Cellular and Molecular Biology
E-ISSN : 1165-158X / P-ISSN : 0145-5680
www.cellmolbiol.org
Original Research

The Effects of different nitrogen doses on antioxidant and antimicrobial activity of Stevia (*Stevia rebaudiana* Bert.)

Mehmet Atas[1], Nuraniye Eruygur[2], Esra Ucar[3*], Yasar Ozyigit[4], Kenan Turgut[5]

[1] Department of Pharmaceutical Microbiology, Cumhuriyet University Faculty of Pharmacy Sivas; Turkey
[2] Department of Pharmacognosy, Cumhuriyet University Faculty of Pharmacy Sivas; Turkey
[3] Department of Crop and Animal Production, Sivas Vocational School, Cumhuriyet University, Sivas, Turkey
[4] Department of Horticulture, Korkuteli Vocational School, Akdeniz University, Antalya, Turkey
[5] Department of Field Crops, Faculty of Agriculture, Akdeniz University, Antalya, Turkey

Correspondence to: eucar@cumhuriyet.edu.tr

Received October 5, 2017; **Accepted** February 1, 2018; **Published** February 10, 2018

Doi: http://dx.doi.org/10.14715/cmb/2018.64.2.8

Abstract: In this study, the efficiency of the different nitrogen doses (0, 5, 10, 15 and 20 kg ha^{-1}) on biological activity levels (antioxidant and antimicrobial activity) of *Stevia rebaudiana* Bert. was investigated. In addition, methanol extracts were obtained by maceration method from different doses of fertilizer applied stevia. The components in methanol extracts of plants were determined by gas chromatography-mass spectrometry (GC-MS) method. Antimicrobial activities of stevia extracts were investigated by microdilution method. The antioxidant activity evaluated by 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging, ferric thiocyanate (FTC), thiobarbituric acid (TBA), reducing power, total phenol content (TPC), and total flavonoid content (TFC) methods. According to the results, the fertilizer doses effects on antimicrobial activity of stevia were not made much difference. But in antioxidant activity, there were some variations in the activity-dependent on fertilizer amount.

Key words: Antimicrobial; Antioxidant; Essential oil; *Stevia rebaudiana*.

Introduction

Stevia rebaudiana (Bert.), known as sweet herb, is a perennial shrub herb of the Asteraceae family. This plant originated in South America (1-2). The leaves of stevia contain eight diterpene glycosides such as stevioside, steviolbioside, rebaudiosides (A, B, C, D and E) and dulcoside A (3). Extracts obtained from stevia leaves are being consumed as a natural alternative to artificial sweetener (4). These glycosides are 300-350 times sweeter than sucrose and it is zero calorie (5-6). Besides, stevia has nutrient like protein, calcium, phosphorous (7-8), sterols, triterpenes, flavonoids, tannins and essential oil comprising of aromatics, aldehyde, monoterpenes and sesquiterpenes (9). Many studies have been carried out on the chemical composition of essential oil of stevia by the different scientist. According to Siddique *et al.* 62 compounds were identified as major compounds with two different extraction methods (hydro distillation and steam distillation) (9).

Stevia is important due to its natural property which can be safely used specially for diabetes and other people who care about their health (1-10). On the other hand, the glycosides have important effects such as hypoglycemic, oral contraceptive, cardiovascular and biological activities (antioxidants, antimicrobials and antifungals) (10-13).

The glycosides are obtained from the plant leaves and the increase of the plant's green parts has a great importance. According to Brandle *et al.* and Shock, the presence of these components content in stevia leaves depends on basic agricultural techniques (14-15). From this point of view, agronomic activities gain importance in order to increase both the amounts and contents of glycosides. One of the agronomic activities is fertilizing and nitrogen is one of the main macronutrients necessary for plants. In addition, the nitrogen which exists in the structure of proteins, amino acids, nucleic acids, enzymes, chlorophyll, ATP and ADP is certain necessary elements for the formation of new cells and plant growth (16-17). Moreover, nitrogen is the building block in all plant parts (18-19).

Medicinal and aromatic plants contain essential oils, terpenes, alcohols, aldehydes, esters, phenols, nitrogen and sulfur compounds (20-21). The phenols, active components of essential oils presented in medicinal and aromatic plants, can prevent oxidative tissue damage (22) and used as antioxidant, antimicrobial, antidiabetic, and antimutagenic substances (23-24). The protective biochemical functions of natural antioxidants found in spices, herbs and medicinal plants are of great interest (25). Furthermore, these phenols have been used as a disinfectant since ancient times owing to their antimicrobial effects (26). Today, pathogens have developed resistance to available drugs. Therefore, there is a great interest for natural antimicrobial compounds of plant origin to treat diseases (27-29).

The aim of the present work was to identify and quantify the phenolic compounds, the antioxidant and antimicrobial activity levels of methanol extracts of *S.*

rebaudiana were evaluated in different nitrogen doses (0, 5, 10, 15, 20 kg da^{-1}) applied plants.

Materials and Methods

This study was conducted in the experimental fields of Field Crops Department of Agricultural Faculty, Akdeniz University in Antalya and Laboratories of Faculty of Pharmacy, Cumhuriyet University in Sivas during 2014 and 2017 years. Cultivated seedlings in a greenhouse of Department of Field Crops of Agricultural were used as experimental material. The seedlings were transplanted in an open field condition and were applied different nitrogen levels (0, 5, 10, 15, 20 kg.da^{-1}) (ammonium nitrate (% 33 N) form). The experiment was carried out in randomized plots with four replications. The experiments were carried out in field conditions in randomized plots with four replications.

The chemical components

Dry plant leaves were ground in a blender (Blue house). These leaf fragments were extracted with methanol. The obtained extracts were analyzed by GC-MS.

Chemical reagents

The standards (BHT, BHA, Ascorbic acid and gallic acid) and chemicals used in the present study were analytical grades obtained from Sigma Chemical Co, St Louis, USA.

Preparation of extracts

The plant extracts were prepared as below: Dry plant leaves were ground in a blender (Blue house). 10 gr of powder was soaked in 50mL of methanol for 24h with intermittent shaking. At the end of extraction, it was filtrated by No. 1 Whatman filter paper. The filtrate was concentrated to dryness under reduced pressure with a rotary evaporator at 40°C and this was repeated for three times. The obtained extracts were analyzed by GC-MS. Then stored at -20°C until experimental studies (22-23).

Antioxidant assay

The antioxidant activity of the Stevia extracts was tested using different methods namely as DPPH radical scavenging activity, ferric thiocyanate (FTC), thiobarbituric acid (TBA) methods, reducing power, total phenol, and total flavonoid content method.

DPPH radical scavenging activity

The free radical scavenging activity by Stevia extracts was done according to the method reported by Blois (30). The 1 mL of methanol extract was mixed with 3mL of 1.5×10^{-3} M DPPH solution freshly prepared in methanol. 1 mL of Methanol only was used as a control of the experiment. After 30 min of incubation at room temperature (25°C), the reduction of the DPPH free radical was measured reading the absorbance at 517nm with a spectrophotometer. Gallic acid and quercetin used as positive controls. From the absorbance value, percent inhibition was calculated with the following equation:

% Inhibition = [Absorbance of control – Absorbance of test sample / Absorbance of control] × 100

Ferric thiocyanate (FTC) method

The standard method as described by Zahin M, Aqil F and Ahmet I, 2009 was used (31). A mixture of 4mL plant extracts in absolute ethanol (1mg/mL), 4.1mL of 2.5% linoleic acid prepared in absolute ethanol, 8.0 mL of 0.05M phosphate buffer (pH=7.0) and 3.9mL of water was placed in a vial with screw cap and then placed in an oven at 40 °C in the dark. Then, 9.7 mL of 75% ethanol and 0.1 mL of 30% ammonium thiocyanate were added to 0.1 mL of this solution. Precisely 3 min after addition of 0.1 mL of 0.02M ferrous chloride in 3.5% HCl to the reaction mixture, the absorbance of red color was measured at 500nm each 24 hr until the day after absorbance of control reached the maximum. BHT and α-tocopherol were used as positive controls while the mixture without plant sample was used as the negative control.

Thiobarbituric acid (TBA) method

The TBA method was done according to the method reported by Ottolenghi A, 1959 (32). 1 mL of the sample solution, which was prepared in FTC method, mixed with 2 mL of 20% trichloroacetic acid and 2 mL of 0.67% 2-thiobarbituric acid in the test tube. The mixture was placed in a boiling water bath for 10 min and, after cooling, was centrifuged at 3000 rpm for 20 min. The absorbance of the supernatant was read at 552 nm. Antioxidant activity was based on the absorbance on the final day of FTC method.

Reducing power method

The ferrous reducing power of the samples was determined by the method of Oyaizu M, 1986 (33). To 1 mL of various concentrations (20-1000 mL) of test samples and standards (BHT and a-tocopherol) prepared with methanol, 2.5 mL, 0.2 M, pH 6.6 of phosphate buffer and 2.5 mL of 1% potassium ferricyanide solution was added. The mixture was incubated 50°C for 20 min. Then 2.5 mL of 10% trichloroacetic acid was added and centrifuged at 2500 rpm for 10 min. 2.5 mL supernatant was taken and mixed with 2 mL distilled water and 0.5 mL of 0.1% $FeCl_3$. Phosphate buffer was used as blank without samples. The absorbance was measured at 700 nm using a UV-Vis spectrophotometer (UV-1800, Shimadzu).

Determination of total phenolics (TPC)

The spectrophotometric Folin–Ciocalteu method was used to measure the total phenolic content in the extracts, as previously described by Clarke *et al*., 2013 (34). Thus, 10 µL of extract diluted appropriately in DMSO was mixed with 100 µL F-C reagent freshly diluted 1/10 with distilled water. After five minutes, the solution was mixed with 100 µL 7.5% Na_2CO_3 solution, and the whole left for 60 min, before measurement of absorbance at 650 nm in a Multiskan™ FC microplate photometer (Thermo USA). All the analyses were performed in triplicate and the results expressed as mean ± standard deviation. Appropriate blanks (DMSO) and standard (quercetin in DMSO) were run simultaneously, after which the total phenolics content (TPC) was calculated as milligrams gallic acid equivalents per gram of dry extract.

Estimation of total flavonoids (TFC)

The aluminium chloride colorimetric method was used for the determination of total flavonoid concentration, as previously described by Quettier et al., 2000 (35) using quercetin as the reference standard. Briefly, the test sample of ethanol solution (150 µL, 0.3 mg/mL) was mixed with 2% (w/v) $AlCl_3$ (150 µL) in 96-well plates. After 15 min of incubation at room temperature, the absorbance was measured at 435 nm by a spectrometer. All determinations were carried out in triplicates. The content of total flavonoids was expressed as mg of quercetin equivalent per g of dry weight of the sample, using an equation obtained from the standard quercetin calibration.

Antimicrobial activity

The microdilution method was used to determine Minimum Inhibitory Concentration (MIC) value against bacteria and fungi for the Stevia extract (36). In this study *Staphylococcus aureus* (ATCC 29213), *Enterococcus faecalis* (ATCC 29212), *Pseudomonas aeruginosa (*ATCC 27853), *Escherichia coli* (ATCC 25922) and *Candida albicans* (ATCC 10231) strains were used. Stevia extracts were dissolved in 8% Dimethyl sulfoxide (DMSO). 50 µL sample was added in the first line of a microtiter plate which was serially diluted two-fold with sterile distilled water. The concentration of plant extract in wells ranged from 5.00 to 0.02 µg/mL. Final inoculum size was 5×10^5 CFU/mL at bacteria and 0.5-2.5 $\times 10^3$ CFU/mL at Candida every well (37-38). Mueller Hinton Broth and Sabouraud Dextrose Broth were used for dilution bacteria and Candida culture's, respectively. 50 µL bacteria and fungi suspension were added on prepared samples. Samples which added bacteria were incubated at 37 °C and samples which added Candida were incubated at 35 °C for 16-24 hours. Gentamycin and Fluconazole were used as positive control and DMSO were used as negative control. Afterwards, 50 µL 2 mg/mL 2, 3, 5-Triphenyltetrazolium chloride (TTC) (Merck, Germany) was added to each well to indicate microbial growth. The microtiter plates were further incubated at 37 °C for 2h. Reduction in density of formazan's red color after incubation was accepted MIC value. The experiment was performed in duplicate and the standard deviation was zero.

Results

Extracts of *S. rebaudiana* obtained from plants, which were applied different fertilization doses, were tested (Table 1). As a result of GC-MS analyzes, 37 different components were determined. The most common components are as follows;

4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-, Benzoic acid, 2-hydroxy-, methyl ester (CAS), 2-Furancarboxaldehyde, 5-(hydroxymethyl)-(CAS), Phenol, 2-methoxy-4-(2-propenyl)- (CAS), Phenol, 2-propyl-, Benzeneethanol, 4-hydroxy- resveratrol, Cyclododecane, 4-(2,6,6-Trimethylcyclohexa-1,3-dienyl)but-3-en-2-one, Phenol, 2,4-bis(1,1-dimethylethyl), 3-Buten-2-one, 1-(2,3,6-trimethylphenyl)-, 1-Methoxy-1,4-cyclohexadiene, Germacrene, 1,4-Methanoazulene, decahydro-4,8, 8-trimethyl-9-methylene-, [1S- (1.alpha.,3a.beta.,4.alpha.,8a. beta.)], (3E,5E,8Z)-3,7,11-Trimethyl-1,3,5, 8,10-dodecapentanene, Neophytadiene, 3,7,11,15-Tetramethyl-2-hexadecen- resveratrol and 1H-Naphtho[2,1-b]pyran, 3-ethenyldodecahydro-3,4a,7,7,10a-pentamethyl-, [3S-(3.alpha.,4a.alpha.,6a.beta.,10a.alpha.,10b.beta.)]- in 5 kg da^{-1} nitrogen fertilization.

There were differences in the chemical contents between the control group and the nitrogen applied group. The compounds that established at only control group, are Furfural, 1,2-Benzenediol (CAS), Benzene, 1-(1,5-dimethyl-4-hexenyl)-4-methyl-(CAS), Cyclohexane,1,5-diethenyl-3-methyl-2-methylene-, (1.alpha.,3.alpha.5.alpha.)-, Naphthalene, 1,2,3,5,6,7,8,8a-octahydro-1,8a-dimethyl-7-(1-methylethenyl)-, [1S-(1.alpha.,7.alpha.,8a.alpha.)], Azulene, 1,2,3,4,5,6,7,8-octahydro -1,4-dimethyl-7-(1-methylethylidene)-Valencene, 1H-Cycloprop[e]azulene, decahydro- 1,1,7-trimethyl-4-methylene-, (1a, 3-C(9),4-C(9)-epoxytricyclo[4.2.2, resveratrol. 0(1,5)] decane, Eudesma-4(14),11-diene and 1H-Cyclopropa[a] naphthalene, decahydro-1,1,3a-trimethyl-7-methylene-[1as-(1a.alpha.,3a.alpha.,7a.beta.,7b.alpha.)]-.Whereas there are components that obtained at only nitrogen applied, total 23 components such as mainly 2-Furancarboxaldehyde, 5-(hydroxymethyl)- (CAS), Phenol, 2-methoxy-4-(2-propenyl)- (CAS), Cyclododecane, Phenol, 2,4-bis(1,1-dimethylethyl).

Antioxidant activity

DPPH radical scavenging activity of methanol extracts from different doses of nitrogen applied Stevia was analyzed (Figure 1) and the scavenging activities were found in the range of 40-70 % which are nearly same with Gallic acid and quercetin, which were in accordance with the results obtained in previous studies (12).

Flavonoids that are phenolic compounds have antioxidant activities (23). The present work was conducted to evaluate the total flavonoid and phenolic contents of the different fertilizer applied stevia leaves, and the data were shown in Figure 2. The total phenolic contents of the extract were in the range of 99.19 and 129.01µg, while total flavonoid content was in the range of 73.9 and 113.4 µg in the dry weight basis of crude methanol extract. The obtained data demonstrated that there was a negative correlation between total flavonoid and phenolic compounds with dose of nitrogen fertilizer (Figure

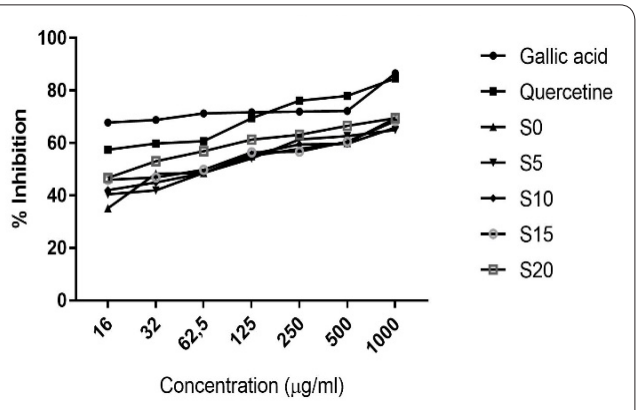

Figure 1. Determination of DPPH radical scavenging activity. All experiments were conducted in triplicate. Data are expressed as mean + SD (n=3, $p < 0.05$) for all tested concentration.

Table 1. The Chemical Composition of Sweet Herb (*Stevia rebaudiana*).

Chemical Component	RT	Control Group	5 kg da^{-1}	10 kg da^{-1}	15 kg da^{-1}	20 kg da^{-1}
Furfural	5.839	0.95				
Pyrazole, 1,4-dimethyl-	5.856					0.33
3-fluoro-2,5-dimethyl-2,4-hexadiene	13.175					1.53
4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-	16.551	2.47	8.71		6.46	9.19
Benzoic acid, 2-hydroxy-, methyl ester (CAS)	18.055	1.41	9.77		22.49	10.16
1,2-Benzenediol (CAS)	18.170	2.04				
2-Furancarboxaldehyde, 5-(hydroxymethyl)-(CAS)	19.183		19.13			21.62
Phenol, 2-methoxy-4-(2-propenyl)- (CAS)	24.321		2.06		1.75	2.82
Phenol, 2-propyl-	27.537		0.19			
Benzeneethanol, 4-hydroxy- resveratrol.	27.697		0.95			
Cyclododecane	28.195		5.37		8.44	7.84
1-Dodecanol (CAS)	28.201				7.14	
1-Tridecanol	28.212					5.61
4-(2,6,6-Trimethylcyclohexa-1,3-dienyl)but-3-en-2-one	28.538		2.52			
1-Benzosuberone	28.704			3.26		
Phenol, 2,4-bis(1,1-dimethylethyl)	29.265		4.94	33.89	7.37	6.87
3-Buten-2-one, 1-(2,3,6-trimethylphenyl)-	29.499		1.24			
1-Methoxy-1,4-cyclohexadiene	29.866		1.14			
Benzene, 1-(1,5-dimethyl-4-hexenyl)-4-methyl-(CAS)	31.021	2.61				
Camphene	31.176				5.84	
Cyclohexane,1,5-diethenyl-3-methyl-2-methylene-, (1.alpha.,3.alpha.5.alpha.)-	31.182	17.44				
Naphthalene, 1,2,3,5,6,7,8,8a-octahydro-1,8a-dimethyl-7-(1-methylethenyl)-, [1S-(1.alpha.,7.alpha.,8a.alpha.)]	31.290	5.80				
Azulene, 1,2,3,4,5,6,7,8-octahydro -1,4-dimethyl-7-(1-methylethylidene)-Valencene	32.217	1.81				
1H-Cycloprop[e]azulene, decahydro-1,1,7-trimethyl-4-methylene-, (1a.	32.435	2.67				
Germacrene	32.452		1.64			
Bicyclo[4.4.0]dec-1-ene, 2-isopropyl-5-methyl-9-methylene-	32.463				2.83	
3-C(9),4-C(9)-epoxytricyclo[4.2.2, resveratrol. 0(1,5)]decane	32.566	1.17				
Eudesma-4(14),11-diene	32.687	6.34				
1H-Cyclopropa[a]naphthalene, decahydro-1,1,3a-trimethyl-7-methylene- [1as-(1a.alpha.,3a.alpha.,7a.beta.,7b.alpha.)]-	32.744	2.89				
1,4-Methanoazulene, decahydro-4,8, 8-trimethyl-9-methylene-, [1S- (1.alpha.,3a.beta.,4.alpha.,8a.beta.)]	32.767		1.92			
Valencene	32.778				2.82	
(3E,5E,8Z)-3,7,11-Trimethyl-1,3,5,8,10-dodecapentanene	33.104	4.19	2.65			
Neophytadiene	36.206	3.17	3.49		9.78	
2H-Furo[2,3-H]-1-benzopyran-2-one	36.858			33.01		
3,7,11,15-Tetramethyl-2-hexadecen- resveratrol.	36.972		1.85			
Butanoic acid, 3-methyl-, 3,7-dimethyl-6-octenyl ester	36.984					3.76
1H-Naphtho[2,1-b]pyran, 3-ethenyldodecahydro-3,4a,7,7,10a-pentamethyl-, [3S-(3.alpha.,4a.alpha.,6a.beta.,10a.alpha.,10b.beta.)]-	39.084		6.38			

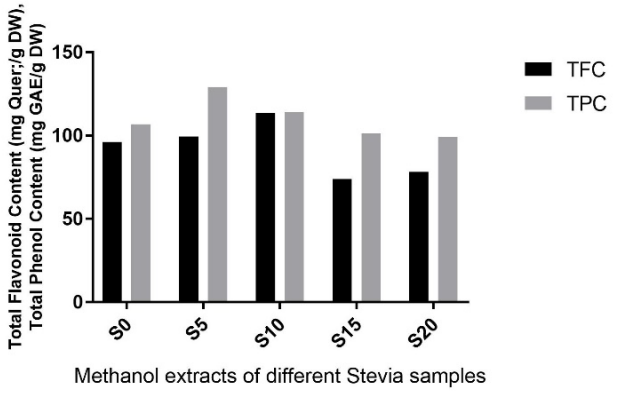

Figure 2. Total flavonoid and polyphenolic content of the methanol extracts of samples.

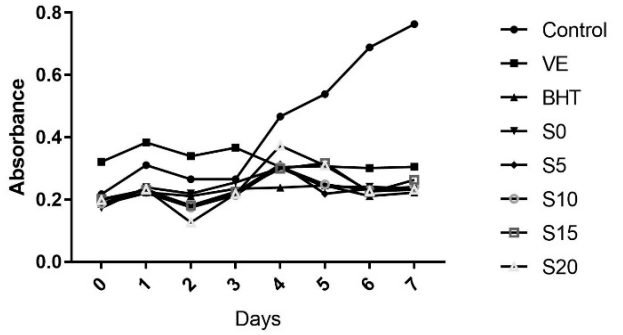

Figure 3. Antioxidant activity of methanol extracts of Stevia leaves and standard BHT, V_E as measured by the FTC method.

3, 4 and 5).

Antimicrobial activity

Plant extracts have been used by the humans for different aims for thousands of years and because of the developing antimicrobial resistance plant extracts have a more importance today. Antimicrobial properties of various solvent extracts of *S. rebaudiana* have been known (29- 42,43).

The MIC values of the Stevia methanol extract against *Bacillus subtilis, Klebsiella pneumonia, Proteus vulgaris, Streptococcus pneumoniae, Staphylococcus aureus* and *Pseudomonas fluorescence* were found to be in the range of 0.781 to 6.25 µg/ml while the in the leaf extract was limited to 3.125 to 12.5 µg/ml for all organisms. (42).

Discussion

When the obtained data are evaluated; the different fertilizer doses application has been shown to be effective in the chemical content of the plant. In GC-MS results of the Stevia methanol extract, the compound phenol, 2,4-bis(1,1-dimethylethyl) was ascended with the increasing of nitrogen than control group, but after 10 kg da^{-1}, the content of the compound was decreased vigorously. The nutrient content of the plant from the soil is also influential on the chemical components that the plant synthesizes. The secondary metabolites such as total flavonoid and phenolic compounds influenced by environmental conditions such as light intensity, CO_2 level, temperature, and fertilization. The nitrogen is the main component required for the plant growth and in turn it can change a number of the active secondary metabolites (39). When studies of Stevia's chemical composition have been examined, it is often seen that the essential oil of the plant is evaluated. For example, according to Siddique *et al.* leaves of *S. rebaudiana* extracted by different extraction methods (hydro distillation and steam distillation) (9). As a result; while they obtained major components like Cyclohexasiloxane (4.40%), α-Cadiol (2.98%) and (-)-spathulenol (2.21%) with hydro distillation method, they determined major compounds like Silanediol (4.49%), Cyclopentasiloxane (7.00%) and Cyclohexasiloxane (7.10%) with the steam distillation.

In this study, both the plants obtained with the nitrogen fertilizer application and the chemical composition values of the methanol extracts of these plants were evaluated. Another study on the assessment of the chemical components of stevia extracts, Tadhani *et al.* and Liu *et al.* were found that, the methanolic extracts of leaf and callus of *S. rebaudiana* contained a certain amount total phenols (respectively 25.18 mg/g, 25.25 mg/g) and flavonoids (respectively 21.73 mg/g, 23.46 mg/g) compounds (40-41).

The total flavonoid content was highest in S10 sample, while the total phenol content was found in S5 sample. From the results, it can be deduced that the nitrogen fertilizer is playing a vital role in producing of total flavonoid and phenolic compounds, the increase in nitrogen fertilizer demonstrated negative correlation with total flavonoid and phenolic compounds.

Recently, a group researchers carried out a study which was investigated antibacterial activity of different extracts (carbon tetrachloride, hexane, ethanol, and aqueous extracts) of stevia leaves against *Staphy-*

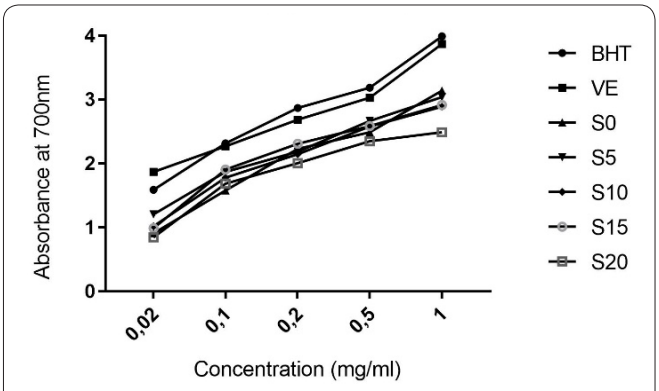

Figure 4. Antioxidant activity of methanol extracts of Stevia leaves, standard BHT and V_E as measured by the TBA method.

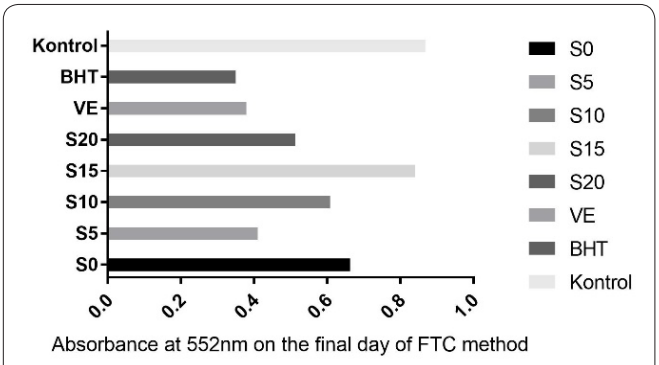

Figure 5. Ferrous reducing antioxidant capacity of methanol extracts of Stevia leaves and standards.

Table 2. Antimicrobial activity results for different doses of fertilizer applied Stevia samples (mg/mL).

	Test sample	*E. coli*	*S. aureus*	*P. aeruginosa*	*E. faecalis*	*C. albicans*
1	Stevia 0	1.25	2.5	5.0	>5	5.0
2	Stevia 5	2.5	5.0	5.0	>5	5.0
3	Stevia 10	2.5	5.0	5.0	>5	5.0
4	Stevia 15	1.25	2.5	5.0	>5	>5
5	Stevia 20	2.5	2.5	5.0	>5	5.0

lococcus aureus, *Staphylococcus epidermidis* and *Pseudomanas aeruginosa* (44). According to their results, the MIC value of ethanol extracts was detected in the range of 5.36-0.161 mg/mL. Our results showed that the MIC values of the methanol extract vary between in the range of >5-1.25 mg/mL (Table 2). The most secondary methabolites are biosynthesied via glycoside pathway in stevia (45-51).

This is the first study to determine the antimicrobial activity of 5 extracts obtained from *S. rebaudiana* that was planted at different doses of fertilizer. The MIC values of the stevia extracts were not found significant differences.

In conclusion, the free radicals are known as the cause of some important disease such as cancer, diabetes mellitus, hypertension etc.. Therefore, quenching of the free radicals produced in the biological systems by natural antioxidants, can extend life expectancy. We have demonstrated that the methanol extract prepared from *S. rebaudiana* leaves contains high levels of phenolic and flavonoid contents (99.19 - 129.01 mg gallic acid equivalents/g and 73.9 - 113.4 mg quercetin equivalents /g respectively) at the dry base of extracts. This indicates that the extract capable of inhibiting free radicals due to the contained a higher amount of total phenolic and flavonoid compounds. In addition, the results demonstrated that fertilization with increasing nitrogen fertilizer can decrease the content of total flavonoid and phenolic compounds in Stevia. At the same time, in the plant extracts which was obtained from *S. rebaudiana* that was planted at different doses of fertilizer were observed antibacterial and antifungal activity. These results are important because it was the first report about screening the *in vitro* antioxidant and antimicrobial activity of the *S. rebaudiana* plants growing in different nitrogen as fertilizer.

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