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Preliminary In Vitro Investigations on The Inhibitory Activity of The Original Dietary Supplement Oxidal® On Pathogenic Bacterial Strains

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Abstract

The antimicrobial action of the dietary supplement Oxidal® was tested using the classic Bauer and Kirby agar-gel diffusion method. Clinical and reference strains of *Staphylococcus aureus* and *Escherichia coli* were used in the studies.

The tested dietary supplement showed a well-pronounced inhibitory effect against the microbial strains commensurable with that of the broad-spectrum chemotherapeutic agent Enrofloxacin and showed even higher activity than the broad spectrum antibiotic Thiamphenicol. The proven inhibitory effect of the tested dietary supplement against the examined pathogenic bacteria is in accordance with the established clinical effectiveness standards for antimicrobial agents.

Keywords: Oxidal®, Staphylococcus Aureus, Escherichia Coli, Antibacterial Activity

Introduction

Due to the increasing antibiotic resistance of pathogenic bacteria, a current problem of modern microbiology is the search for alternative antimicrobials, non-toxic to animals and humans, that are capable of effectively suppressing the multiplication of pathogens and are suitable for the prevention and treatment of various infections. In recent years, evidence has emerged that the activated (acidic) water solution anolyte is a promising antimicrobial agent (Suzuki et al., 2002; Ignatov and Mosin, 2013; Karadzov et al., 2014; Gluhchev et al., 2015; Popova et al., 2016 a, b; et al., 2020). Another opportunity in this regard is provided by Oxidal®, a dietary supplement containing methylene blue as the main ingredient (methylthioninium chloride), which is characterized by a pronounced antioxidant action (Deiana, 2009). It is a thiazine dye, included in the World Health Organization's list of essential medicines (WHO, 2019). Methylene blue is a component of the drug Prosed DS (2011), commonly used against genitourinary infections, which contains phenyl salicylate, benzoic acid, hyoscyamine sulfate, and methenamine. The use of methylene blue in aquaculture by tropical fish hobbyists for the treatment of fungal infections is well-known. It inhibits the respiration of the fungi as it binds the hydrogen ions formed during the process. It is also used to protect newly hatched fish eggs from infection with fungi or bacteria. The interest in using it as an antimalarial means has recently been revived, especially because of its low cost (Meissner et al., 2006). Its antiviral activity has been studied against AIDS-related Kaposi's sarcoma, West Nile virus, HIV-1 virus, and others (Wagner et al., 2000; Floyd et al., 2004; Papin et al., 2005; Tardivo et al., 2006). The virucidal properties of photoactive phenothiazine dyes, such as methylene blue, have been found to occur after binding of the dye to the viral nucleic acid, absorption of light, generation of reactive oxygen species, and oxidation of guanine in the viral genome (Wagner (2002). Zolfaghari et al. (2009) reported about an inhibitory effect of methylene blue on *Staphylococcus aureus.*

The aim of the present work is to determine 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 from clinical veterinary practice patients treated with antibacterial means, as well as its effects 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 medicament 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.

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.

The antibacterial effect was investigated by the classical Bauer-Kirby agar-gel diffusion method (Bauer et al., 1966), designed for rapidly growing aerobic microorganisms, on Mueller Hinton agar (BUL BIO NCIPD EOOD - Sofia) with a pH of 7.2-7.4 and a layer thickness of 4 mm. The Oxidal® preparation was administered by 0.1 ml in wells with a diameter of 9 mm, undiluted (100%), and diluted 50% in sterile saline solution. In parallel with Oxidal®, comparative studies were conducted with neutral anolyte applied in the same amount in two variants - undiluted (100%) and diluted 50% in sterile saline solution. Two of the classic antibiotics used in clinical practice (Thiamphenicol and Enrofloxacin) were tested as a controls. These were aplied diluted in sterile saline at standard final concentrations of 30 µg for Thiamphenicol and 5 µg for Enrofloxacin respectively, in 0.1 ml per well. Inoculation of bacterial suspensions at a dose of 2.10^6 cells/ml was followed by cultivation at 35-37 ° C for 18-24 and 72 hours. The results were readed by measuring the diameters of the inhibitory zones in millimeters accurate to the nearest whole millimeter, including the diameter of the well, with a transparent ruler on the outside of the bottom of the petri dishes. A complete suppression of growth was considered as a boundary of the inhibitory zone. The inhibitory zones measured were interpreted using the three-step Bauer and Kirby categorization system. The susceptibility of the tested microorganisms to the examined preparations was determined as for non-antibiotic agents such as sulfonamides, namely: resistant (R) - in areas with diameters <12 mm, medium sensitive - intermediate (I) - in areas within 13 - 16 mm and sensitive (S) $\underline{\geq}$ 17 mm. For Thiamphenicol, the corresponding limits are: R <12 mm, I - 13 - 17 mm and S $\underline{\geq}$ 18 mm (NCCLS). According to NCCLS (1997, 1999), high susceptibility of microorganisms to Enrofloxacin was found in growth inhibition zones ≥ 21 mm, medium sensitivity in zones with diameters from 16 to 20 mm, and resistance in zones ≤ 15 mm.

The statistical processing of the results was performed using the classic Student-Fisher t-test.

Results and Discussion

The physical indicators pH and oxidation-reduction potential of the antimicrobial agents tested are presented in Table 1.

Figure 1 shows the relationship between the acidity and basicity (pH) solutions and the oxidation-reduction potential (ORP). The pH value within the interval from 3 to 10 units and the ORP within the interval from -400

mV to +900 mV characterize the area of the biosphere of microorganisms. Outside these ranges of pH and ORP, the microorganisms will hardly survive. The point 1 is of Anolyte, and point 2 is of Oxidal®.

Table 1. Physical indicators of anolyte and Oxidal® tested

Outbound composition		**pH**	**ORP, mV**
Aqueous sodium chloride solution (0.5%)	Anolyte	6,86	733
Oxidal®	50% solution	7,3	65

ORP - oxidation-reduction potential

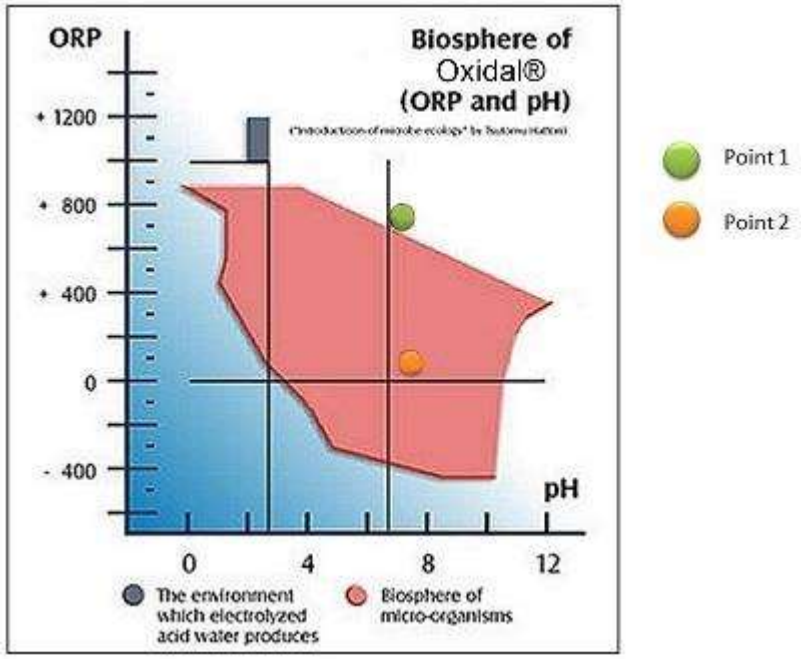

Figure 1. Dependence between pH and ORP of the tested solutions.

Summarized results reflecting the effect of Oxidal® on the experimental strains are presented in Table 2.

The data in the table shows that the growth of all strains was successfully suppressed. The inhibitory zone diameters of Oxidal® administration ranged from 25.57 $\pm$ 1.19 to 41.26 $\pm$ 3.46 mm.

The well-expressed inhibition zones around the wells are illustrated in Fig. 2.

The results show that Oxidal® exhibits strong inhibitory effect on the pathogenic microorganisms tested, with clearly defined inhibitory zones and a lack of secondary colonies in them.

The experiments carried out herein show that Oxidal® had the same or, in many cases, better inhibitory effect *in vitro* when compared to some of the most commonly used antibiotics (Table 2).

In all strains tested, there were no colonies in the inhibitory zones, which is evidence of high antimicrobial activity. The sensitivity of all strains tested to both examined Oxidal® concentrations was high. The results

were higher in the concentrated preparation than in the diluted one (50%), with significant differences in *E. coli* (P <0.05). The tested strains of *S. aureus* showed a higher sensitivity to Oxidal® than those of *E. coli*. The differences in the diameters of the inhibitory zones between *S. aureus* and *E. coli* were statistically significant (P <0.05 for the undiluted preparation and P <0.001 for 50% Oxidal®). This was expected since Gram-positive bacteria such as staphylococci are more sensitive to the action of aniline dyes than Gram-negative bacteria such as *E. coli*. The sensitivity of all tested microorganisms to Oxidal® at a concentration of 50% was very similar to that to the undiluted preparation. Considering Oxidal® does not have particularly high or low pH and ORP values, its antibacterial activity is not due to them, but to the chemical structure of its ingredients.

Table 2. Antimicrobial effect *in vitro* of Oxidal® against *Staphylococcus aureus* and *Escherichia coli*

Antimicrobial means	**Inhibitory zones in mm**	
	Staphylococcus aureus	***Escherichia coli***
Oxidal® 100%	41,26 ± 3,46	32,75 ± 2,16
Oxidal® 50%	37,33 ± 2,43	25,57 ± 1,19
Anolyte 100%	11,70 ± 1,93	10,88 ± 0,52
Anolyte 50%	10,97 ± 1,29	10,00 ± 0,23
Enrofloxacin	41,20 ± 1,17	34,25 ± 0,83
Thiamphenicol	21,60 ± 5,86	17,00 ± 3,18

The sensitivity to Oxidal® of the bacteria tested in this study was similar to that of the quinolone Enrofloxacin, which is currently one of the most effective antibacterial agents used in veterinary medicine. The differences in the diameters of the inhibitory zones were small and insignificant (P> 0.05). However, the broad-spectrum antibiotic Thiamphenicol showed significantly weaker inhibitory effect on the tested strains of *S. aureus* and *E. coli* compared to Oxidal® (P <0.001). These data are, in our view, important because clinical strains are highly resistant to treatment.

The acidic water solution anolyte, which in the suspension method shows high antimicrobial activity against the same bacteria (Popova et al., 2016 a, b) tested in the agar-gel diffusion method, does not have an inhibitory effect. This is probably due to the neutralization of its active ingredients upon penetration into the agar nutrient medium. The differences in the diameters of the inhibitory zones of Oxidal® and anolyte in *S. aureus* and *E. coli* were statistically significant (P <0.001).

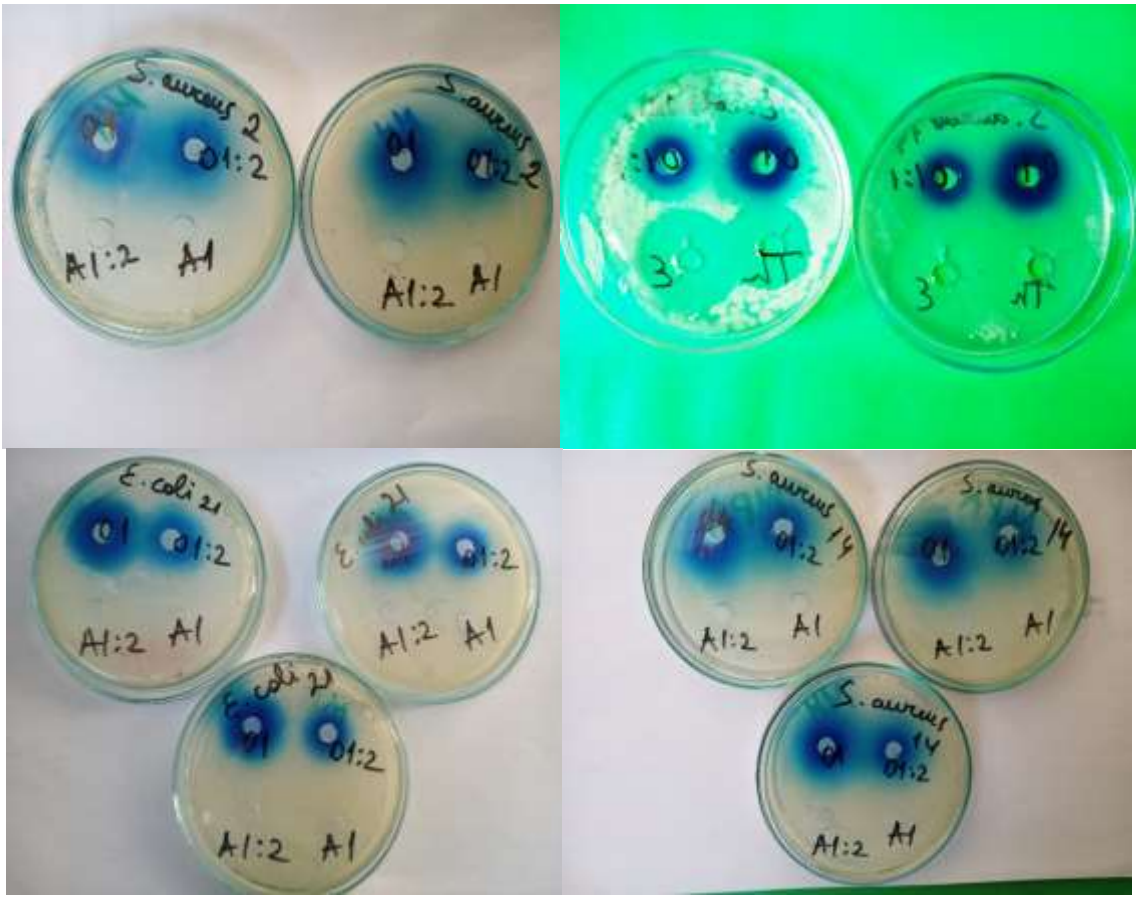

Figure 2. In vitro sensitivity of some of the tested strains of S. aureus and E. coli to Oxidal®, anolyte, Enrofloxacin, and Thiamphenicol.

However, after the application of Oxidal® and anolyte in adjacent wells, where simultaneous cross-diffusion occurs, a marked synergistic effect was observed against some of the strains. It was more pronounced at interaction with 50% anolyte.

The results we have obtained showed significant activity of the tested preparation Oxidal® against *S. aureus* and *E. coli* and were indicative of significant antibacterial activity commensurate with and higher than that of some of the most widely used broad-spectrum antibacterial agents, which may be subject to further research.

Conclusions

The dietary supplement Oxidal® shows an *in vitro* highly pronounced inhibitory effect against clinical and control reference strains of *S. aureus* and *E. coli*, even at 50% dilution. Its effectiveness is comparable to that of the chemotherapeutic Enrofloxacin and significantly higher than the effect of the broad-spectrum antibiotic Thamphenicol.

The inhibitory effect of Oxidal® was more pronounced against *S. aureus* compared to that against *E. coli*.

Oxidal® can and should be used as a concomitant mean in the treatment of bacterial infections caused by *S. aureus* and *E. coli*.

Conflict of interests

The authors have declared that no conflict of interests exists.

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