World Journal of Agricultural Research, 2016, Vol. 4, No. 6, 162-165
Available online at http://pubs.sciepub.com/wjar/4/6/2

DOI:10.12691/wjar-4-6-2

Distribution of Phytochemicals and Bioactivity in Different Parts and Leaf Positions of *Stevia Rebaudiana* (Bertoni) Bertoni- a Non-caloric, Natural Sweetener

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Abstract *Stevia rebaudiana* (Bertoni) Bertoni (Asteraceae) is a small perennial herb which is widely cultivated for its sweet leaves and possesses 250-300 times the sweetness than sucrose due to the presence of steviol glycosides (mainly stevioside and rebaudioside). It is commonly known as candy leaf, sweet leaf and sugar leaf. Even though, this plant has been studied extensively for its sweetness, information on therapeutically important active components presence in stevia is scattered or lacking. Therefore, the present study was undertaken to determine the distribution of Total Phenolic Content (TPC), Total Flavonoid Content (TFC) and Total Antioxidant Capacity (TAC) of different parts and different leaf positions of *S. rebaudiana*. Leaf fresh weight, dry weight and leaf area at different leaf positions were recorded. The TAC, TPC and TFC were determined using Ferric Reducing Antioxidant Power Assay (FRAP), modified Folin-Ciocalteu colorimetric method and calorimetric method respectively. Leaf fresh weight and leaf dry weight were increased with the maturity. All tested plant parts demonstrated the presence of TPC, TFC and TAC. The significantly higher TPC, TFC and TAC were reported in leaves than other parts of plant. The order of increase of active components was leaf>flower>stem>branch>root. TPC, TFC and TAC of different leaf positions revealed that TAC, was decreased gradually from immature to mature leaf (1^{st} leaf>2^{nd} leaf>3^{rd} leaf>4^{th} leaf>5^{th} leaf). Moreover, all tested phytochemicals (phenolics and flavonoids) and antioxidant capacity were significantly higher in extracts prepared from the first leaf. Interestingly, a strong significant correlations were observed between TAC and tested secondary metabolites (TFC, R^2=0.85). The results of the present study are vital important in cultivation, harvesting and quality control aspects of *S. rebaudiana*.

Keywords: *stevia rebaudiana, antioxidant capacity, flavonoids, phenolics, leaf positions*

Cite This Article: K.L.N.W. Kumari, D.C. Abeysinghe, and R.M. Dharmadasa, "Distribution of Phytochemicals and Bioactivity in Different Parts and Leaf Positions of *Stevia Rebaudiana* (Bertoni) Bertoni- a Non-caloric, Natural Sweetener." *World Journal of Agricultural Research*, vol. 4, no. 6 (2016): 162-165. doi: 10.12691/wjar-4-6-2.

1. Introduction

*Stevia rebaudi*ana (Bertoni) is a small perennial herb belonging to the family Asteraceae [1]. The plant is native to Paraguay and Brazil, and produces high potency low calorie sweeteners, stevioside and rebaudioside which are about 250-300 times sweeter than sucrose [2,3,4,5]. These natural sweeteners are gaining very high popularity amongst all type of sweetener users as the most ideal substitute for sugar [6]. Other than, its sweetening property stevia is also known for its therapeutic properties including antidiabetic [7] antimicrobial [8], antiviral [9], antifungal [10], anti-hypertensive [11,12,13], anti-hyperglycaemic [14,15], anti-tumour [8], anti-human rota-virus activities [16,17], anti-HIV [18], hepatoprotective [19] and immune-modulatory effects [20,21]. Even though there are many information are available on sweetening capacity, information on health benefits due to the presence of therapeutically active secondary metabolites are scattered. However, phytochemical distribution in different parts of the plant and different leaf positions play an important role on therapeutic potential of the plant. Therefore, present study was undertaken to determine the Total Antioxidant Capacity (TAC), Total Phenolic Content (TPC) and Total Flavonoid Content (TFC) of different parts (leaves, stems, roots, flowers and branches) of S. rebaudiana and different leaf positions of the plant (1^{st} leaf, 2^{nd} leaf, 3^{rd} leaf, 4^{th} leaf and 5^{th} leaf).

2. Materials and Methods

2.1. Chemicals and Regents

Folin-Ciocalteu reagent, Gallic acid, Rutin, 2, 4, 6-trypyridyl-2-try-azine (TPTZ), 6-hydroxy-2, 5, 7, 8-tetramethy-chroman-2-carboxilic acid (Trolox) and ferric chloride ($FeCl_3.6H_2O$) were purchased from sigma Aldrich

Chemical Co. (St. Louis, Mo). All other chemicals used were of analytical grade.

2.2. Preparation of Samples

Samples of leaves, flowers, roots, stems parts and leaves from different leaf positions (1st leaf, 2nd leaf, 3rd leaf, 4th leaf and 5th leaf) of *S. rebaudiana* were collected from same aged, similarly maintained plants grown under same soil and climatic conditions (Low Country Intermediate Zone (IL_{1a}), at an elevation of 25 m above mean sea level with soil type of red yellow podosolic at experimental plots of Wayamba University of Sri Lanka, Makandura, Gonawila (NWP).

2.3. Extraction of Samples

Collected samples were cut into small pieces and air dried for three days at room temperature (28±2°C). Then samples (Three samples from each category) were powdered using motor and pestle and sieved with 0.25 mm mesh. Powdered samples (0.1 g) were mixed with 5 mL of 80% methanol and vortexed for 15 min. Then it was placed in a water bath at 60 °C for 40 min and vortex procedure was repeated at 10 min interval. After centrifugation at 4,000 rpm for 5 min, the supernatant was decanted into a 15 mL centrifuge tube and the remaining was re-extracted with 5 mL of 80% methanol. Supernatants were pooled and stored at -20 °C prior to analysis.

2.4. Total Phenolic Content (TPC)

Total phenolic content was quantified using a modified Folin-Ciocalteu method. Briefly, 4 mL of distilled water and 0.5 mL of properly diluted leaf extract were mixed with 0.5 mL of 0.5 N Folin-Ciocalteu reagent (FCR) and allowed to react for 3 min. Then 1 mL saturated sodium carbonate solution was mixed and incubated in a water bath for 2 h at 30°C. The absorbance was measured at 760 nm using UV visible spectrophotometer (Shimadzu, UV Mini 1240, Japan). Gallic acid was used as the standard and data were expressed as mg of Gallic Acid Equivalent (GAE) per gram of dry weights.

2.5. Quantification of Total Flavonoid (TFC)

Total flavonoid content (TFC) was determined by a colorimetric method, with slight modifications [22]. Briefly, 0.5 mL of the plant extract was diluted with 3.5 mL of distilled water. Then 0.3 mL of 5% $NaNO_2$ solution was added to the mixture. After 6 min, 0.3 mL of a 10% $Al(NO_3)_3.6H_2O$ solution was added, and the mixture was allowed to stand for another 6 min. Then 2 mL of 2 M NaOH was added, and top up to 8 mL with distilled water. After thoroughly mixing, the absorbance was measured at 510 nm using UV visible spectrophotometer (Shimadzu UV-160, Japan). Rutin was used as the standard and data were expressed as mg of Rutin Equivalent (RE) /g DW.

2.6. Determination of Total Antioxidant Capacity (TAC)

Total antioxidant capacity was determined using Ferric Reducing Antioxidant Power (FRAP) assay [23] with slight modifications. Methanolic extract of sample (100 µL) was mixed with 900 µL of freshly prepared FRAP reagent of pH 3.6 containing 2.5 mL of 10 mol/L, 2,4,6-Tripyridyl-s-Triazine (TPTZ) solution in 40 mmol/L, HCl plus 2.5 mL of 20 mmol/L $FeCl_3$ and 25 mL of 300 mol/L acetate buffer. Absorbance was measured at 593 nm using the spectrophotometer (Shimadzu, UV Mini 1240, Japan) after incubating for 4 min. Trolox was used as the standard solution and TAC was expressed as mg Trolox Equivalents (TE) /g DW.

2.7. Determination of Leaf Area

Leaf area was measured using a leaf area meter (LI-3100C, USA). Samples were collected from 10 plants and ten leaves from each category (part from first to fifth leaves).

2.8. Determination of Fresh to Dry Weight Ratio

Samples were collected from 10 plants (part from first to fifth leaves) and fresh weight was measured then the samples were air dried. After three days dry weight of the samples were measured.

2.9. Statistical Analysis

To verify the statistical significance of all parameters the values of means and ±SD were calculated. Statistical comparison of mean values was performed by General Linear Model (GLM) of ANOVA followed by Turkey Multiple Range Test using SAS (SAS institute, 1999). The P values of less than 0.05 were adopted as statistically significant.

3. Results and Discussion

In the present study attempts were made to determine economical yield, therapeutically important phytochemicals (phenolics and flavonoids) and Total Antioxidant Capacity (TAC) of different parts and different leaf positions of *S. rebaudiana*.

As demonstrated in Table 1, current study exhibited the presence of major phytochemical secondary metabolites (TPC and TFC) and TAC in all five main parts (leaves, stems branches, flowers and roots) of *S. rebaudiana*. Significantly higher TAC, TPC and TFC were reported in leaf extracts. Order of increase was leaves>flowers>stem>branches>roots. The higher content of TAC in leaf and flower extract and TPC and TFC in leaf extract might be due to presence of high content of phenolics, flavonoids and variety of other pigments in the leaf. Systematic investigation of distribution of important phytochemicals is important for the quantitative estimation and locating of pharmacologically active chemical compounds [24]. Presence of biologically active phytochemicals in all parts of the plant is important for the therapeutic activity of plant. Further, presence of higher content of investigated phytochemicals and antioxidant activity is in agreement with [25], who observed the higher antioxidant capacity in leaf extracts of *S. rebaudiana*. Further, the highest therapeutically active components presence in leaf extracts are in agreement with [26,27] and [28], who demonstrated the presence of

higher content of TAC in leaf extracts of *Acmella oleraceae*, and *Withania somnifera* respectively. Economical yield of any plant depend on the physical and chemical yield of the plant. As shown in Figure 1 and Table 2, in the current study, leaf fresh weight, leaf dry weight, fresh to dry weight ratio, leaf area, TPC, TFC and TAC of different leaf positions were investigated. The results revealed that TAC was decreased gradually from immature to mature leaf (1st leaf>2nd leaf>3rd leaf>4th leaf>5th leaf). Further, all tested phytochemicals (TPC and TFC) were significantly higher in extracts prepared from the first leaf (Bud).

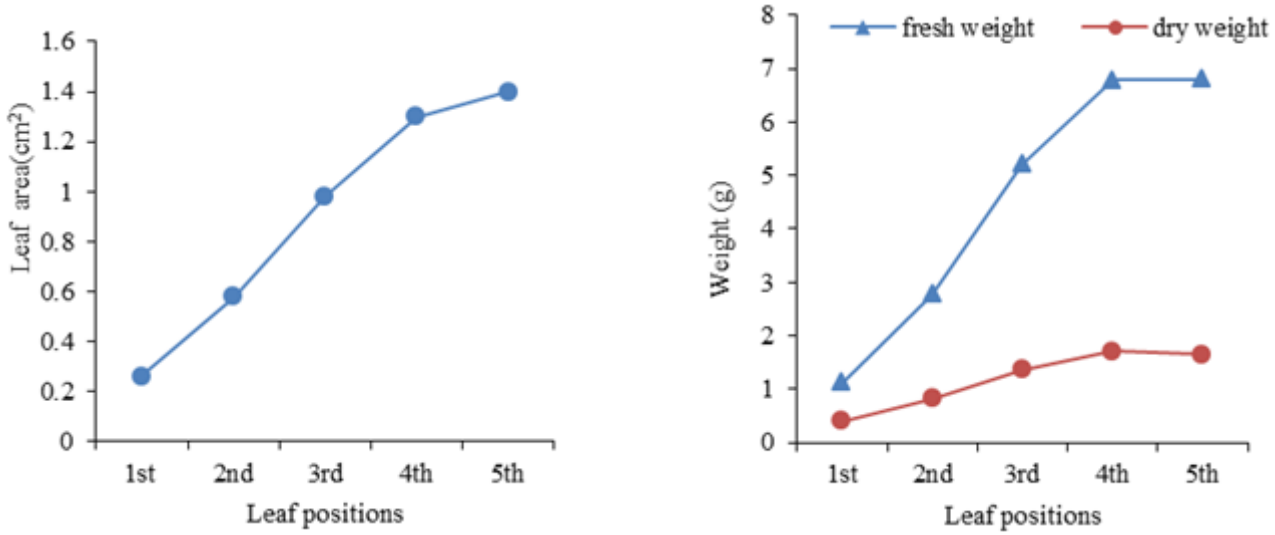

Figure 1. Changes in area of leaves (A) and fresh weight, dry weight (B) at different leaf positions of *Stevia rebaudiana*

Table 1. Distribution of Total Antioxidant Capacity (TAC), Total Phenolic Content (TPC) and Total Flavonoid Content (TFC) in different parts of *Stevia rebaudiana*

Plant part	TAC (mg TE / g DW)	TPC (mg GAE/ g DW)	TFC (mg RE / g DW)
Leaves	27.67±0.32^{a}	12.44±0.02^{a}	29.33±0.22^{a}
Flowers	27.57±0.95^{a}	11.99±0.03^{b}	27.19±0.08^{b}
Roots	03.45±0.30^{d}	03.18±0.04^{e}	01.95±0.30^{e}
Stems	16.10±0.49^{b}	08.49±0.01^{c}	14.38±0.16^{c}
Branches	10.76±0.33^{c}	07.23±0.04^{d}	13.76±0.08^{d}

Means denoted by the same letters in a column represent non-significant differences ($p< 0.05$); TE=Trolox equivalent; GAE=Gallic acid equivalent; RE=Rutin equivalent; DW=Dry weight.

Table 2. Total Antioxidant Capacity (TAC), Total Phenolic Content (TPC) and Total Flavonoid Content (TFC) and fresh to dry weight ratio of leaves at different positions of *Stevia rebaudiana*

Leaf position	TAC (mg TE/g DW)	TFC (mg RE/g DW)	TPC (mg GAE/g DW)	Fresh to dry weight ratio
1st	34.24±0.78^{a}	34.86±0.25^{a}	13.64±0.04^{a}	3.2:1
2nd	30.01±0.07^{b}	23.29±0.14^{d}	11.56±0.04^{d}	2.9:1
3rd	28.10±0.68^{c}	29.62±0.08^{b}	12.67±0.02^{b}	2.6:1
4th	24.80±0.44^{d}	25.00±0.29^{c}	11.23±0.06^{e}	2.5:1
5th	21.15±0.49^{e}	29.52±0.30^{b}	11.77±0.05^{c}	2.4:1

Means denoted by the same letters in a column represent non-significant differences ($p< 0.05$); TE=Trolox equivalent; GAE=Gallic acid equivalent; RE=Rutin equivalent; DW=Dry weight.

4. Conclusions

Present study investigated Total Antioxidant Capacity (TAC), Total Phenolic Content (TPC) and Total Flavonoid Content (TFC) of different plant parts and different leaf positions of *Stevia rebaudiana*. Results indicated, all plant parts of *S. rebaudiana* possess TPC, TFC and TAC. The higher TPC, TFC and TAC were recorded in leaf extracts than other parts of plant. Moreover, all tested secondary metabolite contents (TPC and TFC) and antioxidant capacity were reduced with the leaf maturity. Strong significant correlations were observed between TAC and tested secondary metabolites (TFC, R^2=0.85).

Acknowledgements

Authors wish to express their gratitude to all staff of the Department of Plantation Management, Wayamba university of Sri Lanka for their help during study period and Dr. J Ranatunga, who provided the planting materials for the establishment of research plot.

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