

# Effects of Selected Wound Dressings and Remedies on Planktonic Bacteria and Bacterial Biofilms of *Staphylococcus aureus* and *Pseudomonas aeruginosa*

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**Abstract:** The overall aim of this research is to evaluate the effect of some approaches of treating wounds and the use of wound dressings of on the planktonic bacteria and bacterial biofilms of *Staphylococcus aureus* and *Pseudomonas aeruginosa*, two major bacterial species found in wounds. The wound treatments were dressings containing either honey or silver, and commercial hand gels with alcohol. Results indicate that dressings that contain Manuka honey were efficacious against planktonic bacteria of *S. aureus*, whereas, dressings with silver were most effective against planktonic *Pseudomonas aeruginosa*. Panaderm (antibiotic) showed the strongest activity against both planktonic *S. aureus* and *P. aeruginosa*. The effect of wound treatments on non-established and established bacterial biofilms after 24 and 48 hours after application, show that dressings were effective against the biofilm of *S. aureus*. However, a non-adherent dressing with Activon Manuka Honey was ineffective against *Pseudomonas aeruginosa* biofilm. Overall, except for hand gels, the wound remedies tested show remarkable activity against the biofilms of *S. aureus* and *P. aeruginosa*.

## Introduction

Wounds arise when the skin suffers internal or external injuries. External injuries involve cutting or piercing the skin, which causes an open wound. Internal injuries are caused by contusion of the skin that generates a closed wound. Wounds provide bacteria with an opportunity to invade the body, although wound entry can be managed through methods such as cleaning, closure, and application of dressing that contain antibiotics. Antibiotics can fail in most cases in chronic wounds with the formation of bacterial biofilm that are very hard to eliminate and expensive to treat (Bjarnsholt *et al.*, 2008, Davis *et al.*, 2008, James *et al.*, 2008).

Dressings provide good healing and dry conditions where wounds are protected from contamination, in addition to the antimicrobial activity added by the dressing (Benbow, 2005).

The effects of silver dressing against pathogenic bacteria differ from that of antibiotics. Silver

dressings act on the respiratory system of the cell, transmembrane electrolyte transport, cell membrane integrity, enzyme activities and cell proliferation (Lansdown, 2002). In addition, silver dressings lessen the ability of bacteria to adhere to surfaces (Chaw *et al.*, 2005, Klueh *et al.*, 2000), and acts against the formation of bacterial biofilm (Percival *et al.*, 2007).

Manuka honey is widely used in wound dressing. Manuka honey has shown marked activity on wound because of its osmolarity, acidity, and content of hydrogen peroxide content, flavonoids, phenolic acid compounds, and methylglyoxal, a unique compound (Moore *et al.*, 2001, Gethin *et al.*, 2008, Kwakman *et al.*, 2010, Escriche *et al.*, 2013, Kwakman and Zaat, 2012).

Treatment with honey was used successfully in wounds, ulcers, abdominal pain and burns (Sharp, 2009, Topham, 2002). It was reported that Medihoney™ dressing succeeded in curing some

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chronic wounds after drug therapy failure after 12 weeks (Dunford and Hanano, 2004).

In this study, the effect of different wound dressings and wound remedies on planktonic bacteria and bacterial biofilms was studied.

### Materials and Methods

Two types of the most common causing wound infecting bacteria were used in the tests, namely *Staphylococcus aureus* SH1000 and *Pseudomonas aeruginosa* PA01. The bacteria were grown in five different media. Biofilms of *S. aureus* were grown on brain and heart infusion agar (BHI), while biofilms of *P. aeruginosa* were grown on agar base ISO 13720 (ISO). Nutrient agar (NA) was used for bacterial plate counts, while nutrient broth and Muller Hinton Agar (MHA) were employed for the sensitivity tests.

### Effects of dressings and creams on *S. aureus* and *P. aeruginosa* development

Four types of wound dressings were tested on planktonic bacteria as follows: (1) Askina® Calgitrol® Ag, (2) Actilite® (a non-adherent dressing containing Activon Manuka honey), (3) Algivon® alginate containing active Manuka honey, and (4) Algivon® alginate dressing impregnated with 100% Manuka honey. The dressings were cut into 12 mm pieces and kept in sterile, empty Petri dishes until use. Muller Hinton agar plates were inoculated with bacterial broth and incubated at 37°C for 30 minutes. The dressings were then arranged equidistantly on the plate and incubated at 37°C for 24 hours.

Three types of wound creams, Panaderm®, Activon® Manuka honey and Flamazine, and a hand sterilizer (with isopropanol) were tested for their antibacterial properties. Panaderm cream consists of three types of antibiotics (neomycin sulphate 2.5 mg, Nystatin 100.000 units and gramicidin 0.25 mg), while Flamazine contains silver as active ingredient. The creams and the hand gel were dispensed in 4 wells on the MHA plates previously inoculated with bacterial suspension. The plates were incubated for 24 hours at 37 °C.

The activities of the dressings Askina® Calgitrol® Ag, Actilite® and Algivon® impregnated with 100% Manuka honey were tested against biofilm formation, and established biofilm 24 and 48 hours after dressing application.

### Colony biofilm assay

Nitrocellulose membranes of 25 mm diameter (Millipore) were used to create the biofilm. Development of biofilms as described in Protocol 3 described by Merritt et al., (2005) was followed.

Membranes were soaked into phosphate buffer solution for 24 hours and then transferred into adjusted bacterial suspension media and left for 15 minutes. Using sterile forceps, membranes were placed onto the surface of nutrient agar plates and incubated side down at 37 °C for 48 hours, transferred to new plates and incubated for additional 48 hours incubation. After 96 hours, three membranes were removed from the media, and washed in sterile phosphate buffer to remove all planktonic cells. Washed membranes were transferred separately into 10 ml tubes with sterile phosphate buffer solution, and then transferred to a sonicator bath for 1 hour to release all viable cells. The tubes were vortexed for 30 seconds to homogenize the suspension. One ml of the suspension was transferred to 9 ml phosphate buffer to make dilutions of 10<sup>-1</sup>; serial dilutions were then made up to 10<sup>-6</sup>. Aliquots (100 µl) were taken from all tubes and spread on nutrient agar plate by use of a spreader and incubated for 24 hours to obtain the colony forming unit count (CFU). All counts are given as the mean of triplicates.

Remaining membranes from the agar plates were removed after incubation for 96 hours, and then washed with sterile phosphate buffer saline solution. Washed membranes were then distributed equidistant from each other onto new agar plates. Wound dressings were cut into squares and placed on the plates with the membranes. The plate thus contained four membranes with three pieces of the same dressings as follows:

1. Negative control - membrane soaked in phosphate buffer without bacteria
2. Non-established biofilm - membrane neutralized in phosphate buffer for 24 hours then soaked in bacterial suspension for 15 minutes
3. Established biofilm- membrane with established biofilm, and tested after 24 hours
4. Established biofilm- membrane with established biofilm and tested after 48 hours

All the determinations were done in triplicate.

Twenty-four hours after incubation, all dressings were removed except one which was examined after 48 hours. After taking out the membranes from the media, these were washed in sterile phosphate buffer to remove all planktonic cells, and then transferred into 10 ml separate tubes of sterile phosphate buffer solution, and then transferred to a sonicator bath for 1 hour to release all viable cells. Tubes were vortexed for 30 seconds to homogenize the suspension. One ml was then transferred to 9 ml phosphate buffer to make a dilution of 10<sup>-1</sup>, serial dilutions were then

made up to  $10^{-6}$ . An aliquot (100  $\mu$ l) was taken from all tubes and spread on nutrient agar plate and incubated for 24 hours to obtain the colony forming unit (CFU) of the main tubes. All counts are the means of triplicates. The same procedure was performed on the dressings sampled after 48 hours.

#### **Determination of the effect of wound creams on bacterial biofilms**

Four wound treatments, Panaderm<sup>®</sup> cream (effective against Gram negative and Gram positive bacteria as well as fungi), Flamazine<sup>®</sup> cream (silver as active component), Activon<sup>®</sup> cream (active component is Manuka honey), and a hand sterilizing agent. The minimum biofilm eradication concentration (MBEC) was determined using the MBEC<sup>™</sup> High-Throughput (HTP) Assay (Innovotech, Canada). In summary, more than 7 colonies of the same morphological appearance of a fresh subculture of the test bacteria were picked off with a sterile loop and dipped into 1.5 ml sterile nutrient broth. One ml of prepared inocula was transferred into 29 ml of nutrient broth. This 30-fold dilution of the 1.0 McFarland standard serves as the inocula for the MBEC plate. A new MBEC plate was opened and the first row and the second row of pegs were removed by using sterile metal pliers to use these rows as negative controls afterward. Twenty-two ml of the previous prepared fold dilution was added to the MBEC plate and incubated in 37°C for 48 hours in the case of *S. aureus* and 96 hours for *P. aeruginosa* (Wirtanen et al., 2001).

Concentrations of the treatment were prepared previously in sterile tubes and then transferred to wells depending on the used treatment. The vertical well line was used for one type of honey from the third row to the eleventh row. The test for each type of honey was done in duplicate. The twelfth row used as positive control. A sterile microtiter plate with 200  $\mu$ l of physiological saline was setup in every well. This plate was used to rinse the pegs to remove loosely adherent planktonic cells from the biofilm.

After the incubation period of the MBEC plate, the peg lid from the trough was removed and the pegs

submerged in the wells of the rinse plate. The peg lid was let for 1 to 2 minutes. This step was repeated in a new rinse plate. After washing, the peg lid of the MBEC was inserted into the challenge plate properly and incubated at 37°C for 24 hours. After the incubation period, the peg lid of the MBEC plate was washed twice using the same mentioned protocol in two different rinse plates. A 96-well plate was prepared by adding 200  $\mu$ l of nutrient broth in all wells and this was the recovery plate. The washed MBEC plate was transferred to the recovery plate and closed tightly to prevent any possibility of the contamination. The plate was transferred onto the tray of the sonicator. The plate was left for 1 hour to allow the vibrations to disrupt the biofilms from the surface of the remaining pegs into the recovery plate. The plate was then incubated for 24 hours and checked for the visible growth.

The horizontal wells were used for one type of treatment from the ninth column to the twelfth column. The test for each type of treatment was done in the whole column. The fifth and sixth columns were removed by using sterile metal pliers to use these rows as negative controls afterward whereas the first, second and third columns were used as positive controls.

#### **Results and Discussion**

##### **a. Effects of some wound dressing on planktonic bacteria of *Staphylococcus aureus* and *Pseudomonas aeruginosa***

When *Staphylococcus aureus* bacteria was tested against dressings impregnated with honey, they showed greater activity compared with that of silver dressing which agrees with the findings of Kostenko *et al.* (2010) about the diversity of effectiveness of dressings that associated with dressing base material. The activity of Actilite was highest, followed by Algivon with active Manuka honey and then Algivon with 100% Manuka honey (Figure 1). However, silver dressing was more effective in inhibiting the growth of *Pseudomonas aeruginosa* compared with Manuka honey (Figure 2).

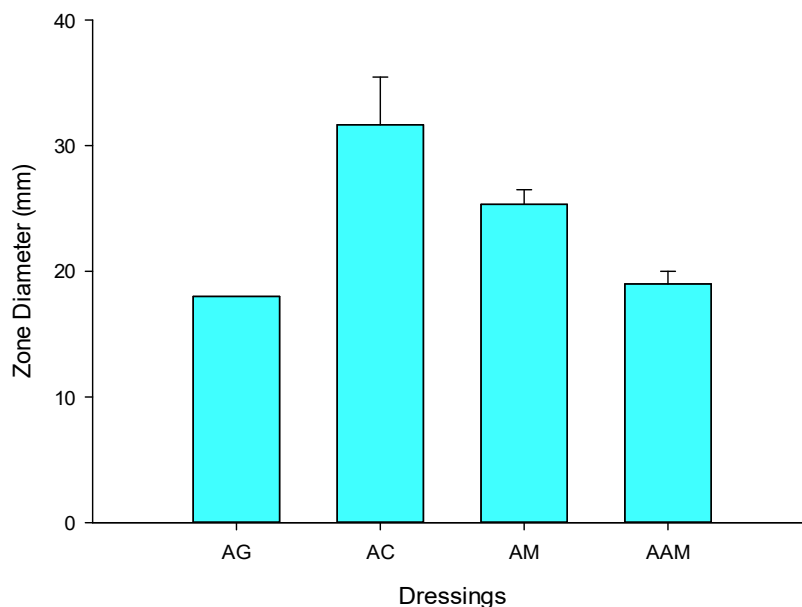


Figure 1. **Effect of wound dressings on the growth of *Staphylococcus aureus*.** Ag-Askina Calgitrol Ag; AC- Actilite; AM- Algivon (alginate containing Activon Manuka honey); AAM -Algivon (alginate dressing impregnated with 100% Manuka honey).

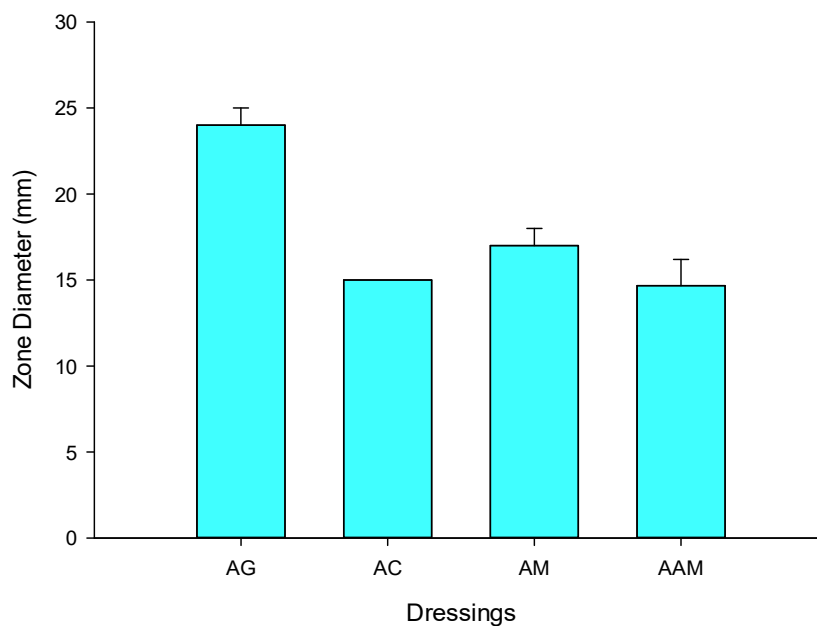


Figure 2. **Effect of wound dressings on the growth of *Pseudomonas aeruginosa*.** Ag-Askina Calgitrol Ag; AC- Actilite; AM- Algivon (alginate containing Activon Manuka honey); AAM -Algivon (alginate dressing impregnated with 100% Manuka honey).

**b. Effects of some wound remedies on planktonic bacteria of *Staphylococcus aureus* and *Pseudomonas aeruginosa***

Some wound treatments including antibiotics, honey, silver and hand gel were tested on the viable cells of *S. aureus* and *Ps. aeruginosa*. Panaderm was the most

effective one against *S. aureus* and *P. aeruginosa*. Activon Manuka showed remarkable activity against *S. aureus* and intermediate activity against *P. aeruginosa*. Flamazine exhibited moderate activity against *S. aureus* and *P. aeruginosa*. The hand gel showed the least activity on both types of bacteria (Figures 3 and 4).

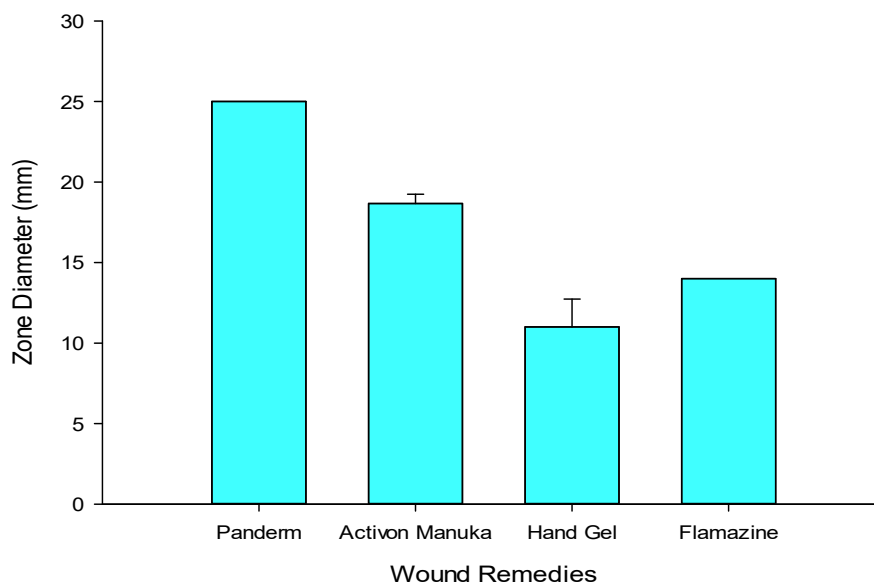


Figure 3. The effect of wound treatments on the planktonic *Staphylococcus aureus*.

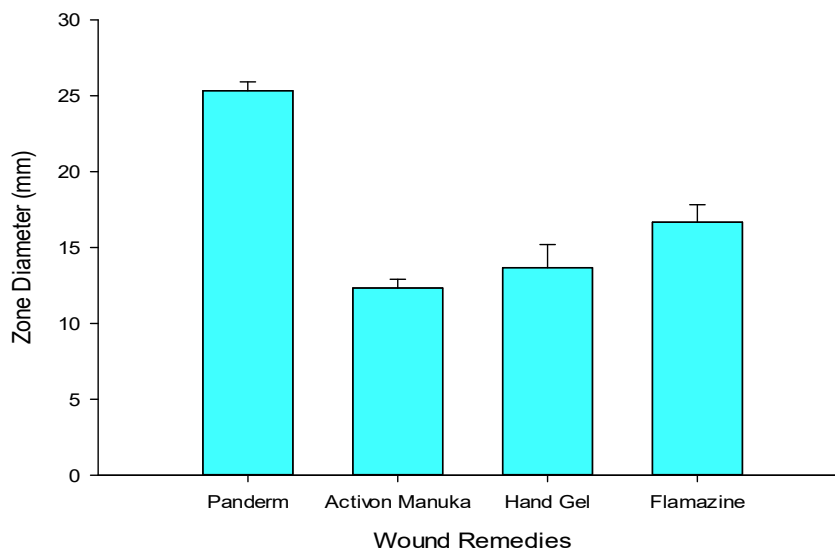


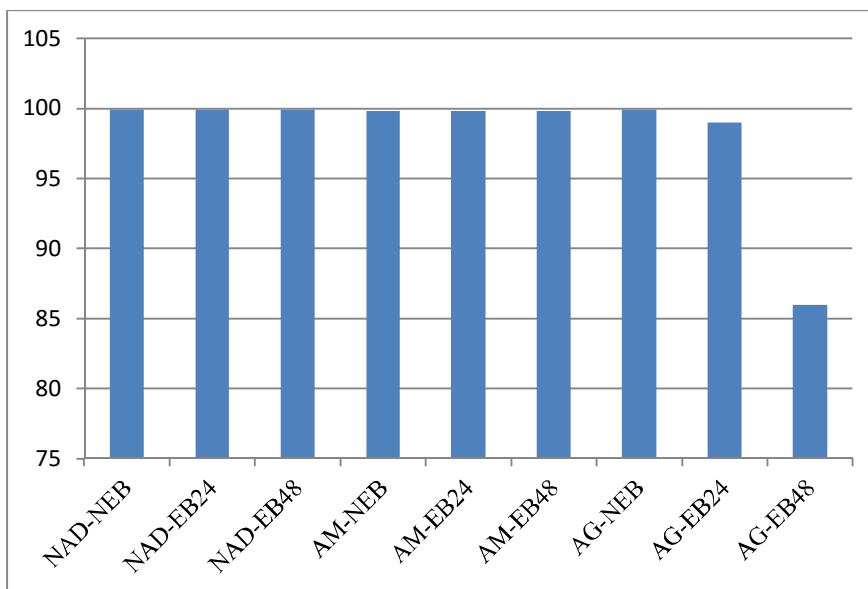
Figure 4. The effect of wound treatments on planktonic *Pseudomonas aeruginosa*

c. **Activity of some wound dressings on the bacterial biofilm of *Staphylococcus aureus* and *Pseudomonas aeruginosa***

The killing activities of three types of dressings were tested against biofilms of *S. aureus* and *P. aeruginosa*. Biofilms were tested in three different stages of development, before biofilm formation, and at 24 and 48 hours after formation.

Against *S. aureus*, a variety of antibacterial dressings of the three types of biofilms brought about complete biofilm inhibition after 0, (representing biofilm initiation) 24 and 48 hours. In contrast the silver dressings achieved only 99% and 86 % inhibition of biofilm formation after 24 and 48 hours (Figure 5). Against *Pseudomonas aeruginosa*, all of the dressings killed all the biofilm formed at 0, 24 and 48 hours except for the NAD dressing (Figure 6). Similar results were obtained by Percival *et al.*, (2008). However, this finding did not correlate well with *in vivo* observations (Hegggers *et al.*, 2005).

Kostenko *et al.*, 2010 claimed that silver content within medical dressings in general is insufficient to eliminate chronic wound biofilms, where the complete killing happens when a silver concentration is greater by 10 – 100 times than that used to treat the planktonic bacteria. It was also reported that *in vitro* *P. aeruginosa* biofilm can be successfully eradicated with silver concentrations of 5-10µg/ml (Bjarnsholt *et al.*, 2007). The biofilm is a population that is very hard to eliminate because the cells inside the biofilm are inert and the atmosphere is anaerobic and acidic, so that antimicrobial agents do not work effectively (Davey and O'Toole, 2000, Davies, 2003, Sternberg *et al.*, 1999) . Moreover, the diversity of bacterial species within wounds makes the exchange of drug resistance determinants easier between bacterial cells (Cookson, 2005, Davies, 1994). So more than one antibiotic with high concentration is needed based on a study by Hill *et al.* (2010) who reported that a mixture of *S. aureus* and *Ps. aeruginosa* biofilm was not treated *in vitro* by using high concentrations of Flucloxacillin (15mg/L) or Ciprofloxacin (5 mg/L).



(Figure 5): The killing activity of selected wound dressings on the biofilm of *S. aureus*. **Ag**= Askina<sup>®</sup> Calgitrol<sup>®</sup> Ag, **NAD** = Actilite<sup>®</sup> (a non-adherent dressing with Activon Manuka honey), **M**= Algivon<sup>®</sup> (alginate dressing impregnated with 100% Manuka honey) , **NEB**= Non Established Biofilm, **EB24**= Established Biofilm for 24 hours, **EB48**= Established Biofilm for 48 hours

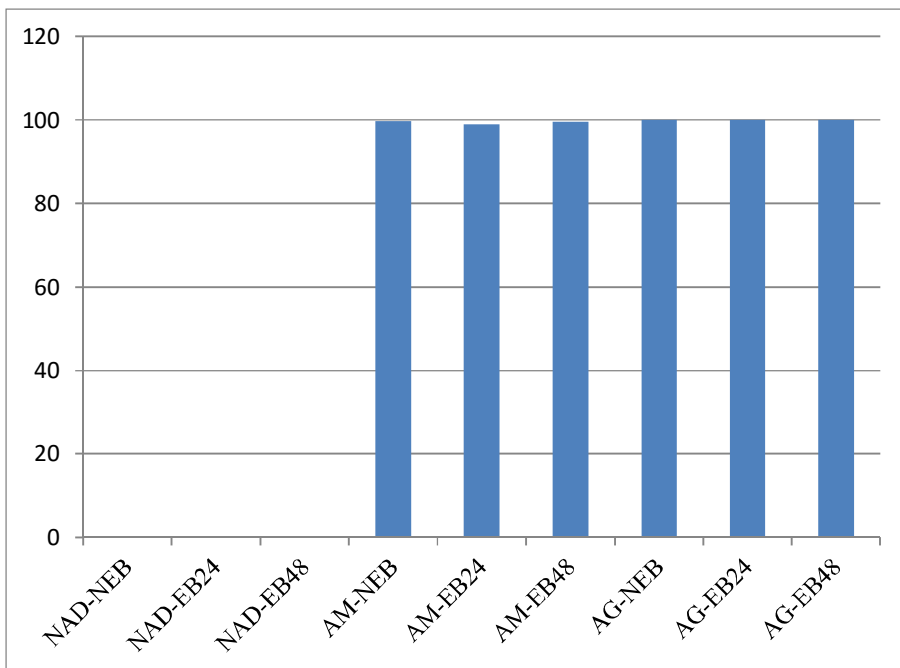


Figure 6. The killing activity of some dressings on the biofilm of *P. aeruginosa*. **Ag**= Askina<sup>®</sup> Calgitrol<sup>®</sup> Ag, **NAD** = Actilite<sup>®</sup> (a non-adherent dressing with Activon Manuka honey), **M**= Algivon<sup>®</sup> (alginate dressing impregnated with 100% Manuka honey), **NEB**= Non Established Biofilm, **EB24**= Established Biofilm for 24 hours, **EB48**= Established Biofilm for 48 hours

**d. Effect of wound treatments on the bacterial biofilm**

Figures 7 and 8 show the effects of wound remedies on bacterial biofilms of *S. aureus* and *P. aeruginosa*. Panaderm cream, which is a mixture of different antibiotics, showed the highest activity against the two bacterial species tested. The antimicrobial activity of the hand gel was the weakest. In contrast, Flamazine, and Activon have approximately similar activity. Among previously used antimicrobial agents, honey is gaining popularity among doctors and medical specialists. In a comparative study between ampicillin ointment, saline treatment and honey dressing, honey was the leading antibacterial agent showing least epithelization, inflammation and

the most active fibroblastic and angioblastic activity (Gupta, 1992). In a similar observation honey was found to be more active on multidrug resistant bacteria such as methicillin resistant *S. aureus* (MRSA) than both antibiotics and antiseptics in the treatment of wounds (Blaser *et al.*, 2007a). The results of the hand gel correlate well with Pietsch (2001) allegation when he claimed that alcoholic gels are not always preferable in hand sterilization as they are not compatible with the European standard for hand disinfectants (EN 1500). *In vitro*, Al Zahrani and Baghdadi, (2012) showed that only two out of seven tested types of hand sanitizers were active and can be used as anti-bacterial agent.

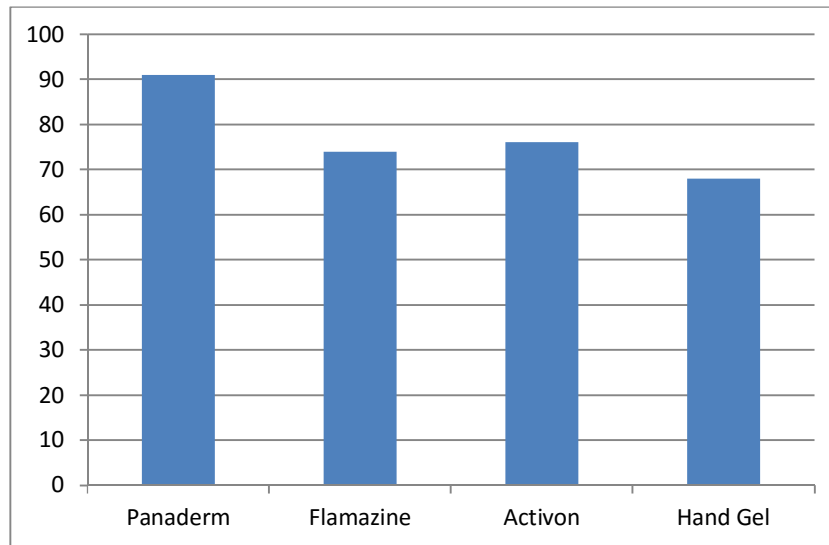


Figure 7. Effect of various cream products and a hand gel on biofilm of *S. aureus*.

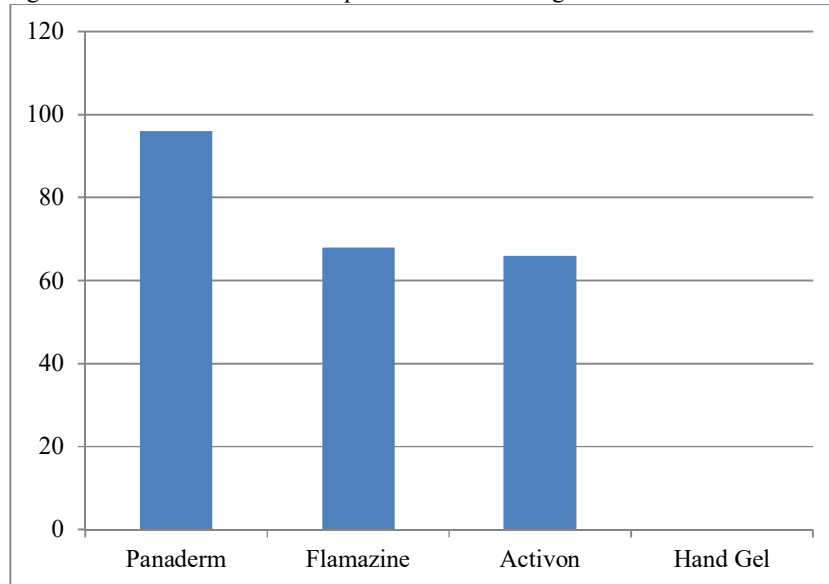


Figure 8. Effect of various antibacterial cream products and a hand gel on biofilm of *P. aeruginosa*

## CONCLUSIONS

Results obtained from the present study indicate that silver and honey exhibited good outcomes as antimicrobial agents for wound dressings. The use of silver to dress wounds causes inhibition of bacterial growth, because silver inhibits bacterial respiration and the activities of enzymes required for viability. Further, silver causes inhibition of bacterial biofilms. Similarly, honey is an active dressing agent for treating wounds because its acidity, hydrogen peroxide content and methylglyoxal which can be found in Manuka honey. We strongly recommend using silver and honey as dressing agents, because they reduce inflammation, accelerate healing, kill

planktonic bacteria, and inhibit the growth of the bacterial biofilms.

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