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Research Article

Antimicrobial activity of ozonated sunflower oil against *Pseudomonas aeruginosa* strains isolated from mares, and interactions with antimicrobial drugs

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Abstract: Infectious endometritis in mares can result in reduced fertility, early embryonic loss, and general health decline, implicating extensive economic loss. *Pseudomonas aeruginosa* is an opportunistic pathogen that is among the most frequent bacterial pathogens associated with infectious endometritis in mares. Here we explored the antimicrobial potential of ozonated sunflower seed oil, compared to the conventional form (both widely explored for wound healing), against clinical isolates of *Pseudomonas aeruginosa* obtained from mares with endometritis. The effects of combining the oils with clinically relevant antimicrobial drugs were also investigated. The conventional form of the oil was active against the strains, whereas the ozonated oil was not. When combined with the antimicrobials, both oils significantly decreased the pharmacological activity of the drugs. This study opens doors for discussions on clinical protocols for treating fertility issues in mares, as well as bacterial resistance in the intrauterine environment.

1. INTRODUCTION

Infectious endometritis is a disease characterized by infection and an inflammatory process at the endometrium. In breeding mares, bacterial pathogens are the most prevalent microorganisms associated with the disease, but fungal and viral pathogens might be associated as well (Prete *et al*., 2024; Virendra *et al*., 2024). Different factors contribute to explaining the infection, including impaired uterine clearance, alteration in perineal and vulvovaginal conformation, immunosuppression, natural breeding, and inappropriate handling such as the use of contaminated semen for fixed-time artificial insemination (Köhne *et al*., 2020; Morris *et al*., 2020). As a result, the disease can result in difficulties such as reduced fertility, early embryonic loss, foaling complications, and general health decline (Morris *et al*., 2020).

Pseudomonas aeruginosa is among the most frequent bacterial pathogens associated with infectious endometritis in mares (Gil-Miranda *et al*., 2024; Virendra *et al*., 2024). This Gram-negative species is considered opportunistic, and it has been suggested that the stallion can

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harbor and transmit this pathogen in the breeding process (Guimarães *et al*., 2012). Providing an effective treatment for such infections is of interest to avoid further reproductive problems. The most common treatment consists of using antimicrobial drugs, which poses risks of dysbiosis and of promoting bacterial resistance (Köhne *et al*., 2020; Nocera *et al*., 2023a).

Bacterial resistance to antimicrobial drugs is a relevant health issue due to the complications associated with the lack of therapeutic options to treat diseases caused by resistant pathogens (Dias-Souza *et al*., 2023). In Veterinary Medicine, the context can be even more complex: the microbiota of animals and humans share some similarities, but they can differ enough to result in zoonotic diseases (Nocera *et al*., 2023b; Ito *et al*., 2024). Although infectious endometritis is not expected to cause a zoonotic disease, it can cause significant financial impacts, due to impaired reproductive function of the females and the high cost of the antimicrobial treatment (Köhne *et al*., 2020; Da Silva-Álvarez *et al*., 2022). Thus, new treatments should be safe and of low cost.

Sunflower (*Helianthus annus*) seed oil (SSO) is widely known for its wound healing properties and low cost, and thanks to its safety, it is largely used in human and animal food (Nakonechna *et al*., 2024). Fixed oils such as sunflower oil present varied nonpolar phytomolecules, which are classified as saponifiable and unsaponifiable compounds (Salehi *et al*., 2020; Landolsi *et al*., 2024). Saponifiable compounds like triglycerides and phospholipids contain fatty acids in their molecular structure and can form soap when exposed to strong alkalis such as KOH. Unsaponifiable compounds such as phytosterols, tocopherols, and carotenoid isoforms, in contrast, lack fatty acids in their structures and thus do not form soap (Salehi *et al*., 2020; Landolsi *et al*., 2024). Their health benefits are largely attributed to antioxidant properties; however, given that most of the relevant nonpolar secondary metabolites present in fixed oils are also unsaponifiable compounds, other biological and pharmacological activities are likely (Landolsi *et al*., 2024; Okafor *et al*., 2024). Nonpolar molecules such as phytosterols and sesquiterpene lactones have been investigated for their antimicrobial properties (Poli *et al*., 2021; Spring, 2021).

In spite of the exposure, the antimicrobial potential of sunflower oil in its conventional and ozonated forms remains scarcely investigated in a proper quantitative approach (Silva *et al*., 2021). Ozonation is a chemical process in which a substance is exposed to known quantities of ozone (O3) in gas form. In oils, ozonation targets carbon-carbon double bonds in fatty acids, both in their free form and as part of triglycerides. As a result, unstable intermediates are formed, and depending on the molecule they interact with next, different reactive oxygen species with antimicrobial properties can be obtained, such as peroxides (Ugazio *et al*., 2020).

Following a recent study of our group on the antimicrobial activity of the ozonated sunflower oil against bacterial strains isolated from mares with endometritis (Dos Santos *et al*., 2023), we hypothesized that the oil could be effective against other bacterial species involved in endometritis, especially if combined with clinically relevant antimicrobial drugs. Here we compared the antimicrobial potential of the ozonated and conventional sunflower seed oil on *Pseudomonas aeruginosa* strains isolated from mares with endometritis. We also investigated the behavior of combinations of the oils to clinically relevant antimicrobial drugs. Given the scarcity of data concerning safe and efficient alternative treatments to infectious endometritis in mares, our data becomes even more relevant.

2. MATERIAL and METHODS

2.1. Microorganisms

Ten clinical isolates of *P. aeruginosa* from mares with endometritis (obtained from intrauterine lavage fluids) were selected from the bacterial collection of the research laboratory of Anhanguera College (MG, Brazil). The isolates were cultivated in sterile brain-heart infusion broth (BHI, Difco) at 35±2 °C for 18 h before the experiments (Dos Santos *et al*., 2023).

2.2. Preparation of the Oils in Aqueous Solution

The conventional and ozonated (91.13 mEq peroxides/Kg) sunflower oils were purchased from local pharmaceutical companies in reagent grade. For the antimicrobial activity assays, the oils were prepared as a stock solution (4.1 mg/mL) in 0.9% sterile saline solution with 0.5% Tween 80, as described previously by our group (Dos Santos *et al*., 2023).

2.3. Minimal Inhibitory Concentration Test

The conventional and ozonated (91.13 mEq peroxides/Kg) sunflower oils were purchased from local pharmaceutical companies in reagent grade. For the antimicrobial activity assays, the oils were prepared as a stock solution (4.1 mg/mL) in 0.9% sterile saline solution with 0.5% Tween 80, as described previously by our group (Dos Santos *et al*., 2023).

The plates were incubated overnight at 35±2°C, and following, resazurine staining (0.1 g/L, 20 µL) was performed. The lowest concentration at which no color change from blue to pink was observed was considered the MIC. A 0.9% sterile saline solution with 0.5% Tween 80 was used as a negative control.

2.4. Minimal Bactericidal Concentration Test

The minimal bactericidal concentration (MBC) of the oils was also determined in triplicate using a method described by our group (Da Costa *et al.*, 2023). Aliquots of 10 µL of each well in which resazurine staining result was negative (indicating bacterial death) were spotted in Mueller-Hinton agar (Difco) plates and inoculated overnight at 37°C. The 0.9% sterile saline solution with 0.5% Tween 80 was used as a negative control. MBC was established as the lowest concentration that yielded no bacterial growth of all strains in agar plates.

2.5. Interactions of the Oils and Antimicrobial Drugs

The possible interference of the oils on antimicrobial drugs was assessed in duplicate using the method standardized by our group (Dias-Souza *et al*., 2013). Antimicrobial disks (10 µg ampicillin, 30 µg ceftriaxone, 10 µg tobramycin, all from Sensifar, Brazil) were distributed in Mueller-Hinton agar (Difco) plates for performing an antimicrobial susceptibility assay. Then, briefly, 10 µL of the oils in their MBC were dispensed in each disk. As controls, we used plates with disks without the addition of any oil (control group 1) and plates with disks with the addition of 10 µL of the sterile saline solution with 0.5% Tween 80 (control group 2).

All plates were incubated overnight at 37 °C, and the inhibition zone mean diameter was compared to the controls. Synergism was considered if the inhibition zone mean diameter was at least 2 mm larger than control 1, and antagonism was considered if the inhibition zone mean diameter was at least 2 mm shorter than control 1. Both synergism and antagonism required further statistically significant differences to be confirmed. If the inhibition zone mean diameters were larger or shorter than the control, but lacked statistical significance, the data were characterized as a trend of synergism or antagonism.

2.6. Statistics

Interference on antimicrobial drugs was analyzed using the Bartlett test for homocedacistiy and Shapiro-Wilk for normality. Following, we used Kruskal-Wallis with Student-Newman-Keuls post hoc tests. Results were considered significant if $p < 0.05$.

3. RESULTS

The ozonated sunflower was not active against any of the isolates, whereas the conventional oil was active. Negative control was also not active. The MIC of the conventional oil was 16 µg/mL, and the MBC was of 256 µg/mL. This difference of MIC and MBC values suggests a bacteriostatic profile of the conventional oil (Levison and Levison, 2009).

Concerning the interactions of the oils with the antimicrobial drugs, the conventional oil was tested at its MBC, but the ozonated oil was tested at its highest concentration (1024 µg/mL), as it presented no antimicrobial activity. For both oils, the interactions with antimicrobials were antagonistic in all tested strains (p<0.05, Table 1).

Table 1. Results of the interaction test of the oils and antimicrobial drugs

Isolate	AMP	AMP+O_3	AMP+CSO	CEF	CEF+O_3	CEF+CSO	TBR	TBR+O_3	TBR+CSO
P1	21	16	19	28	20	23	35	26	29
P2	28	12	17	25	21	22	35	20	23
P3	23	15	18	26	18	21	34	27	24
P4	25	21	23	28	22	25	35	25	22
P5	28	20	25	29	18	21	31	26	25
P6	27	19	24	27	15	19	30	22	20
P7	22	16	19	25	13	20	32	27	23
P8	29	20	25	22	17	19	30	24	27
P9	26	20	22	26	20	22	34	20	28
P10	28	20	22	21	15	18	33	27	21

Isolates are identified from 1 to 10 (P1-P10). Values correspondto inhibition zones measured in millimeters. AMP – ampicillin, CEF – ceftriaxone, TBR – tobramycin. +O_3: addition of ozonated oil. +CSO: addition of conventional sunflower oil.

4. DISCUSSION

Here we provide evidence on the antimicrobial potential of sunflower oil against *P. aeruginosa* strains isolated from mares with infectious endometritis. Sunflower oil is rich in secondary nonpolar metabolites such as phytosterols, sesquiterpenes, fatty acids, and triglycerides. Phytosterols have been investigated for their antimicrobial properties, beyond their potential to regulate endogenous cholesterol synthesis (Poli *et al*., 2021). There is evidence on the antimicrobial activity of β-sitosterol, campesterol, and stigmasterol (Uttu *et al*., 2022; Lestari *et al*., 2024), which are among the most prevalent phytosterols in sunflower oil (García-González *et al*., 2021). More recently, a study confirmed the detection of sesquiterpene lactones such as costunolide and dehydrocostuslactone in sunflower oil (Spring, 2021), and their antimicrobial activity has been described (Kim *et al*., 2019; Li *et al*., 2022). Taken together, these phytomolecules might be directly involved in the antimicrobial effects reported here for the conventional sunflower oil.

Surprisingly, the ozonated sunflower oil was not effective against *P. aeruginosa* isolates. In a recent study from our group exploring bacterial pathogens from infectious endometritis in mares, both conventional and ozonated were not active against *Escherichia coli* isolates, whereas the ozonated sunflower oil was active against *Staphylococcus aureus* isolates at 512 μg/mL (Dos Santos *et al*., 2023), consistent with other findings (Sechi *et al*., 2001). Beyond the mentioned expected nonpolar phytomolecules with antimicrobial potential, ozonated fixed oils present reactive oxygen species (ROS) with antimicrobial potential, such as lipoperoxides, hydroperoxides, and peroxides. ROS can cause damage to the lipid membrane and to organelles (Travagli & Iorio, 2023), what helps to explain their antimicrobial potential. Nevertheless, antioxidant systems in *P. aeruginosa* may have had a role in protecting the isolates against ROS from the ozonated oil (Kim *et al*., 2024), what partially explains the susceptibility profile of the isolates.

A common popular practice among patients in several countries is the combined use of allopathic medication and plant extracts, generally aiming to reach the expected therapeutic outcomes more rapidly (Cota *et al*., 2024; Welz *et al*., 2018). However, the safety and the results of these combinations are poorly predictable due to the diversity of phytochemicals in plant extracts. Research on drug interactions with nonpolar plant extracts or isolated molecules is scarce, mostly due to technical limitations such as lack of water solubility and incompatibilities with reagents. In this study, the combination of ozonated and conventional sunflower oils with antimicrobial drugs resulted in statistically significant antagonism (Table 1). To the best of our knowledge, this is the first investigation of such combinations. We believe that two situations have developed in the experiments. First, the addition of the diluted oils might have worked as a physical barrier to proper diffusion of the drugs, due to the solubility difference. Furthermore, it is possible that the nonpolar phytomolecules are interacting with the drugs (Borzyszkowska-Bukowska *et al*., 2023; Zhao *et al*., 2024), but in a way that hampers binding to their molecular targets. Reports on antagonistic combinations of antimicrobials and nonpolar phytomolecules are scarce, given that they present drug-like antimicrobial mechanisms of action (Dias *et al*., 2022; Kanokmedhakul *et al*., 2005).

The intrauterine administration of sunflower oil (conventional and ozonated forms) alongside antimicrobial drugs for the treatment of subfertility is a common practice in equine breeding (Carneiro *et al*., 2023; Ferreira *et al*., 2021), thus, the rationale for this experiment. We believe that this practice, which lacks real-world evidence, can contribute to increasing bacterial resistance in the uterine environment in different ways, including stimulation of biofilm formation (Dias-Souza *et al*., 2023). *In vivo* experiments are necessary to confirm our hypothesis.

Our group has been exploring the interactions of natural products and antimicrobial drugs, including nonpolar phytomolecules. We described that lycopene and β-carotene, water-insoluble carotenoids, abrogated the activity of different antimicrobial drugs, including nitrofurantoin, erythromycin, gentamicin, and oxacillin against *S. aureus* and *E. coli* (Dos Santos *et al*., 2015). Nevertheless, lycopene and β-carotene combinations to chloramphenicol and aztreonam against *P. aeruginosa* isolates resulted in synergism (Dos Santos *et al*., 2016). Lycopene and vitamin D also decreased the antimicrobial potential of some β-lactams and chloramphenicol (Dias *et al*., 2022). Dichloromethane extracts of *Banisteriopsis argyrophylla* and *Davilla rugosa* presented antimicrobial activity against veterinary and human strains of *Staphylococcus aureus*, with lower MIC values for veterinary strains (Dias-Souza *et al*., 2024). Phytomolecules isolated from dichloromethane fractions of *Colocasia affinis* and *Colocasia gigantea* were described to be active against *Shigella dysenteriae* and different *Bacillus* species (Alam *et al*., 2024).

5. CONCLUSION

This study highlights the antimicrobial potential of conventional sunflower oil against *Pseudomonas aeruginosa* isolates, with insights on the role of phytosterols and sesquiterpene lactones in these effects. The antagonistic interactions observed when combining sunflower oils with antimicrobial drugs raise important concerns regarding their clinical application, particularly in equine breeding practices. Further *in vivo* investigations are necessary to elucidate the mechanisms of interaction between natural products and antimicrobial agents, as well as their implications for bacterial resistance and biofilm formation. However, this does not interfere with our assessment of the potential consequences of such interactions in a real-world context.

Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research and publishing ethics.

Authorship Contribution Statement

Arthur Azevedo Perpétuo: performed the experiments and drafted the manuscript. **Gabriel Souza dos Santos:** performed the experiments. **Marcus Vinícius Dias-Souza:** conceptualization of the study, performed statistical analysis, preparation of the final version of the manuscript.

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