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Antimicrobial Activity of Pregnenolone *in vitro*

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Authors' contributions

This work was carried out in collaboration among all authors. Author TPP designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors II and TP managed the analyses of the study. Author GD managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

The antimicrobial effect of pregnenolone on clinical and reference strains of *Escherichia coli* and *Staphylococcus aureus* was tested. Pregnenolone was found to have inhibitory activity against all tested bacterial strains. Slightly higher sensitivity is shown by the strains of *S. aureus*. When applied directly, pregnenolone has a weak antimicrobial effect due to its very low water solubility, as it is in oleose state because the formulation studied in this experiment contains tocopherol (a fat-soluble vitamin) as a co-solvent. When emulsified with lecithin, as well as with methanol, its solubility in water increases and penetrates over a longer distance in the agar around the points of its application. Applied as an emulsion, it shows significantly higher inhibitory activity against *E. coli* and *S. aureus*. Even the non-emulsified version should still be useful *in vivo* due to the fact that intracellular environments are much more lipophilic than serum, the target of most antimicrobial substances is the intracellular space, and non-emulsified pregnenolone has been shown to have very high intracellular uptake.

Keywords: Pregnenolone; antimicrobial activity; E. coli; S. aureus.

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1. INTRODUCTION

Pregnenolone is involved in the synthesis of all steroids by cholesterol, including corticosteroids (mainly cortisol and aldosterone), androgens and estrogens. Cholesterol is converted to pregnenolone in cellular mitochondria, from which progesterone or dehydroepiandrosterone (DHEA) is then synthesized. Progesterone can be transformed into cortisol, deoxycorticosterone or aldosterone, and DHEA is a precursor to androgenic and estrogenic steroids such as testosterone, dihydrotestosterone, estradiol, estrone, etc. [1]. There is evidence of antimicrobial activity of pregnenolone [2, 3]. One of the first direct experiments with pregnenolone was performed by Lamb et al. [2], who reported successful treatment with pregnenolone acetate of *Nocardia asteroides*, a pathogen with very high mortality, especially in immune-compromised patients such as with AIDS disease. The authors describe a case of extremely resistant *N. asteroides* infection in a child, and successful treatment with pregnenolone acetate (200mg-300mg daily doses orally) after traditional treatment with sulfonamides did not give the desired effects and the patient's condition worsened. The authors also emphasize the absolute absence of side effects in the treatment with pregnenolone (acetate salt), which is of great importance given that all known interventions for the treatment of such highly resistant bacterial infections have pronounced side effects / toxic effects. In 1966 Yotis and Stanke [3] described a pronounced antibacterial effect of pregnane-derivative steroids such as pregnenolone and progesterone against a wide range of pathogens, including *Staphylococci* sp. Banday et al. [4] reported an efficient and easy synthesis of 17-chalconyl derivatives of pregnenolone and their antimicrobial activity against various microbial strains. Lone et al. [5] also synthesized oxides of steroidal chalcones and established their activity *in vitro* against various bacterial and fungal strains by the agar diffusion method. The activity of the tested compounds against individual microorganisms varies due to the structural differences between them. Pregnenolone has also been used as a template for the development of new anti-cancer compounds. Mohareb and Al-Omran [6] reported cytotoxic properties of newly synthesized heterocyclic steroids against three human tumor cell lines. In 2018 Chepkirui et al. [7] isolated five previously undescribed pregnenolone-type triterpenes 1–5 from a mycelial culture of basidiomycete *Fomitiporia aethiopica* fermented on rice and extracted with methanol. They have shown moderate cytotoxic effects against various human cancer cell lines, but have no significant antimicrobial activity.

Steroid-antibiotic conjugates have recently been developed as potential therapeutic agents for infectious diseases. Figueroa et al. [8] reported antibacterial activity of two pregnenolone derivatives (hemisuccinate-pregnenolone and ethylenediamine-hemisuccinate-pregnenelolone) on *S. aureus*, *E. coli* and *Klebsiella pneumoniae*, with minimal inhibitory concentrations MIC = 0.25 - 1 mg / ml. Other authors [9] synthesized a conjugate of pregnenolone with carbamazepine and found that it exhibited a significant antimicrobial effect on *Proteus mirabilis*. Their data indicate that the conjugate pregnenolone-carbamazepine can be used as an antibiotic for the treatment of infections caused by *P. mirabilis*. In 2013 Kakati et al. [10] synthesized a new class of chalconoyl pregnenolones, some of which show significant inhibitory activity against certain species of bacteria (*Bacillus subtilis* and *E. coli*) as well as against filamentous and oval fungi (*Aspergillus niger* and *Candida albicans*).

Testing the antimicrobial potential of steroids such as pregnenolone and its derivatives is a new and promising approach in the search for effective remedies for infections caused by multidrug-resistant bacteria, which are a major problem in medical practice today. In this regard, the aim of the present work is to test the antibacterial activity of pregnenolone with tocopherols against Gram-negative (*E. coli*) and Gram-positive (*S. aureus*) pathogenic bacteria.

2. MATERIALS AND METHODS

Antimicrobial agent. The antimicrobial effect of Pregnenolone (Sigma-Aldrich) with tocopherols (Sigma-Aldrich) was tested. Soya Lecithin 1360 MG (SXENB1611201B, Solgar, UK, Ltd., Herts, HP 23, Inc., Leonia N. J.), as well as 70% methanol were used as an emulsifiers in some of the experiments.

Control. As a positive control, the broad-spectrum antibiotic thiamphenicol (Nikovet - Sofia) was used, to which the tested microorganisms did not show resistance.
Microorganisms. Pure cultures of 10 pathogenic strains were tested - five Gram-negative (*Escherichia coli*) and five - Gram-positive (*Staphylococcus aureus*). Two of them are

reference, obtained from the Bulgarian National Bank for Industrial Microorganisms and Cell Cultures (NBIMCC): *Esherichia coli* ATCC- 8739 (NBIMCC 3397) and *Staphylococcus aureus subsp. aureus* ATCC - 6538 (NBIMCC 3359). The others are four strains of *E. coli* and four of *S. aureus* are isolated from inflammatory skin secretions from dogs in the laboratory of microbiology at the University Clinic in the Faculty of Veterinary Medicine at the University of Forestry in Sofia.

Nutrient media. Mueller Hinton agar and broth (BUL BIO NCIPD - Sofia) and Columbia blood agar (Biolab Zrt. H-1141, Budapest Ov. Utra 43) were used, as well as selective media: Endo agar (Antisel - Sharlau Chemie SA, Spain) for *E. coli* and Mannitol Salt agar for *S. aureus* (Biolab Zrt. H-1141, Budapest Ov. Utra 43).

The cultivation of microorganisms was carried out at 35-37° C for 24-48 hours under aerobic conditions.

The studies were performed by the *classical agar diffusion method* of Bauer et al. [11] and according to NCCLS [12,13]. Suspensions of test microorganisms were inoculated in exponential growth phase at a dose of $2,10^6$ cells / ml in an amount of 0.1 ml in 9 cm diameter Petri dishes on Mueller-Hinton agar with a pH of 7.2 - 7.4 and a layer thickness 4 mm. Endo agar and Mannitol Salt agar for the respective bacteria were also used in some of the experiments. Pregnenolone (with and without emulsifier) and the control antibiotic were administered by instillation of 0.1 ml in 9-mm wells in the agar at concentrations of the active substances per well of 0.5 mg for pregnenolone and 30 μg for thiamphenicol, respectively. After incubation for diffusion for 3-4 hours at room temperature, the dishes were cultured at 35-37° C for 18-24 and 72 hours. The results were read by measuring the diameters of the inhibitor zones in millimeters, including the diameter of the well to the nearest 1 mm, with a transparent ruler on the outside of the bottom of the plates, with full growth inhibition being taken as the boundary zone. According to the three-stage Bauer-Kirby system, an inhibitory effect of pregnenolone was observed in areas > 12 mm and of thiamphenicol at > 17 mm. The susceptibility of the test micro-organisms to pregnenolone was determined as for non-antibiotic preparations such as sulphonamides, namely: resistant (R) - in areas with diameters < 12 mm, moderately sensitive - intermediate (I) - in areas in the range 13 - 16 mm and sensitive (S) - > 17 mm. For thiamphenicol, the corresponding limits are as follows: R <12 mm, I - 13 - 17 mm and S - > 18 mm [12,13].

The experiments were performed in three variants. • In one of them, pregnenolone was tested without emulsifier (pure product). • In the second, it was emulsified in saline using methanol in a ratio of 1: 4: 5. • In the third variant, the pregnenolone was emulsified in saline using lecithin and methanol in a ratio of 2: 2: 1: 2 with homogenization for 1 min on a Vortex apparatus (Heidolph - Labimex, Bulgaria) to achieve some permeability in the agar gel nutrient media. In the variant with the methanol emulsion, after instillation into the wells, the Petri dishes with the samples were kept with the lid slightly open at room temperature for at least 2 hours to evaporate the methanol so as not to affect the results. In these experiments, selective media were used to prevent the development of any external microorganisms accidentally entered during the methanol evaporation.
All experiments were performed three times.

The statistical processing of the results was performed according to the classical method of Student and Fisher.

3. RESULTS

In the studies performed by the agar diffusion method, a different inhibitory effect of pregnenolone to the tested bacteria was found depending on whether it was administered in pure form or in emulsion.

The data in Table 1 show that when testing the pure product without emulsification, the growth of S. aureus strains was inhibited by a minimum dose of 150 mg (inhibitory zone diameters averaged 12.0 + 0.6 mm at this dose), while of those of *E. coli* - from 200 mg (diameters of the inhibitory zones on average 13.0 + 0.9 mm). However, the differences in the mean diameters of the zones without growth between the strains of *S. aureus* and *E. coli* were not significant - P> 0.05, t-criteria of Student.

All microorganisms studied showed high sensitivity to thiamphenicol used as a positive control. The differences in the diameters of the inhibitory zones of all strains between the antibiotic and pregnenolone administered directly without emulsification were statistically significant (P <0.01, t-criteria of Student).

Significantly higher sensitivity to pregnenolone was shown by the studied Gram-negative and

Gram-positive strains when it is applied in an emulsion. The summarized results are presented in Tables 2 and 3.

The data show that in an emulsion in physiological saline with methanol (Table 2) pregnenolone showed a significantly higher inhibitory effect on the studied bacterial strains than the oily product.

The differences in the diameters of the inhibitory zones with pregnenolone tested without emulsification were significant (P> 0.05, t-criteria of Student). When administered at a dose of 200 mg, the inhibitory zones of the Gram-positive and Gram-negative strains tested were comparable to those of the control broad-spectrum antibiotic (P> 0.05, t-criteria of Student).

The results of the test of the inhibitory activity of pregnenolone were highest when it was emulsified in saline with lecithin and methanol (Table 3).

The differences with the results obtained in the other test variants were statistically significant (P <0.001, t-criteria of Student). As can be seen from the aggregated data, when pregnenolone in this emulsion was administered at a dose of 112 mg, the inhibitory zones of the studied Gram-positive and Gram-negative strains were comparable to those of the control broad-spectrum antibiotic (P> 0.05, t-criteria of Student).

In all experimental variants used, *S. aureus* strains showed higher susceptibility to pregnenolone compared to those of *E. coli*, although the differences were not significant (P> 0.05, t-criteria of Student).

4. DISCUSSION

The results of the present studies show that pregnenolone exhibits inhibitory activity against both Gram-positive and Gram-negative bacteria. Of great practical importance is the high susceptibility of the tested *E. coli* strains - both clinical and control reference strains. Slightly higher susceptibility is shown by all studied strains of *S. aureus*, which is also of great importance for clinical practice. However, our results show that pregnenolone administered directly has a weak antimicrobial effect. This is due to its very low solubility in water, as it is in an oily state due to the content of tocopherol (fat-soluble vitamin). However, upon emulsification, its solubility in water increases and penetrates over a longer distance into the agar around its application points. The present results show that methanol is effective as an agent in this regard, but by the use of lecithin a better emulsion and higher water solubility of pregnenolone is achieved. However, when the conditions of the intracellular environment are considered (e.g. *in vivo* effects), the lipophilic nature of non-esterified pregnenolone is actually an advantage for its use as an anti-microbial intervention. The intracellular environment is much more lipophilic than serum, and most anti-microbial substances (e.g. antibiotics) exert their beneficial effects inside the cell. McManus et al. [14] have demonstrated that non-esterified steroids have very good intracellular uptake. In fact, in their experiment non-esterified pregnenolone had the highest cellular uptake than any other of the nine common steroids they tested, and it reached intracellular concentrations ~100 times higher than the concentrations in the culture media. Therefore, non-esterified formulations of pregnenolone such as the tested tocopherol formulation are likely to display strong anti-microbial effect *in vivo* comparable to the effects *in vitro* demonstrated in the present study.

Our results are in accordance with those of other authors. Figueroa et al. [8] found that the effect caused by pregnenolone derivatives may be due to the interaction with certain bacterial membrane factors that are specific for bacterial resistance. In this sense, the antibacterial activity of pregnenolone derivatives may depend on the nature of the functional groups involved in their chemical structure.

Table 1. Antimicrobial effect of pregnenolone against Gram-positive and Gram-negative bacteria in the agar-gel diffusion method

Microorganisms	Inhibitory zones in mm				
	50 mg	100 mg	150 mg	200 mg	Thiamphenicol
Escherichia coli	10.0 + 0.0	11.2 + 0.8	11.6 + 1.0	13.0 + 0.9	20.3 + 2.3
Staphylococcus aureus	10.6 + 0.5	11.4 + 0.5	12.0 + 0.6	13.2 + 1.0	20.9 + 2.5
Total	10.3 + 0.5	11.3 + 0.6	11.8 + 0.9	13.1 + 0.9	20.6 + 0.3

Table 2. Antimicrobial effect of pregnenolone emulsified with methanol against Gram-positive and Gram-negative bacteria in the agar-gel diffusion method

Microorganisms	Inhibitory zones in mm				
	50 mg	100 mg	150 mg	200 mg	Thiamphenicol
Escherichia coli	15.4 + 1.9	15.4 + 1.2	16.2 + 0.8	19.6 + 1.0	19.8 + 2.3
Staphylococcus aureus	14.8 + 1.7	15.6 + 1.5	17.8 + 0.8	19.4 + 1.0	20.8 + 1.2
Total	15.1 + 1.8	15.5 + 1.4	17.0 + 1.1	19.0 + 1.2	20.3 + 2.2

Table 3. Antimicrobial effect of pregnenolone emulsified with lecithin and methanol against Gram-positive and Gram-negative bacteria in the agar-gel diffusion method

Microorganisms	Inhibitory zones in mm				
	14 mg	28 mg	56 mg	112 mg	Thiamphenicol
Escherichia coli	13.8 + 1.8	15.3 + 1.2	17.6 + 2.8	19.3 + 2.9	20.8 + 3.1
Staphylococcus aureus	14.4 + 1.0	16.0 + 1.1	18.6 + 1.0	20.4 + 0.5	20.3 + 2.4
Total	14.1 + 1.4	16.1 + 1.7	18.1 + 2.1	19.9 + 2.1	21.6 + 0.3

Kakati et al. [10] found that the presence of α, β-unsaturated carbonyl moiety in the chalconoyl pregnenolones synthesized by them is essential for their antimicrobial activity. Prabpayak et al. [15] also reported antibacterial action of pregnenolone compounds. Valverde et al. [16] synthesized pregnenolone derivatives and found antibacterial activity *in vitro* on *S. aureus, K. pneumoniae* and *E. coli*. Growth of test microorganisms has been inhibited in a dose-dependent manner. These results suggest that the quaternary amine group of hemisuccinate-pregnenolone-vitamin B1 requires the hydrophobic region of the steroid to interact with certain components of the bacterial cell, thereby violating bacterial growth and causing cell death. These data indicate that in oily form the effect of pregnenolone in direct contact with microorganisms would be facilitated and correspondingly higher than found in our experiments, because the *in vitro* test methods require higher water solubility of the examined substance to read the antimicrobial effect.

Figueroa-Valverde et al. [9] also reported antibacterial activity of the synthesized by them pregnenolone-danazol-ethylenediamine conjugate on *S. aureus* and *Vibrio cholerae*. The growth of the test microorganisms has been inhibited in a dose-dependent manner. Their data indicate that the degree of lipophilicity may affect the inhibitory activity of the pregnenolone-danazol-ethylenediamine conjugate. Our results also show that the lipophilicity of pregnenolone affects its antibacterial activity. In order for it to manifest *in vitro*, it is necessary to obtain a water-soluble emulsion. However, *in vivo*, the lipophilicity of pregnenolone may not be an issue and may even be an advantage considering its extremely high intracellular uptake by both active and passive transport.

5. CONCLUSION

Pregnenolone exhibits inhibitory activity against both Gram-positive (*Staphylococcus aureus*) and Gram-negative bacteria (*Escherichia coli*).

The inhibitory activity of pregnenolone in the oleic state (in combination with tocopherol) is lower due to lack of water solubility. However, the antimicrobial activity of pregnenolone is significantly increased in emulsion in saline and methanol. Even higher water solubility of pregnenolone is achieved in an emulsion in saline with lecithin and methanol, which results in a significant increase in its antimicrobial properties *in vitro* in the agar-gel diffusion method. Pending future in-vivo experiments, we suspect that in living organisms even non-esterified pregnenolone would display strong antibacterial effects due to its high intracellular uptake.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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