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In Vitro Determination of Total Phenolics, Flavonoids and Free Radical Scavenging Activities of *Stevia rebaudiana* Dry Leaves Powder in Different Solvents Extract

Md Moinul Abedin Shuvo, Mohammad AL-Mamun* and Nurul Absar

Department of Biochemistry and Biotechnology, Faculty of Basic Medical and Pharmaceutical Sciences, University of Science and Technology Chittagong (USTC), Foy's Lake, Chittagong, Bangladesh

***Corresponding Author:** Mohammad AL-Mamun, Fellow (Research) - Nutritional and Clinical Biochemistry, Department of Biochemistry and Biotechnology, Faculty of Basic Medical and Pharmaceutical Sciences, University of Science and Technology Chittagong (USTC), Foy's Lake, Chittagong, Bangladesh. E-Mail: mbbt_ustc2013@yahoo.com

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Abstract

The core objective of this study was to evaluate the total phenolic contents, flavonoids and antioxidant activity as scavenging of free radical like DPPH (2, 2 - diphenyl-1-picrylhydrazyl) using *in vitro* propagated *Stevia rebaudiana* dry leaves powder in different solvents extract. Water at 100^{0}C and 50%, 70% and Pure (100%) reagent grade- methanol, ethanol and acetone (v/v) were used for the phenolic extractions as well as for the determination of flavonoids and free radical scavenging activity, where 70% methanol was found to be the most suitable solvent with highest phenolic extraction as 3.52 gm% tannic acid equivalent from Stevia leaves powder, and that also showed the maximum inhibition of DPPH as 67.85% rather than others. Lowest amount of phenolics and minimum inhibition of DPPH were determined in 50% ethanol as 0.17 gm% and 16.51% respectively. Flavonoids content was determined as uppermost in water (100^{0}C) and pure acetone extract at the amount of 0.14 gm% quercetin equivalent and the lowermost in 50% Acetone as 0.05gm% quercetin equivalent, respectively. Findings obtained from this study have also been found significant as compared with other reported values and clearly indicates as potential as natural antioxidant.

Keywords: *Stevia rebaudiana; Phenolics; Flavonoids; Antioxidant; Free Radical Scavenging Activity*

Abbreviations

TAE: Tannic Acid Equivalent; GAE: Gallic Acid Equivalent; QE: Quercetin Equivalent

Introduction

Since few decades phytochemicals have drawn the attention of many researchers and scientists and be an extensively studied matter due to their wide variety of structures and different biological roles against numerous disorders. Scavenging of free radical has found as the most potential one. Free radicals are the metabolic byproduct of cells and transition-metal ions, responsible for oxidative modification and cellular damages [1]. Increased formation of free radicals inside the body may be the cause of diseases like atherosclerosis, chronic inflammation, cardiovascular and neurological disorders [2,4]. Many *in vitro* and *in vivo* studies suggest that antioxidant can reduce the production of free radicals [3-4]. Antioxidants are mainly the phenolic compounds that are abundant in fruits and vegetables including medicinal plants and have scavenging activity by binding with free radicals and eliminate them from the body [5-7].

Stevia rebaudiana, can also be referred to as sweet leaf or honey leaf, is considered as a significant source of natural sweetener due to its 100 to 300 times more sweeter [8,9] than sucrose and also popular in use as a sugar substitute in tea or coffee by the diabetic patients [10,11]. It is an herbaceous perennial shrub derived from the family of Asteraceae. Growths are available in semi- humid subtropical regions and preferred 24^0C temperature for cultivation [12,13]. It is found as high in phyto-constituents and showed significant activities as antimicrobial, antitumor, anti-inflammatory, hepatoprotective and immunomodulatory activities in both *in vitro* and *in vivo* studies [14-16]. Therefore this research was initiated to evaluate the total phenolic contents, total flavonoids and free radical scavenging activities of *in vitro* propagated *Stevia rebaudiana* leaves powder in different solvent extracts.

Materials and Methods

Sample Collection and Preparation

Due to low germination, tissue culture methods are widely followed for the mass propagation of Stevia plants in some nurseries during rainy season (June to August) in Bangladesh. For experimental purposes, Stevia plants with leaves were collected from the Green Nursery, Mirsorai, Dhaka - Chittagong highway at October, 2014.

Sample Preparation

Mature fresh green leaves were removed from the plants and washed in clean water and spread on trays for sundry for five days. Then they were dried again at 37^0C in an incubator (Binder E28, Germany) for 24 hrs. Fine powders were prepared through 3 times blending through blender (Miyako BL152, 25000 rpm, China), and then again grounded with mortar and pestle and kept in 4^0C temperature to avoid moisture absorption and possible microbial damages prior to analysis.

Solvent choice and extraction

Phenolic compounds have diverse structure and their affinity of binding in solution is also varied in different solvents. As Stevia is usually used in tea or coffee as sugar substitute, considering this water at 100^0C including 50%, 70% and Pure - methanol, ethanol and acetone were used for the extraction of phenolics from Stevia powder by following the extraction procedure of Makkar., *et al* [17]. 0.2 gm of powder was digested in 10 ml of solvents in different test tubes for 20 minutes at room temperature. Then centrifuged at 3000 rpm for 10 minutes and filtered through Whatman No.1 filter paper and the filtrate were kept at 4^0C temperature.

Determination of total phenolic content (TPC)

Folin - Ciocalteu (FC) method, described by Makkar., *et al*. 1993 [17] was followed for the determination of TPC from stevia powder. A 100 µl of each extract was mixed with 0.25 ml of FC reagent and 1.25 ml of 20% sodium carbonate solution and then incubated for 40 minutes at room temperature (27^0C). The absorbance was measured at 725 nm. TPC was calculated from the standard curve of Tannic acid and the results were expressed as gm TAE (Tannic Acid Equivalent) per 100 gm.

Determination of total flavonoid content (TFC)

500 µl of each extract was mixed with 500 µl of 2% methanolic AlCl3.6H2O by following the procedure of Christel., *et al* [18]. The mixture was then incubated for 10 min at room temperature and then absorbance was measured at 430 nm. TFCs were calculated from the standard curve of Quercetin and expressed as gm QE (Quercetin Equivalent) per 100 gm.

Free radical scavenging activity

Free radical scavenging activities of each extract were measured according to the procedure of Brand Williams., *et al*. [19] against DPPH with slight modification. DPPH (2.4 mg) was diluted in 100 ml of methanol in a dark bottle and immediately the absorbance (A_0)

was recorded at 515 nm. Then 1 ml of each extract was mixed with 1 ml of DPPH and kept for 30 minutes incubation and the absorbance (A1) was measured at 515 nm. The scavenging activity was calculated in percent (%) by following the below equation.

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

Data Analysis

All experiments were executed duplicate. The optical density (OD) of each sample was measured in a spectrophotometer and all the ODs were plotted on the graph against respective standard. Concentration was calculated in 1 ml and then converted into 100 gm correspondingly and the results were expressed as the means of duplicate ± Standard Deviation (SD).

Results and Discussion

In analysis of total phenolic contents (Table 1, Figure 1), 70% methanol was revealed as the most effective solvent for phenolic extraction which extracted 3.52 gm% TAE phenolics from Stevia leaves powder which is 46.67%, 802.56%, 357.14% and 39.68% higher than the reported values of TPC by Abou-Arab., *et al.* (2010) in water [20]; Katarzyna., *et al.* (2015) in water and pure ethanol [21]; and Tadhani., *et al.* (2007) in pure methanol extracts [22] but found 42.76% lower than the reported value of Serio., *et al.* (2010) in pure ethanol extract [23], respectively. 50% ethanol was found as low in phenolic extraction that extracted phenolics as 0.175 gm% TAE which is 95.03% decrease than the maximum extraction. Efficacy of solvents in phenolic extraction was found as per following order:

70% Methanol (3.52 gm%) > Pure Methanol (2.74 gm%) > Pure Ethanol (2.41 gm%) > 50% Methanol (1.86 gm%) > Water (100^{0}C) (1.84 gm%) > 70% Ethanol (1.50 gm%) > 70% Acetone (1.11 gm%) > 50% Acetone (1.02 gm%) > Pure Acetone (0.87 gm%)> 50% Ethanol (0.17 gm%).

Solvent	Total phenolic content (gm TAE/100gm)	Abou-Arab., *et al*. 2010 (gm GAE/100gm)	Katarzyna G.B *et al*. 2015 (gm GAE/100gm)	Tadhani., *et al*. 2007 (gm GAE/100gm)	Serio., *et al*. 2010 (gm GAE/100gm)
Water					
Water (100°C)	1.84 ± 0.29	2.4g%	0.39g%	……	
Methanol					
50% Methanol	1.86 ± 0.41	……		2.52g%	……
70% Methanol	3.52 ± 0.12				
Pure Methanol	2.74 ± 0.36				
Ethanol					
50% Ethanol	0.17 ± 0.09	……	0.77g%	……	6.15g%
70% Ethanol	1.50 ± 0.17				
Pure Ethanol	2.41 ± 0.23				
Acetone					
50% Acetone	1.02 ± 0.31	……			
70% Acetone	1.11 ± 0.21				
Pure Acetone	0.87 ± 0.16				

Table 1: *Total phenolic contents (TPC) of Stevia rebaudiana dry leaves powder.*

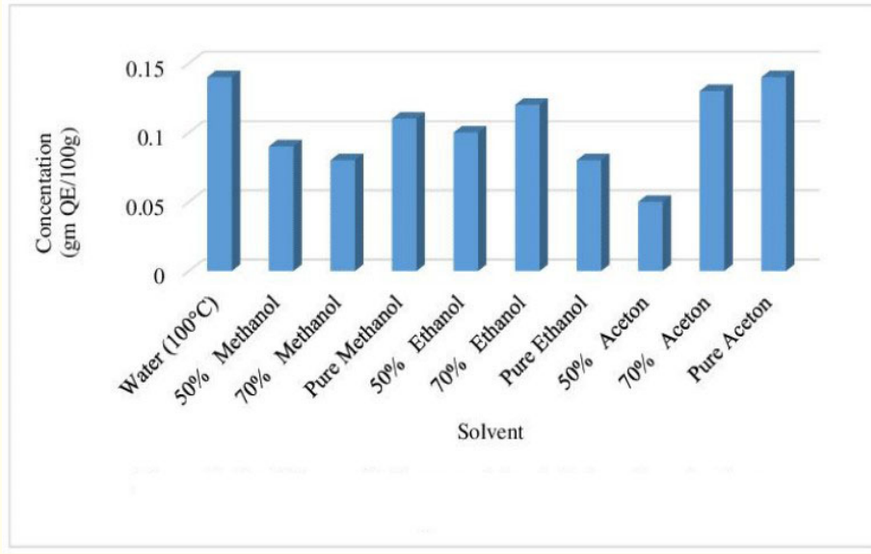

Figure 1: *Total phenolic content of Stevia rebaudiana dry leaves powder.*

Table 2 represents the Total Flavonoid Contents (TFC) of Stevia leaves powder in different solvent extracts (Figure 2) where water at 100^{0}C and pure acetone were evaluated as suitable for extraction of maximum flavonoids at the amount of 0.14 gm% QE, which is 92.96%, 87.93%, 95.27%, 93.55%, and 94.04% less than the findings of TFC by Abou-Arab., *et al.* (2010) in water [20], Katarzyna., *et al.* (2015) in water and pure ethanol [21], Tadhani, *et al.* (2007) and Liu., *et al.* (2003) in pure methanol extract [22,24], consequently. Minimum Flavonoids was observed in 50% acetone extraction as 0.052 gm% QE that is 62.85% less than the highest TFC in water (100^{0}C) and pure acetone extracts. Solvents were evaluated as their suitability in extraction of flavonoid contents as per following sequences:

Water (100^{0}C) and Pure Acetone (0.14 gm%) > 70% Acetone (0.13 gm%) > 70% Ethanol (0.12 gm%) > Pure Methanol (0.11 gm%) > 50% Ethanol (0.10 gm%) > 50% Methanol (0.094 gm%) >Pure Ethanol (0.084 gm%) > 70% Methanol (0.083 gm%) > 50% Acetone (0.052 gm%).

Solvent	Flavonoids Results (g QE/100g)	Abou-Arab., *et al.* 2010	Katarzyna GB., *et al.* 2015	Tadhani., *et al.* 2007	Liu., *et al.* 2003
Water					
Water (100°C)	0.14 ± 0.02	1.99g%	1.16g%	……	
Methanol					
50% Methanol	0.09 ± 0.04	……		2.17g%	2.35g%
70% Methanol	0.08 ± 0.05				
Pure Methanol	0.11 ± 0.08				
Ethanol					
50% Ethanol	0.10 ± 0.03	……	2.96g%	……	1.5g%
70% Ethanol	0.12 ± 0.06				
Pure Ethanol	0.08 ± 0.06				
Acetone					
50% Acetone	0.05 ± 0.09	……			
70% Acetone	0.13 ± 0.07				
Pure Acetone	0.14 ± 0.08				

Table 2: *Total flavonoid contents (TFC) of Stevia rebaudiana dry leaves powder.*

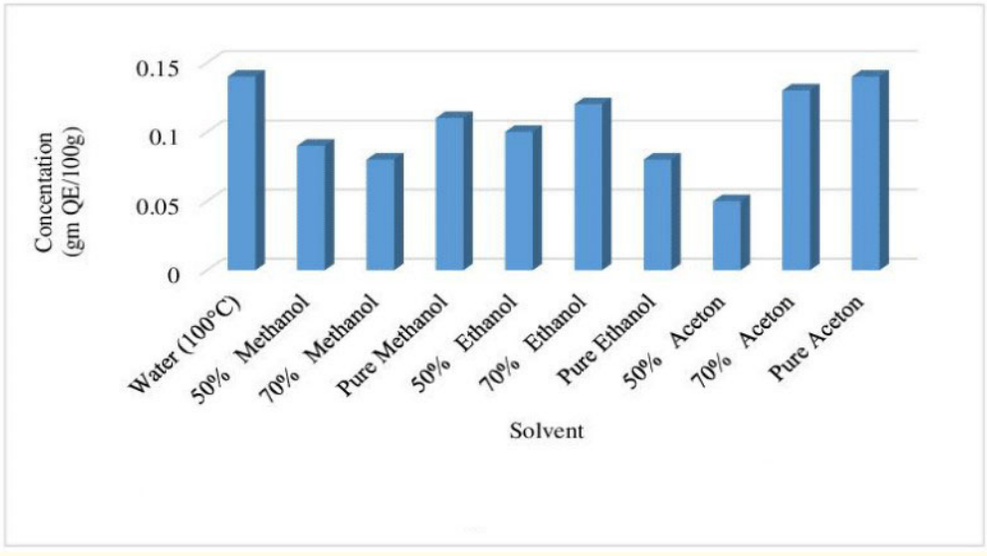

Figure 2: *Total flavonoid content of Stevia rebaudiana dry leaves powder.*

In analysis of free radical scavenging activities against DPPH (Table 3), a correlation has been observed in between phenolic contents and antioxidant properties (Figure 3). DPPH scavenging activity was found high in 70% Methanol extraction like the solvent that was high in phenolic extraction, which inhibited 67.85% DPPH and found 81.61% and 114.65% higher than the findings in DPPH inhibition by Abou-Arab., *et al*. (2010) in water and pure methanol [20]. 50% Ethanol extract was evaluated as low in DPPH inhibition, as like as low in phenolic extraction, at the amount of 16.51% and showed 75.67% decrease than the maximum inhibition. Solvents were manifested as maximum to minimum in inhibition of DPPH as per following sequences:

70% Methanol (67.85%) > Pure Methanol (54.23%) > Pure Ethanol (49.27%) > 50% Methanol (45.22%) > Water (100^{0}C) 41.79% > 70% Ethanol (39.85%) > 70% Acetone (31.09%) > Pure Acetone (29.53%) > 50% Acetone (27.07%) > 50% Ethanol (16.51%).

Solvent	Inhibition (%) of DPPH	Abou-Arab., *et al*. 2010
Water		
Water (100°C)	41.79 ± 1.08	37.36%
Methanol		
50% Methanol	45.22 ± 0.16	31.61%
70% Methanol	67.85 ± 0.07	
Pure Methanol	54.23 ± 0.04	
Ethanol		
50% Ethanol	16.51 ± 0.21	.....
70% Ethanol	39.85 ± 0.09	
Pure Ethanol	49.27 ± 0.19	
Acetone		
50% Acetone	27.07 ± 0.14	.....
70% Acetone	31.09 ± 0.04	
Pure Acetone	29.53 ± 0.02	

Table 3: *Free radical scavenging activities (FRSA) of Stevia rebaudiana dry leaves powder.*

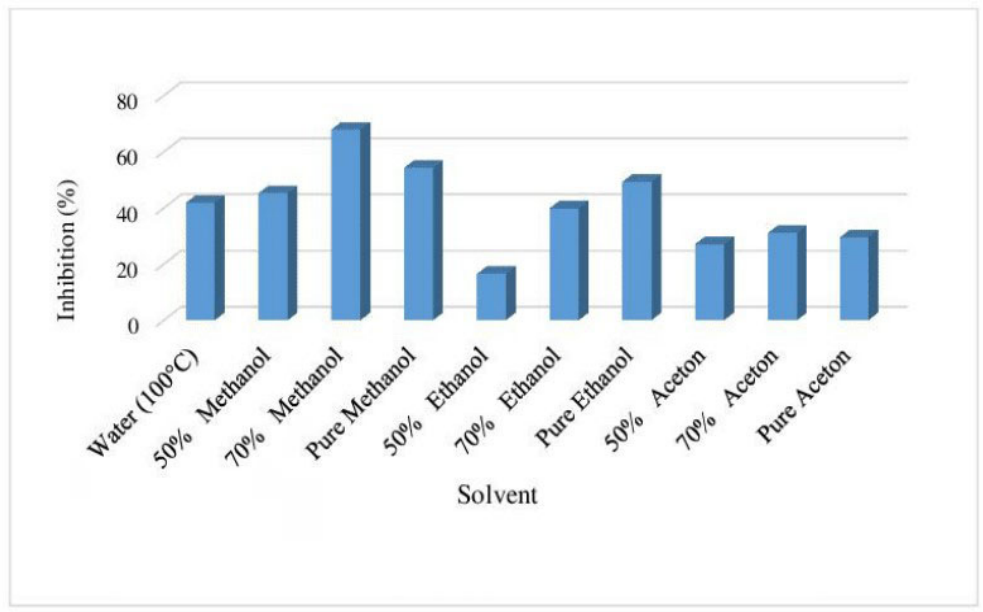

Figure 3: *DPPH scavenging activities of Stevia rebaudiana dry leaves powder.*

Conclusion

High in phenolic compounds have high antioxidant activity that has been evident from this experiment and also has consistent with several reports. Considerable amount of phenolics and flavonoids were estimated from *in vitro* propagated Stevia plant dry leaves powder and that also showed significant antioxidant activity. So, Stevia leaves could be a potential source of natural antioxidant and can be used as food additives in different food stuffs. Aqueous mixture of methanol (70%) can be used as prime solvent for maximum phenolic extraction whereas phenolics mainly contain the most bioactive molecules that exhibit the therapeutic activities. Hot water (100^0C) is also found significant for phenolics and flavonoids extraction as well as in inhibition of free radical (DPPH). So, it can be mentioned that uses of Stevia in hot water like tea or coffee is beneficial for health. Further phytochemical studies are needed to isolate and characterize its most bioactive molecules from *in vitro* propagated Stevia plants and to identify their biological activities *in vivo* in order to use them as pharmaceutical drug component and in cosmetic products. A complete nutritional database on *Stevia rebaudiana* in compare with other available researches on different species of Stevia will also make a significant impact to choose the suitable one for mass production and uses in Bangladesh.

Conflict of Interest

The authors declare no conflict of interest.

Bibliography

1. Rahman K. "Studies on free radicals, antioxidants, and co-factors". *Clinical Interventions in Aging* 2.2 (2007): 219-236.

2. Pham-Huy LA., *et al*. "Free Radicals, Antioxidants in Disease and Health". *International Journal of Biomedical Science: IJBS* 4.2 (2008): 89-96.

3. Lobo V., *et al*. "Free radicals, antioxidants and functional foods: Impact on human health". *Pharmacognosy Reviews* 4.8 (2010): 118-126.

4. Randhir Singh, *et al*. "Role of free radical in atherosclerosis, diabetes and dyslipidaemia: larger-than-life". *Diabetes/Metabolism Research and Reviews* 31.2 (2015): 113-126.

5. Jau-Tien Lin., *et al*. "Composition of phenolic compounds and antioxidant attributes of Cycleagracillima Diels extracts". *Journal of Food and Drug Analysis* 26.1 (2017): 193-200.

6. Działo M., *et al*. "The Potential of Plant Phenolics in Prevention and Therapy of Skin Disorders". *International Journal of Molecular Sciences* 17.2 (2016): 160.

7. Dai J and Mumper RJ. "Plant Phenolics: Extraction, Analysis and Their Antioxidant and Anticancer Properties". *Molecules* 15.10 (2010):7313-7352.

8. MBA Glória., *et al*. "In Encyclopedia of Food Sciences and Nutrition (Second Edition)". Edited by Benjamin Caballero. Academic Press, Oxford (2003): 5695-5702.

9. Giri A., *et al*. "Effect of partial replacement of sugar with stevia on the quality of kulfi". *Journal of Food Science and Technology* 51.8 (2014): 1612-1616.

10. Renu Mogra and Versha Dashora. "Exploring the Use of Stevia rebaudiana as a Sweetener in Comparison with Other Sweeteners". *Journal of Human Ecology* 25.2(2009): 117-120.

11. Madhumita Kumari and Sheela Chandra. "Handbook of Medicinal Plants and their Bioactive Compounds". Editor: Nidhi Gupta, Stevia rebaudiana: Beyond sweetness (2014): 11-26.

12. JE Brandle and N Rose. "Heritability for yield, leaf: stem ratio and stevioside content estimated from landrace cultivar of Stevia rebaudiana". *Canadian Journal of Plant Science* 72.4 (1992): 1263-1266.

13. K Ramesh., *et al*. "Cultivation of Stevia [Stevia rebaudiana (Bert.) Bertoni]: A Comprehensive Review". *Advances in Agronomy* 89 (2006): 137-177.

14. S Jayaraman., *et al*. "In-vitro Antimicrobial and Antitumor Activities of Stevia rebaudiana (Asteraceae) Leaf Extracts". *Tropical Journal of Pharmaceutical Research* 7.4 (2008): 1143-1149.

15. Noosud J., *et al*. "Invitro and invivo assessment of inhibitory effect of stevioside on pro-inflammatory cytokines". *Avicenna Journal of Phytomedicine* 7.2 (2017): 101-106.

16. Ena Gupta., *et al*. "Nutritional and therapeutic values of Stevia rebaudiana: A review". *Journal of Medicinal Plants Research* 7.46 (2013): 3343-3353.

17. Makkar HPS., *et al*. "Gravimetric determination of tannins and their correlations with chemical and protein precipitation methods". *Journal of the Science of Food and Agriculture* 61 (1993): 161-165.

18. Christel QD., *et al*. "Phenolic compounds and antioxidant activities of buckwheat (Fagopyrume sculentum Moench) hulls and flour". *Journal of Ethnopharmacology* 72.1-2 (2000): 35-42.

19. Brand-Williams W., *et al*. "Use of a free radical method to evaluate antioxidant activity". *LWT Food Science and Technology* 28.1 (1995): 25-30.

20. Abou-Arab., *et al*. "Evaluation of bioactive compounds of Stevia reabudiana leaves and callus". *African Journal of Food Science* 4.10 (2010): 627-634.

21. Katarzyna GB., *et al*. "Stevia reabudiana Bert. Leaf Extracts as a Multifunctional Source of Natural Antioxidants". *Molecules* 20.4 (2015): 54568-5486.

22. MB Tadhani., *et al*. "In vitro antioxidant activities of Stevia reabudiana Leaves and callus". *Journal of Food Composition and Analysis* 20.3-4 (2007): 323-329.

23. Serio L. "La stevia rabaudiana, une alternative ausurce". *Phytotherapie* 8.1 (2010): 26-32.

24. Liu JC., *et al*. "Mechanism of the antihypertensive effect of stevioside in anesthetized dogs". *Pharmacology* 67.1 (2003): 14-20.

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