

# Dexpanthenol and propolis extract in combination with local antibiotics for treatment of Staphylococcal and Pseudomonas wound infections.

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## Abstract

**Background:** Managing bacteria and their biofilms in wounds is vital in achieving wound healing. Determination of antimicrobial and antibiofilm activity of different remedies such as dexpanthenol and propolis- natural bee product- alone and/or in combination with topical antimicrobial agents on *Pseudomonas* and *Staphylococcus* wound infection was the aim of this study.

**Methods and Findings:** Following up on our previous study, six *Staphylococcus* and *Pseudomonas* clinical strains selected from 80 strains isolated from wound swab cultures through 2007-2008 were included in this study. Selection was made according to their biofilm forming capacity (strong biofilm forming single isolates) and the magnitude of change in their biofilm production (for isolate mixtures). The effect of dexpanthenol on the minimum inhibitory concentrations (MICs) of four selected topical antimicrobial agents was determined by broth micro-dilution technique using checkerboard method as well as its antibiofilm activity. Dexpanthenol showed a great effect on the MIC values of the tested antibiotics, as well, demonstrating high antibiofilm activity. Dexpanthenol showed mild efflux inhibition property upon testing with serial dilutions of ethidium bromide. The effect of ethanolic extract of propolis (EEP) on activity of different antimicrobial agents was determined. The outcome differs between tested isolates. Estimation of the antibiofilm activity of EEP by modified microtiter plate method revealed a great decrease in adhesion at low concentrations of EEP. *In vivo* study of the effect of selected dexpanthenol-drug combinations on induced wound infection in rats, revealed complete healing with resolution of infection, as evaluated histopathologically, in a shorter time.

**Conclusions:** Dexpanthenol and propolis alone or in combination with topically applied antibiotics were proved effective in prevention of biofilm formation, thus favoring wound healing in a short time.

## Introduction

When skin becomes broken due to an injury or trauma to the tissue, the normal wound healing process begins. Basically, three phases of wound healing occur in healthy individuals, which include the inflammatory, proliferation and remodeling phase. Chronic wounds seem to be arrested in a stage dominated by inflammatory processes [1]. Bacteria can establish a complex community within the wound. These communities are organized as biofilms. Wound bacterial biofilms offer an explanation for the pathophysiology of non-healing wounds. Aerobic bacteria such as *Pseudomonas aeruginosa* (*P. aeruginosa*)

and *Staphylococcus aureus* (*S. aureus*) have long been recognized as major wound pathogens. The ability of colonizing bacteria to establish and proliferate in a biofilm in the wound leads to the increasing emergence of multidrug-resistant *P. aeruginosa* and *S. aureus*. This might contribute to the lack of successful antibiotic treatment [2, 3].

The bacteria in a micro-colony divide and reach a critical density, which has been termed a quorum. These cells produce extracellular molecules used to communicate from cell to cell (quorum sensing molecules), that is important for biofilm development. Therefore, manipulating the cell-cell signaling of

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**Running head:** Dexpanthenol and Propolis with topical antibiotics against Wound Infection.

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the bacterial community can also alter the defenses and virulence of the bacterial community [4, 5]. Controlling bacteria in wounds is a vital factor in achieving wound healing. Stringent management regimens that combine multiple strategies into a single, concomitant approach, aimed at promoting the patient's normal healing functions and also killing the bacteria and degrading the community, which has afforded added defenses to the bacteria are required for chronic wound healing [5]. Combining anti-biofilm strategies with traditional strategies as debridement in non-healing and potentially infected wounds can be used to weaken the biofilm until it reaches a level at which the impaired host's immunity can fight off the chronic infection. The most exciting area of research in anti-biofilm agents is quorum sensing inhibitors [6].

Pantothenic acid (B<sub>5</sub> vitamin) is essential to normal epithelial function. It is a component of coenzyme A, which serves as a cofactor for a variety of enzyme-catalyzed reactions that are important in the metabolic processes. The topical use of dexpanthenol, the stable alcoholic analog of pantothenic acid, is based on good skin penetration and high local concentrations. Topical dexpanthenol acts like a moisturizer, improving stratum corneum hydration, maintaining skin softness and elasticity. The stimulation of epithelization, granulation and mitigation of itching were the most prominent effects of formulations containing dexpanthenol [7].

Propolis is a multifunctional substance used by bees to maintain the safety of their hives. It is worldwide used for its potential therapeutic effects. Propolis is a chemically very complex resinous bee product collected by bees from tree buds. The main constituents of propolis are beeswax, resin and volatiles. The insects secrete beeswax, while the other two constituents are obtained from plants [8]. Along with other honey bee products, propolis has outstanding therapeutic properties. It has been shown to exhibit pharmacological activities such as antibacterial, antiviral, antifungal, antioxidant, anti-inflammatory, anti-cariogenic and immune-stimulating properties [9-13].

As the in-depth knowledge of chronic wounds and searching new ways of fighting biofilm is urgently required and would significantly improve treatment and prognosis. The aim of the present study involved practical management of *Pseudomonas* and *Staphylococcus* wound infections through determination of activity of different remedies such as dexpanthenol and propolis on the selected pathogens and study of the effects of drug combinations on the formation of single and mixed bacterial biofilms as well as their effects on induced infected wounds in rats (*in vivo* models).

## Methods

Following up on our previous study [14], Six *Staphylococcus* and *Pseudomonas* clinical strains selected from 80 strains isolated from wound swab cultures at the Microbiology Laboratory of Medical Research Institute, Alexandria University through 2007-2008 were included in this study. Two were present as

single entities and four were in mixture of two each. Isolates were identified using conventional methodologies. These strains were selected according to their biofilm forming capacity (strong biofilm forming single isolates) and the magnitude of change in their biofilm production (for the isolate mixtures) as tested by modified microtiter plate method [15]. Possible mechanisms involved in such change among which *N*-acyl-homoserine lactone (quorum sensing molecule) production intensity in *Pseudomonas* isolates were previously investigated [14]. The tested isolates were: N31 *P. aeruginosa* [strong biofilm former, optical density (O.D.)= 0.39; *N*-acyl-homoserine lactone producer, O.D.= 0.353], N31 mixture isolates (O.D.= 0.869) [N31 *P. aeruginosa* and N31 *S. aureus* (weak biofilm former, O.D.=0.093)], N6 *S. aureus* [strong biofilm former, O.D.=0.508]; N6 mixture isolates (O.D.= 0.216) [N6 *S. aureus* and N6 *P. aeruginosa* (weak biofilm former O.D.= 0.074; *N*-acyl-homoserine lactone producer, O.D.= 0.467)], N4 *P. aeruginosa* [strong biofilm former O.D.= 0.4; *N*-acyl-homoserine lactone producer O.D.= 0.467], N25 *S. aureus* [strong biofilm former, O.D.= 0.414]. Antimicrobial susceptibility testing of the isolates was performed by Kirby-Bauer method onto Mueller-Hinton agar and interpreted according to Comité de l'Antibiogramme de la Société Française de Microbiologie (CASFM) 2008. In the present study, these selected strains were challenged against certain antibiotics applied alone and in combination with local wound healing agent as dexpanthenol and propolis extract.

## Effect of dexpanthenol on the minimum inhibitory concentration [MIC] of four selected topical antimicrobial agents

Gentamicin, ciprofloxacin, oxytetracycline (El-pharaonia pharmaceutical company, Alexandria, Egypt) and colistin sulphate (Arab company for gelatin and pharmaceutical products, Alexandria, Egypt) as powder were used to prepare stock solutions. The rationale behind using these topically applied antibiotics was as follows: Gentamicin and oxytetracycline are widely used as topical antimicrobials in Egypt, for colistin it is coming back into action [16], and for ciprofloxacin; it is a recently topically applied antimicrobial in treatment of soft tissue infections [17]. Dexpanthenol gel (Luna cosmetics company, Egypt) was used to prepare stock solutions.

## Preparation of stock solutions

### Stock solutions of the selected antibiotics were prepared as follows

Four hundred milligrams of gentamicin sulphate powder were dissolved in sterile distilled water to prepare 0.4% solution and the same was done for colistin sulphate and oxytetracycline. For ciprofloxacin, 400 mg of the drug was dissolved in minimal amount of 0.1N NaOH, and then completed to the required volume with sterile distilled water. **Stock solution of dexpanthenol was prepared as follows:** Twenty grams of dexpanthenol were dissolved in sterile distilled water to prepare 20% dexpanthenol solution.

## Checkerboard method

MICs of each of the selected antibiotics and dexpanthenol alone and in combination with the antibiotics were determined by broth micro-dilution technique using checkerboard method [15] carried out in 96 wells sterile microtiter plates (Greiner bio 1, cellstar). Antibiotics were diluted to a final concentration ranging between 1000 and 0.9 µg/ml and distributed along the horizontal wells (2-12), 50 µl/well. Vertical wells received final concentrations of dexpanthenol [3%, 2.5%, 2%, 1.5%, 1% and 0.5%] (50µl/well). A1 served as negative control (100µl sterile distilled water and 100µl un-inoculated double strength broth). Each of the 84 wells received 100µl of sterile double strength broth inoculated with the organism (s) under test ( $10^6$  CFU/ml), OD=0.2 at 600 nm. H1 served as positive control (100µl sterile distilled water and 100µl inoculated double strength broth). Plates were covered and incubated at 35-37°C for 24 hours and MIC was calculated as the lowest antimicrobial agent concentration with lack of visual turbidity. Results of the combinatorial effect of dexpanthenol and the antimicrobial agent were interpreted as activity index as follows: Activity index = MIC of the antimicrobial agent in the combination/ MIC of the antimicrobial agent alone. The obtained activity index values were translated into log activity index values for ease of interpretation. The experiment was repeated three times and results were reproductive.

## Determination of antibiofilm activity of certain antibiotics alone and in combination with Dexpanthenol against selected bacterial isolates using modified microtiter plate method [15, 18]

The same Checkerboard arrangement was used as mentioned previously. After 24 hours incubation, plates were emptied and washed three times with sterile saline and fixed with 200 µl of 99% methanol for 15 min. Wells were emptied and air dried. After that, 200 µl of 2% crystal violet solution was dispersed in each well, left for 5 minutes then washed and dried. Each well was eluted with 160 µl of 33% glacial acetic acid and read using ELISA plate reader at 590 nm. Results were interpreted and plotted into figures.

## Determination of efflux inhibition capacity of Dexpanthenol

A simple qualitative method according to Martins et al. (2009) [19] was applied. It was done by a 96-well micro-plate screening assay, where serial dilutions of ethidium bromide (EB) starting at 4µg/ml up to 0.003 µg/ml, were dispensed to the wells (2-12) in the rows A, B, C. The wells B1- B12 and C1 -C12 received dexpanthenol with a final concentration of 0.5 % and 3 %, respectively. Then an inoculum of  $10^6$  CFU /ml of the selected organisms in double strength broth was added to the wells A (2-12), B (2-12) and C (2 -12). A1 (EB, sterile distilled water and inoculated broth), B1 and C1 (EB, dexpanthenol and un-inoculated broth) served as controls. The plates were incubated at 35-37°C, overnight and the lowest concentration of EB that

promotes fluorescence under ultraviolet trans-illuminator was visually interpreted and photographed.

## Determination of antimicrobial activity of propolis extract

The chemical composition of propolis (American propolis) is four flavonoids: kaempferol, galangin, 3,3'-dimethoxyquercetin and 3-methoxykaempferol, also contains caffeic acid, chrysin, ferulic acid [20] (Arab company for gelatin and pharmaceutical products, Alexandria, Egypt)

## Preparation of propolis extract

Twenty grams of propolis powder was mixed with 100 ml of 95% ethanol to obtain 20% W/V propolis suspension. Extraction was carried at room temperature in dark for 7 days with frequent shaking. After extraction the mixture was filtered and the filtrate was designated as ethanolic extract of propolis [EEP] [21]. Antimicrobial activity of EEP was determined by agar diffusion against N31 *Staphylococcus* and *Pseudomonas* isolates as representative of Gram positive and Gram negative bacteria, respectively [21] using EEP in different dilutions (10, 5, 2.5, 1.25 and 0.625%).

## Determination of the MIC of EEP by agar dilution technique

The test plates were prepared with 19 ml of Mueller-Hinton agar and 1 ml of 2-fold serial dilution of EEP in sterile distilled water starting at 10 mg/ml and up to 0.019 mg/ml. The plates were spot inoculated with 10 µl of  $10^6$  cells/ml of the tested isolates and incubated for 24 hours at 35-37°C. The MIC was defined as the lowest concentration that prevented visible growth of micro-organisms spots [21]. Parallel control plates using ethanolic solutions in the same concentrations (95% ethanol in serial dilutions) were done.

## Determination of the effect of EEP on different antimicrobial agents

Kirby-Bauer method with modifications according to Stepanovic et al. (2003) [21] instead of broth micro-dilution technique using checkerboard method due to clouding of the wells upon EEP addition. The test was carried out using the six bacterial isolates. The following antibiotics were challenged: polymyxin B, colistin sulphate, vancomycin, Sulphamethoxazole-trimethoprim, erythromycin, tetracycline, chloramphenicol, bacitracin, fusidic acid, ampicillin-sulbactam, amoxicillin-clavulanic acid, methicillin, cefaclor, cefoperazone, cefipime, imipenem, ciprofloxacin, ofloxacin, levofloxacin, gentamicin, neomycin, tobramycin (Oxoid Ltd., UK). Two milliliters of appropriate concentration [sub-inhibitory concentration (0.25 MIC of EEP of the corresponding isolate)] of EEP were mixed with 38 ml of Mueller-Hinton agar to obtain concentration of EEP that did not inhibit microbial growth. Parallel controls with same concentrations of the ethanolic solution were carried out. The

plates were incubated and diameter of inhibition zones was measured and compared to that in absence of EEP and plotted into figures.

### Determination of antibiofilm activity of EEP

Biofilm assay was performed according to the modified microtiter plate methods [15] using a range of final EEP concentrations from 0.05 to 100 mg/ml. Parallel controls of the corresponding ethanolic solution in two fold serial dilutions was carried out.

### In vivo study of drug combination effects on the formation of mixed bacterial biofilms

#### Induction of wound infection in rat using a mixture of *P. aeruginosa* and *S. aureus* (N31) culture-mate isolates

To establish an animal model of *P. aeruginosa* and *S. aureus* biofilm associated with wound infection and investigate the pathogenic effects of biofilm and the outcome of different treatment regimen, Four Para-vertebral incision wounds; were made by sterile surgical blades on the dorsal surface of 30 Sprague- Dawley albino female rats of 3 months age and weighing about 170 grams. All surgical procedures were carried out under anesthesia using thiopental 40 mg/kg body weight administered intraperitoneally. Each wound received 50  $\mu$ l of mixture of  $10^6$  cells/ml *P. aeruginosa* and  $10^6$  cells/ml *S. aureus*; originally isolated together from a wound. The wounds were stitched under aseptic conditions [22]. All experimental procedures were approved by the Institutional Ethics Committee in accordance with the Guide to the Care and Use of Experimental Animals by Canadian Council for Animal Care (1993).

#### Treatment of wounds using two antibiotics each alone or in combination with dexpanthenol: Preparation of gel

Gel was prepared using carbopol 934 NF (Luna cosmetics company, Egypt) in a 0.5% concentration. Gel was soaked overnight in the prepared solution of antibiotic and alkalized in the next day with triethanolamine or 0.1 N NaOH except for ciprofloxacin gel which was prepared in a 0.1 N NaOH solution from the start. The dose of dexpanthenol was dissolve in the gel, mixed and kept in small containers at room temperature. The prepared EEP gel darkened upon standing as a result we continued in the *in vivo* experiment with dexpanthenol.

**Rat experiment:** Rats were divided into six groups; five rats each; that received the following treatments: **Group 1:** Carbopol gel 0.5% base, **Group 2:** 4% dexpanthenol gel (Dexpanthenol in the chosen concentration here has no cytotoxic activity according to Klöcker et al. (2004) [23] who reported that Dexpanthenol is considered to be safe up to 5%), **Group**

**3:** 0.4% gentamicin gel, **Group 4:** 0.4 % gentamicin and 4% dexpanthenol gel, **Group 5:** 0.4% ciprofloxacin gel, **Group 6:** 0.4% ciprofloxacin and 4% dexpanthenol gel. One rat was kept as no treatment control and sacrificed under anesthesia on the second day post infection. All treatments began from day one post infection. Gels were applied once daily for all rats until complete healing of the wounds, then rats were sacrificed under anesthesia and the tested skin areas were excised and kept in freshly prepared 10% formaldehyde solution and examined histopathologically [22].

## Results

### Effect of dexpanthenol on MIC of the selected topically applied antibiotics

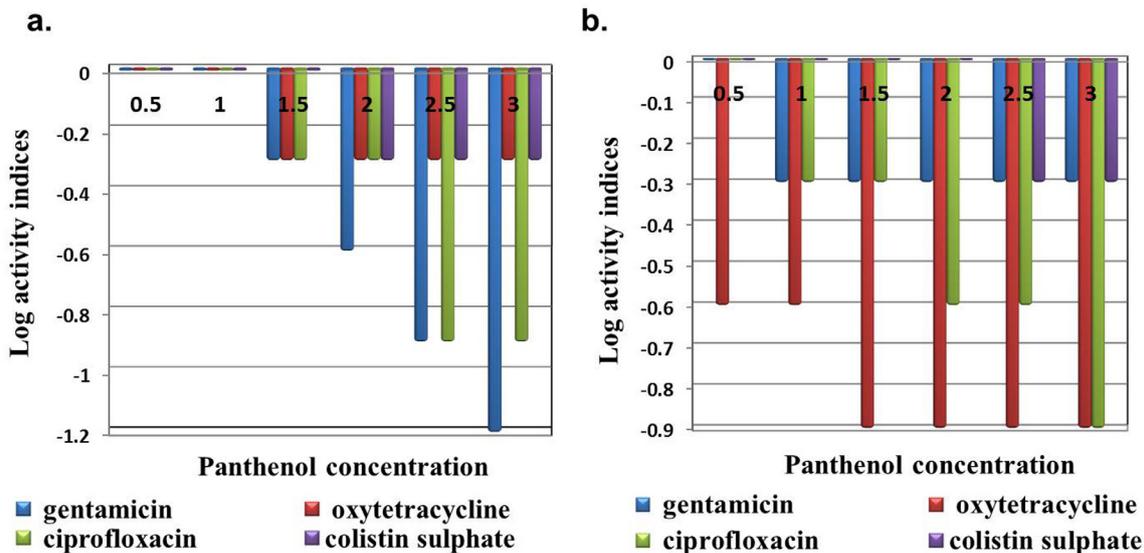
Results of the combinatorial effect of dexpanthenol and the antimicrobial agent were interpreted as activity index instead of fractional inhibitory concentration (FIC) as dexpanthenol showed no antimicrobial activity under the tested concentration range. The obtained activity index values were translated into log activity index values for ease of interpretation.

### Effect of different dexpanthenol concentrations on the MIC of four topically applied antibiotics against *P. aeruginosa* present in isolate mixture N31

The effect of dexpanthenol started at concentration 1.5 % showed 2 fold decreases in the MIC of all tested antibiotics and continued till reach 16 fold and 4 fold decreases in case of gentamicin and ciprofloxacin respectively at 3% dexpanthenol. All dexpanthenol concentrations showed 2 fold decreases in MIC for oxytetracycline and colistin [Table1]. On calculation of log activity indices of the 4 selected antibiotics, the synergistic effect of dexpanthenol was evident in case of gentamicin sulphate [Fig.1a].

**TABLE 1.** Effect of different dexpanthenol concentrations on the MIC of four topically applied antibiotics on *P. aeruginosa* present in isolate mixture N31.

Dexpanthenol concentration (g%)	Gentamicin sulphate	Oxytetracycline HCl	Ciprofloxacin	Colistin sulphate
	MIC in $\mu$ g/ml			
0	250	62.5	15.625	1.9
0.5	250	62.5	15.625	1.9
1	250	62.5	15.625	1.9
1.5	125	31.25	7.8	$\leq 0.9$
2	62.5	31.25	7.8	$\leq 0.9$
2.5	31.25	31.25	3.9	$\leq 0.9$
3	15.625	31.25	3.9	$\leq 0.9$



**FIGURE 1.** Effect of dexpanthenol concentrations on activity indices of four topically applied antibiotics against *P. aeruginosa* isolate N31 (a) and *P. aeruginosa* and *S. aureus* mixture N31 (b).

**Effect of different dexpanthenol concentrations on the MIC of four topically applied antibiotics against *P. aeruginosa* and *S. aureus* isolates mixture N31**

A decrease of the MIC started at 0.5% for oxytetracycline (MIC=125 µg/ml) and at 1% for gentamicin (MIC=1000 µg/ml) and ciprofloxacin (MIC=62.5µg/ml), reaching 8 fold decrease in MIC at 3% dexpanthenol for oxytetracycline and ciprofloxacin while remained constant at 2 fold for gentamicin. On the other hand, 2 fold decrease in MIC for colistin starts at 2.5%. Synergism was evident for oxytetracycline and ciprofloxacin reaching its maximum effect at 1.5% for oxytetracycline and 3% for ciprofloxacin [Fig 1b].

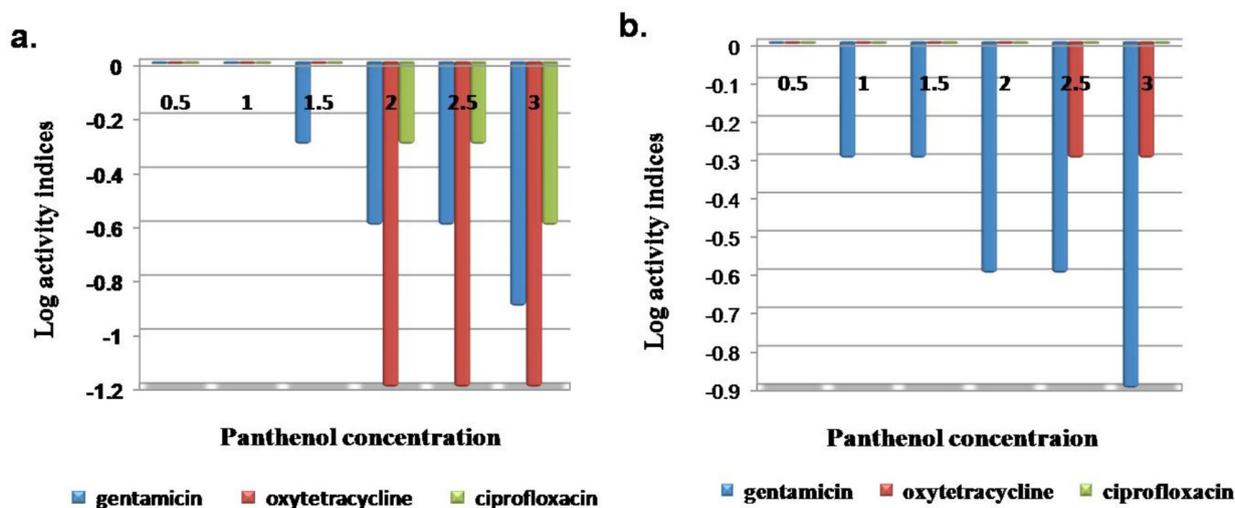
**Effect of different dexpanthenol concentrations on the MIC of three topically applied antibiotics on *S. aureus* present in isolate mixture N6**

An abrupt decrease in MIC of oxytetracycline occurred at 2% dexpanthenol that reached 32 fold, while a gradual decrease occurred in MIC from 1.5% dexpanthenol for gentamicin and from 2% dexpanthenol for ciprofloxacin up to 8 fold for genta-

micin and 4 fold for ciprofloxacin at 3% dexpanthenol [Table2]. Synergistic effect of dexpanthenol started at 1.5% with gentamicin and 2% with other tested antibiotics [Fig 2a].

**TABLE 2.** Effect of different dexpanthenol concentrations on the MIC of three topically applied antibiotics on *S. aureus* present in isolate mixture N6.

Dexpanthenol concentration (g%)	Gentamicin sulphate	Oxytetracycline HCl	Ciprofloxacin
	MIC in µg/ml		
0	62.5	15.625	31.25
0.5	62.5	15.625	31.25
1	62.5	15.625	31.25
1.5	31.25	15.625	31.25
2	15.625	≤0.9	15.625
2.5	15.625	≤0.9	15.625
3	7.8	≤0.9	7.8



**FIGURE 2.** Effect of dexpanthenol concentration on activity indices of three antibiotics against *S. aureus* isolate N6 (a) and *S. aureus* and *P. aeruginosa* mixture N6 (b).

### Effect of different dexpanthenol concentrations on the MIC of three topically applied antibiotics against *S. aureus* and *P. aeruginosa* isolates mixture N6

A 2 fold decrease in the MIC of gentamicin (MIC=500 µg/ml) and oxytetracycline (MIC=62.5 µg/ml) started at 1% and 2.5% respectively, the decrease remained constant for oxytetracycline and reached 8 fold at 3 % dexpanthenol for gentamicin. On the other hand, it had no apparent effect on the MIC of ciprofloxacin (MIC=31.25µg/ml). Synergistic Effect of dexpanthenol was more apparent for gentamicin reaching its maximum at 3% dexpanthenol [Fig 2b].

### Effect of different dexpanthenol concentrations on the MIC of four topically applied antibiotics on *P. aeruginosa* isolate N4

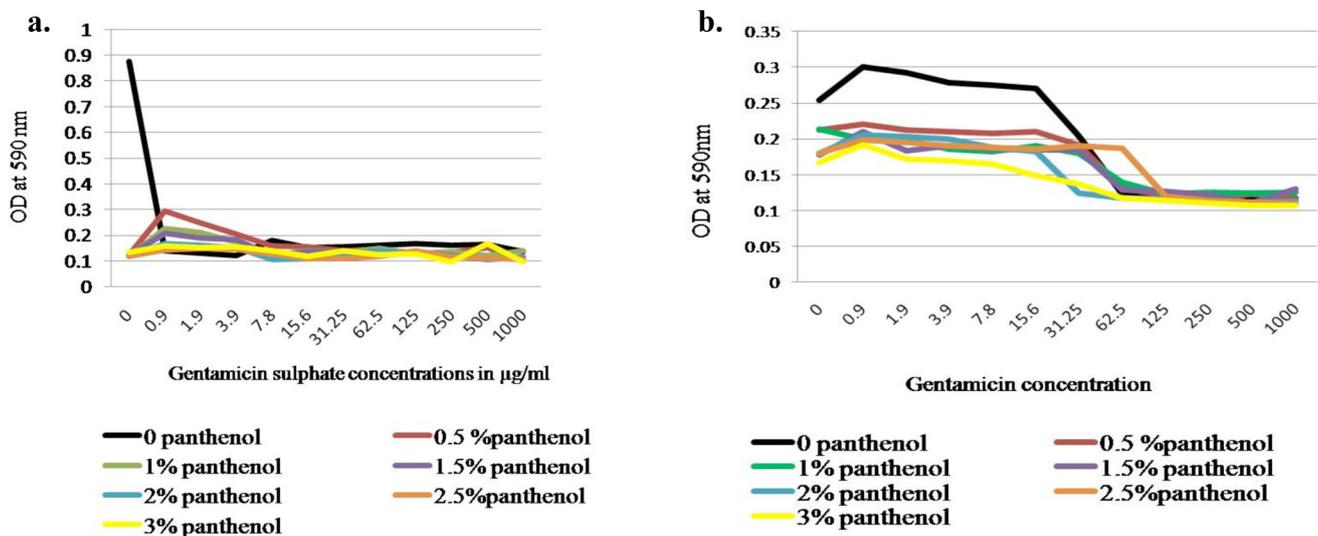
A 2 fold decrease in the MIC of gentamicin and ciprofloxacin (62.5 up to 31.25 µg/ml and 1.9 up to ≤0.9 µg/ml, respectively) started at 0.5 % dexpanthenol and remained constant for the different concentrations applied for dexpanthenol, while reached 4 fold decrease (15.625 µg/ml) at 3% dexpanthenol in case of gentamicin. Dexpanthenol at different concentrations had no effect on the MIC of colistin and oxytetracycline. An evident synergistic effect of dexpanthenol with gentamicin sulphate appeared at concentrations ranged between 1 and 3% and negligible for the other antibiotics.

### Effect of different dexpanthenol concentrations on the MIC of three topically applied antibiotics on *S. aureus* isolate N25

Two fold decrease started at 0.5% and 1% for ciprofloxacin (31.25 up to 15.625 µg/ml) and gentamicin (250 up to 125 µg/ml) respectively and reached 8 fold for ciprofloxacin (3.9 µg/ml) and 4 fold for gentamicin (62.5 µg/ml) at 3 % dexpanthenol, an abrupt decrease (32 fold) was noticed in oxytetracycline (31.25 up to 0.97 µg/ml) upon dexpanthenol addition. Maximum synergistic effect of dexpanthenol with oxytetracycline was apparent in all tested dexpanthenol concentrations. These results were found to be statistically significant by Paired T test and confidence interval (CI) = 95% using MiniTab 16 taking effect of dexpanthenol on activity of applied antibiotics (P=0.03 to < 0.3)

### Effect of presence of dexpanthenol alone and in combination with the four topically applied antibiotics on the biofilm forming capacity of the tested isolates

Regarding gentamicin sulphate, in case of *Pseudomonas* isolate N31, a great decrease in biofilm formation by gentamicin alone occurred gradually with the lowest value at 3.9 µg/ml, this decrease in biofilm was augmented by the presence of dexpanthenol at the different concentrations used. Concerning the biofilm forming capacity of isolates mixture N31, an abrupt



**FIGURE 3.** Effect of different concentrations of gentamicin sulphate alone and in combination with six dexpanthenol concentrations on the biofilm forming capacity of *P. aeruginosa* and *S. aureus* mixture N31 (a.) and *Pseudomonas* and *Staphylococcus* isolate mixture N6 (b.).

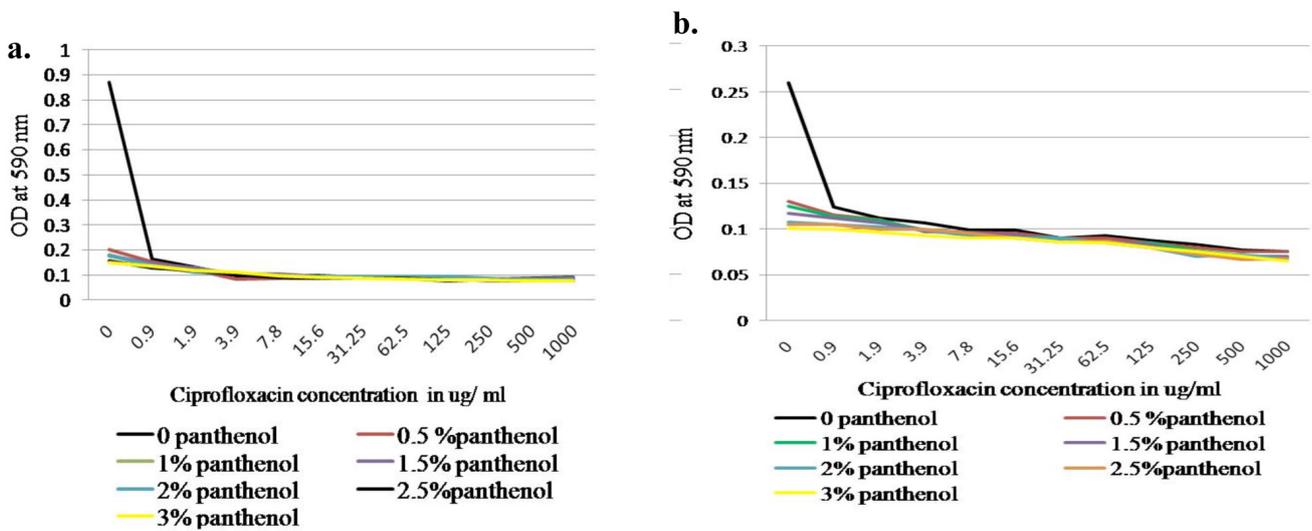
decrease in the biofilm formation was observed among the different concentrations of gentamicin alone and in combination with dexpanthenol. Dexpanthenol alone showed also a great decrease in the biofilm formation in all concentrations under test [Fig 3a.]. The same was observed for *Pseudomonas* isolate N4 and *Staphylococcus* isolate N25. In case of *Staphylococcus* isolate N6, a marked inhibition of the biofilm formation capacity was observed among the different concentrations used for dexpanthenol and gentamicin. Dexpanthenol alone was very effective at preventing the biofilm formation at all concentrations used (1-3%). On the other hand, slight decrease in the biofilm forming capacity of isolates mixture N6 was observed among the different concentrations of dexpanthenol used either alone or in combination with gentamicin reach maximum decrease at 125 µg / ml and above, while gentamicin alone at low concentrations showed an enhancement of the biofilm formed mass that started to decrease by increasing concentration [Fig 3b.].

Concerning oxytetracycline Hcl, a gradual decrease in biofilm formed mass by the *P. aeruginosa* N31 was achieved in presence of oxytetracycline; presence of dexpanthenol potentiated the antibiotic effect in prevention of biofilm formation. For isolates mixture N31, dexpanthenol led to a drastic decrease in biofilm forming capacity of the isolate under test at all its concentrations, maximum effect in combination with oxytetracycline is at 15.6 µg/ml and above. A gradual decrease in the biofilm forming capacity of the tested isolate was detected by oxytetracycline alone and weak biofilm was formed under the

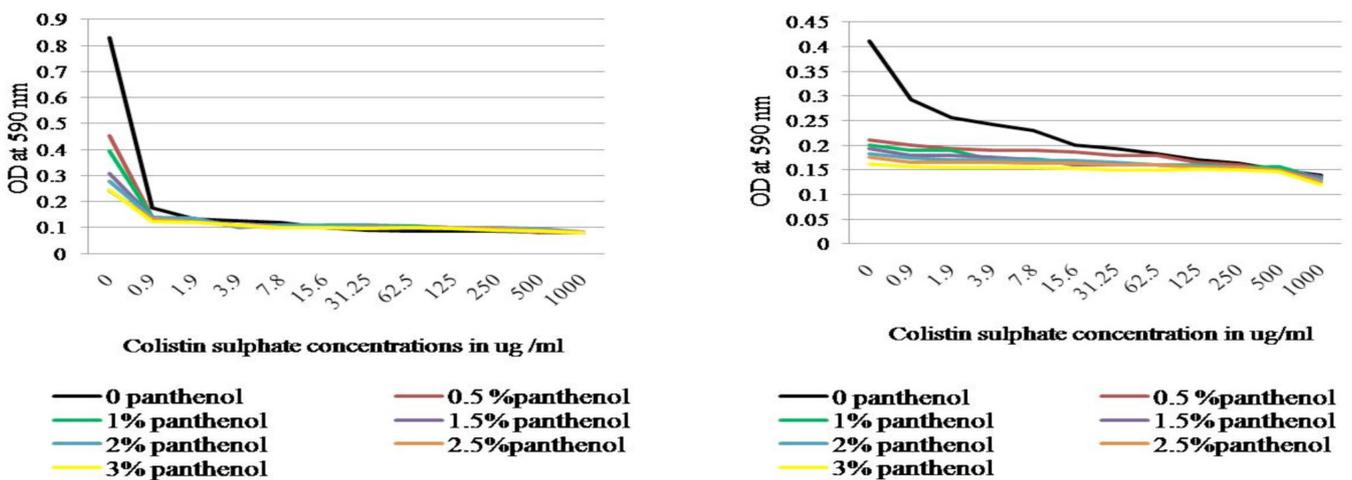
effect of presence of dexpanthenol alone and when combined with oxytetracycline in case of *S. aureus* present in mixture N6. The same was observed for the isolates mixture N6. A steep decrease in the formed biofilm mass of *Pseudomonas* isolate N4 was noticed and started from antibiotic concentration, 1.9 µg/ml, a negligible biomass is allowed to form. Furthermore, a more intensified behavior is noticed at different dexpanthenol concentrations alone and in combination with antibiotic. The same was applied for *Staphylococcus* isolate N25.

Regarding ciprofloxacin, the presence of ciprofloxacin decreased greatly biofilm formation of *P. aeruginosa* N31. Such decrease was prominent at 1.9 µg/ml and above, a gradual decrease in biofilm forming capacity by dexpanthenol alone at the different concentrations used was detected. For isolates mixture N31, steep decrease in the biofilm forming capacity by all antibiotic concentrations alone or in presence of dexpanthenol. Dexpanthenol alone caused a marked decrease in the biofilm forming capacity [Fig 4a.]. Nearly, the same pattern was observed for *S. aureus* N6, isolate mixture N6 [Fig 4b.] as well as the other isolates.

For colistin sulphate, a marked decrease in biofilm forming capacity of *P. aeruginosa* N31 was detected by all dexpanthenol concentrations alone, and in combination with colistin, while, a gradual decrease in biofilm forming capacity was noticed when colistin was applied alone. On the other hand, a marked decrease in the biofilm forming capacity of isolates mixture N31 was noted in all dexpanthenol and colistin alone or in com-



**FIGURE 4.** Effect of different concentrations of ciprofloxacin alone and in combination with six dexpanthenol concentrations on the biofilm forming capacity of *P. aeruginosa* and *S. aureus* mixture N31 (a.) and *Pseudomonas* and *Staphylococcus* isolate mixture N6 (b.).

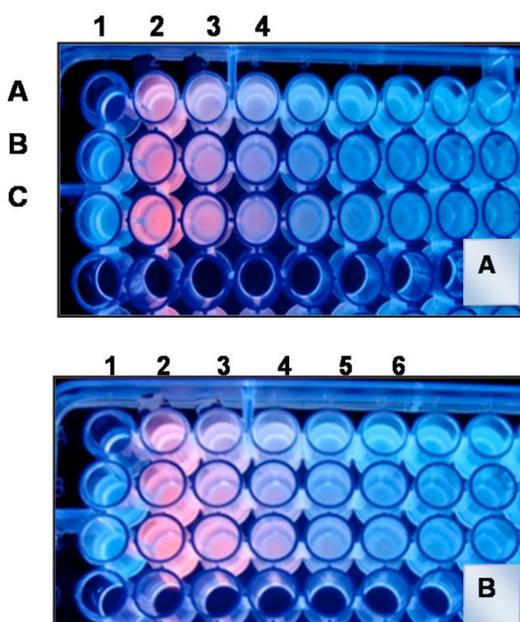


**FIGURE 5.** Effect of different concentrations of colistin alone and in combination with six dexpanthenol concentrations on the biofilm forming capacity of *Pseudomonas* isolate present in mixture N31(right) and *P. aeruginosa* and *S. aureus* mixture N31 (left).

bination [Fig 5.]. For *Pseudomonas* isolate N4, great decrease in biofilm forming capacity by dexpanthenol alone was observed that increased gradually when combined with colistin. The colistin alone resulted in abrupt decrease in biofilm forming capacity that increased gradually by increasing concentration. Regarding the effect of dexpanthenol on antibiofilm activity of the antibiotics, results were found to be statistically highly significant by Paired T test and CI =95% using MiniTab 16 especially in case of gentamicin and ciprofloxacin ( $P=0.001$  to  $< 0.09$ ).

### Determination of efflux inhibition activity of dexpanthenol using ethidium bromide

A simple qualitative method was applied just to shed light on one of the proposed mechanisms for the dexpanthenol being able to convey synergistic effect with the tested antibiotics despite being without an antimicrobial activity of its own in the concentrations tested in the current study. For *P. aeruginosa* present in isolate mixture N31, an increase in the fluorescence intensity is observed in wells B2 (0.5% dexpanthenol) and C2 (3% dexpanthenol) as compare to A2 (No dexpanthenol). From left to right in rows B and C, the fluorescence emitted by the bacteria grown in decreasing concentration of EB is up to well B4 (1 $\mu$ g/ml) and C5 (0.5 $\mu$ g/ml) while in absence of dexpanthenol is up to A3 (2  $\mu$ g/ml) [Fig 6A.]. For *P. aeruginosa* present in isolate mixture N6, in rows B and C, the fluorescence emitted by the bacteria grown in decreasing concentration of EB is up to well B4 (1 $\mu$ g/ml) and C4 (1 $\mu$ g/ml), while in absence of dexpanthenol is up to A3 (2 $\mu$ g/ml) [Fig 6B.].



**FIGURE 6.** Fluorescence of *P. aeruginosa* present in isolate mixture N31(A) and N6 (B) in ethidium bromide containing media at 0.5 % and 3 % dexpanthenol concentrations.



**FIGURE 7.** EEP antibacterial activity against *S. aureus* (A) and *P. aeruginosa* (B) isolates of N31 mixture. Inhibition zones developed around the wells containing EEP in different dilutions (10, 5, 2.5, 1.25 and 0.625%).

### Propolis antimicrobial activity

EEP showed antibacterial activity against *S. aureus* and *P. aeruginosa* isolates of N31 mixture [Fig.7].

### Determination of the MIC of EEP by agar dilution technique

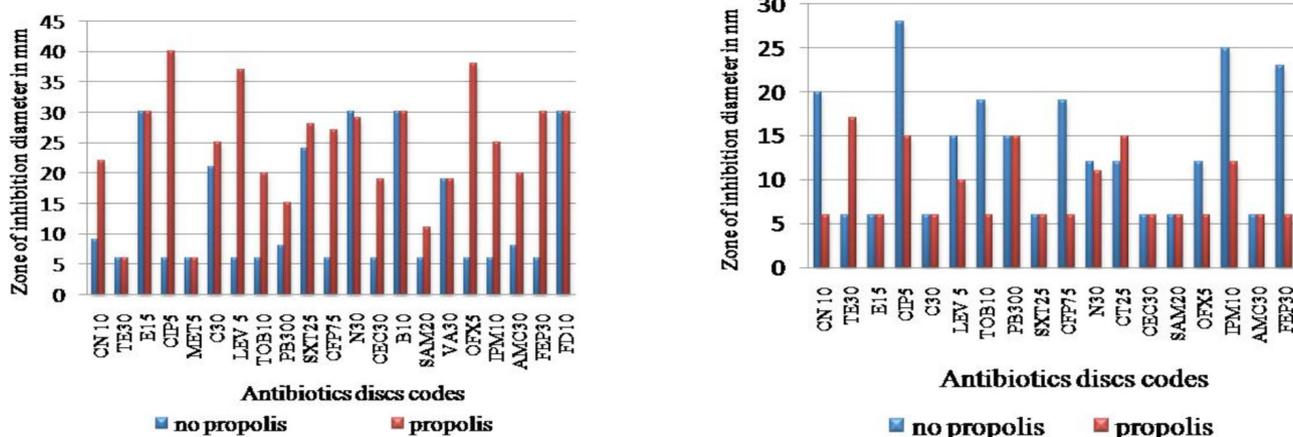
*Pseudomonas* isolate in mixture N31 demonstrated the highest degree of resistance against EEP (MIC = 5000  $\mu$ g/ml) while *Pseudomonas* N4 was the least resistant among the selected isolates (MIC = 625  $\mu$ g/ml). The other MIC values for the remaining isolates were: 2500  $\mu$ g/ml for *Pseudomonas* isolate N6 and *Staphylococcus* isolates N31 and N25 and 1250  $\mu$ g/ml for *Staphylococcus* isolate N6.

### Effect of EEP on the different antibiotic sensitivity pattern of the selected isolates

The effect of sub-inhibitory concentration (0.25MIC) of EEP on the zone of inhibition diameter of tested antibiotics applied against the selected isolates revealed a synergistic effect for most of antibiotics applied in case of *S. aureus* isolate N31. The organism which was resistant to fluoroquinolones, cephalosporins and imipenem became sensitive to antibiotics in presence of EEP compared to the tested antibiotics alone. For *P. aeruginosa* isolate N31, no effect was detected upon testing with the applied antibiotics [Fig.8]. The same was observed for the other selected isolates.

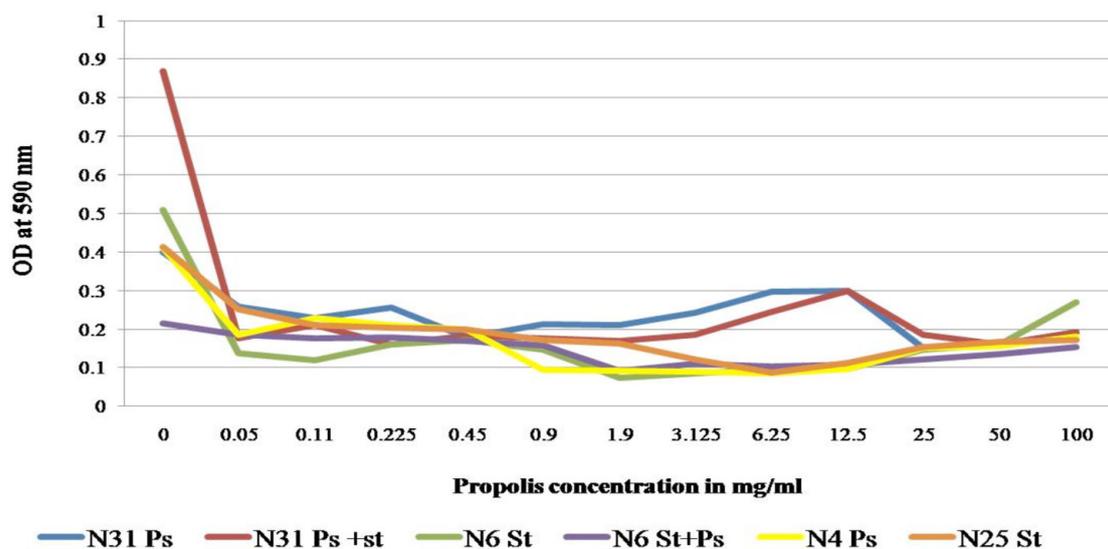
### Effect of EEP on the capacity of biofilm formation by the selected isolates

An abrupt decrease in the biofilm formation was observed for all isolates and mixtures being more markedly observed in case of isolates mixture N31 [Fig.9].



CN, gentamicin; TE, Tetracycline; E, Erythromycin; CIP, Ciprofloxacin; MET, Methicillin; C, Chloramphenicol; LEV, Levofloxacin; TOB, Tobramycin; PB, PolymixinB; SXT, Sulphamethoxazole/ Trimethoprim; CFP, Cefoperazone; N, Neomycin; CEC, Cefaclor; B, Bacitacin; SAM, Ampicillin /Sulbactam; VA, Vancomycin; OFX, Ofloxacin; IPM, Imipenem; AMC, Amoxicillin /Clavulanic acid; FEP, Cefipime; FD, Fucidic acid.

**FIGURE 8.** Effect of sub-inhibitory concentration (0.25 MIC) of EEP on the zone of inhibition diameter of some antibiotics applied against *S. aureus* isolate N31 (left) and *P. aeruginosa* isolate N31 (right).



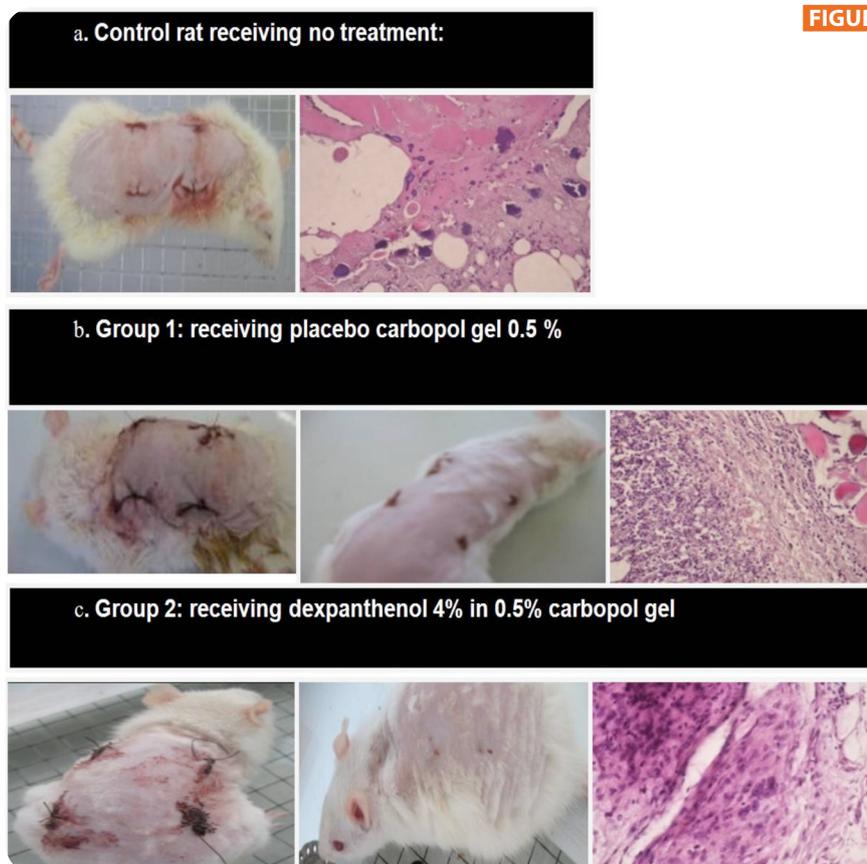
**FIGURE 9** Inhibition of biofilm-forming capacity of the selected isolates at different EEP concentrations.

### In vivo application of different gel formulation on induced wound infections in albino rat models

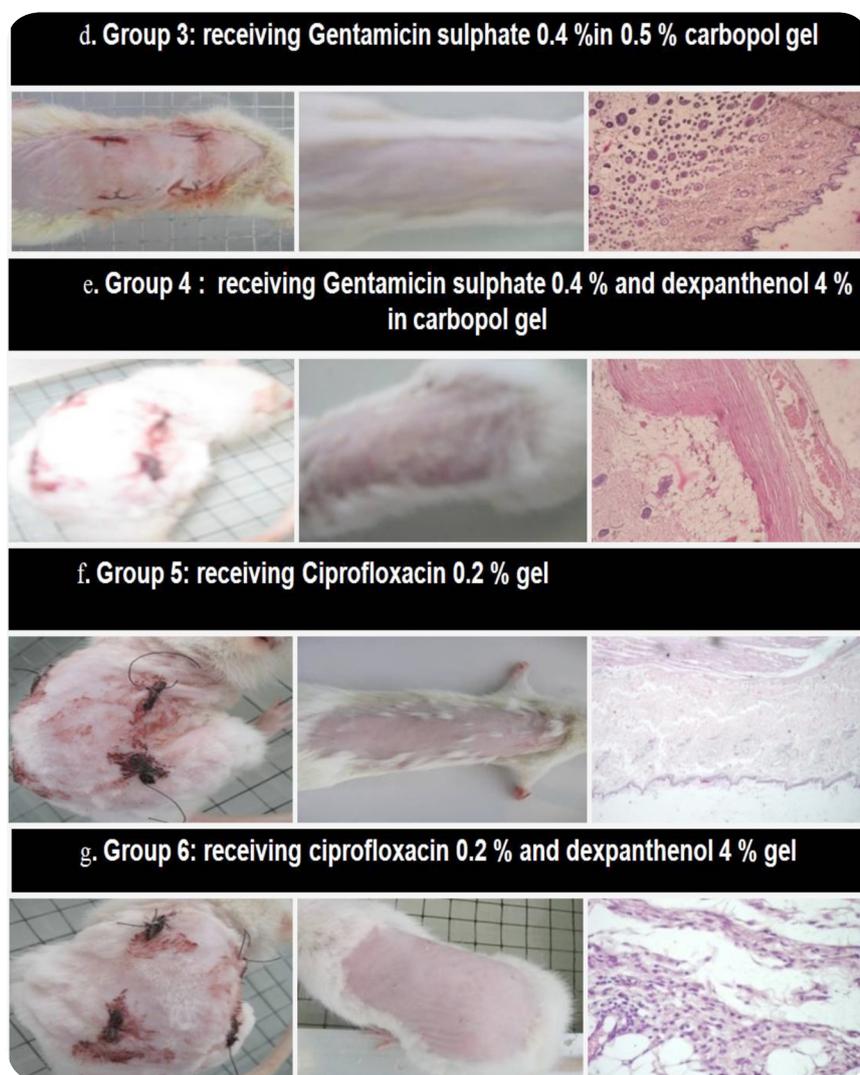
**Fig.10** illustrates the images as well as the histopathological sections of representative rats, infected with *P. aeruginosa* and *S. aureus* N31 culture mates; one day after infection and when healing occurred upon using different treatment regimen. **Fig.10 a.** illustrates the control rat received no treatment showing no healing. Histopathological examination of the wound section (H&E, X400) reveals infected subcutaneous tissue with aggregates of basophilic grape-like clusters of *S. aureus*, and few rods of *P. aeruginosa*. The bacterial colonies are surrounded by prominent capillary proliferation with fibroblasts and infiltrated by neutrophils with cuffing of neutrophils around the capillaries (septic granulation tissue) associated with marked congestion. Degenerative muscular changes and fat necrosis are also noticed (hyalinization). **Fig.10b.** demonstrates group (1) rat received placebo carbopol gel 0.5% showing incomplete healing with scar formation. Histopathological examination (H&E, X200) shows that infection extends from the epidermis with gaping (abscess formation) and through the dermis to subcutaneous tissue. The inflammatory infiltrate is made up

mainly of sheets of polymorphonuclear leukocytes and foamy histiocytes. Markedly congested proliferating capillaries are seen with hemorrhage and extension of inflammation to the skeletal muscle fibers. **Fig. 10c.** demonstrates rat from group (2) treated with dexpanthenol 4% in 0.5% carbopol gel showing partial healing with scar formation. Histopathological examination (H&E, X400) shows granuloma formation made up mainly of collection of epithelioid histiocytes admixed with few neutrophils, lymphocytes and foamy histiocytes. The dermis and subcutaneous tissue show frequent mast cells. Prominent capillary proliferation is noted both in dermis and subcutaneous tissue.

**Fig. 10d.** demonstrates a rat from group (3) treated by gentamicin 0.4 % in 0.5 % carbopol gel. A complete healing without scar was noticed. Histopathological examination (H&E, X200), showing a section involving the epidermis and the upper dermis, with complete resolution of the inflammation. **Fig.10e.** demonstrates a rat from group (4) treated with gentamicin-dexpanthenol gel; complete healing without scar was noticed. However, the application of dexpanthenol with gentamicin shortened the time taken for the wound to heal completely



**FIGURE 10** (a-c) **a. Control group:** Dorsal aspect of albino rat model with 4 incisions infected with *P. aeruginosa* and *S. aureus* mixture isolates N31 (left). Histopathological examination of the wound section (H&E, X400) shows aggregates of basophilic grape-like clusters of *S. aureus*, and few rods of *P. aeruginosa* surrounded by prominent capillary proliferation with fibroblasts and infiltrated by neutrophils (right). **b. Group 1:** Dorsal aspect of albino rat model with 4 incisions infected with isolate mixture N31 at day 1 post infection (left). Dorsal aspect of the albino rat model at day 8 after treatment with placebo carbopol 0.5 % gel show incomplete healing (Middle). Histopathological examination of skin (H&E, X200) at day 8 shows the inflammatory infiltrate made up mainly of sheets of polymorphonuclear leukocytes and foamy histiocytes with markedly congested proliferating capillaries (right). **c. Group 2:** Dorsal aspect of albino rat model with 4 incisions infected with isolate mixture N31 at day 1 post infection (left). Dorsal aspect of the albino rat model after 8 days treatment with dexpanthenol 4 % in 0.5 % carbopol gel base shows partial healing (Middle). Histopathological examination at day 8 of treatment (H&E, X400) shows granuloma formation (right).



**FIGURE 10** (d-g) **d. Group3:** Dorsal aspect of albino rat model with 4 incisions infected with isolate mixture N31 at day 1 post infection (*left*). Dorsal aspect of the albino rat model at day 8 of treatment with gentamicin 0.4 % gel shows complete healing (*Middle*). Histopathological examination (H&E, X200) at day 8 treatment shows complete resolution of the inflammation (*right*). **e. Group4:** Dorsal aspect of albino rat model with 4 incisions infected with isolate mixture N31 at day 1 post infection (*left*). Dorsal aspect of the albino rat model at day 6 of treatment shows complete healing (*Middle*). Histopathological examination at day 6 treatment shows intact subcutaneous tissue and skeletal muscles with no sign of inflammation or infection (*right*). **f. Group5:** Dorsal aspect of albino rat model with 4 incisions infected with isolate mixture N31 at day 1 post infection (*left*). Dorsal aspect of the albino rat model at day 8 of treatment treated with ciprofloxacin 0.2 % in carbopol gel shows complete healing (*Middle*). Histopathological examination at day 8 of treatment shows no evidence of inflammation (*right*). **g. Group6:** dorsal aspect of albino rat model with 4 incisions infected with isolate mixture N 31 at day 1 post infection (*left*). Dorsal aspect of the albino rat model at day 6 of treatment with ciprofloxacin 0.2% and dexpanthenol 4% in carbopol gel shows complete scarless healing (*Middle*). Histopathological examination at day 6 of treatment demonstrates mild chronic mononuclear inflammatory infiltrate (*right*).

without scar as histopathological examination at day 6 treatment showed intact subcutaneous tissue and skeletal muscles with no sign of inflammation or infection. There is mild capillary congestion in subcutaneous tissue. **Fig. 10f.** demonstrates a rat from group (5) treated with ciprofloxacin 0.2% in carbopol gel; a complete healing without scar was noticed. Histopathological examination shows intact epidermis, dermis & skeletal muscles with no evidence of inflammation. Fig.10g. demonstrating a rat from group (6) treated with ciprofloxacin 0.2 % and dexpanthenol 4 % in carbopol gel; complete scarless healing was noticed. However, the application of dexpanthenol with ciprofloxacin shortened the time taken for the wound to heal completely with no scar. Histopathological examination (H&E, X 400) demonstrates subcutaneous tissue showing mild chronic mononuclear inflammatory infiltrate.

## Discussion

In order for wounds to heal normally, specific issues must be addressed such as aggressive debridement that allows the wound to move more rapidly from inflammation to proliferation minimizing the risk of infection. However, a regimen of topical antimicrobial agents should be considered to reduce an unacceptable level of bacteria and other pathogens. Adjunct microbial chemotherapy was also a field of investigation [24].

In the current work, the antimicrobial activity of dexpanthenol that was assessed against the selected isolates and mixtures revealed that dexpanthenol tested concentration has no antimicrobial activity. A previous study [25] confirmed the bacteriostatic properties of the dexpanthenol at MIC amounting to

over 10% which is very high. In the present study, the effect of different dexpanthenol concentrations was tested on the MIC of four selected topically applied antibiotics including colistin sulphate. Colistin sulphate was chosen as an old antibiotic coming into action [16]. It has attracted more interest recently because of its significant activity that proved encouraging in comparison with previous experience [26].

In the present study, variable synergistic effects appeared as gradual or abrupt decrease in the MIC of the challenged antibiotics against the selected isolates at different dexpanthenol concentrations was noticed. This could be explained as pantothenic acid is essential for the growth of pathogenic microorganisms. Dexpanthenol is one of the analogues of pantothenic acid that compete with pantothenate. Dexpanthenol could be up-taken by bacteria instead of pantothenate leading to inhibition of fatty acid synthesis essential for cell wall lipid manufacturing resulting in a weak cell wall that increases passage of antibiotics. Spry et al. [27] stated that this could represent a potential target for the development of novel antibacterial therapeutics.

Currently, by comparing the MIC values of gentamicin against *Pseudomonas* N31 with that of the isolates mixture N31, a 4 fold increase of the MIC was observed. Such interesting result could be explained by the production of aminoglycoside acetyltransferase by *Staphylococcus* culture mate as stated by Martel et al. [28] leading to development of resistance to aminoglycosides. Therefore, the production of aminoglycosides modifying enzymes by *Staphylococcus* protected both *Staphylococcus* and *Pseudomonas* isolates from the effect of gentamicin.

Furthermore, in the present work, testing the prevention of biofilm formation using the selected antibiotics either alone or in combination with the six dexpanthenol concentrations revealed that gentamicin alone showed a great decrease in biofilm formation of *Pseudomonas* isolate N31 at 0.9 µg/ml, while original MIC was 250 µg/ml, meaning that in a range of sub-inhibitory concentration, the drug was able to prevent the adhesion of *Pseudomonas* single isolate and mixture N31 and therefore counteracting their pathogenicity. Similar finding reported for *Staphylococcus* isolate and mixture N6. The reduction in biofilm formation was also noticed when the other selected antibiotics were assayed against the selected isolates and mixtures. Inhibition of biofilm formation at sub-inhibitory concentrations of antimicrobial agents was reported previously [29-33].

In the present study, dexpanthenol by its own decreased greatly the adhesion of isolates and mixtures under test and assisted the antibiotic in its action in prevention of adhesion of the tested isolates. This capacity is thought to be due to the importance of pantothenic acid as a vitamin in the growth media in the procedure of biofilm formation and production of exopolysaccharides. As a result to structure similarity between dexpanthenol and pantothenic acid, the bacteria are tricked and the reaction stops and a decrease in the biofilm mass is the result as stated by Grobbsen et al. [34].

Biofilm-dwelling bacteria are particularly resistant to antibiotics, making it hard to eradicate. Bacteria rely on efflux pumps to get rid of toxic substances. Kvist et al. [35] discovered that efflux pumps are highly active in bacterial biofilms, thus making efflux pumps attractive targets for antibiofilm measures. Since many types of antibiotic resistance in bacteria are known to be conferred by efflux pumps, it is not surprising that enhanced efflux pump activity occurs concomitantly with enhanced antibiotic resistance. In line with this reasoning, interference with efflux pump activity during biofilm growth should be predicted to impact the biofilm-forming capacity. Werle [36] considered acyclic amines including dexpanthenol are drug efflux inhibitors. The production of *N*-acyl homoserine lactone- one of the signaling molecules mediate the biofilm formation- in medium is assisted by efflux pump in *P. aeruginosa*. Thus a decrease in biofilm formed mass and consequently reduction of invasiveness of the organism is associated with efflux pump inhibition [37]. From the present work, dexpanthenol could have mild efflux inhibition activity in case of *Pseudomonas* isolate N31 and to a lesser extent in case of *Pseudomonas* isolate N6. Therefore, it could affect the ability of the organism to extrude both the antibiotics and the *N*-acyl homoserine lactone, thus disturbing its ability to form biofilm. Interestingly, *Pseudomonas* isolate N6 produced a slightly higher *N*-acyl homoserine lactone compared to *Pseudomonas* isolate N31. Therefore, efflux pump inhibition could be a possible mechanism that needs further investigation putting into consideration the role played by dexpanthenol in weakening the cell wall.

Propolis or Bee glue is a newly introduced old remedy for treatment of wound and proved to have antibacterial action [10]. In the Egyptian market, propolis is stuck in its role as a dietary supplement; however, we tried here to shed light on its antimicrobial anti-biofilm efficacy. In present study, propolis demonstrates a slightly higher antibacterial action against *Staphylococcus* (Gram positive) than *Pseudomonas* (Gram negative) isolates. Our results were in accordance with previous reports [38, 39]. Mechanisms of activity of propolis against microorganisms are still controversial. Some components present in propolis extracts like flavonoids and caffeic acid, benzoic acid, cinnamic acid, probably act on cell wall, causing functional and structural damages [40]. In the present work, EEP in sub-inhibitory concentration showed synergism with some antibiotics such as those interfere with bacterial protein synthesis and antagonism with others and this finding was in accordance with previous studies [22, 41]. Oner et al. [12] confirm the safety and efficacy of intraarticular propolis and synergistic effect of propolis when used with cefazolin in an experimental septic arthritis model. Moreover, Arslan et al. [13] reported that Propolis was found to be effective on *Candida albicans* and *Enterococcus faecalis*, being more effective in lower concentrations on *C. albicans* than on *E. faecalis*. In the current study, EEP at low concentrations (0.05mg/ml) showed a drastic decrease in biofilm formation by *Pseudomonas* and *Staphylococcus* isolates and mixtures under test. Similar effect was detected by Scazzocchio et al. [42], as well; Koudhi et al. [11] found that EEP possessed excellent protective effects against biofilms activity of oral streptococci.

Since the reduction of wound bioburden should reduce adverse influences that impede healing. Therefore, *in vivo* studies help clarify the role of bacterial biofilms in wound infection and healing. It also provides a tool to study antimicrobial efficacy of topical agents in the complex wound environment. In the present work, six groups, each of five rats were assayed using five different topical formulations and control placebo gel. Healing was considered achieved when scar-less complete healing of the induced infected wound occurred. Histopathological examination of the untreated wound section revealed infected subcutaneous tissue with aggregates of basophilic grape like clusters of *S. aureus* and few rods of *Pseudomonas* surrounded by prominent capillary proliferation; such effect matches to the colonization step cited by Kennedy et al. [43]. When carbopol gel alone was applied as placebo control, after 8 days, histopathology revealed extended infection through the skin layers with inflammation and congestion and these finding was matching with Smith et al. [44] who tested the role of acyl homoserine lactones (AHLs) *in vivo* as after injection into the skin of mice, they found a significant influx of white blood cells and subsequent tissue destruction. Therefore, the quorum-sensing systems of *P. aeruginosa* contribute to its pathogenesis both by regulating expression of virulence factors and by inducing inflammation. The marked congestion revealed histopathologically in non treated rats and those treated with only dexpanthenol gel is matching the inflammatory response induced by the presence of AHL as reported by Lawrence et al. [45]. Yet, application of dexpanthenol with either gentamicin or ciprofloxacin shortened the time taken for the wound to heal completely without scar formation.

## Conclusion

Consequently, we can conclude that, antibacterial action of the topically applied antibiotics is improved greatly in presence of dexpanthenol. It could be formulated in the same vehicle and administered locally to assist in prevention of wound infections putting into consideration the dexpanthenol antibiofilm activity, favoring healing in a short time. However, further investigations is needed about the mechanisms by which dexpanthenol could prevent biofilm formation. Propolis extract proved here its effective *in vitro* antimicrobial- antibiofilm activities; however, further *in vivo* study for propolis alone and in combination with antibiotics is essential as it could be of benefit in local wound management. Therefore, dexpanthenol and propolis alone or in combination with topically applied antibiotics proved effective in prevention of biofilm formation and hence could have a great impact in wound management.

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