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Research Article

Quantitative analysis of biologically active substances and the investigation of antioxidant and antimicrobial activities of some extracts of Osage orange fruits

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Abstract

Biologically active substances, the antioxidant and antimicrobial activity of aqueous, 70% ethylate and ethyl acetate extracts of fruits of maclura orange (Osage Orange), a representative of the *Moraceae* genus growing in the Ararat region of Armenia have been studied. It is found that plant extracts are rich in a wide range of pharmacologically active substances, in particular, tannins, flavonoids, organic acids, vitamins, anthocyanins, micro- and microelements, etc.

Based on the investigations performed, extracts of maclura orange can be recommended as a source of biologically active substances (BAS), as well as a preventive and corrective agent in deficiency or imbalance of macro- and microelements in tissue and cellular structures in various pathological processes in the body.

An ethanol extract of maclura fruits exhibits the antimicrobial activity against *Bacillus subtilis* 1820, *E. Coli* 5002, *Serratia marcescens* 5251 and *Staphylococcus aureus* ATCC-6538 strains. Maclura extracts can be used as an environmentally friendly source of antioxidants against early aging.

Keywords

Biologically active substances, antioxidant, tannins, flavonoids, organic acids, vitamins, antimicrobial activity

Introduction

Despite advances in synthetic pharmaceuticals, the interest in herbal medicine continues throughout the world. A fairly high adherence to treatment with plant components in different parts of the world is largely due to social and cultural peculiarities. For the population of low-income countries, herbal medicine is attractive for its relatively low cost, wider availability compared to drugs used by the official medicine (Heinrich et al. 2004).

Moreover, in the developing countries the number of certified doctors is small, while the number of practitioners

of traditional methods of treatment is disproportionately higher (Jamison et al. 2006; Jenssen et al. 2006). Herbal medicine remains a popular method also in developed countries (Russian National Standard 1995; Russian National Standard 1999; Ates et al. 2003). The interest of the population in states with a high level of income is largely due to the influence of mass media promoting alternative methods of treatment as more natural, accordingly, safer (Russian National Standard 1994). The search for medicinal substances in nature is still relevant today (Heinrich et al. 2004; Jenssen et al. 2006).

Their pharmacological properties have not been completely determined and the antimicrobial potential and biochemical compositions of many plant species have not been analyzed yet (Heinrich et al. 2004). World Health Organization (WHO) has predicted increasing antimicrobial resistance as a major threat for the public health for the 21st century. In order to prevent spreading of antibiotic resistant infections, scientists have been conducting intensive researches to determine new antimicrobial agents. One way to prevent antibiotic resistance of microorganisms is using new compounds that are not based on existing antimicrobial agents (Jamison et al. 2006; Jenssen et al. 2006). In the last three decades, antimicrobial activity-related experiments have been applied by using plant extracts (Ates et al. 2003).

In developing countries, traditional herbs have crucial significance for disease treatment and they aid to detect novel antibiotics. Maclura pomifera (Raf.) Osage orange, is a tree of the Moraceae or mulberry family. There are antimicrobial researches on M. pomifera in the literature and results are promising.

It has been used as an insect repellent for many years. Pioneer researchers placed the ripe fruit of this tree in cupboards to repel roaches and other insects (Sand 1991, Brandies 1979). Past research indicates that there may be scientific justification for this well-popularized use. Karr and Coats (1991) found fragments of the fruit, as well as its hexane and methanol extracts, to be significantly repellent to the German cockroach Blattella germanica. The wood of the osage orange tree resisted termites and had antifungal properties. Antimicrobial activity has been found in extracts containing osajin or pomiferin, two of the major angular isoflavonoid components present in osage orange fruits.

The aim of the work is to study biologically active substances, antioxidant and antimicrobial activity of aqueous, 70% ethanol and ethyl acetate extracts of fruits of maclura orange, a representative of the *Moraceae* genus growing in the Ararat region of Armenia.

Materials and methods

In the presented work we used aqueous, aqueous-ethanol, and ethyl acetate extracts of maclura orange. To obtain extracts, the dried raw material was ground in a ceramic mortar to a powdery state (particle size ≤1 мм), passed through a sieve with a hole diameter of 1 mm and 50 ml of extractant was poured into 1 g (precise weight) of the raw material, and extracted with reflux for 30 min at 50–70 °C. Then the contents of the flask were filtered through a paper filter, cooled and the volume of the extract was adjusted with extractant to 50 ml.

Determination of the amount of BAS (the total amount of extractives, flavonoids, tannins, carboxylic acids, vitamins) that had the highest physiological and therapeutic activity in the samples analyzed was carried out according to generally accepted procedures (Russian National Standard 1994, 1995, 1999).

In the investigated samples, the quantitative composition of vitamins P, C, B_{12} and organic acids was also studied by the HPLC.

Determination of flavonoids by HPLC

The HPLC analysis was carried out at the Laboratory of Biologically Active Compounds Purification and Certification. It was performed with Aqueouss HPLC Alliance 2695 Separation Module HPLC. The analysis process was carried out with a ZORBAX Eclipse C18, 4.6 × 250 mm, 5 μm particle size column, volume injection of 10 μl and a flow rate of 1.0 ml/min. All analyses were performed with a mobile phase of Methanol: H_2O:acetic acid of 50:48:2 ratios and detection at 254 nm.

Determination of ascorbic acid by HPLC

Quantification of ascorbic acid content was performed on an Aqueous Alliance 2695 Separation Module HPLC system. Chromatographic separation was achieved on a ZORBAX Eclipse C18, 4.6 × 250 mm column through isocratic delivery of a mobile phase (Methanol:H_2O (0.1% TFA) of 5:95 ratios) at a flow rate of 0.5 mL/min. UV absorbance was recorded at 254 nm.

Determination of Vitamin B_{12} by HPLC

HPLC analysis was performed with the Aqueous Alliance 2695 Separation Module HPLC system including a UV detector. For B_{12} quantification, the Phenomenax C18 column was used (10 μm, 3.9 × 300 mm) with an acetonitrile-aqueous (70:30) solvent separations with a flow rate of 0.8 ml per min. UV absorbance was recorded at 214 nm and 230 nm.

Determination of the antioxidant activity of maclura orange extracts

To determine the antioxidant activity of maclura orange, we studied the extracts (samples 1–3), taking into account the data obtained (Fig. 1, Table 1),

For this purpose, 8 ml of freshly distilled boiled water of room temperature, 1 ml of 20% sulfuric acid solution, 1 ml of 0.05 *N* potassium permanganate solution were poured into a glass with a 50 ml volume. The whole

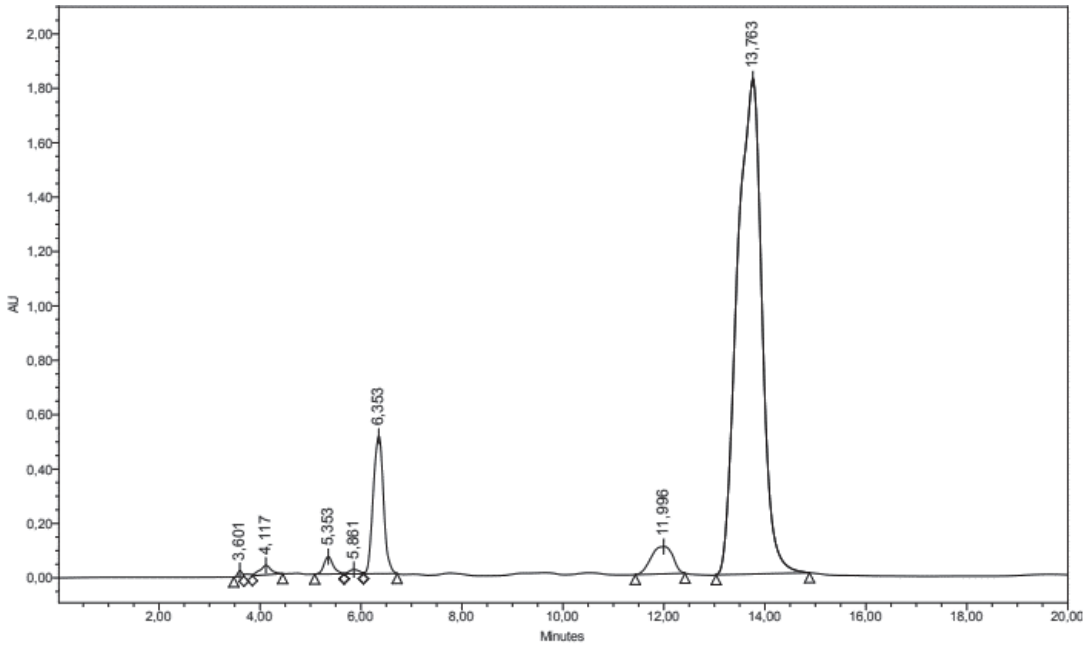

Figure 1. Results of chromatography of organic acids in aqueous extract of Osage Orange.

Table 1. Results of chromatography of organic acids in aqueous extract of Osage Orange.

	Name	Retention time	Area	% Area	Height	Conc. mg/mL
1		3,601	128642	0.18	21803	
2	Oxalic acid	4,117	540825	0.74	34876	0.134
3		5,353	819280	1.12	64471	
4	Malic acid	5,861	221155	0.30	15699	0.63
5		6,353	7178674	9.81	506320	
6	Succinic acid	11,996	3003186	4.10	99935	12.93
7		13,763	61311921	8376	1822144	

was stirred and titrated with extracts of maclura orange (samples 1–3) from microburette (with 1 ml volume with 0.01 ml graduation) until the pink color disappeared.

For the control experiment about 0.05 g (precise weight) of quercetin (FS 42-1290-79) was dissolved in 40 ml of ethyl alcohol, transferred to a 100 ml measuring flask, adjusted with alcohol to the mark and stirred. 8 ml of freshly boiled and cooled distilled aqueous, 1 ml of 20% sulfuric acid solution, 1 ml of 0.05 *N* potassium permanganate solution were poured into a titration glass with a 50 ml volume, stirred and titrated with a quercetin solution from a microburette (with 1 ml volume with 0.01 ml graduation) until the pink color disappeared. 1 ml of 0.05 *N* potassium permanganate solution corresponds to 0.25 mg of quercetin.

The calculation of the antioxidant activity index (AOA), which corresponds to the BAS concentration of a reducing nature in terms of quercetin (in mg/g) was carried out according to the formula $B = C_k x V_k x V_o / V_x x m$, where B is the concentration of BAS of the reducing nature of the object under study, consumed for titration of 1 ml of 0.05 *N* potassium permanganate solution, mg/ml (0.5 mg/ml); V_k is the volume of quercetin solution consumed for titration of 1 ml of 0.05 *N* potassium permanganate solution, ml (1.4 ml); Vo is the volume of the test solution, ml (50 ml); Vx is the volume of the solution of the test object consumed for titration of 1 ml of 0.05 *N* potassium permanganate solution, ml (0.4 ml); m is the mass of the precise weight of the object under study, g (1 g).

The total amount of BAS of a reducing nature was determined in terms of quercetin in 1 g of the preparation.

Screening of the antimicrobial activity of maclura orange extracts

The screening of antimicrobial activity of extracts was carried out using the micromethod of diffusion in agar. It was designed at the Department of Microbiology, Virology and Immunology of Ivano-Frankivsk National Medical University (Kutsyk 2004).

To estimate the activity of extracts, test cultures of the following strains were used: *Pseudomonas aeruginosa* 5249, *Bacillus subtilis* 1820, *Staphylococcus aureus* ATCC 6538, *Escherichia coli* 5002, *Candida albicans* ATCC 10231, *Streptococcus faecalis* 5242, *Mycobacterium roseus* 5239, *Mycobacterium* sp. 5237, *Bacillus coagulans* 1906, *Enterococcus faecalis* 5254, *Serratia marcescens* 5251, *Micrococcus luteus* 5270.

Test cultures were provided by the Microbial Depository Center of the SPC "Armbiotechnology". 1 ml of the medium contained 10^7 bacteria. The cultivation was carried out in a bacteriological box on nutrient agar. Bacterial suspensions were prepared in sterile water and then added to a liquid nutrient medium. Agar was transferred into Petri dishes until solidification, then small disks (0.3 ml) of filter paper soaked with extract were placed on nutrient agar using sterile needles. After drying at room temperature for 30–40 min, the dishes were transferred to a thermostat for 24–48 h at 25–27 °C. At the end of incubation, the zones of inhibition of bacterial growth were measured.

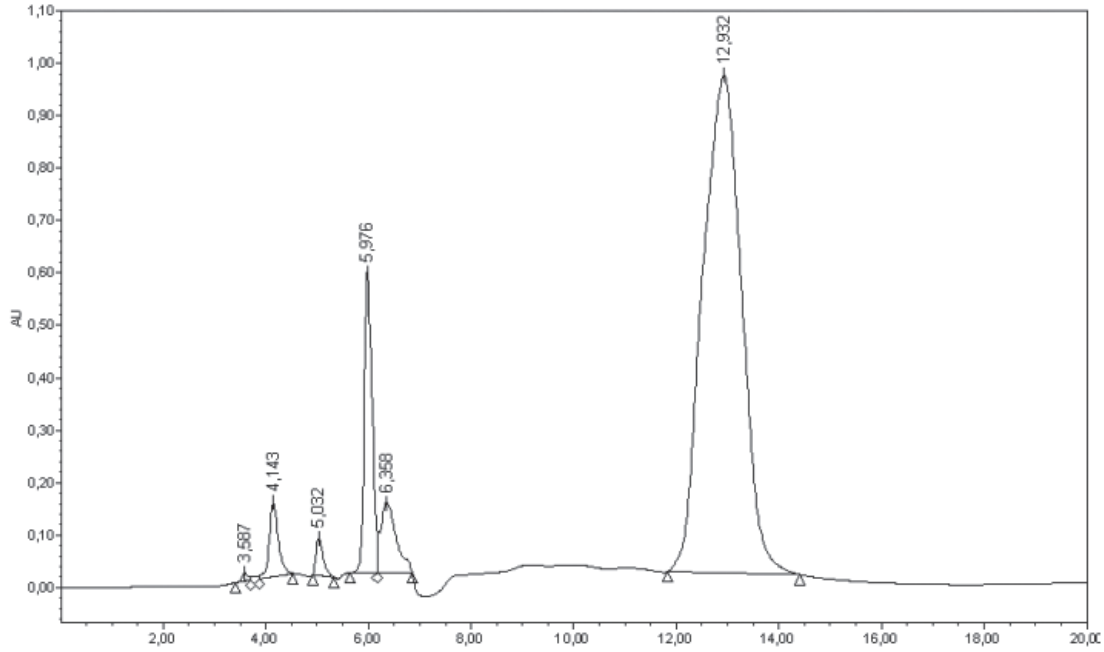

Figure 2. Results of chromatography of organic acids in ethanol extract of Osage Orange.

Table 2. Results of chromatography of organic acids in aqueous extract of Osage Orange.

	Name	Retention time	Area	% Area	Height	Conc. mg/mL
1		3,587	115940	0.19	16097	
2	Oxalic acid	4,143	1727685	2.81	142915	0.23
3		5,032	680874	1.11	71449	
4	Malic acid	5,976	6194818	10.08	578497	17.56
5		6,358	2885196	4.69	133464	
6		12,932	49877120	81.13	950770	

The actual material was processed using the method of variation statistics with the calculation of the average arithmetic and its standard error, the reliability of the comparable values was estimated according to Student, Wilkinson, Mann Whitney criteria; the probability level was determined as $p \leq 0.05$ using the Image Tool 2.0 (UTHSCSA ImageTool 2.0, The University of Texas Health Science Center in San Antonio, 1995–1996) and MS Excel (State Pharmacopeia 2015).

Results

The qualitative and quantitative contents of biologically active substances, antioxidant and antimicrobial activity of aqueous, 70% ethanol and ethyl acetate extracts of fruits of maclura orange, a representative of the *Moraceae* genus growing in the Ararat region of Armenia have been studied. The results of the study are presented in Figs 1–4 and Tables 1, 2.

At the first stage, a quantitative analysis of BAS by HPLC and titration methods was performed. As a result, it was established that the composition of extracts differed depending on the type of extractant. Quantitative indices of BAS are shown in Figs 1–9.

Figs 1–3 show the results of chromatography of organic acids in aqueous, ethanol and ethyl acetate extracts of Osage Orange.

Figs 4–6 show the results of quantification and identification of certain flavonoids in aqueous, ethanol and ethyl acetate extracts of Osage Orange.

Figs 7–9 show the results of vitamin C determination in aqueous, ethanol and ethyl acetate extracts of Osage Orange.

Taking into account the rich composition of the extracts, the next stage was study of the antioxidant activity. 0.05% Solution of quercetin was used as a control. In accordance with the results obtained during titration, in the case of quercetin (1.4 ml), the consumption of maclura extracts was respectively 1.95, 0.55 and 0.48 ml, which indicates that extract of maclura orange can be considered as an efficient preventive agent against the antioxidant aging of the body.

The amount of antioxidant substances was determined using the following formula:

In the case of aqueous extract:

$$B = \frac{0,5 \cdot 1,4 \cdot 100}{1,95 \cdot 1} = 35,89 \text{mg} / \text{g}$$

In the case of 70% ethanol extract:

$$B = \frac{0,5 \cdot 1,4 \cdot 100}{0,55 \cdot 1} = 127,27 \text{mg} / \text{g}$$

In the case of ethyl acetate extract:

$$B = \frac{0,5 \cdot 1,4 \cdot 100}{0,48 \cdot 1} = 145,83 \text{mg} / \text{g}$$

Studies have shown that ethanol and ethyl acetate extracts are richer in the above mentioned biologically active substances and have more pronounced antioxidant activity.

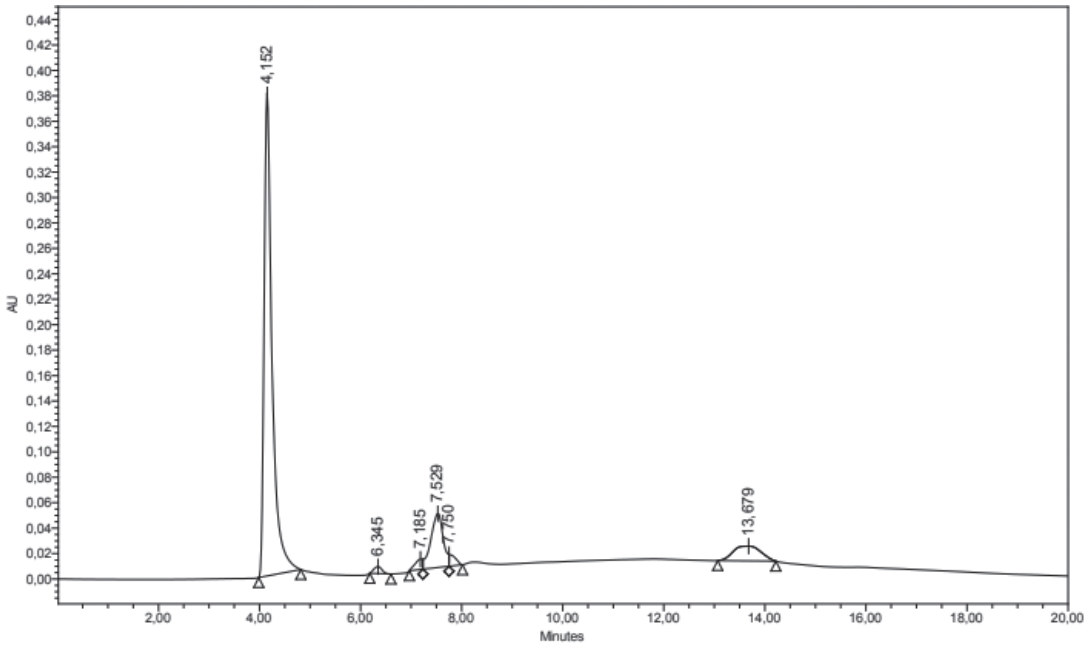

Figure 3. Results of chromatography of organic acids in ethyl acetate extract of Osage Orange.

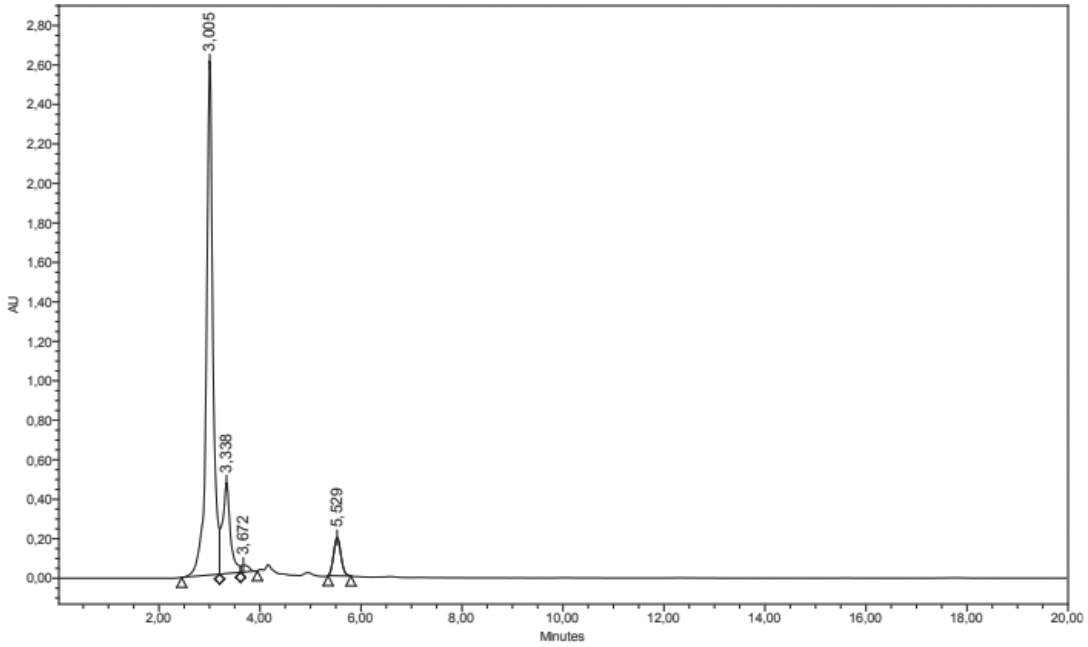

Figure 4. Results of quantification and identification of flavonoids in aqueous extract of Osage Orange.

Table 3. Results of chromatography of organic acids in ethyl acetate extract of Osage Orange.

	Name	Retention time	Area	% Area	Height	Conc. mg/mL
1	Oxalic acid	4,152	4157399	75.01	379271	1.029
2		6,345	60470	1.09	5413	
3		7,185	83573	1.51	8100	
4		7,529	719133	12.98	42597	
5		7,750	80286	1.45	9223	
6		13,679	441279	7.96	11583	

Table 4. Results of quantification and identification of flavonoids in aqueous extract of Osage Orange.

	Name	Retention time	Area	% Area	Height	Conc. mg/mL
1	Tannic acid	3,005	23515994	76.14	2609491	3,016
2		3,338	5060321	16.38	459709	
3		3,672	356487	1.15	33379	
4		5,529	1954134	6.33	193737	

The presence of high antioxidant activity in extracts can be explained by the fact that the investigated extracts contained multifunctional BAS together with easily oxidized functional groups (for example, -SH, $(CH_3)_2CH$-), that relatively faster bind free radicals formed in living organisms.

The results of HPLC and quantitative analysis of substances with regenerative properties are presented in Table 10.

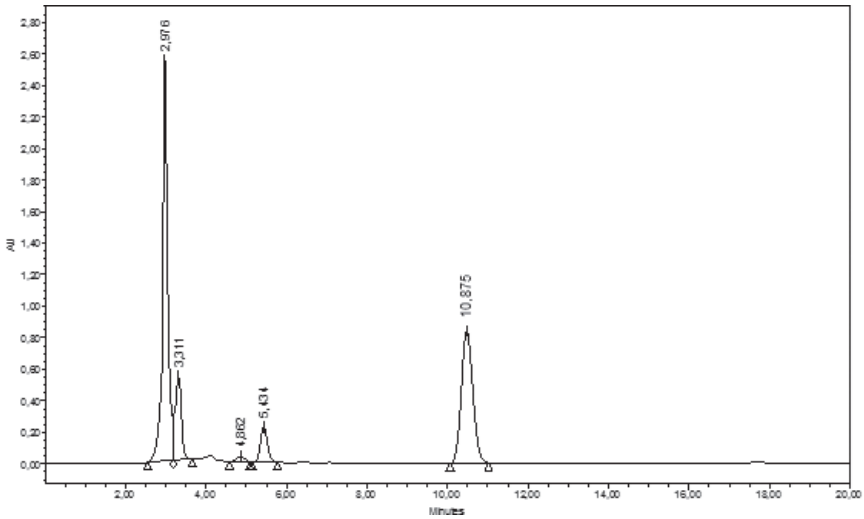

Figure 5. Results of quantification and identification of flavonoids in ethanol extract of Osage Orange.

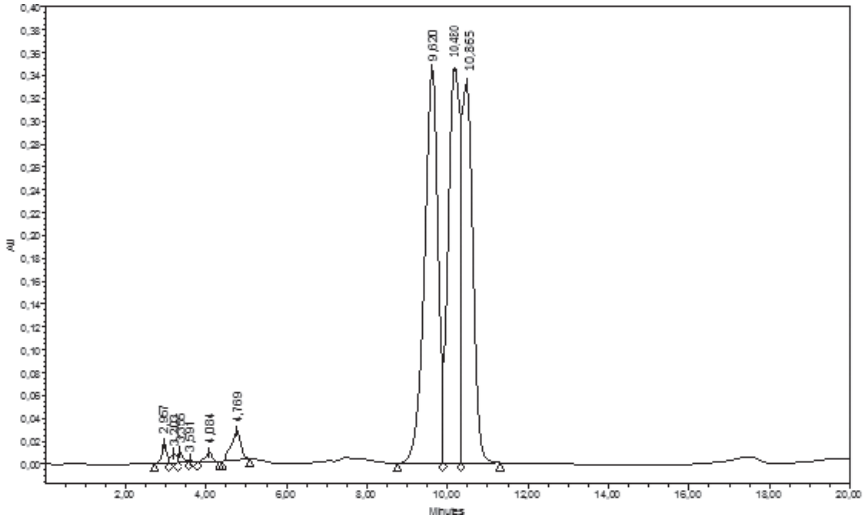

Figure 6. Results of quantification and identification of flavonoids in ethyl acetate extract of Osage Orange.

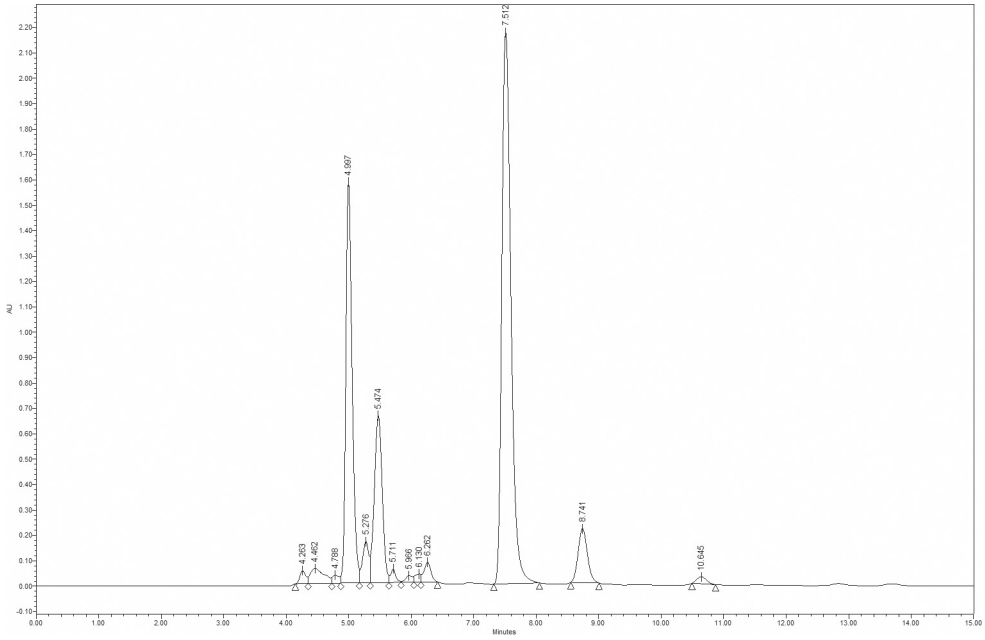

Figure 7. Quantitative content of vitamin C in aqueous extract of Osage Orange.

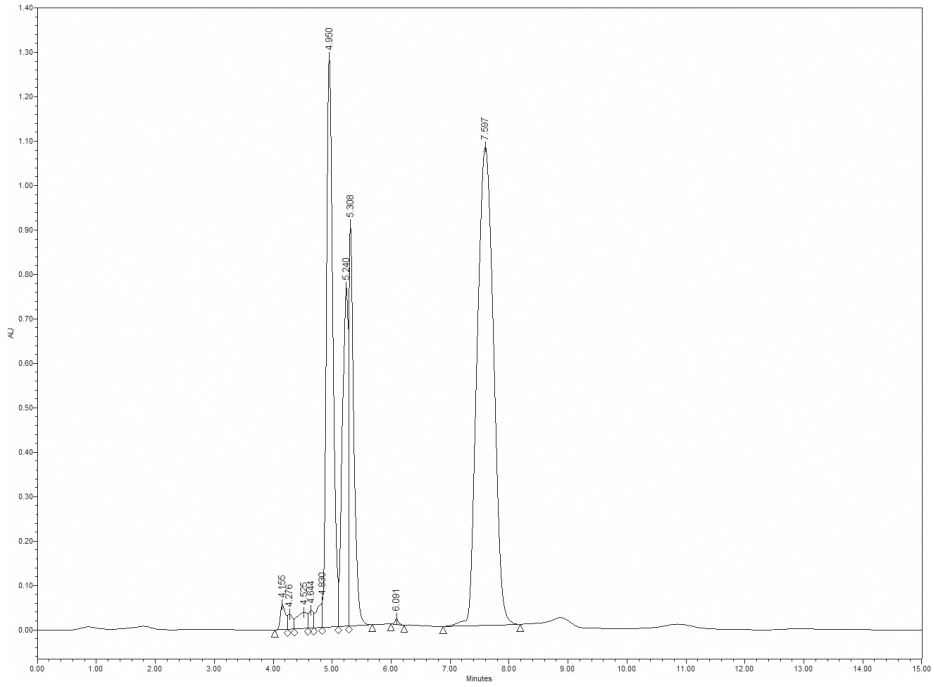

Figure 8. Quantitative content of vitamin C in ethanol extract of Osage Orange.

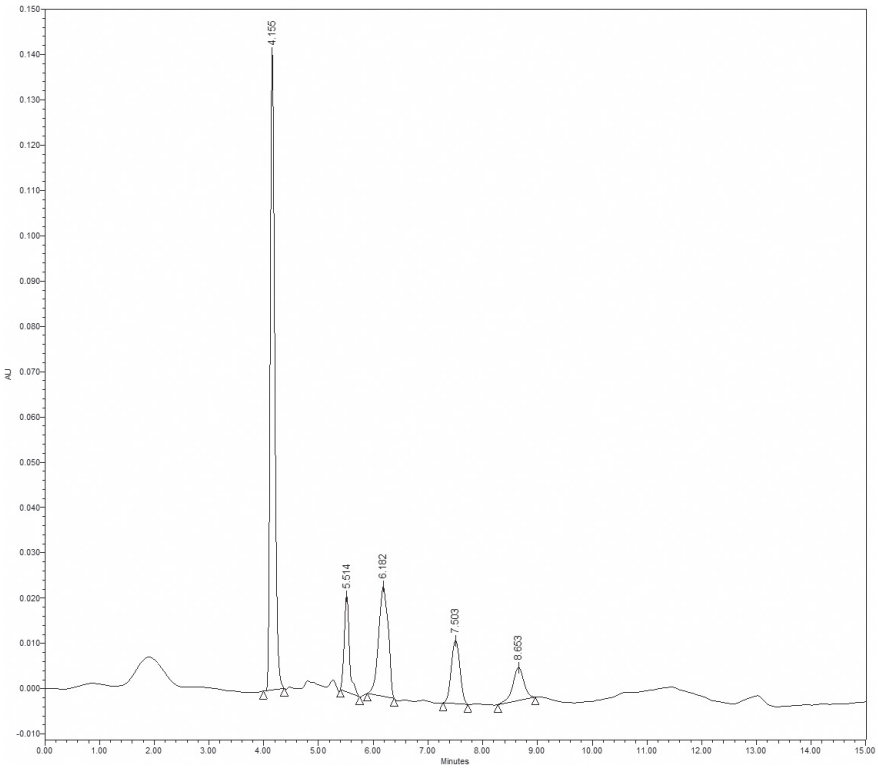

Figure 9. Quantitative content of vitamin C in ethyl acetate extract of Osage Orange.

Table 5. Results of quantification and identification of flavonoids in ethanol extract of Osage Orange.

	Name	Retention time	Area	% Area	Height	Conc. mg/mL
1		2,976	22533959	46.97	2573678	
2		3,311	5766580	12.02	530493	
3		4,862	435700	0.91	32132	
4		5,434	2673574	5.57	223008	
5	Quercetin	10,875	16569058	34.53	841168	0.885

As can be seen from the data presented in the Table 10, flavonoids, tannins, vitamins C, P, carboxylic acids and other biologically active substances were found in the extracts obtained from the fruits of maclura orange growing in Armenia (Yerevan, Nor Nork). Experimental studies show that aqueous and ethanol extracts of maclura are distinguished for high content of the above mentioned biologically active substances.

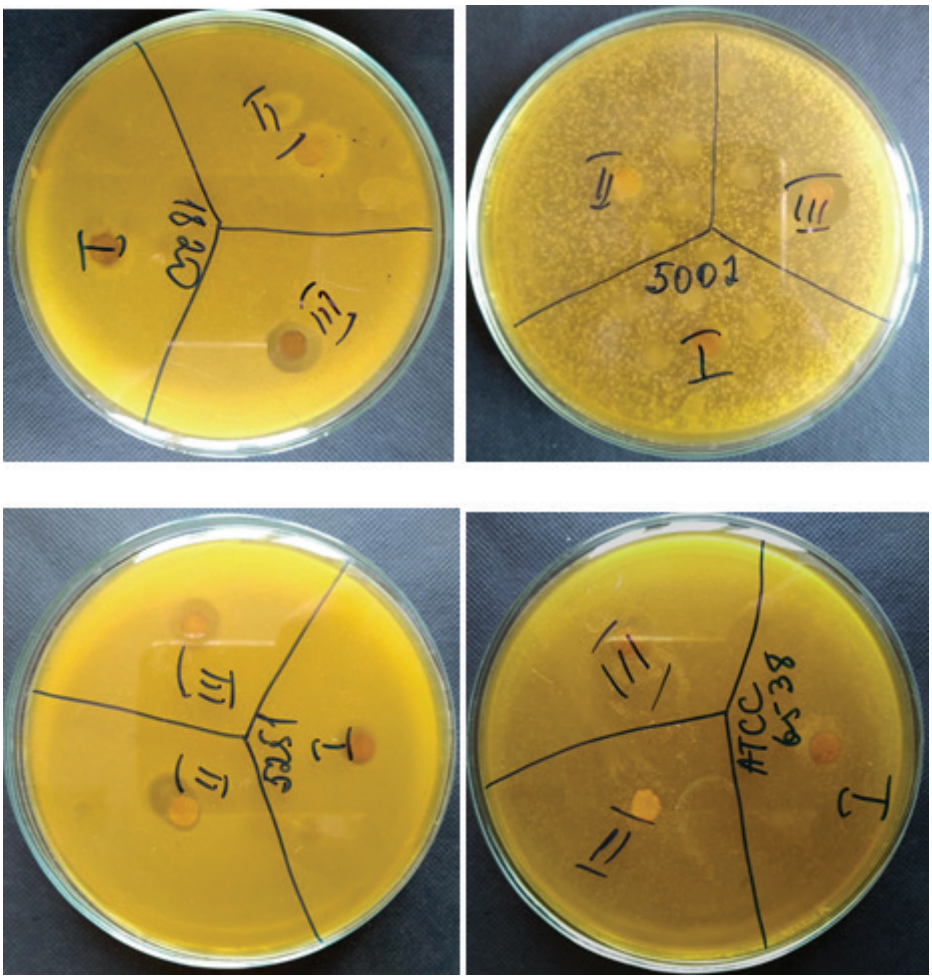

Figure 10. Evaluation of the antimicrobial activity of Osage Orange extracts on strains of *Bacillus subtilis* 1820, E. Coli 5002, *Serratia marcescens* 5251 and *Staphylococcus aureus* ATCC-6538.

Table 6. Results of quantification and identification of flavonoids in ethyl acetate extract of Osage Orange.

	Name	Retention time	Area	% Area	Height	Conc. mg/mL
1		2,957	145294	0.66	18258	
2		3,203	82294	0.37	7876	
3		3,355	78481	0.36	9281	
4		3,591	12516	0.06	2007	
5		4,084	90515	0.41	7968	
6		4,769	411845	1.87	25032	
7		9,620	7982247	36.18	344978	
8		10,480	6897562	31.27	347672	
9	Quercetin	10,865	6360149	28.83	331168	0.340

Table 7. Quantitative content of vitamin C in aqueous extract of Osage Orange.

	Name	Retention time	Area	% Area	Height	Conc. mg/mL
1		4,263	342839	0.76	50870	
2		4,462	908973	2.01	58952	
3		4,788	239527	0.53	31166	
4	Vitamin C	4,997	10991905	24.30	1579617	0.27
5		5,276	1173897	2.59	162786	
6		5,474	5774006	12.76	657596	
7		5,711	303754	0.67	53656	
8		5,966	206448	0.46	26028	
9		6,130	194335	0.43	31603	
10		6,262	609509	1.35	78206	
11		7,512	22012404	48.66	2172692	
12		8,741	2187446	4.84	213267	
13		10,645	296205	0.65	27386	

At the next stage, we studied the antimicrobial activity. The extracts were evaluated using clinical strains of microorganisms characterized by varying degrees of resistance to modern antimicrobial drugs. The research results are presented in Fig. 10. We found that maclura orange contained compounds that could inhibit the growth of microorganisms *Bacillus subtilis* 1820, *E. Coli* 5002, *Serratia marcescens* 5251 and *Staphylococcus aureus* ATCC-6538. The presence of compounds with antimicrobial properties in extracts depended on the nature of the solvent.

I. Aqueous
II. Ethyl acetate
III. Ethanol

As can be seen from the figures, only 70% ethanol extract of maclura had the antimicrobial effect on the strains of *Bacillus subtilis* 1820, *E. coli* 5002,

Table 8. Quantitative content of vitamin C in ethanol extract of Osage Orange.

	Name	Retention time	Area	% Area	Height	Conc. mg/mL
1		4,155	343799	0.81	55852	
2		4,276	198768	0.47	33680	
3		4,525	442036	1.04	36387	
4		4,644	223404	0.53	39602	
5		4,830	367434	0.87	57371	
6	Vitamin C	4,950	9797336	23.07	1282931	0.24
7		5,240	5336482	12.56	762387	
8		5,308	4911080	11.56	904638	
9		6,091	54602	0.13	13663	
10		7,597	20796881	48.97	1076773	

Table 9. Quantitative content of vitamin C in ethyl acetate extract of Osage Orange.

	Name	Retention time	Area	% Area	Height	Conc. mg/mL
1		4,155	806177	53.92	140581	
2		5,514	139621	9.34	21144	
3		6,182	293835	19.65	24296	
4		7,503	152459	10.20	13890	
5		8,653	103081	6.89	7275	

Serratia marcescens 5251 and *Staphylococcus aureus* ATCC-6538 causing the growth inhibition zone of 1.1–1.3 cm. Ethyl acetate extract specifically inhibited only the *Serratia marcescens* 5251 strain, causing the growth inhibition zone of 1.1 cm. and the aqueous extract had no effect on the studied strains.

Table 10. Results of HPLC and quantitative analysis of substances with regenerative properties.

BAS	Extracts		
	Aqueous	Ethanol	Ethyl acetate
Oxalic acid (mg/ml)	0.134	0.23	1,029
Malic acid (mg/ml)	0.63	17.56	0
Succinic acid (mg/ml)	12.93	0	0
Tannic acid (mg/ml)	3,016	0	0
Quercetin (mg/ml)	0	0.885	0.34
Vitamin C (mg/ml)	0.27	0.24	0
Antioxidants (mg/g)	35.89	127,27	145,83

Conclusions

The aqueous, ethanol, ethyl acetate extracts of maclura were obtained, and quantitative analysis of a number of biologically active substances was carried out by HPLC.

The study of the antioxidant activity showed that ethyl acetate extract was richer in substances with antioxidant activity.

Evaluation of the antimicrobial activity of the extracts showed that 70% ethanol extract exhibited the greatest antibacterial activity.

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