

## Article

# Identification of bioactive compounds and antioxidant activity in leaves and fruits of *Actinidia arguta* accessions from northeastern China

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**Abstract:** *Actinidia arguta* (Sieb. et Zucc.) Planch. ex Miq. is abundant of vitamin C and bioactive compounds with high antioxidant activities. In this study, eight wild *A. arguta* accessions from different areas in Northeast China were collected. Some bioactive compounds were examined on the different tissues of different germplasms including four kinds of leaves, petioles and fruits. The method of UPLC-MS was used to detect the flavonoid compounds. The results showed that some bioactive compounds including vitamin C, soluble sugar, free amino acid, total phenolics and flavonoids content showed significant differences between six tissues of *A. arguta* accessions and showed significant variability with maturity. In eight accessions, the highest vitamin C content was found in young apical leaves of 'CBS-6' (7.47 mg/g fresh weight), and the highest soluble sugar content was in fruits of 'CJ-1' (196.52 mg/g fresh weight) and the highest total phenolic content and total flavonoids content were in young apical leaves of 'CBS-11' (3.48 mg/g fresh weight) and of 'CBS-3' (2.00 mg/g fresh weight), respectively. Ten flavonoid compounds including kaempferol, isorhamnetin and quercetin were detected in leaves, petioles and fruits. The total content of flavonoids were highest in young apical leaves (10219.84 µg·g<sup>-1</sup>) and the lowest in fruits (78.75 µg·g<sup>-1</sup>). Based on the comparison of the contents of several bioactive compounds, the two accessions 'CJ-1' and 'CBS-8' had relatively outstanding performance, and in the comprehensive evaluation of the antioxidant activity among different tissues, the young leaves had the strongest antioxidant activity. These results highlighted the antioxidant potentialities of *A. arguta* leaves as a major source of phenolics and vitamin C as well as flavonoids. It provided a theoretical basis for the utilization of leaves of *A. arguta*.

**Keywords:** *Actinidia arguta*; leaf; bioactivity; antioxidant activity; LC-MS/MS

## 1. Introduction

*Actinidia arguta* (Sieb. et Zucc.) Planch. ex Miq. is well known as hardy kiwifruit with good cold resistance, which is native to East Asia and different from other commercial kiwifruit [1]. *A. arguta* is widely distributed from 22° N to 47° N in latitude throughout of China [2]. It has a smooth and edible skin, good taste and flavour [3]. *A. arguta* is known to be very high vitamin C content and rich bioactive and volatile compounds with high antioxidant activities [4], such as flavonoids, phenolic compounds, etc [5,6]. At present, its growing commercially has become very popular in several countries including New Zealand, some European countries (Canada, Japan, the United States) and China [7-9].

Kiwifruit has been recorded in China for more than 2,000 years. It has rich nutritional value, strong protective activity and good medicinal value [10-12]. In recent years, many of the previous studies have focused on the fruits of *A. arguta* [13-15] and kiwifruit [16], their fruits have a high antioxidant capacity [17,18] and are also an important source of bioactive substances [19], such as high levels of vitamins, phenolics and flavonoids, etc. Some researchers have been studying flavonoid metabolites and other polyphenol content and evaluated the nutritional values of *A. arguta* fruits [20,21]. Yu *et al.* investigated the flavonoid compounds in *A. chinensis* and *A. arguta* fruits and identified a total of 125 flavonoids and 39 metabolites in two kiwi berries, which indicated that flavonoid components were abundant in kiwifruit and kiwi berry [22]. Although the bioactivity and antioxidant activity of the *A. arguta* fruit has been well characterized [4,23], there have been few studies investigating the bioactivity of *A. arguta* leaves.

*A. arguta* leaves are accumulated during the pruning each year, which are an affordable and abundant raw material as a kind of byproducts of farming. As early as 1977, *A. arguta* was recorded that it could be used as a medicinal food homologous plant. Its roots and leaves had the effects of clearing heat and promoting diuresis and strengthening stomach [24]. Cyboran *et al.* evidenced the effect of *A. arguta* leaf extract which could be used as an effective natural antioxidant protecting the body against external oxidizing agents and also be food additives or dietary supplements against food aging from oxidation-induced. So they are expected applied in the cosmetic, medicine, and food industries because of rich bioactive compounds. [25]. Webby identified a new flavonol triglycoside and isolated the kaempferol analogues in leaves of *A. arguta* [26]. Almeida *et al.* evaluated the leaves of *A. arguta* regarding antioxidant and antimicrobial activity, as well as radical scavenging activity for the first time, identified and quantified phenolic compounds [19]. In this study, eight wild *A. arguta* accessions from different areas were collected. They were growing strong, six of them were female plants, the fruits were all green, and the quality was good, and there were two male accessions, with a large amount of blooms and concentrated flowering period. Using LC-MS/MS and a series of physiological and biochemical substances to determine the leaves at different developmental stages of eight accessions, it was expected that new germplasm could be screened out, which would lay the foundation for the utilization of leaves of *A. arguta*.

## 2. Results

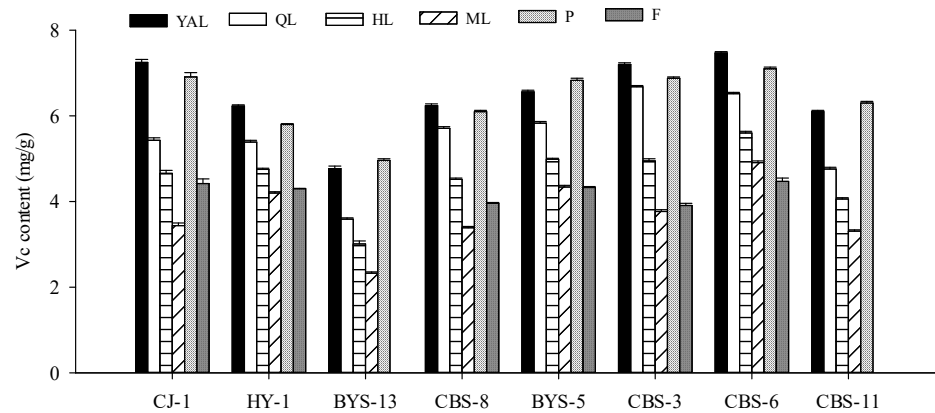
### 2.1. Determination of bioactive compounds in leaves and fruits of *A. arguta*

#### 2.1.1. Vitamin C (Vc) content

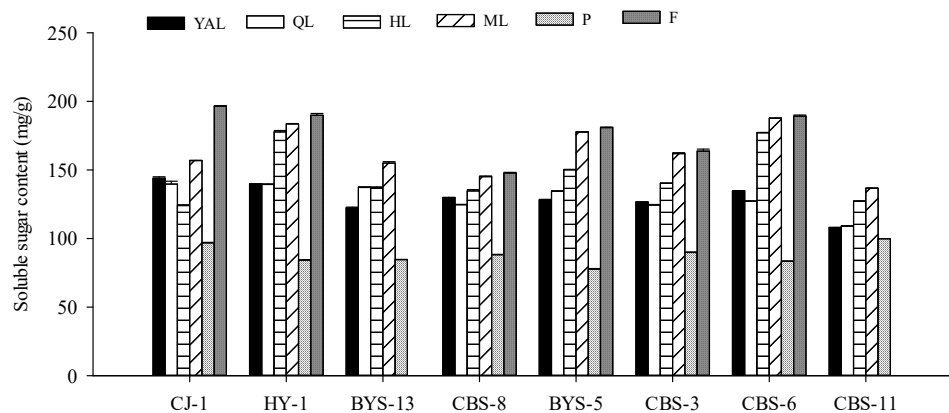
Overall, the Vc content in the eight *A. arguta* accessions leaves at different stages of maturity performed significant differences. The Vc content in young tissues (eg. YAL, P and QL) was slightly higher than that of old tissues (eg. HL and ML), and declined with leaf maturity. A continuous decrease was observed in Vc content of eight accessions leaves as leaf maturity (from YAL to ML), such as 'CJ-1' where it ranged from 7.25 to 3.44 mg/g fresh weight from young apical leaves to mature leaves, as well as 'HY-1', 'BYS-13', 'CBS-8', etc. Interestingly, the Vc content in the petioles of young leaves was similar to that in young leaves, and three accessions were even higher than those in young leaves, such as 'BYS-13', 'BYS-5' and 'CBS-11'. On the other hand, Vc content in fruits was lower than those of immature leaves and petioles and higher than mature leaves. By comparing the Vc content of leaves, petioles and fruits of the eight accessions in Figure 1, it was found that the Vc contents in YAL leaves and fruits of 'CBS-6' were the highest among the eight accessions, being 7.47 mg/g and 4.47 mg/g fresh weight, respectively, followed by 'CBS-3' and 'CJ-1', while 'BYS-13' was the lowest.

#### 2.1.2. Soluble sugar content (SSC)

The results of the SSC in six tissues of the eight accessions confirmed that the highest content was in the fruits, followed by the leaves, and the petioles had the lowest content (Fig. 2). While, the SSC in the leaves of each accession mostly increased with the gradual maturity of the



**Figure 1.** Comparison of Vc content of different tissues in eight *A. arguta* accessions.



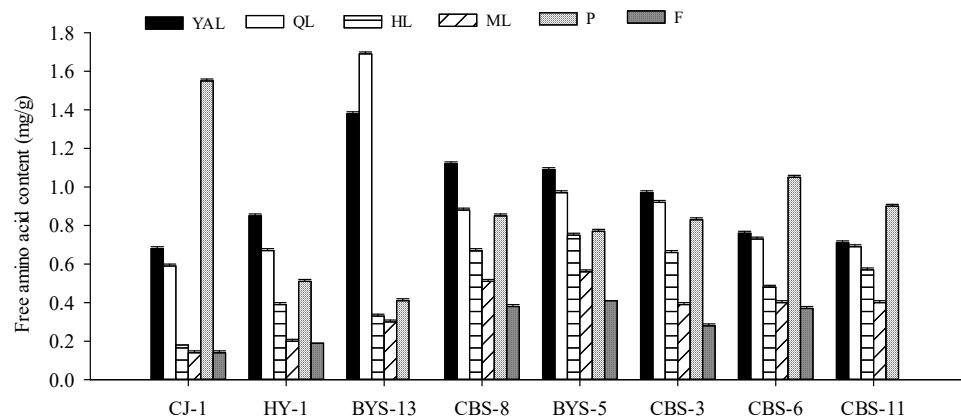
**Figure 2.** Comparison of soluble sugar content of different tissues in eight *A. arguta* accessions.

leaves and the highest content of leaves was in mature leaves (ML). In 'CJ-1', the SSC in mature leaves (ML, 156.85 mg/g) was lower than that in fruits, higher than that in other tissues, and 61.88% higher than that in petioles. The highest SSC (196.52 mg/g fresh weight) was obtained from the mature fruits of 'CJ-1'. The other five female accessions were similar to 'CJ-1', the highest SSC was in fruits and the lowest content in petioles. In male accessions, the highest SSC was in mature leaves. On the other hand, by comparing the SSC of the eight accessions in Figure 2, the content of 'CJ-1', 'HY-1', and 'CBS-6' in different tissues was relatively higher than the other five accessions, and 'CBS-11' was the lowest.

### 2.1.3. Free amino acid (FAA) content

The FAA content in young tissues (eg. YAL, P and QL) was significantly higher than that of old tissues (eg. HL and ML), and declined with leaf maturity, the lowest content was in fruits. In 'CJ-1', the content of FAA in petiole was the highest, which was 1.55 mg/g fresh weight, extremely higher than that in other tissues. The lowest content was observed in mature leaves and fruit (ML and F of 'CJ-1', 0.14 mg/g fresh weight), which was also the lowest among the eight accessions. Furthermore, it was found that the highest content was in QL of 'BYS-13' with 1.69 mg/g fresh weight, which was

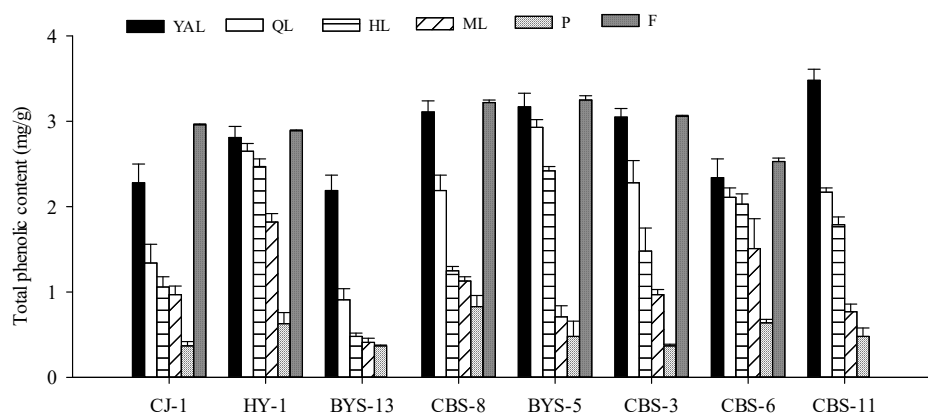
also the highest among the eight accessions. By comparing the FAA content of the eight accessions in Figure 3, it was found that the contents in P of 'CJ-1', and in YAL and QL of 'BYS-13' were higher than the other six accessions, while there was no significant difference among other six accessions.



**Figure 3.** Comparison of free amino acid content of different tissues in eight *A. arguta* accessions.

#### 2.1.4. Total phenolic content (TPC)

A continuous decrease was observed in TPC of the eight accessions in leaves as leaf maturity (from YAL to ML). While the highest content was in fruits of six female accessions, and the values in petioles of each accession were the lowest. In 'CJ-1', the TPC in fruits was higher than that in leaves and petioles with significant differences, that in petioles was the lowest (0.37 mg/g fresh weight), and the highest content in fruits was 2.96 mg/g fresh weight, the difference was eight times. By comparing the TPC of the eight accessions in Figure 4, it was found that the contents of different tissues in 'BYS-5' were the highest in fruits (3.25 mg/g fresh weight), while 'BYS-13' were lower than the other accessions, meanwhile the highest value (3.48 mg/g) among the eight accessions was observed in the YAL of 'CBS-11'.

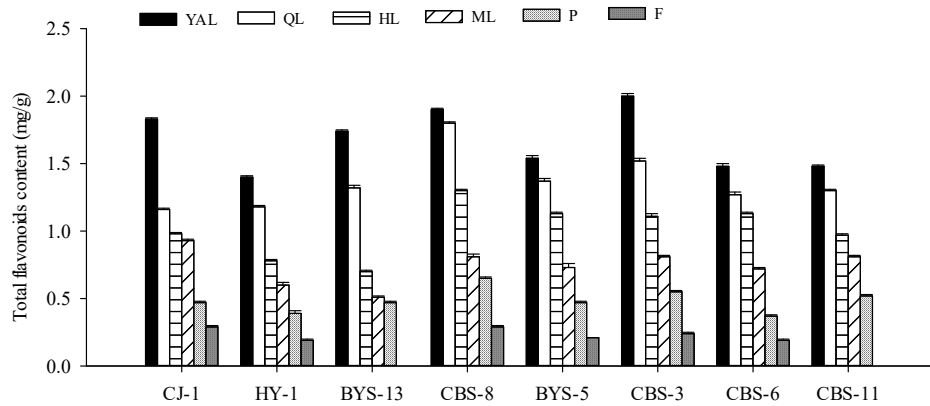


**Figure 4.** Comparison of total phenolic content of different tissues in eight *A. arguta* accessions.

#### 2.1.5. Total flavonoids content (TFC)

Overall, the TFC in the eight *A. arguta* accessions leaves performed continuous decrease with the leaf maturity. The TFC of the young leaves were higher than that of the old leaves, while those in petioles were higher than fruits, but lower than leaves among the eight accessions, the lowest content was in fruits. The TFC of YAL in the eight accessions were highest, followed by QL, HL and

ML. When compared to ML among the eight accessions, the TFC of YAL was higher from 1.83-fold to 3.41-fold. In 'CJ-1', the TFC in different tissues were in the order of leaf>petiole>fruit, and the content in YAL (1.83 mg/g) was 6.3 folds higher than that in fruit (F, 0.29 mg/g). By comparing the TFC of the eight accessions (Figure 5), it was found that there was no significant difference among the eight accessions; 'CJ-1' 'CBS-8' and 'CBS-3' were slightly higher than the other six accessions.

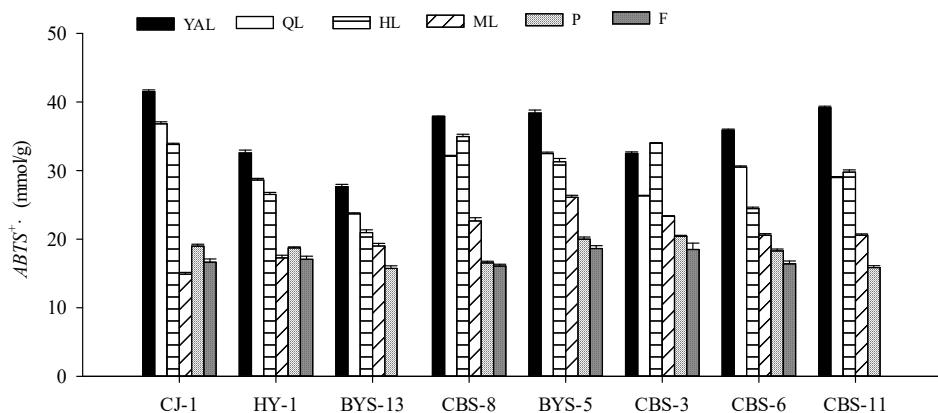


**Figure 5.** Comparison of total flavonoids content of different tissues in eight *A. arguta* accessions.

## 2.2. Antioxidant activity determinations

### 2.2.1. ABTS free radical scavenging assay

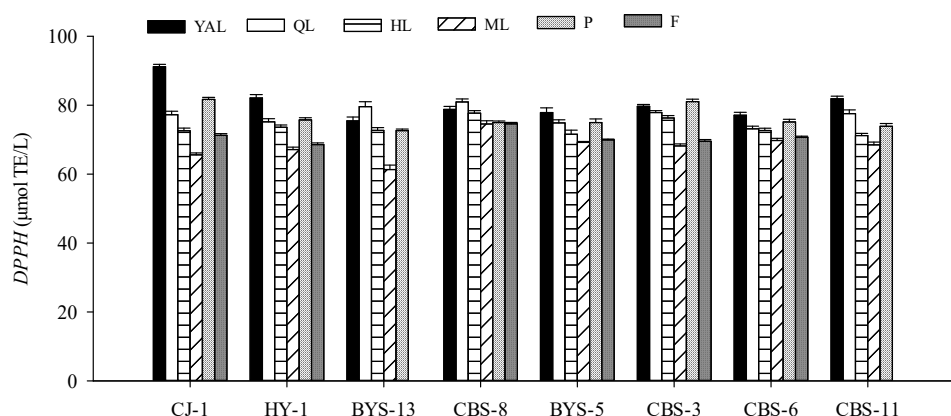
It was observed that ABTS radical scavenging activity in the eight *A. arguta* accessions leaves performed continuous decrease with the leaf maturity. The ABTS radical scavenging activity of the young leaves were higher than that of the old leaves, and higher than that of petioles and fruits, the lowest values were in fruits. Interestingly, ABTS radical scavenging activity in the petioles of young leaves was almost the lowest not similar to that in young leaves. The ABTS radical scavenging activity of YAL in the eight accessions were highest, followed by QL, HL and ML, such as 'CJ-1' where it ranged from 41.51 to 14.90 mmol/g fresh weight from young apical leaves to mature leaves, as well as 'HY-1', 'BYS-13', 'BYS-5' and 'CBS-6', were also showed similar trend. On the other hand, in 'CJ-1', except for ML, the ABTS radical scavenging activity of leaves was greater than that of petioles and fruits, and the ABTS radical scavenging activity of YAL (41.51 mmol/g) was 2.5 times the fruit (16.65 mmol/g). By comparing ABTS radical scavenging activity of the eight accessions (Figure 6), it was found that 'CJ-1' was slightly higher than the other accessions and the lowest was 'BYS-13'. The highest value in fruits was in 'CBS-3', that was 18.51 mmol/g.



**Figure 6.** Comparison of ABTS+ antioxidant activity of different tissues in eight *A. arguta* accessions.

## 2.2.2. DPPH free radical scavenging assay

It was observed that DPPH radical scavenging activity in the eight *A. arguta* accessions leaves showed continuous decrease with the leaf maturity as similar to that of ABTS radical scavenging activity. The DPPH radical scavenging activity of the young leaves and petioles was higher than that of the old leaves. While the values in fruits were only higher than mature leaves. The DPPH radical scavenging activity of YAL in eight accessions was highest except 'BYS-13', 'CBS-3' and 'CBS-8', followed by QL, HL and ML, such as 'CJ-1' where it ranged from 91.22 to 71.29  $\mu\text{mol TE/L}$  from young apical leaves to mature leaves. In 'CJ-1', the DPPH radical scavenging activity of YAL (91.22  $\mu\text{mol TE/L}$ ) was 1.3 times than the fruit, and the value of fruits (71.29  $\mu\text{mol TE/L}$ ) was only higher than ML (65.60  $\mu\text{mol TE/L}$ ). By comparing DPPH radical scavenging activity of leaves and petioles with different maturity of the eight accessions (Figure 7), it was found that 'CJ-1' was slightly higher than the other accessions, while there was no significant difference among these accessions.



**Figure 7.** Comparison of DPPH antioxidant activity of different tissues in the *A. arguta* accessions.

## 2.2.3. Correlation between bioactive compounds and antioxidant activity

To identify the relationship between the main bioactive substances in the leaves of *A. arguta* and their relationship with the ability to scavenge free radicals, a correlation analysis was done and the results were presented in Table 1. The Vc content and the soluble sugar content had significantly

**Table 1.** Correlation analysis between main bioactive substances and free radical scavenging ability of *Actinidia arguta*

Parameter	Vc content	Soluble sugar content	FAA content	Total phenolic content	Total flavonoids content	ABTS <sup>+</sup>
Soluble sugar	-0.936					
FAA	0.957*	-0.969*				
Total phenolic	0.997**	-0.917	0.933			
Total flavonoids	0.999**	-0.943	0.968*	0.993**		
ABTS <sup>+</sup>	0.943	-0.905	0.849	0.956*	0.932	
DPPH <sup>•</sup>	0.989*	-0.967*	0.952*	0.986*	0.987*	0.970*

Note: Data are statistically analysed using Pearson's correlation coefficient test. The correlation coefficient  $r$  value in the table; \* indicates significant correlation at  $\alpha = 0.05$  level (both sides); \*\* indicates significant correlation at  $\alpha = 0.01$  level (both sides);  $r > 0$  is positive correlation,  $r < 0$  is negative correlation

negative correlation ( $r^2 = -0.936$ ;  $p < 0.05$ ), and was extremely positive correlation with FAA content ( $r^2 = 0.957$ ;  $p < 0.05$ ), TPC ( $r^2 = 0.997$ ;  $p < 0.01$ ) and TFC ( $r^2 = 0.999$ ;  $p < 0.05$ ). The soluble sugar content and FAA content ( $r^2 = -0.969$ ;  $p < 0.05$ ) exhibited significantly negative correlation. An extremely positive correlation was found between the FAA content and TFC ( $r^2 = 0.968$ ;  $p < 0.05$ ). The strong positive correlations was observed between TPC and TFC, which accounted for,  $r^2 = 0.993$  at  $p < 0.01$ .

The results showed that Vc content, FAA content as well as TPC and TFC were significantly positively correlated with DPPH free radical scavenging rate ( $r^2 = 0.989, 0.952, 0.986, 0.987$ , respectively;  $p < 0.05$ ), while significant negative correlation was found between soluble sugar content and DPPH free radical scavenging rate ( $r^2 = -0.967$ ;  $p < 0.05$ ). A strong positive correlation was found between TPC and ABTS free radical scavenging rate ( $r^2 = 0.956$ ;  $p < 0.05$ ). Regarding the ABTS and DPPH radical scavenging activities, the correlation determined was positive ( $r^2 = 0.970$ ;  $p < 0.05$ ).

### 2.3. Analysis of Flavonoids Compounds Using LC-MS/MS

By LC-UV-MS analysis, the composition of flavonoids in *A. arguta* could be identified by the UV-vis spectrum, elution order, retention time and MS fragmentation pattern by reference with published data [27]. A total of ten peaks were detected in the flavonoid extract of *A. arguta*, which were identified by the retention time in LC-MS/MS system, elution order,  $\lambda_{max}$  in the visible region, main  $MS^2$  fragments and molecular ion (Figure S1). Some components had similar absorption UV spectra. The maximum absorption peaks were at 240-280 nm and 330- 380 nm. Therefore, it was inferred that these components belonged to the flavonol glycoside compound (Figure S1). As shown in Figure S1, for example, a series of strong  $[M+H]^+$  ions were observed in the (+) ESI-MS spectrum of the flavonoid extract of *A. arguta* leaves, including  $m/z$  579,  $m/z$  595,  $m/z$  625,  $m/z$  653,  $m/z$  757, etc., as well as stronger  $Y_0^+$  ions,  $[Y_0+H]^+$  ions and other fragment ions, such as  $m/z$  285,  $m/z$  300,  $m/z$  301,  $m/z$  314,  $m/z$  315,  $m/z$  316 plasma and  $m/z$  271,  $m/z$  255,  $m/z$  243,  $m/z$  227 and other fragment ions (Table 2). It was preliminarily determined to be aglycone components belonging to isorhamnetin, quercetin and kaempferol. From the above information, it could be inferred that the compound was replaced by one glycoside and two glycosides. On the basis of the UV-vis spectrum, retention time, main  $MS^2$  fragments and molecular ion, F1, F2 and F3 were tentatively established as Kaempferol-3-O-Rutinoside (cis), Kaempferol-3-O-Rutinoside (trans) and kaempferol-3-O-neohesperidoside (P), respectively. The possible structure of F4 or F5 was isorhamnetin-3-O- $\alpha$ -L-rhamnosyl-(1 $\rightarrow$ 3)- $\alpha$ -L-rhamnosyl-(1 $\rightarrow$ 6)- $\beta$ -D- Galactoside. F6 was identified as isorhamnetin-3-O-neohesperidoside (P) (cis) for the moment, F7 might be isorhamnetin-3-O-neohesperidoside (P) (trans) and F8 might be isorhamnetin-3-O-rutinoside. The possible structure of F9 was isorhamnetin-3-O-neohesperidoside or isorhamnetin-7-O-(4"-O-acetyl-glucosyl)-3 -O-xyloside. F10 was tentatively determined as Quercetin-3-O-rhamnoglycoside (Figure S2).

The content of ten flavonoid compounds in different tissues of 'CJ-1' was quantitatively analyzed (Table 4). Quercetin -3-O- rhamnoglycoside was among the highest content of total flavonoids in the five tissues, accounting for 32.62% in YAL, 29.71% in QL, 31.58% in ML, 58.95% in P, and 56.55% in F, respectively. Kaempferol, isorhamnetin, and quercetin compounds were found in leaves (YAL, QL, HL and ML), petioles (P) and fruits (F). The highest level of flavonoid accumulation was detected in leaves, and flavonoids in P and F were  $151.63 \mu\text{g}\cdot\text{g}^{-1}$  and  $78.75 \mu\text{g}\cdot\text{g}^{-1}$ , respectively. It varied significantly in different types of leaves, which was  $10219.84 \mu\text{g}\cdot\text{g}^{-1}$ ,  $4977.83 \mu\text{g}\cdot\text{g}^{-1}$ ,  $3986.94 \mu\text{g}\cdot\text{g}^{-1}$  and  $350.55 \mu\text{g}\cdot\text{g}^{-1}$  in YAL, QL, HL and ML, respectively. The highest total flavonoid content was found in YAL and the flavonoid in YAL, which was 29.2 folds of that in ML, 67.4 folds of that in P and 129.8 folds of that in F. On the other hand, it was interesting to find that the concentration of some flavonoids from different tissues, such as isorhamnetin-3-O-glucoside, kaempferol-3-O-Rutinoside and quercetin-3-O-glucoside, showed significant differences in

different maturity. Quercetin compounds were the most widely distributed in *A. arguta* (Table 3). However, the levels of isorhamnetin and kaempferol compounds were higher than those of quercetins in HL. The content of isorhamnetin compounds in YAL was the highest followed by quercetin and kaempferol compounds. Among the flavonoids in QL, kaempferol compounds were present at the highest level, followed by isorhamnetin and quercetin compounds (relatively low). The content of isorhamnetin compounds was the highest in HL, followed by kaempferol and quercetin compounds. The content of isorhamnetin compounds was highest in ML, followed by quercetin and kaempferol compounds. However, the levels of quercetin compounds were the highest in P and F, followed by isorhamnetin and kaempferol compounds.

**Table 2.** Chromatographic and spectral data of flavonoid glycosides from *Actinidia arguta* leaves

Constituent	Retention time/min	$\lambda_{\text{max}}$ in the visible region (nm)	Molecular ion / $[M+H]^+$	Fragment ions/ (m/z)	M.W.(Da)
F1	12.188	266,350	595	287	594
F2	13.276	266,350	595	287	594
F3	13.693	267,354	595	287	594
F4	10.772	256,356	757	317	756
F5	11.339	257,358	757	317	756
F6	14.363	266,350	625	317	624
F7	14.602	254,358	625	317	624
F8	14.915	263,356	625	317	624
F9	14.609	259,357	653	317	652
F10	21.799	260,359	579	301	578

**Table 3.** The contents of flavonoids in different parts of leaves in CJ-1.

Type	YAL (µg·g <sup>-1</sup> )	QL (µg·g <sup>-1</sup> )	HL (µg·g <sup>-1</sup> )	ML (µg·g <sup>-1</sup> )	P (µg·g <sup>-1</sup> )	F (µg·g <sup>-1</sup> )
Kaempferol-3-O-Rutinoside (+)	121.19±8.53 f	—	397.11±24.59 c	7.36±0.52 d	—	—
Kaempferol-3-O-Rutinoside (-)	775.95±45.80 c	456.45±28.86 cd	—	14.33±1.07 cd	0.56±0.03 e	—
Kaempferol-3-O- Neohesperidoside (P)	1999.91±125.80 b	1326.07±82.84 b	990.58±59.92 a	24.05±2.19 c	10.68±0.62 cd	3.23±0.19 c
isorhamnetin-3-O-α-L-rhamnopyranosyl - (1→3) - α - L - rhamnopyranosyl - (1→6) - β - D - galactopyranoside	572.36±34.78 cd	499.85±29.61 c	1101.83±70.97 a	56.44±4.58 b	13.53±0.81 c	5.65±0.33 c
isorhamnetin-3-O-α-L-rhamnopyranosyl - (1→3) - α - L - rhamnopyranosyl - (1→6) - β - D - galactopyranoside	401.43±24.92 def	323.55±20.53 de	697.98±43.18 b	57.35±4.03 b	1.32±0.01 e	0.95±0.05 c
isorhamnetin-3-O-neohesperidoside (P) (+)	212.87±13.58 ef	—	28.36±1.89 d	8.59±0.59 d	—	—
isorhamnetin-3-O-neohesperidoside (P) (-)	2103.58±133.21 b	592.69±36.25 c	115.43±9.24 d	53.39±3.59 b	30.46±1.75 b	12.36±0.74 b
isorhamnetin-3-O-rutinoside	446.09±31.49 de	210.33±13.04 ef	62.35±4.23 d	11.27±0.93 d	5.69±0.32 de	—
isorhamnetin-3-O-neohespeidoside	252.63±16.26 ef	89.95±5.65 f	—	7.05±0.45 d	—	—
Quercetin -3-O- rhamnoglycoside	3333.83±221.73 a	1478.94±86.92a	593.31±44.91 b	110.72±7.69 a	89.38±5.16 a	56.55±3.32 a
TF contents (µg·g <sup>-1</sup> )	10219.84±1136.36	4977.83±526.03	3986.94±448.49	350.55±44.39	151.63±15.07	78.75±8.04

Note: YAL, Young apical leaves; QL, Leaves expanded to 1/4 to 1/3 of the full leaf size; HL, Leaves expanded to 1/2 to 2/3 of the full leaf size; ML, Mature leaves; P, Petioles of young apical leaves; F, Fruits; — means failure to be detected.

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**3. Discussion**

*3.1 Nutrient content and changing law of different parts of Actinidia arguta*

In previous report, the maturity was important to the nutritional quality of fruits and vegetables [28]. The content of bioactive compounds and antioxidant capacity in leaves were impacted by the maturation degree, such as Vc [29], total carotenoids, total polyphenol [30], etc. Lisiewska *et al.* noted diminishing Vc concentrations with dill growth [31]. In our study, the Vc content decreased as the leaf became mature, and Vc concentrations of fruits were lower than those of immature leaves and petioles, while higher than mature leaves. However, Yamada *et al.* [32] and Omary *et al.* [33] found an opposite tendency in leaf maturity development of broccoli and spinach, respectively.

In some plants, the polyphenols concentrations also decrease with the leaf maturity, such as in aronia [34]. The polyphenols in levels of *Cosmos caudatus* were decreased with maturity [35], and the highest phenolic compounds were observed at the young leaves stages of *Rosmarinus officinalis* [36]. In blackberry, raspberry, and strawberry leaves, Wang and Lin [37] also found the same tendency. At present, there were few reports on the evaluation of *A. arguta* leaves. According to Thi and Hwang [34] total polyphenol content of aronia leaves was lower than that of the aronia fruit, while the TFC of leaves was approximately twice that of fruits. In our study, the TFC and TPC decreased with the increase of maturity, while in *A. arguta* fruits, the TPC was higher than that of leaves and petioles, but TFC was lower than leaves and petioles. The FAA content in different parts of the leaves and different tissues showed a trend of declining with the maturity. The FAA content in the petiole of young leaves was not much different from that in young leaves, but they were higher than that of in fruits. This was consistent with the result of Song *et al.*, [38] which reported that the total FAA showed decreasing tendency with the maturity.

*3.2 The leaf flavonoids identification of Actinidia arguta*

The LC-MS/MS was a useful technique to conduct structural identification of flavonol aglycone type [39,40,27]. The LC-MS/MS method had been developed for the rapid screening and determination of bioactive compounds in fruit efficiently and sensitively [41]. Singh *et al.* [40] identified quercetin-O-pentohexoside, quercetin-3-diglucoside, quercetin-O-xylo-pentoside and kaempferol-O-glucoside using LC-MS/MS and found some molecular ions in the full scan mass spectra, such as m/z 625, m/z 595, m/z 579, and some other ion fragments, including m/z 301, m/z 285. The LC-MS/MS analysis was used to determine the overall composition of the leaf flavonoids in *A. arguta* in this study and in the light of the UV-vis spectrum, ten peaks had characteristic absorption peak at 240-280 nm and 330- 380nm, which indicated that these compounds were all flavonol glycoside compound [42,43]. It was found that the concentration of some flavonoids from different tissues showed significant differences in different maturity.

*3.3 The potentialities of A. arguta leaves*

In recent years, the antioxidant phytochemicals, have been reported to restrain free radical reactions propagating, such as flavonoids and other polyphenols to defend the human body against diseases and increase life expectancy [44,45]. Simultaneously, considerable regard has been devoted to the development of natural antioxidants of plants origin, including *Salvia* [46], and *Prunus salicina* [47]. Our study indicated that the flavonoid components in young leaves of *A. arguta* contained quercetin, kaempferol and isorhamnetin compounds, and the content of total flavonoid was higher than that of mature leaves. Maybe the young leaves would be processed into tea in the future, like *Ginkgo biloba* [48], pear jujube [49], and *Lycium barbarum* [50]. Therefore, if the leaves can also be processed and utilized, *A. arguta* would usher in greater business opportunities in addition to producing fresh fruit.

54 4. Materials and Methods

55 4.1 Plant Materials

56 Eight *A. arguta* accessions were collected from Northeast of China, which were *A. arguta* cv.  
57 Changjiang No.1 ('CJ-1', female plant), *A. arguta* cv. Huanyou No.1 ('HY-1', female plant), BYS No.  
58 13 ('BYS-13', male plant), CBS No. 3 ('CBS-3', female plant), CBS No. 6 ('CBS-6', female plant), CBS  
59 No. 11 ('CBS-11', male plant), CBS No. 8 ('CBS-8', female plant) and BYS No. 5 ('BYS-5', female plant)  
60 (Table 4, Figure S3). They were grown in the open field in the Germplasm Resource Repository of  
61 Wild Kiwifruit in Shenyang Agricultural University, with the space of 2×5 m arranged with drip  
62 irrigation. The fresh leaves of the eight accessions were collected at summer pruning stage in the  
63 middle of June, and mature fruits were collected from the middle of August to the middle of  
64 September, 2018. Six tissues were collected for analysis, including young apical leaves (YAL), leaves  
65 expanded to 1/4 to 1/3 of the full leaf size (QL), leaves expanded to 1/2 to 2/3 of the full leaf size  
66 (HL), mature leaves (ML), petioles of young apical leaves (P) and fruits (F) (Figure 8). The samples  
67 were collected and wrapped in tinfoil, temporary frozen with liquid nitrogen and kept in -80 °C  
68 refrigerator. Triplicate samples were made with each 10 plants, and 10g leaves and 100 g mature  
69 fruits in total were sampled randomly and pooled to constitute the replicates.  
70

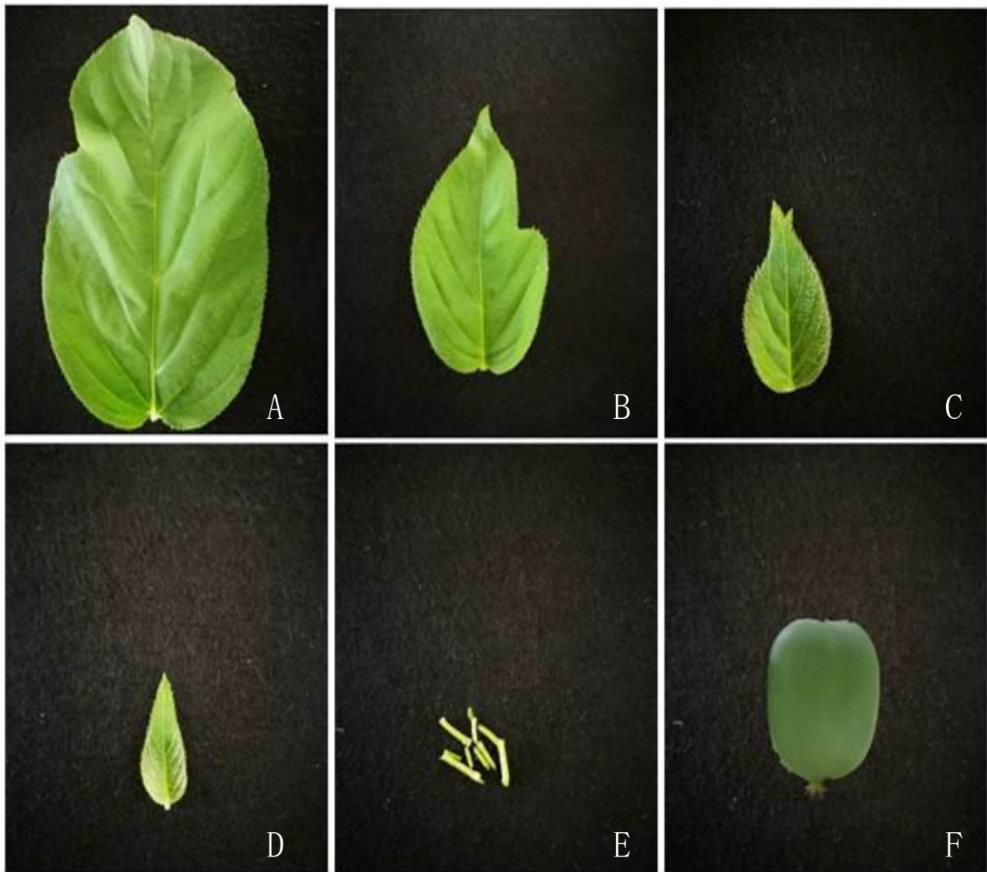
**Table 4.** The geographical description of the eight *Actinidia arguta* accessions examined in this study

Accessions	Accessions sources	Latitude and longitude	Altitude (m)	Plant sex
CJ-1	Shenyang,Liaoning	123°43'E, 41°80'N	55	female
HY-1	Benxi,Liaoning	125°35'E, 41°27'N	282	female
BYS-13	Zhuanghe,Liaoning	122°98'E, 39°70'N	1078	male
CBS-3	Changbaishan,Jilin	100°12'E, 40°15'N	2579	female
CBS-6	Changbaishan,Jilin	100°13'E, 40°18'N	2623	female
CBS-11	Changbaishan,Jilin	100°11'E, 40°15'N	2637	male
CBS-8	Changbaishan,Jilin	100°13'E, 40°17'N	2583	female
BYS-5	Zhuanghe,Liaoning	122°97'E, 39°73'N	1074	female

71 4.2 Determination of bioactive compounds in leaves and fruits of *A. arguta*

72 4.2.1 Vitamin C (Vc) content

73 The Vc contents in leaves and fruits were determined according to the method of Yoon *et al.* with  
74 some improvement [51]. Freeze-dried sample (0.2 g) was ground into homogenate with 5 mL of  
75 oxalic acid-Ethylene Diamine Tetraacetic Acid (EDTA), then the distilled water was used to make  
76 total volume to 10 mL. The extract (5 mL) was mixed with 0.5 mL of metaphosphoric acid-acetic acid  
77 solution, 1 mL of 1:19 sulfuric acid, and 2 mL of 5% ammonium molybdate solution. After 15 min,  
78 the mixture was centrifuged for 15 min at 4000 r/min. The absorbance at 760 nm was recorded using  
79 spectrophotometer. The total content was calculated using Vc as standard.  
80



**Figure 8.** Four types of leaves, petioles and fruits of *A. arguta* accessions. (A) Mature leaves (ML); (B) Leaves expanded to 1/2 to 2/3 of the full leaf size (HL); (C) Leaves expanded to 1/4 to 1/3 of the full leaf size (QL); (D) Young apical leaves (YAL); (E) Petioles of young apical leaves (P); (F) Fruits (F).

4.2.2 Soluble sugar content (SSC)

SSC of the leaves and fruits were determined according to the method of Liu *et al.* with some modifications [52]. Freeze-dried sample (0.2 g) was finely powdered and added 15 mL of distilled water and placed in boiling water bath 15 minutes. After that final volume was made to 25 mL with distilled water. The extract (1 mL) was mixed with 5 mL of anthrone reagent. After 10 min of boiling in water bath, the absorbance at 620 nm was recorded using spectrophotometer. The glucose was used as standard. The result was represented as mg/g fresh weight (mg /g . FW).

4.2.3 Free amino acid (FAA) content

The FAA contents of the leaves and fruits were determined with some modification of the method proposed by Avino *et al.* [53]. Freeze-dried sample (0.1 g) was ground into homogenate with acetic acid (10%), and the ammonia-free distilled water was used to make total volume to 10 mL. The extract (2 mL) was mixed with 3 mL of ninhydrin reagent and 0.1 mL of ascorbic acid solution and placed in boiling water bath for 15 min. The absorbance was recorded at 570 nm using spectrophotometer. The leucine was used as standard.

4.2.4 Total phenolic content (TPC)

The TPC in *A. arguta* extracts was determined according to Folin Ciocalteu method of Navajas-Porras *et al.* with slight improvement [54]. Freeze-dried sample (0.2 g) was chopped and added 10 mL of distilled water and placed in boiling water bath for 10 min. After that, the solution was centrifuged for 10 min at 3000 r/min. The extract of *A. arguta* (250 µL) was added to 1 mL distilled water, followed by Folin-Ciocalteu reagent (250 µL) and then 7% sodium carbonate (2.5 mL) was

added to the mixture. The distilled water was used to make total volume to 10 mL and placed at room temperature for 1.5h. The absorbance at 760 nm was recorded using spectrophotometer. The gallic acid was used as standard.

#### 4.2.5 Total flavonoids content (TFC)

The TFC was determined by the method of Zainudin *et al.* with minor modifications [30]. The sample (0.5 g) was finely powdered and extracted with 10 mL formic-methanol (0.2%), centrifuged at 5000 r/min for 10 min. The extract (5 mL) was added to 0.3 mL of sodium nitrite (NaNO<sub>2</sub>, 5% w/v), and then 0.3 mL of aluminium chloride (AlCl<sub>3</sub>, 10% w/v) and 4% sodium hydroxide solution (2 mL) were added every 6 min, respectively. The distilled water was used to make total volume to 10 mL, which kept for 10 min. The absorbance at 510 nm was recorded using spectrophotometer. The rutin was used as standard. The result was represented as mg /g fresh weight (mg/g . FW).

### 4.3. Antioxidant activity assay

#### 4.3.1 ABTS radical scavenging activity

The ABTS radical scavenging activity was performed with minor modifications according to the spectrophotometric method of Chandel *et al.* [55]. The reaction mixture containing ratio of 1: 2 including 2.45 mmol/L potassium persulfate solution and 7 mmol/L aqueous solution of ABTS (ABTS<sup>+</sup>). The mixture was kept in dark for 14 h at 29 °C, and then it was diluted with ethanol in order to obtain an absorbance at 734 nm of 0.7±0.02 units. The extracts or reference substances (Trol ox) (200 µL) were added to 3 mL of ABTS radical solution in a dark condition and the absorbance was measured at 734 nm after six min.

$$\text{ABTS radical-scavenging activity (\%)} = ((A_0 - A_1)/A_0) \times 100\%$$

where A<sub>0</sub> is the absorbance of ABTS radical absolute ethanol solution, and A<sub>1</sub> is the absorbance of the mixture ABTS radical extract.

#### 4.3.2 DPPH radical scavenging activity

The DPPH method was used to determine the free radical scavenging activity of the extract [56]. In order to obtain the antioxidant activity, 0.2 g sample was extracted with 10 mL of absolute ethanol and filtered. Then 2 mL solution was taken and mixed with 2 mL of 0.2 mmol/L DPPH absolute ethanol solution. The mixture was kept at room temperature and protected from light for 30 min, and the absorbance was recorded at 517 nm.

$$\text{DPPH radical scavenging activity (\%)} = ((A_0 - A_1)/A_0) \times 100\%$$

Where A<sub>0</sub> is the absorbance of DPPH ethanol solution, and A<sub>1</sub> is the absorbance of the mixture of DPPH and extract.

### 4.4. Analysis of Flavonoids Compounds Using LC-MS/MS

The flavonoid compounds were determined using LC-MS/MS analysis (Figure 9). The content of ten flavonoid compounds in different tissues of 'CJ-1' was quantitatively analyzed. Chromatographic analysis was conducted on an Agilent 6410 Triple Quad LC-MS/MS (Agilent Technologies, USA) with 1260 liquid chromatography system and electrospray ionization source (electronic spray ionization, ESI). The chromatographic separation column was Agilent Poroshell 120 SB-C18 column (2.1 × 100 mm, 2.7 µm, Agilent Technologies, USA). A volume of 10 µL sample solution was injected for LC-MS/MS analysis with flow rate of 0.3 mL/min. Flavones and flavonols were detected at 350 nm. The mobile phases A and B were composed of 2.5% (v/v) formic acid in water and 2.5% (v/v) formic acid in acetonitrile, respectively. The column was eluted using linear gradients at 30 °C: 0 min, 100% A, 0% B; 3 min, 90% A, 10% B; 5 min, 86.5% A, 13.5% B; 7 min, 86.5% A, 13.5% B; 8 min, 86% A, 14% B; 9 min, 85% A, 15% B; 10 min, 85% A, 15% B; 11 min, 84.5% A, 15.5% B; 12 min, 83.5% A, 16.5% B; 12.5 min, 82% A, 18% B; 13 min, 70% A, 30% B; 13.5 min, 69.5% A, 30.5% B; 14 min, 68.5% A, 31.5% B; 17 min, 60% A, 40% B; 20 min, 0% A, 100% B; 23 min, 95% A, 5% B; 25 min, 95% A and 5% B. The MS

conditions were: electrospray ionization, positive ion mode; gas (N<sub>2</sub>) temperature 300 °C; nebulizer pressure, 15 psi; capillary voltage, 4000 V; capillary exit, 200 V. Mass spectra from m/z 100 to 1000 nm were recorded. The analysis of data was conducted using LC/MSD Trap Software 5.3. Naringenin, quercetin 3-O-rutinoside, myricetin, quercetin, dihydroquercetin, kaempferol, dihydromyricetin, dihydrokaempferol, (+)-catechin, (–)-epicatechin obtained from the National Institutes for Food and Drug Control, Beijing, China, and the standard for quantification was quercetin 3-O-rutinoside. The contents of total flavonoids were calculated at 350 nm. Each sample was analyzed in triplicates.



**Figure 9.** Four types of leaves of eight *A. arguta* accessions used in LC-MS/MS.

#### 4.5. Statistical Analysis

Analyses of variance was performed using SPSS 19.0 statistical analysis software. All experimental data was presented as means from triplicates standard deviations. The data significance was checked at  $p \leq 0.05$ . A Pearson's correlation analysis was conducted between the antioxidant capacity and bioactive compounds.

#### 5. Conclusions

*A. arguta* is rich in vitamin C and bioactive and volatile compounds with high antioxidant activities, such as flavonoids, phenolic compounds, etc. This study evaluated the eight wild *A. arguta* accessions and different tissues according to the bioactive compounds and antioxidant activities. The contents of different bioactive compounds in the eight accessions varied greatly, and 'CJ-1' and 'CBS-8' had relatively outstanding performance. According to the comprehensive evaluation of the antioxidant activity among different tissues, the young leaves had the strongest antioxidant activity. These results provided a theoretical basis for further utilization of leaves of *A. arguta*.

**Supplementary Materials:** The following are available online at [www.mdpi.com/xxx/s1](http://www.mdpi.com/xxx/s1), Figure S1: LC-MS analysis of the crude extract from leaves of *Actinidia arguta* in positive ion mode. Selected ion chromatograms of m/z 595,757,625,653,579 respectively, with the retention time of corresponding constitutions, Figure S2: LC-MS2 spectra of [M+H]<sup>+</sup> ion for the ten constituents, Figure S3: Fruits (female) or flowers (male) of eight accessions in *Actinidia arguta*. a; CJ-1; b; HY-1; c; CBS-3; d; CBS-11; e; CBS-6; f; CBS-8; g; BYS-13; h; BYS-5.

**Author Contributions:** conducted the experiment, analyzed the data, and wrote the manuscript, C.T.; writing—review and editing, Z.W., M.I.; methodology, X.F.; supervision, C.L. All authors have read and agreed to the published version of the manuscript.

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