Extracting Sweetening and Bioactive Compounds From Stevia Rebaudiana Using Cellulase Enzyme

Extracting Sweetening and Bioactive Compounds From Stevia Rebaudiana Using Cellulase Enzyme

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Abstract

This study was conducted to find out the sweetener and bioactive compounds in the *Stevia* plant's enzyme extract and its antioxidant and antibacterial effect. The enzyme extract was used in a ratio of 1:15 (w: v) with the use of sodium phosphate buffer solution (pH = 4) in an equal mixing ratio with the enzyme. Extraction was carried out at 55°C with a time of 25 minutes to extract and determine steviol glycosides using HPLC. Stevioside and Rebaudioside-A (5.101 and 3.027 mg/g) were obtained, respectively. The study showed that *Stevia* contains high amounts of phenols and flavonoids (83.052 and 71,765) mg/ml, respectively. The enzyme extract gave an antioxidant activity of 71.367% compared to BHT which gave an activity of 77.267%. It gave the highest inhibitory activity against *Bacillus subtilis*, which was 14 mm, followed by *Staphylococcus aureus* and *E. coli*. The bioactive compounds were diagnosed by GC-MS and contained important bioactive compounds such as Hydroxydehydrostevic acid (steviol), 1,2-Benzenediol, 4-Ethylcatechol, 9-Octadecenamide, (Z)-, Isosteviol methyl ester, gamma-Sitosterol.

Keywords: Stevioside, Rebaudioside-A, Antioxidant, Antibacterial, HPLC, GC-MS.

1. Introduction

Functional products are in high demand due to their beneficial health effects [1]. Therefore, food manufacturers are constantly interested in replacing synthetic sweeteners with natural sources to supply a segment of consumers that are calorie-conscious, diabetics, and unable to consume calorie-rich sweeteners to meet their requirements [2].
Stevia rebaudiana Bertoni is a natural plant grown in many countries such as Brazil, Paraguay, Central America, Thailand, Korea, China, and India. It has gained increasing industrial and scientific interest in the past 20 years as a suitable nutritional alternative to sucrose and artificial sweeteners [3,4].
Stevia leaves are the source of diterpenes steviol glycosides and the sweetness given to *Stevia* results from them: Stevioside (%4-13), Rebaudioside-A (%2-4), Dulcoside A (%0.4-0.7), Rebaudioside-C (%1-2), and other species less abundant such as Rebaudioside-B, Rebaudioside-F, and Steviolbioside [4]. *Stevia* is a source of active compounds such as (phenols, alkaloids, flavonoids, polysaccharides, amino acids, essential oils, lipids, proteins, free sugars, and minerals) with antimicrobial and antioxidant properties [5-7].
Several studies have shown that *Stevia* extracts have various functional properties such as anti-hyperglycemic, anti-hypertensive, anti-diarrheal, anti-tumor, anti-inflammatory, and anti-immune properties [8]. Therefore, there is no effect on public health when steviol glycosides are used in moderation and many international organizations have determined that consumption of high-quality *Stevia* products within the recommended doses is safe for every one [9].
Innovative methods for extracting high-value compounds from *Stevia* leaves have been developed and improved. In contrast to conventional alternatives, innovative extraction methods allow for higher yields in a shorter time, and lower use of organic solvents [10]. Including Enzyme-assistance extraction, as enzymes are ideal catalysts to assist extract the bioactive compounds. Various enzymes such as cellulases, pectinases, and hemicellulase are often required to degrade the cell walls and plant membranes, thus enabling the bioactive substances inside the cells easier to extract from plants, as it degrades the components of the cell wall it increases the permeability of the cell wall and thus leads to an increase in the productivity of the extraction from compounds [11,12]. This study aims to diagnosis the sweetener and bioactive compounds and measures the antioxidant and antibacterial activity of the enzymatic extract.

2. Materials and Methods

2.1.Stevia plant leaves

The *Stevia* plant (*Stevia rebaudiana* Bretoni) was obtained from Jannat Al-Nakheel for Tissue Culture, Baghdad, Iraq.

2.2. Enzymatic extraction

Cellulase enzyme from *Trichoderma reesei*, CMC ≥300,000u/ g was used to extract *Stevia* leaves according to the method of [13]. The enzyme was prepared with a weight of 1 g/unit in 100 ml of water and extracted at a ratio of 1:15 (plant: buffer) with the use of a buffer solution of sodium phosphate (pH = 4) at an equal mixing ratio with the enzyme. The extraction was carried out at 55°C with a time of 25 minutes. They were placed in a vibrating water bath and the extract was filtered using a Whatman No.4, then the filtration process was carried out using 0.22 mm filters and stored at refrigerator temperatures until use.

2.3.Determination of steviol glycosides by High-performance liquid chromatography (HPLC)

Analysis of Stevioside and Rebaudioside-A in the enzymatic extract was performed using (HPLC) according to the method [14]. The standard Stevioside and Rebaudioside-A were prepared in a solution of 1 mg / mL of methanol using five concentrations (0.1-0.3 mg / mL). The device used from a company Shimadzu, Analysis Column Type (NUCLEODUR C18 Gravity, 5 µm, Germany), the operating conditions were a mobile phase consisting of water: acetonitrile with a ratio of 20:80 (v / v), a wavelength of 210 nm, and a flow rate of 0.7 mL/min.

2.4. Determination of Total Phenol Content

The amount of phenolic compounds in the enzymatic extract was determined using the Folin–Ciocalteu method, as described by [15]. Briefly, 0.5 ml of the extract was mixed with 2 ml of 20% sodium carbonate solution, 1.5 ml of Folin's reagent was added and the volume was supplemented with distilled water to 5 ml. It was incubated at 55 °C for 1.5 hours and the absorbance was measured at a wavelength of 760 nm using a spectrophotometer. Gallic acid was used to establish the graphic relationship between the acid concentration and the absorbance at a wavelength of 760 nm and the concentrations ranged between (0-100 mg/ml).

2.5.Determination of Total Flavonoid Content

The amount of flavonoids in the enzymatic extract was estimated following the method reported by [16]. Briefly, 1.5 ml of the extract was mixed with 1.5 ml of aluminum chloride at a concentration of 2% dissolved in 100 ml of methanol. The mixture was shaken and incubated for 10 minutes, then it was measured at a wavelength of 367 nm and rutin compound was used at concentrations (0-100 mg/ml), to calculate the amount of flavonoids by the relationship between acid concentration and absorbance.

2.6. DPPH Radical Scavenging Activity

The antioxidant activity was estimated using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) method, prepared by dissolving 0.005 g in 100 ml methanol, according to the method mentioned in [17], 60 µl of the enzyme extract were taken and 3 ml of DPPH solution were added to it, mixed well and incubated at room temperature for 20-30°C, then the sample was measured at a wavelength of 517 nm and the control sample was prepared by adding water instead of the extract. Butylated Hydroxy Toluene (BHT) was used for comparison, and the effectiveness was calculated from the following equation:

$$\text{DPPH scavenging} = (AD - AS / AD) \times 100\%$$

* AD: absorbance of DPPH and AS: absorbance of the samples.

2.7. Antibacterial activity

The Agar Well diffusion method was followed by [18], the three isolates, *Staphylococcus aureus*, *Bacillus subtilis*, and *Escherichia coli* were activated, then the tubes were incubated for 24 hours at 37 °C, Nutrient Agar culture medium was added in Petri dishes, and left to solidify for several minutes, 0.1 ml of the activated bacterial suspension was taken and spread on the surface of the N.A culture medium was mediated by L-shape. Then, drills were made using a cork borer with a diameter of 6 mm on the solid culture media, and 75 µl of the enzymatic extract at a concentration of 100 mg/ml was placed

in the holes. For comparison, pits containing control were made. The dishes were incubated at 37°C for 24 hours, after which the inhibition diameters were measured to determine the ability of the extract to inhibit bacteria.

2.8. Gas chromatography-mass spectrometry (GC-MS) analysis

The bioactive compounds in the enzymatic extract were diagnosed using a GC-MS device (Shimadzu GC-MS 2010, Gas Chromatography-Mass Spectrometry), Equipped with a type separation shaft (Rtx-5MS capillary column (cross bond 5% diphenyl-95% dimethylpolysiloxane), 30 m(L) × 0.32 mm (i.d.) with a 0.25 µm film thickness), The used gas was helium at 99.99% purity, A-type mass spectrometer (Electron Impact Ionization (E.I.); recorded in intervals from 50 to 800 m/z), And under the following operating conditions: The sample volume upon injection 1 µl, injection temperature 280 °C, mass spectrometer temperature 200 °C, the column temperature starting from 50 °C and stabilizing for 5 minutes, then increasing by 50 °C until it reaches 100 °C, then increases by 9 °C until it reaches 280 °C, to settle there. The results are processed with GC-MS solutions and the separated peaks were applied to the spectra database of the program library NIST08. LIB.

3. Results and Discussion

3.1. Diagnosis of steviol glycosides in the enzymatic extract

Figure (1) and Table (1) showed the effect of Cellulase enzyme and sodium phosphate buffer solution (pH= 4), ratio 1:15 (leaves: water), the temperature of 55 °C and a time of 25 min on the concentration of Stevioside and Rebaudioside-A in the enzymatic extract. The two compounds Stevioside and Rebaudioside-A were determined in the enzymatic extract by comparing the retention time with the two standard compounds.

It appears that the use of equal amounts of the enzyme and buffer solution (pH = 4) gave a concentration ratio of (5.101 and 3.027) mg/g of Stevioside and Rebaudioside-A, respectively. Thus, the addition of the enzyme improved Rebaudioside-A, which gives a sweet taste compared to Stevioside, which gives a bitter taste in *Stevia* [4]. This addition has benefited the *Stevia* plant, some companies are seeking *Stevia* production due to commercial demand to improve the *Stevia* yield from Rebaudioside-A and reduce Stevioside in the *Stevia* plant by the method of enzymatic conversion from Stevioside to Rebaudioside-A through pre-treatment of *Stevia* leaves using Cellulase, and confirmed that the conversion led to an increase in Rebaudioside-A yield [19-21].

These results are in agreement with [22]. who mentioned that steviol glycosides are stable in acidic and neutral conditions, but not in the basic conditions. The best time for extraction was 25 min, which agrees with [13]. He studied three types of enzymes and found that the increase in the extraction times did not lead to an increase in the yield. It agrees with the latter regarding the effect of temperature at 55 °C, when using the Cellulase enzyme, the yield improved at 50 °C. However, the increase in temperature led to the disruption of the enzyme's work, because the enzymes had good activity at warm temperatures, but they were inactivated at a high temperature. Much of the sweetening compounds present in *Stevia* are extracted to the enzyme's action at the optimum temperature.

In general, the reason for the ability of the Cellulase enzyme to improve the yield of steviol glycosides is due to its ability to degrade polysaccharides present in the cell wall such as xyloglucans and heteroxylans. Thus disrupting or degrading cell walls and plant membranes, which facilitates the access of solvents to the active compounds inside the cells, thus leading to increased extraction productivity of the bioactive compounds [13].

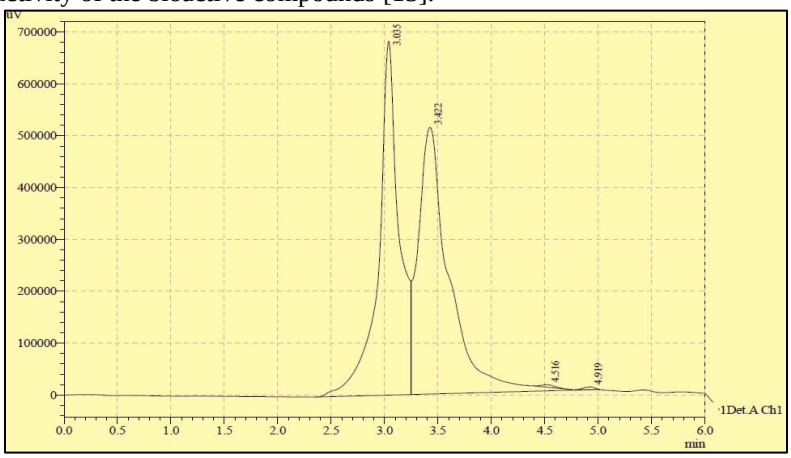

Figure 1. HPLC chromatogram of Stevioside and Rebaudioside-A in the enzymatic extract.

Table 1. Retention time and concentration of Stevioside and Rebaudioside-A in enzymatic extract.

Peak	compound	Retention time (minute)	Area	Area%
1	Rebaudioside-A	3.035	9209215	47.032
2	Stevioside	3.422	10293259	52.568
3	/	4.516	41540	0.212
4	/	4.919	36846	0.188
	Total		19580859	100.000

3.2. Phenols and flavonoids

The enzyme extract gave high amounts of phenols 83.052 mg/ml. In comparison, the amount of flavonoids was 71.765 mg/ml. This may be because enzymes are ideal catalysts to assist in the extraction of bioactive compounds, enzymes work to disrupting cell walls and plant membranes. It makes the bioactive substances easier to extract from plants and lead to an increase in the productivity of extraction from compounds [11,12]. Previously, the *Stevia* plant is a good source of phenolic compounds, and mainly its high leads to stronger antioxidant activity, so *Stevia* is a rich source of natural antioxidants.

3.3.DPPH Radical Scavenging Activity

The enzymatic extract showed the ability to scavenge free radicals using DPPH, compared to BHT. The results showed that the enzymatic extract gave 71.367% antioxidant activity, which is a good percentage compared to BHT, which gave 77.267%. The reason for the effectiveness of the extract may due to the high content of phenolic compounds responsible for ending the reaction of free radicals because they contain hydroxyl groups capable of giving electrons to the free radical and converting it to more stable products. This is what [23], showed in his study of the relationship between a high content of phenols and an increase in antioxidants. It agreed with the results of the quantitative determination of the phenolic content, as the extract has high concentrations of phenols.
The antioxidant activity of *Stevia* leaf extracts is due to the scavenging of free radicals [24]. In his study of five different medicinal plants with different solvents, [25], found the highest antioxidant activity in *Stevia* and for three types of solvents.

3.4. Antibacterial activity

The enzymatic extract gave inhibitory activity against some types of bacteria, the diameter of inhibition was 14 mm against *B. subtilis* bacteria, followed by *S. aureus* with an inhibition diameter of 11 mm, and the lowest was *E. coli* with a diameter of 9 mm. The antibacterial activity of the extracts is attributed to flavonoids, phenols, aromatic compounds, and terpenes [26]. Found [27], that the secondary metabolites of *Stevia* had antimicrobial activity against the types of bacteria studied. The presence of Stevioside and Rebaudioside-A compounds prevents the growth of many bacteria [28].

3.5. Diagnosis of the bioactive compounds in the enzymatic extract

Figure (2) and Table (2) show the bioactive chemical compounds present in the enzymatic extract diagnosed by the GC-MS device. The results showed that the extract contained 37 bioactive compounds with their ratios, the most important of which was Hydroxydehydrostevic acid (Steviol) with a percentage of 53.96%, followed by the active phenolic compound 1,2-Benzenediol with a percentage of 9.84%, which is considered one of the compounds with a health benefit, the enzymatic extract also contained 4-Ethylcatechol (2.46%), 9-Octadecenamide, (Z)- (Oleamide) (3.05%), Isosteviol methyl ester (1.37%) and gamma-Sitosterol common in many plants by 1.62%.
Therefore, based on the presence of plant bioactive compounds, it can be said that *Stevia* contains a group of primary and secondary compounds that belong to different groups. This tends to justify its medicinal properties and its application in both the food and pharmaceutical industries.

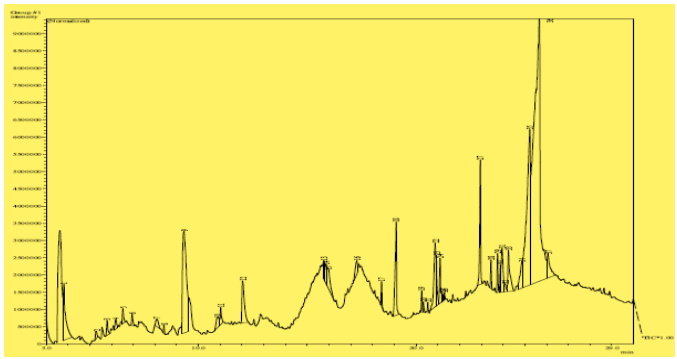

Figure 2. Chromatogram of bioactive compounds in the enzymatic extract.

Table 2. Retention time and concentration of Stevia bioactive compounds in the enzymatic extract.

Peak	R.Time	Area	Area%	Name
1	3.818	12956667	3.39	2-Furanmethanol
2	5.306	1084242	0.28	1,2-Cyclopentanedione
3	5.801	1750006	0.46	2,4-Dihydroxy-2,5-dimethyl-3(2H)-furan-3-one
4	6.194	1421776	0.37	1H-Pyrazole, 1-vinyl-
5	6.525	1824835	0.48	2-Hexenoic acid, 2-methyl-
6	6.950	1265124	0.33	2,5-Dimethyl-4-hydroxy-3(2H)-furanone
7	8.090	1856361	0.49	4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-
8	8.412	636174	0.17	Methyl salicylate
9	9.336	37613612	9.84	1,2-Benzenediol
10	10.858	3064675	0.80	3-n-Butylthiolane
11	11.024	1989776	0.52	Eugenol
12	12.026	9414912	2.46	4-Ethylcatechol
13	15.758	2273972	0.59	Cyclopropanecarboxylic acid, 2,2-dimethyl-3-(2-methyl-1-propenyl)-, 2-methyl-4-oxo-3-
14	15.875	3135524	0.82	D-Allose
15	15.992	5223553	1.37	1-Naphthalenol, decahydro-4a-methyl-
16	17.276	3106568	0.81	2,3-Bis(1-methylallyl)pyrrolidine
17	18.417	1620138	0.42	Hexadecanoic acid, methyl ester
18	19.091	13011058	3.40	n-Hexadecanoic acid
19	20.263	1470338	0.38	cis-13-Octadecenoic acid, methyl ester
20	20.334	921151	0.24	cis-13-Octadecenoic acid, methyl ester
21	20.542	915366	0.24	Octadecanoic acid, methyl ester
22	20.881	11309619	2.96	17-Octadecynoic acid
23	20.933	4220415	1.10	trans-13-Octadecenoic acid
24	21.106	4487105	1.17	Octadecanoic acid
25	21.192	674247	0.18	Benzene, (1-methyl-1-propylpentyl)-
26	21.305	524689	0.14	Nopyl acetate
27	22.956	11649982	3.05	9-Octadecenamide, (Z)-
28	23.445	2234824	0.58	Isosteviol methyl ester
29	23.748	3011860	0.79	Isosteviol methyl ester
30	23.877	2412610	0.63	Kaur-16-en-18-oic acid, 13-hydroxy-, methyl ester, (4.alpha.)-(.+/-.)-
31	23.952	7476696	1.96	3,3a-Epoxydicyclopenta[a,d]cyclooctan-4.beta.-ol, 9,10a-dimethyl-6-methylene-3.beta.-i
32	24.100	921670	0.24	Tetradecanedioic acid
33	24.248	7825428	2.05	Hexadecanoic acid, 2,3-dihydroxypropyl ester, (.+/-.)-
34	24.867	6183488	1.62	gamma.-Sitosterol
35	25.230	55027814	14.39	Hydroxydehydrostevic acid
36	25.669	151318006	39.57	Hydroxydehydrostevic acid
37	26.075	6533577	1.71	Octadecanoic acid, 2,3-dihydroxypropyl ester
Total		382367858	100.00	

Conclusions

The study concluded that the enzymatic extract of the *Stevia* plant has a high ability to enhance functional products because of its health and nutritional benefits and because it contains important active compounds.

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