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INVESTIGATION OF BIOLOGICALLY ACTIVE SUBSTANCES AND ANTIMICROBIAL ACTIVITY IN *STEVIA REBAUDIANA* EXTRACTS

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The World Health Organization, taking into account available scientific studies, recognized Stevia (Sweet Clover) in 2006 as a harmless, beneficial crop and allowed its widespread use in food and pharmaceutical production. This decision has served as a basis for various scientific groups to continue large-scale studies on the composition and bioactivity of stevia.

Considering the nearly complete absence of studies on the stevia cultivated in Armenia, this work investigates the quantitative content of certain biologically active substances in stevia grown in Armenia. The following data were obtained: diterpene glycosides 19.69%, fructose, glucose, and sucrose. The study of the antimicrobial properties of the water extract from the raw material showed that it possesses pronounced antimicrobial activity.

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***Keywords*:** diterpene glycosides, stevioside, HPLC method, stevia leaves.

Introduction. *Stevia rebaudiana Bertoni* is a perennial plant belonging to the Asteraceae family and growing in South and Central America: Paraguay, Chile, Argentina [1].

Stevia rebaudiana Bertoni was botanically classified in 1899 by Moisés Santiago Bertoni, who described it in more detail. Initially called Eupatorium rebaudianum, its name was changed to *S. rebaudiana* (*Bertoni*) *Bertoni* in 1905. The sweet principle was first isolated in 1909 and only in 1931 was the extract purified to produce stevioside, the chemical structure of which was established in 1952 as a diterpene glycoside. Stevioside is described as a glycoside comprising

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three glucose molecules attached to an aglycone, the steviol moiety. During the 1970s, other compounds were isolated, including rebaudioside A, with a sweetening potency even higher than stevioside [2–4].

Stevia leaves contain low-calorie diterpene glycosides, united under the general name "stevioside", the total content of which in dry leaves ranges from 4 to 20% (stevioside – 5–10%, rebaudioside A – 2–4%, rebaudioside C – up to 1.4%, dulcoside A – up to 1%, etc.). The mentioned substances are 210, 242, 30, and 30 times sweeter than sugar. Currently, several dozen glycosides have been identified in stevia, which differ only in the amount and type of monosaccharides attached to the R1 and R2 positions of aglycone steviol. The total amount of sweet glycosides in dry leaves depends on the genotype and growing conditions [5–10].

Stevioside isolated from stevia has minimal calories, does not increase blood sugar levels, moreover, stimulates the production of insulin by the pancreas, has an antimicrobial, antifungal effects, strengthens the immune system, slows down the aging process, thus making life more vigorous and long-lasting, does not harm tooth enamel, lowers blood cholesterol, poisons, heavy metals, radionuclides, etc. [11–16].

Stevia contains essential oil, tannins, minerals and other active bioelements, which show anti-inflammatory and regenerative, antioxidant and other effects, so it is effective for burns, cuts, eczema, frostbite, trophic ulcers [4, 17].

Recently, interest in stevia has increased greatly, as there is a huge demand around the world for low-calorie nutrients endowed with beneficial properties. For the widespread use of stevia, in-depth and more specific studies are needed, some of which have been carried out in this work, taking into account the scarcity of studies on the phytochemical composition of stevia cultivated in the Republic of Armenia [18].

The purpose of the research is the identification and quantitation of some biologically active substances of stevia cultivated in the RA, and determination of antimicrobial activity of raw materials.

Materials and Methods. The collection of raw stevia, drying, pre-preparation of raw materials and its quality control were carried out in accordance with state standards [19].

Reagents. High-Performance Liquid Chromatography (HPLC) grade acetonitrile (ACN), chloroform ($CHCl_3$), methanol, acetic acid and analytical grade reference compounds, D-(−)-fructose (≥ 99% GC), D-(+)-glucose (≥ 99.5% GC), and D-(+)-sucrose (≥ 99.5% GC), stevioside (≥ 99% GC), tannic acid, quercetin, rutin were purchased from Sigma Aldrich (Milan, Italy), whereas deionized water (>18 *Ohm* resistivity) was obtained from Milli-Q SP Reagent Water System (Millipore, Bedford, MA, USA).

To determine the biologically active substances present in stevia, two solvents were selected: ethanol at concentrations of 40%, 70%, 90%, and water.

The data were processed through Microsoft Excel software.

For the detection and identification of bioactive substances, various high performance liquid chromatography methods were used, which allow a reliable judgment about the quantitative composition of the test substance.

The quantitative and qualitative content of stevioside, some mono- and disaccharides, tannic acid, rutin and quercetin in the aqueous and alcohol (90%)

extracts obtained from raw stevia was determined by the HPLC method according to the standards of the mentioned compounds; devices and the analysis conditions are presented below.

The separation of sugars by the HPLC method was carried out on KNAUER-GERMANY liquid chromatograph with refractometric detection. As a chromatographic column, Shodex-RP-NH2 150×4.6 *mm*, RI 2300 detector KNAUER (Germany) were used; flow rate – 0.5 *mL*/*min*, column temperature – 30°C, mobile phase – 75% CH_3CN / 25% H_2O, duration of chromatography – 25 *min*, injection volume – 30 μL, pump operating mode – isocratic.

The determination of the authenticity of stevioside was carried out by Shimadzu Prominence-I LC-203°C, 3D plus- (with PDA detection) device, as a chromatographic column, Alltima C18 250×4.6 *mm*, 5 μm, detector PDA 210 *nm* were used; flow rate – 0.4 *mL*/*min*, column temperature – 40°C, mobile phase – 85% CH_3OH / 15% H_2O, duration of chromatography – 25 *min*, injection volume – 10 μL.

Preparation of Standard. To prepare a model solution of a mixture of fructose, glucose, sucrose, maltose and lactose, the following amounts were taken from each standard: fructose – 1.74 *mg*; glucose – 1.45 *mg*; sucrose – 3.73 *mg*; maltose – 0.88 *mg*; lactose – 0.86 *mg* and dissolved in 1 *mL* of extractant. For stevioside – 0.997 *mg* in 1 *mL* of extractant. Ethanol–water mixture in the ratio of 60:40 was used as an extractant. The vial was shaken until the contents were dissolved, then stirred with Vortex axial stirrer for 3 *min*. Then, the standard solution was filtered through 0.22 μm pore size filters and placed in the automatic injection system of the HPLC device on the platform provided for the samples, which had a certain numbering.

Identification and Quantitation of Tannic Acid, Quercetin and Rutin in Stevia Leaves. The content of tannic acid, quercetin and rutin in aqueous and alcohol (90%) extracts obtained from raw stevia was determined by the HPLC method. The separation of the above-mentioned substances by the HPLC method was carried out with a liquid chromatograph Waters Separation module e2695 (USA), and as a chromatographic column Alltima C18 250×4.6 *mm*, 5 μm was applied. A mixture of solvents with a methanol/water/acetic acid volume ratio of 50/48/2 was used as an eluent.

Determination of the Antimicrobial Activity of Aqueous and Alcohol Extracts Obtained from Raw Stevia. To investigate the antimicrobial activity of biologically active substances in the composition of stevia, test cultures such as *Pseudomonas aeruginosa* 9056, 9059, and 9150 strains were selected. Meat peptone culture medium was used for the experiment.

Results and Discussion. At the first stage, quantitation of the total content of diterpene glycosides in stevia leaves was carried out by spectrophotometric method. For the quantitation of the total content of diterpene glycosides, extraction from the plant raw material was implemented. To determine the optimal extractant (solvent), 90%, 70%, 40% ethanol solutions and water were chosen (Tab. 1).

From the data presented in the Table, it follows that 90% alcohol is an extractant with the optimal working concentration, where the maximum total content of diterpene glycosides of 19.69% was found. This corresponds to the literature data, according to which the total sugar amount should be 4% to 20% [5–10].

Table 1

Total content of diterpene glycosides determined using different extractants

No	Extractant	Total content of diterpene glycosides, %
1	90% alcohol	19.69
2	70% alcohol	2.83
3	40% alcohol	2.21
4	water	3.12

Then, it was decided to continue the research using 90% alcohol and aqueous extracts. Quantitation of stevioside, side sugars, tannic acid, rutin, quercetin was carried out by the HPLC method; the research results are presented in the form of chromatograms and tables.

Quantitative content (x) of substances identified in the test samples, expressed as a percentage, was determined by the following formula and then recalculated per 100 g: $Area_{(smpl)}$

$$x = \frac{Area_{(smpl)} \cdot m_{STD} \cdot V_{smpl}}{Area_{(STD)} \cdot V_{STD} \cdot m_{smpl}} \cdot 100\%,$$

where $Area_{(smpl)}$ is the peak area of the test sample; m_{STD} is the mass of the standard sample, g; V_{smpl} is the solubility volume of the test sample, mL; $Area_{(STD)}$is the peak area of the standard sample; V_{STD} is the solubility volume of the standard sample, mL; m_{smpl} is the mass of the test sample, g.

Fig. 1 shows the chromatogram of a model solution of stevioside, from which the retention time was determined.

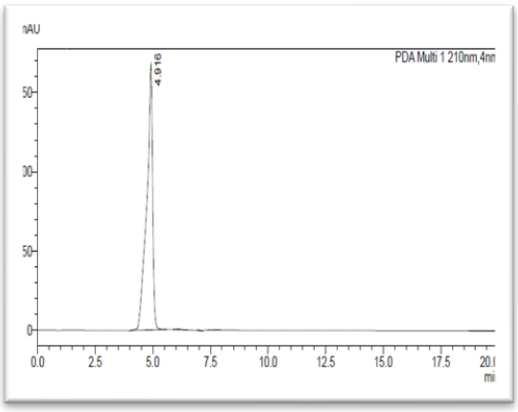
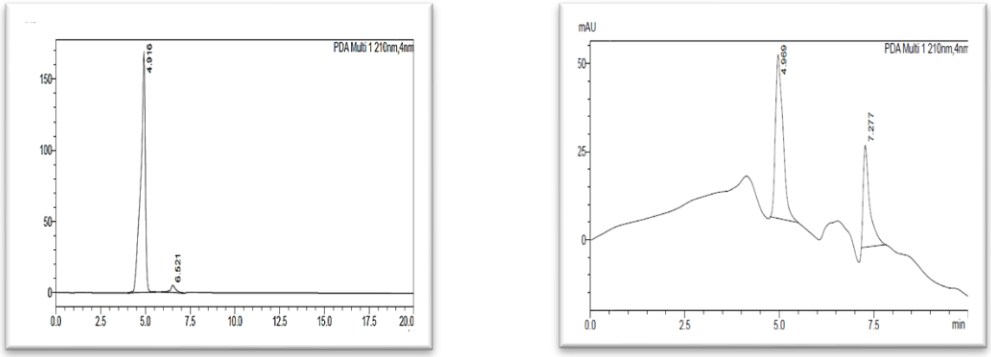


Fig. 1. Chromatogram of a model solution of stevioside.

Based on this, the presence of stevioside in both alcohol and aqueous extracts was confirmed, with corresponding peaks observed in Fig. 2.

The qualitative identification was performed by comparing the retention times of the compounds (Figs. 1 and 2), while the quantitative analysis was carried out using the peak area ratio method.



a b

Fig. 2. Chromatogram of alcohol (a) and aqueous (b) extracts of stevioside.

The quantitative analysis of the results obtained is presented in Tab. 2.

Table 2

Quantitative content (%) of stevioside in 100 g of raw material

Sample Name	Retention time	Amount, %
standard	4.916	9.87
90% alcohol	4.939	9.74
water	4.969	2.7

As can be seen from the presented data, a significantly higher quantitative yield of stevioside is observed in the 90% alcohol extract.

Fig. 3 shows the chromatogram of the model solution of mono- and disaccharides.

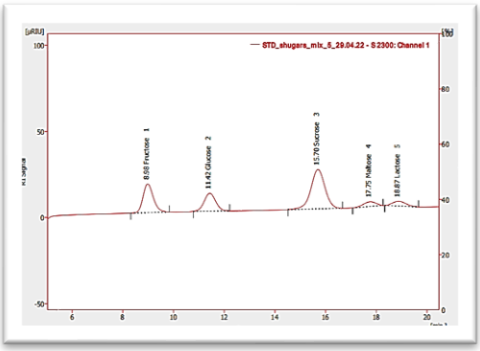
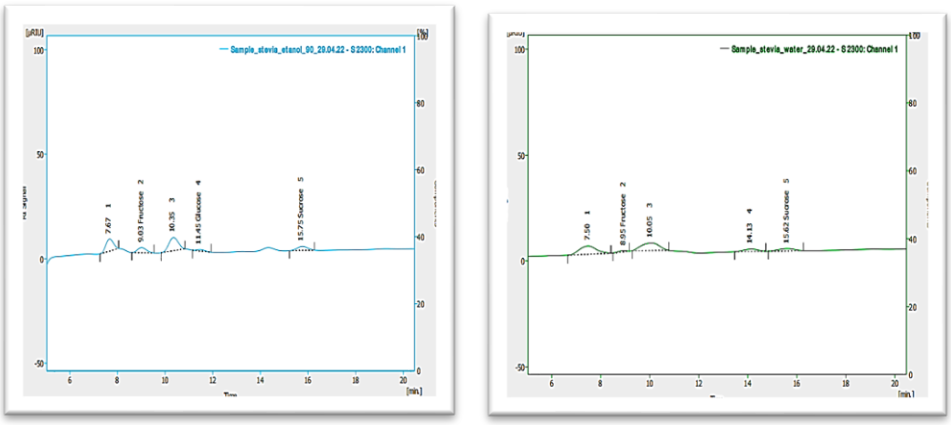


Fig. 3. Chromatogram of a model solution of fructose, glucose, sucrose and maltose mixture.

The presence of other sugars in aqueous and alcoholic extracts was determined in a similar manner using model chromatograms of mono- and disaccharides (Fig. 3).

Fig. 4 presents the chromatographic analysis of the sugar composition in stevia extracts: 90% ethanol extract (a) and aqueous extract (b).



a b

Fig. 4. Chromatograms of the test alcohol (a) and aqueous (b) extracts for sugar determination.

Table 3

Chromatogram parameters for the test alcohol extract

No	Name	Amount, %	
		alcohol extract	aqueous extract
1	Fructose	2.295	0.240
2	Glucose	0.709	–
3	Sucrose	2.537	1.036

From the chromatogram data in Tab. 3 and Fig. 4, it follows that fructose, glucose, sucrose and two other unknown sugars are present in the 90% alcohol extract, and three unknown sugars – in the aqueous extract.

Thus, the studies show that diterpene glycosides are the main sweeteners. The data obtained correspond to the data available in the literature, according to which the amount of stevioside should be 4–20%. The analysis of the literature data allows to conclude that the three unknown sugars are probably rebaudioside A – 2–4%, rebaudioside C – up to 1.4%, dulcoside A – up to 1%, the amounts of which also correspond to [5–10].

Table 4

Chromatogram parameters for a model solution of rutin, tannin and quercetin mixture

No	Name	Retention time	Amount, *mg/mL*
1	Tannic acid	3.903	0.125
2	Rutin	7.945	0.115
3	Quercetin	17.875	0.155

Fig. 5 shows the chromatogram of a model solution of rutin, tannin and quercetin, from which the retention time was determined (Tab. 4).

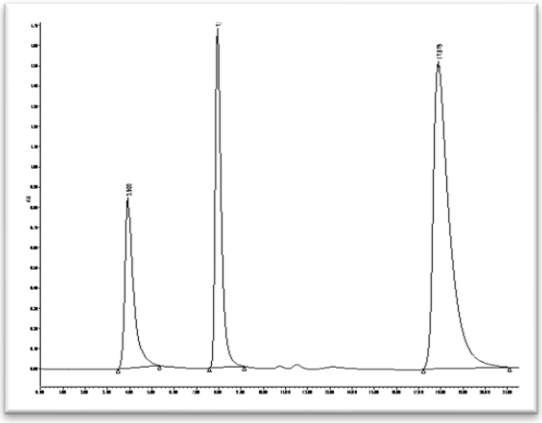
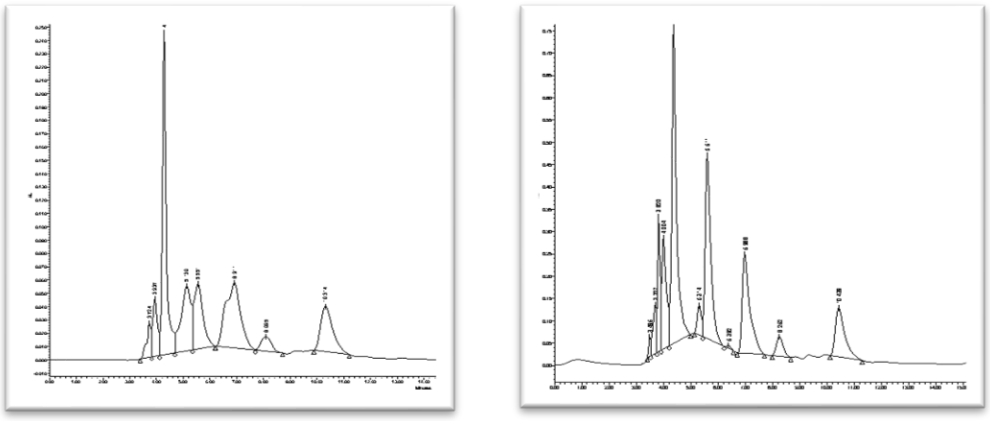


Fig. 5. Chromatogram of a model solution of rutin, tannin and quercetin mixture.

The chromatography used to determine the presence of tannin, rutin, and quercetin in the aqueous and ethanolic extracts of stevioside is presented in Fig. 6, while the quantitative analysis is shown in Tab. 5.



a b

Fig. 6. Chromatograms of the test alcohol (a) and aqueous (b) extracts.

Table 6

Chromatogram parameters for the test alcohol and aqueous extracts

No	Name	Amount, *mg*/*mL*		Amount, %	
		alcohol extract	aqueous extract	alcohol extract	aqueous extract
1	Tannic acid	0.03	0.109	3	10.9
2	Rutin	0.01	0.03	1	3

It follows from the obtained data, that tannic acid and rutin were identified by the HPLC method in aqueous and alcohol (90%) extracts obtained from 1 *g* of raw stevia, while quercetin was not detected (Tab. 6). In addition to diterpene glycosides, the plant is also rich in both flavonoids and bitter substances. The results obtained show that the content of tannins and rutin in the aqueous extract is significantly high.

Determination of Antimicrobial Activity of Aqueous and Alcohol Extracts Obtained from Raw Stevia. The research on antimicrobial activity of biologically

active substances present in the composition of stevia was also carried out. For this purpose, antibiotic-resistant test cultures were selected: *Pseudomonas aeruginosa* 9056 – sensitive; *P. aeruginosa* 9059 – kan^r, cm^r, amp^r, amx^r, $augm^r$, cfx^r, azm^r, cip^r; *P. aeruginosa* 9150 – kan^r, cm^r, amp^r, amx^r, $augm^r$, cfx^r, gen^r, str^r, pcn^r, which were obtained from the Microbial Depository Center of SPC "Armbiotechnology" of NAS RA, SNPO. To check the antimicrobial effect of aqueous and alcohol (90%) extracts obtained from raw stevia against the mentioned strains, inoculation of these strains was carried out in a solid culture medium. For comparison, filter papers moistened with 90% alcohol and kanamycin solution were placed in parallel as a standard. As a result, the following data were obtained (Tab. 7).

Table 7

Effect of the test samples on microbial colonies

No	*Pseudomonas aeruginosa* strains	Test samples			
		Aqueous extract	90% alcohol extract	90% alcohol	Kanamycin solution
		range of influence, *cm*			
1	9056	3.2 ± 0.01	0.5 ± 0.02	–	3.5 ± 0.01
2	9059	2.8 ± 0.01	2.0 ± 0.01	–	3.0 ± 0.01
3	9150	3.3 ± 0.01	0.4 ± 0.02	–	4.0 ± 0.01

In the course of research, it was found that 90% alcohol extract of stevia has an antibiotic-resistant effect on antibiotic-resistant *P. aeruginosa* 9059 – kan^r, cm^r, amp^r, amx^r, $augm^r$, cfx^r, azm^r, cip^r strain.

It is of great interest that an aqueous extract obtained from dry stevia, like control kanamycin, exhibits antimicrobial activity against *P. aeruginosa* 9150 – kan^r, cm^r, amp^r, amx^r, $augm^r$, cfx^r, gen^r, str^r, pcn^r strain. The effect of the aqueous extract is high for all selected strains, which is probably due to the presence of other bioactive substances and sugars in the extract that are absent in the alcohol solution. For example, the antibacterial effectiveness of tannic acid is explained by its ability to pass through the bacterial cell wall up to the internal membrane, interference with the metabolism of the cell, and – as a result – its destruction [20, 21].

Conclusion. Thus, based on the results obtained and the phytochemical research carried out, it can be concluded that the medicinal plant material of stevia cultivated in Armenia is rich in a number of biologically active substances: flavonoids, diterpene glycosides and other side sugars (fructose, glucose, sucrose), and the aqueous extract has a pronounced antimicrobial activity.

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Ա. Մ. ՀՈՎՀԱՆՆԻՍՅԱՆ

ՄԵՂՐԱԽՈՏԻ ԷՔՍՏՐԱԿՏՆԵՐՈՒՄ ԿԵՆՍԱԲԱՆԱԿԱՆ ԱԿՏԻՎ ՆՅՈՒԹԵՐԻ ԵՎ ՀԱԿԱՄԱՆՐԷԱՅԻՆ ԱԿՏԻՎՈՒԹՅԱՆ ՈՒՍՈՒՄՆԱՍԻՐՈՒԹՅՈՒՆԸ

Առողջապահության համաշխարհային կազմակերպությունը, հիմք ընդունելով առկա գիտական հետազոտությունները, 2006 թ.-ին ճանաչել է ստևիան (մեղրախոտը) որպես անվնաս և օգտակար մշակաբույս՝ թույլատրելով դրա լայնածավալ օգտագործումը սննդի և դեղագործական արտադրության մեջ: Այդ որոշումը հիմք է հանդիսացել, որպեսզի տարբեր գիտական խմբեր շարունակեն ստևիայի բաղադրության և կենսաբանական ակտիվության լայնածավալ ուսումնասիրությունները: Հաշվի առնելով Հայաստանում մշակված ստևիայի վերաբերյալ հետազոտությունների գրեթե լիակատար բացակայությունը՝ տվյալ աշխատանքում ուսումնասիրվել է Հայաստանում աճեցված ստևիայի մեջ որոշ կենսաբանորեն ակտիվ նյութերի քանակական պարունակությունը: Ստացվել են հետևյալ տվյալները՝ դիտերպենային գլիկոզիդներ՝ 19,69%, ինչպես նաև ֆրուկտոզ, գլյուկոզ և սախարոզ: Հումքից ստացված ջրային թուրմի մանրէազերծող հատկությունների ուսումնասիրությունը ցույց է տվել, որ այն ունի արտահայտված հակամանրէային ակտիվություն:

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ИССЛЕДОВАНИЕ БИОЛОГИЧЕСКИ АКТИВНЫХ ВЕЩЕСТВ И ПРОТИВОМИКРОБНОЙ АКТИВНОСТИ ЭКСТРАКТОВ СТЕВИИ МЕДОВОЙ

Всемирная организация здравоохранения, основываясь на имеющихся научных исследованиях, в 2006 г. признала стевию медовую безопасной и полезной сельскохозяйственной культурой, разрешив ее широкое применение в пищевой и фармацевтической промышленности. Это решение послужило основанием для продолжения крупномасштабных исследований состава и биологической активности стевии со стороны различных научных групп. С учетом практически полного отсутствия исследований стевии, выращенной в Армении, в данной работе проведено исследование количественного содержания некоторых биологически активных веществ в армянской стевии. Были получены следующие данные: дитерпеновые гликозиды – 19,69%, а также наличие фруктозы, глюкозы и сахарозы. Исследование антимикробных свойств водного экстракта из растительного сырья показало, что он обладает выраженной антимикробной активностью.