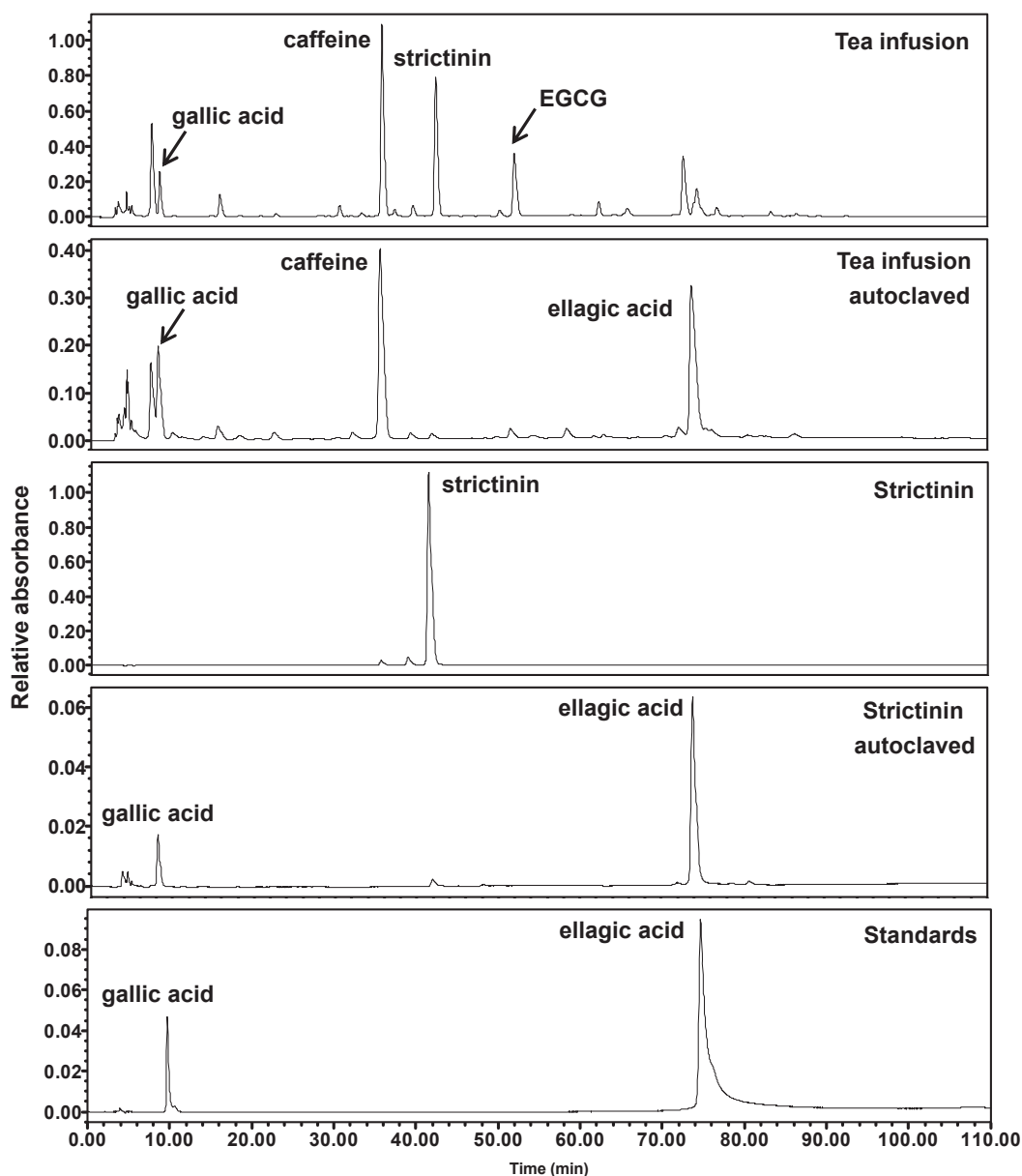


Fig. 5 – High-performance liquid chromatograms of the infusion of wild Pu'er tea and purified strictinin before and after autoclave. The infusion of wild Pu'er tea or purified strictinin was autoclaved for 7 minutes, and their chemical changes were analyzed and compared by high-performance liquid chromatography. Pure gallic acid and ellagic acid (0.1mM for each) are shown in the bottom panel as standard compounds for comparison.

strictinin and that of EGCG when these two phenolic compounds in cultivated and wild Pu'er tea plants are compared (Fig. 2).

It was reported that strictinin strongly inhibited influenza virus after infection for 12 hours [6]. However, strictinin failed to prevent plaque formation sufficiently in the plaque reduction assay of this study after infection for 48 hours (Fig. 7). Instead, the decomposed product of strictinin, ellagic acid was found to inhibit plaque formation effectively. Taken together, it is suggested that strictinin may impede viral replication at a relatively early stage

after infection, while ellagic acid may interfere with the life cycle of the virus differently and generate longer persistence. The detailed molecular mechanism for the antiviral activity of ellagic acid remains to be investigated.

It was noticed that the contents of polyphenols, such as catechins and strictinin, were substantially reduced when raw Pu'er teas were converted to ripened ones in the process of postfermentation [20]. However, the degradation of strictinin did not lead to the accumulation of ellagic acid in the ripened Pu'er teas examined. Similar outcomes were also obtained

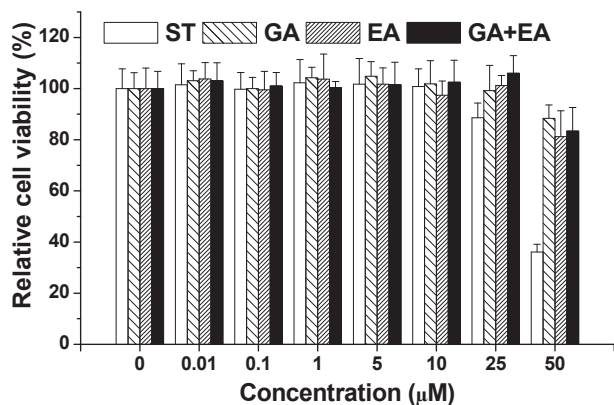


Fig. 6 – Cellular toxicity of strictinin (ST), gallic acid (GA), and ellagic acid (EA). Cytotoxicity of ST, GA, EA, and combination of GA+EA of various concentrations (0–50 μM) on Madin–Darby canine kidney cells was determined by XTT assay. Data are presented as mean ± standard error of the mean of three independent experiments.

when we analyzed the phenolic compounds of several ripened Pu'er teas (data not shown). Possibly, strictinin might not be degraded to form ellagic acid or the ellagic acid was temporarily formed and degraded as well in the current protocols for the preparation of ripened Pu'er teas. It may be worthwhile to develop an adequate process to convert strictinin to ellagic acid efficiently in the preparation of Pu'er teas for the purpose of enhancing antiviral activity.

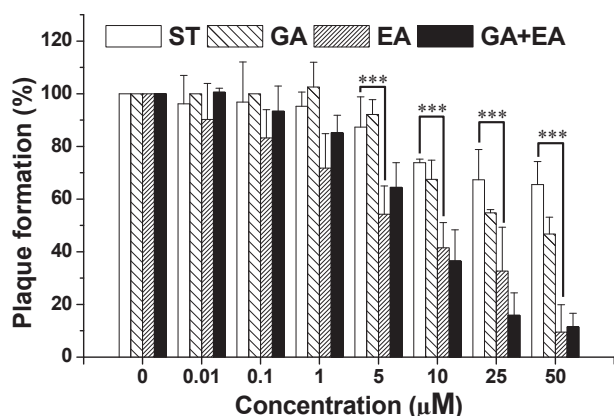


Fig. 7 – Effects of strictinin (ST), gallic acid (GA), and ellagic acid (EA) on the infection of influenza virus. Influenza virus A/Puerto Rico/8/34 was preincubated with ST, GA, EA, and combination of GA+EA of various concentrations (0–50 μM) for 60 minutes before they were inoculated to Madin–Darby canine kidney cells and covered with 6% agarose gel. After infection for 48 hours, the percentage of plaque formation was compared with the control. Data are presented as mean ± standard error of the mean of four independent experiments. The asterisk indicates a significant difference between the ST and EA groups (** $p < 0.001$, Student t test).

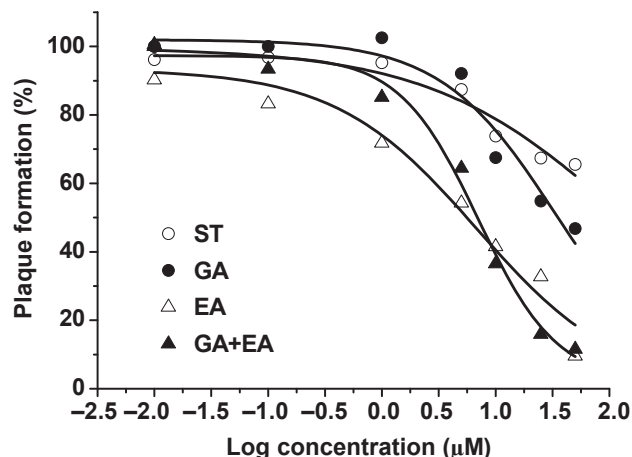


Fig. 8 – The inhibitory potency of strictinin (ST), gallic acid (GA), and ellagic acid (EA) on virus infection. The inhibitory potency of ST, GA, EA, and combination of GA+EA was determined by the plaque reduction assay.

Conflicts of interest

All authors declare no conflicts of interest.

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