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ANALYSIS OF COMPOSITION AND ANTIMICROBIAL ACTIVITY OF POMEGRANATE (*PUNICA GRANATUM*) FRUIT EXTRACT AGAINST GRAM-NEGATIVE MULTI-DRUG RESISTANT BACTERIA FROM CLINICAL ISOLATES

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ABSTRACT

The basic therapy against microbial infections is application antibiotics. However, overuse of antibiotics has become the major factor for the emergence and dissemination of multi-drug resistant strains. The purpose of our work was to study the phytochemical composition of a thick extract of pomegranate fruits, as well as to investigate *in vitro* and *in silico* antimicrobial activity against clinical multidrug-resistant strains of *A. baumani*, *P. aeruginosa*, *K. pneumonia* and *E. cloacae*. The quantification of biologically active substances (BAS) was accomplished with spectrophotometric, titrimetric and HPLC methods of analysis; antimicrobial effects were evaluated by the well method and minimum inhibition concentration. The total content of phenolic compounds was 0.40 and 10.10%, organic acids – 5.80 and 1.60% for pomegranate fruit thick and green tea leaf extract. The total content of anthocyanins in the pomegranate fruit thick extract was 1071.0 mg/kg, where cyanidin-3-O-glucoside was dominated (417.4±8.4 mg/kg). Theoretical studies have found that neither single antibiotic nor anthocyanins highly effectively inhibits all antimicrobial mechanisms of resistant gram-negative bacteria. Thick pomegranate fruit extract actively inhibits all resistant strains of *A. baumannii*, *K. pneumonia*, *P. aeruginosa*, and *E. cloacae*. These findings have shown that to inhibit resistant strains of bacteria, you need to use only a complex drug or jointly apply dietary supplements of pomegranate, and in turn, herbal medicines are a "lifeline" for their creation and there is a chance to return old antimicrobial drugs in life.

Keywords: pomegranate fruit; anthocyanins; multi-drug resistant; Gram-negative strains; molecular docking

INTRODUCTION

Today, antimicrobial resistance is the number one problem worldwide. One of the first mentions of the emergence of antibiotic-resistant strains of bacteria in humans was obtained during military conflicts in Iraq and Afghanistan 20 years ago **(Mende *et.al.*, 2022)**.

To date, no statistics have been officially published on the resistant strains of bacteria that have been isolated from combat wounds during the current conflict in Ukraine. However, between 2014 and 2020, statistics have shown that the detection rate of multi-resistant strains of bacteria in combat wounds was significantly higher than in civilian hospitals **(Kondratiuk *et.al.*, 2021)**. In addition, according to the latest data, it has found that *Acinetobacter baumani*, *Pseudomonas aeruginosa* and *Klebsiela pneumonia* are predominant among all isolated pathogens. Among all gram-negative bacteria (*A. baumani*, *P. aeruginosa and K. pneumonia*), 71.3% were resistant to the antibiotic carbapenem, which is the last "line of defense" against resistant strains **(Petrosillo *et.al.*, 2023)**. In March 2022, the European Center for Disease Prevention and Control reported that Ukrainian refugees with traumatic wounds may have resistant strains of *A. baumani*, *K. pneumonia*, and made recommendations for isolating isolates and conducting screening studies **(WHO/Europe, 2022)**. At a German clinic in Frankfurt am Main, staff reported treating traumatic wounds in 103 Ukrainian patients between March and June 2022. Among all admitted patients, 17% had resistant gram-negative strains of bacteria **(Vučić *et.al.*, 2019)**. Thus, in light of data on the rapid spread of resistant strains of bacteria, it is necessary to search for new antimicrobial compounds.

The perspective source of anthocyanins was chosen pomegranate fruits, whereas a green tea leaf is the source of catechins. Pomegranate (*Punica granatum* L.) is a fruit-bearing deciduous tree that belongs to the family *Lythraceae*, the cultivation has carried out in the region with a warm climate such as South Caucasus, Transcaucasia, South and Central Asia and in Mediterranean region **(Vučić *et.al.*, 2019)**. The chemical composition of peel is represented by ellagotannins, catechins, flavonoids, whereas arils contain mostly anthocyanins and organic acids **(Maslov *et.al.*, 2023)**. The composition of green tea leaf (*Camellia sinensis* L.) contains: catechins (epigallocatehin-3-O-gallate, epicatehin, (+)-catechin, epigallocatechin), organic acids (oxalic acid), flavonoids (rutin) and hydroxycinnamic acids (caffeic acid) **(Maslov *et.al.*, 2021)** .

Thus, the purpose of our work is to study the phytochemical composition of pomegranate fruit thick extract, as well as to investigate *in vitro* and *in silico* antimicrobial activity against clinical multidrug-resistant strains of *A. baumani*, *P. aeruginosa*, *K. pneumonia* and *E. cloacae*.

MATERIAL AND METHODS

Plant material

The study focused on *P. granatum* fruits, which were harvested from areas where they are naturally grown. The collection took place in 2022 following the fruiting season, Abkhazia (42°95′29″ N, 41°13′43″ E). The study focused on the leaf of *C. sinensis* from the Chun Myn variety, which was gathered as raw material in the Anhui province of China during the months of March through May.

Reagents

"Acetonitrile (purchased from "Allchem", Kharkiv), acetic acid (purchased from "Allchem", Kharkiv), phosphoric acid (purchased from "Allchem", Kharkiv), cyanidine-3-O-glucoside (≥98.0%), cyanidin-3-O-rutinoside (≥98.0%), cyanidin-3-O-malonyl glycoside (≥98.0%), cyanidin-3-O-xyloside (≥98.0%), cyanidin-3,3'-diglucoside (≥98.0%) were purchased in Sigma Aldrich Company, Lublin, Poland.

Phytochemical analysis

The total content of phenolic compounds was measured by the Folin-Ciocaltau assay, the absorbance was measured at 760 nm **(Maslov *et.al.*, 2023)**. The vanillin reagent assay was applied to determine the total catechins **(Maslov *et.al.*, 2023)**; the absorbance was measured at 505 nm. The total anthocyanin content was determined by the molecular adsorption analysis, the absorbance was measured at 546 nm **(Maslov *et.al.*, 2023)**. The total organic acids content was determined by acid-base titration with the fixation end-point by potentiometric method **(Maslov *et.al.*, 2021)**.

Extraction procedure

A 100.0 g (exact mass) of *P. granatum* fruits was pressed, then it was added of 96% ethanol in a threefold amount to the extraction, after that filtration, then obtained filtrate was concentrated by a vacuum-evaporator at a temperature of 50-60°C until the humidity of the extract is 25%.

HPLC analysis of *P. granatum* fruit thick extract

Antocyanins content was quantified by HPLC Perkin-Elmer Series 400 and a semi-preparative Dynamax Rainin Model SD-300 Liquid Chromatograph, equipped with a Hewlett-Packard 1040A photodiode array detector. The analytical HPLC system used a 250 4.6-mm inner dia Prodigy 5 ODS 3 column (Phenomenex). The mobile phases used were set up as a binary solvent system, which consisted of: (A) 100% acetonitrile and (B) 5% acetonitrile, 10% acetic acid, and 1% phosphoric acid. The two solvents were used in a gradient of:0–5 min 100% B, 5–20 min 20–80% A/B, 20–25 min 40–60% A/B, and 25–30 min 100% B. The flow rate was 1 mL/min; injection volume was 50 µL. Samples were filtered through a 0.45-m Millipore filter type HA (Millipore Corp., Bedford, Mass., U.S.A.). Detection was at 520 nm with amounts of individual peaks being reported as the percentage of total peak area.

Test organisms

A three clinical isolates of multidrug-resistant Gram-negative bacteria were chosen for research: *Acinetobacter baumannii 150, Klebsiella pneumonia 18* and *Pseudomonas aeruginosa 18.* Isolates from clinical samples including tracheal aspirate and broncoalveolar lavage, were provided by Mechnikov Institute of Microbiology and Immunology of the NAMS of Ukraine, Kharkiv. All strains are stored and accepted by the Head of Museum of strains – O.G. Peretyatko. *Acinetobacter baumannii 150, Klebsiella pneumonic 18, Pseudomonas aeruginosa 18* and *E. cloacae 18* were accepted at 01 November 2022.

Screening antimicrobial activity of extracts

The method of diffusion of the drug into agar carried out using the method of "wells" **(Maslov *et.al.*, 2022)**. as a standards it was applied following antibiotics: 30 µmg/disk of chloramphenicol, 1.86 mM; 5 µmg/disk levofloxacin, 0.28 mM, 30 µmg/disk gentamycin, 0.42 mM; 30 30 µmg/disk doxycycline, 1.35 mM; 30 µmg/disk ceftriaxone, 1.08 mM; 30 µmg/disk ceftazidime, 0.94 mM; 30 µmg/disk cefepime, 1.25 mM; 5 µmg/disk moxifloxacine, 0.23 mM; 30 µmg/disk netilmycine, 0,42 mM; 30 µmg/disk amoxicillin, 1.64 mM.

Table 1 Interpretation criteria for microbial sensitivity

Microbial sensitivity	Diameter of the growth retardation zone, mm
High sensitivity	>25
Sensitive	15-25
Low sensitivity	10-15
Not sensitivity	<10

Assay of determination of minimum inhibitory concentration (MIC)

MIC is the lowest concentration of antibacterial agent that completely inhibits the bacterial growth. The MIC of the different extracts was assessed using the broth microdilution method **(Mbarga *et.al.*, 2021)**.

Molecular docking

A molecular docking study was conducted using the tool known as AutoDockTools 1.5.6. The preparation of the protein involved an optimization process, which included the removal of water and other atoms, followed by the addition of a polar hydrogen group. Autogrid was used to configure the grid coordinates (X, Y, and Z) on the binding site. Genetic algorithm parameters were applied for ligand interaction, with 10 runs of this criterion.
DNA-gyrase (PDB ID: 1KIJ), DHFR (PDB ID: 1RX3), deacytelese (PDB ID: 3UHM), acyl-homoserinelactone synthase (AHS) LasI (PDB ID: 1RO5), acyl-homoserinelactone synthase (AHS) RhI (PDB ID: 1KZF), diguanylate cyclase (PDB ID: 3BRE) structures were obtained from PDB database **(RCSB PDB, 2023)**. The resolution of 1KIJ was 2.30 Å, 1RX3 – 2.20 Å, 3UHM – 2.20 Å, 1RO5 – 2.30 Å, 1KZF – 2.20 Å, 3BRE – 2.40 Å. For docking experiment protein structure is selected if resolution above 2 Å. So, all mentioned proteins can be used for the experiment. The ligand structures of cyanidin-3-O-glucoside (CID_12303220), cyanidin-3-O-rutinoside (CID_29231), cyanidin-3-O-malonyl glycoside (CID_443915), cyanidin-3-O-xyloside (CID_87948385) and cyanidin-3,3'-diglucoside (CID_44256727) were obtained from PubChem database **(PubChem, 2024)**. The active site of the docking protein was identified utilizing the Computed Atlas for Surface Topography of Proteins (CASTp) **(Tian *et.al.*, 2018)**.

RESULTS

Phytochemical analysis of BAS

According to obtained results shown in table 3, the green tea leaf extract (10.10±0.25%) had higher content of phenolic compounds, than in *P. granatum* thick fruit extract (0.40±0.02%).
Table 3 demonstrates that the total content of anthocyanin in *P. granatum* thick fruit extract was 0.11±0.002%, whereas in green tea leaf extract anthocyanin was not presence. The percentage of anthocyanin out of total polyphenols was 28% in *P. granatum* extract.
The highest amount of organic acids was determined in *P. granatum* thick fruits extract (5.80±0.50%), whereas in the green tea leaf extract it was lower 72% (1.60±0.10%). In *P. granatum* extract, the total organic acids were in 14.5 times higher than polyphenols, whereas in the green tea leaf, the total organic acids were in 6.3 times lower than polyphenols. (Table 3)

Table 3 Quantitative content of total phenolic compounds, anthocyanin and organic acids in *P. granatum* fruit thick extract

Sample	Total phenolic compounds, %±SD	Total anthocyanin, %±SD	Total catechin, %±SD	Total of organic acids, %±SD
P. granatum fruit thick extract	0.40±0.02	0.11±0.002	—	5.80±0.50
Green tea leaf extract	10.10±0.25	—	10.47±0.25	1.60±0.10

Legend: SD – standard deviation, n=3

The HPLC method was used to carry out a qualitative and quantitative analysis of anthocyanins in the obtained extracts of *P. granatum* fruits extract. According to the results of the study, 6 anthocyanins were identified in *P. granatum* fruits extract (Fig. 2).
As shown in Table 4, cyanidin-3-O-glucoside dominated among all anthocyanins (38.98% out of the total anthocyanins), pelargonidin-3,5-diglucoside (19.49% out of the total anthocyanins) was in second place, and the lowest content was delphinidin-3-O-glucoside (4.66% out of the total anthocyanins).

Table 4 Chemical composition of antocyanins in *P. granatum* fruit thick extract by HPLC analysis

№	Antocyanins	Retention time, min	Content of antocyanins in extract, mg/kg of extract ±SD	part out of total antocyanins
1	Cyanidin-3,5'-diglucoside	6.250	127.0±2.5	11.86
2	Pelargonidin-3,5-diglucoside	7.650	208.7±4.2	19.49
3	Delphinidin-3,5-diglucoside	9.320	190.6±3.8	17.80
4	Cyanidin-3-O-glycoside	12.470	417.4±8.4	38.98
5	Pelargonidin-3- O -glycoside	16.120	77.1±1.5	7.20
6	Delphinidin-3-O-glucoside	21.700	49.9±1.0	4.66
	THE TOTAL ANTOCYANINS		**1071.0**	

Legend: SD – standard deviation, n=3

Molecular docking analysis

The next stage of our research was to conduct a theoretical investigation of the antimicrobial activity of the identified compounds using molecular docking for realsing their promising capabilities for suppressing the growth of gram-negative strains of bacteria. The assessment the antimicrobial effect was conducted with six key enzymes: DNA-gyrase, DHFR, Deacytelese, AHS LasI, AHS RhI and Diguanylate cyclase. A six groups of the most applied antimicrobial drugs were chosen as standards of comparison in theoretical study such as a group of tetracyclines (Doxycycline), aminoglycosides (Gentamycin, Netilmycine), fluoroquinolones (Moxifloxacine, Levofloxacin), β-lactames (Cefepime, Ceftazidime, Ceftriaxone), penicillins (Amoxicillin), and amphenicols (Chloramphenicol).
In the indexed scientific journals Scopus and Web of Science, there are a large number of works with molecular docking on the study of the pharmacological activity of different groups of compounds. But, the main problem of these studies is the lack of rating assessment of the efficiency of binding of the ligand to the active site. A number of scientific works have used comparison standards, but in

our view, this method is not promising as since more than one standard may be used for the enzyme protein being studied. Thus, this method of assessment will lead to confusion in the data among scientists. In this work, we will use a conditional rating classification based on the value of binding energy. This rating is based purely on our results obtained during our theoretical research. We propose to divide the binding energy value into three levels: low (binding energy up to -4.5 kcal/mol), medium (binding energy from -4.5 to -9.5 kcal/mol) and high (binding energy > -9.5 kcal/mol).

Molecular modeling of the identified compounds was carried out with the active site of DNA-gyrase. The active site was represented by the following amino acids: Arg75, Lys102, Arg135, Asp80. Trp387, Lys109, Asp72 and Thr166. According to the results of the study and conditional rating, it was established that doxycycline, cyanidin-3,5'-diglucoside, delphinidin-3,5-diglucoside, moxifloxacine, pelargonidin-3-O-glycoside, delphinidin-3-O-glucoside, cyanidin-3-O-glycoside had high affinity to the active site, whereas the lowest level had gentamycin. (Table 5)

The next enzyme that was studied was DHFR. The active center of this enzyme was represented by the following amino acids: NADP, Tyr110, Asp30, Ile8, Phe34, Ile104, Arg55, Arg60. According to the results shown in Table. 5, the following compounds had high binding energies: doxycycline, cyanidin-3-O-glycoside, pelargonidin-3-O-glycoside, moxifloxacine, cyanidin-3,5'-diglucoside, delphinidin-3-O-glucoside, delphinidin-3,5-diglucoside and netilmycine. (Table 5)

Molecular modeling of the studied compounds was carried out with the active site of Deacytelese. The active center was represented by the following amino acids: Thr190, Lys238, Gly92. Phe191, Leu18, Ala206. According to the results of the study and conditional rating, it was established that pelargonidin-3,5-diglucoside, netilmycine, doxycycline, delphinidin-3,5-diglucoside, ceftazidime, moxifloxacine, cyanidin-3,5'-diglucoside, cyanidin-3-O-glycoside had high affinity to the active site. (Table 5)

Table 5 Results of molecular docking of the compounds identified by the HPLC in the *P. granatum* thick extract and antimicrobials drug standards with the DNA-gyrase, DHFR and deacytelese structures

№	Ligand	DNA-gyrase	Ligand	DHFR	Ligand	Deacytelese
		ΔGbind[a] (kcal/mol)				
1	**Doxycycline**	**-11.59**	**Doxycycline**	**-11.59**	Pelargonidin-3,5-diglucoside	-11.48
2	Cyanidin-3,5'-diglucoside	-11.59	Cyanidin-3-O-glycoside	-11.42	**Netilmycine**	**-11.09**
3	Delphinidin-3,5-diglucoside	-11.57	Pelargonidin-3-O-glycoside	-11.11	**Doxycycline**	**-11.03**
4	**Moxifloxacine**	**-10.29**	**Moxifloxacine**	**-10.89**	Delphinidin-3,5-diglucoside	-10.99
5	Pelargonidin-3-O -glycoside	-10.15	Cyanidin-3,5'-diglucoside	-10.79	**Ceftazidime**	**-10.37**
6	Delphinidin-3-O-glucoside	-10.10	**Netilmycine**	**-10.70**	Cyanidin-3,5'-diglucoside	-10.21
7	Cyanidin-3-O-glycoside	-9.69	Delphinidin-3-O-glucoside	-10.57	**Moxifloxacine**	**-9.78**
8	Pelargonidin-3.5-diglucoside	-9.15	Delphinidin-3,5-diglucoside	-10.36	Cyanidin-3-O-glycoside	-9.74
9	**Netilmycine**	**-9.00**	**Ceftazidime**	**-9.49**	Delphinidin-3-O-glucoside	-9.61
10	**Levofloxacin**	**-8.69**	**Levofloxacin**	**-8.98**	**Cefepime**	**-8.77**
11	**Cefepime**	**-8.27**	**Cefepime**	**-8.37**	Pelargonidin-3- O -glycoside	-8.66
12	**Amoxicillin**	**-7.24**	Pelargonidin-3.5-diglucoside	-8.22	**Levofloxacin**	**-8.34**
13	**Ceftazidime**	**-6.48**	**Chloramphenicol**	**-7.97**	**Gentamycin**	**-7.45**
14	**Chloramphenicol**	**-6.38**	**Amoxicillin**	**-7.87**	**Chloramphenicol**	**-7.19**
15	**Ceftriaxone**	**-4.61**	**Gentamycin**	**-6.78**	**Amoxicillin**	**-6.64**
16	**Gentamycin**	**-4.08**	**Ceftriaxone**	**-6.36**	**Ceftriaxone**	**-6.09**

Legend: red colour – low level of affinity ΔG <4.5 kcal/mol; orange colour – medium level of affinity 4.5<ΔG<9.5 kcal/mol; green colour – high level of of affinity ΔG>9.5 kcal/mol

The AHS LasI was next enzyme that was studied by molecular docking. The active center of this enzyme was represented by the following amino acids: Thr142, Thr144, Val143, Phe27, Arg30, Arg104, Met79, Leu102, Phe106, Ser103. According to the results shown in Table. 6, the following compounds had high binding energy: chloramphenicol, whereas levofloxacin had the lowest level of free energy as well as cyanidin-3-O-glycoside, cyanidin-3,5'-diglucoside, pelargonidin-3,5-diglucoside, delphinidin-3,5-diglucoside, pelargonidin-3-O-glycoside gentamycin, ceftazidime, cefepime and netilmycine were not interact with active center of AHS LasI. (Table 6)

Molecular modeling of the studied compounds was carried out with the active site of AHS RhI. The active center was represented by the following amino acids: Asp48, Tyr54, Met42. Leu63, Leu56. According to the results of the study and conditional rating, it was established that cyanidin-3,5'-diglucoside, pelargonidin-3-O-glycoside, delphinidin-3-O-glucoside, doxycycline, pelargonidin-3,5-diglucoside, whereas ceftriaxone had the lowest level of binding as well as cyanidin-3-O-glycoside and gentamycin were not interact with protein. (Table 6)

The diguanylate cyclase was the last protein enzyme that was assessed by molecular docking. The active center was represented by the following amino acids: Glu254, Glu253, Glu252, Lys327, Arg331, Thr262, Arg198, Arg194. The obtained results showed that there was any compound possessing high affinity energy. All compounds except gentamycin and cefepime had medium level of energy affinity. (Table 6)

Table 6 Results of molecular docking of the compounds identified by the HPLC in the P. granatum thick extract and antimicrobials drug standards with the AHS LasI, AHS RhI, diguanylate cyclase structures

№	Ligand	AHS LasI	Ligand	AHS RhI	Ligand	Diguanylate cyclase
				ΔGbind[a] (kcal/mol)		
1	**Chloramphenicol**	**-10.76**	Cyanidin-3,5'-diglucoside	-12.85	**Doxycycline**	**-9.14**
2	**Ceftriaxone**	**-6.56**	Pelargonidin-3-O -glycoside	-12.60	Pelargonidin-3- O -glycoside	-8.10
3	**Amoxicillin**	**-6.55**	Delphinidin-3-O-glucoside	-12.30	**Ceftazidime**	**-8.06**
4	**Moxifloxacine**	**-6.34**	Pelargonidin-3,5-diglucoside	-12.13	Cyanidin-3.5'-diglucoside	-7.86
5	**Doxycycline**	**-4.99**	Delphinidin-3.5-diglucoside	-11.12	Cyanidin-3-O-glycoside	-7.63
6	**Levofloxacin**	**-4.11**	**Doxycycline**	**-10.99**	Delphinidin-3.5-diglucoside	-7.44
7	Cyanidin-3-O-glycoside	—	**Netilmycine**	**-8.36**	Delphinidin-3-O-glucoside	-7.39
8	Cyanidin-3,5'-diglucoside	—	**Moxifloxacine**	**-8.27**	**Chloramphenicol**	**-6.59**
9	Pelargonidin-3,5-diglucoside	—	**Amoxicillin**	**-7.41**	**Moxifloxacine**	**-6.3**

10	Delphinidin-3,5-diglucoside	—	**Levofloxacin**	**-6.62**	Pelargonidin-3.5-diglucoside	-6.13
11	Pelargonidin-3- O -glycoside	—	**Ceftazidime**	**-6.43**	**Netilmycine**	**-6.06**
12	**Gentamycin**	—	**Chloramphenicol**	**-5.88**	**Amoxicillin**	**-5.89**
13	**Ceftazidime**	—	**Cefepime**	**-5.05**	**Levofloxacin**	**-5.32**
14	**Cefepime**	—	**Ceftriaxone**	**-4.48**	**Ceftriaxone**	**-5.19**
15	**Netilmycine**	—	Cyanidin-3-O-glycoside	—	**Gentamycin**	**-4.49**
16	Delphinidin-3-O-glucoside	—	**Gentamycin**	—	**Cefepime**	**-4.40**

Legend: red colour – low level of affinity ΔG <4.5 kcal/mol; orange colour – medium level of affinity 4.5<ΔG<9.5 kcal/mol; green colour – high level of of affinity ΔG>9.5 kcal/mol

Further, all antibiotics and anthocyanins were conditionally divided into two categories. The first category included compounds that had a high affinity for the active site, and the second category included compounds that had medium and low binding energies. This compound separation approach was necessary to clearly identify compounds that interact highly effectively with antimicrobial mechanisms and which compounds work below this level. According to the results obtained shown in Table 7, it can be seen that among all antibiotics, doxycycline works best, which inhibits all enzymes of the "first line of defense", and in the case of biofilm formation, only one mechanism was inhibited – AHS LasI. Amoxacillin, cefepime, ceftriaxone, gentamicin, levofloxacin was not highly effectively inhibiting any of the mechanisms of antimicrobial action presented above. In the case of anthocyanins, among all the compounds presented, cyanidin-3,5'-diglucoside, delphinidin-3,5-diglucoside and delphinidin-3-O-glucoside inhibit the largest number of mechanisms, namely this compound actively inhibits the "first line of defense" of bacteria, and one mechanism of biofilm formation – AHS RhI. In turn, pelargonidin-3,5-diglucoside was the most low active compound to inhibit the mechanisms of action.

Table 7 Schematic division of antimicrobial drug standards and identified anthocyanins in two categories

№	Compound	DNA-gyrase	DHFR	Deacytelese	AHS LasI	AHS RhI	Diguanylate cyclase	№ of inhibition enzymes of "First line of protection"	№ of inhibition enzymes of "Biofilm"
	Antimicrobial drug standards								
1	**Chloramphenicol**							0	1
2	**Levofloxacin**							0	0
3	**Gentamycin**							0	0
4	**Doxycycline**							3	1
5	**Ceftriaxone**							0	0
6	**Ceftazidime**							1	0
7	**Cefepime**							0	0
8	**Moxifloxacine**							3	0
9	**Netilmycine**							2	0
10	**Amoxicillin**							0	0
	Identified anthocyanins								
11	Cyanidin-3-O-glycoside							3	0
12	Cyanidin-3,5'-diglucoside							3	1
13	Pelargonidin-3,5-diglucoside							1	1
14	Delphinidin-3,5-diglucoside							3	1
15	Pelargonidin-3- O -glycoside							2	1
16	Delphinidin-3-O-glucoside							3	1

Legend: green colour – high level of inhibition; red colour – lower and medium of inhibition

Antimicrobial and anti-fungi activity

In this research work, the antimicrobial and antifungal activity of the obtained *P. granatum* thick fruit extract was investigated against the following antimicrobial resistance strains of *A. baumani*, *K. pneumonia*, *P. aeruginosa*, *E. cloacae*. According to the obtained results, extract obtained from the *P. granatum* fruit had an effective antimicrobial effect.

Among all Gram-negative strains *P. granatum* fruit extract was the most active against *E. cloacea*, whereas the lowest inhibition had against *K. pneumonia*. However, the strains of *A. baumani* and *P. aeruginosa* were also sensitive to the action of *P. granatum* fruit extract. Comparing obtained results with reference standards of antimicrobial drugs, it was established that *A. baumani*, *K. pneumonia*, *P. aeruginosa* were absolutely resistant to the action of antimicrobial drugs. In the case of *E. cloacae* only 3 antibiotics inhibited the growth of resistant strains as well as other reference standards were not affect to the growth of *E. cloacae* strain at all. (Table 8)

Table 8 Inhibition diameter (mm) resulting from the screening of antimicrobial effect against resistance strains of *A. baumani*, *K. pneumonia*, *P. aeruginosa*, *E. cloacae* by well diffusion method with of pomegranate thick extract and drug standards (Chloramphenicol, Levofloxacin, Gentamycin, Doxycycline, Ceftriaxone, Ceftazidime, Cefepime, Moxifloxacine, Netilmycine, Amoxicillin)

Sample	Concentration, mM	Diameter of the growth retardation zone, mm±SD			
		A. baumani 150	*K. pneumonia 18*	*P. aeruginosa 18*	*E. cloacae 17*
P. granatum thick extract	0.007[a]	15.50±0.50	17.00±0.20	16.00±0.50	16.50±0.20
Chloramphenicol	1.86	**Growth**	**Growth**	**Growth**	**Growth**
Levofloxacin	0.28	**Growth**	**Growth**	**Growth**	**Growth**
Gentamycin	0.42	**Growth**	**Growth**	**Growth**	**12.00**
Doxycycline	1.35	**Growth**	**Growth**	**Growth**	**12.00**
Ceftriaxone	1.08	**Growth**	**Growth**	**Growth**	**Growth**
Ceftazidime	0.94	**Growth**	**Growth**	**Growth**	**Growth**
Cefepime	1.25	**Growth**	**Growth**	**Growth**	**Growth**
Moxifloxacine	0.23	**Growth**	**Growth**	**Growth**	**Growth**
Netilmycine	0.42	**Growth**	**Growth**	**Growth**	**11.00**
Amoxicillin	1.64	**Growth**	**Growth**	**Growth**	**Growth**

Legend: SD – standard deviation, n=3, a – molar concertation of total phenolic compounds in terms of gallic acid

Minimum Inhibitory Concentrations (MIC)

The investigated extract *P. granatum* significantly inhibit the antibiotic resistant strains with MIC. In the previously above conducted antimicrobial study, the extract of *P. granatum* fruit extract was the most active independently of the tested strains. Table 8 shows, the *P. granatum* fruits extract with MIC value of 0.88 μM was the most active against *E. cloacae*. (Table 9)

Table 9 Minimal inhibitory concentration (μM) of the *P. granatum* thick extract against the resistance strains of *A. baumani*, *K. pneumonia*, *P. aeruginosa*, *E. cloacae*

Sample	MIC			
	A. baumanii	*K. pneumonia*	*P. aeruginosa*	*E. cloacae*
P. granatum thick extract	1.18	1.18	1.18	0.88

DISCUSSION

The content of BAS in *P. granatum* fruit extracts was quantified by spectrophotometric, titrimetric and HPLC methods of analysis. The organic acids were present in both extracts, where the highest content of organic acids was determined in *P. granatum* fruits extract than in *C. sinensis* leaf extract. In our view, it relates with different purpose of accumulation organic acids. The organic acids are precursor for biosynthesis of sugars in fruits, whereas in leaf, organic acids only play a role in photosynthesis as result there is no purpose of high accumulation organic acids in leaf **(Arena *et al.*, 2023)**. **Russo (2018)** investigated anthocyanin content of *P. granatum* fruit 50% methanol extract by HPLC method. According to their results, it was detected following anthocyanins (mg/kg per extract): cyanidin-3-O-glucoside (28.0 mg/kg), cyanidin-3-O-rutinoside (46.0 mg/kg), cyanidin-3-O-xyloside (42.0 mg/kg), pelargonidin-3-O-glucoside (17.0 mg/kg) and delphinidin-3-O-glucoside (11.0 mg/kg). Compering with our results, the content of anthocyanins in our research was lower, but cyaniding-3-O-glucoside was dominated in both extracts. The chemical composition of fruit is changed during fruits development, its ripening, and different cultivars.

The search of new antimicrobial drugs has become a very important task for scientific community. One of the way to create effective antimicrobial drugs is focused on the application of natural compounds such as catechins and anthocyanins. Nowadays, a large number of multidrug-resistant bacteria, also called "superbacteria," have been reported worldwide. Most of the "superbacteria" are represented by gram-negative bacteria such as *A. baumannii, K. pneumonia, P. aeruginosa* and *E. cloacae* **(Abinaya *et.al.*, 2019)**. In order to inhibit the growth of any bacteria, you need to effectively influence 3 main mechanisms: DNA gyrase, DHFR and inhibition of membrane formation. DNA gyrase is an enzyme responsible for the temporary division of bacterial DNA into two strands, subsequently the replication stage begins. The next important enzyme is DHFR; this enzyme is responsible for the formation of folic acid, which is necessary for the existence of bacteria **(Jogula *et.al.*, 2020)**. One of the main defense mechanisms of any bacteria is its membrane, and gram-negative strains are no exception to the rule. The membrane of gram-negative bacteria contains a special liposaccharide that causes an immune system response and fever. The enzyme UDP-3-O-(R-3-hydroxymyristoyl)-N-acetylglucosamine deacetylase is responsible for the synthesis of liposaccharide; this enzyme has no homologs in humans and mammals and is present only in bacteria **(Zuo *et.al.*, 2017)**.

But, the main problem of multi-resistant strains of bacteria is that they can form biofilms, thereby preventing the bacteria from penetrating antibiotics into the bacterial cell itself. The mechanism of biofilm formation in gram-negative bacteria is the formation of a quorum system. The quorum system is a type of cellular signaling that relies on the production and perception of chemical signaling molecules called autodoctors. For the formation of these signal molecules, the protein acyl-homoserine lactone synthetase LasI and RhI is responsible **(Abinaya *et.al.*, 2019)**. Also, one of the main stages of biofilm formation is the cell adhesion of bacteria to the surface. Adhesions require a signaling molecule, cyclic di-guanylate monophosphate (c-di-GMP). This molecule coordinates "the transition of the bacterial lifestyle from motile to immobile." c-di-GMP is synthesized from two molecules of guanylate triphosphate by the enzyme guanylate cyclase **(Valentini and Filloux, 2016)**. Thus, in order to inhibit the growth and development of "superbugs" it is necessary to act on six mechanisms: DNA gyrase, DHFR, deacetylases (membrane synthesis), AHS Las and RhI (biofilm formation), and diguanyl cyclase (cell adhesion).

According to the results obtained, it was found that not a single antibiotic and not a single anthocyanin highly effectively inhibits all mechanisms of antimicrobial action, which suggests that in order to inhibit the growth of "superbacteria," a complex antimicrobial drug or jointly apply with dietary supplements of pomegranate should be used. According to our results, chloramphenicol works highly effectively through only one mechanism - AHS LasI; doxycycline is effective against DNA gyrase, DHFR, deacetylase and AHS RhI; ceftazidime works well against deacetylase; Moxifloxacin is effective against DNA gyrase, DHFR and deacetylase, while netilmicin works only against DHFR and deacetylase.

A serious threat to human health is the emergence of "superbacteria". This issue is especially relevant in relation to *A. baumannii, K. pneumonia, P. aeruginosa* and *E. cloacae*. These bacterial strains are capable of causing nosocomial infections and respiratory associated pneumonia. The above-mentioned bacteria have been isolated that are resistant to aminoglycosides, fluoroquinolones, as well as to the action of the "last line of defense" - carbapenems **(Jean *et.al.*, 2022)**. The scientific community has identified 3 main mechanisms of resistance to antibiotics: internal, acquired and adaptive resistance. Internal resistance consists of low membrane permeability, as well as the expression of genes responsible for the production of enzymes, which are inactivated by antibiotics. Acquired resistance is based on mutational changes or horizontal gene transfer. Adaptive resistance of bacteria is expressed in the formation of biofilms, which prevent the penetration of antibiotics into the bacterial cell **(Aranaga *et.al.*, 2022)**.

In our experimental studies showed that no antibiotic has an effect on *A. baumannii, K. pneumonia* and *P. aeruginosa*, and in the case of *E. cloacae*, the antibiotics gentamicin, doxycycline and netilmicin work, but *E. cloacae* is low sensitive to these antibiotics. At the same time, the thick *P. granatum* extract actively inhibits all of the above-mentioned resistant strains of bacteria. In theoretical studies, it was found that no single antibiotic highly effectively inhibits all antimicrobial mechanisms; in the case of the identified anthocyanins, it was also shown that anthocyanins also did not inhibit all mechanisms of action. But, we want to note that *P. granatum* extract is a complex drug, therefore, in experimental studies, *P. granatum* extract inhibited the growth of all bacteria, and in turn, antibiotics only inhibited the bacterial strain *E. cloacae*.

The next important point is that the dose of anthocyanins in the thick *P. granatum* extract was an order of magnitude less than the dose of antibiotics, and the *P. granatum* extract effectively inhibited resistant bacteria. But, this raises the question of how anthocyanins could so actively inhibit bacterial growth, despite the fact that in molecular docking studies they do not inhibit all mechanisms of antimicrobial action. Phytochemical analysis showed that the thick *P. granatum* extract contains a high content of organic acids, which exceeds the content of phenolic compounds by 6 times. In our opinion, organic acids can inhibit those mechanisms that anthocyanins cannot or have a weak effect on. Organic acids are promising antimicrobial compounds, so in our previously published work we studied a lipophilic extract of green tea leaves, where the main group of compounds were organic acids. As a result of our research, it was shown that it was organic acids that made the main contribution to the antimicrobial effect of the extract. Although, the main advantage of *P. granatum* extract – minimal side effects. Pharmaceuticals based on natural compounds do not lead to liver cirrhosis, as with the use of tetracyclines; do not have nephrotoxicity and do not lead to deafness, as in the case of taking aminoglycosides. According to obtained theoretical and practical results, it was concluded that obtaining a single drug that will inhibit the growth of resistant strains of bacteria is impossible.

CONCLUSION

It has been established that thick pomegranate fruit extract contains phenolic compounds, anthocyanins and organic acids. Cyanidin-3-O-glucoside is one of the main anthocyanins in pomegranate fruit extract. Theoretical studies have found that no single antibiotic highly effectively inhibits all antimicrobial mechanisms of resistant gram-negative bacteria. Thick pomegranate fruit extract actively inhibits all resistant strains of *A. baumannii, K. pneumonia, P. aeruginosa,* and *E. cloacae*. These studies show that to inhibit resistant strains of bacteria, you need to use only a complex drug or jointly apply with dietary supplements of pomegranate, and in turn, herbal medicines are a "lifeline" for their creation and there is a chance to return old antimicrobial drugs in life.

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