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A comparison of the production of polyphenol contents and the expression of genes involved in Vietnamese tea cultivars

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Abstract

Tea (*Camellia sinensis*) is a popu*lar* health beverage which is consumed all over the world due to its good aroma and taste. Tea consumption is also considered to reduce the risk of several diseases in humans, including cardiovascu*lar* diseases, diabetes and cancers. Recent studies have shown that polyphenols derived from tea may contribute to the majority of these pharmaceutical properties. Among all the tea polyphenols, catechins are the main components that include (−)-epicatechin (EC), (−)-epicatechin gallate (ECG), (−)-epigallocatechin (EGC), (−)-epigallocatechin-3 gallate (EGCG), (+)-catechin (C), (−)-catechin gallate (CG), (−)-gallocatechin (GC), and (−)-gallocatechingallate (GCG). In the present work, four catechins (C, EGC, ECG, and EGCG) and two anthocyanidins (cyanidin 3-O-glucoside and delphinidin 3-O-glucoside) in two Vietnamese tea cultivars, *Trungduxanh* and *Trungdutim*, were quantitatively detected by high-performance liquid chromatography. The total catechin content in *Trungduxanh* was generally higher than that in *Trungdutim*. By contrast, the concentrations of the two anthocyanidins were lower in *Trungduxanh* than that in *Trungdutim*, suggesting that *Trungdutim* tea accumulates anthocyanins to produce purple colour in buds and leaves, rather than converting them into catechins. Real-time PCR was also performed to analyse the expression levels of leucocyanidin reductase (*LAR*) and anthocyanidin reductase (*ANR*) genes, which are involved in catechin biosynthesis. In accordance with the HPLC analysis, the qPCR results showed that the transcripts of both genes in *Trungduxanh* tea were more abundant than in *Trungdutim* tea.

Introduction

Tea (*Camellia sinensis*) is one of the most widely consumed beverages. It was first discovered in China about 2700 BC, after which it spread throughout the world. Recently, tea in China has been classified into seven primary types based on the different processing methods: green tea, black tea, oolong tea, white tea, yellow tea, aged pu-erh and ripened pu-erh tea (Yi *et al*., 2015). Among these, the former three types are more popu*lar* than the others. Green tea (non-fermented) is commonly consumed in Asian countries, black tea (fermented) is favoured in the Western countries, and oolong tea (semi-fermented) is consumed in southern China (Khan and Mukhtar, 2007). Vietnam is one of the *lar*gest tea producing and exporting countries in the world. In Vietnam, tea is grown mainly in the Northern midland and mountainous regions, and the Central Highlands. According to the General Statistics Office of Vietnam (http://www.gso.gov.vn), the total tea growing area had reached 131.5 thousand hectares with an output of 1,033.6 thousand tons in 2016. In the same year, Vietnam tea export reached 138 thousand tons which were valued at USD 228 million.

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Polyphenols that comprise approximately 30% - 42% of green tea dry weight are responsible for tea's health benefits. Among polyphenols, catechins (also known as flavan-3-ols) are the most abundant substances with a concentration of 70% of the total tea polyphenol content. Catechins consist of two main types, epicatechins and epicatechin epimers. Epicatechins include (−)-epicatechin (EC), (−)-epicatechin gallate (ECG), (−)-epigallocatechin (EGC), and (−)-epigallocatechin-3 gallate (EGCG); and epicatechin epimers consist of (+)-catechin (C), (−)-catechin gallate (CG), (−)-gallocatechin (GC), and (−)-gallocatechin gallate (GCG). Along with caffeine and several amino acids, these catechins contribute to the quality and taste of tea (Yamamoto *et al*., 1997). Catechins are known for their potential health benefits, including antioxidant activities (Shahidi *et al*., 2008), prevention or reduction of some types of cancer (Beltz *et al*., 2006), immune system improvement, and prevention of cardiovascu*lar* disease, diabetes and dental decay (Hamilton-Miller, 2001; Yang *et al*., 2004; Nagao *et al*., 2007). Furthermore, tea catechins have also been widely used in food and cosmetic industries.

In addition to catechins, anthocyanins are important biological compounds in plants. More than 670 anthocyanins have been identified in nature, among which the following six anthocyanins are primarily found in fruits and vegetables: pe*lar*gonidin, cyanidin, peonidin, delphinidin, petunidin and malvidin. Anthocyanins contribute to the colours of numerous plants, such as grapes (Rivero-Pérez *et al*., 2008), berries (Nicoué *et al*., 2007), and *Hibiscus* flowers (Lo *et al*., 2007). Studies have reported that anthocyanins possess several valuable bioactive compounds such as antioxidants (Bae and Suh, 2007), anticarcinogens (Lee *et al*., 2009), and antimicrobials (Viskelis *et al*., 2009).

High-performance liquid chromatography (HPLC) is a useful method for the quantitative determination of polyphenol contents in different tea types. Numerous studies have been conducted to improve the separation efficiency and to simultaneously determine the polyphenol content in green, black, oolong and pu-erh teas (Yashin *et al*., 2015). The efficiency of HPLC analysis is also dependent on the method of extraction, the type of solvent used, and the method of sample preparation (Lorenzo and Munekata, 2016).

Several studies have investigated the biosynthetic pathway of catechins and anthocyanins in the tea plant using genetic and biochemical approaches over the recent few decades. Some genes involved in the biosynthesis of catechins and anthocyanins have been discovered and characterised (Punyasiri *et al*., 2004). A recent study had elucidated the biosynthetic and regulatory mechanisms of catechins in tea using a combination of transcriptomic analysis and HPLC (Zhang *et al*., 2018). Key enzymes that directly catalyse catechin producing reactions are leucoanthocyanidin reductase (*LAR*) and anthocyanidin reductase (*ANR*). *LAR* is responsible for the formation of catechins and gallocatechins, and *ANR* plays an important role in the production of epicatechins (Figure 1) (Liu *et al*., 2015; Zhang *et al*., 2018). Furthermore, anthocyanins share their precursors with catechins. In particu*lar*, leucoanthocyanins can be converted into anthocyanins by anthocyanidin synthase (ANS)

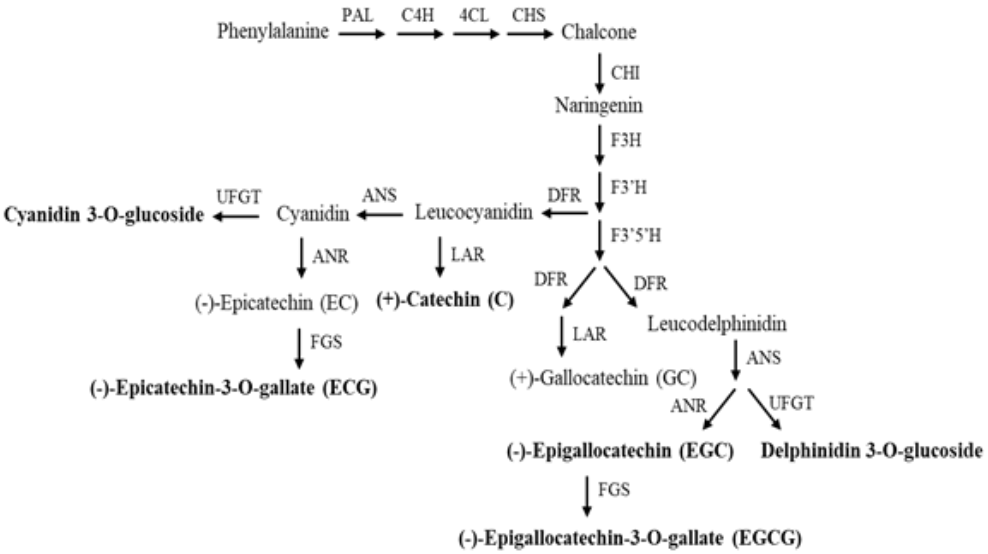

Figure 1. Putative biosynthetic pathways of catechins and anthocyanins in tea (*C. sinensis*) leaves (Liu *et al*., 2015; Zhang *et al.*, 2018). PAL: phenylalanine ammonia-lyase; C4H: cinnamate 4-hydroxylase; 4CL: 4-coumarate CoA ligase; CHS: chalcone synthase; CHI: chalcone isomerase; F3H: flavanone 3-hydroxylase; F3'H: flavonoid 3'-hydroxylase; F3'5'H: flavonoid 3',5'-hydroxylase; DFR: dihydroflavonol 4-reductase; LAR: leucocyanidin reductase; ANR: anthocyanidin reductase; ANS: anthocyanidin synthase; FGS: flavan-3-ol gallate synthase; UFGT: anthocyanidin 3-O-glucosyltransferase.

or into non-epicatechins by *LAR*. Anthocyanins such as cyanidin and delphinidin are catalysed by *ANR* to form epicatechins (Liu *et al*., 2015).

In the present work, the contents of two anthocyanins and four catechins that significantly contribute to the valuable properties of tea were analysed in two Vietnamese local tea cultivars, *Trungduxanh* and *Trungdutim*. *Trungduxanh* belongs to the green-coloured tea cultivar, whereas *Trungdutim* is a purple tea that has purple buds and young leaves. The present work also investigated the relationship between the expression of *LAR* and *ANR* genes and catechin accumulation. The results of the present work could provide more valuable information about these tea cultivars and support to enhance tea conservation and planting activities.

Materials and methods

Plant materials

Two tea cultivars (*C. sinensis* var. *macrophylla* cultivars *Trungduxanh* and *Trungdutim*) were grown at the tea garden of Thai Nguyen University of Agriculture and Forestry, Thai Nguyen Province, Vietnam. The buds and young leaves (the first two to three leaves) were collected in September 2017. The tea samples were divided into two portions. The first portion used for the analysis of anthocyanins and catechins was directly extracted with a solvent following collection. The remaining samples were immediately frozen in liquid nitrogen and stored at -80°C for RNA isolation.

Extraction of catechins, anthocyanins and HPLC analysis

The standard chemicals, including (−)-epigallocatechin-3 gallate (EGCG), (−)-epicatechin gallate (ECG), (−)-epigallocatechin (EGC), (−)-epicatechin (EC), (+)-catechin (C), cyanidin 3-O-glucoside, and delphinidin 3-O-glucoside (Sigma, USA) were dissolved in methanol to make 2 mg/mL stock solution.

Buds (5.0 g) and young leaf samples were accurately weighed and cut into small pieces. Tea samples were extracted using 15 mL methanol by a sonicator (Elmar, Germany) for 15 min. The supernatant was obtained after centrifugation at 6,000 g for 15 min. The extraction step was repeated twice for each sample. The final extract was placed in a 50 mL volumetric flask and the volume was made using the same solvent. The extract was filtered through a 0.45 µm Millipore filter prior to HPLC analysis.

The extract was analysed using the Agilent 1260 LC/MS system (Agilent, CA, USA) equipped with a quaternary HPLC Agilent 1260 pump and a single quadrupole Agilent 6120 mass spectrometer. The extract was injected at different concentrations into a reversed phase Eclipse XDB C18 column (250 × 4.6 mm, 5 µm) with an XDB C18 guard column (Agilent Technologies Inc., USA). Mobile phases consisted of 1.0% acetic acid in water (v/v) (eluent A) and a gradient of acetonitrile (ACN) in methanol from 95/5 to 5/95 (v/v) for 45 min (eluent B). Detection was carried out at 280 nm. The flow rate was set at 1.0 mL/min. The column was maintained at a temperature of 25°C. The sample injection volume was 5 µL, and all samples were run in triplicate.

RNA isolation and quantitative real-time PCR

Total RNA was extracted from the bud and young leaf samples using the GeneJET RNA Purification Kit (Thermo Fisher Scientific, USA) according to the manufacturer's instruction. The integrity and quality of the isolated RNA were assessed by electrophoresis in 1% agarose gel and measurement of the optical density. First strand cDNA was synthesised from 0.5 µg of total RNA using the USB® First-Strand cDNA Synthesis Kit for Real-Time PCR (Affymetrix Inc., USA) with the reaction set-up according to the product's manual. The synthesised cDNA was used immediately or stored at -20°C for further experiments.

Table 1. Primers of catechin biosynthetic genes used for real-time PCR analysis in the two tea cultivars.

Gene	Primer sequence 5'-3'
LAR	Forward: ACTAGACCAACTCACCCTAGTCC
	Reverse: CACCCCACACTCTTCTATCAATC
ANR	Forward: TCCGAGGATCCAGAGAATGAC
	Reverse: TCCAGTGACTCTCATCCATGAC
GADPH	Forward: TCAAGCAAGGACTGGAGAGG
	Reverse: ACAGTGGGAACGCGGAAAG

Primers for quantitative real-time PCR (Table 1) were designed and synthesised by Phusa Biochem Ltd., Vietnam. The PCR analysis was conducted using Luna® Universal qPCR Master Mix (New England Biolabs, USA) in the LightCycler® 96 System (Roche, Switzerland). Each 20 µL reaction mixture contained 10 µL Luna Universal qPCR Master Mix, 0.5 µL of 10 µM of each primer, and 1 µL cDNA. The amplification conditions consisted of one cycle of 60 s at 95°C, followed by 45 cycles of 15 s at 95°C and 30 s at 60°C. Melting curves were obtained to verify primer efficiency by the default setup of the LightCycler® 96 system. The qPCR assay included three technical and biological replicates. Data were analysed using the LightCycler® 96 software version 1.1.

Results

Analysis of catechins and anthocyanins by HPLC

Tea samples of two tea cultivars, *Trungduxanh* and *Trungdutim*, were extracted using 100% methanol immediately after harvest. The normal epi structure of catechins was confirmed to be unstable at the temperature > 80°C (Chen *et al*., 1998). Thus, the controlled temperature extraction method was applied in the present work.

The anthocyanins and catechins detected in the two tea cultivars were as follows: delphinidin 3-O-glucoside, cyanidin 3-O-glucoside, (−)-epigallocatechin-3 gallate (EGCG), (−)-epicatechin gallate (ECG), (−)-epigallocatechin (EGC), and (+)-catechin (C). The order of elution of these anthocyanins and catechins was: delphinidin 3-O-glucoside, 10.2 min; cyanidin 3-O-glucoside, 12.7 min; EGC, 15.1 min; C, 16.7 min; EGCG, 18.8 min; and ECG, 24.9 min (Figure 2). Furthermore, the separation of the reference standards and the two tea cultivar extracts by HPLC was consistent and could be achieved within 25 min using the conditions described above (Figure 2).

The individual content of each anthocyanin and catechin in the two tea cultivars is presented in Table 2. It was observed that EGCG had the highest abundance, followed by ECG and EGC, in both the tea extracts. Meanwhile, the C content was the lowest among the tested catechins. However, the contents of all catechins in *Trungduxanh* were higher than those in *Trungdutim*. Regarding anthocyanins, both delphinidin 3-O-glucoside and cyanidin 3-O-glucoside in *Trungdutim* exhibited a higher concentration than those in *Trungduxanh*. Unlike green-coloured tea, *Trungdutim* is a special type of tea whose buds and leaves are purple, upside leaves are pale purple then turn to green upon maturity while downside leaves retain this dark colour. Studies have also reported about anthocyanin accumulation in other purple-coloured tea cultivars, which contributed to their special colour (Saito *et al*., 2011; Lai *et al*., 2016). A previous study that investigated six tea samples also reported that the total anthocyanin content in purple tea samples was approximately 3.1 times higher than that in green tea samples (Wei *et al*., 2016). Therefore, the colour of the buds and leaves in *Trungdutim* might be due to anthocyanin

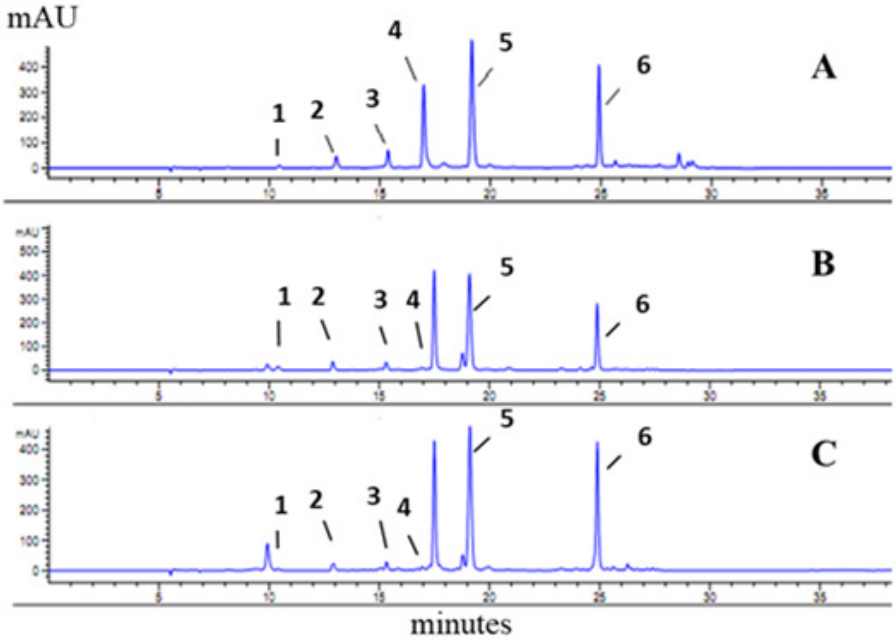

Figure 2. HPLC chromatogram of catechin components in the two tea cultivars. (A) catechin standard, (B) *Trungdutim* and (C) *Trungduxanh*. The peaks were identified as 1. Delphinidin 3-O-glucoside, 2. Cyanidin 3-O-glucoside, 3. EGC, 4. C, 5. EGCG, and 6. ECG.

Table 2. The concentration of catechin components in tea leaves (mg/g).

Cultivar	Anthocyanins*		Catechins*			
	Delphinidin 3-O-glucoside	Cyanidin 3-O-glucoside	EGC	C	EGCG	ECG
Trungdutim	0.46 ± 0.12	2.81 ± 0.07	5.65 ± 0.49	0.78 ± 0.11	16.51 ± 1.23	6.18 ± 0.48
Trungduxanh	0.13 ± 0.08	2.68 ± 0.08	6.81 ± 1.05	0.82 ± 0.07	20.15 ± 2.12	9.57 ± 1.45

Data are means of triplicate (n = 3) ± standard deviation.

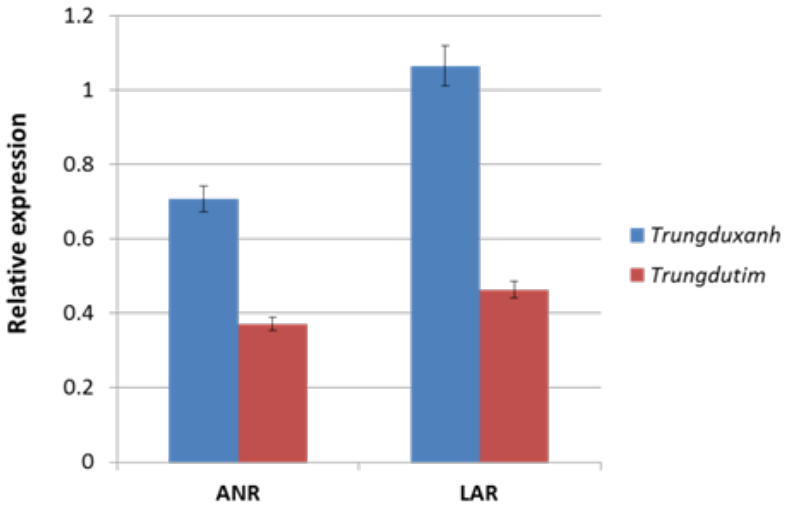

Figure 3. Relative expression of catechin biosynthetic pathway genes in tea leaves of the two different cultivars. The expression of each gene was standardised to the expression of GADPH cDNA and normalised to 1 in *Trungduxanh* tea cultivar.

accumulation. Furthermore, anthocyanin biosynthesis is a part of the biosynthesis of phenylpropanoids and flavonoids. Leucoanthocyanin is the precursor of not only anthocyanin but also non-epicatechin. It has been reported that a number of anthocyanidins such as cyanidin and delphinidin are directly catalysed to produce epicatechins (Liu *et al*., 2015). On this basis, the HPLC analysis results suggested that *Trungdutim* preferred anthocyanidin accumulation over catechin formation. To verify this hypothesis, the expression levels of the genes involved in catechin synthesis in the two tea cultivars, *Trungduxanh* and *Trungdutim*, was investigated.

Expression levels of ANR and LAR genes

Both anthocyanins and catechins are derived from multiple branches of the flavonoid metabolic pathway. Catechin and gallocatechin are produced from leucocyanidin and leucoanthocyanidin, respectively, by the catalysis of *LAR*. Meanwhile, epicatechin and epigallocatechin share direct precursors with anthocyanins. The biosynthesis of epicatechin and epigallocatechin first requires ANS to convert leucoanthocyanidins into anthocyanidins. Thereafter, anthocyanidins can be converted into epicatechins by *ANR* or into its derivatives by UFGT enzyme (Liu *et al*., 2015). In the present work, the relative expression levels of *LAR* and *ANR* genes, which play important roles in the formation of catechins was examined. Quantitative real-time PCR was performed using specific primers of the two genes of interest, and a housekeeping gene, GADPH, was used as an internal control. Total RNA samples isolated from the two tea cultivars were used to synthesise cDNA, which was used as the template in the qPCR experiments.

The comparison of *LAR* and *ANR* expression levels between *Trungduxanh* and *Trungdutim* tea cultivars is depicted in Figure 3. It could be observed that the transcript levels of both genes in *Trungduxanh* were higher than those in *Trungdutim*. The expression of *LAR* in *Trungduxanh* was 2.3 times higher than that in *Trungdutim*. However, when compared with *Trungduxanh*, *Trungdutim* tea exhibited an approximately 2-fold lower level of *ANR* relative expression. This result was consistent with the HPLC analysis in which catechins were more abundant in *Trungduxanh* than in *Trungdutim*.

Discussion

In the present work, the polyphenol content as well as the expression of genes associated with catechin synthesis in two Vietnamese tea cultivars, *Trungduxanh* and *Trungdutim*, were investigated. Four catechins (C, EGC, EGCG, and ECG) and two anthocyanins (delphinidin 3-O-glucoside and cyanidin 3-O-glucoside) were quantitatively determined by HPLC analysis. Polyphenols are prone to degradation or form complexes with different tea contents during the extraction process (Yao *et al*., 2004). In the present work, methanol was selected as a solvent for extraction to maximise the efficiency of polyphenol extraction. Among the investigated catechins, EGCG had the highest concentration, and C had the lowest in both tea cultivars. In general, the total catechin content in *Trungduxanh* was higher than that in *Trungdutim*. In contrast, the concentration of anthocyanins was found to be lower in *Trungduxanh* than in *Trungdutim*. This difference may be explained by an increase in the accumulation of anthocyanidins

in purple tea cultivars. Accordingly, Wei *et al*. (2016) reported that the concentration of anthocyanins in purple-coloured tea was more than 3-fold higher than that in ordinary tea. Another previous study reported that the antioxidant ability of anthocyanins was simi*lar* to that of catechins in unaerated tea samples and approximately 23 times higher than that of catechins in aerated ones due to the loss of catechins during tea processing (Kerio *et al*., 2013). In fact, a recent study demonstrated the relationship between anthocyanin hyper-accumulation and the action of a transcription factor (CsMYB75) and glutathione transferase (CsGSTF1). These genes play a vital role in the vacuo*lar* deposition of anthocyanins in tea. The total anthocyanin content of purple tea cultivar (ZJ) was 16-fold higher than the content of normal tea cultivar (LJ43). The abundance of CsGSTF1 transcript in the ZJ transcriptome was 5-fold greater than that in the LJ43 transcriptome (Wei *et al*., 2019). The contents of catechins and anthocyanins vary depending on factors such as the tea variety, the collection time, the development stage, and the processing method. Zeng *et al*. (2016) had reported that the content of tea polyphenols, especially catechins, was influenced by time, temperature, and pH of the processing and storing methods. The total catechin concentration of *Longjing43* cultivar was approximately 150 mg/g in dry tea buds, consisting of approximately 5 mg/g of EGC, 13 mg/g of C, 52 mg/g of EGCG, and 65 mg/g of ECG (Zhang *et al*., 2016). In comparison, the ratios between catechin compounds in *Trungdutim* and *Trungduxanh* tea cultivars in the present work are inconsistent with those reported by Zhang *et al*. (2016). The reasons could be the differences in cultivars, collection time, and the processing procedure for HPLC analysis.

This hypothesis was supported by qPCR that was conducted to analyse the expression levels of *ANR* and *LAR* genes involved in catechin synthesis in the two tea cultivar. Results indicated that the expression levels of these genes positively correlated with catechin concentrations in tea leaves. *Trungduxanh* exhibited higher expression levels of *LAR* and *ANR* genes than *Trungdutim*, which may explain why the catechin content in *Trungduxanh* was higher than that in *Trungdutim* in the HPLC analyses. In each tea cultivar, *LAR* exhibited a transcription level that was approximately 1.3 to 1.5-fold higher than that of *ANR*. It has been reported that *LAR* plays an important role in the conversion of leucocyanidin into non-epicatechin (C and GC) (Ashihara *et al*., 2010). However, another study indicated that the expression of *LAR* had a negative relationship with non-epicatechin concentrations in the autumn season (Liu *et al*., 2015). Furthermore, Zhang *et al*. (2016) reported that *LAR* had no significant involvement in the synthesis of C and GC, but they demonstrated a positive correlation with ECG and total catechins, suggesting the possible conversion of C into EC in tea plants. In addition, the transgenic tobacco overexpressing CsLAR had EC as the primary product with a minor amount of C (Wang *et al*., 2018). The results obtained in the present work are consistent with these findings because C showed the lowest content in both *Trungduxanh* and *Trungdutim* tea cultivars. Furthermore, Pang *et al*. (2013) showed that CsANR1 and CsANR2 could convert cyanidin into a mixture of EC and C. Therefore, it is deduced that cyanidin was catalysed from leucocyanidin by ANS and then converted into C by *ANR*. The formation of C by *ANR* may occur more frequently than the direct conversion of leucocyanidin into C by *LAR*. Otherwise, *ANR* is also known to convert anthocyanins into epicatechin and epigallocatechin (EC, EGC, ECG, and EGCG) (Punyasiri *et al*., 2004). In the present work, the transcription level of *ANR* showed a positive correlation with epicatechin but anthocyanin concentration. Therefore, this finding supported the hypothesis that anthocyanins were mainly accumulated in *Trungdutim* tea cultivar.

Conclusion

To our knowledge, the present work is the first report about the content of anthocyanins and catechins and the relative expression of the genes involved in catechin synthesis in the two tea cultivars, *Trungduxanh* and *Trungdutim*, in Vietnam. The present work demonstrated that *Trungduxanh* contained higher catechin concentrations but lower anthocyanin contents as compared to *Trungdutim*. Unlike *Trungduxanh*, *Trungdutim* may have a tendency to accumulate anthocyanins to form purple-coloured buds and leaves, rather than converting them into catechins. Results of the qPCR analysis were consistent with this hypothesis because both *LAR* and *ANR* genes exhibited weaker expression levels in the purple-coloured leaf tea than in the ordinary tea. Catechins and anthocyanins have numerous health benefits. Therefore, the findings obtained in the present work could enhance conservation activities and the development of these local tea cultivars.

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