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Antibacterial Activity of the Original Dietary Supplement Oxidal® *in Vitro*

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Abstract

Studies have been conducted to determine the antibacterial effect of Oxidal® and anolyte against *Staphylococcus aureus* and *Escherichia coli*. The bacterial strains tested showed high sensitivity to the dietary supplement Oxidal® and to anolyte, as well as to the control antibiotic Thiamphenicol. The mean MPC_{50} values of Oxidal® for Gram-positive bacteria *S. aureus* (0.21 ± 0.13 mg/ml) were lower than those for Gram-negative *E. coli* (0.55 ± 0.24 mg/ml). The dose that fully suppressed *S. aureus* growth was 0.86 ± 0.52 mg/ml, while for *E. coli* this dose was significantly higher - 2.40 ± 0.80 mg/ml ($P<0.05$). For the antibiotic Thiamphenicol, the results were opposite - the mean MPC_{50} values for *E. coli* (2.40 ± 0.80) were lower than those for *S. aureus* (3.71 ± 2.86). Under the influence of 10% Oxidal® solution, the amount of viable *S. aureus* and *E. coli* decreased by more than 30% over the untreated control after 20 min. After 45 minutes, only 37% of *S. aureus* cells and 19% of *E. coli* cells developed upon cultivation, and after 90 minutes of exposure, about 20% of the cells of the microorganisms under study of both species remained viable. The fresh anolyte and the one stored for 1 year inactivated *S. aureus* and *E. coli* for 2 min in a suspension at a density of 10^6 cells/ml.

Keywords: Oxidal®, Anolyte, S. Aureus, E. Coli, Antibacterial Activity, Minimum Inhibitory Concentrations

Introduction

Since 1990, the World Health Organization has held regular meetings on the increasing spread of antibiotic resistance of microorganisms, which has increasingly hampered the treatment of infections. With increasing rates of antibiotic resistance, treatment options diminish because the antibiotics available are no longer effective. After decades of overuse and abuse of antibiotics, the emergence and spread of resistant bacteria is a pervasive fact. At the same time, no success has been reported in the discovery of sufficient number of new antibiotics effective against resistant bacteria (WHO, 2019).

Today, as a result of the efforts to develop new effective antimicrobial agents, the inhibitory action of various substances is being tested. One of them is methylene blue, which is being tested by many researchers in combination with various materials. Many experiments confirmed its considerable antimicrobial activity, especially in combination with various polymers and nanoparticles. Naik et al. (2011) applied methylene blue and 2 nm gold nanoparticles to impregnate polyurethane polymer sheets. The dye-impregnated polymers show significant bactericidal activity against suspensions of *S. aureus* upon irradiation with white light. Perni et al. (2009) reported testing polysiloxane polymers impregnated with methylene blue and gold nanoparticles. These polymers show significant antimicrobial activity against methicillin-resistant *S. aureus* and *E. coli* when exposed to light from a low-power 660 nm laser for 5 min. This bactericidal activity is due to the light-induced production of singlet oxygen and other reactive oxygen species from methylene blue. In addition to light, the presence of 2 nm gold nanoparticles significantly increases the ability of methylene blue to kill bacteria. Methylene blue is also used for photodynamic therapy - for the inactivation of viral particles, fungal, bacterial

and cancer cells using photosensitizers and light of varying wavelengths. There is evidence that important pathogenic bacteria such as *Helicobacter pylori* can be easily inhibited in vitro in this way. The bactericidal effect increases in proportion to the concentration of methylene blue and the duration of the irradiation. The effect is due to oxidative damage to the DNA of the bacteria (Choi et al., 2010). In the last decade, methylene blue has been used in some European countries for photoinactivation of hepatitis viruses in fresh frozen plasma, while other phenothiazine derivatives with greater affinity for nucleic acids and the ability to inactivate intracellular virus have been tested for photochemical decontamination of suspensions of red cells. The toxicity of methylene blue is well characterized and the compound has been used for many years to treat methaemoglobinaemia, using higher concentrations than those used for photoinactivation of the virus (Wagner et al., 2000; Wagner, 2002). The purpose of this work was to investigate *in vitro* the extent of the inhibitory effect of the dietary supplement Oxidal® on the development of pathogenic strains of *Staphylococcus aureus* and *Esherichia coli* isolated by clinical veterinary practice from patients treated with antibacterials, as well as on reference strains.

Materials and methods

Microorganisms. Pure cultures of 11 pathogenic strains were tested: 6 strains of *Staphylococcus aureus* and 5 strains of *Esherichia coli*. The microorganisms were isolated from skin inflammatory secretions of dogs in the laboratory of microbiology at the University Clinic of the Faculty of Veterinary Medicine at the University of Forestry in Sofia. Two control strains were also included - 1 of *S. aureus subsp. aureus* ATCC - 6538 (NBIMCC 3359) and 1 of *E. coli* ATCC - 8739 (NBIMCC 3397), obtained from the Bulgarian National Bank for Industrial Microorganisms and Cell Cultures (NBIMCC).

Antimicrobial preparations

Original dietary supplement Oxidal® (IdeaLabs, LLC, Washington, USA; author Georgi Dinkov), containing methylene blue, salicylic acid and caffeine. *Neutral anolyte*, prepared with 0.5% NaCl and activation time 12 min. We tested both a freshly prepared solution and an anolyte solution stored at room temperature in the dark for 1 year. *Physical parameters* pH and oxidative-reduction potential (ORP) of the investigated compounds were determined using Manual multi-parameter analyser Consort C1010 (Consort bvba, Belgium) for pH, mV and temperature measurement. *Minimum inhibitory concentrations* (MIC) were determined by the two-fold serial dilution method from 0.016 to 16 mg/ ml for Oxidal® and from 0.016 to 16 µg/ml for the control antibiotic Thiamphenicol in Mueller-Hinton agar (BUL BIO NCIPD EOOD - Sofia) with a pH of 7.2-7.4, described by Ericsson and Sherris (1971) and NCCLS (1999). Bacterial suspensions were applied at a dose of 10^6 cells/ml. After incubation at 35-37° C for 24-48 hours, the number of developing colonies was determined. The MIC of the test agents causing a reduction in the number of colonies of the microorganisms by 50% and 90% compared to the untreated controls were calculated, as well as the range of growth suppression (D) - the minimum concentrations at which the growth of the respective microorganism is completely suppressed. At MIC <0.5-4 µg/ml, the bacteria tested were evaluated as being susceptible to thiamphenicol. They are moderately sensitive at reported MIC values of 8 to 64 µg/ml, and at MIC = 64 µg/ml resistance was determined (NCCLS, 1999).

Experimental settings of studies in the suspension method.

● Oxidal® antimicrobial activity assay. To the 10% solutions of Oxidal® in sterile distilled water were added suspensions of the tested strains of *S. aureus* at a concentration of 10^7 cells/ml in an amount of 1 ml, to receive a final concentration of 10^6 cells/ml. *E. coli* strains at final concentrations of 10^6 cells/ml in 10% Oxidal® solutions in sterile distilled water were similarly examined. Controls were also analyzed – placed either in sterile distilled water (without Oxidal®) with the same content of the tested bacterial strain, or placed in 10% Oxidal® without microorganisms.

● Antimicrobial activity assay for anolytes used as a control to compare to the effect of Oxidal®. Fresh and aged anolyte were placed in tubes of 9 ml each. To each of these was added a suspension of the studied

strains of *S. aureus* at a concentration of 10^7 cells/ml in an amount of 1 ml for receiving a final concentration of 10^6 cells/ml. *E. coli* strains at final concentrations of 10^6 cells/ml in fresh and stored anolyte were similarly examined. The following controls were also analyzed - placed either in sterile distilled water (without anolyte) with the same content of each of the bacterial strains tested, or placed in 100% anolyte without microorganisms.

After different intervals of exposure to Oxidal® and the anolytes (2 min, 5 min, 10 min, 15 min, 20 min, 30 min, 45 min, 60 min and 90 min), seedings of each of the samples on Mueller-Hinton agar were made, which were incubated at 37° C for 24 - 48 h under aerobic conditions. After cultivation, the growth of the examined bacteria treated with the antimicrobials tested as well as the controls applied were recorded. All experiments were performed three times. The statistical processing of the results was performed according to the classical Student-Fisher method.

Results

The summarized results obtained in regards to the minimum inhibitory concentrations are shown in Tables 1 and 2 and the cumulative curves of the MIC_{90} - in Figures 1 and 2. As can be seen from the summarized data, the bacterial strains tested showed a higher sensitivity to Oxidal® than to the control antibiotic, but the differences were not significant (P> 0.05). All the strains tested showed sensitivity to the antibiotic Thiamphenicol.

Table 1. Mean values of the minimum inhibitory concentrations of Oxidal®

Microorganisms	**Strains No**	**Minimum inhibitory concentrations in mg/ml**		
		MIC_{50}	**MIC_{90}**	D
E. coli	5	0,55 ± 0,24	1,20 ± 0,40	2,40 ± 0,80
S. aureus	7	0,21 ± 0,13	0,43 ± 0,26	0,86 ± 0,52
Total examined	12	0,38 ± 0,11	0,82 ± 0,048	1,63 ± 0,77

MIC - Minimum inhibitory concentrations; D – Diapason/range of growth suppression

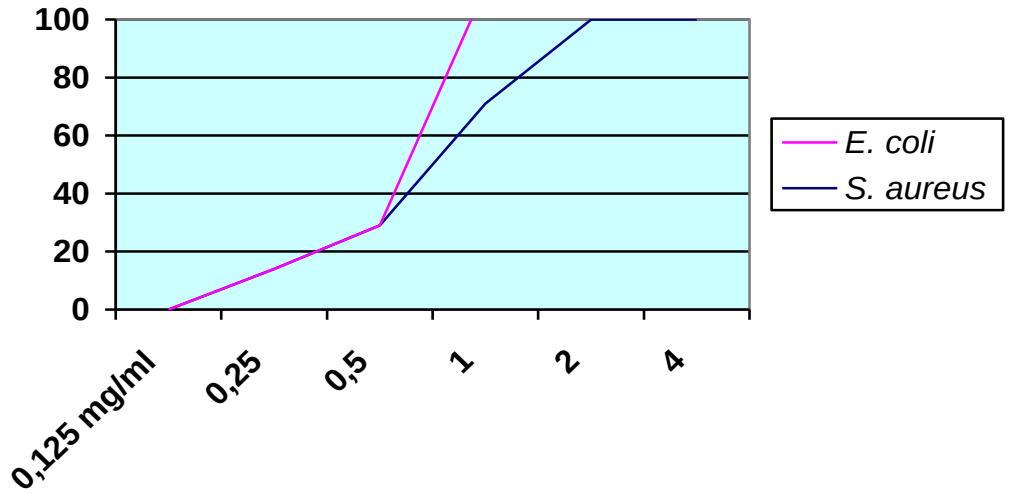

Fig. 1. Cumulative curves of MPC_{90} of Oxidal® for the bacteria tested.

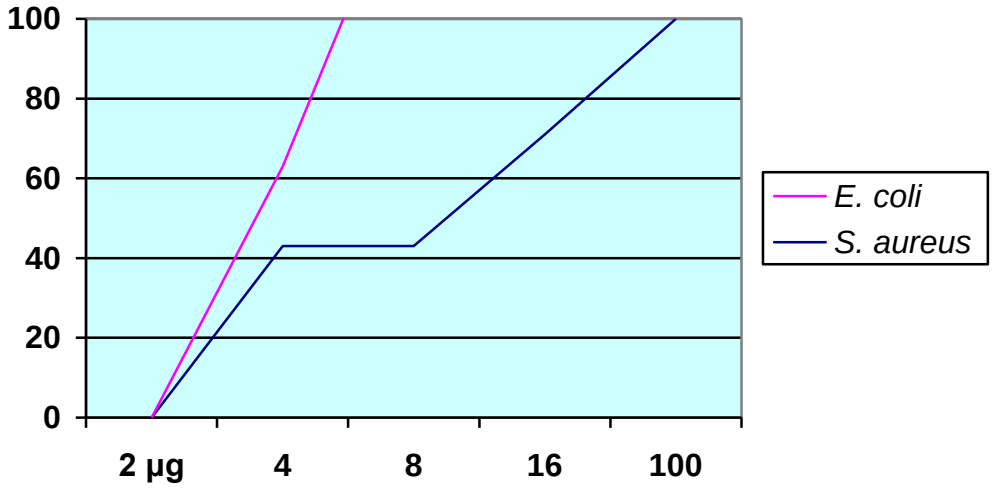

Fig. 2. Cumulative curves of MPC_{90} of Thiamphenicol for the bacteria tested.

Table 2. Mean values of the minimum inhibitory concentrations of Thiamphenicol

Microorganisms	Strains No	Minimum inhibitory concentrations in µg/ml		
		MIC_{50}	MIC_{90}	D
E. coli	5	2,40 ± 0,80	4,80 ± 1,60	9,60 ± 3,20
S. aureus	7	3,71 ± 2,86	4,00 ± 2,62	8,00 ± 5,24
Total examined	12	3,06 ± 0,66	4,40 ± 0,40	8,80 ± 0,80

MIC - Minimum inhibitory concentrations; D – Diapason/range of growth suppression

The mean MIC_{50} values of Oxidal® for the Gram-positive bacteria *S. aureus* (0.21 ± 0.13 mg/ml) were lower than those for the Gram-negative *E. coli* (0.55 ± 0.24 mg/ml), with statistically significant differences ($P<0.05$). The dose that fully suppressed *S. aureus* growth was 0.86 ± 0.52 mg/ml, while for *E. coli* this dose was significantly higher - 2.40 ± 0.80 mg/ml ($P<0.05$). For the antibiotic Thiamphenicol, the results were opposite - the mean MPC_{50} values for *E. coli* (2.40 ± 0.80) were lower than those for *S. aureus* (3.71 ± 2.86), but the differences were not statistically significant ($P> 0.05$). The results of the studies performed to determine the susceptibility of *S. aureus* and *E. coli* to anolyte by the suspension method are presented in Table 3. The data show that not only the fresh anolyte but also the one stored for 1 year inactivated *S. aureus* and *E. coli* strains after 2 min of exposure.

Table 3. Study of the effect of anolyte on *S. aureus* and *E. coli* in suspensions with concentration 10^6 cells/ml

Antimicrobial agent	Growth of *S. aureus* and *E. coli* (number of colonies) after different intervals of anolyte exposure					
	2 min		5 min		10 min	
	S. aureus	*E. coli*	*S. aureus*	*E. coli*	*S. aureus*	*E. coli*
Anolyte fresh	0	0	0	0	0	0
Anolyte old	0	0	0	0	0	0
Control without anolyte	many	many	many	many	many	many
Control (anolyte without bacteria)	0	0	0	0	0	0

The results of the studies performed to determine the susceptibility of *S. aureus* and *E. coli* to Oxidal® by the suspension method are presented in Fig. 3. Data show that the number of viable micro-organisms decreased by more than 30% compared to the untreated control after 20 minutes of exposure to 10% Oxidal® solution. After 45 minutes, only 37% of *S. aureus* cells and 19% of *E. coli* cells developed upon cultivation, and after 90 minutes of exposure, about 20% of the cells of the tested microorganisms of both species remained viable. However, no complete bactericidal effect of 10% Oxidal® solution on *S. aureus* and *E. coli* was reported during this period. After longer exposure (20-60 min), *E. coli* showed a higher sensitivity than *S. aureus*, but after 90 minutes the results for both strains converged.

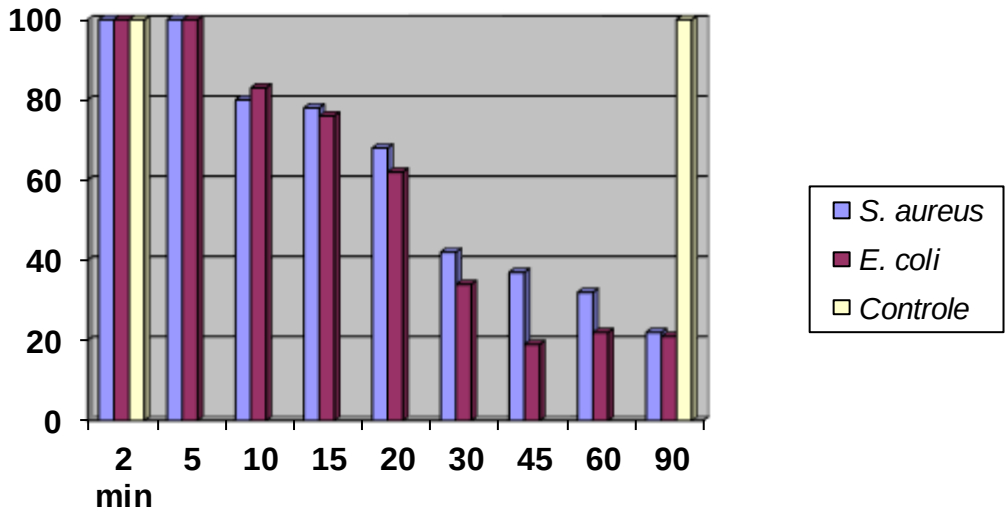

Figure 3. Inhibitory effect of Oxidal® at a final concentration of 10% on *S. aureus* and *E. coli* in suspension with a density of 10^6 cells/ml.

Discussion

Our preliminary studies of the antimicrobial action of Oxidal® in the agar-gel diffusion method (Popova et al., 2020) showed high inhibitory activity against *S. aureus* and *E. coli*. Data from the present studies indicate a significant antibacterial effect of Oxidal®. It is probably due mainly to the aniline dye methylene blue contained in the preparation. Our results are largely in line with those obtained by Authman and Shatti (2015) in examining the effect of methylene blue on bacteria isolated from patients with atopic dermatitis. They reported that all isolates (*Staphylococcus aureus, Staphylococcus haemolyticus, Pseudomonas fluorescens* and *Enterobacter aerogenes*) had lost their ability to grow at a concentration of 10 mg/ml methylene blue. In their studies, the MIC of methylene blue against *S. haemolyticus* and *P. fluorescens* was 1 mg/ml, while against *S. warneri* and *S. aureus* were 10 mg/ml.

Other authors have also reported significant inhibitory activity of methylene blue. In treating a suspension of *Helicobacter pylori* with 0.2 mg/ml methylene blue Sung et al. (2010) achieved a 100-fold reduction in viable cell counts after 10 min. However, when exposed to light, ten times lower dose of the dye (0.02 mg/ml) was sufficient to achieve the same result in the same period of time. Piccirillo et al. (2009) found significant bactericidal activity of methylene blue, covalently bound to an activated silicone polymer by an amide condensation reaction. Significant bactericidal activity against *E. coli* and *Staphylococcus epidermidis* was reported with a 99.999% reduction in viable bacteria after four minutes of low power laser exposure.

Obviously, when irradiated with light, the antimicrobial action of methylene blue is increased several-fold. Elumalai et al. (2015) demonstrated that the photodegradation of this aniline dye under ultraviolet light for 90 minutes with a 30 mg ZnO catalyst and plant extract of *Vitex trifolia* results in a significant increase in its antimicrobial activity. Methylene blue turns out to be a promising agent with broad-spectrum antimicrobial activity, especially when activated by light. Gao et al. (2016) have successfully implemented methylene blue for the photodynamic inactivation of pathogenic fungi. They found that photodynamic *in vitro* treatment with methylene blue and LEDs was effective for inhibiting the growth of *Fusaruim* spp. and *Exophiala* spp., both in cultures and in biofilms.

The antimicrobial effect of the anolyte is already known. Antibacterial activity on *E. coli* and antiviral effect on Classical Swine Fever Virus of anolyte have been reported by Atanasov et al. (2014), Gluhchev et al. (2015), Ignatov et al. (2018), Karadzhov et al. (2019) and others. Ignatov. (2020) also described the possible antiviral effects of Oxidal®, water Ccatholyte and nano colloidal silver over Coronaviruses SARS-CoV, SARS-CoV-2 and disease, caused by COVID-19. The results we have obtained are consistent with theirs and those of our previous studies (Popova et al., 2016 a, b). Some of the major effects of the electrochemically activated aqueous solutions anolyte and catholyte on human health have been studied and described by Ignatov (2019), Ignatov and Gluhchev (2019) and Ignatov et al. (2020 a, b). The data show that these solutions are extremely promising not only as antimicrobial agents but also as environmentally friendly tools for human health restorations with anti-inflammatory and antioxidant effects. Their positive health effects are similar to those of the mineral spring waters studied by Valcheva (2019).

Conclusions

The tested strains of *S. aureus* and *E. coli* showed a higher sensitivity to Oxidal® than to the control antibiotic Thiamphenicol, but the differences were not significant. The mean MIC_{50} values of Oxidal® for the Gram-positive bacteria *S. aureus* (0.21 ± 0.13 mg/ml) were significantly lower than those for the Gram-negative *E. coli* (0.55 ± 0.24 mg/ml). The dose that completely suppressed *S. aureus* growth was 0.86 ± 0.52 mg/ml, and for *E. coli* this dose was significantly higher - 2.40 ± 0.80 mg/ml ($P < 0.05$). For the antibiotic Thiamphenicol, the mean MPC_{50} values for *E. coli* (2.40 ± 0.80) were lower than for *S. aureus* (3.71 ± 2.86), but the differences were not statistically significant.

Under the influence of 10% Oxidal® solution, the amount of viable cells of *S. aureus* and *E. coli* decreased by over 30% compared to the untreated control after 20 minutes. After 45 minutes of exposure to 10% Oxidal® solution, only 37% of *S. aureus* cells and 19% of *E. coli* cells developed on cultivation, and after 90 minutes about 20% of the cells of the bacteria of both species remained viable. The fresh anolyte and the one stored for 1 year inactivated *S. aureus* and *E. coli* after 2 min of exposure.

Conflict of interests

The authors have declared that no conflict of interests exists.

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