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In vitro Applications of *In Silico* Designed Antibiofilm Agents for *Staphylococcus epidermidis*

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ABSTRACT

In recent years anti-virulence agents have been used widely to reduce bacterial resistance and prevent damage to host cells and normal flora. Six molecules were used in this study (according to previous *in silico* studies) to detect their antibiofilm activity in lab. Four molecules gave positive results and were as follows : Acetaminophen inhibited biofilm production 100% at 11000µg/ml concentration, Acetylsalicylic Acid inhibition was 100% at 1600µg/ml , Ibuprofen inhibited 35% of biofilm production at 600µg/ml and Acetic Acid inhibition was 25% at 1000 µg/ml concentration .All the molecules at the used concentrations were found to affect biofilm production without significant change in bacterial growth. It was concluded that a structure based drug design strategy using Ligand Based Virtual Screening had a success score of about 60% and that Acetaminophen, Acetylsalicylic Acid, Ibuprofen and Acetic Acid can be used as antibiofilm molecules . Also Non-Steroidal Anti-Inflammatory Drugs family can be a useful library for antibiofilm future investigations.

Keywords: Drug design, antibiofilm, *S. epidermidis*, biofilm, acetaminophen, acetic acid, acetylsalicylic acid, ibuprofen.

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INTRODUCTION

Biofilm is an extracellular polymeric substance (EPS) that allows adhesion of bacteria to various material surfaces and cohesion to each other. This material includes polysaccharides, and nucleic acids (Flemming and Wingender, 2010)[1]. In *Staphylococcus epidermidis, biofilm formation is regarded* as a major pathomechanism as it renders the bacterium highly resistant to conventional antibiotics and host defenses. This can be caused by slow diffusion of these compounds through the extracellular polymeric matrix and slow growth of the bacteria (Mah and O'Toole, 2001; Schoenfelder *et al.*, 2010)[2,3]. In general the antibiotic affect the beneficial bacteria/microbiota and disturb the balance state of human health and it would be a great chance for emerging of resistant organisms. The arguments are that resistance to compounds targeting the virulence factors cannot evolve and spread in the resident flora, as these bacteria lack virulence targets. It is also proposed that resistance to virulence blocking agents is likely to result in nonfunctional virulence systems, and consequently nonvirulent bacteria (Keyser *et al.*, 2008)[4]. So if these virulence factors could be neutralized by the use of small organic molecules, virulence blockers, it is possible that the infection could be inhibited and cleared by the immune system , this would allow to design a completely novel set of antibacterial agents with a potential to act as alternatives to antibiotics (Salyers and Whitt, 2002)[5]. In recent years *in silico* strategies have been used to predict anti-virulence agents,and the main aim of *in silico* drug design is to bring the best chemical entities to experimental testing by reducing costs and time (Kapetanovic, 2008)[6].

MATERIALS AND METHOD

Bacterial Isolation and Identification

Eighty seven isolates of staphylococci where collected from wound swaps, ear swaps and from urine samples of patients from different hospitals of Baghdad city (Baghdad hospital, Al-Yarmook hospital, Al-Karama hospital and Ibn Al-Baladi hospital), for the interval started from 18-10-2012 to 28-2-2013. These isolates were subjected to different routine tests; microscopic and biochemical tests. Biofilm production was studied, methods for biofilm detection used were modified to proceed under microaerobic conditions (Al-Mulla *et al.*, 2013).[7]

Measurement of Antibiofilm Activity

This was done by the tissue culture plate method according to Christensen *et al* (1985)[8] with some modifications (Al-Mulla *et al*., 2013)[7].

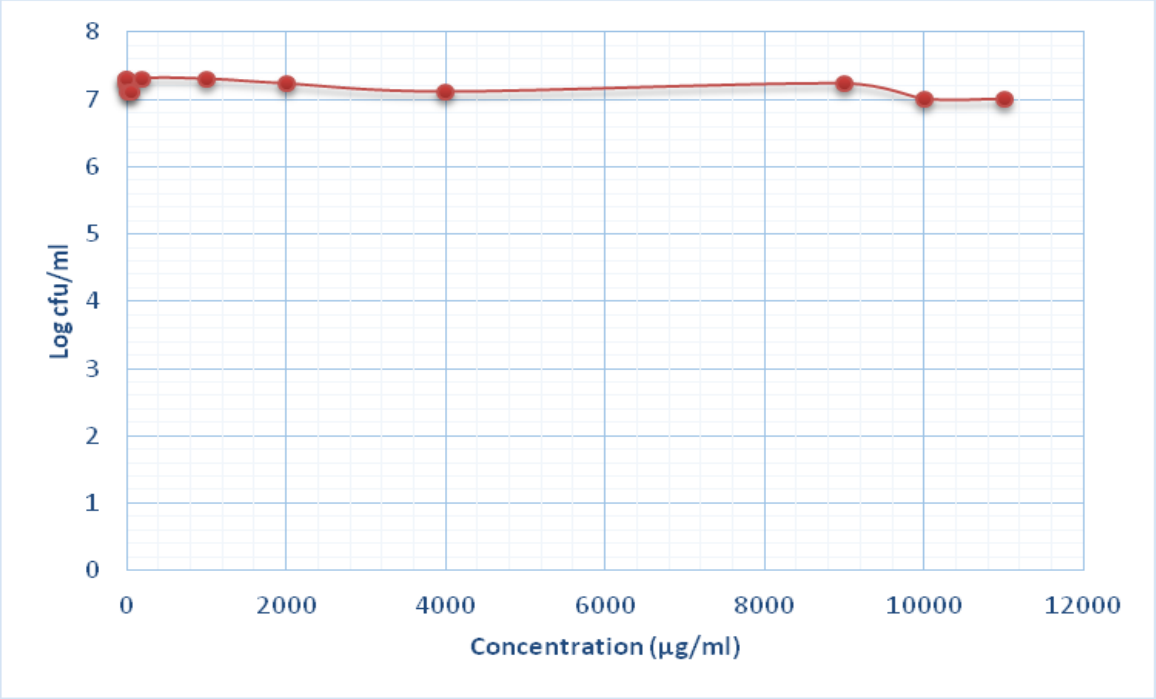
RESULTS AND DISCUSSION

A designed molecules in previous *in silico* study for prediction of anti-biofilm agents for

Staphylococcus epidermidis (Al-Khafaji and Al-Mulla, 2014)[9] were used as a starting point in this study. It concluded that 37 small molecules can be used as anti-biofilm agents. Out of these molecules, only seven molecules were selected to be tested *in vitro* as an anti-biofilm. These molecules were: Acetaminophen, Acetylsalicylic acid, Acetic acid, Diacetyl, Ferric ammonium citrate , Ibuprofen , Thymol. The selection was made on the basis of: drug likeness, low side effects in humans, market availability, low cost, ease of handling in the laboratory. The experiments were done using these molecules to investigate the biofilm production and viable count of the bacteria after exposure to gradient concentrations of each molecule. Biofilm monitoring only is not enough because the aim of these experiments was to disarm the virulence factor of the bacteria, not to kill them.

Acetaminophen

The following are schematic diagrams for biofilm production represented by micro ELISA OD_{570nm} readings and viable count (Log values) of bacterial growth versus different concentrations of the selected molecules.



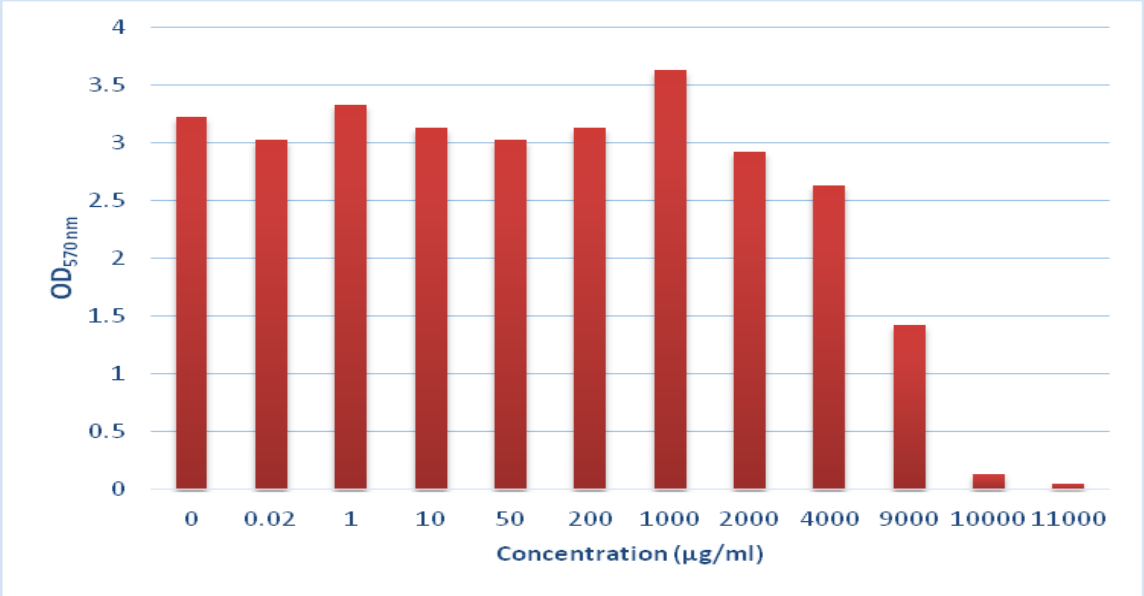
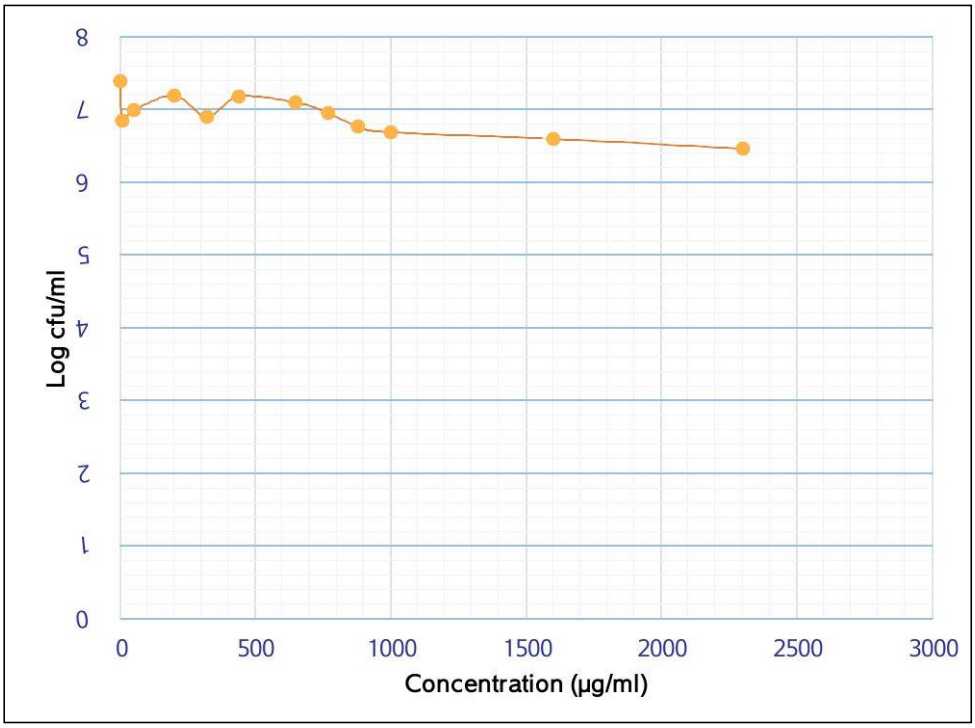


Figure 1: Effect of Acetaminophen on growth of *Staphylococcus epidermidis* (upper section), and biofilm production (lower section).

Acetylsalicylic Acid



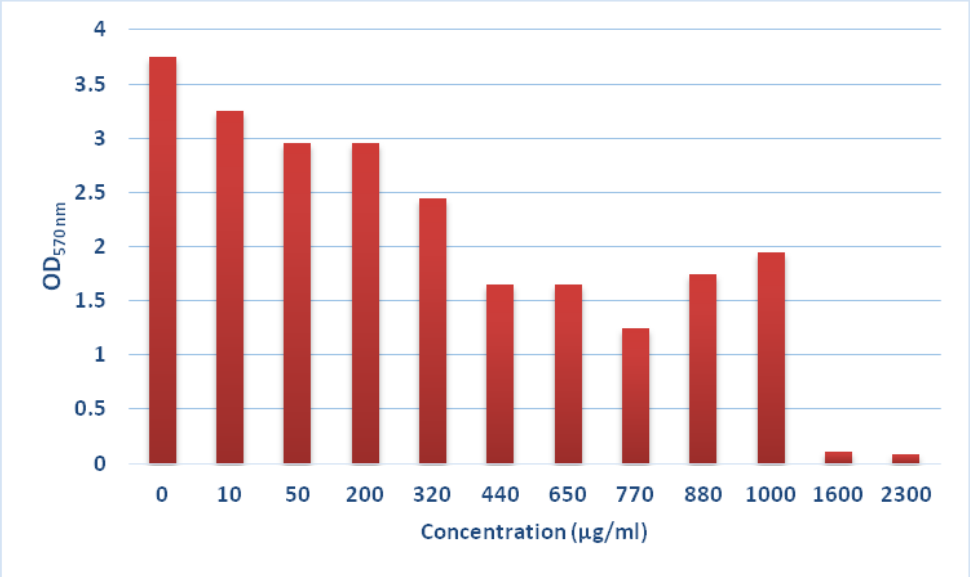
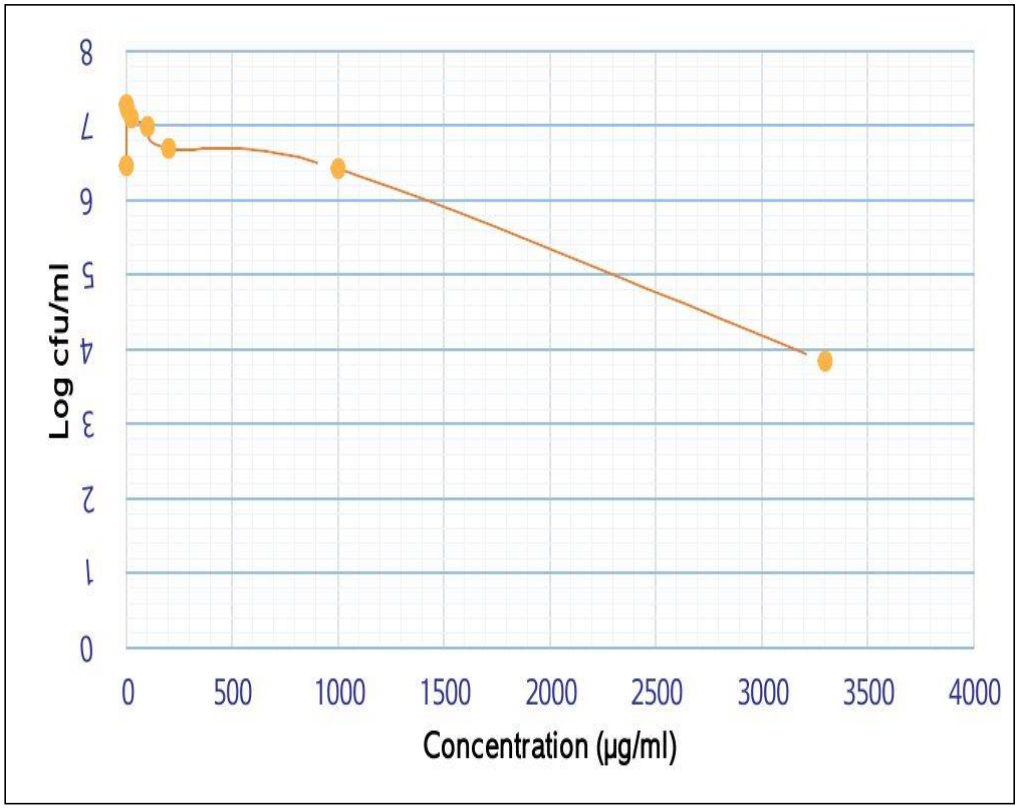


Figure 2: Effect of Acetylsalicylic Acid on growth of *Staphylococcus epidermidis* (upper section), and biofilm production (lower section).

Acetic Acid



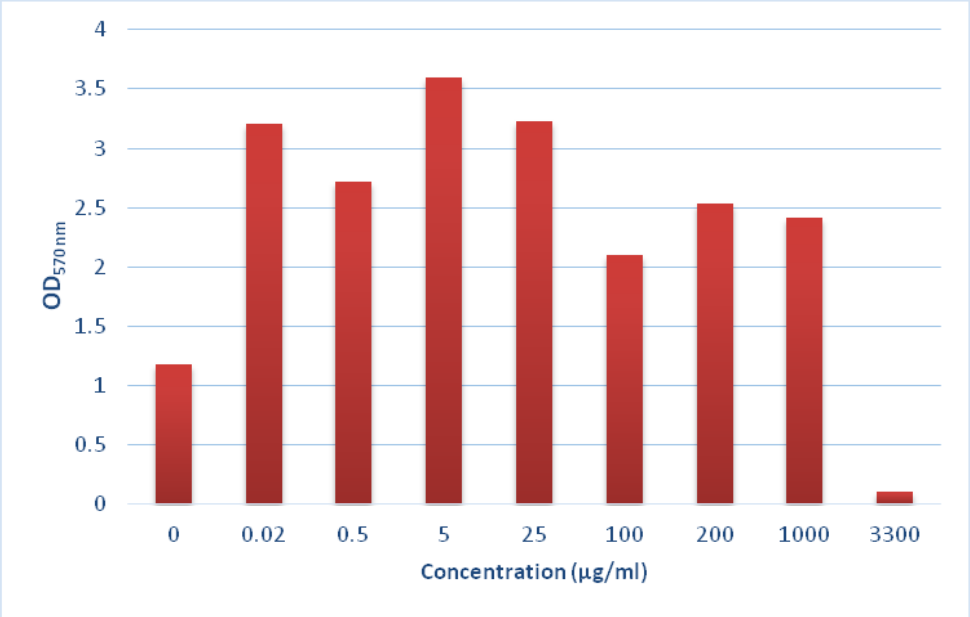
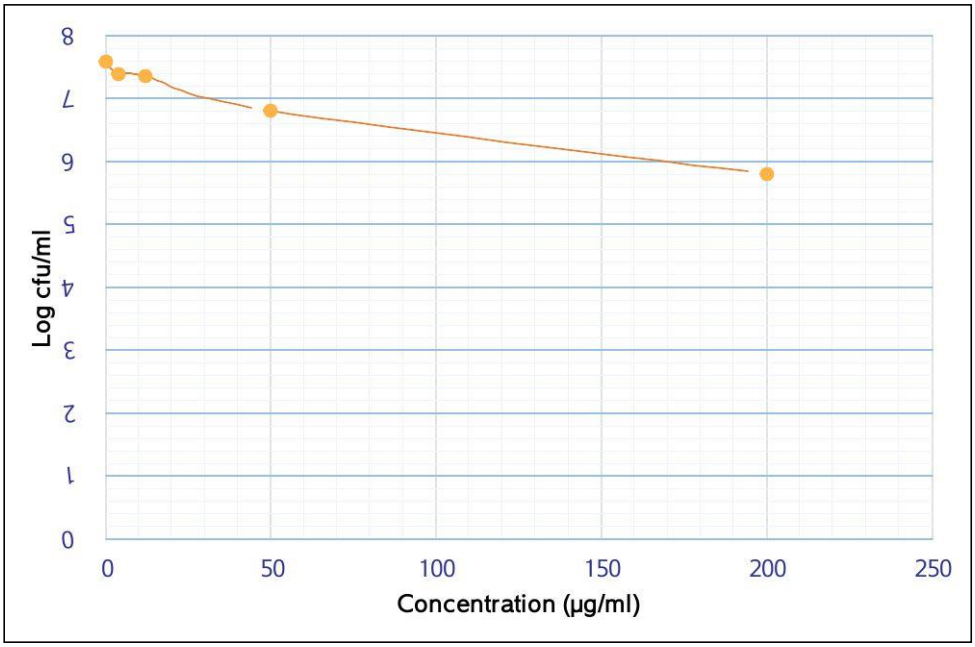


Figure 3: Effect of Acetic Acid on growth of *Staphylococcus epidermidis* (upper section), and biofilm production (lower section).

Ferric Ammonium Citrate



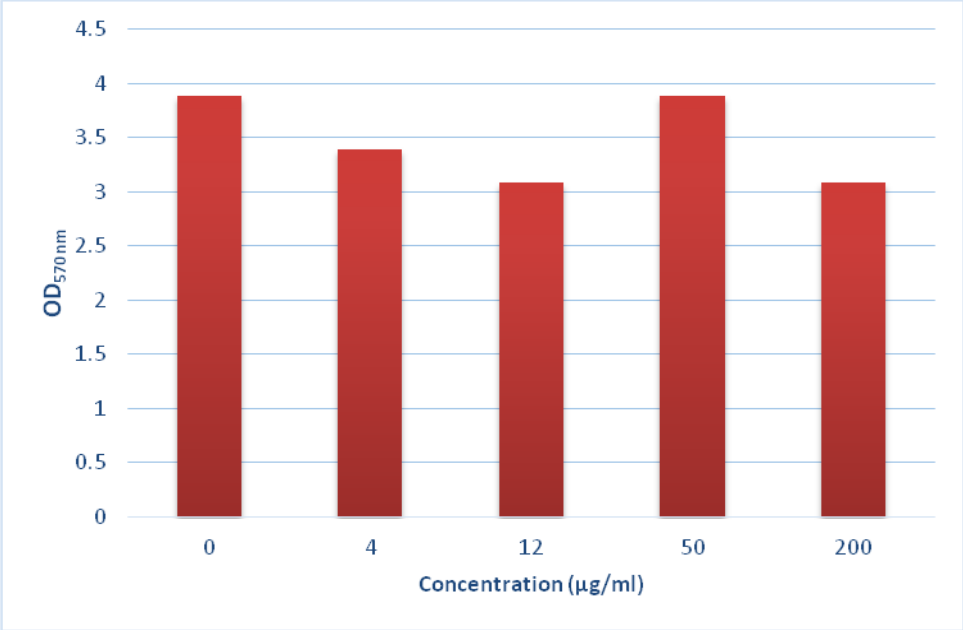
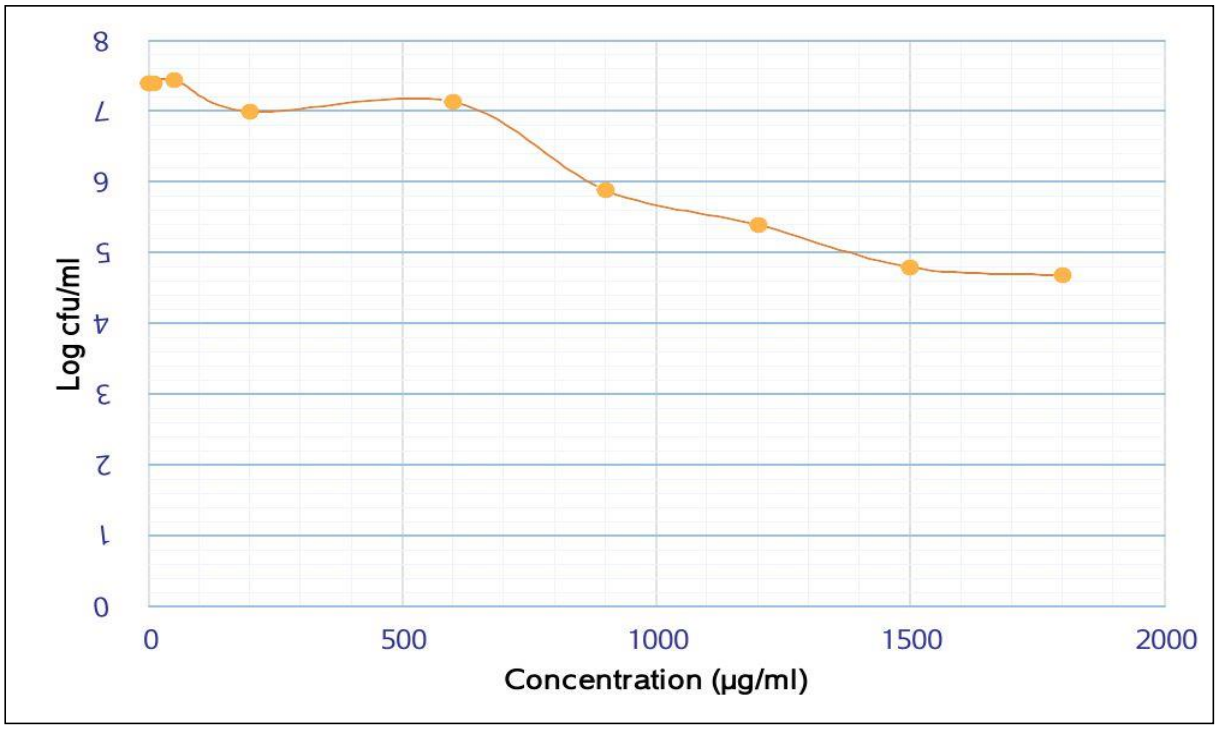


Figure 4: Effect of Ferric Ammonium Citrate on growth of *Staphylococcus epidermidis* (upper section), and biofilm production (lower section).

Ibuprofen



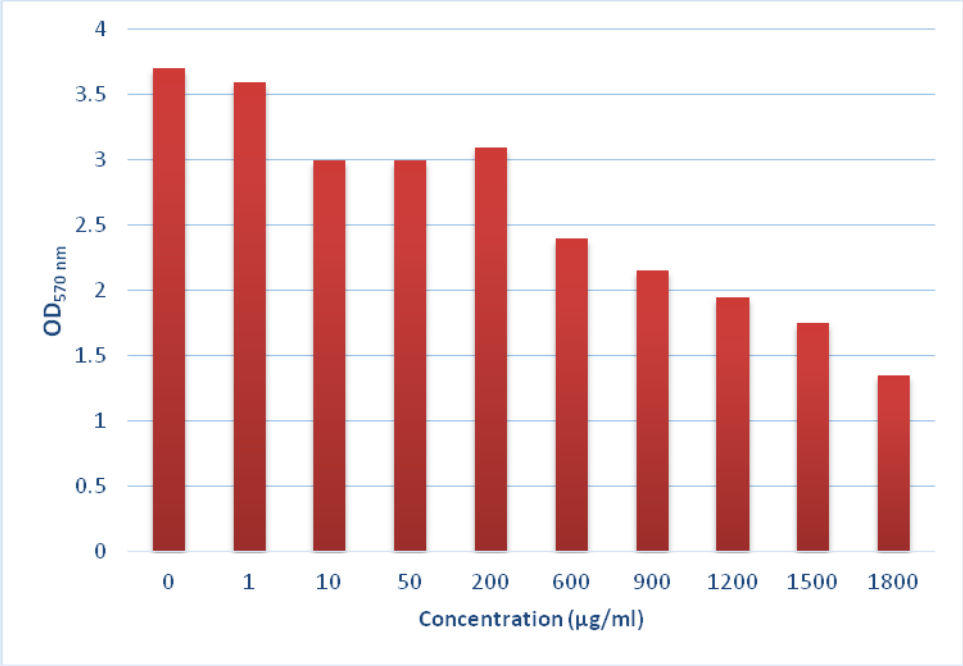
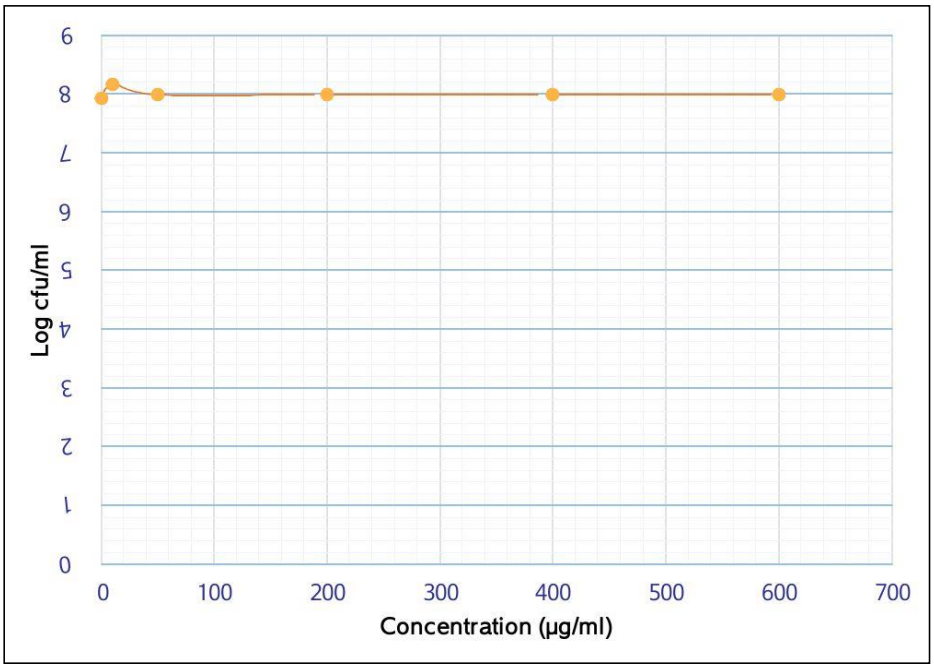


Figure 5: Effect of Ibuprofen on growth of *Staphylococcus epidermidis* (upper section), and biofilm production (lower section).

Thymol



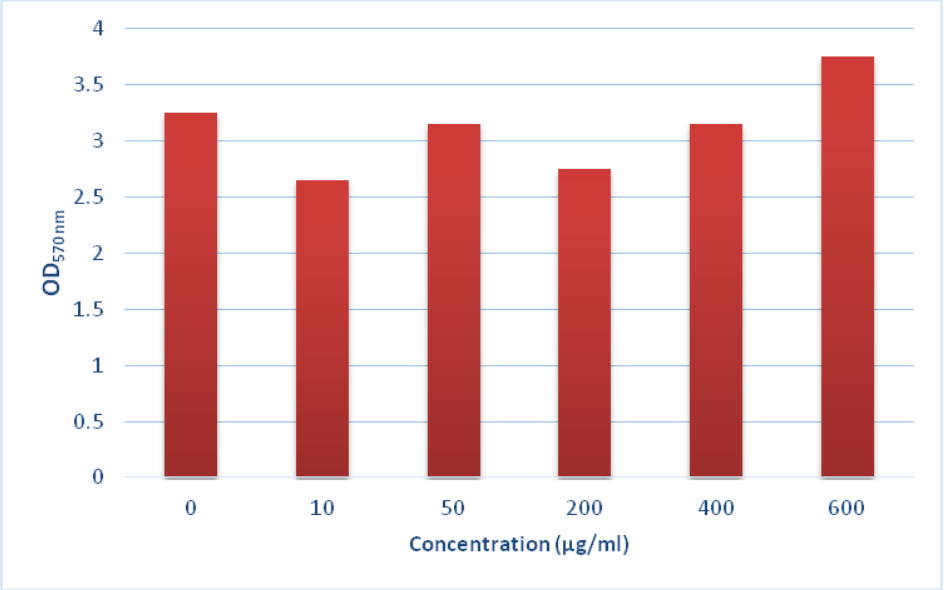


Figure 6: Effect of Thymol on growth of *Staphylococcus epidermidis* (upper section), and biofilm production (lower section).

The molecules selected in the study have high binding affinity to sarA protein (one of the most active proteins in *Staphylococcus epidermidis* biofilm production process). This affinity was predicted by measuring binding free energy with the target protein using *in silico* programs (Al-Khafaji and al-Mulla, 2014)[9]. The results may lead to a conclusion that Acetaminophen and Acetylsalicylic Acid gave 100% antibiofilm activity, and that Ibuprofen and Acetic Acid gave 35%, 25% antibiofilm activity respectively. The other molecules were poor biofilm suppressors. This leads to proposal that Non- Steroidal Anti Inflammatory Drugs (NSAID) constitute a good family for searching antibiofilm drugs. Farber and Wolff (1992)[13] reported that Salicylic Acid inhibited adherence (55%) and biofilm production of *S. epidermidis* (Farber and Wolff, 1992)[10]. A second study by Farber *et al.* (1995) further illustrated that NSAIDs, including sodium salicylate , inhibit *S. epidermidis* and *P. aeruginosa* biofilm production on contact lenses, lens cases, and commonly used medical polymers in a dose-related manner. This manner has also been observed by other authors (Tomlinson *et al.*, 2000; Bandara *et al.*, 2004)[11,12]. Alem and Douglas (2004)[15] found in their studies on *Candida albicans* that seven of nine (NSAID) drugs tested at a concentration of 1 mM inhibited biofilm formation. Aspirin, etodolac, and diclofenac produced the greatest effects, aspirin causing up to 95% inhibition. Celecoxib, nimesulide, ibuprofen, and meloxicam also inhibited biofilm formation, but to a lesser extent. Aspirin was active against growing and fully mature (48-h) biofilms; its effect was dose dependent, and it exhibited significant inhibition (20 to 80%) at pharmacological concentrations. Abd El-Aziz *et al.* (2012)[14]

found that Salicylate at a concentration of 10 µg/ml reduced biofilm synthesis by 57.01% and eradicated pre-adhered biofilms by 29.19% while at 100 µg/ml biofilm synthesis was reduced by 68.35% and pre-formed biofilms was disrupted by up to 62.73%. In comparison with the previous studies the results of Acetylsalicylic Acid at therapeutic levels (50-200 µg/ml) involved inhibition to pre-formed biofilm by 22%. This difference in results is due to difference in experiment conditions, and bacterial genera (as *P. aeruginosa* was used). The concentrations of the molecules as antibiofilms do not meet the pharmacological limits for human use (maximum therapeutic plasma concentrations for Acetaminophen, Acetylsalicylic Acid and Ibuprofen are 150, 225, 50 µg/ml respectively) (Mueller and Lieberman, 1970; Hall *et al*., 1988; Rumack, 2002)[16,17,18] . However *In vitro* studies and results must not be taken as final results for clinical applications because differences in environmental conditions may affect the results, where lab tools, nutrient media, temperature and solubility status differ from plasma conditions, body circulation, body temperature, body defenses. Also bacterial behavior in lab differs from that in human body, and drug molecule characteristics in lab differ from those in human body where they may undergo degradation, conformational changes, re-arrangement, plasma protein binding. Therefore, all lab experiments are considered as a beginning step and must be completed with *in vivo* studies. In this study four of six molecules were proposed to suppress biofilm production by inhibiting sarA protein activity. This means that the percent of success in drug design was about 66.7 % %. This percent is good if considered the hypothetical processes and prediction and modeling of protein and the error percent in each drug design step. Although the results gave an initial view of novel antibiofilm drugs and need to be completed with *in vivo* studies in future but there are no limitations on using these compounds in other aspects away from human body, as an antiseptic or medical equipment surface protectant.

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