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Isomiddin BOBAYEV
*Tashkent Institute of Chemical Technology, Tashkent, Uzbekistan*, bobaev-isom@mail.ru

Asaloy JUMANIYOZOVA
*Tashkent Institute of Chemical Technology, Tashkent, Uzbekistan*, asaljumaniyozova29@gmail.com

Nozima KARIMOVA
*Tashkent Institute of Chemical Technology, Tashkent, Uzbekistan*, lobarduos@gmail.com

Anvar NORMATOV
*Tashkent Institute of Chemical Technology, Tashkent, Uzbekistan*, anvar8184@mail.ru

Namuna ALIYEVA
*Kokon Pedagogical Institute, Kokon, Uzbekistan*, aliyeva.namuna@mail.ru



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# PREPARATION OF STEVIA EXTRACT USED IN THE FOOD INDUSTRY AND ITS ANTIMICROBIAL ACTIVITY

***Isomiddin BOBAYEV[1] (bobaev-isom@mail.ru)***
***Asaloy JUMANIYOZOVA[1] (asaljumaniyozova29@gmail.com)***
***Nozima KARIMOVA[1] (lobarduos@gmail.com)***
***Anvar NORMATOV[1] (anvar8184@mail.ru)***
***Namuna ALIYEVA[2] (naleyiva@mail.ru )***
***[1]Tashkent Institute of Chemical Technology, Tashkent, Uzbekistan***
***[2]Kokon Pedagogical Institute, Kokon, Uzbekistan***

*A dry extract of the leaves of stevia, belonging to the Asteraceae family, grown in Uzbekistan, was extracted with hot water for use as a sweet flavor additive in the food industry. The content of the extract was determined by thin layer chromatography. The extract turned out to be a mixture of various steviol glycosides. It has been established that the main part of the mass of the extract is steviol glycosides - stevioside, rebaudioside A, rebaudioside C, rebaudioside B. When studying the biological activity of the resulting composition, its antimicrobial activity was determined.*

**Keywords:** stevia, extract, sweet flavor additive, steviol glycosides, stevioside, rebaudioside, thin layer chromatography, antimicrobial activity

# ПРИГОТОВЛЕНИЕ ЭКСТРАКТА СТЕВИИ, ИСПОЛЬЗУЕМОГО В ПИЩЕВОЙ ПРОМЫШЛЕННОСТИ, И ЕГО АНТИМИКРОБНАЯ АКТИВНОСТЬ

***Исомиддин БОБАЕВ[1] (bobaev-isom@mail.ru)***
***Асалой ДЖУМАНИЁЗОВА[1] (asaljumaniyozova29@gmail.com)***
***Нозима КАРИМОВА[1] (lobarduos@gmail.com)***
***Анвар НОРМАТОВ[1] (anvar8184@mail.ru)***
***Намуна АЛИЕВА[2] (naleyiva@mail.ru )***
***[1]Ташкентский химико-технологический институт, Ташкент, Узбекистан***
***[2]Коконский педагогический институт, Кокон, Узбекистан***

*Сухой экстракт листьев стевии, принадлежащей к семейству сложноцветных, выращиваемой в Узбекистане, экстрагировали горячей водой для использования в качестве сладкой вкусоароматической добавки в пищевой промышленности,. Содержание экстракта определяли методом тонкослойной хроматографии. Экстракт оказался смесью различных гликозидов стевиола. Установлено, что основную часть массы экстракта составляют гликозиды стевиола - стевиозид, ребаудиозид А, ребаудиозид С, ребаудиозид В. При изучении биологической активности полученной композиции была определена ее антимикробная активность.*

**Ключевые слова:** гидрогенизация, стевия, экстракт, сладкая вкусоароматическая добавка, стевиоловые гликозиды, стевиозид, ребаудиозид, тонкослойная хроматография, антимикробная активность

# OZIQ-OVQAT SANOATIDA QO'LLANILADIGAN STEVIA EKSTRAKTINI OLISH VA UNING MIKROBLARGA QARSHI FAOLLIGI

***Isomiddin BOBAYEV[1] (bobaev-isom@mail.ru)***
***Asaloy JUMANIYOZOVA[1] (asaljumaniyozova29@gmail.com)***
***Nozima KARIMOVA[1] (lobarduos@gmail.com)***
***Anvar NORMATOV[1] (anvar8184@mail.ru)***
***Namuna ALIYEVA[2] (naleyiva@mail.ru)***
***[1]Toshkent kimyo-texnologiya instituti, Toshkent, O'zbekiston***
***[2]Qo'qon pedagogika instituti, Qo'qon, O'zbekiston***

*O'zbekistonda yetishtiriladigan oziq-ovqat sanotida shirin ta'm beruvchi qo'shimcha sifatida qo'llash uchun Asteraceae oilasiga mansub stevia o'simligining barglarini issiq suv yordamida ekstraktsiya qilib quruq ekstrakti ajratib olindi. Ekstrakt tarkibini tahlil qilish uchun yupqa qatlamli xromatografiya usuli yordamida ekstrakt tarkibi tekshirildi. Ekstrakt turli xil steviol glikozidlari aralashmasidan iborat ekanligi aniqlandi. Ekstrakt tarkibidagi steviol glikozidlar - steviozid, rebaudiozid A, rebaudiozid C, rebaudiozid B massani asosiy qismini tashkil etishi aniqlandi. Olingan yig'ma tarkibi biologik faolligi o'rganilganda mikroblarga qarshi faolligi aniqlandi.*

**Kalit so'zlar:** gidrogsteviya, ekstraktlar, shirin ta'm beruvchi qo'shimcha, steviol glikozidlar, steviozid, rebaudiozid, yupqa qatlamli xromatografiya, mikroblarga qarshi faollik

**DOI: 10.34920/cce2023310**

## Introduction

*Stevia Rebaudiana* Bertoni - is a perennial plant belonging to the Asteraceae family, which grows mainly in Paraguay and Brazil. Currently, the leaves of the *Stevia rebaudiana* plant have been used in the food industry since ancient times due to their sweet taste [1].

There are currently about 200 species of Stevia plants, but only one of them, Stevia rebaudiana, contains steviol glycosides. The results of studies on steviol glycosides contained in *Stevia rebaudiana* showed that steviol glycoside - stevioside is found in all above-ground parts of the plant, but stevioside is 6-15% (relative to the obtained dry mass) in the leaves of the plant. There is very little in the stem [2]. Stevioside is registered in the food industry as a sweet flavoring E960 food additive.

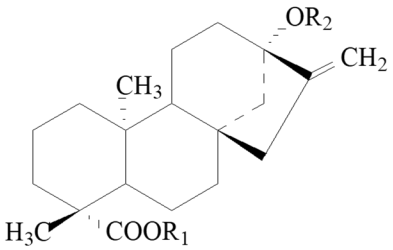
Steviol glycosides are useful as sweeteners for people with impaired carbohydrate metabolism and especially for people with diabetes because they have hypoglycemic properties [3, 4].

Stevia has been used in the food industry for over 40 years [5]. Highly purified steviol glycosides began to be widely used by the public after they were recognized as generally safe in the United States in 2008. Also, on November 11, 2011, the European Food Safety Authority approved the use of purified steviol glycosides in food products equivalent to 4 mg of steviol per kilogram of human body weight per day [6]. Extracts from the Stevia plant are used directly in the form of powders and tablets to produce various sweeteners. The consumption of stevia has increased significantly in the production of pickled vegetables, dried seafood, soy sauces and meat products [7, 8]. In addition, stevia leaves and steviol glycosides are used in the production of carbonated and non-carbonated soft drinks, teas, yogurts, pastries and ice cream. They are also a sweet flavor additive in toothpastes, mouthwashes, and chewing gums [6, 9-12].

The growing demand for natural ingredients with sweetening and antioxidant properties is the main reason why the stevia market is growing in popularity. Increasing use of stevia in the pharmaceutical industry is also fueling its growth. According to data, the stevia market was valued at $490.1 million in 2017, and will reach $771.5 million by 2022 [13]. Stevia leaf extract, powder, stevioside and rebaudioside A of various degrees of purity, depending on the type of product and its characteristics, the international prices of sweeteners vary from 10 to 150 US dollars per kg [14]. China is currently the world's largest producer and supplier of stevia sweeteners, but the industry has seen significant growth worldwide in recent years.

The biochemical composition of stevia leaves, rich in natural components, has been found to have beneficial effects on human health [15]. Some of the beneficial effects of stevia consumption have been reported in a number of scientific articles.

Stevia extract has been shown to have beneficial effects on blood glucose levels [12, 16 - 21]. A review article reported that steviol glycosides contained in stevia extract control the production of insulin and glucagon hormones in the blood [22], have a hypoglycemic effect by reducing the release of glucagon and increasing the release of glucose in the urine.




To date, more than 40 steviol glycosides have been identified from the composition of the stevia plant, which differ only in the number and type of monosaccharides attached to the aglycone steviol ring in the R1 and R2 states. At the C-13 and C-19 positions, the monosaccharides are glucose, rhamnose, xylose, fructose, and deoxyglucose through the 1,2-oxygen; 1,3-; 1,4- or 1,6-α- or β-glycosidic bonds are attached [27-29].

The extraction of steviol glycosides was shown to be significantly less in hexane, chloroform, and ethyl acetate fractions, and 60.3% in the isobutanol fraction. Methanol, ethanol, and ethyl acetate extracts from stevia plant leaves were more rich in phenolic compounds with antioxidant activity than the water extract. According to research data, organic compounds and mineral elements obtained from stevia leaves constitute the main composition [10].

**Research methods**

The leaf of the plant "Stevia - *Stevia rebaudiana* Bertoni (Asteraceae family)" grown in Uzbekistan was used as a source for obtaining the sweetened purified dry extract in laboratory conditions. Stevia plant leaf grown in Uzbekistan was used as a source to obtain the sweetened purified dry extract in laboratory conditions.

Extraction using water. To obtain stevia dry extract samples, 2.0 kg of the above-ground part of the plant was crushed (fineness level 0.3-0.5 cm) and extracted with distilled water (in a ratio of 1:10) at 70-80 °C (repeated 5 times).

When the composition of the aqueous extract was analyzed by the YUQX (Thin-Layer xpomatography) method, it was found that it consists of a small amount of diterpene glycosides (stevioside, rebaudioside A, rebaudioside B, rebaudioside C, etc.) along with water-soluble phenolic

compounds, monosaccharides and other additives. .

The condensed extract was dried to a powder in a desiccator vacuum cabinet and a 1:1 ratio of dis. when diluted in water and treated with organic solvents - chloroform, n-butanol, fractions with the following mass were obtained.

Butanol fraction. After washing the aqueous extract with chloroform and ethyl acetate, it was washed with n-butanol. As a result, a collection of sweet-tasting compounds consisting of 7.55% of dry stevol glycosides in relation to the raw mass was isolated.

Fraction with chloroform - 19.7 g (0.985%);

Ethyl acetate fraction - 20.5 g (1.05%);

Butanol fraction - 150 g (7.55%).

Fertigplatten Kieselgel 60 (Merck) plates were used for thin-layer chromatography, with a suitable chloroform:methanol:water (60:30:6) system selected. A 20x10 cm long plate was placed in the chamber until it rose to the front line in the system and was removed from the chamber and dried at room temperature. A 50% aqueous solution of H2SO4 was used to remove spots on the plate.

Stevioside Rf = 0.44, water solubility 0.13%, rebaudioside A Rf = 0.40, water solubility 0.80%, rebaudioside B Rf= 0.52, water solubility 0.10%, rebaudioside C Rf=0.17, water solubility is 0.80%.

In conclusion, the aqueous extract of the Stevia plant leaf was obtained, for chemical analysis 19.7 g (0.985%) with chloroform, 20.5 g (1.05%) with ethyl acetate, 150 g (7, 55%) were allocated.

Analytical method in high performance liquid chromatography.

High-performance liquid chromatography "Agilent 1200", complete with Degasser G1379A degasser, QuatPump G1311A pump, ALS G1313A autosampler, Colcom G1316A thermostatic column, data processing Agilent ChemStation Rev system with refractometric RID G1362Ai detector developed in USA B.01.03. SupelcosilLC-NH2 5micron 4.6x250 mm "Supelco" column. "VWR" Poland 100 and 1000 µl micropipette. 5 ml pipette, "Biohit" Finland. AnD GR-202 (accuracy of 0.00001 g) analytical balance, "AnD" Japan. Millipore Simplicity, a water deionizer from Millipore, France. C 30 H Elmasonic "Elma" German ultrasonic bath. 0.45 micron 13 mm nylon filter. Standards Fructose, Glucose, Sucrose, Maltose (Sigma-aldrich USA). Acetonitrile for YuSSX "Sigma-aldrich" USA.

A method for determining the antimicrobial activity of aqueous, alcoholic extract and chloroform fraction isolated from stevia leaves against typovoy strains of conditionally pathogenic microorganisms. Antimicrobial activity of the oil isolated from chestnut seeds against typical strains of conditionally pathogenic microorganisms was determined according to the methodical manual OFS.1.2.4.0010.15 "Method for determination of antimicrobial activity by agar diffusion method".

Test microorganisms: conditional pathogenic strains of Escherichia coli NS 101, Pseudomonas aeruginosa 003841/114, Proteus mirabilis 6, Bacillus subtilis VKM, Klebsiella oxitoca 1, Staphylococcus aureus D2, Candida albicans were used as test microorganisms.

The cups were kept at +4 $^{o}$C for 24 hours so that the extracts and fractions were well absorbed into the agar. Then it was grown at a temperature of 36±1 °C for 16-18 hours. After 18 hours, using a ruler, the diameter of the zones where the growth of test microorganisms was stopped was measured and recorded in a notebook.

**Results and Discussion**

When the composition of the aqueous extract was analyzed by the YUQX method, it was determined by the YUSSX method that, along with water-soluble monosaccharides, phenolic compounds and other additives, steviol glycosides consist of stevioside, rebaudioside A, rebaudioside B, and rebaudioside C.

When performing high performance liquid chromatography analysis, the standard solution was prepared by accurately weighing it during development. To ensure long-term storage, the standard solution is mixed with acetonitrile in a ratio of 1/1. Working solutions of standards were prepared by diluting the standard solution with a 1/1 water-acetonitrile mixture.

During the development of the method, the analysis conditions were determined: - isocritical elution mode, the composition of the mobile phase is acetonitrile/water in volume 82/18, two separate vessels without mixing. The composition

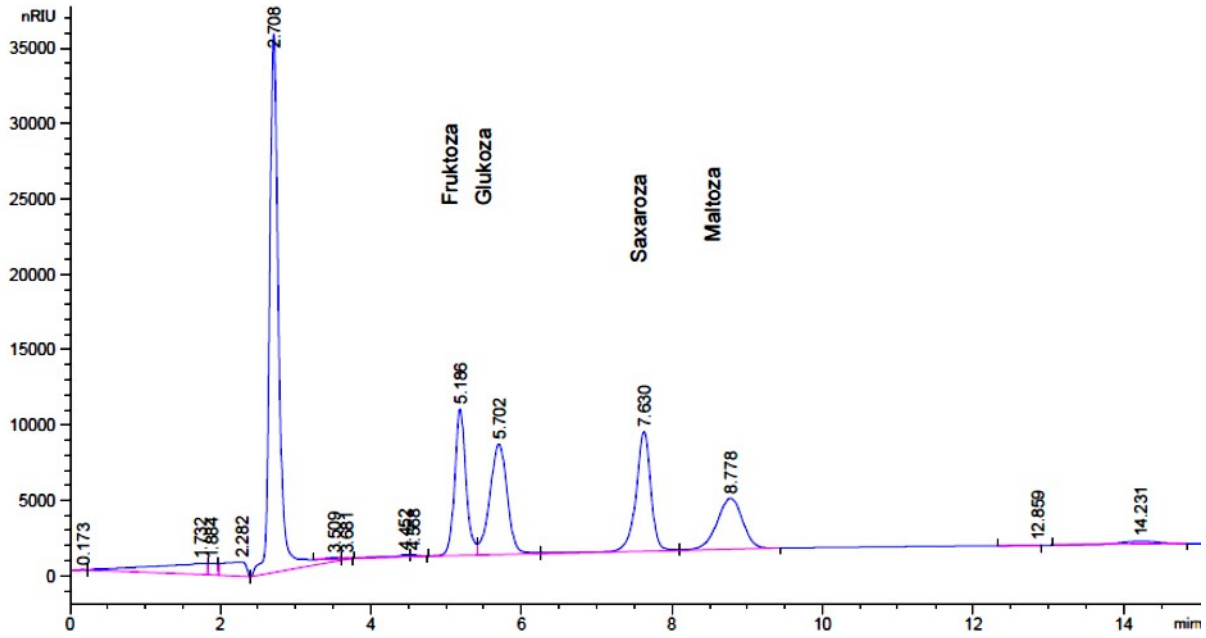



**Figure 1. Derived diagram of standard monosaccharides YuSSX.**

of the mobile phase can be varied to achieve complete separation of glucose and fructose peaks. Volume speed of the washer is 1.0 ml/min.; injection volume 10 µl; the temperature of the column thermostat is 35 °C; retention times of standards: fructose – 5.18±0.2 min, glucose – 5.70±0.2 min, sucrose – 7.63±0.2 min, maltose – 8.77±0.2 min. The detector signal of each carbohydrate content was determined in 0.1 concentration range. 10.0 mg/ml at 6 points (corresponding to 10-1000 g/kg extract), repeated 3 times for each point (Table 1).

In the analysis of the aqueous extract of the

**Table 1**

**Indicators of standard monosaccharides obtained in YuSSX**

| Peak# | RetTime [min] | Type | Width [min] | Field [nRIU*s] | Height [nRIU] | Field % |
|---|---|---|---|---|---|---|
| 1 | 0.173 | BB | 0.1200 | 297.81693 | 39.66695 | 0.0378 |
| 2 | 1.732 | BV | 0.6254 | 3.79169e4 | 754.19446 | 4.8171 |
| 3 | 1.884 | VV | 0.1161 | 6407.14844 | 772.96533 | 0.8140 |
| 4 | 2.282 | VB | 0.2821 | 2.00485e4 | 936.66089 | 2.5470 |
| 5 | 2.708 | BV R | 0.1271 | 3.03288e5 | 3.57517e4 | 38.5304 |
| 6 | 3.509 | VV E | 0.2457 | 1022.83710 | 65.19008 | 0.1299 |
| 7 | 3.681 | VB X | 0.0811 | 258.67029 | 48.93864 | 0.0329 |
| 8 | 4.452 | BV | 0.2055 | 1713.75867 | 111.33444 | 0.2177 |
| 9 | 4.568 | VB | 0.1057 | 578.38495 | 80.18887 | 0.0735 |
| 10 | 5.186 | BV | 0.1583 | 1.01248e5 | 9715.90625 | 12.8628 |
| 11 | 5.702 | VV | 0.2507 | 1.18126e5 | 7328.91797 | 15.0071 |
| 12 | 7.630 | VV | 0.2092 | 1.09680e5 | 7912.44922 | 13.9340 |
| 13 | 8.778 | VB | 0.3647 | 8.02477e4 | 3390.02026 | 10.1949 |
| 14 | 12.859 | BB | 0.4043 | 117.37947 | 3.49894 | 0.0149 |
| 15 | 14.231 | BV R | 0.4455 | 6187.49658 | 182.37737 | 0.7861 |

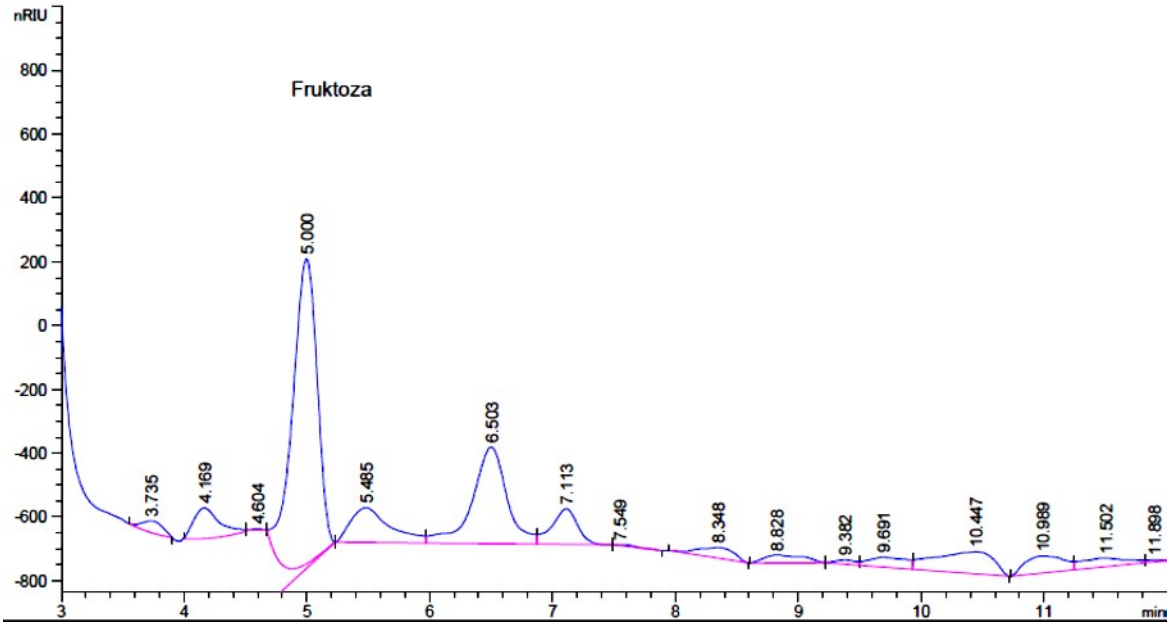



**Figure 2. Schematic diagram of aqueous extract of stevia leaf.**

stevia plant, the solutions were prepared as follows: 1 g of the studied (exactly weighed) dried extract of stevia was dissolved in 50 ml of deionized water. The solution is mixed in an ultrasonic bath at room temperature in the "mixing" mode until the extract is completely homogeneous. Then the solution is filtered on a membrane filter with a diameter of 0.45 microns. The filtrate was mixed with acetonitrile in a ratio of 1/1 and analyzed using YuSSX (Fig. 2).

Volume speed of the washer is 1.0 ml/min.; injection volume 10 µl; the temperature of the column thermostat is 35 °C; retention times of standards: fructose – 5.00±0.2 min, glucose – 5.48±0.2 min, sucrose – 7.11±0.2 min, maltose – 8.34±0.2 min. The detector signal of each carbohydrate content was determined in 0.1 concentration range. 10.0 mg/ml at 6 points (corresponding to 10-1000 g/kg extract), repeated 3 times for each point (Table 2).

Antimicrobial activity of aqueous, alcoholic extract and chloroform fraction isolated from stevia leaves against typovoy strains of conditionally pathogenic microorganisms.

During the experiment, the antimicrobial activity of the studied substances - the aqueous, ethanolic extract of stevia leaves, and the chloroform fraction showed a moderate level of antimicrobial activity against all 7 tested microorganisms. During the experiment, the diameter of the zone of stopping the growth of the test microorganisms tested with ethanol extract of stevia: Proteus mirabilis 6 – 18 mm, Pseudomonas aeruginosa 003841/114 – 15 mm, Staphylococcus aureus D2 – 15 mm, Candida albicans – 18 mm, Escherichia coli NC 101 - 18 mm and Bacillus subtilis VKM - 18 mm. This substance did not show antimicrobial activity against Klebsiella oxitoca test strain.

Aqueous extract of stevia showed antagonistic activity against 3 tested test microorganisms, and the diameter of their growth arrest zone was: Pseudomonas aeruginosa 003841/114 - 12 mm, Candida albicans - 19 mm, Klebsiella oxitoca - 15 mm. This substance did not show antimicrobial activity against the test strains of Proteus mirabilis 6, Staphylococcus aureus D2, Escherichia coli NC 101 and Bacillus subtilis VKM (Table 1).

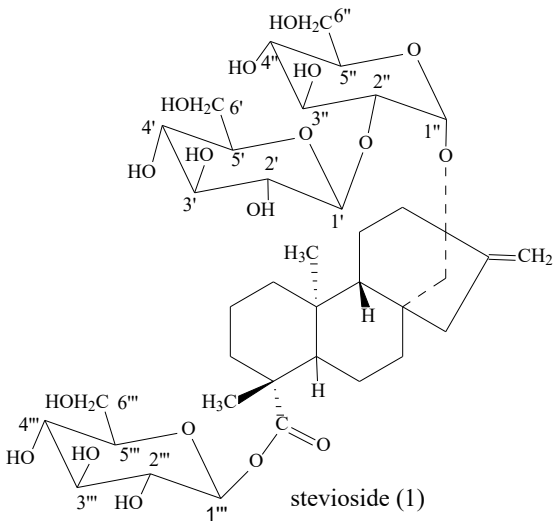
During the experiment, the chloroform fraction showed antagonistic activity against 4 of the tested test microorganisms, and the diameter of their growth arrest zone was: Proteus mirabilis 6 - 15 mm, Staphylococcus aureus D2 - 13 mm, Candida albicans - 12 mm, Escherichia coli NC 101 - was 15 mm. This substance did not show antimicrobial activity against Pseudomonas aeruginosa 003841/114, Escherichia coli NC 101 and Bacillus subtilis VKM test strains.

From the results presented in Table 3, it is known that the gram-positive, spore-forming rod-shaped bacterium - Bacillus subtilis BKM is sen-

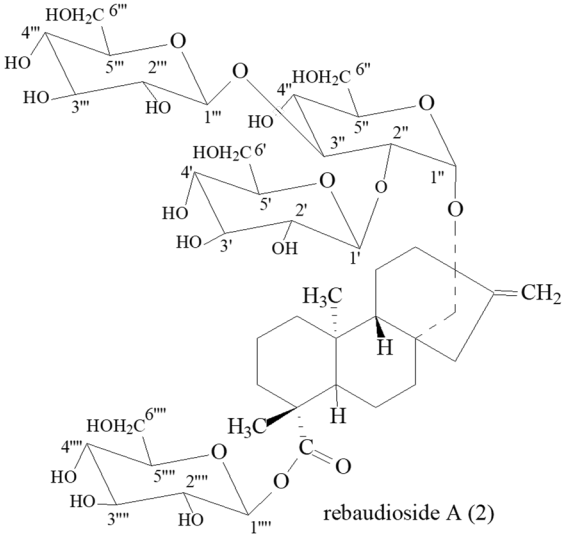
**Table 2**

**YuSSX indicators of aqueous extract of stevia leaves**

| Peak | RetTime [min] | Type | Width [min] | Field [nRIU*s] | Height [nRIU] | Field % |
|---|---|---|---|---|---|---|
| 1 | 0,025 | BV | 0,2804 | 139,88625 | 8,31485 | 8,810e-3 |
| 2 | 0,153 | VB | 0,0829 | 202,65739 | 40,73582 | 0,0128 |
| 3 | 0,583 | BB | 0,3341 | 536,96326 | 20,18726 | 0,0338 |
| 4 | 0,957 | BV | 0,1550 | 619,00653 | 55,94121 | 0,0390 |
| 5 | 1,055 | VB | 0,1163 | 340,93765 | 47,42666 | 0,0215 |
| 6 | 1,425 | BV | 0,2255 | 3038,43262 | 187,81677 | 0,1914 |
| 7 | 1,717 | VV | 0,2766 | 1,00019e4 | 490,91568 | 0,6299 |
| 8 | 2,289 | VB | 0,3049 | 2,21052e4 | 938,94025 | 1,3922 |
| 9 | 2,678 | BV R | 0,1592 | 1,51929e6 | 1,55691e5 | 95,6850 |
| 10 | 3,735 | VV E | 0,1983 | 445,76321 | 37,14242 | 0,0281 |
| 11 | 4,169 | VV E | 0,2221 | 1407,73315 | 96,44479 | 0,0887 |
| 12 | 4,604 | VV X | 0,0750 | 18,64134 | 3,07648 | 1,174e-3 |
| **13** | **5.000** | **VB X** | **0.2185** | **1.34676e4** | **955.30316** | **0.8482** |
| 14 | 5,485 | BV | 0,3218 | 2428,89063 | 108,44365 | 0,1530 |
| 15 | 6,503 | VV | 0,2933 | 5874,00195 | 302,72379 | 0,3699 |
| 16 | 7,113 | VB | 0,2133 | 1625,62854 | 111,47227 | 0,1024 |
| 17 | 7,549 | BB | 0,1817 | 54,01439 | 3,64062 | 3,402e-3 |
| 18 | 8,348 | VB R | 0,2772 | 678,30914 | 33,20749 | 0,0427 |
| 19 | 8,828 | BB | 0,2835 | 549,66370 | 24,90006 | 0,0346 |
| 20 | 9,382 | BV | 0,1481 | 143,34172 | 13,21794 | 9,028e-3 |
| 21 | 9,691 | VV | 0,2836 | 661,51904 | 30,45890 | 0,0417 |
| 22 | 10,447 | VB | 0,4476 | 2250,69067 | 68,98055 | 0,1417 |
| 23 | 10,989 | BV | 0,2871 | 1159,75586 | 53,58004 | 0,0730 |
| 24 | 11,502 | VV | 0,3236 | 715,26611 | 26,68050 | 0,0450 |
| 25 | 11.898 | VBA | 0.1104 | 48.66902 | 5.96125 | 3.065e-3 |



stevioside (1)



rebaudioside A (2)

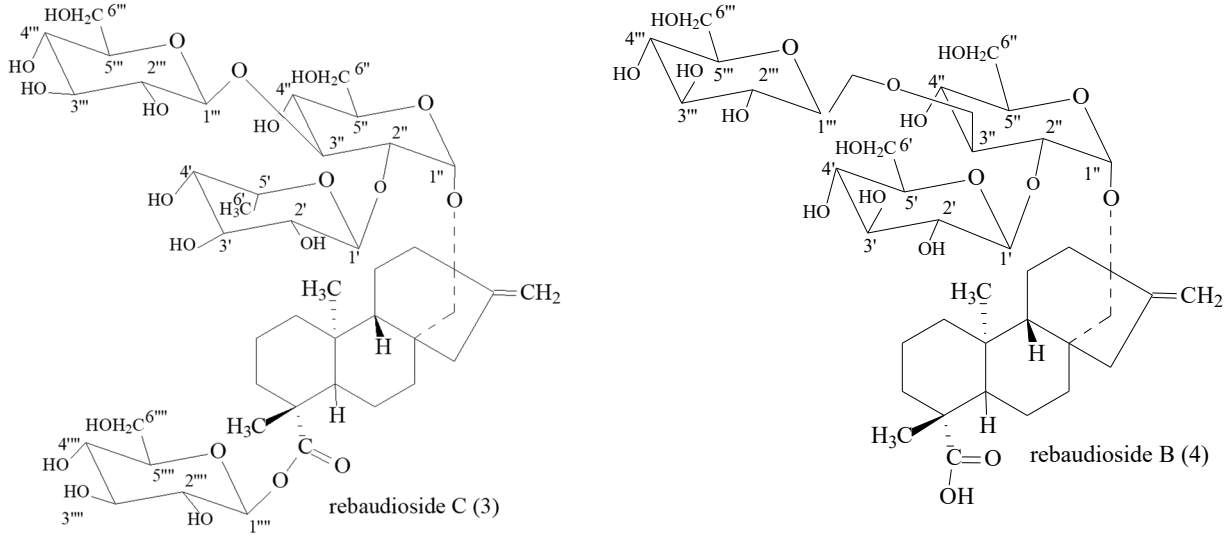



**Figure 3. Steviol glycosides present in Stevia rebaudiana leaves.**

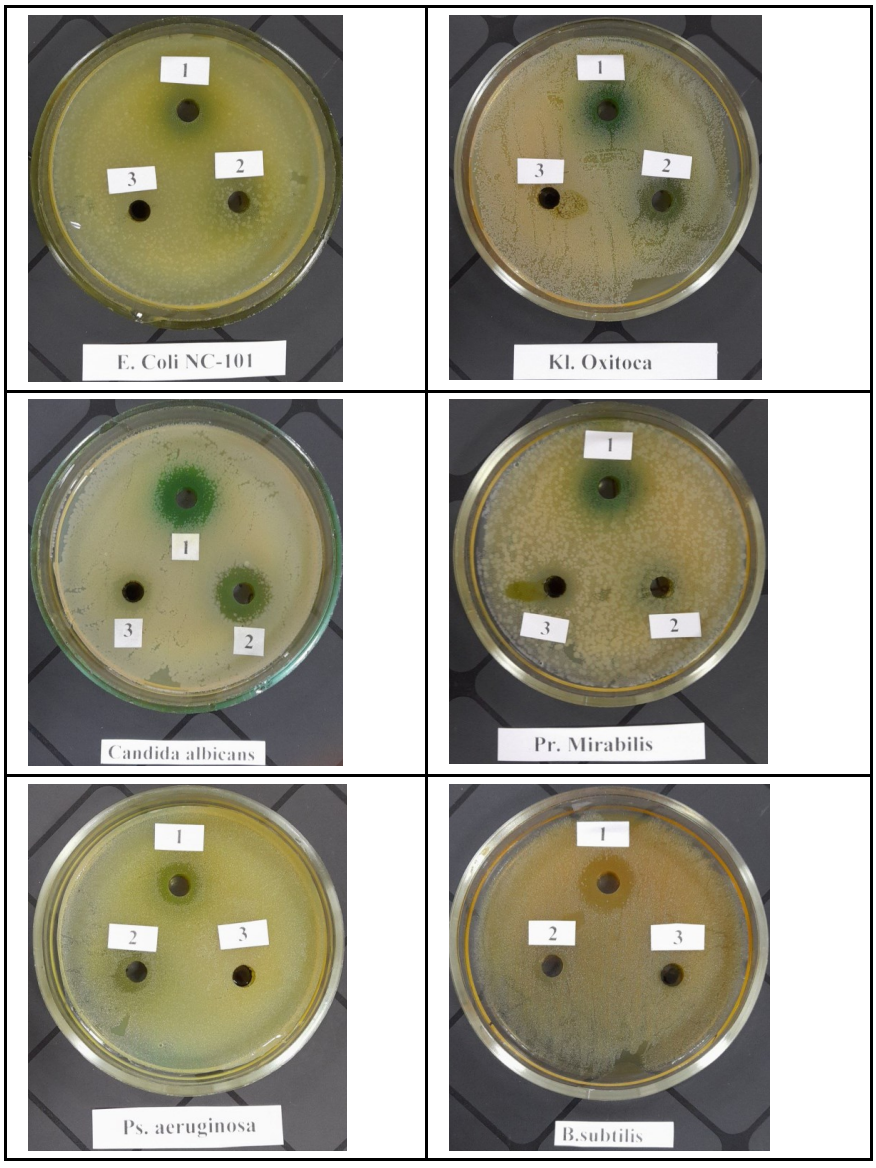



**Figure 4. Antimicrobial activity of stevia leaf extracts and fra against opportunistic pathogenic microorganisms. 1. Ethanol extract; 2. Aqueous extract; 3. Fraction with chloroform.**

**Table 3**

**Antimicrobial activity of stevia leaf extracts and fraction against conditionally pathogenic microorganisms**

| № | Conditionally pathogenic microorganism strains | Diameter of the zone of antimicrobial activity of the tested substance, mm | | |
|---|---|---|---|---|
| | | Ethanol extract | Aqueous extract | Chloroform fraction |
| 1 | Escherichia coli NC 101 | 18 | 0 | 15 |
| 2 | Proteus mirabilis 6 | 18 | 0 | 15 |
| 3 | Pseudomonas aeruginosa 003841/114 | 15 | 12 | 0 |
| 4 | Klebsiella oxitoca 1 | 0 | 15 | 0 |
| 5 | Staphylococcus aureus D2 | 15 | 0 | 13 |
| 6 | Bacillus subtilis BKM | 18 | 0 | 0 |
| 7 | Candida albicans | 18 | **19** | 12 |

sitive to the ethanolic extract of stevia, and other substances do not have an antimicrobial effect on this test microorganism. Also, among the substances tested, only the aqueous extract of stevia showed activity against gram-negative, rod-shaped bacteria Klebsiella oxitoca 1. All tested compounds show the same level of antagonistic activity against the yeast Candida albicans (Fig. 3).

## Conclusion

An aqueous extract of stevia plant leaf was taken, and for chemical analysis, chloroform 19.7 g (0.985%), ethylacetate 20.5 g (1.05%), and n-butanol fraction were divided into 150 g (7.55%).

Using high-performance liquid chromatography, monosaccharides in aqueous extract of stevia plant were compared with standard samples.

Chemical analysis of alcoholic and aqueous extracts and chloroform fraction from the stevia plant grown in Uzbekistan was carried out, as a result of which their biochemical composition was determined. In the obtained analysis, the analysis of the main compounds of the sweetener used in the food industry, water-soluble compounds, monosaccharides, stevol glycosides and other natural substances is carried out using YuSSX. Antimicrobial activity of aqueous and alcoholic extracts and fractions obtained from Styvia plant was studied, as a result, it was found that the aqueous extract has anti-Candida albicans activity.

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