Studii şi Cercetări Ştiinţifice
Chimie şi Inginerie Chimică, Biotehnologii, Industrie Alimentară
2025, **26** (2), pp. 141 – 153
https://doi.org/10.29081/ChIBA.2025.598
Scientific Study & Research
Chemistry & Chemical Engineering, Biotechnology, Food Industry
ISSN 1582-540X

ORIGINAL RESEARCH PAPER

DESIGN OF RHAMNOLIPID-ETHYL THIOSULFANILATE NANOPARTICLES, PHYSICOCHEMICAL CHARACTERISTICS, BIOLOGICAL ACTIVITY

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Received: July, 04, 2024
Accepted: June, 06, 2025

Abstract: Nowadays, the urgent task of pharmacy and chemistry is to increase the bioavailability and effectiveness of medicines to overcome the resistance of pathogens. Nanoparticles based on biologically active substances have a special potential for solving this problem. The purpose of this work was the synthesis of nanoparticles of ethyl thiosulfanilate (ETS) with rhamnolipids (RL), establishment of physicochemical properties and antimicrobial action, comparison of the activity of nanoparticles and ETS-RL compositions. Using the methods of dynamic light scattering and scanning electron microscopy, it was established that aqueous solutions of the obtained ETS-RL compositions and nanoparticles formed spherical micelles, with hydrodynamic radii of 138 nm and 109 nm, respectively. The influence of the size of nanoparticles on their properties is shown, in particular, solutions of nanoparticles have a higher emulsifying and wetting ability compared to compositions. Solutions of ETS-RL compositions and nanoparticles show antimicrobial activity against *Staphylococcus aureus, Micrococcus luteus, Escherichia coli, Alternaria alternata, Fusarium oxysporum, Candida tenuis*. The obtained results testify to the effectiveness of the developed compositions and nanoparticles of ETS-RL and confirm the perspective of their use in pharmacy.

Keywords: *antimicrobial activity, dynamic light scattering, ethyl thiosulfanilate, nanoparticles, physicochemical properties, rhamnolipids, scanning electron microscopy, UV spectroscopy*

INTRODUCTION

The resistance of pathogenic microorganisms to existing medicines is an urgent problem in the world. [1] The low water solubility of many active pharmaceutical ingredients is a major obstacle to the development of new drugs. Regardless of the method of application of pharmaceutical preparations, their active substance must be water-soluble or solubilized. A promising approach to solving this problem is the creation of compositions and nanosized preparations of poorly soluble biologically active substances with biogenic surfactants (biosurfactants) to increase the effectiveness and bioavailability of drugs. Due to their amphiphilic nature, biosurfactants have surface activity, emulsifying, solubilizing, wetting ability, low critical concentrations of micelle formation [2]. Among biosurfactants, the most studied are rhamnolipids (RL), which exhibit antibacterial, antifungal and antiviral effects, are low-toxic and environmentally safe [3, 4]. The physicochemical properties of RL contribute to the stabilization of liposomes [5] and metal nanoparticles [6 – 11].

An important property of biosurfactants is their effect on the permeability of cell membranes, which helps to increase the bioavailability and effectiveness of biologically active substances [12, 13]. The interaction of biosurfactants with cell membranes was established at the molecular level using mass spectrometry, which was confirmed by studies of paired systems of RL with dipalmitoylphosphatidylcholine (a model membrane phospholipid) [14]. From the literature, it is known about the composition of the fungicidal component (syringomycin or pseudomycin) and with RL biosurfactant [15]. RL enhance the activity of aminoglycosides against *S. aureus* by inducing PMF-independent uptake (PMF - proton motore force) of aminoglycosides to restore susceptibility to other persisters, biofilms, small colony variants and anaerobic populations of *S. aureus*, as well as other high-quality microorganisms, resistant clinical isolates and gram-positive pathogens [16]. The efficacy of a number of antibiotics such as niclosamide, clindamycin and lincomycin has been improved in the form of RL micelles [17, 18].

The high activity of nanoparticles is associated with their large surface area. This contributes to the protection of biologically active components from degradation, prolonged release of the active substance and specific interaction with biological structures. Thus, on the basis of RL, nanoparticles with model biologically active agents - Nile red, dexamethasone and tacrolimus (up to 30 % load) were obtained. Experiments on human skin models showed effective penetration of nanoparticles with Nile red into the stratum corneum and partially into the lower epidermis of the skin [19]. Nanoparticles based on RL were used to deliver the synthetic peptide ParELC3, an inhibitor of bacterial topoisomerases, which contributed to increased activity against *E. coli* and *S. aureus* [20]. The functional properties of RL nanoparticles with hydrophobic curcumin were determined in different ratios. It was shown that with an increase in the mass fraction of RL, the encapsulation efficiency of curcumin increased from 44.59 % to 81.12 %, and the load increased from 10.14 % to 31.67 % [21]. The high efficiency of RL nanoparticles with the hydrophobic photosensitizer pheophorbide in inducing photodynamic damage under the action of laser irradiation has been demonstrated [22].

Representatives of the class of disulfide organic compounds - S-esters of thiosulfonic acids of synthetic or natural origin deserve attention among promising substances with a wide spectrum of biological activity. Thiosulfonates of natural origin are phytoncides

isolated from onion plants (garlic, leek), as well as various types of cabbage. Some of them may act on platelet receptors, inhibiting ADP- and collagen-induced platelet aggregation (in a dose-dependent manner). The regulation of platelet activation and aggregation processes was confirmed *in vitro* experiments using platelet-enriched plasma [23]. Experiments on laboratory animals have established the toxic effect of propylpropane thiosulfanilate, potential natural additive to food products and feed with a sufficient safety profile. This indicates their prospects for the agro-food sector [24]. According to the principles of "green chemistry", several S-esters were synthesized, and their antibacterial and antiviral activity as well as cytotoxicity against A-549 cells, were established [25].

It has been established that ethyl thiosulfanilate (ETS), an effective and more chemically stable alternative to plant phytoncides, possesses antioxidant, antimicrobial, antiviral, and anticancer activities, but is characterized by low water solubility [26]. It inhibits the growth of opportunistic fungi *C. tropicalis*. At fungistatic and subfungicidal concentrations, ETS exhibits membranotropic action and ensures a high degree of cooperativity in membrane structural transitions [27]. In both *in vitro* and *in vivo* experiments, it has been shown that the addition of thiosulfonate preparations as feed additives for rats did not cause pathological changes and positively affected the antioxidant levels in the blood of the experimental animals [28].

In vitro experiments have also determined the anthelmintic effect of S-methyl-(2-methoxycarbonylaminobenzimidazole-5) on the development of ascariasis (*Ascaris suum*) in pigs, making this compound a potential ovicidal anthelmintic agent [29]. The effects of allyl thiosulfanilate, ethyl thiosulfanilate, and methyl thiosulfanilate on the physico-mechanical properties of textile materials after exposure to sunlight have been established. It has been shown that the mechanical and physical properties of cotton and polyester-cotton sewing materials are preserved under these conditions [30]. After treating cellulose-containing textile materials with thiosulfonate preparations, the fabrics exhibit prolonged antimicrobial activity, which persists even after washing the treated materials [31].

It has been established that a complex liposomal preparation based on ethyl thiosulfanilate normalizes hematological and biochemical blood parameters in cows suffering from catarrhal mastitis. Specifically, it enhances the bactericidal and lysozyme activity of blood serum and the enzyme glutathione peroxidase, reduces the levels of lipid peroxidation products, and maintains metabolic homeostasis in the organism, positively affecting the activity of the body's natural defense mechanisms [32].

Since the use of thiosulfonates is limited by their low solubility and consequently low bioavailability, the properties of RL have been utilized to enhance their solubility and bioavailability. We previously demonstrated the increased antimicrobial activity of ETS in compositions with RL against a range of phytopathogenic and test microorganism cultures [33, 34]. Currently, RL biosurfactants as nanocarriers for hydrophobic preparations are insufficiently studied.

The purpose of this work was the synthesis of nanoparticles and compositions of rhamnolipids-biosurfactant with ETS, studying physicochemical properties and estimation of antimicrobial activity, comparison of ETS-RL nanoparticles and compositions efficiency.

MATERIALS AND METHODS

In the study was used ethylthiosulfanilate synthesized at Lviv Polytechnic National University according to the scheme (Figure 1).

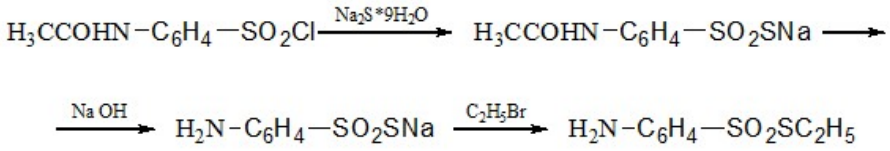


Figure 1. *Scheme of synthesis of ethyl thiosulfanilate*

Study of solubility of ethyl thiosulfanilate

To determine the solubility, 50 mg of ETS was crushed in a porcelain mortar and water (*p*H 7.2) was gradually added, transferred to a flask, bringing the volume to 50 mL, and left overnight. The obtained suspension was centrifuged (10 min, 6000 rpm, 3985 g). The sediment after centrifugation and the undissolved residue in the flask were dried to a constant mass and weighed.

To determine the effect of ultrasound on the solubility of ETS, the suspension was placed in an ultrasonic bath for 15 minutes at 308 K. After 24 hours, the treatment was repeated. The resulting suspension was centrifuged (10 min at 6000 rpm, 3985 g), and the sediment was dried to constant weight and weighed.

Rhamnolipids production

Rhamnolipids were obtained by biosynthesis using the strain *Pseudomonas* sp. PS-17 (collection of microorganisms of Department of Physical Chemistry of Fossil Fuels) in an optimized medium containing glycerol [35].

The biomass was separated by centrifugation for 20 minutes at 6000 rpm (3985 g). The sediment containing RL was obtained by acidifying the supernatant with 10 % HCl to *p*H 3.0, followed by settling and centrifugation at 7084 g (8000 rpm). RL were extracted from the sediment using a chloroform/methanol mixture (2:1), followed by evaporation under vacuum [36].

Synthesis of nanoparticles and compositions of ETS-RL

ETS-RL composition was prepared according to the method [19] with our modification: 0.03 g of ETS was dissolved in 30 mL of an aqueous solution of RL (1 $g \cdot L^{-1}$, *p*H 7.2). The compositions were treated in an ultrasonic bath for 15 minutes at 308 K, left overnight in the dark at 300 K, and then subjected to another round of ultrasonic treatment.

To obtain the solution of ETS-RL nanoparticles, the obtained composition was passed through a PTFE membrane filter with a pore size of 0.22 μm [19].

The quantitative determination of sulfur in solutions of ETS-RL nanoparticles was determined by the micromethod based on the content of sulfur in organic compounds [37].

Surface tension

The surface tension of solutions of compositions and nanoparticles of RL with ETS was determined by Du Noüy ring method with a platinum ring on the Tensiometer KRÜSS K6 ("KRÜSS" Gmbh, Germany).

Emulsification

The emulsification of the developed preparations was carried out with sunflower oil [38].

Ultraviolet spectrometry

Ultraviolet spectrometry of the solutions performed using a Shimadzu UV 3660i Plus device (Shimadzu Corporation, Japan) of the Lviv Polytechnic National University.

Hydrodynamic dimensions

Determination of the hydrodynamic dimensions of micellar structures of compositions and nanoparticles of RL with ETS was carried out by the method of dynamic light scattering (DLS) on a DynaProNanoStar device (Wyatt Technology, USA) using non-invasive backscattering technology at a temperature of 298 K. The sizes of micellar structures of ETS-RL were measured in an aqueous medium in 3 replicates.

Scanning Electron Microscopy

Scanning electron microscopy was used for investigation of structures RL with ETS compositions and nanoparticles (Zeiss EVO 40-XVP, Zeiss, Germany). A drop of obtained nanoparticle solution was applied to a silicon wafer. The samples were dried at room temperature for 3 days, after which a gold coating was applied.

Antimicrobial activity

Antimicrobial activity was evaluated based on the minimum inhibitory concentration (MIC) and bactericidal concentration (MBC) of the preparation. The serial dilution method in liquid nutrient medium was used, conducted in 24-well plastic plates (Sarstedt, USA). The diluted media were inoculated with the test culture. Daily cultures were diluted to a concentration of $1 \cdot 10^9$ CFU·cm^{-3}. The minimum concentration of the preparation that resulted in complete visible inhibition of culture growth (clear broth) corresponded to the MIC of this preparation concerning the tested strains. To determine the degree of bactericidal activity of the preparation, the last clear wells were plated on solid agar medium. The MBC of the tested preparations was defined as the lowest concentration in the wells from which no growth was observed on agar after incubation [39].

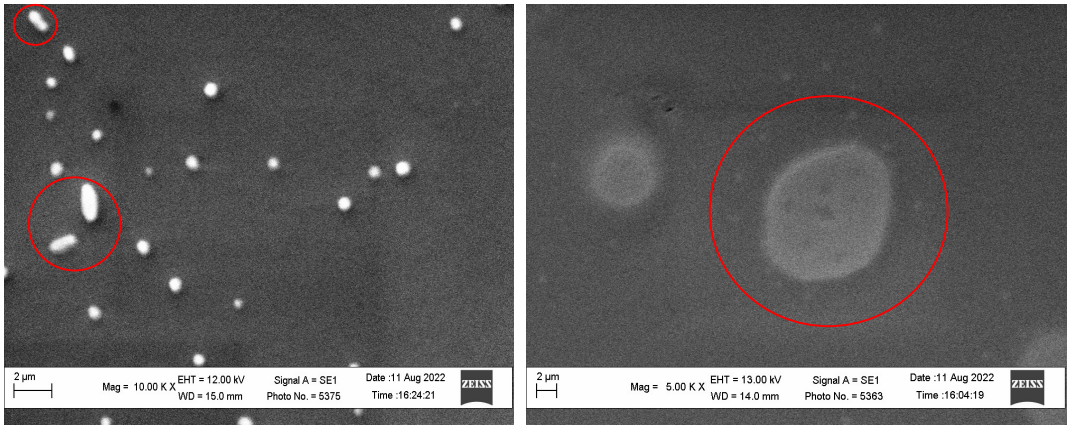
Statistical analysis

The results are presented as means ± standard deviations. Experimental dates were statistically processed using the Microsoft Excel-2010 software package. Differences between the experimental results were statistically analyzed using the Statisticasoftware package version 12.0 (StatSoft, Tulsa, OK, USA). Differences were considered as statistically significant at $P < 0.05$ [40].

RESULTS AND DISCUSSION

It is known that ETS belongs to the category of poorly soluble compounds (State Pharmacopoeia of Ukraine) [41]. It was established that the solubility of ETS in water is 0.69 $g \cdot L^{-1}$. When processing an aqueous suspension of ETS in an ultrasonic bath with heating, the solubility increases to 0.83 $g \cdot L^{-1}$. However, the obtained solution turned out to be unstable, the sediment fell out within 24 hours. RL biosurfactant was used to stabilize the ETS solution, by using which the corresponding ETS-RL compositions and nanoparticles were obtained. Besides, the use of RL increased the solubility of ETS up to 1 $g \cdot L^{-1}$.

It was established that the obtained nanoparticles have a predominantly spherical shape with a size of about 200 nm. However, during the preparation of SEM samples, agglomerates are formed on the silicon wafer. The process of agglomeration of two nanomicelles is clearly visible in Figure 2A - in the upper left corner, resulting in the formation of nanotubes. Also, in the process of drying the drop on SEM plate, large spherical agglomerates are formed - Figure 2B.



A B

Figure 2. *Sizes and shapes of ETS-RL nanomicelles determined by using scanning electron microscopy*

Using the DLS method, it was established that both developed formulations contain nanosized micelles. The solutions of the ETS-RL composition exhibit a bimodal particle size distribution, predominantly containing nanoparticle fractions of 2.5 nm and 138 nm with a polydispersity index of 0.57. This can be explained by the simultaneous presence in the solution of free RL and RL micelles loaded by ETS (Figure 3).

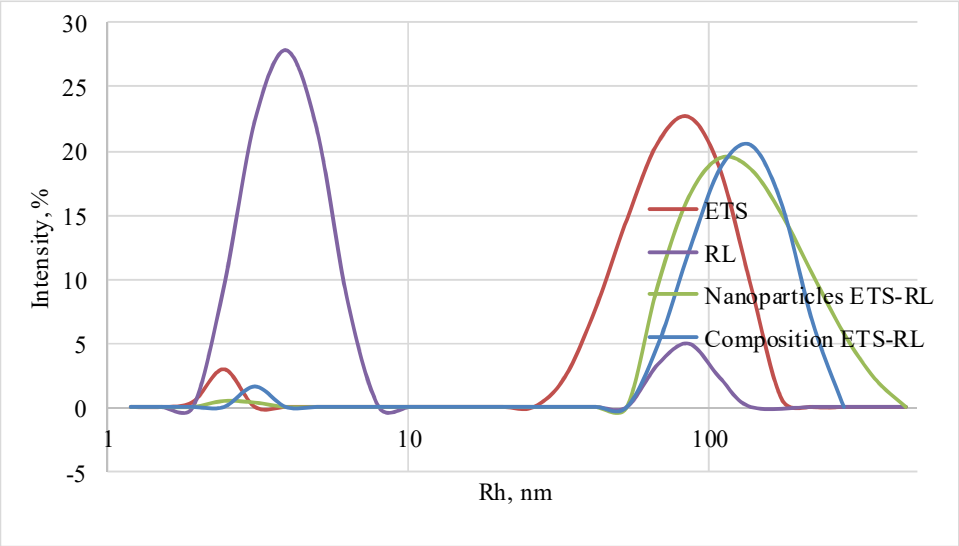


Figure 3. *Dynamic light scattering data of the obtained micellar structures of ETS-RL in aqueous solution (pH 7.2)*

It was established that the ETS-RL nanoparticle solution is more homogeneous and mainly contains a fraction with a size of 109 nm, while the polydispersity index decreases to 0.40. Probably, this effect is caused by a decrease in the hydrodynamic radius of the micellar structures after the separation of the composition solution using a membrane filter (Figure 3). The surface tension and emulsifying activity solutions of the composition and nanoparticles ETS-RL with respect to sunflower oil were determined (Table 1).

Table 1*. Surface tension and emulsifying activity of aqueous solutions of ETS-RL compositions and nanoparticles in relation to sunflower oil.*

Preparations	**Concentration ETS-RL [mg·L^{-1} / mg·L^{-1}]**	**σ [mN·m^{-1}]**	**E_{24} [%]**
RL	0/50	30.7±0.1	54±1
	0/25	30.8±0.1	52±2
	0/12,5	30.7±0.1	49±2
ETS	100/0	59.3±0.1	50±3
	50/0	69.3±0.2	45±2
Compositions ETS-RL	50/50	30.8±0.1	55±2
	25/25	31.2±0.2	50±1
	12.5/12.5	31.2±0.2	44±1
Nanoparticles ETS-RL	50/50	30.7±0.1	58±2
	25/25	30.8±0.1	54±1
	12.5/12.5	31.0±0.2	46±1

It was established that aqueous suspensions of ETS at concentrations of 50 and 100 mg·L^{-1} have emulsifying activity with respect to sunflower oil. In aqueous solutions

of nanoparticles and compositions of ETS-RL the emulsification index increases with an increase in the concentration of RL.

The wetting angles of the aqueous solutions of the studied preparations with respect to different types of surfaces were determined (Figure 4). Aqueous solutions of ETS-RL nanoparticles more effectively wet the surface of glass and St3 steel relative to the wetting activity of ETS-RL compositions. The action of the studied preparation when wetting Plexiglas and PTFE practically does not differ. According to the literature, such effect depends on both the nature of the surface and the size of the nanoparticles [42].

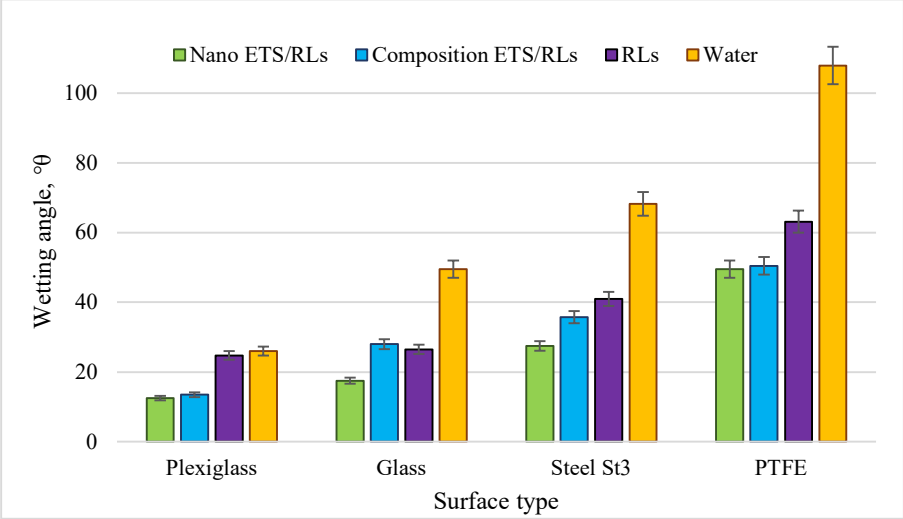


Figure 4. *Wetting angles of various surfaces by aqueous solutions of ETS-RL compositions and nanoparticles*

Note: Aqueous solutions of compositions and nanoparticles were used in 1:1 ratio at concentration of 1 $g \cdot L^{-1}$.

Thus, it is shown that aqueous solutions of ETS-RL nanoparticles, which have a smaller hydrodynamic size, more effectively wet various surfaces than solutions of the corresponding compositions.

The structure of the studied drugs was analyzed by UV spectroscopy (Figure 5). It was established that a clear peak at λ 291 nm is observed on the spectrum of ETS (5 $mg \cdot L^{-1}$ solution). A new peak at a wavelength of λ 254 nm appears on the spectra of solutions of both nanoparticles and ETS-RL compositions (ratio 1:1 at concentration of 5 $mg \cdot L^{-1}$).

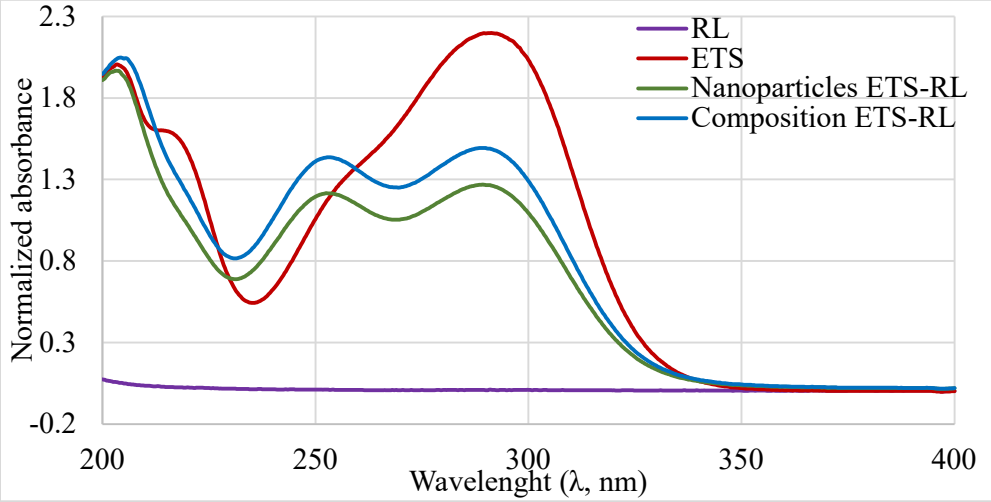


***Figure 5**. UV spectra of solutions of ETS-RL compositions and nanoparticles*

Analysis of the spectral data indicated the formation of composition of RL and ETS, probably, by donor-acceptor mechanism according to the scheme (Figure 6).

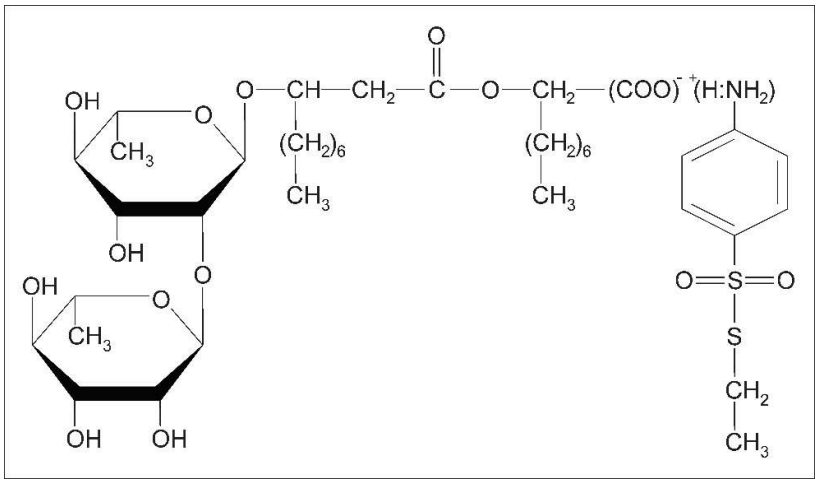


***Figure 6.** Complex RL with ETS*

The content of sulfur in solutions of ETS-RL nanoparticles was determined and the amount of ETS in the preparations was calculated. It was established that the ETS content in ETS-RL solutions of compositions and nanoparticles is 1 $g \cdot L^{-1}$ and is practically the same.

The antimicrobial activity of the developed ETS-RL preparations was determined. It is shown that solutions of nanoparticles and compositions show the same bactericidal and fungicidal activity. Therefore, the difference in the size of nanomicelles of nanoparticles and compositions in the range of 109 - 138 nm (at the chosen concentration of ETS-RL) practically does not affect their antimicrobial activity (Table 2).

Table 2. *Antimicrobial activity of aqueous solutions ETS-RL nanoparticles and compositions*

Test cultures	Nanoparticles ETS-RL		Composition ETS-RL	
	MIC [mg·L^{-1}]	MBC [mg·L^{-1}]	MIC [mg·L^{-1}]	MBC [mg·L^{-1}]
Staphylococcus aureus	0.78±0.04	1.56±0.02	1.56±0.03	6.25±0.03
Micrococcus luteus	1.56±0.02	6.25±0.03	1.56±0.02	6.25±0.04
Escherichia coli	9.30±0.05	18.70±0.06	9.30±0.04	18.70±0.04
Alternaria alternata	4.60±0.03	9.30±0.04	4.60±0.04	9.30±0.04
Fusarium oxysporum	2.30±0.02	4.60±0.04	2.30±0.04	4.60±0.04
Candida tenuis	2.30±0.02	4.60±0.03	2.30±0.02	4.60±0.04

Note: Aqueous solutions of compositions and nanoparticles were used in a 1:1 ratio.

Thus, the results of the experiments indicate that it is more appropriate to use the ETS-RL composition than nanoparticles as an antimicrobial preparation, which is not only environmentally safe and low-toxic, but also economically beneficial, as it does not require additional stages of microfiltration.

CONCLUSIONS

Nanoparticles and compositions of the biocide ethyl thiosulfanilate with a rhamnolipids biosurfactant were synthesized. It was shown that both the ETS-RL compositions and nanoparticles contain nanosized micelles, predominantly spherical in shape, with hydrodynamic radii of 138 nm and 109 nm, respectively. The ETS-RL nanoparticle solutions exhibit better wetting properties surfaces of different materials than the composition solutions, which can be explained by their smaller hydrodynamic sizes. It was also shown that the ETS-RL nanoparticle solutions have a higher emulsification index compared to the corresponding compositions.

The UV spectra of the obtained compositions and nanoparticles indicate an interaction between ETS and RL suggesting the formation of a complex, likely through a donor-acceptor mechanism.

The antimicrobial activity of the ETS-RL preparations against test microorganisms was determined. It was shown that the activity of the ETS-RL composition and nanoparticle solutions does not depend on the obtained size of their nanomicelles.

Thus, the obtained ETS-RL nanoparticles and compositions with high antimicrobial activity are promising for use in modern pharmacy.

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