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Synthesis and Characterization of Antibacterial activity from *Danazol-aminoca- proic acid derivative* on both Gram negative and Gram positive bacteria

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Abstract: In this work, *danazol-aminocaproic acid derivative* was synthesized and their antibacterial activity on both Gram negative and Gram positive bacteria was evaluated, using dilution method and the minimum inhibitory concentration (MIC). The results indicate that bacterial growth of *S. aureus* was inhibited in presence of *danazol-derivative* (MIC=1.04 × 10^{-3} mmol). Additionally, another data showed that *E. coli* was susceptibly to *danazol-aminocaproic acid derivative* with a MIC of 2.08 × 10^{-3} mmol. Finally, the growth bacterial of *K. pneumoniae* was blocked in presence of *danazol-derivative* (MIC=2.08 × 10^{-3} mmol). In conclusion, experimental data suggest that molecular mechanism involved in the antibacterial activity induced by *danazol-derivative* could be because the *steroid* require of the hydrophilic region of spacer arm with free carboxyl group, in order to interact with some factors of the cell surface and integrate into the cytoplasmic membrane and induce growth bacterial inhibition.
Key Words: Danazol, aminocaproic acid, antibacterial activity.

Introduction

Several causal agents, such as *S. aureus*[1], *K. pneumoniae*[2] and *E. coli*[3] among others[4], have been shown to accelerate the progression of infectious diseases. Although there are many therapeutic agents for the treatment of these bacterial microorganisms[5-7], unfortunately, prolonged antibiotic therapy may induce bacterial-resistance[8,9], because some bacteria have developed ways to circumvent the effects of antibiotics[10,11]. As a consequence several infectious diseases are reemerging, causing a serious public health problem[12,13]. This fact requires an international approach to its management, in this sense; new drugs have been developed for control of bacterial resistance[14-16]. For example, there has been a resurgence of interest in steroids as potential therapeutic agents for infectious diseases[17]. In this context, several steroid-antibiotics have been developed to mimic the antibacterial behavior of endogenous peptide antibiotics[18]. This task includes selective association of the steroid-antibiotic with disruption of bacterial membranes. The association relates to the chemical structural characteristics of the

steroid-antibiotic agents such as, cationic forms and facially amphiphilic conformations,
which seems to be the key required for antibacterial activity[19]. It has also been suggested that membrane selectivity is primarily derived from ionic recognition of negatively charged bacterial membranes[20]. In addition, several studies suggest that functional groups of steroid-derivative are involved in the bacterial activity[21]. In present study, the objective was to synthesize a new drug that can be used for treatment of infectious diseases. Therefore, our initial design included the synthesis of *danazol-aminocaproic acid derivative*. It is important to mention that this *steroid-derivative* have an arm with free carboxyl group involved in their chemical structure.
On the other hand, the *danazol-aminocaproic acid derivative* was used to evaluate their antibacterial activity on *S. proteus*, *K. pneumoniae* and *E. coli* using the microbial minimal inhibitory (MIC 90) method[22].

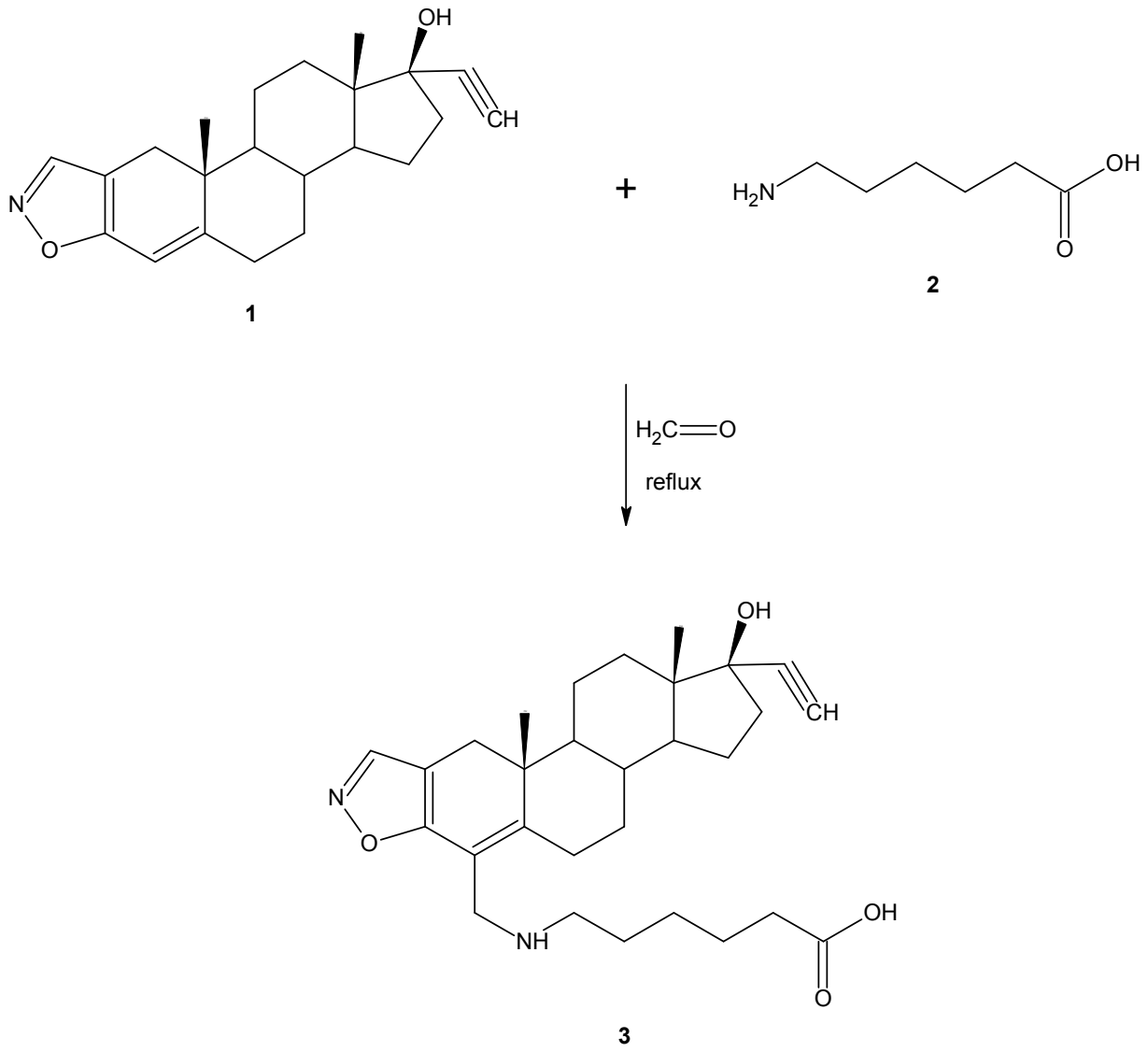

Figure 1. Synthesis of *danazol-aminocaproic acid derivative.* Reaction of *danazol* (1) with 6-*aminocaproic acid* (2) to form *danazol-derivative* (3).

Experimental

Danazol and other compounds evaluated in this study were purchased from Sigma-Aldrich Co., Ltd. The melting points for the different compounds were determined on an Electrothermal (900 model). Ultraviolet spectroscopy (UV) was carried out in dry methanol on a Perkin-Elmer model 552 spectrophotometer and infrared spectra (IR) was recorded using KBr pellets on a Perkin-Elmer Lambda 40 spectrometer. ^{1}H and ^{13}C NMR spectra were recorded on a Varian VXR-300/5 FT NMR spectrometer at 300 and 75.4 MHz in $CDCl_3$ using TMS as internal standard. EIMS spectra were obtained with a Finnigan Trace GCPolaris Q. spectrometer. Elementary analysis data were acquired from a Perkin-Elmer Ser. II CHNS/0 2400 elemental analyzer.

***Synthesis of* 6-[(2-ethyl-1-hydroxy-10a,12a-dimethyl-2,3,3a,3b,4,5,10,10a,10b,11,12a-dodecahydro-1*H*-7-oxa-8-aza-dicyclopenta[a,h]phenanthren-6-yl-methyl)amino]hexanoic acid.**

A solution of danazol (17-pregna-2,4-dien-20-yno[2,3-d]-isoxazol-17-ol) 200 mg (0.59 mmol), 6-aminocaproic acid 118 mg (1.18 mmol), in 10 mL of formaldehyde was gently refluxed for 24 h and then cooled to room temperature. The reaction mixture was evaporated to a smaller volume, diluted with water and extracted with chloroform. The organic phase was evaporated to dryness under reduced pressure, the residue was purified by crystallization from hexane:methanol:water (2:1:1), yielding 50 % of product; m.p. 76-78 ºC; UV (MeOH) λ_{max} (log) 208 (2.68) 233 (1.65) nm; IR V_{max} 3300 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$) δ_H:0.78 (3H, s, 35-CH_3), 0.78 (1H, m), 0.80 (1H, m), 1.01 (3H, s, 36-CH_3), 1.22-1.29 (3H, m), 1.40-1.81 (11H, m), 1.94-2.09 (4H, m), 2.22 (1H, m), 234-243 (4H, m), 3.08 (1H, m, C≡C**H**), 3.76 (2H, s, C-C**H**$_2$-NH), 5.66 (3H, m, -N**H**, -O**H**), 8.03 (1H, s, *isoxazol-ring*). ^{13}C NMR (74.5 MHz, $CDCl_3$) δ_C:14.04 (**C**-35), 19.10 (**C**-36), 20.97 (**C**-14), 22.54 (**C**-7), 22.90 (**C**-15), 23.30 (**C**-25), 25.48 (**C**-26), 28.95 (**C**-24), 30.98 (**C**-8), 31.75 (**C**-13), 33.57 (**C**-27), 33.93 (**C**-6), 37.00 (**C**-9), 37.22 (**C**-16) 38.00 (**C**-5), 45.50 (**C**-21), 46.28 (**C**-12), 48.60 (**C**-23), 52.88 (**C**-11), 54.41 (**C**-10), 75.14 (C≡**C**H), 79.85 (**C**-17), 85.85 (**C**≡CH), 109.80 (**C**-1), 116.80 (**C**-3), 141.96 (**C**-4), 148.16 (**C**H=N), 159.70 (**C**-2), 175.02 (**C**O_2H). EIMS 480.60 [M+, 18] m/z, 462.62 (57), 319.44(19), 439.12. Anal. Calcd. to $C_{29}H_{40}N_2O_4$: C, 72.47; H, 8.39, N, 5.83, 0, 13.32. Found. C, 72.45; H, 8.37; N, 5.78.

Biological evaluation

Strains: The microorganisms in this study belonged to the strain bank at the Department of Pharmaco-Chemistry at the Facultad of Ciencias Quimico-biologicas of the Universidad Autonoma de Campeche. The strains are certified by the Center for Disease Control in Atlanta and were as follows. *S. aureus* (ATCC 25923), *K. pneumoniae* (ATCC 700603) and *E. coli* (ATCC 25922). The strains are kept under refrigeration at 4 ºC in special gel (BBL).

Antimicrobial agents: The steroids derivatives and the other compounds studied were dissolved in methanol and diluted with distilled water. Cefotaxime, gentamycin, methicillin and ciprofloxacin were used as control drugs.

Antimicrobial activity: The evaluation of antimicrobial effect of the different compounds on the bacterial species was made by method described by Chiong *et al.*[22]. The bacterial species were incubated on McConkey (*E. coli* and *K. pneumoniae*) and *Staphylococcus* 110 (*S. aureus*) agars for 24 h at 37 ºC. After 24 h, it was determined whether growth had taken place or not. In addition, a series of tubes were prepared, the first of which contained 2 mL of culture medium (tripticase soye) at double concentration and the remainder (11 tubes), contained the same quantity of medium at single concentrations. From the first tube (double concentration) an aliquot of 2 mL of the studied compound (1 mg/mL) was added and stirred, from this tube an aliquot of 2 mL was taken and added to the following tube (simple concentration) and the process was successively repeated until the last 2 mL of dissolution had been used up. After this process, each tube was inoculated with 0.1 mL of the bacterial suspension, whose concentration corresponded to McArland scale (9×10^8 cells/mL) and all the tubes were incubated at 37 ºC for 24 h. Subsequently, a loop was taken from each of them and inoculated into the appropriate cultures for different bacterial organisms and were incubated for 24 h at 37 ºC.

After such time, the minimum inhibitory concentration (MIC) was evaluated to consider the antimicrobial effect of all compounds. In order to discard the effect of methanol (solvent) on the bacterial species studied, a series of the same number of tubes was prepared in parallel, to which 2 mL of methanol at 60 % was added to the first and corresponding successive dilutions were added in the same way as before. In addition, a control series was also performed using distilled water at pH 7.0.

Results and Discussion

In this study, the Synthesis and Characterization of Antibacterial activity from *Danazol-aminoca proic acid derivative* on both Gram negative and Gram positive bacteria were made. The first step involves the coupling of 6-*amino- caproic acid* to *danazol* by the method reported by Mannich[28], using formaldehyde to form *danazol-derivative* which has characteristic an spacer arm with free carboxyl group involved in their chemical structure. The results indicate that ^{1}H NMR spectrum of *danazol-aminocaproic acid derivative* showed signals at 0.78 and 1.01 ppm corresponding to methyls presents in the heterocycles rings. In addition, another signal at 234 ppm for methylene bound to carboxyl group and 3.08 ppm for proton of alkyne (C≡C**H**) were found. Additionally, another signal at 3.76 ppm for methylene involved in arm spacer bound to *ring-A* of *danazol*. Another signals at 5.66 ppm for protons of both amine group and the acidic hydrogen of C(=O)-OH were display. Finally, a signal at 8.03 ppm for proton corresponding to *isoxazol-ring* was found.

On the other hand, ^{13}C NMR spectra displays chemical shifts at 14.04 and 19.10 ppm for the carbons of methyls groups presents in the *danazol* fragment. Another chemical shifts at 23.30-28.95 ppm for carbons of methylenes involved in spacer arm bound to *danazol* were found. In addition, several signals were display at 46.28 ppm for methylene of spacer arm bound to *ring-A* of *danazol*; two signals at 75.14 and 85.85 ppm for carbons of alkyne. Additionally, other signals at 109.80-159.70 for carbons corresponding to heterocycles. Finally, at 175.02 for the carbon of CO_2H was found. In addition, the presence of the *danazol-aminocaproic acid derivative* was further confirmed from mass spectrum which showed a molecular ion at m/z 480.60.

On the other hand, in the second step the antibacterial activity of *danazol-aminocaproic acid derivative* on *S. aureus*, *K. pneumoniae* and *E. coli* was evaluated by means of dilution method and the minimum inhibitory concentration (MIC)[22], using *gentamycin*, *ampicillin*, *cefotaxime* and *cifroflaxin* as control in this study. The results obtained (Fig. 2) indicate that bacterial growth of *S. aureus* was inhibited with *cefotaxime* (MIC = 5.23×10^{-4} mmol), *gentamycin* (MIC = 2.68×10^{-5} mmol) and *ciprofloxacin* (MIC = 3.77×10^{-4} mmol). It is important to mention, that in presence of *ampicillin*, the bacterial growth of *S. aureus* was not blocked (data not shown). Additionally, the bacterial growth of *S. aureus* in presence of *danazol-aminocaproic acid derivative* (MIC = 1.04×10^{-3} mmol) was blocked. All this data indicate that antibacterial activity induced by *danazol-derivative* was lower in comparison with *cefotaxime* (β-lactam antibiotic), *gentamycin* (inhibitor of synthesis of protein) and *ciprofloxacin*. This phenomenon can be due mainly to the different molecular mechanism involved and the characteristic chemical structure of the compounds studied in this study.

Therefore, was interesting to consider the molecular mechanism involved in the antibacterial activity induced by *danazol-derivative*. It is important to mention that this compound contains a spacer arm with free carboxyl group involved in their chemical structure, in addition involve a quaternary amine in the *oxazol-ring*. Several reports have shown that drugs with quaternary amine exert antibacterial activity against both Gram-positive and Gram-negative bacteria through perturbation of lipid bi-layer membranes that constitute the bacterial cytoplasmic membrane and the outer-membrane of bacteria[23]. To evaluate this premise, we used the *danazol*, since the nature of functional groups contained in their chemical structure have a quaternary amine in the *oxazol-ring*. The results showed that in presence of *danazol* the bacterial growth of *S. aureus* was not blocked (data not showed). The experimental data suggest that quaternary amine of danazol by itself, does not have antibacterial activity on the pathogen microorganism studied. Those experimental data indicate that spacer arm with free carboxyl group involved in the chemical structure of *danazol-derivative*, could be the responsible of the antibacterial activity. In order to analyze this possibility, the antibacterial effect of *6-aminocaproic acid* compound on *S. aureus* was evaluated to compare with the antibacterial activity induced by the *danazol-aminocaproic acid derivative* on this pathogen. The results showed that the bacterial growth of *S. aureus* was not blocked (data not showed) in presence of *6-aminocaproic acid* compound. It is important to mention that when *danazol* is bound with *6-aminocaproic acid* to form the *danazol-derivative*, the bacterial growth of S. aureus is blocked, possibly because the quaternary amine group involved in oxazol-ring requires the hydrophilic region of spacer arm with free carboxyl group in order to interact with the cell surface and integrate into the cytoplasmic membrane. Such integration into the membrane is sufficient to perturb bacterial growth to cause the membrane to lose fluidity and for the cell to die. This phenomenon can be associated by interaction of *danazol-drivative* with teichoic acid that is an element of Gram-positive bacteria[24].

On the other hand, in alternative experiments on the antibacterial activity of *danazol-aminocaproic acid derivative* was evaluated on Gram negative bacteria using the same controls. The results showed (Fig. 4)

that bacterial growth of *E. coli* in presence of *cefotaxime* (MIC = 5.23 × 10^{-4} mmol), *gentamycin* (MIC = 1.34 × 10^{-5} mmol) and *ciprofloxacin* (MIC = 3.01 × 10^{-3} mmol) was inhibited. Additionally, the bacterial growth of *E. coli* in presence of *danazol-aminocaproic acid derivative* was blocked (MIC = 2.08 × 10^{-3} mmol).

Other results, showed indicate that bacterial growth of *K. pneumoniae* in presence of *cefotaxime* (MIC = 2.61 × 10^{-4} mmol), *gentamycin* (MIC = 2.68 × 10^{-5} mmol) and *ciprofloxacin* (MIC = 1.5 × 10^{-3} mmol) was inhibited. Nevertheless, the bacterial growth of *K. pneumoniae* in presence of *danazol-aminocaproic acid derivative* was blocked (MIC = 2.08 × 10^{-3} mmol). It is important to mention, that in presence of *ampicillin*, the bacterial growth of both *E. coli* and *K. pneumoniae* was not blocked (data not shown). This data indicate that *danazol-aminocaproic acid derivative* have different antibacterial activity on *E. coli* and *K. pneumoniae* in comparison with the controls. Possibly, the molecular mechanism implied in the antibacterial activity induced by *steroid-derivative* can be shown by the intermolecular interaction of carboxyl group with the cations (Mg^{2+} and Ca^{2+}), involved in the membrane cell providing a substantial increase the permeability of the outer membrane of Gram-negative bacteria and induce cell death. This premise is availed by other reports made several investigators which suggest that spacer arm with free carboxyl group involved in other types of steroids may be key requirement for their antibacterial activity[25,26]. Nevertheless, the antibacterial activity of *danazol-derivative* can also depend on the intermolecular interaction with the lipopolysaccharide of Gramnegative bacteria. This premise is based on the works by several investigators which developed a class of steroid antibiotics with the intent of mimicking the antibacterial activities of polymyxin B on Gram-negative bacteria[27]. In addition, this phenomenon can induce, as consequence, an increase in the permeability of the outer membrane and induce growth bacterial inhibition on these pathogen microorganisms.

Conclusions

Experimental data suggest that molecular mechanism involved in the antibacterial activity induced by *danazol-derivative* could be because the *steroid* require of the hydrophilic region of spacer arm with free carboxyl group, in order to interact with some factors of the cell surface and integrate into the cytoplasmic membrane and induce growth bacterial inhibition.

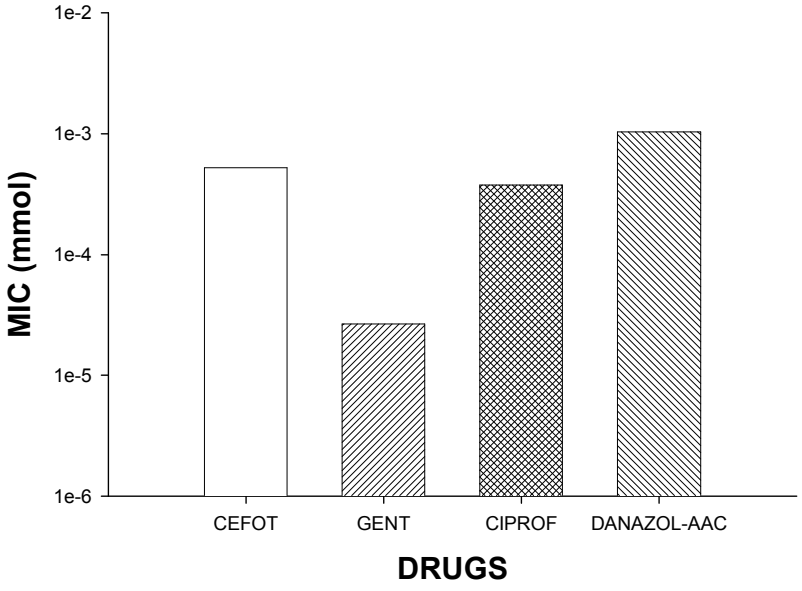

Figure 2. Antibacterial effects induced by *danazol-aminocaproic acid derivative* **(**DANAZOL-AAC**)** and controls (cefotaxime, CEFOT; gentamycin, GENT and ciprofloxacin CIPROF) on *S. aureus*. Data showed that *S. aureus* was susceptibly to cefotaxime (MIC of 5.23 × 10^{-4} mmol), gentamycin (MIC = 2.68 × 10^{-5} mmol) and CIPROF (MIC = 3.77 × 10^{-4} mmol). Additionally, the bacterial growth of *S. aureus* in presence of *danazol-derivative* (MIC = 1.04 × 10^{-3} mmol) was inhibited. MIC = minimum inhibitory concentration.

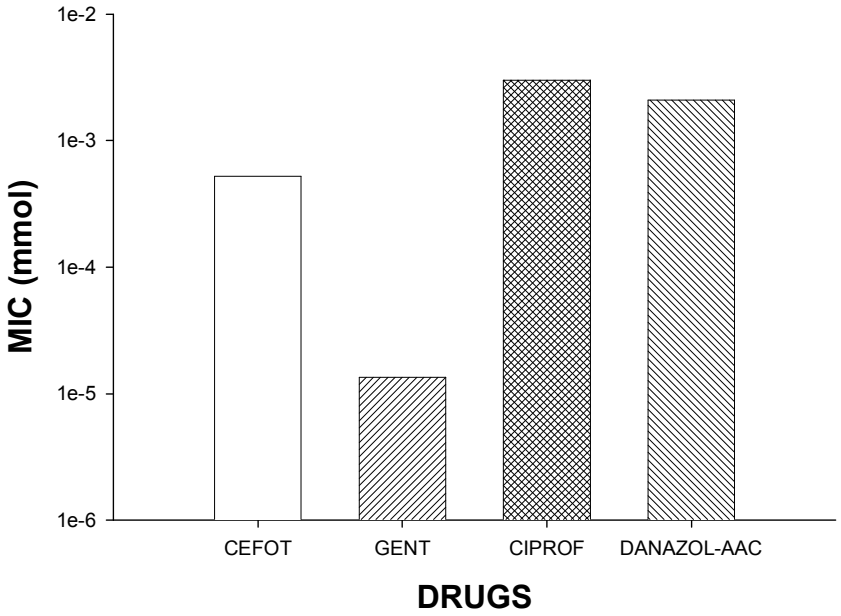

Figure 3. Antibacterial effects induced by *danazol-aminocaproic acid derivative* **(**DANAZOL-AAC**)** and controls (cefotaxime, CEFOT; gentamicin, GENT and ciprofloxacin CIPROF) on *E. coli*. It is showed that exist differences of antibacterial activity of CEFOT (MIC = 5.23×10^{-4} mmol), GENT (MIC = 1.34×10^{-5} mmol) and CIPROF (MIC = 3.01×10^{-3} mmol) on *E. coli* in comparison with the *danazol- derivative* (MIC = 2.08×10^{-3} mmol). MIC = minimum inhibitory concentration.

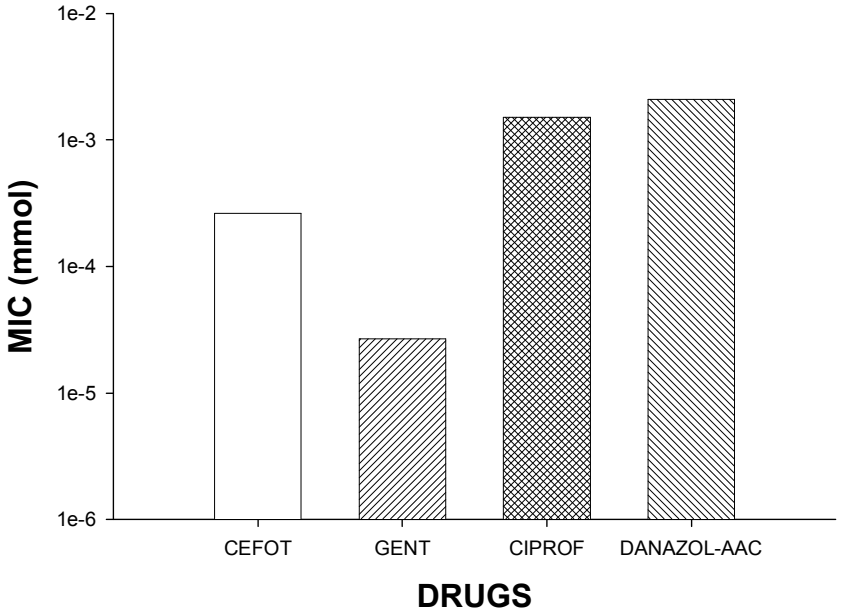

Figure 5. Antibacterial activity induced by *danazol-aminocaproic acid derivative* **(**DANAZOL-AAC**)** and control (cefotaxime, CEFOT; gentamicin, GENT and ciprofloxacin CIPROF) on *K. pneumoniae*. Data showed that *K. pneumoniae* was susceptibly to cefotaxime (MIC = 2.61×10^{-4} mmol), gentamycin (MIC = 2.68×10^{-5} mmol) and CIPROF (MIC = 1.50×10^{-3} mmol). Additionally, the bacterial growth of K. *pneumoniae* in presence of *danazol-derivative* (MIC = 2.08×10^{-3} mmol) was inhibited. MIC = minimum inhibitory concentration.

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