ARTICLE / INVESTIGACIÓN

Efficacy of Bio-fertilizer and Chemical Fertilization on Flavonoids Distribution in Different Plant Parts of *Stevia rebaudiana* (Bertoni.)

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Abstract: This study aims to investigate the effect of the biological and chemical fertilizers on the content of the flavonoid compounds distributed within the different plant parts (leaves, stems, branches, and roots) of *Stevia rebaudiana* (Bertoni.) grown in Iraq. The results showed that the treatments of the biological fertilizers, including Mycorrhiza (C2) achieved the highest content of the most flavonoids in different parts of the plant. The treatment C2 recorded a rise of the flavonoid compounds Naringin, Naringenin and Luteolin 7-glucose in the leaves, Naringin, Rutin, and Acacetin7-neorutinoside in the stems and branches, and Apiening6-rhamnose8- glucose, Apigenin7-o neohespiroside, Kampferol3-7dirmmoside, Quercetrin, Narengenin, Acacetin7-neorutinoside, Kampferol, and Luteolin 7-glucose in the roots. On the other hand, treatment C1 recorded the highest content of Quercetin in the leaves, Quercetrin3-O glucose in the stems and branches, and Quercetrin3-O glucose, Naringenin, and Acacetin7-neorutinoside in the leaves .

Key words: Flavonoid, Stevia, Mycorrhiza and chemical Fertilization.

Introduction

Flavonoid compounds are among the most significant secondary metabolic compounds naturally produced by plants. They consist of more than 1000 structurally varied compounds[1,2]. Half of the flavonoid compounds are accumulated in the plant vacuoles as a form of aglycone, glycosides, and methylated derivatives. Some flavonoids are released from roots to the aria surrounding the roots, known as rhizospheres. Some flavonoids can release from roots and make biological contact with the microorganism in the rhizospheres and establish a symbiotic relationship with them, such as rhizobia, arbuscular mycorrhizal fungi, and plant growth-promoting rhizobacteria, Pathogens, and nematodes in addition to some other plants. This has been confirmed by finding aglycones and glycosides in the flavonoid compounds, the root extract, and the soil containing these organisms[3-5]. *Stevia rebaudiana* is classified among the perennial herbaceous plants of the Asteraceae family. It is a medicinal and nutritional plant that has gained particular nutrient and healthy importance in terms of nutrition and health for its leaves containing Steviol Glycosides that led to using the plant leaves as calorie-free food additives in Japan, Brazil and Europe[6,7].Containing a group of flavonoid compounds essential for the human body as antioxidants that have a role in protecting against cancer, diabetes and diseases of cardiovascular and kidney, increased the importance of Stevia as a medicinal plant; furthermore, it is considered a fungal and bacterial antibiotic and it is used as a diuretic as well as it has many other benefits[8,9]. Creating biologically activated compounds in plants is affected by various environmental and agricultural factors, including fertilizers such as NPK[10]. Flavonoid contents in plant tissues of plant parts were increased when the chemical fertilizers were used while other research proved the revers[11-15]. 80% of plants can form a symbiotic relationship with the mycorrhiza fungus[16]. Mycorrhiza is one of the bio-fertilizers characterized by the capability to increase the secondary metabolism compounds in plants, and 80% of plants have the ability to form a symbiotic relationship with the mycorrhizal fungus[16,17]. Adding Mycorrhiza to medicinal plant roots changed primary metabolism processes, including photosynthesis, water absorption, and plant tolerance to drought[18]. In addition, the changes in secondary metabolism compounds such as the change in the dynamics of plant hormones and the structural manipulation and activating the defensive mechanism[19].The reason was attributed to the excellent compatibility between the Mycorrhiza and the host plant, As well as the nutritional status of the plant. This increased the plant content of secondary metabolites such as phenols and total flavonoids[20].

Due to the nutritional and medicinal importance of the Stevia plant and the absence of studies about it in Iraq, this research aims to probe the effect of the chemical and biological fertilizers on the content of flavonoid compounds in different parts of the Stevia grown in Iraq.

Materials and methods

The experiment was conducted in the greenhouse at research station B affiliated with the Department of Horti-

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culture and Garden Engineering, College of Agricultural Engineering Sciences, the University of Baghdad during the period from 1/3/2017 to 15/7/2017. The design Randomized Complete Block Design (RCBD) was used to lay out this experiment with three replicates. Each replicate contained 10 plants 6 weeks old, knowing that the seedlings had been produced through the tissue culture and been adapted previously. The experiment treatment included: Control treatment: without applying any fertilizer. C0 symbolizes it. Chemical fertilizer treatment: according to the NPK fertilization recommended[21] where the fertilizer contains the combination of 30 N: 10P: 10K. The treatment was symbolized by C1. Biological fertilizer treatment: the *Glomus mosssaea* was added at 15 g .plant^{-1} in the pits in contact to the seedling roots[22] and 250 g.plant^{-1} of sterilized Peat moss[23]. The Mycorrhizae were obtained from the Ministry of Sciences and Technology, Department of Agricultural Research. It was loaded on the soil at 51 spores. g^{-1} soil. C2 symbolized the treatment. The seedlings were planted on terraces of 50 cm in width in a non-warmed greenhouse covered by polyethylene. The distance between plants from each other was 20 cm[24]. Inside the greenhouse was equipped with a thermometer and a hygrometer, also was equipped with non-woven polypropylene spun bounded (characterized by thickness GSM 17 and UV3%) was placed 2.5 m above the plants to decrease the temperature[25] (Table 1)

Estimate the concentration of the flavonoid compounds in different plant parts(roots, stems and leaves)

The flavonoids concentration was estimated with High-Performance Liquid Chromatography (HPLC) according to (26) included the following steps:

Extraction and separation of flavonoids

When leaves reached the full enlargement stage preceding the flowering and after the last batch of the soil NPK fertilization, samples including the leaves, stems, branches, and roots were collected separately after the first harvest in the evening in order to analyze the flavonoid compounds. The plant parts were dried at the room temperature of 25-30 C° until the weight stabile in a room equipped with an evacuator. The samples were ground using a special industrial grinder and kept in airtight paper bags. The bags were also placed inside sealed zipper freezer bags (type Falcon) for food preservation. Then, they were kept in a refrigerator under 4-5 C°. The sample extraction was prepared according to (26). The standard solutions of the compounds studied were imported from the Sigma- Aldrich Co.

The device conditions

All concentrations of flavonoid compounds in the Stevia plant were assessed at the National Agricultural Research Center laboratories - Food Technology Research Institute / Arab Republic of Egypt. The Chromatography approach

Inside the greenhouse					
Month	dayss	Maximum Temperature C°	Minimum Temperature C°	relative humidity %	Soil Temperature
March	1*	21.5	12.8	70.3	25
	2*	24.5	14.2	68.2	25
	3*	27.5	15.1	62.2	25
April	1*	32.6	16.5	58.9	26
	2	30.9	18.4	59.4	26
	3	31.6	19.9	39.9	26
May	1	33.8	20.9	38.1	26.5
	2	36.1	23.7	35.4	28
	3	35.2	26.1	36.7	27.5
June	1	38.1	27.3	35.1	28
	2	40.1	28.1	33.2	28.5

Table 1. Maximum and minimum temperature (C°) and relative humidity (%) as well as the soil temperature inside the greenhouse. * (1) The average of the first ten days of the month .* (2) The average of the second ten days of the month.* (3) The average of the last ten to eleven days of the month.

was used to estimate the compounds of flavonoids studied . HPLC device (type Agilent, model 1200) was used for determining the retention time and area of the standard and sample solutions. The separation column type C18 (4.5 x 250 mm) was used, and the movable phase acetonitrile: Phosphoric acid was pushed (at a Flow rate 14: 80 ml. min^{-1}). The results were measured at the wavelength of 330 nm and a temperature of 35C°. Next, the measurements of the compounds of the package space of the samples were compared to those the standards to recognize the compounds Apiening6-rhamnose-glucose, Naringin, Rutin, Quercetrin 3-O glucose, Apigenin7-o-neohespiroside, Kampferol3-7dirmmoside, Quercetrin, Quercetin, Naringenin, Acacetin7-neorutinoside, Hespirtine, Kampferol, Apeginen, and Luteolin 7-glucose according to the approach followed by (26).

Statistical analysis

The experiment was designed using the Randomized Complete Block Design (RCBD) with three replicates for each treatment[27]. Genastat software was used to compare the means relying on the L.S.D 5 %.

Results

Effect of the chemical and biological fertilizers on the flavonoid content in Stevia plant leaves (ppm)

Results in Table 2 show non -significant effect of the chemical fertilizer treatment C_1 and the biological fertilizer treatment C_2 compared to the control treatment on the compounds Apiening6-rhamnose8-glucose,Rutin,Quercetrin 3-O glucose, Apigenin 7 glucose, Apigenin7-o- neohespiroside, Kampferol3-7dirmmoside, Acacetin7-neorutinoside, Hespirtine, Kampferol, and Apeginen. The treatment of the biological fertilizer C_2 showed superiority in the leaf content of the flavonoid compound Naringin reached 1669.7 ppm compared to the C_1 and C_0 (1430.5 and 978.6 ppm), respectively. The treatment C_2 was superior in the content of Narengen in the Stevia plant leaves (32.6 ppm) compared to C_0. (13.4 ppm) . The treatment C_2 also showed superiority in the plant leaf content of Luteolin 7-glucose (5670.5 ppm) compared to C_1 and C_0 (1562.3 and 1665.0 ppm, respectively). The treatment C_1 recorded the highest value of Quercetin in the leaves (58.6 ppm) compared to the treatments of C_2 and C_0 (39.77 and 34.84 ppm, respectively).

Effect of bio and chemical fertilizers on the flavonoid compounds content of *S. rebaudiana* (Bertoni.) leaves (ppm) Table 2

Effect of bio and chemical fertilizers on the flavonoid compounds content in the stems and branches of Stevia plants

Results in Table 3 illustrate that the treatments C1 and C2 were non-significant on the content of the compounds Apiening6-rhamnose8-glucose, Apigenin7-o-neohespiroside, Kampferol3-7dirmmoside, Quercetrin, Narengenin, Hespirtine, Kampferol, Apeginen, and Luteolin 7-glucose in the stems and branches of Stevia plant. The treatment of C2 recorded an increase in the Naringin content in the stems

Treatment / Flavonoids compounds content	C0	C1	C2	LSD 5%
Apiening6-rhamnose8- glucose	519.5	561.2	598.5	N.S
Naringin	978.6	1430.5	1669.7	39.91
Rutin	4763.9	5764.1	10153.3	N.S up normal
Quercetrin3-O glucose	-	-	-	-
Apigenin 7 glucose	291.0	439.0	346.3	N.S up normal
Apigenin7-o neohespiroside	386.1	420.0	415.1	N.S up normal
Kampferol3-7dirmmoside	173.2	205.0	145.1	N.S up normal
Quercetrin	743.0	1114.1	1116.2	N.S up normal
Quercetin	34.84	58.63	39.77	6.63
Narengenin	13.4	26.4	32.6	16.49
Acacetin7-neorutinoside	165.1	242.4	268.1	N.S up normal
Hespirtine	66.0	68.1	83.0	N.S up normal
Kampferol	74.0	96.4	108.2	N.S up normal
Apeginen	19.1	32.4	23.9	N.S up normal
Luteolin 7-glucose	1665.0	1562.3	5670.5	483.1

C0:control treatment,C1: chemical fertilizer treatment and C2: Biological fertilizer treatment

Table 2. Effect of bio and chemical fertilizers on the flavonoid compounds content of *S. rebaudiana* (Bertoni.) leaves (ppm)

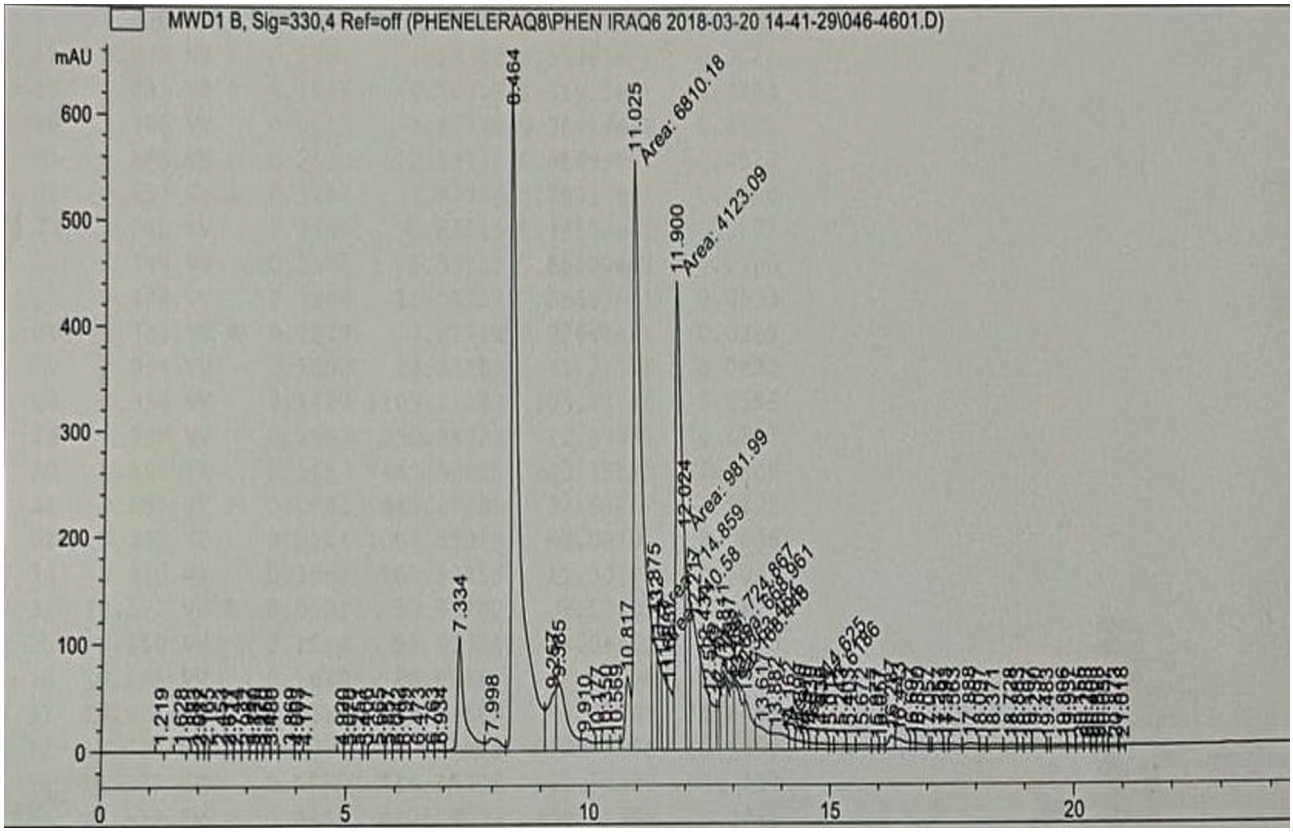

Figure 1. The flavonoid compounds content of *S. rebaudiana* (Bertoni.) leaves in cntrol treatmeant (ppm).

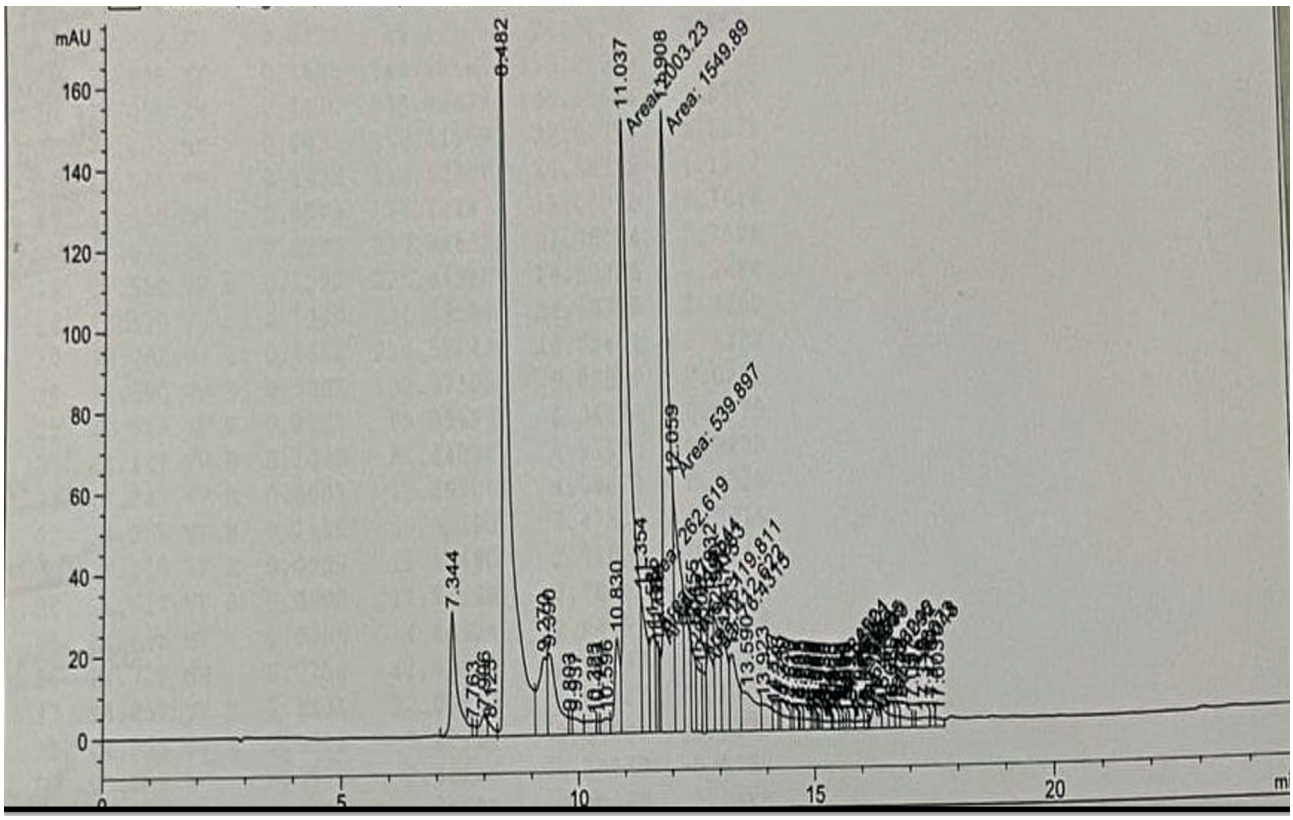

Figure 2. The flavonoid compounds content of *S. rebaudiana* (Bertoni.) leaves in chemical fertilizer treatmeant (ppm).

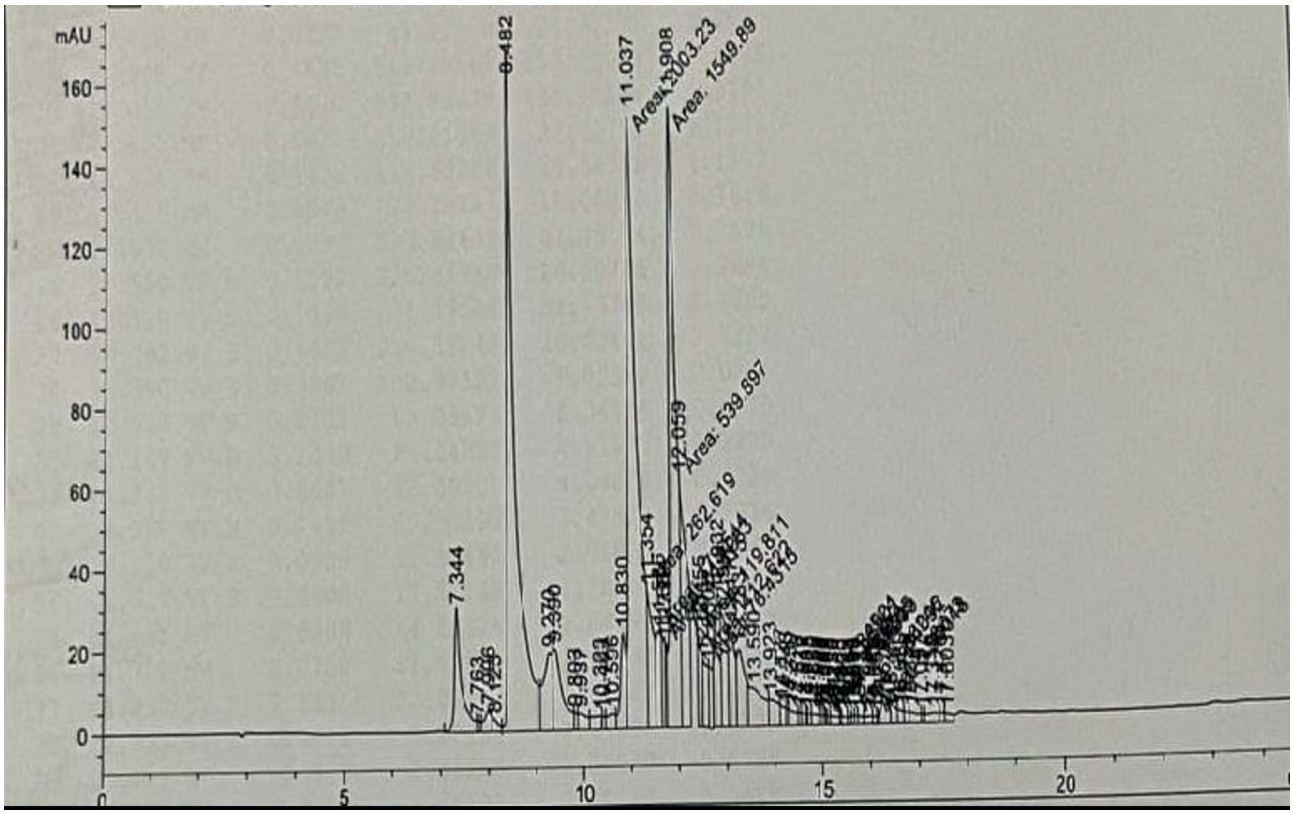

Figure 3. The flavonoid compounds content of *S. rebaudiana* (Bertoni.)leaves in Biological fertilizer treatment fertilizer (ppm).

Treatment / Flavonoids compounds content	C0	C1	C2	LSD 5%
Apiening6-rhamnose8- glucose	184.0	137.4	117.0	N.S up normal
Naringin	320.0	222.2	2175.4	1043.4
Rutin	272.4	611.4	1222.3	629.7
Quercetrin3-O glucose	11.0	18.1	0.0	14.51
Apigenin7-o neohespiroside	43.0	62.5	89.1	N.S up normal
Kampferol3-7dirmmoside	59.0	61.0	99.1	N.S up normal
Quercetrin	40.0	40.1	95.1	N.S up normal
Quercetin	4.6	10.3	8.6	N.S up normal
Narengenin	2.12	2.68	2.82	N.S up normal
Acacetin7-neorutinoside	28.9	33.8	80.3	13.00
Hespirtine	6.5	10.4	10.5	N.S up normal
Kampferol	5.2	5.3	8.2	N.S up normal
Apeginen	1.87	2.83	2.10	N.S up normal
Luteolin 7-glucose	337.1	193.0	239.0	N.S up normal

C0: control treatment, C1 : chemical fertilizer treatment and C2: Biological fertilizer treatment.

Table 3. Effect of bio and chemical fertilizers on the flavonoid content in the stems and branches of *S. rebaudiana* (Bertoni.) (ppm).

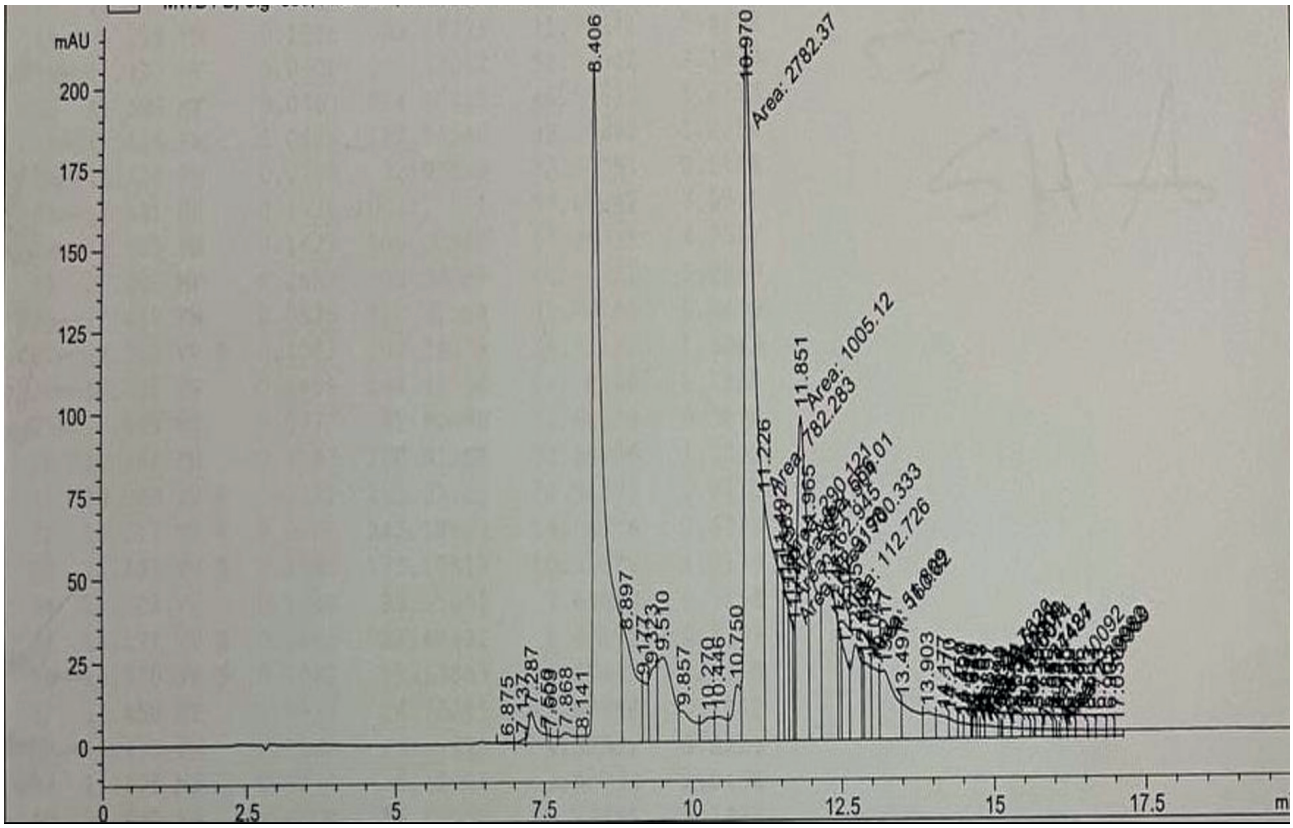

Figure 4. The flavonoid compounds content of *S. rebaudiana* (Bertoni.) stem and branches in cntrol treatmeant (ppm).

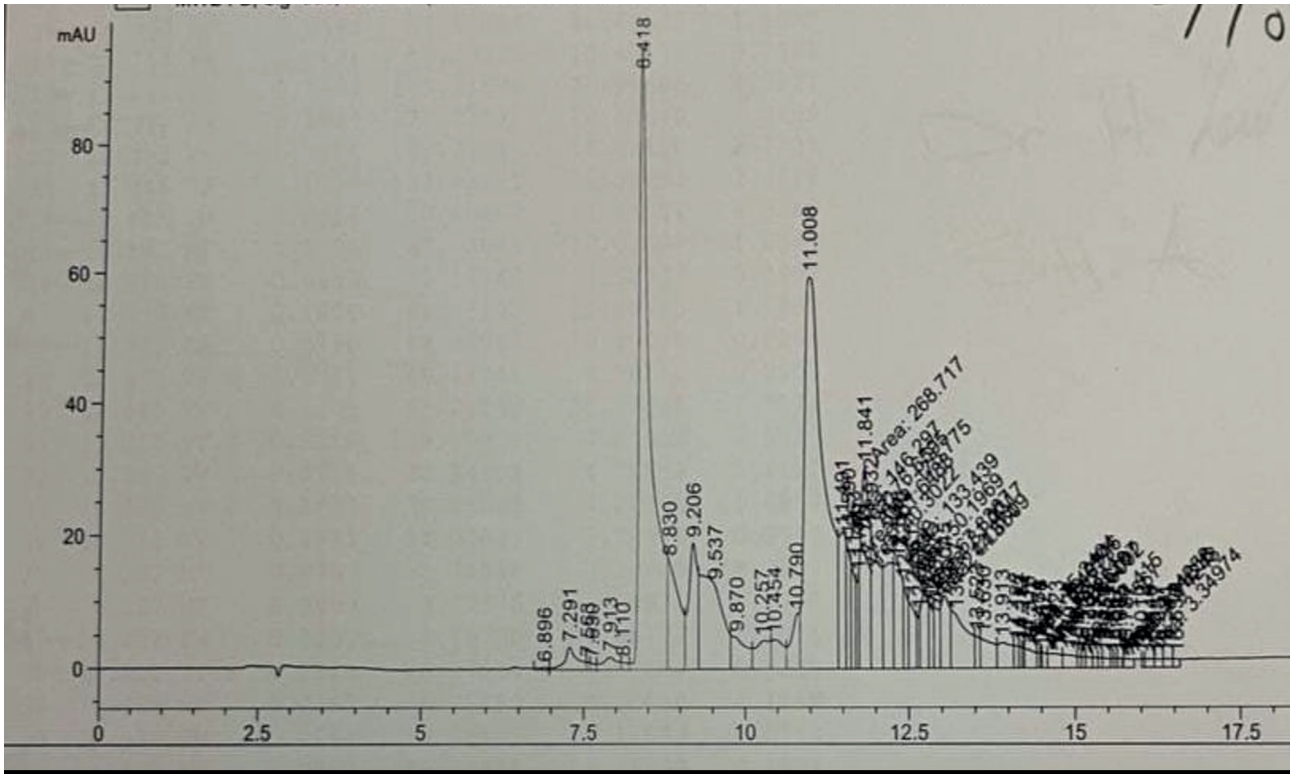

Figure 5. The flavonoid compounds content of *S.rebaudiana*(Bertoni.)stem and branches in chemical fertilizer treatmeant (ppm).

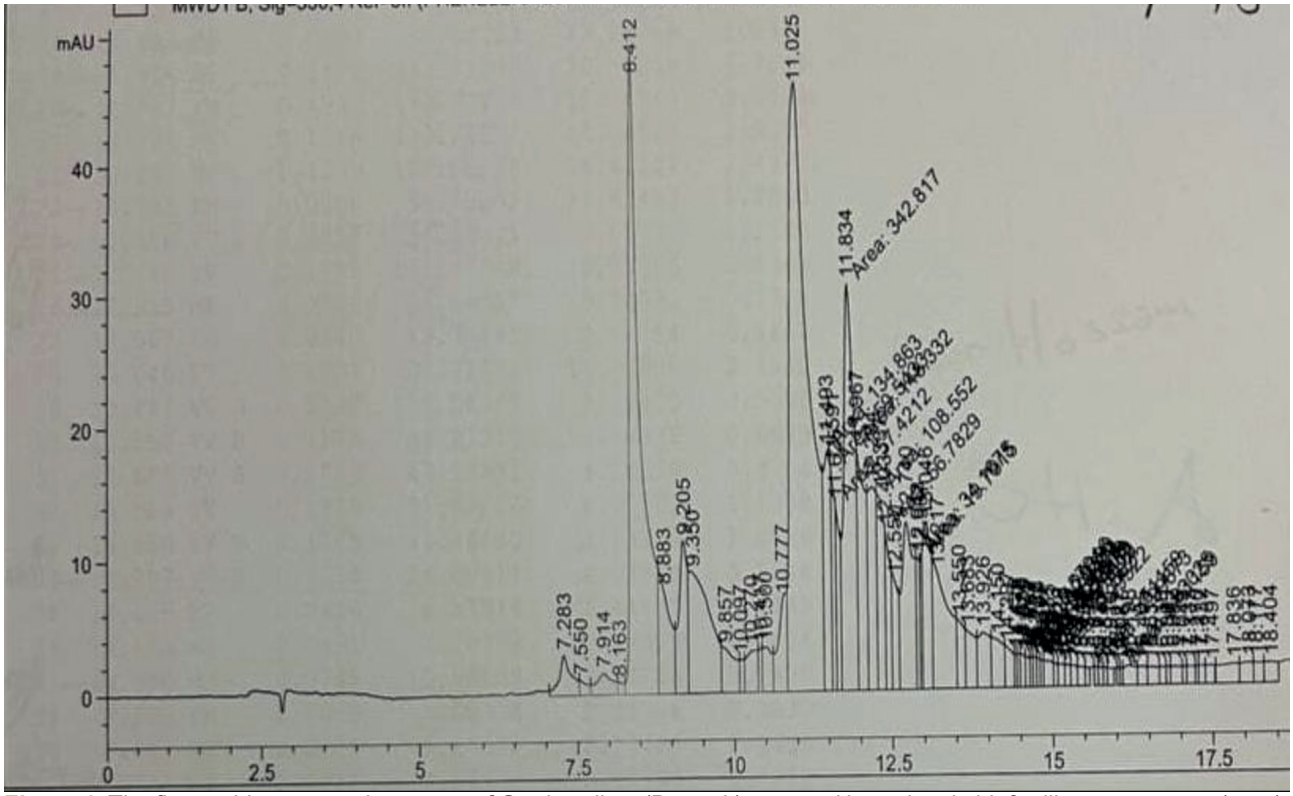

Figure 6. The flavonoid compounds content of *S.rebaudiana*(Bertoni.)stem and branches in biofertilizer treatmeant (ppm).

and branches (2175.4 ppm) compared to the treatments C0 and C1 (320.0 and 222.2 ppm respectively). The treatment C2 also recorded an increase in the Rutin content in the stems and branches (1222.3 ppm) compared to C0 (272.4 ppm). On the other hand, the two treatments, C1 and C0 recorded a noticeable increase of the compound Quercetrin3-O glucose content in the stems and branches to 18.1 and 11.0 ppm respectively. The C2 treatment was superior recording the highest content of Acacetin7-neorutinoside of 80.3 ppm compared to C1 and C0 which recorded 33.8 and 28.9 ppm respectively.

Effect of bio and chemical fertilizers on the flavonoid content in the roots of Stevia plants (ppm)

Results in Table 4 showed a non-insignificant difference among the study treatments in the content of the compounds Naringin, Rutin, Apigenin 7 glucose, Hespirtine, and Apeginen. However, treatment C2 recorded an increase in the content of Apiening6-rhamnose8- glucose compared to C1 and C0 which recorded 251.2 and 146.0 ppm, respectively. The treatment was also superior in the content of Apigenin7-o neohespiroside compared to C0 that recorded 40.9 ppm and recorded an increase in Quercetrin to 88.6 ppm compared to C1 and C0 (44.8 and 43.3 ppm, respectively). The treatment C2 recording 88.6 ppm also increased the Quercetrin content compared to C1 and C0 recording 44.8 and 43.3 ppm, respectively. Moreover, the treatment C2 had the highest in the content of Kampferol recording 15.8 ppm compared to C1 which recorded 6.2 ppm. The treatment C1 was superior in the content of Quercetrin 3-O glucose recording 75.8 ppm compared to C2 and C0 which recorded 30.4 and 3.80 ppm respectively. The superiority of the treatment C2 in the content of Luteolin 7-glucose was observed in comparison to C0 and C1 which recorded 948.2, 497.3, and 478.1 ppm respectively. The two treatments C2 and C1 were superior in the content of Acacetin7-neorutinoside recording 97.3 and 60.3 ppm respectively compared to C0 which recorded (35.0 ppm). Concerning the content of Narengenin, the two treatments C2 and C1 were superior recording 1.67 and 1.66 ppm respectively compared to C0 which recorded 0.71 ppm.

Discussion

The results illustrated in Tables 2, 3, and 4 showed a significant non-effect of the studied treatments on Hespirtine and Apeginen in all plant parts (leaves, stems, branches, and roots). Also the treatments did not significantly affect the content of Apiening6-rhamnose8- glucose, Apigenin 7 glucose, Kampferol3-7dirmmoside, Quercetrin, Hespirtine, Hespirtine, Kampferol, and Apeginen found in the leaves, stems, and branches. Moreover, they did not significantly affect the content of Apigenin 7 glucose and Rutin in the leaves and roots. Further, the content of Quercetrin and Acacetin7- neorutinoside in the leaves, and Quercetin, Naringenin, and Luteolin 7-glucose in the stems and branches were also not significantly affected.

The former results illustrated in Tables 2, 3, and 4 show an insignificant effect of the studied treatments on the content of the compounds this is due to the plant tissue content of the flavonoid compounds in any plant part was not affected by the use of any type of fertilizer[14,15]. Whether they are treated or not by the NPK fertilizers, the medicinal plants' flavonoids in the plant tissues are not affected significantly; as a result, each plant needs a certain critical NPK level for the synthesis and metabolism the flavonoid compounds[10]. Among the flavonoid compounds contained in all Stevia plant parts are Apiening6-rhamnose8- glucose, Apigenin 7 glucose, Kampferol3-7dirmmoside, Quercetrin, Hespirtine, Hespirtine, Kampferol, and Apeginenwhich are not affected significantly by the studied treatments. The treatment of

Treatment / Flavonoids compounds content	C0	C1	C2	LSD 5%
Apiening6- rhamnose8- glucose	146.0	251.2	506.4	N.S **up normal**
Naringin	561.0	563.1	983.1	N.S **up normal**
Rutin	92.0	219.3	247.1	33.26
Quercetrin3- O glucose	3.80	75.8	30.4	N.S **up normal**
Apigenin 7 glucose	15.5	24.1	25.6	18.52
Apigenin7- o neohespiroside	40.9	91.2	113.4	26.50
Kampferol3- 7dirmmoside	30.0	41.4	50.5	26.83
Quercetrin	43.3	44.8	88.6	0.75
Quercetin	3.59	5.50	4.50	0.20
Narengenin	0.71	1.66	1.67	42.14
Acacetin7- neorutinoside	35.0	60.3	97.3	N.S **up normal**
Hespirtine	13.3	13.8	14.1	9.08
Kampferol	12.3	6.2	15.8	N.S **up normal**
Apeginen	2.72	6.34	5.82	167.2
Luteolin 7- glucose	497.3	478.1	948.2	N.S **up normal**

C0:control treatment , C1 :chemical fertilizer treatment and C2: Biological fertilizer treatment

Table 4. Effect of bio and chemical fertilizers on the flavonoid content in the roots of S.rebaudiana(Bertoni.)(ppm).

biological fertilizer also did not show any significant effect on the flavonoids mentioned above which may be due to the type of the biological fertilizer or to the plant part ability extent to synthesize these flavonoid compounds[28]. Results listed in Tables 2-4 show that some flavonoids are found in specific plant parts but not in others of Stevia for instance the compound Quercetrin3-O glucose was not found in the leaves of a plant treated with both fertilizer types, while it was found in the stems and roots although the plants in study were treated with different fertilizer treatment the biological fertilizer (Table 3). Apigenin 7 glucose was not observed in the studied plant stems of all treatments. The results, on the one hand, maybe due to that the distribution of the flavonoid contents formed within plants are various according to the plant part so they may appear in one part but disappear in another; on the other hand, may be due to the effect of the agricultural operations including the fertilization. It indicates a relation between the studied treatments and flavonoids contained in the specific plant part. The reason also may be due to other factors such as the sampling time and rising the temperature during the second and third ten days of June (Table 1). The treatment with Mycorrhiza increased the plant tolerance to the environmental conditions that led to raising the contents of the most flavonoid compositions[29] in addition to the variation in distribution the compounds may be characterized according to the plant species[11-13] concluded that using chemical fertilizers increases the content of the flavonoids in plant parts and some other research papers proved the opposite[14,15].

Conclusions

Using the biofertilizers, Mycorrhiza achieved the highest content of the flavonoid compounds in different plant parts (leaves, stems, branches, and roots) of Stevia followed by using the chemical fertilizers NPK.

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