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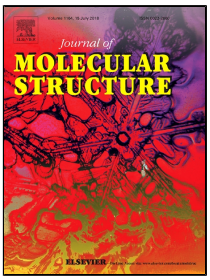

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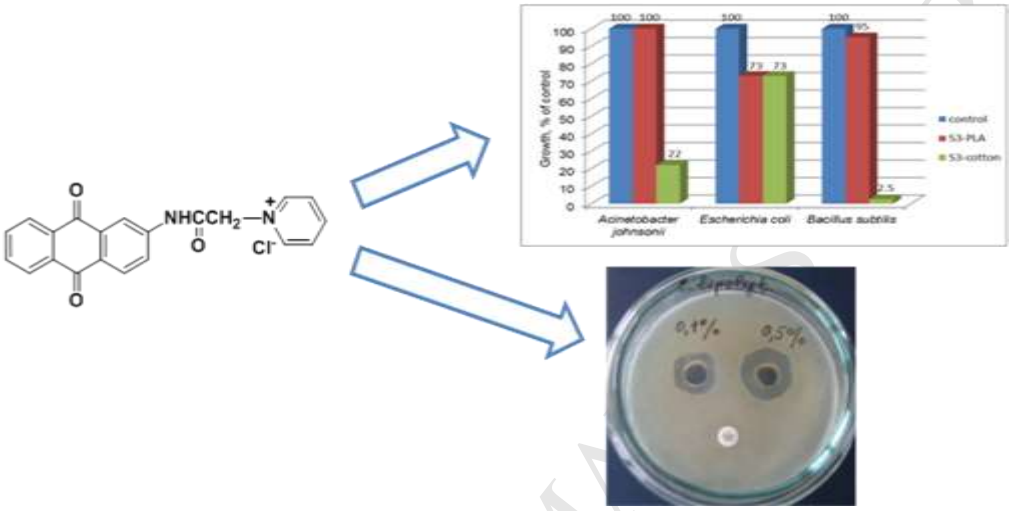

Synthesis and characterization of new water soluble 9,10-anthraquinone and evaluation of its antimicrobial activity

Stanislava Yordanova[1], Evgenia Vasileva-Tonkova[2,*], Desislava Staneva[3], Stanimir Stoyanov[1], Ivo Grabchev[4,*]

[1]Sofia University "St. Kliment Ohridski", Faculty of Chemistry and Pharmacy, 1407 Sofia, Bulgaria

[2]The Stephan Angeloff Institute of Microbiology, Bulgarian Academy of Sciences, 1113 Sofia, Bulgaria

[3]University of Chemical Technology and Metallurgy, 1756 Sofia, Bulgaria

[4]Sofia University "St. Kliment Ohridski", Faculty of Medicine, 1407 Sofia, Bulgaria

Abstract

The synthesis and characterization of a new cationic anthraquinone derivative (**S3**) soluble in water has been described. Its absorption characteristics in aqueous and *N,N*-dimethylformamide solutions have been determined. Antimicrobial activity of the compound **S3** was tested *in vitro* towards eight pathogenic bacteria and two yeasts strains. The results obtained suggest that the newly synthesized compound is effective in treating the relevant pathogens and is suitable in designing new effective antimicrobial preparations. Antimicrobial activity and release of **S3** after its deposition on cotton fabric and incorporation into thin polylactid film have also been investigated.

Keywords: 9,10-anthraquinone, microbiological, antibacterial textile, polylactid,

Corresponding author: Ivo Grabchev: e-mail i.grabchev@chem.uni-sofia.bg

1. Introduction

The cationic compounds and especially the quaternary ammonium salts (QAS) exhibit excellent antimicrobial activity against Gram-positive and Gram-negative bacteria, fungi and certain types of viruses, therefore they can be used as antiseptics, bactericides and fungicides, and as therapeutic agents, as well [1]. These microbial agents damage the cell wall or alter cell membrane permeability, denature proteins, inhibit enzyme activity or inhibit lipid synthesis and all of those factors that are essential for cell survival. Therefore, the introduction of such quaternary groups into certain organic compounds can be a strategy in the design of biologically active compounds with antibacterial and antifungal activity. Recently, we have shown that the introduction of QAS in the structure of benzanthrone leads to the occurrence of antimicrobial activity, which is maintained after deposition into a polylactide thin film. In this way, the effect of dyeing was combined with the antibacterial activity [2, 3].

The derivatives of 9,10-anthraquinone belong to the group of natural quinones which are isolated from different fungi, lichens and some plants [4]. They are the second most important class of dyes which have been used as colorants in textiles industry, cosmetics as hair dyes [5]. Quinones especially anthraquinones, present numerous biological activities such as antiprotozoa, antituberculous, fungicidal, antioxidant, cytotoxic and antitumor activities [6-11].

Polylactide (PLA) is one of the most important commercially available and common biodegradable and biocompatible polymer produced from renewable resources such as corn, potatoes, sugar, etc. Due to transparently and low toxicity it is one of the most promising biopolymers able to replace the petroleum-derived polymers for different practical applications, especially for food packaging when the antibacterial resistance is of particular importance [12,13]. The treatment of textile materials with substances which exhibit antimicrobial activity makes it possible to obtain textile materials with antibacterial or antifungal properties [14, 15]. Such materials could be used as effective wound dressings in the clinical practise.

In this work we describe the synthesis and characterisation of water soluble cationic anthraquinone derivative, comprising quaternary ammonium group. Its antimicrobial activity against gram-positive and gram-negative bacteria and yeasts was investigated. The minimum inhibitory concentration and antimicrobial activity against the tested bacteria and yeasts was

discussed. The antibacterial efficiency in aqueous solution of the thin PLA film and cotton fabric treated with the new compound has also been evaluated.

2. Experimental part

2.1. Materials and methods

Initial 2-aminoanthraquinon has been used as obtained from Sigma Aldrich. UV-Vis spectrophotometric investigations were performed using "Thermo Spectronic Unicam UV 500" spectrophotometer, using 1 cm path length synthetic quartz glass cells. Absorption measurements of the compound were carried out at 10^{-5} mol l^{-1} concentration. ^{1}H (600.13 MHz) and ^{13}C (150.92 MHz) spectra were acquired on an AVANCE AV600 II+NMR spectrometer. The measurements were carried out in $DMSOd_6$ solution at ambient temperature. The chemical shifts were referenced to a tetramethylsilane (TMS) standard. Electrospray mass spectroscopic measurements were carried out using a Hewlett–Packard Series 110 0 MSD. Thin layer chromatographic (TLC) analysis was followed on silica gel (Fluka F_{60} 254 20x20; 0.2 mm) using the solvent system hexane/acetone (1:1) as an eluent. PLA was obtained from Sigma Aldrich (M_w 18,000, T_g 46-50 °C). The thickness of the composite material has been determinate by AirBlast DFT-441 Dry Film Thickness Gauge.

2.2. Synthesis of compound S2

2-aminoanthraqinone (2.23 g, 0.01 mol) was dissolved in 30 ml of dioxan and chloroacetyl chloride (0.81 ml, 1.1 mol) were added. The mixture was stirred vigorously at 50 ^{0}C for 2h. After that liquor was added to 100 ml of ice water. The precipitate was filtered off, washed with water, and dried in vacuum at 40^{0}C. The crude product was purified by recrystallisation from ethanol to give S2 as a yellow solid (2.76g, 96 %)

FT-IR cm^{-1}: 3344, 3066, 2943, 1723, 1668, 1588, 1538, 1422, 1298, 1235, 1172, 926, 723, 712, 662,

^{1}H NMR (DMSO-d6, ppm): d 11.45 (br s, 1H, NH), 8.81-8.78 (d, J = 5.34 Hz, 2H), 8.70-8.68 (d, J = 6.46 Hz, 1H), 8.59-8.42 (d, J = 8.40 Hz, 1H), 8.35-832 (d. J = 6.6 Hz, 2H), 8.09-8.06 (d, J = 8.17 Hz, 1H,), 7.79 (t, J = 7.68, Hz, 1H), 8.87 (t, J = 6.92, Hz, 1H), 7.63 (t, J =7.65 Hz, 1H), 4.57 s, 2H), 3.64-3.55 (2, J =-7.15 Hz, 6H), 1.30 (t, J =7.06 Hz, 9H,).

^{13}C NMR: 182.7, 182.8, 166.0, 144.3, 135.0, 134.7, 134.6, 133.5, 129.0, 128.8, 127.2, 127.1, 124.4, 116.5, 43.9.

Elemental analysis: $C_{15}H_{10}N_1O_3Cl$ (287.6) : Calcd. C 62.58, H 3.47, N 4.86; Found C 62.68, H 3.29, N 4.79.

2.3. Synthesis of compound S3

Compound S2 (0.287 g, 0. 1 mol) was dissolved in 10 ml dioxin, then 1.5 ml of pyridine was added and the solution was stirred for 6 h at 60^0C. After cooling to room temperature, the precipitate was filtered off, washed with acetone and dried in vacuum at 40^0C. Yield: 0.30g (84 %). The product S3 was obtained as light yelow solid and it has been used without any purification.

FT-IR cm^{-1}: 3416, 3054, 2940, 1714, 1673, 1624, 1593, 1553, 1492, 1331, 1293, 1198, 1000. 928, 789, 713, 679, 538,

^{1}H NMR (DMSO-d6, ppm): d 11.45 (br s, 1H, NH), 8.81-8.78 (d, J = 5.34 Hz, 2H), 8.70-8.68 (d, J = 6.46 Hz, 1H), 8.59-8.42 (d, J = 8.40 Hz, 1H), 8.35-832 (d. J = 6.6 Hz, 2H), 8.09-8.06 (d, J = 8.17 Hz, 1H,), 7.79 (t, J = 7.68, Hz, 1H), 8.87 (t, J = 6.92, Hz, 1H), 7.63 (t, J =7.65 Hz, 1H), 4.57 s, 2H), 3.64-3.55 (2, J =-7.15 Hz, 6H), 1.30 (t, J =7.06 Hz, 9H,).

^{13}C NMR: 182.8, 181.8, 164.9, 146.9, 144.2, 135.1, 134.8, 134.7, 133.5, 134.4, 129.1, 129.0, 128.0, 127.2, 127.1, 124.5, 62.8

Elemental analysis: $C_{20}H_{15}N_2O_3Cl$ (362.6) : Calcd. C 66.18, H 4.14, N 7.72; Found C 66.58, H 4.29, N 7.69.

2.4. Preparation of PLA film

Pure PLA and antimicrobial PLA films were prepared by solvent casting method.

0.5 g polylactic acid was dissolved in 10 ml chloroform and 0.05mg compound **S3** was added. After 30 minutes stirring, the homogeneous mixture was poured into a Petri dish and the solvent was evaporated slowly. Thus was obtained a stable polymer film having a thickness of 50 μm (±1 μm). The same method has been used to produce pure PLA film.

2.5. Treatment of cotton fabrics with S3

0. 5mg of compound **S3** was dissolved in 5 ml water. Cotton fabric sample (1g) (weight 140 g/m^2) was immersed in the solution at 25°C for 30 min, and dried at ambient temperature.

2.6. Antimicrobial activity test

The newly synthesized **S3** compound was screened for antimicrobial activity against selected pathogenic bacteria and yeasts by using the agar well diffusion method [16]. The following test cultures were used: Gram-positive bacteria *Bacillus subtilis*, *Bacillus cereus*, *Sarcina lutea* and *Micrococcus luteus*, Gram-negative bacteria *Pseudomonas aeruginosa*, *Escherichia coli*, *Acinetobacter johnsonii* and *Xanthomonas oryzae*, and the yeasts *Candida lipolytica* and *Saccharomyces cerevisiae*. A lawn of each test organism was prepared by spreading aliquots (0.1 ml) of overnight cell suspensions on Mueller-Hinton agar plates. Tests were performed using 0.1% and 0.5% water solutions of the investigated AN compound, of which equal amount (30 µl) was added into 8 mm wells punched aseptically in the agar. Commercial discs with gentamicin (10 µg) and nystatin (Ns, 100 units/disc) were used as a standard antibacterial and antifungal agent, respectively. After incubation of the plates at 26°C for 24-48 h, the diameter of growth inhibition zones (including well/disc) was measured. An inhibition zone diameter ≥ 10 mm indicated that the tested compound is active against the indicator cultures.

2.7. Determination of Minimum Inhibitory Concentration (MIC)

The minimum inhibitory concentration (MIC) of the tested S3 compound was determined by the broth-dilution method [17]. Serial dilutions of the synthesized compound were prepared in meat-peptone broth (MPB) in the range 6-60 µg/mL. Each dilution was inoculated aseptically with 1% of the respective overnight microbial suspension. The culture tubes were incubated at 26°C for 24 h with agitation. Three independent experiments were carried out, and averages are given.

2.8. Test of film antimicrobial activity

The antimicrobial effect of the obtained **S3**-PLA film was evaluated towards model bacteria Gram-negative bacteria *Escherichia coli* and *Acinetobacter johnsonii*, and Gram-positive *Bacillus subtilis*. For antimicrobial tests, square shape speciments (10-mm x 10-mm) were cut from pure PLA film (control) and S3-PLA film -cotton sample under aseptic conditions. The test tubes with 1.5 ml sterile meat-peptone broth (MPB) were inoculated with 1% overnight bacterial cultures and left at room temperature for 15 min. Then, the specimens were inserted into the test tubes. Test tubes without inserted film speciments were also prepared for each bacterial culture. After 24 h incubation at 26°C under shaking, the specimens were removed

and the bacterial growth was determined by spectrophotometric measurement of the optical density (OD) at 600 nm.

3. Results and Discussion

3.6. Synthesis and functional properties of new water-soluble anthraquinone derivative

Scheme 1 illustrates the synthetic route for the synthesis of a new water-soluble anthraquinone derivative. As a starting material, 2-aminoanthraquinone (**S1**) has been used. Acylation of the primary amino group was carried out with chloracetylchloride at 50°C for 2 h in dioxan solution produces compound (**S2**). The activated chlorine atom from S2 can reacts with tertiary amines to forms quaternary ammonium salt. In this case a pyridine has been used as tertiary amine, which reacts with the chlorine atom of S2 in dioxane solution at 60^0C for 4 The precipitate of final product (**S3**) and is isolated in high yield (93%) by filtration and several times was washed with acetone. Products S2 and S3 were characterized with absorption, FT-IR, NMR ^{1}H- and ^{13}C- spectroscopy and elemental analysis.

Scheme 1.

3.7. Functional characteristics of compounds S1-S3.

In order to study the structural dependence, the absorption spectra of the newly synthesized compounds **S2** and **S3** were examined and compared with the starting product **S1** in a solution of water and *N,N*-dimethylformamide (DMF). From Figure 1 it is seen that compound S1 in both solvents absorbs in the visible spectral region with absorption maxima at λ_A = 448 nm (water) and λ_A = 445 nm (DMF). After acylation with chloroacetyl chloride (**S2**) the respective absorption maxima are hypochromatically shifted with $\Delta\lambda_A$ = 80 nm and $\Delta\lambda_A$ = 104 nm, respectively, due to the presence of a electron acceptor carbonyl group that drastically alters the electron donor capability of the primary amino group and the absorption maxima are in the ultraviolet region. The introduction of the cationic pyridine group into the structure of **S3** does not significantly affect the position of the absorption maximum in both solvents, which are λ_A = 362 nm (water) and λ_A = 341 nm (DMF).

Figure 1.

In order to use **S3** in the design of antibacterial wound dressings and to make antibacterial packages, the possibility of separating the active substance **S3** from the surface and mass of the corresponding polymeric materials (cotton cloth and PLA) was investigated. Figure 2 shows the release of compound S3 from the PLA matrix and 100% cotton fabric over the time in aqueous solution. The process was monitored spectrophotometrically by absorption spectroscopy at the absorption maximum λ_A = 362 nm. The figure shows that absorption maximum of S3 increases with time, due to the its release from the polymer matrices to an constant quantity that is different for the different matrices. The Figure 2 shows that compound S3 releases from the cotton surface, for around 20 minutes This indicates that at that time a significant part of the biological active substance will be released and able to show its microbiological activity. In the case of PLA matrix, a significantly longer release time for S3 is required, which may be is due to the rigid polymeric structure and hence the more difficult diffusion of **S3** in the aqueous solution.

Figure 2.

Figure 3.

From Figure 3 it is seen that in pH range 7.7-9.5 compound **S3** absorbs in the near UV region with an absorption maximum at 340 nm. In alkaline media (pH = 9.5), the absorption maximum is batochromicaly shifted by 76 nm (λ = 416 nm) and the colorless solution became yellow. The calculated pKa value is pKa = 10.3 .

3.8. Antimicrobial activity

3.8.1. In vitro antimicrobial activity and MIC

Compound S3 was tested *in vitro* for antimicrobial activity towards eight pathogenic bacteria and two yeasts strains. The results revealed that at the used concentrations the compound possess good antimicrobial activity against most of the test microbial cultures. As seen in Figure 4 and 5, 0.5% **S3**-solution exhibited better zones of inhibition (in the range 17-28 mm) against the test strains than those observed using 0.1% S3-solution (in the range 14-24 mm).

Figure 4.

E. coli, *P. aeruginosa* and *M. luteus* were found to be resistant to 0.1% **S3**-solution while low to moderate activity was observed using 0.5% **S3**-solution. The observed antibacterial activity using 0.5% **S3**-solution was about 2 to 1.4-fold lower than that of the control for six test bacteria or comparable with the control for *B. subtilis* and *X. oryzae*. The compound exhibited good antifungal activity against the test yeasts *S. cerevisiae* and *C. lipolytica* while no growth inhibition was observed by the control drug nystatin. MIC of S3 compound was determined against those test cultures that showed good zones of inhibition in the preliminary screening.

Figure 5.

Table 1

As seen in Table 1, MIC values varied from 6 to 36 μg/ml. The sample was found to be most effective (the lowest MIC) towards *B. subtilis*, and the highest MIC value of 36 μg/ml was determined towards *B. cereus* and *C. lipolytica*. According to Holetz et. al. [18], MIC values less than 100 μg/ml indicated good antimicrobial activity, from 100 to 500 μg/ml the antimicrobial activity was considered moderate, from 500 to 1000 μg/ml – weak, and over 1000 μg/ml the compound was considered inactive. For our result to be interpreted, S3 compound possessed very good antimicrobial activity towards five of the eight test bacteria and both yeast strains. Therefore, **S3** has potential to be used as effective antimicrobial agent in biomedical and agrochemical applications.

*3.8.2. Antimicrobial activity of **S3**-PLA film and **S3**-cotton in aqueous solution*

The results of *in vitro* antimicrobial tests of the obtained **S3**-PLA film and **S3**-cotton samples are shown in Figure 6. After 24-h of treatment, the **S3**-PLA film speciment reduced the growth of *E. coli* and *B. subtilis* by 27% and 5%, respectively, and no growth inhibition of *A. johnsonii* was established. The **S3**-cotton speciment caused a significant decrease in the growth of *A. johnsonii* and *B. subtilis* by 78% and 97.5%, respectively, while for *E. coli* this decrease was much lower (27%). The antimicrobial effect should be due to diffusion of the hydrophilic S3 compound from the hydrophobic PLA matrix into the medium. It has been reported that some other factors such as the level of immobilized compound retained at film surface and the surface area of the film may also have effect the release profile of the

immobilized activity [19]. Preliminary tests showed good antibacterial efficiency of the obtained **S3** incorporated cotton material suggesting its suitability for application as a new additive in wound dressings.

Figure 6.

Conclusion

A new water-soluble anthraquinone **S3** with a quaternary amino group has been synthesized and characterized and its spectral properties in water and DMF solutions have been investigated. The new anthraquinone demonstrated very good antibacterial and antifungal activity against different Gram-positive and Gram-negative bacteria and yeasts. The compound has been deposited on a cotton fabric and in a PLA matrix. In view of the use of these materials as antibacterial products, the release of the active substance in an aqueous solution has been investigated. It was found that anthraquinone deposited on the cotton fabric has been released significantly faster than from the PLA matrix. Antibacterial activity of cotton fabric and PLA film treated with the new anthraquinone was investigated against the strains *A. johnsonii, B. subtilis* and *E. coli*. The modified cotton fabric exhibited better activity against the tested cultures than the **S3**-PLA film, suggesting its suitability for application as a new additive in wound dressings.

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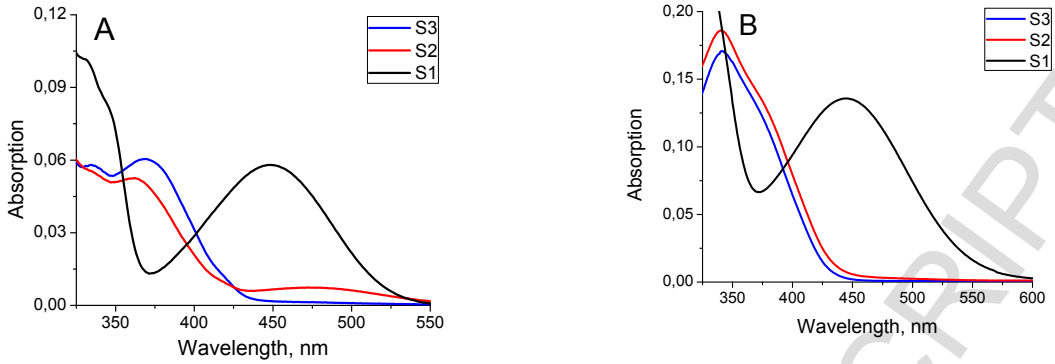


Figure 1. Absorption spectra of compounds S1-S3 in aqueous (A) and DMF (B) solutions.

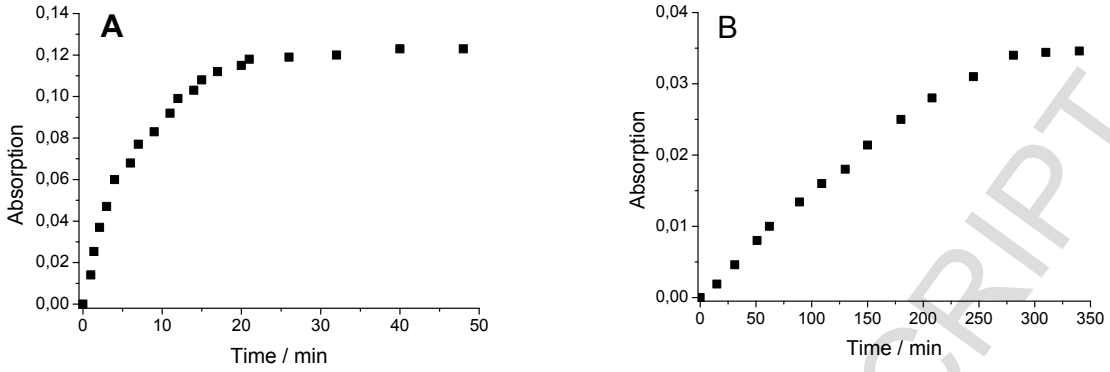


Figure 2. Dependence of the releases of S3 in aqueous solution from cotton fabric (A) and polylactide film (B)

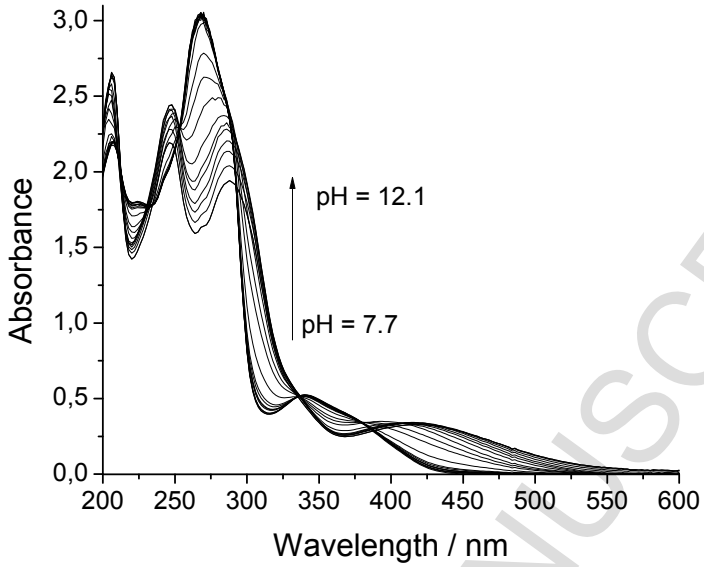


Figure 3. Absorption spectra of compound S3 in aqueous solution at different pH values

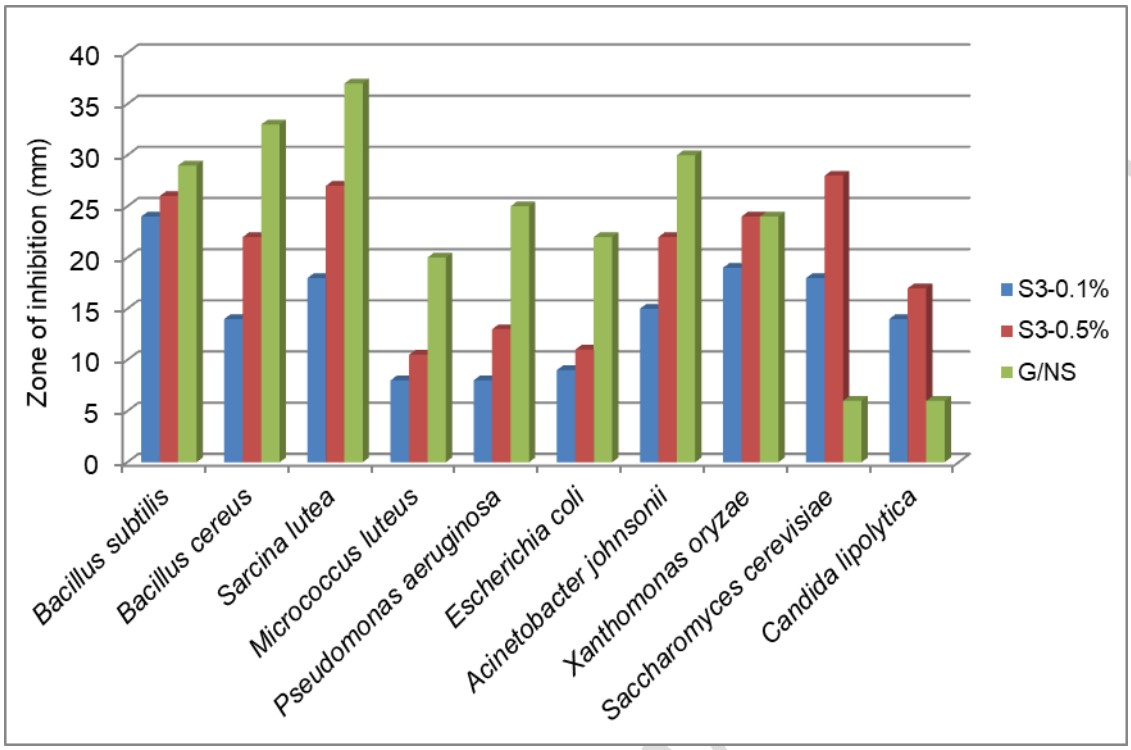


Figure 4. Zones of inhibition of the growth of the tested bacteria and yeasts by 0.1% and 0.5% water solutions of S3 compound. G, Gentamicin used as a standard antibacterial agent; NS, Nystatin used as a standard antifungal agent

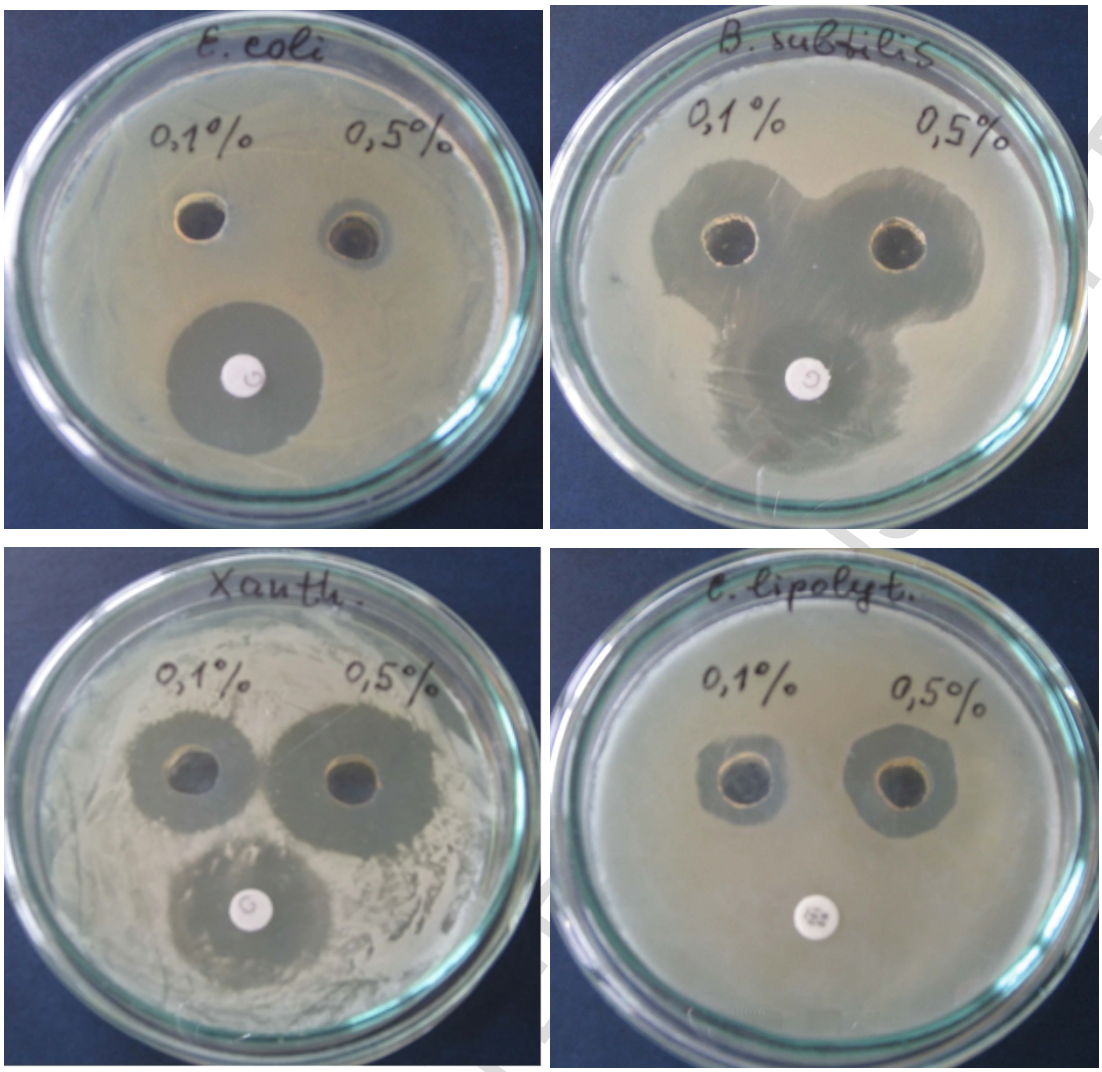


Figure 5. Zones of inhibition of the growth of some test bacteria and yeasts caused by 0.1% and 0.5% water solutions of compound S3.

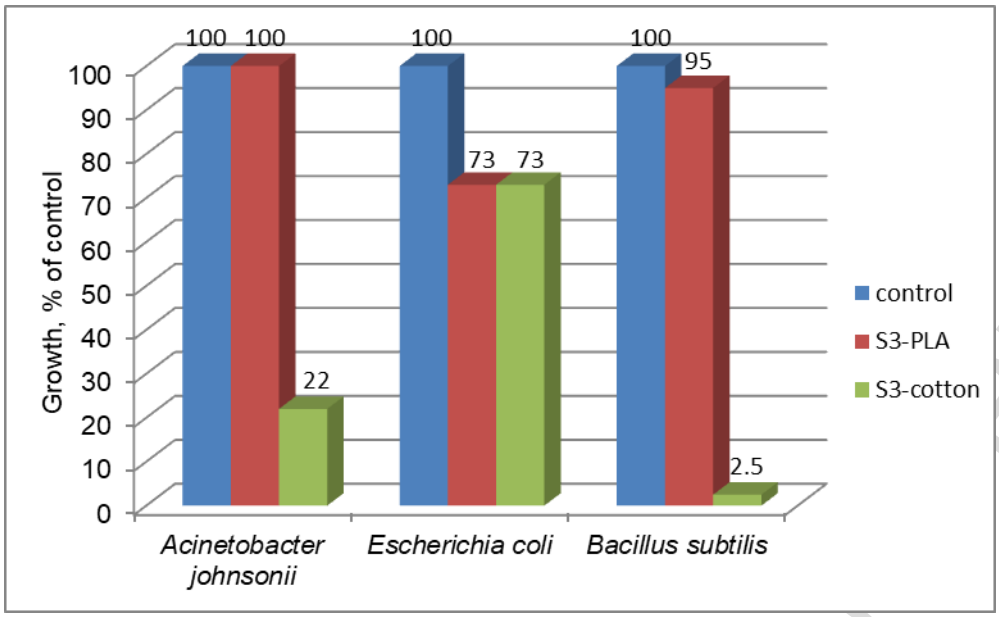
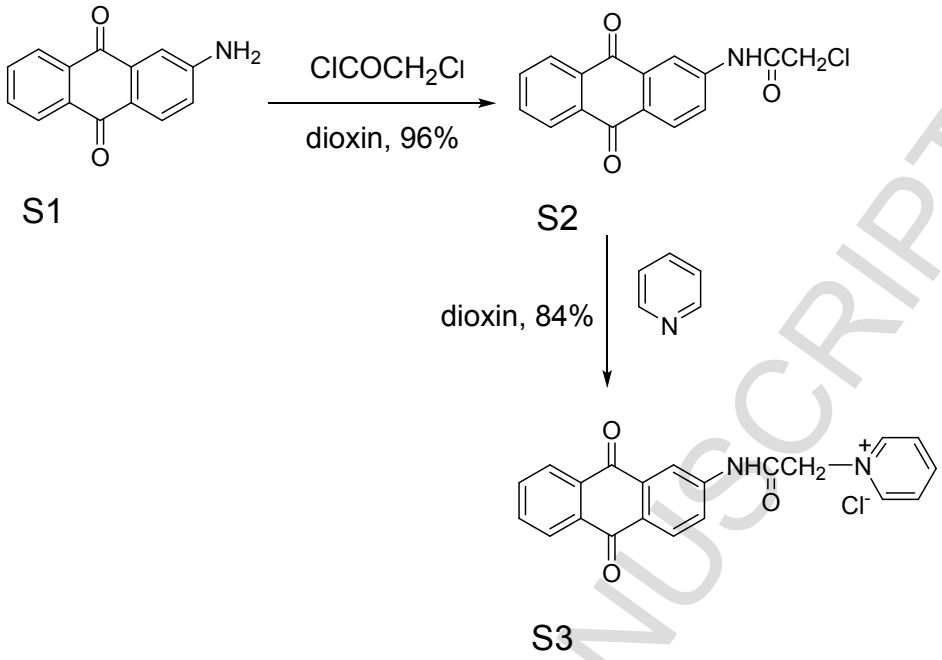


Figure 6. Effect of S3-PLA film and S3-cotton speciments on the growth of *A. johnsonii*, *E. coli* and *B. subtilis* strains tested in meat-peptone broth (MPB).



Scheme 1. Synthesis of new water-soluble anthraquinone S3

1. Synthesis of a new cationic water soluble anthraqinone
2. *In vitro* investigation of antibacterial textile an polylactide
3. Inhibitor activity against Gram-positive and Gram-negative bacteria and yeasts strains.

Table 1. MIC values of compound **S3** towards some test bacteria and yeasts.

Strains	MIC, μg/ml
Bacillus subtilis	6
Bacillus cereus	36
Acinetobacter johnsonii	32
Sarcina lutea	12
Xanthomonas oryzae	24
Saccharomyces cerevisiae	12
Candida lipolytica	36