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Tartaric acid new derivatives as prospective and safe alternative to antimicrobials for food products packing

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Submission Date: August 8th, 2023; **Acceptance Date**: January 2nd, 2024; **Publication Date**: January 9th, 2024

Please cite this article as: Mikaelyan A. R., Babayan B. G., Grigoryan A. L., Grigoryan A. M., Asatryan N. L., Melkumyan . A. Tartaric acid new derivatives as prospective and safe alternative to antimicrobials for food products packing. *Functional Foods in Health and Disease* 2024; 14(1): 33-50. DOI: https://www.doi.org/10.31989/ffhd.v14i1.1195

ABSTRACT

Introduction: Multi-drug resistance of pathogens and food spoilage bacteria is one of the most prevalent problems in contemporary science because the microbial adaptation potential is permanently being stimulated by the excessive antibiotic usage by mankind. In this research, seven new derivatives of tartaric acid (TA) were studied. Their high activity against the multi-drug resistant bacteria and the biodegradation potential by soil microflora was demonstrated.

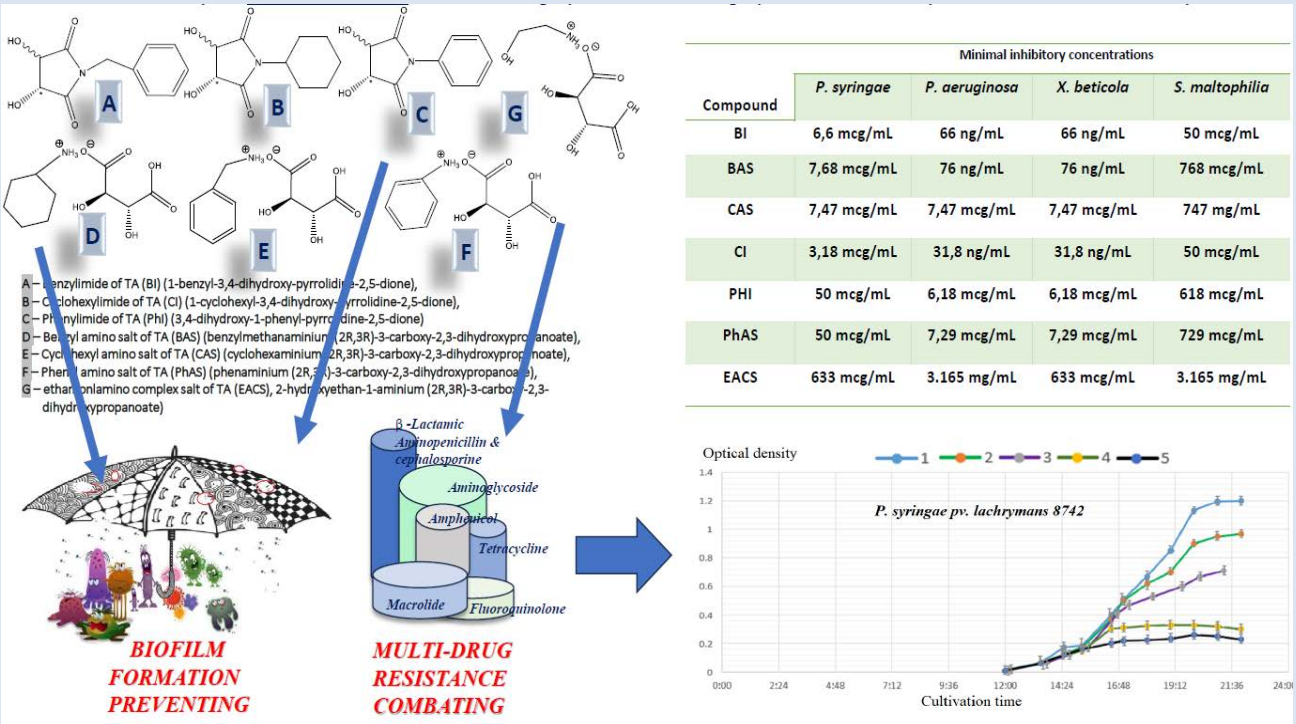
Objective: Based on multiple literature data about aldaric acids antimicrobial properties, for combating pan-drug and especially multi-drug resistant pathogenic microbes, natural L-tartaric acid was target-derivatized by cyclohexyl-, phenyl-, benzyl- and ethanolamine- functional groups into complex amino salts and cyclic imides, which have demonstrated the significant bactericidal and bacteriostatic activity against the spoilage agent bacteria, as well as phytopathogenic and human opportunistic pathogens.

Results: In vitro analyses of TA ethanolamine- benzyl-, cyclohexyl- and phenyl- imides and complex amino salts have shown that the mentioned substances are highly active agents for combating multi-drug resistant Gram-positive and

Gram-negative pathogens and food spoilage microbes, such as: *Pseudomonas aeruginosa, Stenotrophomonas maltophilia, Klebsiella pneumonia, Salmonella enteritidis, Staphylococcus aureus*, etc. These compounds are biodegradable by *P. chlororaphis* group non-pathogenic bacteria and their resistance is ensured by genes of nucleoid as well as are not transmitted by plasmids. In silico analyses have demonstrated their alternative mechanism of action.

Conclusions: The results suggest that TA new derivatives can be potentially recommended as safe alternative antimicrobials for food packaging.

Keywords: tartaric acid imides, tartaric acid complex aminosalts, spoilage microorganisms, antimicrobial activity, multi-drug resistance.



Compound	*P. syringae*	*P. aeruginosa*	*X. beticola*	*S. maltophilia*
BI	6,6 mcg/mL	66 ng/mL	66 ng/mL	50 mcg/mL
BAS	7,68 mcg/mL	76 ng/mL	76 ng/mL	768 mcg/mL
CAS	7,47 mcg/mL	7,47 mcg/mL	7,47 mcg/mL	747 mg/mL
CI	3,18 mcg/mL	31,8 ng/mL	31,8 ng/mL	50 mcg/mL
PHI	50 mcg/mL	6,18 mcg/mL	6,18 mcg/mL	618 mcg/mL
PhAS	50 mcg/mL	7,29 mcg/mL	7,29 mcg/mL	729 mcg/mL
EACS	633 mcg/mL	3.165 mg/mL	633 mcg/mL	3.165 mg/mL

INTRODUCTION

The processes of food products and meal spoilage are being caused by pathogenic bacteria and fungi, such as so-called food-borne microbes of different taxonomies. This unwanted process results in the loss of valuable properties of food components, as well as the formation and accumulation of toxic, dangerous products. [1].

Antimicrobial packaging is a fast-emerging scope of food and beverages production packaging. Unfortunately, there is no single universal antimicrobial agent which can act effectively against all the types of spoilage agents and pathogenic microorganisms. There are different strategies for solving the mentioned problems: the usage of inhibitors of antibiotic chemical modification and degradation enzymes; inhibitors of microbial metabolism, membranes permeability regulators, etc. [2]. Another promising avenue is the development of antimicrobial films, utilizing substances

with alternative targets of effect. This entails seamlessly integrating these active components into the matrix of a target polymer, with the active component itself exhibiting diverse chemical properties. It can be a natural or artificial substance, or the combination of a few substances. In our research, we explore derivatives of the native compound TA, contemplating its potential as a key component within the discussed strategy. The various antimicrobial films can be used for the direct coating of food or can be used as the inner layer of packaging. It's especially important for the protection from the spoilage of probiotic products. The desired active compound should be highly effective against the microbes and simultaneously be harmless for consumers. Also, the ecological safety and biodegradability of the finally elaborated antimicrobial film material is desired [4-5].

The majority of antimicrobial agents, which are being used in food production are elaborated based on their natural origin compound, which ensures minimal harmfulness. Their general sources are various plant species, which produce essential oils and phytoncides [6]. Since the II half of XX century, the general agents against the microorganisms which demonstrate the maximum in usage frequency remain the antibiotics (including microbial sourced natural penicillin, semisynthetic aminoglycosides or completely synthetic fluoroquinolones). Organic acids, especially the aldaric acids of various natural sources, also demonstrate several antimicrobial activities. It is known that organic acids and their salts derivatives do not inhibit the viability of the probiotic *Bifidobacterium*, which is important for digestion [7].

That is why natural L-tartaric acid (natural TA) derivatives of it can become an appropriate basis for elaborating a new generation of substances with improved antimicrobial properties to solve the problem of multi-drug resistant pathogens. It can address this issue without the overburdening of food products by antibiotic increased concentrations. It is known that amino derivatives of mono-/di-amides and polyamides demonstrate the bactericidal properties against the following bacteria: *Klebsiella, Shigella, Escherichia coli, Pseudomonas aeruginosa, Salmonella typhae, Bacillus subtilis, Bacillus cereus*. Also, they provide a fungicidal activity against *Trichophytum rubrum* and some yeasts [8].

Current research is devoted to *in vitro* and *in silico* studies of innovative antimicrobial elaboration. It describes the antimicrobial properties of TA, and its new semisynthetic nitrogen containing derivatives. The issue of our innovation is an investigation of novel classes of substances based on natural plant common component aldaric acids and their derivatives for their potential application as anti-spoilage agent for food and beverage packaging.

Based on simple implementable technology, novel derivatives of natural TA were elaborated. TA, which was used as a row material for all the syntheses, was purified from cream of tartar by the easily implementable and ecologically safe technology of acid hydrolysis. The mentioned cream of tartar is not very expensive, and it is an evaluable resource, because of it being one of the main wastes of wine-making industry, which is sufficiently developed in the Republic of Armenia (RA) [8]. It contains a lot of TA in the form of insoluble tartrates. Using the considered above technology of target derivatization 6 TA cyclic derivatives (benzyl-, phenyl- and cyclohexyl- functionalized compounds) and 1 TA aliphatic derivative (containing completely vital functional group: ethanolamine or colamine) were obtained. They were synthesized: BI or benzylamide of TA; CI or cyclohexylimide of TA; PhI or phenylimide of TA; BAS benzylamino complex salt of TA (also called benzyl complex amino salt of TA); CAS or cyclohexylamino complex salt of TA (also called cyclohexyl complex amino salt of TA); PhAS or phenylamino salt of TA (phenylamino

salt of TA) and EACS or ethanolamine complex salt of TA (or colamine complex amino salt of TA, also known as monoethanolamino complex salt of TA or MEAS) (Figure 1).

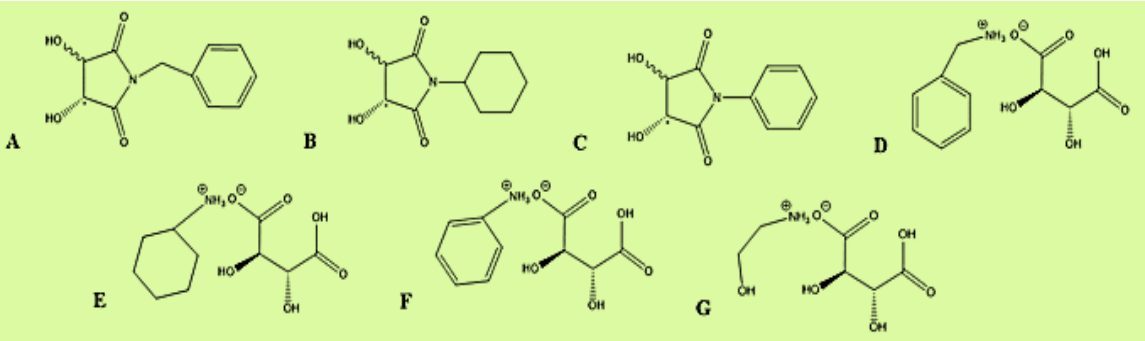


Figure 1. TA new synthetic derivatives.

A – Benzylimide of TA (BI) (1-benzyl-3,4-dihydroxy-3l3-pyrrolidine-2,5-dione), molecular weight: M = 220.20; B – Cyclohexylimide of TA (CI) (1-cyclohexyl-3,4-dihydroxy-3l3-pyrrolidine-2,5-dione), molecular weight: M = 212,23; C – Phenylimide of TA (PhI) (3,4-dihydroxy-1-phenyl-3l3-pyrrolidine-2,5-dione), M = 206,18; D – Benzyl amino salt of TA (BAS) (benzylmethanaminium (2R,3R)-3-carboxy-2,3-dihydroxypropanoate), molecular weight: M = 257,24; E – Cyclohexyl amino salt of TA (CAS) (cyclohexaminium (2R,3R)-3-carboxy-2,3-dihydroxypropanoate), molecular weight: M = 249.26; F – Phenyl amino salt of TA (PhAS) (phenaminium (2R,3R)-3-carboxy-2,3-dihydroxypropanoate), molecular weight: M = 243.22; G – ethamonlamino complex salt of TA (EACS), 2-hydroxyethan-1-aminium (2R,3R)-3-carboxy-2,3-dihydroxypropanoate), molecular weight: M = 211,17.

MATERIALS AND METHODS

Microorganisms: For experiments which were devoted to the evaluation of microbiological properties of TA new nitrogen containing derivatives, there were chosen Gram-positive and Gram-negative bacteria from the National Collection of Microorganisms of Microbial Depository Center (MDC), "Armbiotechnology" Scientific and Production Center (SPC) of National Academy of Sciences of the Republic of Armenia (NAS RA). The following strains of bacteria were used: *Pseudomonas aeruginosa, P. geniculate, Pseudomonas putida, Stenotrophomonas maltophilia, Klebsiella pneumonia, Salmonella enteritidis, Staphylococcus aureus, Escherichia coli*. The mentioned strains were cultivated due to the commonly accepted techniques using solid (nutrient beef-extract agar cultivation medium) and liquid (Luria-Bertani LB medium) nutrient cultivation media, sterilized by autoclaving [8].

Antibiotic resistance tests: For the qualitative assessment of antibiotic-resistance of considered bacteria the selective differential media with protocol concentrations of antibiotics, each of them was used in 50 mcg/mL final concentration and were applied in 60 mm diameter Petri dishes. The cultivation of bacteria was carried out in contribution to the temperature and aeration optimal conditions of the considered bacteria (aerobic condition, 37 °C) by streaking technique. 13

following antibiotics were used: Pcn/Penicillin, Amp/ampicillin, Amx/amoxicillin, Amc/augmentin (combine preparations of Amx and clavulanic acid, which is the classical inhibitor β-lactamases) (from aminopenicillins subtype of β-lactamic antibiotics class), Cfx/cefixime (the I generation cephalosporin representative from β-lactamic antibiotics class, Cro/ceftriaxone (the III generation cephalosporin representative of β-lactams), Kan/kanamycin, Stp/streptomycin, Gnc/gentamicin (form aminoglycosides class antibiotics), Cip/ciprofloxacin (of fluoroquinolone antibiotic class), Tcn/tetracycline (of tetracycline antibiotics), Cam chloramphenicol (from amphenicol class), also Azm/azithromycin (form azalide group of macrolide antibiotics), produced by "Astoria" [9]. The considered antibiotics were selected for this study due to their frequent use in medicine, veterinary agriculture, food industry as well as due to the increase in their residual concentrations after the coronavirus pandemic in 2020.

TA new derivatives antimicrobial activity assessment: Microbiological analyses were carried out on selective cultivation media, containing the tested compounds in appropriate concentrations respectively. To detect the effect of the TA new derivatives, the modified bilayer media technique was applied: 1.8%-agar containing meat peptone agar media (MPA) was applied instead of blood agar. It was poured into 60 mm diameter Petri dishes. Then, in sterile test tubes for each bacterium, the mixtures of culture suspension and molten 0.7%-agar MPA were prepared separately at 37 °C. On the surface of solidified 1.8%-MPA, the mentioned mixtures of each microbial strain (~10^8 CFU) were added as the second layer in the appropriate Petri dish. Then, appropriate 3 mcL of each of the seven derivatives of TA: BI, CI, PhI, CAS, BAS, PhAS, and EACS were sterilely added. There were used the different concentrations of sterile for each of mentioned substances: 0.5 M, 0.1 M, 0.05 M, 0.025 M, 0.01 M and 0.001 M, prepared in water for complex amino salts and in dimethyl sulfoxide (DMSO) for imides, because of their solubility differences. The appropriate control samples were prepared with sterilized water and DMSO. The changes in growth of studied cultures were observed visually at their optimal conditions (37 °C, aerobic conditions) within the periods of: 12 h; 24 h; 48 h; 76 h and 96 h. The detection of the formation of sterile zones, where the bacterial growth was inhibited in an area of applied TA derivative influence, was carried out visually. As a positive control, the growth of same cultures on the same media with the same quantities of sterile pure solvents only (DMSO and water) upon the same aeration and temperature regime was considered. Then, the minimum inhibitory concentration for each TA synthetic derivative was defined in sterile conditions by the multiple dilution procedures for each substance beginning from the 0.5 M to 0.0001 M concentration [10].

Genetical study of bacterial strains: For the comparison of mechanisms, which ensures antimicrobial resistance of the studied bacteria against antibiotics and new TA derivatives, a series of genetical experiments were carried out. The first step of genetic analyses of the observed bacteria was epitomized by the chromosomal and plasmid DNA isolation from the cells of bacteria and further 0.8–2.5%-agarose gel-electrophoresis of all samples. Then, the plasmid analysis was carried out for

antibiotic-resistant and sensitive strains by gel electrophoresis and transformation techniques [11-12]. As the positive control, the following strains of *E. coli* were applied: *E. coli DH5α/VOG16* (Kan-resistance encoding plasmid)*; E. coli DH5α/PEC7* and (Cam-resistance encoding plasmid) *E. coli pUC18* (Amp-resistance encoding plasmid), while *E. coli DH5α* strain was applied as for the negative control, because it is sensitive to all the considered antibiotics and does not carry any plasmid. The mechanism of antibiotic-resistance in studied bacterial strains was defined by microbiological and genetical methods. The dependence between the resistance to antibiotic concentration (dose-dependent effect), as well as PCR (Polymerase Chain Reaction) analyses of 4 genes that encode the enzymes of antibiotic modification were carried out for the evaluation of resistance mechanisms differences (Table 1).

Table 1. PCR analyses conditions.

Enzyme	Gene	Gene primer	Molecular Weight	Subsequence	Temperature regime (T_{lid} 96 ℃),
BlaOXA	*blaOXA-10*	*blaOXA-10*	F: 6117 R: 5736	F:5'TATCGCGTGTCTTTCGAGTA3' R:5'TTAGCCACCAATGATGCCC3'	94 ℃-150 s, 14 cycles: 94 ℃ - 45 s, 63 ℃ 45 s, 72 ℃-90 s, 22 cycles: 94 ℃-45 s, 59 ℃-45 s, 72 ℃-90 s
APH	*aph(3')VI*	*aph(3')VI*	F:1789 R: 12023	F:5'AGGTGACACTATAGAATACGGAAA CAGCGTTTTAGAGC3' R:5'GTACGACTCACTATAGGGAGGTTT TGCATTGATCGCTTT3'	94 ℃-150 s, 14 cycles: 94 ℃-45 s, 66 ℃ 45 s, 72 ℃-90 s, 23 cycles: 94 ℃-45 s, 63 ℃-45 s, 72 ℃-90 s.
AAC	*aac(6')II*	*aac(6')II*	F: 11636 R: 11883	F:5'AGGTGACACTATAGAATATTCATG TCCGCGAGCACCCC3'; R:5'GTACGACTCACTATAGGGAGACTC TTCCGCCATCGCTCT3'	94 ℃ - 150 s, 14 cycles: 94℃-45 s, 64 ℃- 45 s, 72 ℃-90 s, 23 cycles: 94 ℃-45 s, 61 ℃-45 s, 72 ℃-90 s
CAT	*catB7*	*pCAT639*	F: 7455 R: 6954	F:5'AGGGACGACGGTCATATGGGCAA C3', R:5'CCTTCGTCCAAGCTTCAGGCCGT 3'	94 ℃ - 150 s, 14 – cycles: 94 ℃ - 45 s, 66 ℃ - 45 s, 72 ℃ - 90 s, 23 cycles: 94℃ - 45 s, 63 ℃ - 45 s, 72 ℃ - 90 s.

The following primers of genes of antibiotic modification were used: *blaOXA-10* (for the gene of β-lactamase BlaOXA10), *pCAT639* (for the *catB7* gene of CAT chloramphenicol acetyltransferase enzyme) and two primers for aminoglycoside modifying enzymes: *aph(3')IV* (for aminoglycoside-O-phosphotransferase APH) and *aac(6')II* (for aminoglycoside-N-acetyltransferase AAC). The mentioned genes were chosen due to their high prevalence among human and animal pathogens. The following markers for

determining the molecular weight of DNA fragments were used: *M-3788 SIGMAMARKER; M-3913 SIGMAMARKER; D-6293 (pUC18/ Hae III), M-103S λDNA/EcoRI/HindIII*, produced by "SIGMA" [13, 14, 15, 16, 17, 18,19].

Then, for the detection of the resistance transmission potential in studied bacteria, the transformation analysis due to the Mandel's method was used in a modification which was elaborated in our laboratory (Figure 2).

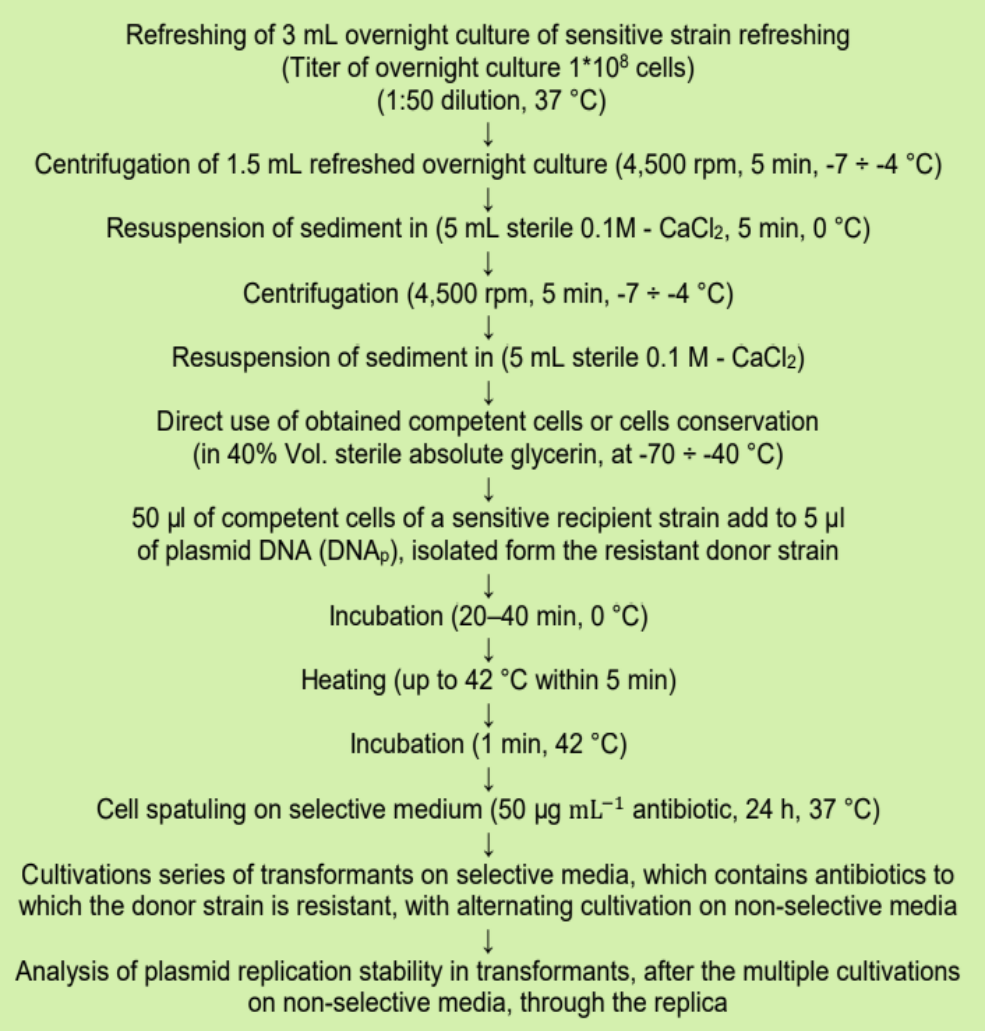


Figure 2. Transformation experiment scheme.

TA synthetic derivatives biodegradation potential analyses: Biodegradation analyses were carried out by carbon source substitution in cultivation media [20]. We tested 3 different concentrations of target compounds: 0.5 M, 1% and 2%. In order to identify the biodegradation properties of the studied bacteria of different genera, a qualitative determination of extracellular polyphenol oxidase activity (tyrosinase and laccase enzymes) was carried out on an agar minimal medium using 3 different substrates of polyphenol oxidases (L-tyrosine ("SIGMA") for tyrosinase, and tannin and α-naphthol ("SIGMA") for laccase) as carbon sources. The presence or absence of extracellular polyphenol oxidase activity was detected visually by the intensity of oxidation of the used substrates. The intensity of the oxidation reaction of L-Tyr, α-naphthol, and tannin was expressed by the

intensity of change in the color of the cultivation medium and by the diameters of the blackout zones formed around the growth zones of the studied strain on a solid medium. The obtained results were recorded visually for 7 days.

For the detection of TA derivatives biodegradation potential, the bacterial strains with the observed resistance to TA complex aminosalts and imides were selected. That bacteria were cultivated on M9 minimal media with addition of appropriate TA derivative as the only one carbon source [21].

In silico analyses: Using the generally accepted cheminformatics protocols and the ChemDraw 18.0 software package, 3D molecular models of the studied ligands and TA derivatives were constructed [22]. To determine their affinity for the structural components of the cell, 3 models of proteins of gram-negative bacteria responsible for the manifestation of pathogenicity and virulence at the stage of recognition during infection of the host organism were selected as targets: transcription regulator (TsaR (3FXQ) in *P. testosteroni* (also known as *Comamonas testosteroni*); benzoate binding effector BenM (2F78) in *P. aeruginosa*; quorum sensing regulation factor in E. coli OXYR (1I69). The considered 3D models of target microbial proteins were taken from the RCSB database with following PDB IDs: 3fxq (TsaR of *P. testosteroni*); 1i69 (OXYR of *E. coli*) and 2f78 (BenM of *P. aeruginosa*) [23, 24, 25].

The molecular docking analysis was carried out using the AutoDock Vina and AutoDock Tools software packages. Statistical significance was obtained by independent analysis 5 times, using the 20 initial conformations for each compound, with a virtual box volume not exceeding 27,000 Å. The value of the continuum of calculations was taken as 500. To identify amino acid residues during complex formation, determine the types of interaction, and compile conformational maps, the software package "BIOVIA Discovery Studio" was used. The processes of visualization and clustering of obtained results were done with the help of FOREL algorithm-based program in Python environment due to the equation presented below:

$$\Delta G_{exp} = -RT \ln(1/K) \quad (1)$$

ΔG - Gibbs free energy, R - gas constant, T - absolute temperature, K - binding constant [26].

Statistical analyses: The carried-out experiments were done in 5 series with 3 repeats for each sample. For data analysis of all the TA new synthetic derivatives effects evaluation MS Excel tool was used. The Standard Error of the Mean (SEM) was ± 0.23–0.37. The significance value was tested by Student t-test application and was estimated as p-value < 0.05.

RESULTS

Antibiotic-resistance study of tested microorganisms: The antibiotic-resistance was analyzed in 225 strains of different species of bacteria from different genera. Antibiotic-resistance analyses of the studied groups of microbes have demonstrated the predominant pan-drug and multi-drug resistance property. Only one from the considered strains of *P. aeruginosa* was absolutely resistant to all the considered antibiotics, while multi-drug resistance is detected for all the bacteria of the studied group of *P. aeruginosa* strains, except the strains *P. aeruginosa 9056*. The genetical analyses have demonstrated the versatile resistance mechanisms in this group of microorganisms. Gel electrophoresis and transformation methods application have convinced the presence of plasmids in all the strains of *P. aeruginosa*, except *P. aeruginosa 9056* sensitive strain. During the experiments two types of plasmids of *P. aeruginosa 9059* there were found. They were responsible for aminoglycoside and β-lactamic resistance, being transmitted and replicated independently (table 1).

Table 2. Antibiotic-Resistance of P. aeruginosa different strains.

Strain	Antibiotic resistance													C
	Gnc	Kan	Stp	Tcn	Cfx	Cro	Amx	Amc	Pcn	Amp	Cam	Azm	Cip	
5249a	R	R	R	R	R	-	R	R	R	R	R	-	R	+
9056	-	-	-	-	-	-	-	-	-	-	-	-	-	+
5249b	-	R	-	R	R*	R	R	R	R	R	R	R	R*	+
9057	-	-	R	-	-	-	R	-	-	R	R	-	-	+
9058	-	-	-	R	-	-	R	-	-	R	R	R	-	+
9059	-	R	-	R	R	-	R	R	R	R	R	R	R	+

"R" – resistance to antibiotic, "-" – sensitivity to antibiotic; "R*" – bacteriostatic effect of antibiotic; "+" – growth of the strain on a nutrient L-agar medium, C - control.

In P. aeruginosa 5249 it was found the plasmid which is able to simultaneous forming β-lactamic and aminoglycoside resistance. In strain *P. aeruginosa 5249a* plasmids are responsible for cephalosporin resistance transmission, while the plasmids *of P. aeruginosa 5249b* are not able to transfer it. In strain P. aeruginosa 9058 the plasmids were detected by gel-electrophoresis, but they are not related to any of 13 considered antibiotics. In all the studied bacteria, chloramphenicol-resistance was encoded by nucleoid genes and not transferring by transformation. PCR analyses have demonstrated the presence of genes which encode the enzymes of antibiotic modification on plasmids and on the bacterial chromosome (nucleoid localization) of different strains. For strains *P. aeruginosa 9059* and in *P. aeruginosa 5249a,* the presence of *catB7* while on plasmids *aac(6')II* gene is detected. On the plasmid isolated from *P. aeruginosa 5249a* the genes *blaOXA-10* and *aph(3')IV* are detected. In strain *P. aeruginosa 9057* plasmids are detected, and they transfer the resistance to Amp and Amx, but this resistance is related to other genes.

The analogous results were obtained from the experiments with *Stenotrophomonas, Xanthomonas*, and other genera representatives. The majority of them were resistant and able to transfer it by plasmids but didn't contain those four genes which were analyzed by the PCS method.

Antimicrobial influence of TA cyclic imides and complex aminosalts: For the preliminary screening of newly synthesized compounds, the effect of growth inhibition on *P. aeruginosa* different strains was compared for seven new TA derivatives and for metal salts of TA, as well as for pure TA. The main goal of that comparison was to analyze whether the derivatization had an effect on an extension of antimicrobial properties in comparison to natural analogous compounds (Table 3 – 5). According to the presented data, EACS in the form of complex salt didn't demonstrate the high activity against the six strains of *P. aeruginosa*. Its antimicrobial effect is comparable to the effects of natural salts of TA. Presumably, low antimicrobial effect is caused by the vital components in the consistence of it, and EACS might be easily decomposed by the microorganisms. Then, the effects of cyclic derivatives were tested on same bacterial strains of different taxonomic groups, which were used for the analyses of aliphatic derivative of TA.

Table 3. EACS and natural compounds effect comparison.

Strain	EACS			TA		Na/K-T		Na-T		C_1	C_2
	0.05M	0.5M	0.5M	0.1M	0.5M	0.1M	0.5M	0.1M	0.5M		
5249a	+	+	-	+	+/-	+	+/-	-	-	+	+
5249b	+	+	+	+	-	+	+/-	+	+	+	+
9056	-	-	-	-	-	-	-	+/-	-	+	+
9057	+	+	+	+/-	+/-	-	-	+	+	+	+
9058	-	-	+	-	-	-	-	+	+	+	+
9059	+/-	+	-	+/-	-	-	-	-	-	+	+

Na-T - sodium tartrate, Na/K-T - Rochelle's salt; Control: C_1 - positive control on nutrient L-agar media, C_2 - control of the nutrient L-agar media with addition of same concentration of DMSO; "+" - growth of the strain, "-" - the presence of growth, "+/-" - bacteriostatic effect.

Table 4. Summary of TA cyclic complex aminosalts and TA natural derivatives antimicrobial effect comparison.

S	BAS			CAS			PhAS			Na-T	Na/K-T	TA	C_1	C_2
	a	b	C	a	B	C	a	b	c	C	C	c		
1	+	+	+	+	+	+	+	+/-	+/-	-	-	+	+	+
2	+	+	+	+	+	+	+	+/-	+/-	+	+	+	+	+
3	-	-	-	-	-	-	+/-	-	-	-	-	-	+	+
4	+	+/-	-	+	+	+/-	+	+	-	+	+	+	+	+
5	-	-	-	-	-	-	+/-	+/-	-	+	+	-	+	+
6	+/-	+/-	-	+	-	-	+	+/-	+/-	-	-	+	+	+

S – bacterial strain: *1 – P. aeruginosa 5249a, 2 – P. aeruginosa 5249b, 3 – P. aeruginosa 9056, 4 – P. aeruginosa 9057, 5 – P. aeruginosa 9058, 6 – P. aeruginosa 9059*; Concentrations tested compounds: a – 0.05, b – 0.1, c – 0.5; "+" – growth of the strain, "-" – the presence of growth, "+/-" – bacteriostatic effect; Na-T – sodium tartrate, Na/K-T – Rochelle's salt; Control: C_1 – positive control samples, cultivated on nutrient L-agar media, C_2 – the positive control samples cultivated on nutrient L-agar media with addition of same concentration of DMSO.

Table 5. TA synthetic imides and natural derivatives effects summary comparison on *P. aeruginosa* different strains.

S	Na-T		Na/K-T		TA		CI			PhI			BI			C_D	C
	a	c	a	c	a	c	a	b	c	a	b	c	a	b	c		
1	-	+/-	-	+/-	+	+	-	+/-	+	-	+/-	+	-	-	+	+	+
2	+	+	+	+	+	+	+	+	+	+/-	+/-	+	+	+	+	+	+
3	-	-	-	+/-	+/-	-	+	+	-	-	+/-	+/-	+	+	-	+	+
4	+	+	+	+	+	+	-	-	+	-	+	+	-	+/-	+	+	+
5	+	+	+	+	+/-	-	-	-	-	-	+/-	+/-	-	-	-	+	+
6	-	-	-	+/-	+	+	-	+/-	+	+/-	+/-	+/-	-	-	+/-	+	+

S – bacterial strain: *1 – P. aeruginosa 5249a, 2 –P. aeruginosa 5249b, 3 – P. aeruginosa 9056, 4 – P. aeruginosa 9057, 5 – P. aeruginosa 9058, 6 – P. aeruginosa 9059*; Concentrations tested compounds: a – 0.5; b – 0.1; c – 0.05; "+" - growth of the strain, "-" – the presence of growth, "+/-" – bacteriostatic effect; Na-T – sodium tartrate, Na/K-T – Rochelle's salt; Control: C_w – positive control on nutrient L-agar cultural media, C_D - control of the nutrient L-agar cultural media with addition of same concentration of DMSO.

In these experiments PhI and PhAS have demonstrated the less activity then in case of CAS, CI, BI, BAS. Analogous results were obtained for *P. putida, St. aureus, P. geniculate, S. maltophilia, K. pneumonia, S. enteritidis*, etc. CI and CAS were maximal active against the majority of the considered strains [27].

Table 6. TA synthetic derivatives minimal inhibitory concentrations.

Compound	Minimal inhibitory concentrations			
	For P. syringae	*For P. aeruginosa*	*For X. Beticola*	*For S. maltophilia*
BI	6,6 mcg/mL	66 ng/mL	66 ng/mL	50 mcg/mL
BAS	7,68 mcg/mL	76 ng/mL	76 ng/mL	768 mcg/mL
CAS	7,47 mcg/mL	7,47 mcg/mL	7,47 mcg/mL	747 mg/mL
CI	3,18 mcg/mL	31,8 ng/mL	31,8 ng/mL	50 mcg/mL
PHI	50 mcg/mL	6,18 mcg/mL	6,18 mcg/mL	618 mcg/mL
PhAS	50 mcg/mL	7,29 mcg/mL	7,29 mcg/mL	729 mcg/mL
EACS	633 mcg/mL	3.165 mg/mL	633 mcg/mL	3.165 mg/mL

Then, the efficiency of antimicrobial influence of the considered TA synthetic cyclic imides and complex aminosalts was compared to the effect of classical antibiotics. For that goal, the minimal inhibitory concentrations were calculated for the strains of different species and genera (table 6). The collected data proved that the minimal effective concentrations of TA imides and complex aminosalts are comparable to antibiotics effective concentrations. Previous research had also demonstrated a high activity of BAS, BI, CAS and CI on various opportunistic pathogens.

Biodegradation potential research: For the evaluation of biodegradation abilities of studied bacteria, the series of qualitative assessment experiments with their some extracellular enzymes were caried out. There were defined extracellular polyphenol oxidases: laccase and tyrosinase (Figure 3).

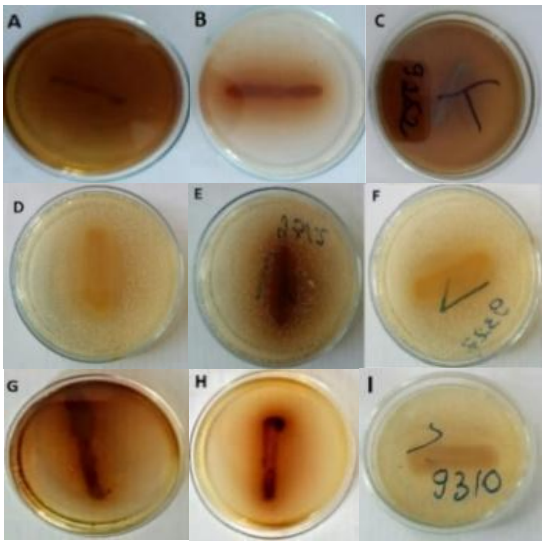


Figure 3. Qualitative assessment of *Pseudomonas* various species representative's extracellular polyphenol oxidases.

A: α-naphthol degradation by laccase (polyphenol oxidase II) *P. fluorescens 9075*; B: (polyphenol oxidase I) tyrosinase *P. geniculata 9335*; C: tannin degradation by *P. putida, var. melanogenes 9252* laccase; D: *P. geniculata 9336* tyrosinase; E: *Pseudomonas sp. 9312 tyrosinase*.; F: *Pseudomonas sp. 9327* tyrosinase; G: *S. maltophilia 9288* tyrosinase; H: *S. maltophilia 9302* tyrosinase; I: tyrosinase of *S. maltophilia 9310*.

At the next step, TA 7 derivatives were tested on non-pathogenic microbe (Table 7). In case of non-pathogenic soil bacteria of *P. chlororaphis group* (*P. taetrolens; subspecies chlororaphis; subspecies aureofaciens; subspecies aurantiaca*), these compounds practically have no effect. Also, *P. fluorescens* have demonstrated essentially the same properties [27]. After that, all those resistant strains were tested for biodegradation abilities. The experiments have demonstrated that the majority of the observed resistant strains are able to degrade complex aminosalts.

Table 7. Biodegradation of TA complex aminosalts by non-pathogenic *P. chlororaphis* three subspecies representatives on solid cultural media.

Strain		BAS	CAS	PhAS	EACS	C_+	C_-	Strain		BAS	CAS	PhAS	EACS	C_+	C_-
9189		+	-	+	+	+	-	*9163*		-	-	-	+	+	-
9171		+	-	+	+	+	-	*9157*		-	+	+	+	+	-
9190		+	-	+	+	+	-	*9178*		-	-	-	+	+	-
9159	1	-	-	+	+	+	-	*9172*		-	-	+	+	+	-
9177		+	+	+	+	+	-	*9168*	1	-	-	-	+	+	-
9174		-	-	-	+	+	-	*9164*		-	-	-	+	+	-
9158		-	-	+	+	+	-	*9165*		+*	-	+	+	+	-
9195		+	+	+	+	+	-	*9066*		+	-	+	+	+	-
9200		-	-	+	+	+	-	*9062*		+	+	+	+	+	-
9199	2	-	-	+	+	+	-	*9064*	3	-	+	+	+	+	-
9197		-	-	+	+	+	-	*9061*		-	-	+	+	+	-

Cultures: *1 – P. chlororaphis subsp. chlororaphis, 2 – P. chlororaphis, subsp. aureofaciens, 3 – P. chlororaphis, subsp. aurantiaca*; "+" – bacterial growth presence, "-" – bacterial growth absence, "*" – the separate colonies growth, "C+" – positive control on nutrient L-agar media, "C-" – negative control on minimal media without carbon source.

Further transformation experiments with all the resistant strains have demonstrated the absence of transmission of resistance to TA new derivatives to any Gram-negative bacteria by plasmids. The experiments convinced the absence of spreading the resistance to all the seven considered compounds by gene horizontal transfer by plasmids on recipients from different genera: *Xanthomonas, Stenotrophomonas, Pseudomonas, Pectobacterium, E. coli, etc*. For further research, a more detailed study of the resistance transmission by the transduction method is planned.

TA new derivatives antibacterial effect mechanism study: For the confirmation of results which were obtained *in vitro*, due to which it is visible the difference in effect of natural and synthetic derivatives of TA, the series of in silico experiment were carried out by the methods of molecular docking. According to the presented data, the targets of all the interactions are differing from classical targets of analogous lactamic and other antibiotics. They are the proteins which are involved in biofilm formation in protein synthesis and the molecules of the membrane mechanisms of cellular recognition. The formation biofilm and the regulation of that process is very important for the resistance profile forming of bacteria [28] (Figures 4 - 6).

In silico results have shown that the interactions of TA new derivatives take place by hydrogen bonds, such as like Van Der Waals and hydrophobic interactions between several groups in radicals of amino acids in polypeptide chains of appropriate target-protein. In zones of interactions, the specific space structures in the shape of holes are notable. Thus, the obtained results demonstrate the key role of substituents for the interactions of all the 7 derivatives with target proteins.

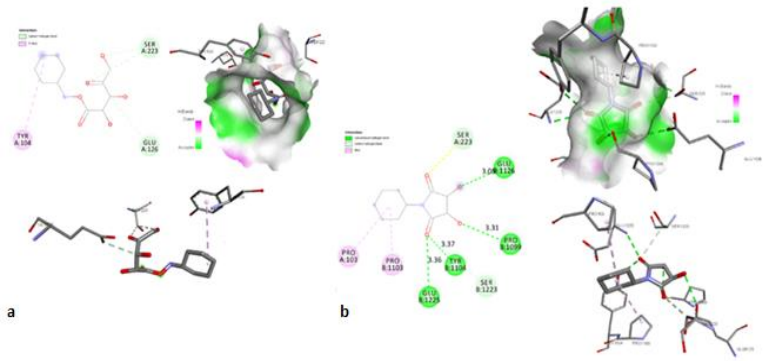


Figure 4. Docking-analysis of CAS (a) and CI (b) interaction with OXYR protein.

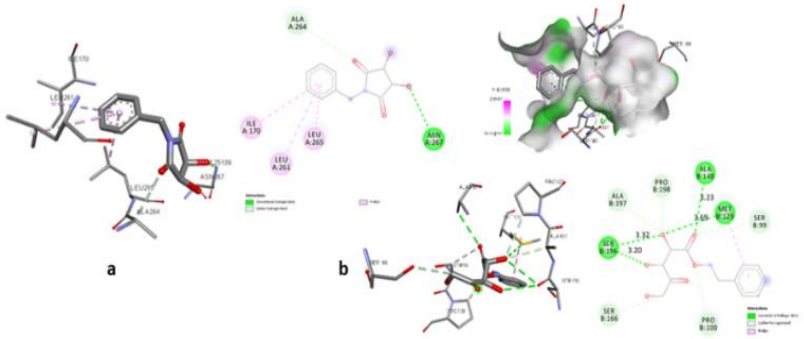


Figure 5. Docking-analysis of BI (a) and BAS (b) interaction with TsaR protein.

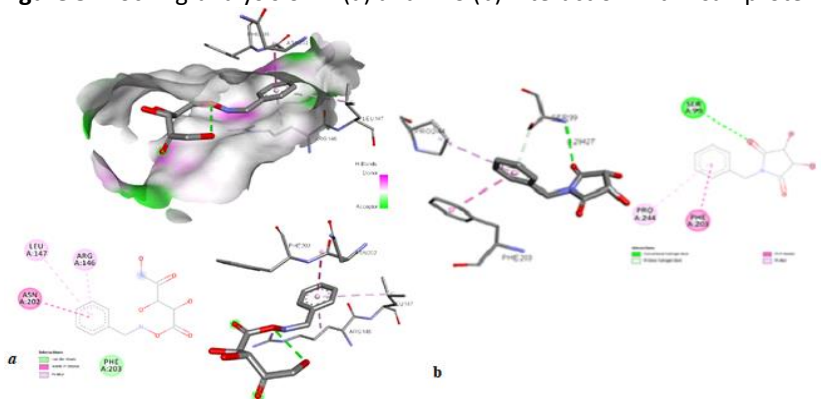


Figure 6. Docking-analysis of BAS (a) and BI (b) interaction with BenM protein.

DISCUSSION

The elaboration of innovative alternative preparations which can potentially replace the classical antimicrobials is very urgent problem, because of extreme need for novel classes of compounds with alternative activity mechanisms targets. The significance of that is high, especially for food production, where the applying of food additive compounds should be harmless for the human body. The potential desired compound should be antimicrobial and simultaneously not have an acute and cumulative immunosuppressive, genotoxic, teratological and other non-desirable side-effects. The importance of

the problem mentioned is notable starting from the stage of food production and up to the packaging of it. In these regards, the elaboration of innovations based on novel safe antimicrobials is extremely important in combined and complex preparations for crop protection in agriculture and farming. Nowadays, more and more corporations are starting to decrease the volumes of the antibiotics used and support green agriculture techniques. Nevertheless, antibiotics still remain the classical antimicrobial preparation with comprehensive usage [29].

One of the promising directions for the solvation of the above-mentioned problem is the elaboration of novel compounds, based on natural sources. For this purpose, organic acids (especially, oxyacids and aldaric acids) such as like their derivatives are one of the useful row materials for the further derivatizations by the target synthons. Pure citric, lactic, acetic, tartaric, and malic acids, such as like their salts have a several levels of involvement in food and beverage production, having the appropriate “E” codes [30]. Also, it’s known that some dicarboxylic and tricarboxylic acids (1%-citric acid, citrates, etc.) have the ability to enhance an antimicrobial effect of some disinfection agents (methylene blue, crystal violet and other disinfecting dyes) and even decrease the resistance of some bacterial pathogens to some antibiotics. Organic acids also increase the antibacterial effect of phytogenic polyphenols, which activate plants' innate immune systems [31, 32]. The elaboration of natural aldaric acids’ target of functionalized derivatives is one of the important directions of new classes of antimicrobial agents’ investigation. The main advantage of that is the possibility to obtain the substances, which are strongly differing from the well-known antibiotics by their action mechanism. The previously conducted *in vivo* research and field tests in lakes have shown that the developed antimicrobial compounds are not toxic for fishes and other lake animals. Due to the results of earlier research aromatic substituent containing TA imides, such as like complex amino salts are effective against *K. pneumonia, S. maltophilia, P. carotovorum, P. geniculate, St. aureus, P. aeruginosa, X. beticola, X. campestris, S. enteritis, X. vesicatoria, Rh. javanica, Rh. fascians* and other pathogens of plants, human and animals, including the antimicrobial influence against entomopathogenic bacteria in some species. The inactivity of the considered substances against some soil normal microflora bacteria such as like *P. taetrolens, P. chlororaphis* such as like *P. fluorescens* and their ability to consume these substances as the carbon source give the potential possibility to consider complex amino salts of TA as a non-stabile xenobiotic which can be biodegraded. Imides also can be degraded by the soil normal pH influence by the hydrolysis processes [,33, 34, 35]. That is why in controversy to various synthetic compounds which are being elaborated for similar purposes, new derivatives of TA cannot be considered as a stabile pollutant of soil and environment in general. Thus, TA complex amino salts and imides can be considered as more prospective substances.

Thus, as a next step of research, more detailed research of mechanisms of action of TA different derivatives with various substituents should be planned. It is very prospective to research the interaction of these compounds with some key enzymes of biodegradation, such as laccase and tyrosinase. The study of natural TA derivatives with biomembrane proteins provide a more detailed definition of antimicrobial effect targets in studied pathogens. The obtained results suggest the potential implementation of CI, BI, CAS, BAS, PhAS, PhI, EACS in various scopes, especially in food production as a comparably ecologically safe alternative antimicrobials for beverages and food product packaging, such as like as antiphytopathogenic agents in agriculture, as well as in veterinary as an agent against the pathogens of animals.

Further safety toxicological research of the mentioned compounds might also disclose the potential of their prospective implementation in medicine for patients with chronic and immunosuppressive disorders (HIV/AIDS, cancer, allergies, autoimmune disorders, etc.).

CONCLUSION

The obtained results provide the evidence of a significant potential of newly synthesized TA cyclohexyl- benzyl-, phenyl- and ethanolamine- substituted imides and appropriate complex amino salts implementation in various scopes as a comparably ecologically safe alternative antimicrobial agent. Especially it might be important for the food and beverages ecologically safe packaging technologies elaboration. Also, the properties of the discovered compounds might be used against the range of pathogenic microorganisms in veterinary and agriculture, for example for plant protection. Further research of mentioned compounds safety might disclose the potential of their usage also in medicine for patients with different etiology chronical and immunosuppressive disorders against the various opportunistic pathogenic bacteria.

Abbreviation: Pcn: Penicillin, Amp: ampicillin, Amx: amoxicillin, Amc: augmentin; Cfx: cefixime,; Cro: ceftriaxone, Kan: kanamycin, Stp: streptomycin, Gnc: gentamicin, Cip: ciprofloxacin, Tcn: tetracycline, Cam: chloramphenicol, Azm: azithromycin, PCR: Polymerase Chain Reaction, DMSO: Dimethyl Sulfoxide, MPA: meat peptone agar, TA: tartaric acid, BI: benzylimide of TA; BAS: benzyl complex aminosalt of TA (or benzyl complex aminosalt of TA), CI: cyclohexylimide of TA, CAS: cyclohexyl complex aminosalt of TA (or cyclohexylamine complex salt of TA), PhI: phenylimide of TA, PhAS: phenyl complex aminosalt of TA (or phenylamine complex salt of TA), EACS: ethanolamine complex aminosalt of TA or MEAS monoethanolamino complex salt of TA, Na-T: sodium tartrate, Na/K-T: Rochelle's salt.

Author's Contributions: All authors contributed to this study.

Acknowledgments: We're thankful to all the stuff of "Agrobiotechnology Scientific Center" Branch of ANAU Foundation, Department of "Creation and Quality Control of Agricultural Preparations", and SPC "Armbiotechnology" NAS RA Laboratory of Ecological Safety. Also, we're very thankful to Russian-Armenian University for all the support.

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