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The role of amino acids and polyamines in increasing the stevioside of the stevia plant (*Stevia rebaudiana* Bertoni) in vitro

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Abstract:

This study was conducted in the Plant Tissue Culture Laboratory at the College of Agriculture, Diyala University, for the period 2022-2023. It aims to identify the role of amino acids and polyamines in increasing the stevioside compound of the stevia plant *in vitro.* The histological and field experiments included the use of the amino acids glutamine and arginine, the polyamine putyrcine at three different concentrations, in addition to the control treatment, which is 0, 50, 100, and 150 mg.L^{-1}. The results showed that putyrcine gave the highest plant height of 12.6 cm. The arginine at a concentration of 50 mg. L^{-1} and putyrcine at 150 mg. L^{-1} gave the highest number of branches reached 2.2 branches. $Plant^{-1}$. The arginine treatment at 50 mg.L^{-1} gave the highest wet weight of 0.483 grams, and the treatment at 150 mg.L^{-1} gave the highest sweetening for all amino acids used. It was the highest for putyrcine, which reached 251.6 micrograms ml, and the lowest sweetening for the control treatment and for all treatments. The highest average content of stevioside in the leaves of the stevia plant was when the plants were sprayed in the field with putyrcine at a concentration of 150 mg.L^{-1}, reaching 462.473 µg ml-1. All experiments were carried out in a completely randomized design (CRD)

Keywords: stevia , stevioside , amino acids , poly amin ,glutamin , arginine , putyrcine

دور الأحماض الأمينية والبولي أمينات في زيادة نسبة الستيفيوسيد في نبات الستيفيا (Stevia rebaudiana Bertoni) في المختبر

علي طالب ابراهيم و هبة احمد جواد

كلية الزراعة – جامعة ديالى / العراق

الخلاصة

أجريت هذه الدراسة في مختبر زراعة الأنسجة النباتية في كلية الزراعة جامعة ديالى للمدة 2022-2023. ويهدف إلى التعرف على دور الأحماض الأمينية والبولي أمينات في زيادة مركب الستيفيوسيد لنبات الستيفيا في المختبر. شملت التجارب النسيجية والحقلية استخدام الأحماض الأمينية الجلوتامين والأرجينين والبولي أمين بوتيرسين بثلاثة تراكيز مختلفة، بالإضافة إلى معاملة المقارنة وهي 0، 50، 100، 150 ملغم.لتر-1. أظهرت النتائج أن البوتيرسين أعطى أعلى ارتفاع للنبات بلغ 12.6 سم. الأرجينين بتركيز 50 ملغ. لتر-1 و بوتيرسين بجرعة 150 ملغ. L-1 أعطى أعلى عدد من الفروع بلغ 2.2 فرع. النبات-1. أعطت معاملة الأرجينين بـ 50 ملغم.لتر-1 أعلى وزن رطب بلغ 0.483 جرام، والمعاملة بـ 150 ملغم.لتر-1 أعطت أعلى نسبة تحلية لجميع الأحماض الأمينية المستخدمة. وكانت أعلى نسبة للبيوتيرسين حيث بلغت 251.6 ميكروجرام مل، وأقل نسبة تحلية لمعاملة السيطرة ولجميع المعاملات. أعلى متوسط لمحتوى الستيفيوسيد في أوراق نبات الستيفيا كان عند رش النباتات في الحقل بالبيوتيرسين بتركيز 150 ملغم.لتر-1 حيث وصل إلى 462.473 ميكروغرام مل-1. أجريت جميع التجارب وفق التصميم العشوائي الكامل (CRD)

الكلمات المفتاحية: ستيفيا، ستيفيوسيد، أحماض أمينية، بولي أمين، جلوتامين، أرجينين، بوتيرسين .

Introduction

The sugar bush plant of the Stevia genus is a sweet herb, which belongs to the Asteraceae family. Its scientific name is *Stevia rebaudiana* Bertoni. It is native to the regions of South America (Paraguay and Brazil). It is known for its sweet taste and is considered to be a vital, natural, calorie-free sweetener that provides a solution to obesity problems in humans [1] [2]. Stevia includes 200 species and the most widely used species are Stevia eupatoria, S.Ovata, S. plummerae, S. salicifollia, S. serrata, and S. rebaudiana [3] [4][5]. At the present, it has become necessary to increase the quantities of secondary metabolites, as they are an effective medicine and an added source of food, in addition to their important role in adapting plants to the environment Plant cell and tissue culture techniques have been used to produce and increase plant secondary metabolites since the late 1960s, This may lead to improved stress, and selection High-production cell lines and the use of factors affecting their production [6]. Many studies have proven that amino acids play a positive role in enhancing plant yield and quality They contribute to reducing the effect of stress resulting from drought and salinity through various physiological activities by changing the osmotic capacity of plant tissues They also significantly reduce injuries caused by biotic stresses. They stimulate physiological and biochemical processes and participate in protein and carbohydrate synthesis amino acids were also believed to be responsible for cell division and the production of some natural growth hormones such as IAA and GA3, thus increasing yield and improving quality [7]. Polyamines are aliphatic amines found in all living cells and are essential for many basic cell activities. Their protective role against various abiotic stressors has been characterized in different plant species, while the mechanism by which polyamines act during plant-microbial interactions is still not well understood [8]. Polyamines cannot be considered as growth regulators because advanced and primitive plants produce them within their tissues and do not dispense with them in their life cycle. They are biosynthesized from amino acids known and identified in the plant and are necessary for cell growth and development [9].

Materials and methods

Experiments were conducted in the tissue culture laboratory in the Department of Horticulture and Landscape Engineering of the College of Agriculture, Diyala University, for the period from 1/9/2022 to 1/6/2023. The experiments were conducted on the stevia plant, Stevia rebandiana bertone. Seedlings of the Spanty variety were brought. The mother plant was taken from the National Palm Garden Company in Baghdad, three months old. The medium was prepared using the ready-made nutrient medium, weighing 4.9 $gm.L^{-1}$, according to the recommendation of the producing company (HIMEADIA), with 30 $gm.L^{-1}$ sucrose and 7 $gm.L^{-1}$ agar. 600 ml of nonionic distilled water was taken, sucrose and MS were added, and mixed the solution well, then the pH was adjusted to 5.7 using sodium hydroxide [10]. After that, the volume was increased to 1000 ml, the agar was added, and it was heated until it boiled in order to dissolve the agar and distribute it in the culture bottles whose capacity is 350 ml with dimensions of 14 cm x 7 cm, designed for tissue culture with heat-resistant and light-permeable covers after preparing hormone-free nutrient media and distributing them in the designated glassware. They were sterilized with an autoclave at a temperature of 121° and a pressure of 1.04 $kg.cm^2$ for 15 minutes. All experiments were conducted under sterilization conditions, where all tools, including scalpels and tweezers, were sterilized after wrapping them in heat-resistant aluminum foil in an autoclave at a temperature of 121°C and a pressure of 1.04 kg cm^2 for 30 minutes. 99% of ethyl alcohol was used to burn the scalpels and tweezers when used inside the hood (Laminar air flow cabinet) and UV sterilization [11].

Tissue Culture experiments

Experiment with the amino acid glutamine:

The MS nutrient medium was prepared with concentrations of glutamate (0, 50, 100 and 150) $mg.L^{-1}$ in ten replicates. The plants were incubated for four weeks in the growth room under a light intensity of 1000 lux for an illumination period of 16/8 light/dark, and at a temperature of 24 ± 1 to study its effect on plant multiplication in terms of plant height, number of branches, leaves, nodes, roots, root length, wet weight, dry weight, and stevioside concentration.

Experiment with the amino acid arginine:

The MS nutrient medium was prepared with concentrations of arginine 0, 50, 100 and 150 $mg.L^{-1}$ in ten replicates. The plants were incubated for four weeks in the growth room under a light intensity of 1000 lux for an illumination period of 16/8 light/dark, and at a temperature of 24 ± 1 to study its effect on plant multiplication in terms of plant height, number of branches, leaves, nodes, roots, root length, wet weight, dry weight, and stevioside concentration.

Polyamine butyrosine experiment:

The MS nutrient medium was prepared with concentrations of putyrcine 0, 50, 100 and 150 $mg.L^{-1}$ in ten replicates. The plants were incubated for four weeks in the growth room under a light intensity of 1000 lux for an illumination period of 16/8 light/dark, and at a temperature of 24 ± 1 to study its effect on plant multiplication in terms of plant height, number of branches, leaves, nodes, roots, root length, wet weight, dry weight, and stevioside concentration.

Field experiments:

Stevia seedlings resulting from tissue culture and acclimatization were taken. The seedlings were two months old after acclimatization and were homogeneous in growth characteristics. The plant was sprayed with glutamic, arginine, and putyrcine at three concentrations of each, with the control treatment sprayed with distilled water (0, 50, 100 and 150) $mg.L^{-1}$ at a rate of five replicates for each treatment. The spraying was from the first day on 3/1/2023, and for three sprays, with five sprays between one spray and the next. Ten days [12]. Thereafter, some physical characteristics (plant height, number of branches, number of leaves) and some chemical characteristics (chlorophyll concentration and stevioside concentration in the leaves) were measured fifteen days after the last spraying.

Estimation of the stevioside content of leaves in plant tissues grown *in vitro*:

This work was conducted in the Environment and Water Laboratory at the Ministry of Science and Technology, and according to the method of [13] in extracting and estimating glycoside compounds (Stevioside) in the leaves. Samples were taken from the field trial plants 15 days after the third spraying, and from the plants grown in the laboratory histologically after 4 weeks from planting in the middle. The samples were dried at 65 degrees for 48 hours until their weight was stable before grinding them with a ceramic mortar. 1.0 g of the sample was taken and 10 ml of methanol was added to it, in a 100 ml glass bottle. The sample was tightly closed and placed in an ultrasonic device in a water bath for ten minutes. Then the mixture was concentrated to a volume of 0.5 ml and 0.5 ml of the mobile phase mixture was added to it. The samples were filtered and were ready for estimation in the HPLC device. After that, the mixture was taken and filtered through a micro filter, and 10 microliters were taken from each sample. Thus, the samples were ready for estimation in the HPLC device (High Performance Liquid Chromatography). 100 microliters were injected and the retention time of the standard solution was determined in terms of the separation conditions as shown in Table 1.

Table 1: Separation conditions used in the determination of stevioside

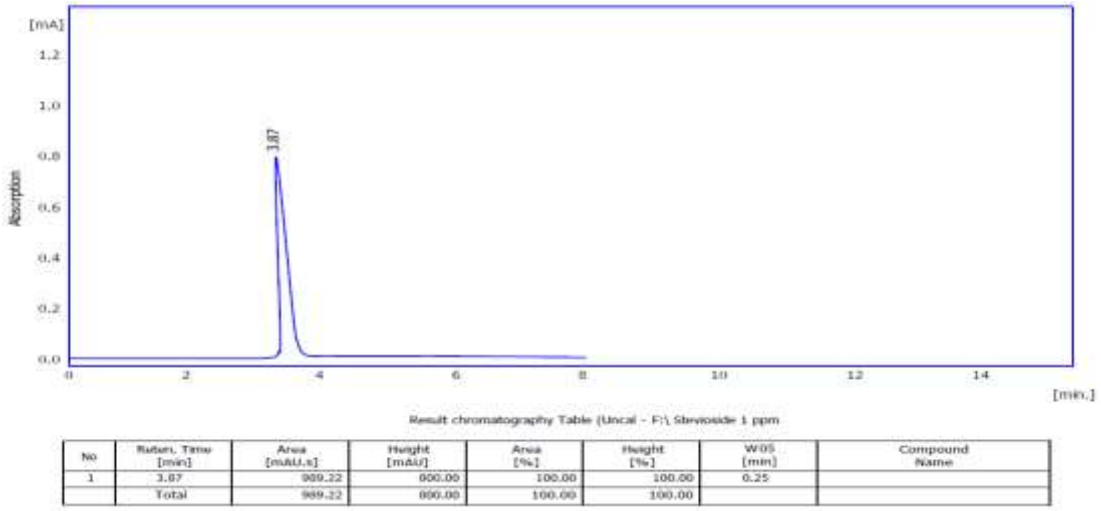
Separation conditions

Mobile phase	Acetonitril :water adjusted to pH 2.6 with orthophosphoric acid
Separation temperature	30 C°
Volume of sample injected	100 ul
Mobile phase flow speed	0.8 ml / min
Detector type	Detector UV set 210 nm
Separation column	Zorbax C18 ,250mm X4.6 mm ID,3.5Mm
Company and model	Sykam – Germany

Preparing samples of the standard compound for stevioside:

Samples of the standard compound were prepared by adding 4.7 mg of Stevioside compound in 50 ml of methanol. It was considered a control sample for the extracts of the different samples. The standard solution was injected and the retention time and area peak height of the sample were determined for the standard solution (Figure 1). Then the separation conditions were determined by fixing the appropriate mobile phase, stationary phase, flow rate, and temperature, as shown in Table 1. The samples were injected for each sample under study in the same conditions. The concentration of each substance was calculated according to the equation:

Sample concentration in sample (µg ml-1) = (standard sample concentration × sample packet area) ÷ number of dilutions [14]



Picture 1: Standard solution curve of stevioside separated by HPLC

3-4 The experimental design used:

All experiments were carried out in a completely randomized design (CRD), with 10 replications of each treatment for the laboratory experiments and 5 replications for the field experiments. The data were analyzed using the ready-made statistical program SPSS, and the means were compared according to the Duncan multiple test at the probability level of 0.05 [15].

Results and discussion

Experiment with amino acids *in vitro*

Effect of treating nutrient medium with different concentrations of glutamine, arginine, and putyrcine on the height of stevia plants

As it can be seen in Table 2 that the highest average plant height was when treating the nutrient medium with a concentration of 50 mg.L^{-1} putyrcine, as it reached 12.60 cm. The case which did not differ significantly from the control treatment and the 100 and 150 mg.L^{-1} with arginine. On the other hand, the lowest plant height was when the medium was treated with a concentration of 150 mg.L^{-1} glutamine, which reached 3.92 cm.

Plant height was significantly affected when different concentrations of glutamine and putyrcine were added. However, it was not significantly affected when different concentrations of arginine were added.

Table 2: Effect of amino acids on growth of stevia plants *in vitro* after four weeks of cultivation.

concentrations (mg L^{-1})	Glutamine	Arginine	Putyrcine
0	9.80A	9.80A	9.80A
50	4.06C	8.0 A	12.60A
100	6.88B	9.0A	10.00B
150	3.92C	9.2 A	10.00B

* Means with similar letters are not significantly different from each other (P≤ 0.05) according to Duncan's multinomial test.

The effect of treating the nutrient medium with different concentrations of glutamine, arginine, and putyrcine on the number of branches of stevia plants *in vitro* after four weeks of cultivation.

obvious from Table 3 that there are significant differences between the treatments. The two treatments, 50 mg.L^{-1} arginine and 150 mg.L^{-1} butyrosine, gave the highest average number of branches, amounting to 2.2 branches plant^{-1} . By contrast, the control treatment gave the lowest number of branches, amounting to 1.4 branches plant^{-1}. The means of this trait were not significantly affected when different concentrations of glutamine were added. However, they were significantly affected when different concentrations of glutamine were added, and they were significantly affected when the medium was treated with different concentrations of arginine.

Table 3: Effect of amino acids on the number of branches (plant branch^{-1}) of the stevia plant *in vitro* after four weeks of cultivation.

concentrations (mg.L^{-1})	Glutamine	Arginine	Putyrcine
0	1.4A	1.4 B	1.4B
50	2.0A	2.2 A	1.2B
100	1.8A	1.8 AB	1.8AB
150	1.6A	1.4 B	2.2 A

* Means with similar letters are not significantly different from each other (P≤ 0.05) according to Duncan's multinomial test.

The effect of treating the nutrient medium with different concentrations of glutamine, arginine, and putyrcine on the number of leaves (leaf plant^{-1}) of the stevia plant *in vitro*.

From Table 4, it appears that the highest number of leaves was in the control treatment, which amounted to 17.6 leaves plant^{-1}, while the treatment with 150 mg.L $^{-1}$ of glutamine gave the lowest average for this trait, which amounted to 11.6 leaves $^{-1}$. On the other hand, the means of this characteristics were not significantly affected when treated the medium with different concentrations of arginine. Whereas, putyrcine decreased significantly when the medium was treated with 150 mg L^{-1} glutamine.

Table 4: Effect of amino acids on the number of leaves (plant leaf^{-1}) of stevia *in vitro*.

concentrations (mg L^{-1})	Glutamine	Arginine	Putyrcine
0	17.6 A	17.6 A	17.6 A
50	14.4 AB	18.8 A	14.0 A
100	12.8 AB	16.4 A	14.8 A
150	11.6 B	13.6 A	16.8 A

* Means with similar letters are not significantly different from each other (P≤ 0.05) according to Duncan's multinomial test.

Effect of different concentrations of glutamine, arginine, and putyrcine on the wet weight (g) of stevia *in vitro* after four weeks of cultivation.

Table 5 shows that there are significant differences between wet weights, as the arginine treatment at 50 mg.L^{-1} gave the highest wet weight of 0.483 g, However, it did not differ significantly from the rest of the treatments except for the arginine treatment in the control treatment, which gave the lowest wet weight of 0.338 g.

Table 5: Effect of amino acids on wet weight (g) of stevia *in vitro*

concentrations (mg L^{-1})	Glutamine	Arginine	Putyrcine
0	0.375 AB	0.388 BC	0.376 A
50	0.251 B	0.483 A	0.347 A
100	0.430 A	0.471 AB	0.458 A
150	0.355 AB	0.381 C	0.497 A

* Means with similar letters are not significantly different from each other (P≤ 0.05) according to Duncan's multinomial test.

The effect of different concentrations of glutamine, arginine, and putyrcine in increasing the sweetener compound stevioside (µg ml^{-1}) *in vitro*.

The results of Table 6 show that there are significant differences between the means of the treatments in the content of stevioside in the leaves. Plants grown in different concentrations of amino acids excelled, and the concentration of 150 mg ml^{-1} gave the highest sweetening of all amino acids, and it was the highest for putyrcine, which reached 251.6 micrograms ml^{-1} and less desalination in control treatment.

Table 6: Effect of treating stevia with amino acids at different concentrations in increasing the stevioside

concentrations (mg L^{-1})	Glutamine	Arginine	Putyrcine
0	**102.6 D**	**102.6 D**	**102.6 D**
50	**122.3 C**	**116.6 C**	**135.0 C**
100	**157.9 B**	**142.3 B**	**205.3 B**
150	**227.0 A**	**176.6 A**	**251.6 A**

Plants that were treated with glutamine, arginine, and butyrecine were sprayed on the leaves in the field, as shown in Table 5. The treatment exceeded 150 mg L-1 for each of glutamine, arginine, and butyrecine, and the comparison treatment for each of them also gave the lowest average content of stevioside in the leaves.

The results of Table 6 and Table 7 show that the highest average content of stevioside in the leaves of the stevia plant was when spraying the plants with pyotrcine at a concentration of 150 mg L-1 in the field, where it reached 462 and 473 micrograms mL-1. The comparison treatment for Stevia plants propagated ex vivo gave the lowest average content in the leaves. Of stevioside blv 102.600 micrograms ml-

Table 7: The effect of spraying stevia with amino acids at different concentrations of each in increasing the sweetening compound stevioside µg ml^{-1} in the field.

concentrations (mg L^{-1})	Glutamine	Arginine	Putyrcine
0	**178.1 D**	**178.1 D**	**178.1 D**
50	**236.2 C**	**207.1 C**	**259.5 C**
100	**338.0 B**	**297.0 B**	**385.0 B**
150	**402.0 A**	**350.2 A**	**462.4 A**

This increase in the averages of some vegetative characteristics and the increase in the plant's content of stevioside can be attributed to the role played by amino acids accumulated in plants in regulating the transport of ions, modifying the opening and closing of stomata, and removing toxins resulting from heavy metals. They also affect the synthesis and activity of some enzymes, gene expression, and the balance of reactions, oxidation and reduction [16]. For amino acids are considered to be biostimulants that provide the plant with energy to compensate for losses resulting from respiration and decomposition processes [17].

Amino acids are well-known biostimulants that have positive effects on plant growth and significantly reduce abiotic stresses. Their addition led to a significant increase in chlorophyll A and B in the leaves, significantly improving the growth rates of shoots and the fresh weight of the plant. Moreover, they led to a significant increase in total nitrogen, phosphorus and potassium in plant leaves, as well as total yield, dry weight, TSS, vitamin C, and total sugar content compared to the control treatment [18].

Providing polyamines, whether through external addition or through genetic engineering, can positively affect the growth and productivity of medicinal plants. However, these effects depend on

the type and quantity of the polyamine added and the plant being treated. In addition, polyamines play a precursor to many groups of alkaloids (pyrrolizidine, tropane and quinolizidine alkaloids) and phenolamides. Therefore, these bioactive compounds can significantly increase the concentration of the above-mentioned natural products [19].

Putrescin affects the modification of metabolic pathways in the roots, as the results of previous studies revealed that putrescine affects the oxidative state of cells by increasing the levels of hydrogen peroxide (H2O2) and malondialdehyde (MDA), and activating the enzymes superoxide dismutase (SOD), catalase (CAT) and peroxidase (POD). Besides, nitric oxide (NO) content increased at the beginning of putrescine treatment. Metabolomics results indicate that treatment with putrescine shifts energy and metabolic fluxes by altering the biosynthesis of carbohydrates and amino acids, towards the production of phenols [20].

The results of the analysis with the HPLC device showed that the stevioside compound reached its maximum in field plants as a function of the package area and detention time. This is due to the fact that field plants have specialized tissues and a higher ability to produce secondary metabolic compounds in larger quantities [21]. These results are consistent with [22] findings. The reason for the increase in the percentage of stevioside in field plants may be attributed to their hormone content and field conditions, which gave optimal growth and led to an increase in relatively high levels of the stevioside compound [23].

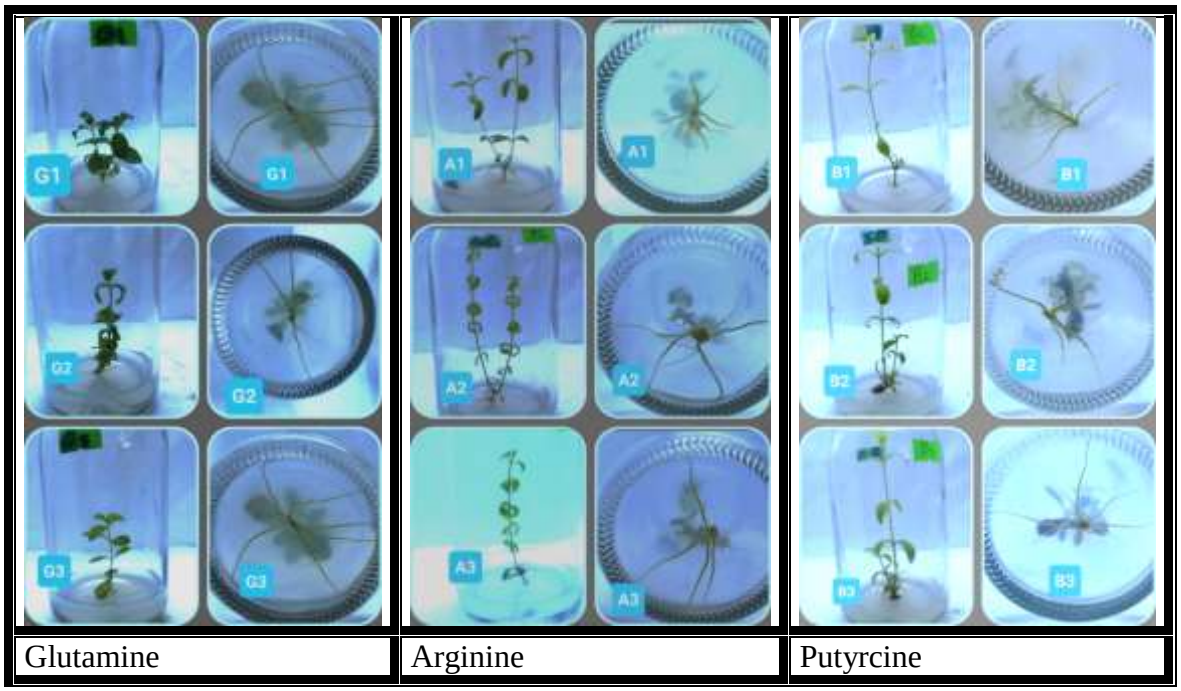


Figure 2: The effect of amino acids and polyamines on the stevia plant in vitro

Conclusions:

The use of amino acids and polyamines in other concentrations and other compounds, with the exception of the stevia leaf content of stevia, improves other physical and chemical treatment.

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