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Analysis of Sweetness Components by Harvest Times and Extract Methods in Stevia

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Abstract

The leaves of *Stevia rebaudiana* Bertoni have attracted much attention as a source of natural sweeteners. This study was conducted to investigate the natural sweeteners contents and analyze antioxidant materials and activity according to harvest times and extraction methods in stevia.

Stevia leaves were extracted using 5 extraction methods to identify the extraction with high extraction efficiency. Extraction yield was high in the order of hot water extraction (HWE), reflux extraction (RE), high temperature and pressure extraction (HTPE), ultrasonic extraction (UE) and vacuum extraction (VE). The contents of rebaudioside A and stevioside in stevia leaves had the highest in HTPE. Also, the total phenolic and flavonoid contents had the highest in HTPE and VE. Using the HTPE method, the result analyzed that the contents of rebaudioside A and stevioside of stevia leaves harvested between April and October exhibited the tendency to increase gradually between July and October. HTPE is considered to be an appropriate method for extracting stevia leaves. Also, it was confirmed that the stevia leaves of July, September and October, except for the high temperature period of August, had superior in quality and quantity.

Keywords: Antioxidant, Extraction efficiency, *Stevia rebaudiana* Bertoni, Steviol glycoside, Sugar substitute

1. Introduction

Currently, obesity and adult diseases as a result of sugar intake have been a consistent problem in Korea (Hwang et al., 2016). Consequently, research on low caloric sweeteners that can substitute sugar has been consistently conducted (Park, 2007; Lee and Kim, 2004; Na et al., 2012). Natural sweeteners as sugar substitutes have many advantages over sugar as a small quantity, but a lot of research is required regarding its application to various foods science it has low preference compared to sugar (Kim and Lee, 2012). Recently, there have been dynamic researches conducted on stevia as natural sweetener substitute for sugar (Esmat et al., 2010).

Stevia (*Stevia rebaudiana* Bertoni) grows in local highland of an altitude over 1000m as in Brazil and Paraquay, belonging to the chrysanthemum family as a perennial herb plant. Sweeteners of stevia were mainly extracted from its leaves with plant height of 40~60cm. Sweetener components in stevia are glycosides of the diterpene derivative steviol, consisting mainly of stevioside and rebaudioside A, as well as with the extremely minor components which includes rebaudioside B, C, D, E, steviolbioside and dulcoside A. Steviol glycosides have low calorie and sweetening potentials 200~300 times than sucrose, with low sugariness index. So, it is used for various processed food as natural sweeteners to substitute sugar (Lim and Oh, 2004). Many researchers have reported that stevia may be used not only for its sweetening purposes, but also for its physiologic and therapeutic effect (Park, 2007). Since the substitute of artificial sweeteners with stevia, stevia has been identified by researchers to have numerous efficiency such as antibacterial and anticancer activity (Tomita et al., 1997), anti-diabetic effect (Dyrskog et al., 2005), antioxidant activity (Tadhani et al., 2007, Kim et al., 2009), and anti-obesity and the effect of regulation of blood glucose (Park, 2007; Park et al., 2010).

Stevia extracts were reported to contain steviol glycosides like stvioside and rebaudiosid A, as well as various polyphenol compounds (Kim et al., 2010). From the analyzed result, polyphenolic compound content in various herb plants showed the highest content in stevia (Yamamoto et al., 2001). Also, stevia was more effective for antioxidant activity than those of other abiotic stresses (Choi et al., 2011) and it observed the highest antioxidant activity compared to trolox of strong antioxidant (Tadhani et al., 2007).

Stevia extract method were conducted by some researchers (Lim and Oh, 2004; Kim et al., 2010; Afandi et al., 2013; Periche et al., 2015). Periche et al. (2015) reported that the greatest yield of steviol glycosides was obtained with microwave treatment, but the conventional high temperature method (90^{o}C, 1min) was the most suitable for antioxidant extraction. In addition, Kim *et al*. (2010) reported that vacuum extraction was proper for higher antioxidant activity. Afandi *et al*. (2013) reported that methanol is the best solvent for the extraction of rebaudidoside A. So, there are some troubles related with extract methods, considering components and antioxidant activity. Although there are lots of researches on the efficiency of stevia, researches related to harvest period in stevia and change components by extraction

method are few. Therefore, this study was conducted to select optimum extraction method based on reported methods and extraction time by measuring the natural sweeteners contents, analyzing antioxidant materials and activity according to harvest times, parts of plant, and extraction methods.

2. Materials and Methods

2.1 Experimental Materials

Stevia 'Dajung' cultivar was purchased from a local market and was cultivated in a plastic house from May to December. The leaves were harvested from April to October and the stem were only harvested in July and September, which reported the high period in component content of stevia. The extraction method experiments only used leaves harvested on September.

2.2 Extraction Yield and Methods

Leaves harvested for experiment on extraction methods were washed and investigated dry weight after drying at room temperature for two weeks. They were ground into fine powder using a mill, and were extracted by 5 methods which are: hot water extraction (HWE), reflux extraction (RE), high temperature and pressure extraction (HTPE), ultrasonic extraction (UE) and vacuum extraction (VE). The extracts were stored at -20℃ until analysis and were used to dilute 10 times of distilled water for experiments. Extraction yield was calculated using the formula below. Extraction yield (%) = (weight after lyophilization (g)/ dry weight (g)) × 100. **Hot water extraction (HWE)**: Dried stevia leaves of 5 g was dispersed in 100 ml of distilled water equivalent to 20 times of dry weight and mixed at 100℃ temperature, and the mixture were extracted by centrifuged with 4000 rpm for 20 min (Lim and Oh, 2004). **Reflux extraction (RE):** Dried stevia leaves of 5 g were extracted using 50 ml of 100% MeOH equivalent to 10 times of dry weight over 3 times for 2 hr using Soxelt extractor (Afandi et al., 2013). **High temperature and pressure extraction (HTPE)**: Dried stevia leaves of 5 g were added by 100 ml of distilled water equivalent to 20 times of dry weight, and the mixture were extracted by autoclave at 121^0C for 15min (Ko et al., 2015). **Ultrasonic extraction (UE)**: Dried stevia leaves of 0.8 g were added by 30 ml of solvent (Acetonitrile:Water 7:3) equivalent to 37.5 times of dry weight, and the mixture were extracted by ultrasonic waves at 70^0C for 15min (Ha et al., 2009).**Vacuum extraction (VE):** Dried stevia leaves of 5 g were added using 150 ml of distilled water equivalent to 30 times of dry weight, and the mixture were extracted under 0.01Mpa pressure at 65^0C for 4hr (Kim et al., 2010).

2.3 Determination of Total Phenolic Contents

Total phenolic contents were analyzed to change by Aronous et al. (2001) method. In a 1.5 ml Eppendorf tube, 790 µl of distilled water and 0.1 ml of sample appropriately diluted, and 50 µl of 1N Folin-Ciocalteu reagent (Sigma Aldrich, St. Louis, Mo, USA) were mixed. After the reaction for 1 minute exactly, 150 µl of 20% sodium carbonate was added, and the mixture was kept in the dark at room temperature, for 120 min. The absorbance was read at 750 nm by spectrophotometer (EZ Read 2000, Biochrome, Cambridge, England). The total polyphenol concentration was calculated from a calibration curve, using gallic acid (Sigma

Aldrich, St. Louis, MO, USA) as a standard. The results expressed as mg gallic acid equivalent (mg GAE) per 100g of dry weight.

2.4 Determination of Total Flavonoid

Total flavonoid contents were analyzed to change by Shen et al. (2009) method. Aliquots (0.5 ml) of appropriately diluted extracts or standard solutions, 2 ml distilled water were mixed with 0.1 5ml 5% $NaNO_2$. After the reaction for 5 min, 0.15 ml 10% $AlCl_3$ $6H_2O$ solution was added, and the mixture reacted and was left for 5 min, and then 1 mL 1 M NaOH was added. The reaction solution was well mixed, after 15 min. The absorbance was read at 415 nm by spectrophotometer (EZ Read 2000, Biochrome, Cambridge, England). The total flavonoid concentration was calculated from a calibration curve, using quercetin (Sigma Aldrich, St. Louis, MO, USA) as a standard. The results were expressed as mg quercetin equivalent (mg QE) per 100g of dry weight.

2.5 DPPH Free Radical Scavenging Activity

Total antioxidant capacity of extracts (DPPH) was analyzed to use a spectrophotometer by the improved DPPH (1, 1-diphenyl-2-picrylhydrazyl, Sigma Aldrich, St. Louis, MO, USA) method as described (Jeong et al., 2010; Yoon et al., 2016). 200 µM DPPH was dissolved in 80% Methanol. 700 µl DPPH was added to 300 µl of the extracts and mixed thoroughly. The mixture was kept at room temperature for 30 min and the absorbance was read at 517 nm by spectrophotometer (EZ Read 2000, Biochrome, Cambridge, England).

2.6 ABTS Free Radical Scavenging Activity

The total antioxidant capacity of extracts (ABTS) was analyzed a spectrophotometer by the improved ABTS (2,2'-azino-bis-3-ethylbenzo-thiazoline-6-sulfonic acid, Sigma Aldrich, St. Louis, MO, USA) method as described (Jeong et al., 2010). ABTS solution (1 ml, absorbance of 0.700) was added to 0.1 ml of the extracts and mixed thoroughly. The mixture was kept at 37^0C for 20 min and the absorbance was read at 734 nm by spectrophotometer (EZ Read 2000, Biochrome, Cambridge, England). Antioxidant activity of DPPH and ABTS was calculated using the formula below and L-ascorbic acid used as control. DPPH and ABTS free radical scavenging activity (%) = (1-sample absorbance/control absorbance) × 100.

2.7 LC-MS/MS Analysis

Liquid chromatography-mass spectrometry/ mass spectrometry was performed by coupling a HPLC system (Agilent 1100, Agilent Technologies, CA, USA) to a Qtrap mass spectrometer (Qtrap, AB Sciex CO, CA, USA) equipped with an electrospray ionization (ESI) source. LC separation was performed on an YMC-Pack ODS-AQ Column (150 x 4.6mm x I.D., 5µm). The mobile phase consisted of H_2O (0.1% formic acid) and acetonitrile of 50:50 (v/v) up to 2min, 40:60 till 15 min, delivered at a flow rate of 0.2 ml/min. The column temperature was maintained at 30℃, and the injection volume was 2 µl. The standard stevioside and rebaudioside A (Wako pure chemical industries, Ltd, Japan) was used as the internal standard for quantitative analysis. The mass spectrometer was operated under negative ion and selected ion monitoring (SIM) modes. ESI was conducted using a spray voltage of 4.5kV.

The capillary voltage and the tube lens offset were fixed at -40 and -130V, respectively. The heated capillary temperature was fixed at 400℃.

2.8 Statistical Analysis

The data were analyzed using one-way analysis of variance (ANOVA) by SPSS Statistics 21 program. The statistical assessments of the differences between mean values were compared using Duncan's multiple range test (DMRT) at p=0.05.

3. Results and Discussion

3.1 Analysis of Sweetness Components According to Extraction Methods

Natural sweetness components like rebaudioside A and stevioside in stevia were extracted by 5 methods such as HWE, RE, HTPE, UE and VE to select the method with high extraction yield. The result showed that the extraction yield was no difference with treatments except for VE treatment, and the content of sweetness was the highest in HTPE, followed by VE, RE, HWE, and UE, in that order (Table 1; Fig. 1). Periche et al. (2015) reported that steviol glycosides and antioxidants were negatively correlated; therefore, there is no single treatment suitable for obtaining the highest yield in both groups of compounds simultaneously. The greatest yield of steviol glycosides was obtained with microwave energy or ultrasonic treatment, and the conventional method (hot water) was the most suitable for antioxidant extraction. Nonetheless, in this study, steviol glycosides content was the highest in HTPE and the lowest in UE and HWE (Fig. 1). It seems that HTPE had a high pressure as well as high temperature in this study. Also, VE was the highest in the content of antioxidants as much as HTPE, but in extraction yield VE was the lower compared to HTPE (Fig. 2). Kim et al. (2010) reported that VE is efficient for the extraction of antioxidants in stevia, although the content of steviol glycosides are not represented. Therefore, despite VE method was suitable for antioxidants extraction, it had some weakness of low extraction efficiency of sweetness components and it requires much equipment. In this study, the content of antioxidants by UE methods is agreement with report of Periche et al. (2015), but the content of steviol glycoside was different in this study (Fig. 1).

Table 1. Comparison of extraction yield in extracts of stevia leaves according to extraction methods.

Extraction methods	Extraction yield (%)
Hot water extraction	51.0 a[z]
Reflux extraction	47.6 a
High Temperature and Pressure extraction	45.1 a
Ultrasonic extraction	48.8 a
Vacuum extraction	18.5 b

[z] Mean separation within columns by Duncan's multiple range at p<0.05.

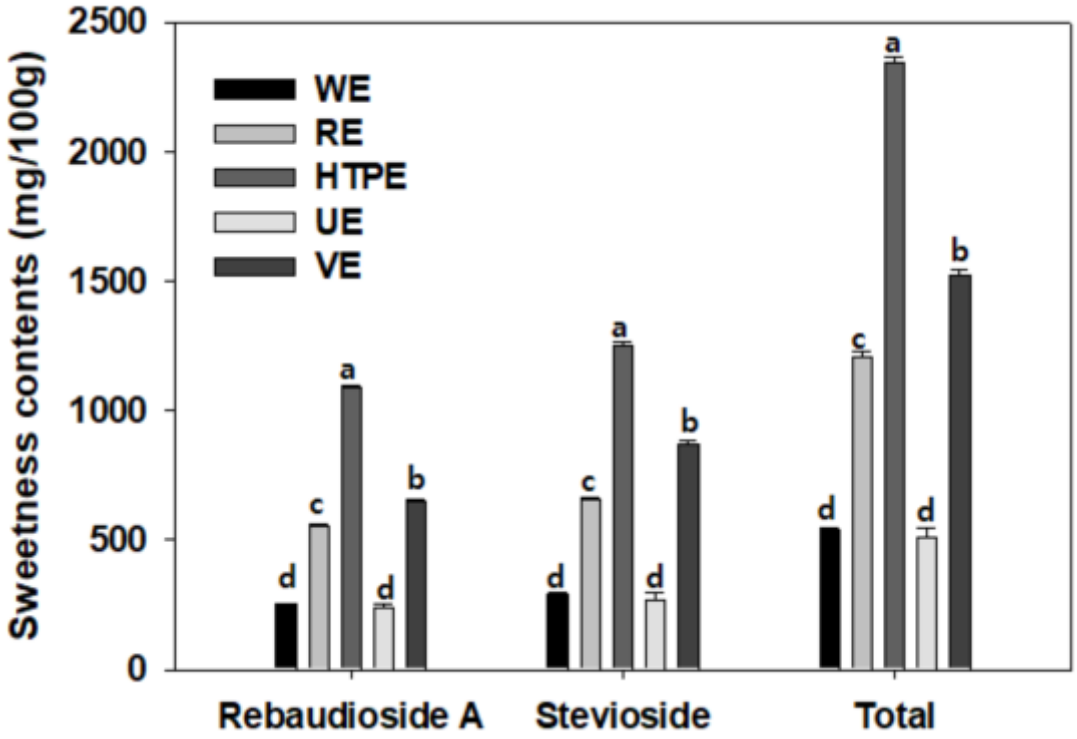

Fig. 1. Comparison of sweetness components in stevia leaves according to extraction methods. Total shows the sum of rebaudioside A and stevioside. WE: Hot water extraction, RE: Reflux extraction, HTPE: High temperature and pressure extraction, UE: Ultrasonic extraction, VE: Vacuum extraction. Data represent the means ± SE(n=3). Different letters are significantly different at $p<0.05$ by Duncan's Multiple Range Test (DMRT)

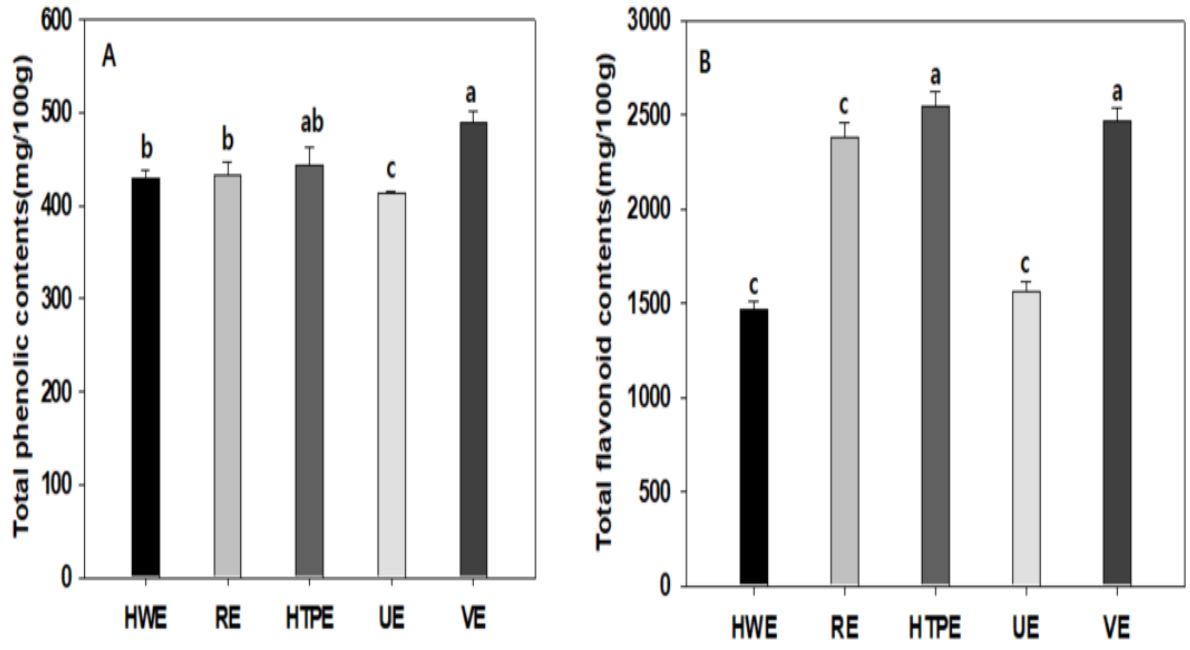

Fig. 2. Total phenolic contents (A) and total flavonoid contents (B) in stevia leaves according to extraction methods. HWE: Hot water extraction, RE: Reflux extraction, HTPE: Pressure heating water extraction, UE: Ultrasonic extraction, VE: Vacuum extraction. Data represent the means ± SE (n=3). Different letters are significantly different at $p<0.05$ by Duncan's Multiple Range Test (DMRT)

Therefore, it seems that HTPE is the most suitable method because this extraction method has the highest content of rebaudioside A, stevioside, extraction yield and antioxidants than other extraction methods.

3.2 Extraction Yield According to Harvest Times and Parts

The extraction yield in stevia leaves according to harvest times was the highest in October, followed by September, and May, in such order. The other harvest times, Apr, June, July and August, were the lowest and these periods have no difference among them. There was no difference between July and September, regarding the extraction yield in stevia stem according to harvest times (Table 2). Also, the leaves in stevia was higher in extraction yield than that of the stem.

Table 2. Comparison of extraction yield in extracts of stevia leaves according to harvest times

Harvest times	Extraction yield (%)	
	Leaves	Stem
Apr.	36.2 c[z]	-
May	39.5 bc	-
Jun.	36.8 c	-
Jul.	33.6 c	18.5[ns]
Aug.	35.6 c	-
Sep.	45.1 b	21.2
Oct.	52.6 a	-

[z] Mean separation within columns by Duncan's multiple range at $p<0.05$.

[ns] No significance at 0.05 level

3.3 Analysis of Sweetness Components and Antioxidants Activity According to Harvest Times and Parts

Steviol glycosides of rebaudioside A and stevioside content according to harvest times in stevia leaves was increased from April to October as rising temperature, and decreased since October (Fig. 3). Wu et al. (2012) reported that contents of rebaudioside A and stevioside was higher in leaves harvested at 95days than in leaves harvested at 65~85days and 105 days after planting. These results have the same tendency with this study. The result showed that it is higher in leaves harvested in late cultivation than in leaves harvested during early cultivation. Nevertheless, it had the tendency to decrease in August. Consequently, the contents of rebaudioside A and stevioside were decreased by extreme high temperature in that season. Lim and Oh (2004) observed that the content of steviol glycoside decreased from the highest peak season of August, but it is the difference in this study. It seems that steviol glycoside content was derived from the different extraction methods such as HTPE and weather condition cultivated in stevia such as the hottest of August in Korea as mentioned above. Contents of rebaudioside A and stevioside in stevia were higher in leaves than in stem (Fig. 4). This was consistent with the general opinion that there are abundant natural sweetness components contained in stevia leaves (Afandi et al., 2013; Periche et al., 2015).

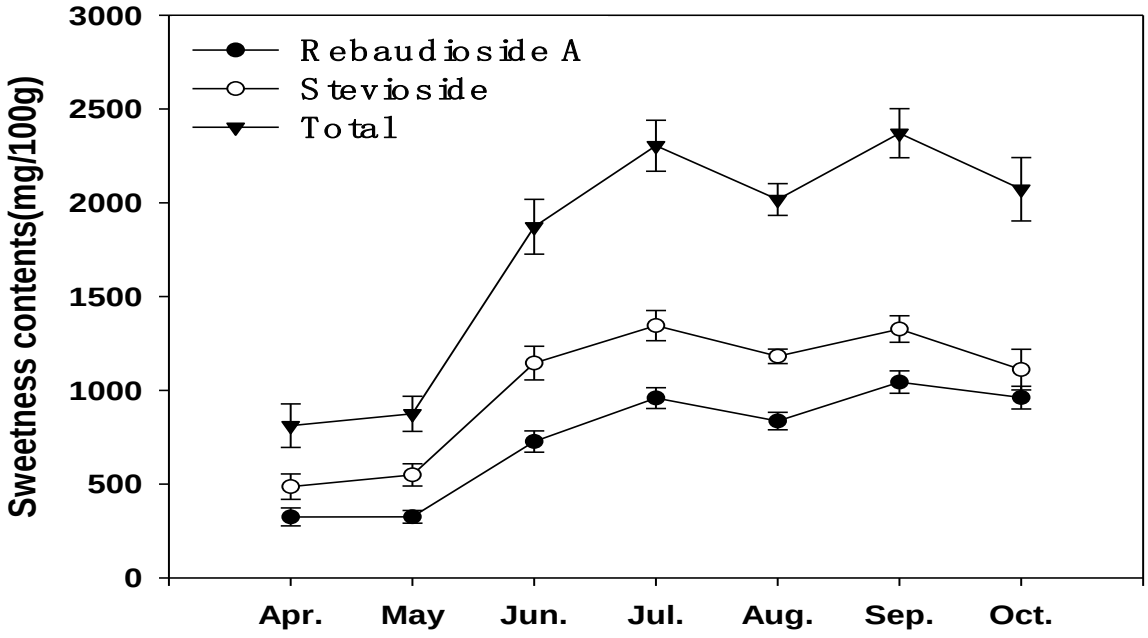

Fig. 3. Seasonal change of sweetness components in stevia leaves according to harvests time. Total shows the sum of rebaudioside A and stevioside. Data represent the means ± SE (n=3)

Table 3. Comparison of antioxidant contents and activity in pressure heating water extraction of stevia leaves according to harvest times

Harvest times	Total phenolic contents	Total flavonoid contents	DPPH	ABTS
	mg·100g^{-1}		%	
Apr.	443.2 ab[z]	2468.4 a	36.0 a	92.9 a
May	477.7 a	2468.4 a	34.9 a	81.1 a
Jun.	463.0 ab	2488.0 a	37.3 a	90.4 a
Jul.	427.0 b	2464.0 a	29.8 ab	86.9 a
Aug.	482.1 a	2512.0 a	34.6 a	88.3 a
Sep.	447.7 ab	2536.0 a	20.8 b	86.3 a
Oct.	448.3 ab	2523.4 a	37.2 a	90.0 a

[z] Mean separation within columns by Duncan's multiple range at $p<0.05$.

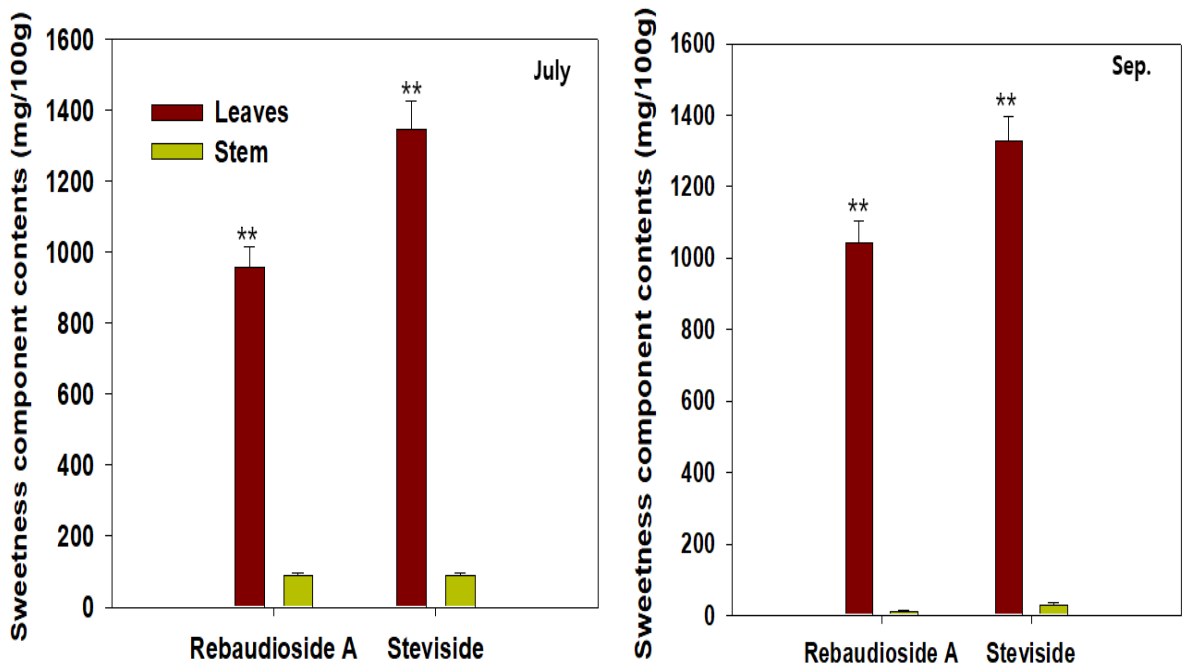

Fig. 4. Comparison of sweetness components in stevia leaves and stem in July and September. Mean values ± SE from triplicate separated experiments are shown. Statistical difference show significantly different at *p<0.05 and **p<0.01 by t-test

The total phenolic compound content in leaves was the highest in August and May, but flavonoid content was not different in harvest times. All harvest times of the DPPH activity in stevia leaves was represented with low activity under 40%, and ABTS activity was not different during harvest times (Table 3). Also, stems, the total phenolic compound was not different with harvest times. Although flavonoid content was higher in July than in September, the content was very low compared to leaves. The DPPH activity by harvest times in stevia stem was higher in September than in July, and ABTS activity was not different in harvest times (Table 4). There were few researches on antioxidant and activity according to harvest times. Although Lim and Oh (2004) observed contents of steviol glycoside according to harvest times, they were not analyzed to antioxidants.

Table 4. Comparison of antioxidant contents and activity in extracts of stevia stem harvested at different time

Harvest times	Total phenolic contents	Total flavonoid contents	DPPH	ABTS
	mg·100g^{-1}		%	
Jul.	409.5^{ns}	2,355.6^{**z}	20.5	94.2^{ns}
Sep.	379.3	1,322.4	68.5	95.5

^{ns} Not significant.

^{z} Stars show significantly different at p<0.05 and p<0.01 by t-test.

In conclusion, stevia leaves are better than stevia stems regarding the use of rebaudioside A and stevioside as natural sweeteners. Also, it was confirmed that the stevia leaves of July, September and October, excluding the high temperature period of August, had superior in quality and quantity.

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