Research Article

L-Phenylalanine Supplementation Effects on Catechin Contents and Tea Quality of Fresh Assam and Chinese Green Tea Leaves

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ABSTRACT

Green tea quality can be assess by the amount of the important chemical constituents such as catechins and chlorophyll. Catechin compounds play an important role in the antioxidant capacity, especially, (-)-epicatechin gallate (ECG) and (-)-epigallocatechin gallate (EGCG), which have stronger radical-scavenging activities than other catechins. Previous research showed that L-phenylalanine is the precursor of the catechin biosynthesis. In this study, L-phenylalanine was used as a fertilizer on Assam tea (*Camellia sinensis var. Assamica*) and Chinese tea (*Camellia sinensis var. Sinensis*) for 1 month. Afterwards, the fresh tea samples were collected and their chemical composition, antioxidant capacity, chlorophyll contents, and the LAB color level were studied. The results showed that L-phenylalanine could increase the catechin concentration, especially ECG and EGCG, in fresh Assam and Chinese green tea leaves. The highest increase of ECG was found in young Chinese green tea leaves (GCN-Y) from 0.02±0.30 mg/g to 0.27±0.01 mg/g. The highest increase of EGCG was found in young Assam green tea leaves (GAS-Y) from undetected to 0.31±0.01 mg/g. The total chlorophyll contents were increased in both Assam and Chinese green tea which the highest increase was found in old Assam green tea leaves (GAS-O) from 4.80±0.07 mg/g to 7.92±0.13 mg/g. The change in the latter correlated with the LAB color level. Moreover, the results of the color change in fresh Assam green tea leaves could be observed by the naked eyes. However, the antioxidant capacity of the fresh green tea leaves has not significantly changed after supplementation with L-phenylalanine.

Keywords: *Camellia sinensis*, L-Phenylalanine, Catechin, Chlorophyll, Green tea

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Introduction

Tea is one of the most popular non-alcoholic beverages consumed worldwide due to its many health benefits [1]. Previous research found that Asians are very fond of green tea and oolong tea, but North Americans prefer black tea [2]. Chiang Rai is one of the most popular places for growing tea because of its climate and topography. There are two common tea types cultivated in Chiang Rai which are Assam tea (*Camellia sinensis var. Assamica*) and Chinese tea (*Camellia sinensis var. Sinensis*).

Both Assam tea and Chinese tea contain similar main chemical components, such as polyphenols, catechins, caffeine, and chlorophyll. Each type of tea has a different ratio of chemical constituents in its leaves. The various ratios of the tea leaves composition contribute to their color, aroma, and flavor. Catechin, a polyphenol compound, is a flavonoid, found in dried tea leaves about 30-42% by weight. Literature reviews showed that catechins are beneficial to health, such as increasing the body antioxidant capacity [3], reducing inflammation [4] and cancer prevention [5]. There are eight main types of catechins found in tea leaves (Figure 1), which are (-)-epigallocatechin gallate (EGCG), (-)-epigallocatechin (EGC), (-)-epicatechin gallate (ECG), (-)-epicatechin (EC), (-)-gallocatechin gallate (GCG), (-)-gallocatechin (GC), (-)-catechin gallate (CG), and (+)-catechin (C) [6]. The green tea uses phenylalanine as a precursor for the biosynthesis of catechins (Figure 2). Phenylalanine is converted to cinnamic acid in the first step by phenylalanine ammonia-lyase (PAL). It is then further converted into *p*-coumaroyl-CoA which will be condensed with 3 malonyl-CoA to produce naringenin chalcone. The naringenin chalcone will undergo in multiple derivatizations and yield the catechin and its analogs [7-9]. Previous research suggested that (-)-epicatechin gallate (ECG) and (-)-epigallocatechin gallate (EGCG) have stronger radical scavenging activities than other catechin derivatives [10,11]. Caffeine is one of the main chemical compounds that are found in tea. It is an alkaloid which is found about 2-5% by weight in tea leaves. It has a stimulating effect on the central nervous system, keeping the body fresh, energetic, increasing energy metabolism, and having a diuretic effect [12]. In addition to catechin and caffeine, there are other known compounds in tea such as theanine, apigenin, and chlorophyll. Theanine is one of the tea compositions which is an amino acid. It affects the flavour of the tea and may help in reducing high blood pressure [13]. Apigenin is a flavonoid compound found in tea leaves. It is highly soluble in water and is the substance that gives the yellow color in tea [14]. Moreover, chlorophyll is the green pigment in the top layers of tea leaves. Chlorophyll content in tea leaves normally decreases during tea fermentation because it will decompose into pheophytin and pheophorbide, which gives the tea darken color [15]. The chemical composition of tea changes throughout the tea production processes which are withering, rocking, fixation, and roll drying [16]. These processes yield different types of tea such as green tea (non-fermentation process), oolong tea (semi-fermentation process), and black tea (fermentation process).

Green tea is a tea product derived from the non-fermentation process. It is obtained by collecting fresh tea leaves through the roasting process to inhibit the activity of the polyphenol oxidase enzyme, which catalyzes oxidation and polymerization reactions [17]. Among the three types of processed tea, green tea contains the highest catechin contents [18]. The antioxidant capacity of tea correlates with the

catechin contents. Chlorophyll is one of the important chemical compound in green tea. Previous studies indicated that chlorophyll content is used to evaluate the quality of green tea [19]. Many studies have investigated the relationship between the antioxidant capacity and catechin compounds but studies on the changing of catechin contents by fermentation with L-phenylalanine (catechin precursor) supplementation in tea plants are limited. In this research, we aimed to study the effects of L-phenylalanine supplementation on the change of catechin and chlorophyll contents, antioxidant capacity, and color level (LAB) in green tea leaves.

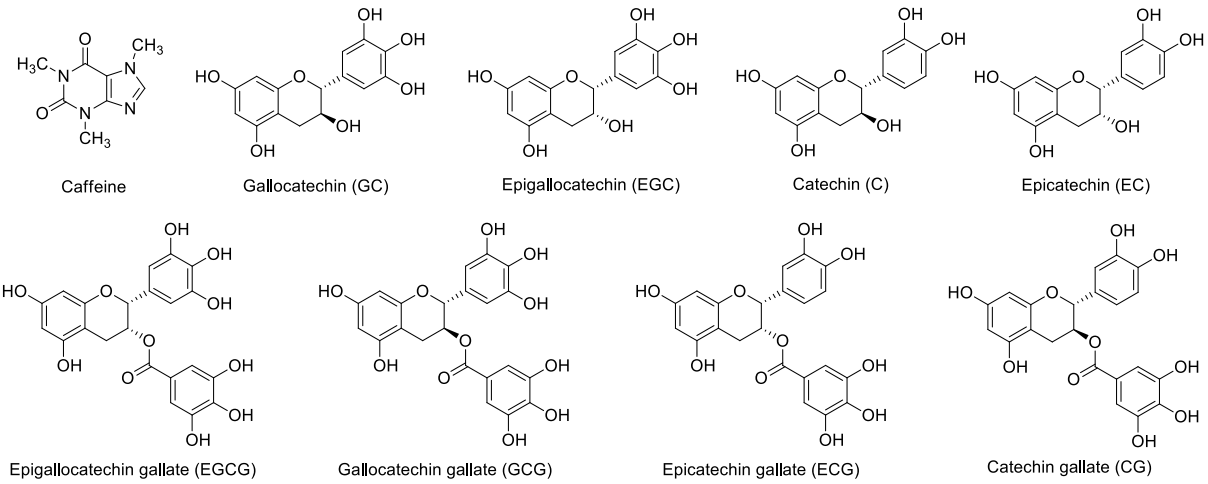

Figure 1 Catechins and caffeine structures.

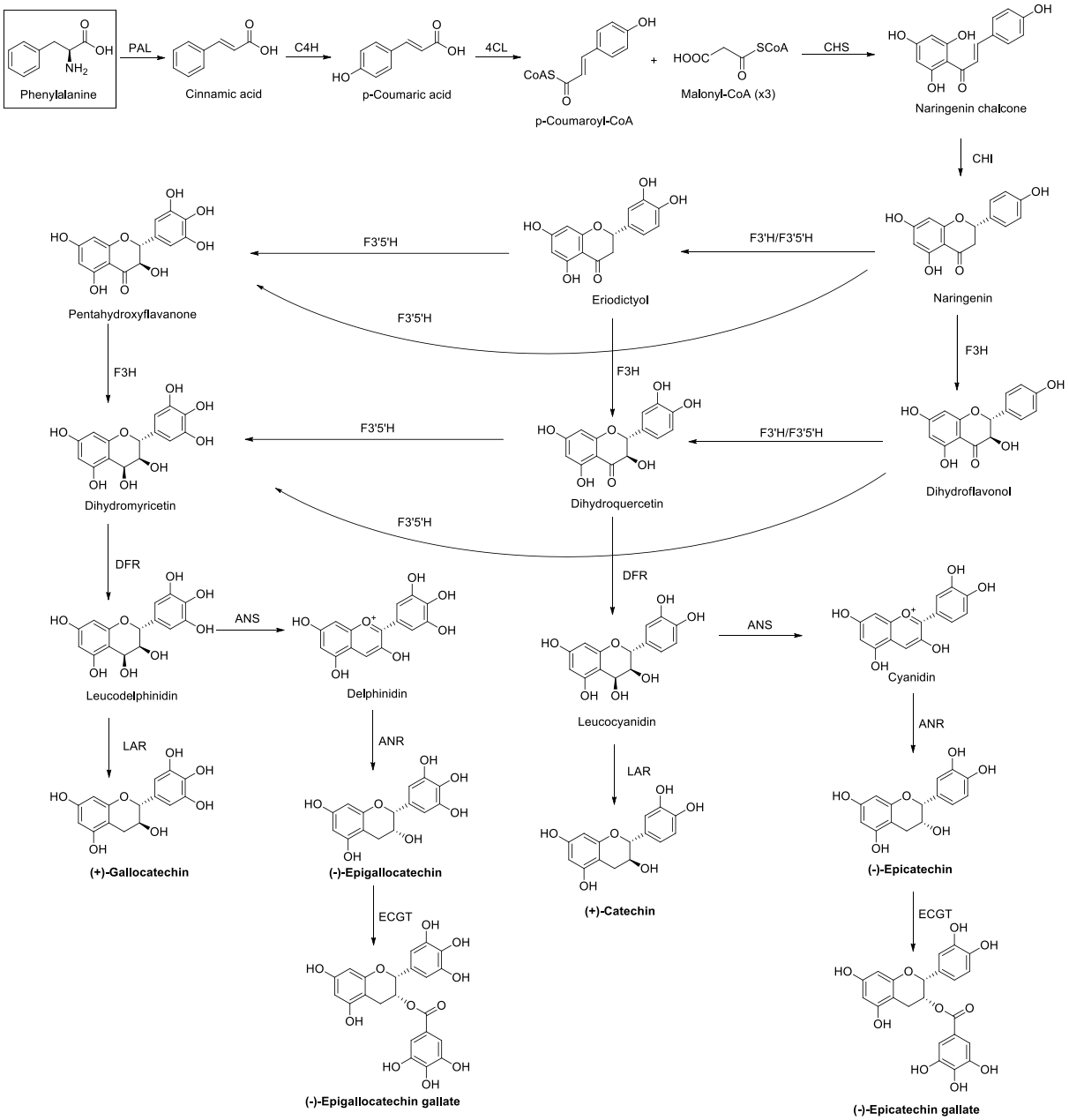

Figure 2 Phenylalanine is the precursor in the biosynthetic pathway of catechin and its derivatives [7-9].

Materials and Methods

Chemical

The standard (+)-catechin (C), (-)-epicatechin (EC), (-)-gallocatechin (GC), (-)-epigallocatechin (EGC), (-)-epicatechin gallate (ECG), (-)-catechin gallate (CG), (-)-epigallocatechin gallate (EGCG), (-)-gallocatechin gallate (GCG), and caffeine (CF) were purchased from Sigma-Aldrich (St-Louis, MO, USA). L-phenylalanine was purchased from HiMedia Laboratories Pvt. Ltd. (Mumbai, India). Methanol and acetone were HPLC-grade obtained from V.C.CHEM HOUSE (Bangkok, Thailand).

Plant material

Both of Assam green tea (*Camellia sinensis var. Assamica*) and Chinese tea (*Camellia sinensis var. Sinensis*) were collected from Thoet Thai, Mae Fah Luang District, Chiang Rai province located in the north of Thailand. The dried tea leaves were prepared by the traditional commercial green tea processing which contains withering, roasting, rolling, and drying. The preparing of these processes were done by the Tea1x2 plantation. The selected green tea plants were 5 years which were fully matured and ready for monthly harvest. There were two sets of fresh green tea leaves; the initial samples and the supplemented samples. In all sets, two samples, young and old green tea leaves, were collected. The young green tea leaves are the 3 top most leaves of the branch. The old green tea leaves are the 5^{th}-8^{th} leaves from the top. All samples were collected and packed in a plastic mesh bag and kept in ice for transportation.

L-Phenylalanine supplementation

After the green tea leaves collection of the green tea plants of the initial set was completed, L-phenylalanine was supplemented to the respective plants. Each plant received 0.5 g of L-phenylalanine which was evenly scattered around the base trunk of the plants. Watering routine and growing condition were kept the same. After 28 days, the green tea leaves of the supplementation sets were collected.

Extracts Preparation

Aqueous extracts preparation for HPLC analysis

Dried tea leaves (2.0 grams) were infused with 200 mL of distilled water at 80 °C for 3 min, and this was done as a triplicate. The samples were then filtered through a 0.45 μm nylon syringe filter. Then, total catechins and caffeine were measured with high-performance liquid chromatography (HPLC).

Methanol extracts preparation for HPLC analysis

Fresh tea leaves (1.0 gram) of each sample were extracted with 20 mL of 70% methanol in distilled water for 30 min at room temperature then filtered through a 0.45 μm nylon syringe filter. All samples were done as a triplicate and the concentrations of C, EC, GC, EGC, ECG, CG, EGCG, GCG and CF were measured with HPLC.

High-performance liquid chromatography analysis of green tea catechin and caffeine

Green tea solutions were prepared in a glass vial and filtered with 0.45 μm nylon syringe filter before injected into HPLC. Column C18 was used and the mobile phase: acetonitrile, 0.05% trifluoroacetic acid, acetonitrile, and distilled H_2O in 0:87:13:0 ratio, flow rate was 2 mL/min at a column temperature of 30°C. The injected volume was 10 μL. The contents of catechins and caffeine in the samples were quantified by HPLC at the Tea and Coffee Institute of Mae Fah Luang University.

DPPH-Radical scavenging activity

For the dried tea leaves, 2 grams of each sample were boiled in 125 mL of distilled water for 10 minutes. The tea solution was filtered and the volume was adjusted to 250 mL with distilled water. For the fresh tea leaves, 10 grams of each sample were soaked in 200 mL of methanol at room temperature. The mixture was blended and filtrated. The solution volume was adjusted to 250 mL with methanol. Both of dried tea and fresh tea solutions were diluted by 50 folds.

A 1,1-diphenyl-2-picrylhydrazyl (DPPH) free-radical scavenging assay was conducted according to the Donlao and Ogawa method [20]. The 5 µL of tea solutions were added into 96-well plate, and then 195 µL of DPPH solution was added. The mixture was placed at room temperature for 30 minutes and the absorbance was measured at 517 nm. Trolox was used as the standard solution.

Determination of chlorophyll

Samples were prepared according to methanol extracts preparation for HPLC analysis but all samples were diluted by 10 folds. The samples were measured by a UV-Vis spectrophotometer. The amounts of chlorophyll a and chlorophyll b were measured at 663 nm and 645 nm, respectively. The contents were calculated using the following formulas [18]:

Content of chlorophyll a (mg/L) $= 12.7A_{663} - 2.95A_{645}$

Content of chlorophyll b (mg/L) $= 22.9A_{645} - 4.67A_{663}$

Total chlorophyll content (mg/L) = Content of chlorophyll a + Content of chlorophyll b

Measurement of color

The sample was prepared using the methanol extract preparation for HPLC analysis, the color of the solution sample was analyzed by ColorQuest XE, HunterLab. According to CIE (L*, a*, b*) system, the value of L* represents lightness. The value of a* indicates redness and greenness, and the value of b* indicates yellowness and blueness [18].

Results and Discussion

High-performance liquid chromatography analysis on individual catechins and caffeine

All dried tea leaves samples (Chiang Rai green tea; Chinese green tea (GCN) and Assam green tea (GAS)) were infused with hot water (80°C) for HPLC analysis. The HPLC results of the chemical components are shown in Table 1. The C, EC, GC, EGC, ECG, CG, EGCG, GCG and CF contents were analyzed by HPLC and calculated to mg/g unit. The results showed that both of GCN and GAS contained low amounts of all catechins and caffeine which were lower than 0.04 mg/g. The reduction of caffeine and catechin contents may be due to the long exposure to the high temperature during the traditional commercial green tea processing. The previous study also had similar results of low catechin contents after green tea processing [18,20].

Table 1 HPLC analysis of chemical contents of dried green tea leaves (mg/g) (n=3).

Analytes	R_t (min)	Sample (mg/g)	
		GCN	GAS
(-)-gallocatechin (GC)	1.06	<0.01	<0.01
(-)-epigallocatechin (EGC)	1.32	0.02±0.01	0.02±0.01
(+)-catechin (C)	1.52	<0.01	<0.01
(-)-epicatechin (EC)	2.07	<0.01	0.02±0.02
(-)-epigallocatechin gallate (EGCG)	2.33	0.02±0.01	<0.01
caffeine (CF)	3.21	0.04±0.01	0.04±0.01
(-)-gallocatechin gallate (GCG)	3.35	<0.01	N/A
(-)-epicatechin gallate (ECG)	4.47	<0.01	<0.01
(-)-catechin gallate (CG)	6.63	<0.01	<0.01

*N/A is not applicable

The young and old leaves of the Assam (GAS) and Chinese green tea (GCN) were collected before and after the supplementation with L-phenylalanine for the analysis of their chemical contents with HPLC. Both the young and old leaves of GAS and GCN after supplementation with 0.5 g of L-phenylalanine for 1 month have a total catechin content higher than the initial samples (Figure 3). As seen in the biosynthesis of catechins (Figure 2), it can imply that increasing the input of L-phenylalanine (catechin precursor) will also increase the output of catechin production. The leaves of Chinese green tea (GCN) have the highest total catechins when compared to the others, especially the old leaves of Chinese green tea (GCN-O) with 1.26 mg/g of total catechins.

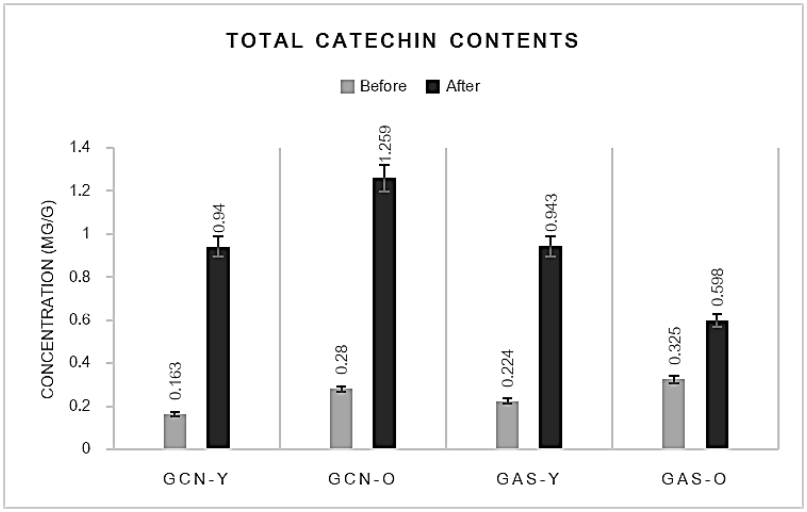

Figure 3 Total catechin contents of young leaves of Chinese green tea (GCN-Y), old leaves of Chinese green tea (GCN-O), young leaves of Assam (GAS-Y) and old leaves of Assam (GAS-O) of initial group and supplemented group (n=3).

The amounts of other catechin derivative contents and caffeine of GCN and GAS before and after supplementation with L-phenylalanine, were analyzed by HPLC. The HPLC results of the catechin (GC, EGC, C, EC, EGCG, GCG, ECG, CG) and caffeine (CF) of young leaves of Chinese green tea (GCN-Y), old leaves of Chinese green tea (GCN-O), young leaves of Assam (GAS-Y) and old leaves of Assam (GAS-O) in the initial group and supplemented group are shown in Table 2 and Figure 4. The concentration of most of the chemical constituents increased after supplementation with L-phenylalanine. The concentration of EGC and EGCG, which are scarce in dried green tea of GCN and GAS, increased after supplementation with L-phenylalanine. In GCN-O, the EGC and EGCG showed the highest increase at 0.512±0.03 mg/g from 0.114±0.10 mg/g of EGC and at 0.484±0.05 from 0.101±0.13 mg/g of EGCG. However, the levels of GC, GCG, and CG were not significantly increased after supplementation. The CF contents of GCN and GAS had a 3-fold increase after supplementation, as shown in Table 2. The previous study showed that the younger tissue of green tea plants had a higher caffeine content and also higher expression of catechin biosynthetic enzymes. It can imply that high amount of the L-phenylalanine (catechin precursor) with high amount of biosynthetic enzymes will produce large quantity of catechin, including caffeine [21].

Table 2 Effect of L-phenylalanine on catechin content in fresh tea leaves (mg/g) (n=3).

Analytes	Group	Sample (mg/g)			
		GCN-Y	GCN-O	GAS-Y	GAS-O
GC	Initial samples	0.02±0.01	0.02±0.01	0.02±0.01	0.02±0.01
	Supplemented samples	0.04±0.02	0.04±0.01	0.04±0.02	0.02±0.01
EGC	Initial samples	0.03±0.02	0.11±0.01	0.02±0.01	0.03±0.01
	Supplemented samples	0.07±0.05	0.51±0.03	0.13±0.02	0.04±0.01
C	Initial samples	0.03±0.05	N/A	0.06±0.01	0.06±0.01
	Supplemented samples	0.14±0.02	N/A	0.11±0.02	0.10±0.08
EC	Initial samples	0.02±0.01	0.03±0.01	0.06±0.02	0.12±0.09
	Supplemented samples	0.18±0.01	0.15±0.05	0.15±0.03	0.18±0.07
EGCG	Initial samples	0.04±0.01	0.10±0.03	N/A	N/A
	Supplemented samples	0.22±0.02	0.48±0.05	0.31±0.02	0.03±0.04
CF	Initial samples	0.12±0.01	0.09±0.08	0.13±0.01	0.11±0.03
	Supplemented samples	0.30±0.06	0.29±0.05	0.31±0.01	0.27±0.01
GCG	Initial samples	N/A	N/A	N/A	N/A
	Supplemented samples	0.01±0.02	0.01±0.01	0.01±0.01	N/A
ECG	Initial samples	0.02±0.01	0.02±0.01	0.07±0.02	0.10±0.02
	Supplemented samples	0.27±0.02	0.07±0.01	0.20±0.02	0.23±0.01
CG	Initial samples	N/A	N/A	N/A	N/A
	Supplemented samples	0.02±0.02	N/A	N/A	N/A

*N/A is not applicable

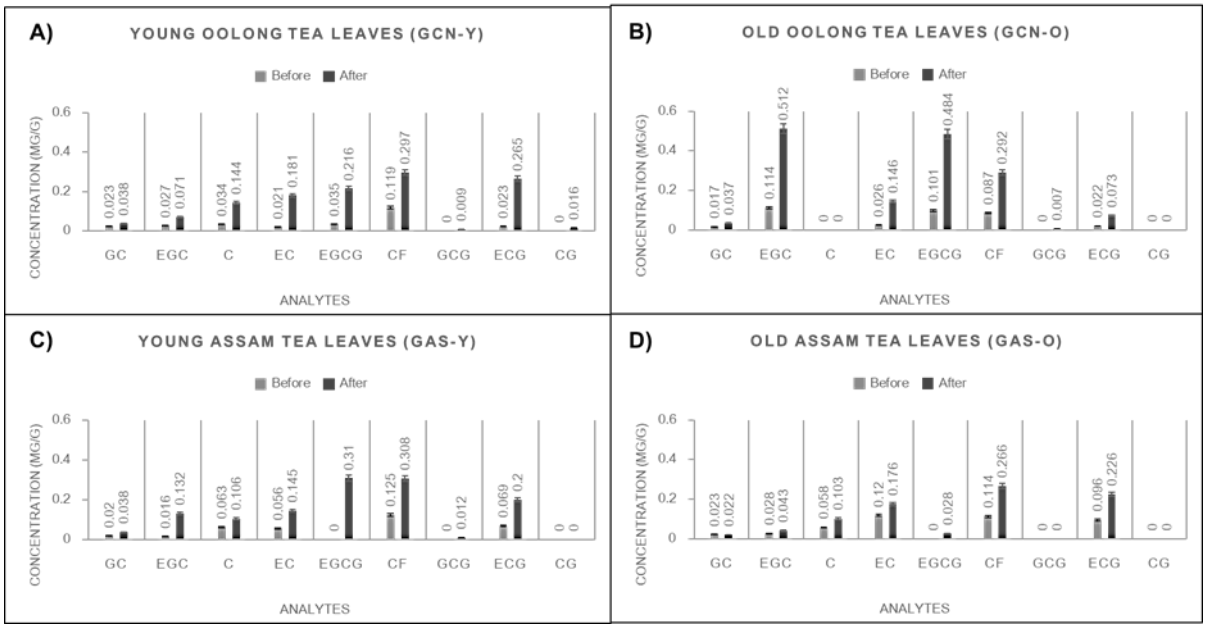

Figure 4 Comparison of the catechins and caffeine concentration in fresh tea leaves extract between initial group and supplementation group. A) young Chinese green tea leaves (GCN-Y), B) old Chinese green tea leaves (GCN-O), C) young Assam green tea leaves (GAS-Y) and D) old Assam green tea leaves (GAS-O) (n=3).

These results suggested that the supplementation with L-phenylalanine can increase the catechin contents in green tea leaves in both Assam green tea (GAS) and Chinese green tea (GCN). Especially, (-)-epicatechin gallate (ECG) and (-)-epigallocatechin gallate (EGCG), which have strong radical-scavenging capacity, increased significantly in the fresh green tea leaves supplemented with L-phenylalanine.

DPPH-radical scavenging activity

The antioxidant activity of green tea samples was measured by the 1,1-diphenyl-2-picrylhydrazyl (DPPH) free-radical scavenging assay [20]. First, we compared the DPPH free-radical scavenging activity of the dried green tea samples. The percent inhibition (%inhibition) on the DPPH assay is shown in Table 3. Trolox was used as a positive control at IC_{50} of 592.08 ppm. The GCN and GAS have lower %inhibition on the DPPH scavenging activity which are 26.85±0.01 and 32.59±0.01, respectively. Moreover, the DPPH scavenging activities of fresh green tea leaves in both young and old green tea of GCN and GAS (GCN-Y, GCN-O, GAS-Y, and GAS-O), in both initial group and supplementation group, were measured. The results showed that the antioxidant capacity of all green tea had no significant change although the amount of ECG and EGCG increased. The sample preparation process of the DPPH free-radical scavenging assay used 100% methanol as the extraction solvent and involved a short period of mixture blending. Methanol is slightly acidic which may trigger the hydrolysis of the ester bond of

certain catechins, such as ECG, and EGCG. The final catechin concentration might be lower than the actual amount. Therefore, the measured antioxidant capacity was also lower than the expected amount.

Table 3 DPPH radical scavenging activity of green tea samples (n=3).

Sample		Fertilized with L-Phenylalanine	%Inhibition
Dried green tea	GCN	-	26.85±0.01
	GAS	-	32.59±0.01
Fresh green tea	GCN-Y	Initial samples	39.82±0.02
		Supplemented samples	37.88±0.01
	GCN-O	Initial samples	36.84±0.01
		Supplemented samples	35.35±0.02
	GAS-Y	Initial samples	45.34±0.01
		Supplemented samples	41.91±0.01
	GAS-O	Initial samples	45.34±0.01
		Supplemented samples	35.35±0.01

*IC_{50} Trolox=592.08 ppm

LAB color value and chlorophyll content of green tea

The LAB color values of infused tea solutions of dried green tea leaves were measured and shown (Table 4). According to the CIE system, the value of L* represents lightness (dark (100) – light (0)), the a* value is the green (-) – red (+) color scales, and the b* value is the blue (-) – yellow (+) color scales. Both of the solutions of dried green tea samples (GCN and GAS) had the LAB results in the dark-red-yellow color which the L* was about 31, a* in positive value (2.22±0.04 and 1.75±0.03, respectively), and b* values were 8.98±0.01 and 5.72±0.03, respectively. These values were linked with the LAB color values of both young and old fresh green tea of GCN and GAS (GCN-Y, GCN-O, GAS-Y, and GAS-O) which were in the dark-red-yellow color. From these results, we supplied L-phenylalanine to the GCN and GAS plants for 28 days and collected the fresh green tea samples. The results showed that for all of the GCN-Y, GCN-O, GAS-Y, and GAS-O after supplementation, the a* values decreased (greener in color) and b* values increased (yellower in color). This suggested that all green teas were greener in color, which correlated with the increase of chlorophyll contents in all samples. However, the L* values of all fresh green tea leaves had no significantly change after supplementation.

Table 4 The LAB color values of all green tea samples (n=3).

Sample		Fertilized with L-Phenylalanine	L*	a*	b*
Dried green tea	GCN	-	31.62±0.02	2.22±0.04	8.98±0.01
	GAS	-	31.04±0.01	1.75±0.03	5.72±0.03
Fresh green tea	GCN-Y	Initial samples	31.21±0.02	0.3±0.01	4.31±0.02
		Supplemented samples	27.77±0.04	-2.19±0.02	5.55±0.04
	GCN-O	Initial samples	28.48±0.01	0.27±0.01	-0.23±0.01
		Supplemented samples	29.19±0.03	-0.66±0.02	0.26±0.01
	GAS-Y	Initial samples	30.73±0.02	1.45±0.04	4.10±0.03
		Supplemented samples	27.32±0.03	-2.25±0.01	6.93±0.03
	GAS-O	Initial samples	28.74±0.02	0.03±0.01	0.43±0.02
		Supplemented samples	25.42±0.04	-0.57±0.02	0.86±0.04

Moreover, the chlorophyll contents of the fresh green tea leaves were studied. This is an evaluation of the relationship between the color value and the chlorophyll contents of fresh green tea samples. The results in Table 5 showed that after supplementation with L-phenylalanine, total chlorophyll contents of the green tea leaves increased which correlated to the LAB color levels. The total chlorophyll contents in both GAS-Y and GAS-O increased from 1.65±0.03 to 2.35±0.04 mg/g and 4.80±0.07 to 7.92±0.13 mg/g, respectively. Chlorophyll content is a well-known indicator for green tea quality [19]. Therefore, L-phenylalanine supplementation may be used for the improvement of the green tea quality in the future. Moreover, Assam green tea (GAS) has a general problem where their fresh green tea leaves color turn red (Figure 5A). After the supplementation with L-phenylalanine, the tea leaves of GAS had a greener color (Figure 5B), compared to the initial sample. Another possible explanation is that L-phenylalanine is a known precursor of the biosynthesis of anthocyanin and its derivatives [22]. Anthocyanins are the group of plant pigments which may affect the color of green tea leaves.

Table 5 The chlorophyll contents of fresh green tea leaves before and after supplementation with L-phenylalanine (n=3).

Samples	Fertilized with L-Phenylalanine	Chlorophyll a (mg/g)	Chlorophyll b (mg/g)	Total chlorophyll content (mg/g)
GCN-Y	Initial samples	1.39±0.01	0.52±0.02	1.90±0.04
	Supplemented samples	1.67±0.01	0.85±0.01	2.52±0.04
GCN-O	Initial samples	4.96±0.01	2.42±0.02	7.37±0.13
	Supplemented samples	1.67±0.02	2.38±0.01	7.21±0.13
GAS-Y	Initial samples	1.17±0.01	0.48±0.02	1.65±0.03
	Supplemented samples	1.48±0.03	0.87±0.02	2.35±0.04
GAS-O	Initial samples	2.93±0.01	1.88±0.03	4.80±0.07
	Supplemented samples	5.14±0.02	2.78±0.01	7.92±0.13

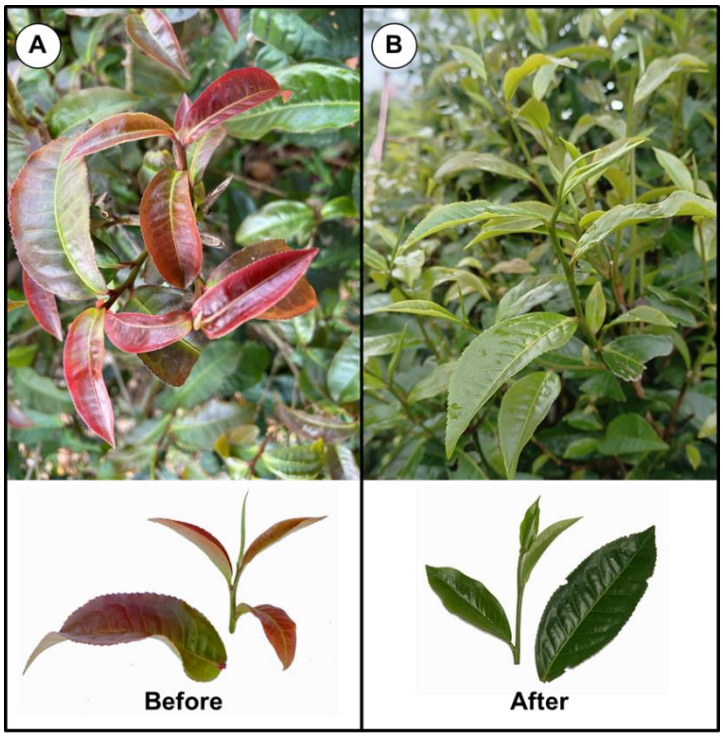

Figure 5 Assam green tea leaves before (A) and after (B) supplementation with L-phenylalanine.

Conclusions

The chemical contents in green teas contribute to their physical properties and biological activities. This study evaluated the change of chemical components in Assam green tea and Chinese green tea after supplementation with L-phenylalanine. The results showed that the supplementation with L-phenylalanine can increase the amounts of (-)-epicatechin gallate (ECG) and (-)-epigallocatechin gallate (EGCG) in fresh green tea leaves. However, the antioxidant capacity of the fresh green tea showed no significant change after supplementation. Moreover, the fresh leaves of Assam green tea and Chinese green tea had a greener color after the supplementation which correlated to the increase of the total chlorophyll contents. These results will be beneficial for the improvement of green tea quality in future research.

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