International Journal of Farming and Allied Sciences
Available online at www.ijfas.com
©2017 IJFAS Journal-2017-6-2/49-55/ 31 March, 2017
ISSN 2322-4134 ©2017 IJFAS

Polyphenolic compounds (HPLC analysis) and Antioxidant Activity of *Stevia Rebaudiana* (Asteraceae) by FRAP and DPPH Assay in greenhouse and free space condition

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ABSTRACT: This paper aimed to establish the content in polyphenolic compounds and antioxidant activity of Stevia Rebaudiana (Asteraceae) by FRAP and DPPH Assay in greenhouse and Free space condition. A high-performance liquid chromatographic method coupled with UV and fluorescence (HPLC) detectors for the determination of polyphenols in Stevia samples are reported. Hesperedin, Ellagic acid, Rosmarinic acid, Chloregenic acid, Eugenol, Coumarin and Vanilin were detected of Stevia. Samples were injected into the chromatograph directly without previous treatment. In the second part of the work the antioxidant activity of the samples were determined by 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay and Ferric ion reducing activity(FRAP) assay. The antioxidant activities of greenhouse essential oil were stronger than those of the essential oil of free space condition with regard to DPPH radical scavenging and FRAP. The correlation between specific polyphenols and the antioxidant activity in samples was also investigated. FRAP assay showed better antioxidant activity than DPPH assay. The FRAP assay, is presented as a novel method for assessing "antioxidant power." The assay is inexpensive, reagents are simple to prepare, results are highly reproducible, and the procedure is straightforward and speedy.

***Keywords*:** Polyphenols, Antioxidant activity, HPLC, FRAP assay, DPPH assay, Stevia

INTRODUCTION

Plants have a high ability to synthesize aromatic substances, a large amount of which are phenols or their oxygen-substituted derivatives (Geissman, 1963). These compounds keep the plant from microbial contamination and deterioration (Cowan, 1999). Some of these phytochemicals can significantly decrease the danger of cancer due to polyphenol antioxidant and anti- inflammatory effects. A number of studies recommend that phytochemicals can stop colorectal cancer and other cancers (Michaud et al., 2000; Birt et al., 2001). One of the effective members of the Asteraceae family is *Stevia rebaudiana* (generally referred to as Honey leaf, Candy leaf and Sweet leaf). It is rich in terpenes and flavanoids. The phytochemicals present in Steviarebaudiana are austroinullin, β- carotene, dulcoside, nilacin, rebaudi oxides, riboflavin, steviol, stevioside and tiamin (Crammer and Ikan, 1986). Stevia has main industrial application in beverages, energizers as well as medicinal uses such as low uric acid treatment vasodilator cardiotonic, anesthetic and anti-inflammatory. The present study was carried out to evaluate the antimicrobial and antitumor activity of *Stevia rebaudiana* leaves extracted using various solvents. Antioxidant compounds in food play an important role as a health protecting factor. Scientific evidence suggests that antioxidants reduce the risk for chronic diseases including cancer and heart diseases (Hu and Willett, 2002).

Antioxidants are compounds or systems that delay autoxidation by inhibiting formation of free radicals or by interrupting propagation of the free radical. The major phenolics of antioxidative plant can be divided into several general groups such as phenolic acids, phenolic diterpenes, flavonoids, and volatile oils (Shan et al., 2005). Phenolic acids generally act as antioxidants by trapping free radicals; flavonoids can scavenge free radicals and chelate metals as well (Geldof and Engeseth, 2002). Phenolic composites are a large various group of secondary plant metabolites that have been widely distributed in plants and are important ingredients of human nutrition. Several antioxidant activity methods have been used to screen and compare the antioxidant activity of foods. A rapid, simple and inexpensive method to measure antioxidant capacity of food involves the use of the free radical, 2, 2-Diphenyl-1-picrylhydrazyl (DPPH). DPPH is widely used to test the ability of compounds to act as free radical scavengers or hydrogen donors, and to evaluate antioxidant activity of foods. It has also been used to quantify antioxidants in complex biological systems in recent years. The DPPH method can be used for solid or liquid samples and is not specific to any particular antioxidant component, but applies to the overall antioxidant capacity of the sample. A measure of total antioxidant capacity helps to understand the functional properties of foods (Huang et al., 2005). However, no much study has been directed toward the antioxidant activity and polyphenolic compounds of the stevia leaves by FRAP and DPPH assay which are locally available. Hence, the objective of this study was to assess the antioxidant activity and polyphenolic compounds of *Stevia Rebaudiana* (Asteraceae) by FRAP and DPPH assay in greenhouse and Free space condition.

MATERIALS AND METHODS

Plant material

Stevia Rebaudiana was collected in June 2014 from plants growing wild in Basht city, near Gachsaran in Kohgiluyeh and Boyer-Ahmad Province in Iran in greenhouse and free space condition. The plant material was identified by staff at the herbarium of Fars Research Center for Agriculture and Natural Resources, Shiraz, Iran. A voucher specimen was deposited in the herbarium of the Research Center for Agriculture and Natural Resources, Shiraz, Iran.

Extraction of Polyphenol and HPLC analysis

The procedure for extraction of polyphenols from plant material was used according to the modified method established by Justesen (Justesen et al,. 1998). HPLC analysis (Fig1) was carried out on a Agilent 1200 series, equipped with a Zorbax Eclipse XDB-C18 column (10cm × 5 µm i.d.; × 150 mm film thickness, RP), and a photodiode array detector (PDA). Elution was monitored at 280 and 230 nm. Gradient elution was selected to achieve maximum separation and sensitivity. The elution was performed by varying the proportion of solvent A (formic acid 1% in deionized water) to solvent B (Methanol (v/v)) as follows: Methanol. Formic acid 1% (10:90), at 0 min; Methanol. Formic acid 1% (25:75), at 10 min; Methanol: formic acid 1% (60:40), at 20 min and finally, Methanol: formic acid 1% (70:30), at 30 min. The total running time was 30 min. The column temperature was 30 ºC. The injection volume was 20µL and it was done automatically using autosampler (Najafian and Rowshan, 2013).

Preparation of plant extraction for antioxidant

20 g of plant dry weight was soaked in 200 ml methanol/water (90/10) for two days. The solvent was changed after one day. The extract was filtered and then concentrated in a rotary evaporator in a period of <10 min time. The yield was determined by weighing the powders. The powders were maintained at temperature of at -20°C before using. Just before each measurement, the powder was dissolved in methanol at the desired concentration and its antioxidant activity was determined (Bejeli et al., 2012).

Using DPPH for determination of antioxidant

The antioxidant activity of plant extract and the standard antioxidants were determined based on the radical scavenging effect of the stable DPPH free radical. The standard solution was prepared by Gallic acid. In a modified assay (Bruits et al., 2001) 200 µl of a 100 mM DPPH radical solution in methanol was mixed with 20 µl of 12.5-3200 µg ml-1 extracts, gallic acid, respectively. The solutions were kept at room temperature for about 30 mins. The inhibition of DPPH radical was measured using a micro-plate reader model Biotek ELx 808 at 515nm. The IC50 of each sample (concentration in µg ml-1 required to inhibit DPPH radical formation by 50%) was calculated by MATLAB software packages. The methanol solution extract without DPPH was considered as a blank and was subtracted from all of the measurements. The antioxidant activity was determined by using the following equation:

$$\text{antioxidant activity} = 100 - \frac{(A_{sample} - A_{blank}) \times 100}{A_{control}}$$

Where "A" is the absorbance of the samples in wells. DPPH (without plant extract) and methanol were used as control and blank, respectively.

The IC50 value for each sample was defined as concentration of the test sample resulting in 50% reduction of the concentration of initial DPPH. The IC50 values were calculated using the non-linear regression between the Log concentrations of the test extract (µg ml-1) against the mean percentage of the radical scavenging activity (Bruits et al., 2001).

Ferric ion reducing activity (FRAP)

The FRAP assay was conducted as described formerly (Benzie and Strain, 1996). Briefly, 180µl of solution of FRAP (10 parts of 300 mM sodium acetate buffer at pH 3.6, 1 part of 10 mM TPTZ solution and 1 part of 20 mM FeCl3. 6H2O solution) was mixed with 20 µl of 12.5-3200 µg/ml methanolic extracts micro plate in oven at ~37°C. Absorbance was determined at 595 nm after 6 min of incubation at room temperature via micro-plate reader (Biotek Lx808).

Inhibition (%) = [(A) sample – (A) blank / (A) blank] x 100 (Bejeli et al., 2012).

RESULT AND DISCUSSION

Antioxidant assays

The results of the antioxidant assays of the two essential oils are stated in fig 2-3. The IC50 values of greenhouse and free space condition were 727.6 mg/L and 824.9 mg/L for DPPH radical scavenging assay, respectively while the IC50 values of greenhouse and free space condition were 6500.1 mg/L and 9233.2 mg/L for FRAP the Ferric Reducing Ability of Plasma assay, respectively. The antioxidant activities of greenhouse essential oil were stronger than those of the essential oil of free space condition with regard to DPPH radical scavenging and FRAP (Fig2 and Fig3).

Polyphenols

The polyphenols as Chloregenic acid, Ellagic acid, Coumarin, Hesperedin, and Rosmarinic acid were detected in greenhouse and Hesperedin, Eugenol, Coumarin and Vanilin were detected of stevia in free space condition (Table 1). The results showed that the maximum polyphenols were Hesperedin. Hesperidin is a natural chemical compound which shown in two plants. The quantity of this component was 2807.1 mg g−1 and 493.4 mg g−1 in greenhouse and free space condition, respectively (Fig4). Hesperidin is believed to play a role in plant defense. (Dolzhenko et al., 2010). The second component which showed a highest amount of polyphenols in stevia was Coumarin. Rosmarinic acid was one of the phenolic compounds found in the plant species in greenhouse condition. This component was 662.3 mg g−1. Rosmarinic acid is an ester of caffeic acid and 3, 4-dihydroxyphenyllactic acid. It is commonly found in species of the Boraginaceae and the subfamily Nepetoideae of the Lamiaceae. However, it is also found in species of other higher plant families and in some fern and hornwort species. Rosmarinic acid has a number of interesting biological activities, e.g. antiviral, antibacterial, antiinflammatory and antioxidant. The presence of rosmarinic acid in medicinal plants, herbs and spices has beneficial and health promoting effects. In plants, rosmarinic acid is supposed to act as a preformed constitutively accumulated defense compound. The biosynthesis of rosmarinic acid starts with the amino acids l-phenylalanine and l-tyrosine. All eight enzymes involved in the biosynthesis are known and characterised and cDNAs of several of the involved genes have been isolated. Plant cell cultures, e.g. from Coleus blumei or Salvia officinalis, accumulate rosmarinic acid in amounts much higher than in the plant itself (up to 36% of the cell dry weight). For this reason a biotechnological production of rosmarinic acid with plant cell cultures has been proposed. Rosmarinic acid is a widely occurring natural product in the plant kingdom with interesting biological activities (Fig5) (Petersen and Simmonds, 2003).

CONCLUSION

This study showed the two plants essential oils contain more antioxidants and poly phenoles and the major compound were ***Hesperedin***, ***Coumarin*** and ***Rosmarinic acid*** in greenhouse .The antioxidant activities of Stevia in greenhouse was stronger than that of free space condition by using DPPH radical and FRAP assay. The antioxidant activity had different results by different assay systems (Tsai et al., 2011). The antioxidant activities of

antioxidants have been attributed to various mechanisms, including the prevention of chain initiation, binding of transition metal ion catalysts, decomposition of peroxides, prevention of continued hydrogen abstraction, and radical scavenging (Diplock, 1997). It could be noticed that the content of total phenolic compounds and antioxidant activity were higher in greenhouse compared to the leaves of free space condition. Phenolic compounds are commonly found in both plants, and they have multiple biological effects, including antioxidant activity. Besides, the contents of flavonoid and other phenolic substance have been suggested to play a preventive role in the development of cancer and heart disease (Kahkonen et al., 1999). There are many different antioxidants present in plants and it is very difficult to measure each antioxidant component separately. Therefore, several methods have been developed to evaluate the total antioxidant activity of fruits or other plants (Van den Berg et al., 1999), total radical absorption potentials (Evelson et al., 2001) and oxygen radical absorption capacity assays (Ou et al., 2001). The ferric reducing ability of plasma (FRAP) assay (Benzie and Strain, 1996) are commonly used and are the representative method frequently used in various investigations. The FRAP and DPPH assay were selected to evaluate the antioxidant activities of the leaves stevia. FRAP assay, Stevia showed better properties than DPPH assay. The FRAP assay offers a putative index of antioxidant, or reducing, potential of biological fluids within the technological reach of every laboratory and researcher interested in oxidative stress and its effects. The results obtained should help to clarify the functional applications of these folk herbs and their essential oils for aroma therapeutic healing and other folkloric uses. There are some reports in the literature on the qualitative and quantitative analyses of some Mentha essential oils from different countries (Gulluce et al., 2007), but not a single report was found to polyphenolic compounds and antioxidant activity of Stevia in free space condition and greenhouse condition. These findings may be extended variation on polyphenolic compounds of stevia. Furthermore, these achievements show that polyphenolic compounds producers and consumers, which utilize these compounds in pharmaceutical and cosmetic industries could benefit from this result. These substances have been suggested to play a preventive role for human health. Also, the results of the present work indicate that stevia leaf extracts may be an ideal candidate for further research into their uses for food preservation as well as pharmaceutical and natural plant-based products due to their antioxidant activities.

Acknowledgments

First of all, I would like to thank the grace of God for completing this work and wish to thank superintendent of Department of Natural Resources, Fars Research Center for Agriculture and Natural Resources Dr. Vahid Rowshan for his kindness and providing the laboratory facilities.

Table1. Phenolic compounds of *Stevia rebaudiana* in greenhouse and free space condition.

Phenolic compounds	Greenhouse	Free space
Chloregenic acid (mg/L)	271.2	
Ellagic acid(mg/L)	491.4	-
Hesperedin (mg/L)	2807.1	493.4
Rosmarinic acid (mg/L)	662.3	-
Eugenol (mg/L)	-	57.4
Coumarin (mg/L)	2185.2	148.7
Vanilin (mg/L)	-	5.2

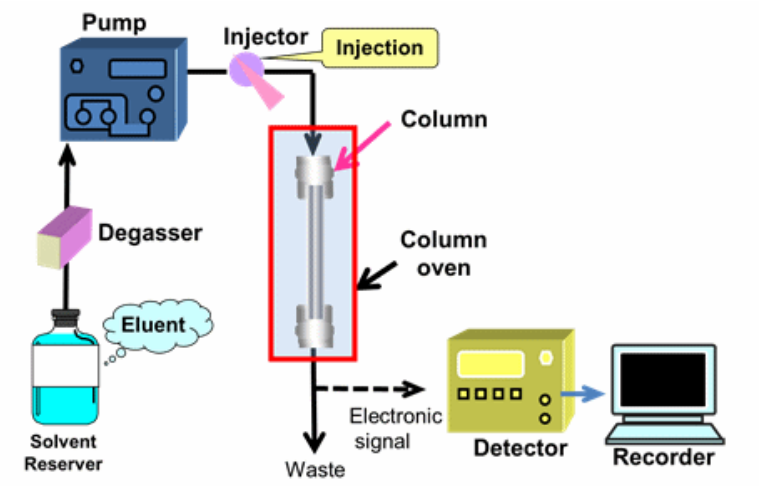

Fig1. Components of HPLC system (Showa Denko America, Inc., 420 Lexington Ave, Suite 2335A New York, NY 10170)

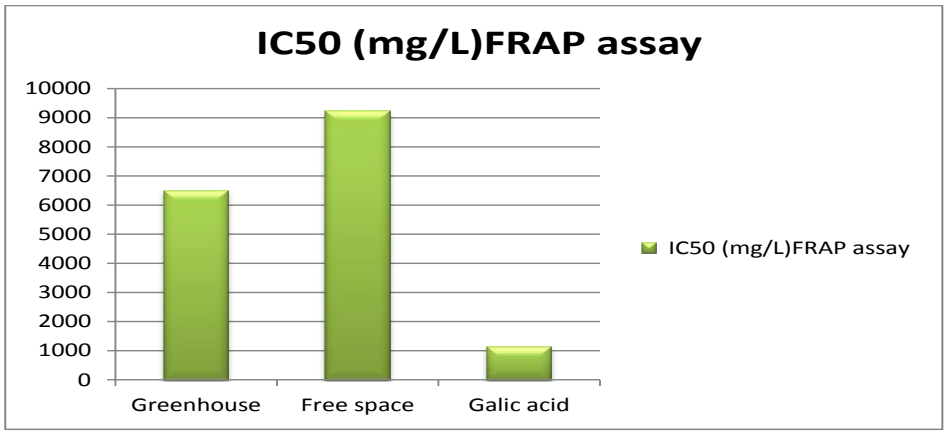

Fig2. Antioxidant activity by FRAP assay.

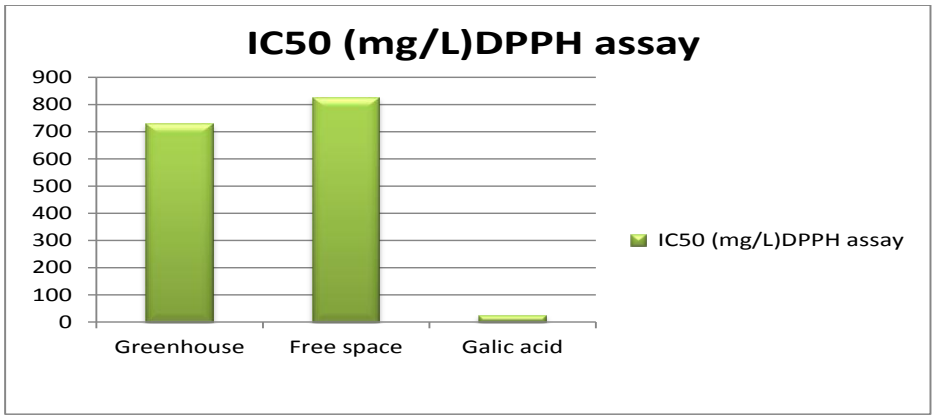

Fig 3. Antioxidant activity by DPPH assay.

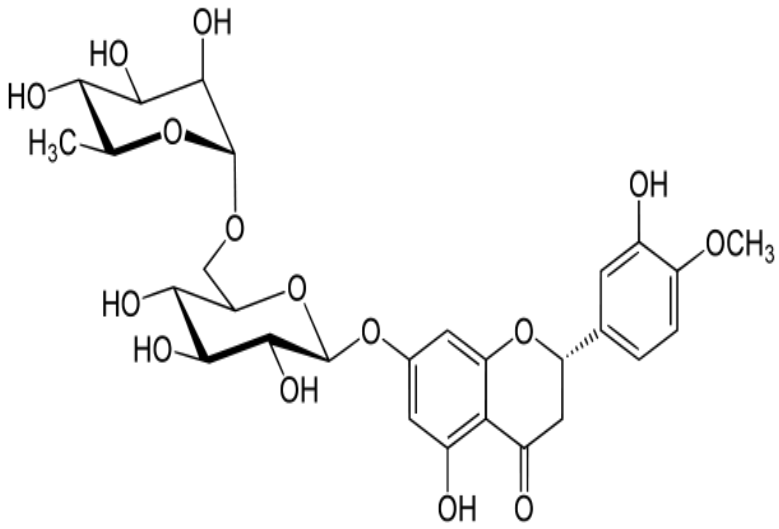

Fig 4. Chemical structure of Hesperidin (http://en.wikipedia.org/wiki/File:Hesperidin_structure.svg).

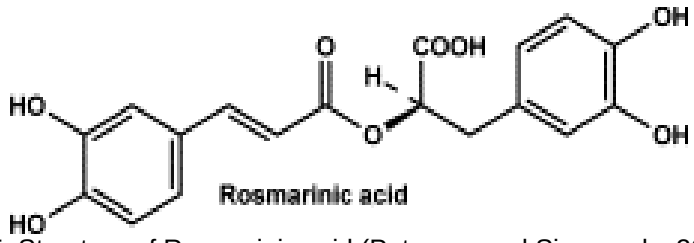

Fig 5. Structure of Rosmarinic acid (Petersen and Simmonds, 2003).

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