

20 **Abstract**

21 Infected chronic wounds heal slowly, exhibiting prolonged inflammation, biofilm formation,
22 bacterial resistance, high exudate and ineffectiveness of systemic antimicrobials. Composite
23 dressings (films and wafers) comprising polyox/carrageenan (POL-CAR) and
24 polyox/sodium alginate (POL-SA), loaded with diclofenac (DLF) and streptomycin (STP)
25 were formulated and tested for antibacterial activity against 2×10^5 CFU/mL of *Escherichia*
26 *coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* representing infected chronic
27 wounds and compared with marketed silver dressings. Minimum inhibitory concentration
28 (MIC) showed higher values for DLF than STP due to non-conventional antibacterial
29 activity of DLF. The DLF and STP loaded dressings were highly effective against *E. coli*,
30 *P. aeruginosa* and *S. aureus*. POL-SA dressings were more effective against the three types
31 of bacteria compared to POL-CAR formulations, whilst the DLF and STP loaded dressings
32 showed greater antibacterial activity than the silver-based dressings. The films, showed
33 greater antibacterial efficacy than both wafers and silver dressings. STP and DLF can act
34 synergistically not only to kill the bacteria but also prevent their resistance and biofilm
35 formation compared to silver dressings, whilst reducing chronic inflammation associated
36 with infection.

37

38 **Keywords:** Antimicrobial dressing; bacterial infection; carrageenan; chronic wound;
39 diclofenac; silver dressing; sodium alginate; streptomycin.

40

41 **1 Introduction**

42 A wound is an interruption in the defensive role of the skin in protecting against
43 harmful environmental agents [1]. Injury evokes wound healing comprising distinct phases
44 (haemostasis, inflammation, proliferation, migration and maturation) involving biochemical,
45 and molecular events that work sequentially towards tissue regeneration [2]. However,
46 wounds can get contaminated by microorganisms, especially during the proliferation stage
47 leading to infection. Persistent infection impairs wound healing causing repeating
48 inflammatory cycle, resulting in chronic wounds [3,4]. Prevention and control of infection
49 have been identified as essential aspects of wound management [5]. Effective management
50 requires reducing exogenous microbial contamination, debridement, using appropriate
51 dressing(s) and administration of topical and systemic broad-spectrum antimicrobial agents
52 [6]. Topical agents such as povidone iodine and chlorhexidine acid are commonly employed,
53 though their use is currently restricted to wound cleansing and skin swabs before surgical
54 incisions [1]. However, antibiotics have high specificity against infection and ultimately
55 improve wound healing at low concentrations [1,7]. Various commercial dressings have
56 been developed that release silver to prevent wound infections both *in vitro* [8] and *in vivo*
57 [9]. The emergence of microbial resistance has resulted in the need for more effective
58 treatments for wound infections [1]. Further, systemic antibiotic treatment is difficult in
59 chronic wounds such as diabetic foot ulcers due to poor blood circulation at the extremities
60 of diabetics [6].

61 Chronic wound infection also causes pain, excessive exudation and patient
62 discomfort and is a major source of cross-infection particularly antibiotic-resistant species.
63 Burns for example provide a protein-rich environment, favourable for microbial colonization
64 [10]. Most infected wounds involve *Staphylococcus aureus*, *Pseudomonas aeruginosa*,

65 *Streptococci* and *Escherichia coli*. *S. aureus* is considered a challenging microorganism in
66 wound infections [6] due to its ability to develop resistance against first line antibiotics.

67 Streptomycin (STP) has been used to treat wound infections [11] and for reducing
68 infection before skin grafting [12]. It's reported that diclofenac (DLF) has antibacterial
69 activity and acts synergistically with STP against *Mycobacterium tuberculosis* after systemic
70 administration [13]. Systemic STP in combination with DLF demonstrated synergistic
71 activity against 45 different strains of mycobacteria [14,15].

72 This paper reports on the evaluation of antibacterial activity of STP and DLF loaded film
73 and wafer dressings against *S. aureus*, *E. coli* and *P. aeruginosa*. Minimum inhibitory
74 concentration (MIC) of STP and DLF in the dressings and *in vitro* antibacterial efficacy
75 (zone of inhibition) against the three microorganisms were evaluated using disk diffusion
76 assay and compared with three commercial silver containing dressings. To the best of our
77 knowledge, this is the first study comparing the antibacterial performance of streptomycin-
78 diclofenac loaded medicated POL-CAR and POL-SA dressings with commercial silver
79 loaded antimicrobial dressings for their antibacterial performance.

80

81 **2 Methods**

82

83 **2.1 Materials**

84 (Polyox™ WSR 301 ≈4000 kDa) was a gift from Colorcon Ltd (Dartford, UK), κ-
85 carrageenan (Gelcarin GP 812) was from IMCD Ltd (Sutton, UK), Aquacel® Ag
86 (ConvaTech, Ltd.), Melgisorb® Ag (Mölnlycke Health Care, Ltd.) were gifted by the
87 manufacturers and Allevyn® Ag (Smith and Nephew, Ltd) obtained from a local pharmacy.
88 Nutrient agar and nutrient broth were purchased from Oxoid, UK. Diclofenac sodium,
89 streptomycin sulphate, glycerol, phosphate buffered saline (PBS) tablets, were purchased

90 from Sigma-Aldrich, (Gillingham, UK). Sodium alginate was purchased from Fisher
91 Scientific (Loughborough, UK). National Collection of Type Culture (NCTC) strains of *S.*
92 *aureus* (A 29213), *E. coli* (DTCC 25922) and *P. aeruginosa* (A 10145), were used for
93 microbiological assays.

94

95 **2.2 Preparation of composite polymer based dressings**

96 Composite films and wafers (Table 1) were prepared as previously reported [16,17].
97 Polymeric gels of POL-CAR and POL-SA gels were prepared as previously reported
98 [16,17]. In brief, blends of POL with CAR and POL with SA (weight ratio of 75/25 and
99 50/50 respectively) yielding 1% w/w of total polymer weight were prepared by stirring on a
100 magnetic stirrer at 70°C to form a uniform gel (POL-CAR-BLK and POL-SA-BLK). The
101 composition of the polymers, drugs used for the preparation of gels are summarised in Tables
102 2 and 3. DL gels of POL-SA and POL-CAR were prepared with 4 ml ethanolic solution of
103 DLF containing 100 mg and 250 mg of the drug to achieve 10% w/w for POL-SA gel and
104 for POL-CAR to achieve 25% w/w of DLF in the polymeric gel respectively. These gels
105 were subsequently cooled to 40°C with constant stirring. Similarly, a 4 ml aqueous solution
106 containing 250 mg and 300 mg of STP was subsequently added to achieve a final STP
107 concentration of 25% w/w (POL-SA) and 30% w/w (POL-CAR) in the DL gels.

108 To obtain films the solutions (25g) were poured into Petri dishes (diameter 90 mm)
109 and dried in an oven at 40°C for 18h, to obtain the films, while unplasticised polymeric
110 solutions (10g) were freeze-dried to obtain wafers. To obtain the wafers, 10 gm of each
111 homogeneous gel was transferred into 6 well moulds (diameter 35 mm) (Thermo-Fisher
112 Scientific Nunc, Leicestershire UK), placed in a Virtis Advantage XL 70 freeze dryer
113 (Biopharma Process Systems, Winchester, UK) and lyophilised using the automated
114 lyophilisation cycle. This involved initially cooling samples from room temperature to -5°C

115 and then -50°C over a period of 10 h (at 200 mTorr). An annealing step at -25°C for 2 h was
116 applied based on the preliminary DSC studies and its effect on the different formulations
117 was investigated. The frozen samples were then heated in a series of thermal steps to -25°C
118 under vacuum (20-50 mTorr) over a 24 h period. Secondary drying of the wafers was carried
119 out at 20°C (10 mTorr) for 7 h.

120

121 **2.3 Bacterial sample preparation**

122 Fresh broth cultures were prepared as reported by Labovitiadi *et al.*, [18] by
123 transferring a single bead unit into 10mL of nutrient broth and incubating for 24h. A loop
124 full of bacterial culture was streaked onto nutrient agar plate and incubated at 37°C for 24h
125 to yield separate colonies. Overnight bacterial cultures were centrifuged at 4000 rpm for 10
126 min in an Accuspin 1 centrifuge (Fisher Scientific, UK), supernatant discarded and pellets
127 suspended in 20mL of simulated wound fluid (SWF) [16]. This process was repeated twice
128 and final pellets re-suspended in 5mL SWF, followed by two fold dilutions in SWF.
129 Bacterial density was determined by measuring the dilute suspension at 500nm to yield the
130 required density of 2×10^5 CFU/mL [18].

131

132 **2.4 Minimum inhibitory concentration (MIC) of STP and DLF**

133 The MIC for STP and DLF was evaluated as previously reported [19]. Briefly, three
134 different stock solutions for each drug were prepared (Table 4) and STP required to obtain
135 10,000mg/L was calculated using equation 1. Antimicrobial susceptibilities of *S. aureus*, *E.*
136 *coli* and *P. aeruginosa* were determined by establishing the MIC using a standard agar
137 dilution method and 0.25-512mg/L calibration solutions of DLF and STP dilutions also
138 prepared. 200µL of stock and diluted solutions (10,000mg/L, 1,000mg/L and 100mg/L
139 respectively) were transferred into a Petri plate and 20mL of nutrient agar (stabilized at

140 45°C) added and mixed. The agar was allowed to set at room temperature and 0.1mL of
141 1×10^5 CFU/mL of *S. aureus*, *E. coli* and *P. aeruginosa* were spread on separate Petri plates.
142 These plates were incubated at 37°C for 24h and ensuring that all microorganisms had grown
143 on drug free control plate. MIC is the lowest concentration of antimicrobial at which there
144 was no visible growth of organisms. Growth of one or two colonies or a fine film of growth
145 was disregarded.

$$W = \frac{1000}{P} \times V \times C \text{ Eq.1}$$

146
147 W is the weight of actives (mg) dissolved in volume V (mL), C is final concentration
148 of solution (multiples of 1,000mg/L), P (785 μ g/mg) is the potency provided by the
149 manufacturer.

150

151 **2.5 *In vitro* antibacterial activity of antimicrobial films, wafers and marketed silver** 152 ***dressings***

153 The disk diffusion method was used for the assessment of the antibacterial activity
154 of the DL films, wafers and commercial silver dressings. Solutions (2×10^5 CFU/mL) of each
155 bacterial strain (*S. aureus*, *E. coli* and *P. aeruginosa*) was prepared as specified above
156 (section 2.3) and 0.1mL of each strain spread separately on set nutrient agar media. The
157 inoculated microorganisms were incubated at $37 \pm 1^\circ\text{C}$ for 4h to initiate growth of
158 microorganisms on the inoculated culture medium before placing the films, wafers and
159 marketed dressings. The films and marketed silver dressings were cut into 2cm diameter disc
160 shapes. However, due to difficulty of cutting thicker wafers into smaller discs, DL gels (2g)
161 were free-dried in 2cm diameter containers to obtain the same diameter as the cut film discs.
162 Further, circular Whatmann[®] paper discs (2cm diameter), each wetted with reference
163 solutions (80 μ L) of STP and DLF at concentrations of 6mg/mL and 5mg/mL respectively
164 were used as positive controls. Negative controls were BLK films and wafers (2cm diameter)

165 without any STP or DLF. The plates were then incubated at $37\pm 1^{\circ}\text{C}$ for 24 h after which the
166 end zones of inhibition (ZOI) in millimetres, formed on the medium ($n = 3$), were measured.

167

168 **2.6 Statistical analysis**

169 Statistical data evaluation was performed using two tailed student t-test at 95%
170 confidence interval (Graph Pad Prism 4 software) with p value < 0.05 as the minimal level
171 of significance.

172

173 **3 Results**

174 **3.1 MIC of STP and DLF**

175 The MIC of STP and DLF was determined for known densities (2×10^5 CFU/mL) of
176 *S. aureus* *P. aeruginosa* *E. coli* commonly associated with infected chronic wounds. The
177 MICs of STP for *S. aureus* and *E. coli* ranged from 4 - 8mg/L but ranged from 8 - 16mg/L
178 for *P. aeruginosa*. MIC for DLF against *P. aeruginosa* was greater than 512mg/L and 256 -
179 512mg/L for *E. coli* and *S. aureus* respectively.

180

181 **3.2 Antimicrobial activity of pure STP and DLF controls**

182 The ZOI of the STP and DLF positive controls for *S. aureus*, *P. aeruginosa* and *E.*
183 *coli* are shown in Figure 1 (N and O). STP showed significantly ($p<0.05$) lower ZOI
184 ($3.2\pm 0.1\text{mm}$) for *S. aureus* compared to *P. aeruginosa* and *E. coli*. The maximum ZOI of *P.*
185 *aeruginosa* was $4.1\pm 0.1\text{mm}$ which was lower compared to *E. coli* ($4.6\pm 0.1\text{mm}$) and was
186 statistically significant ($p<0.05$). DLF did not show ZOI for *S. aureus*, *P. aeruginosa* and *E.*
187 *coli* though there was no bacteria growing directly under the DLF disc (Figure 1, *E. coli*
188 plate O) implying that their effectiveness alone as antibacterial may be limited application
189 to infected wounds.

190

191 **3.3 Antibacterial activity of POL-CAR films (2×10^5 CFU/mL)**

192 Figures 2 (A) and 3(A, B and C) show ZOI of POL-CAR-DL and POL-CAR-DL-
193 20% GLY films against *S. aureus*, *P. aeruginosa* and *E. coli*. There was a significant
194 difference observed for all POL-CAR-DL films against strains of bacteria (compared to
195 wafers and marketed dressing and DLF, STP discs. POL-CAR-DL and POL-CAR-DL-20%
196 GLY films showed a smaller ZOI for *S. aureus* but increased for *P. aeruginosa* and *E. coli*.
197 For *S. aureus* the ZOI for POL-CAR-DL and POL-CAR-DL-20%GLY films was
198 3.6 ± 0.1 mm and 3.5 ± 0.1 mm respectively which was significantly ($p < 0.05$) higher than pure
199 STP (3.2 ± 0.1 mm). For *P. aeruginosa*, the observed ZOI was higher than *S. aureus* but less
200 than *E. coli*. POL-CAR-DL and POL-CAR-DL-20%GLY films showed similar ZOI
201 (4.3 ± 0.1 mm) for *P. aeruginosa* which was higher than the control STP (4.1 ± 0.1 mm),
202 however, the difference was not statistically significant ($p > 0.05$). The maximum ZOI of
203 POL-CAR-DL and POL-CAR-DL-20%GLY films was 4.8 ± 0.2 mm and 4.7 ± 0.1 mm
204 respectively, for *E. coli* which though higher than 4.6 ± 0.2 mm for the control STP were not
205 statistically significant ($p > 0.05$).

206

207 **3.4 Antibacterial activity of POL-SA films (2×10^5 CFU/mL)**

208 Figures 2 (B) and 4 (D, E and F) show the ZOI of POL-SA-BLK, POL-SA-DL and
209 POL-SA-DL-9% GLY) films for *S. aureus*, *P. aeruginosa* and *E. coli*. For *S. aureus*, the
210 observed ZOI for POL-SA-DL and POL-SA-DL-9%GLY films was 4.6 ± 0.2 mm and
211 4.1 ± 0.2 mm respectively which was significantly ($p < 0.05$) higher compared to the STP
212 (3.2 ± 0.1 mm) control. The ZOI increased from 4.6 ± 0.2 mm (*S. aureus*) to 4.8 ± 0.2 mm (*P.*
213 *aeruginosa*) and 5.0 ± 0.2 mm (*E. coli*) for POL-SA-DL films while for POL-SA-9%GLY

214 films it increased from $4.1\pm 0.2\text{mm}$ (*S. aureus*) to $5.1\pm 0.2\text{mm}$ (*P. aeruginosa*) and
215 $5.5\pm 0.2\text{mm}$ (*E. coli*) respectively.

216

217 **3.5 Antibacterial activity of POL-CAR and POL-SA wafers (2×10^5 CFU/mL)**

218 Figures 2 (C) and 5 (G, H, I and J) show the ZOI of POL-CAR and POL-SA (BLK
219 and DL) wafers for *S. aureus*, *P. aeruginosa* and *E. coli* bacterial strains. As was observed
220 for the films, the BLK (no drug) wafers did not show any ZOIs against all three
221 microorganisms (Figure 5, G and I). The ZOI of POL-CAR for *S. aureus* was $3.1\pm 0.1\text{mm}$
222 which increased to $3.3\pm 0.1\text{mm}$ for POL-SA whereas STP had a value of $3.2\pm 0.1\text{mm}$ which
223 was not statistically significant ($p>0.05$). For *P. aeruginosa*, the ZOI was higher than *S.*
224 *aureus* but less than *E. coli*. POL-SA-DL and STP showed similar ZOI of $4.1\pm 0.2\text{mm}$ which
225 subsequently decreased for POL-CAR-DL ($3.9\pm 0.1\text{mm}$). The maximum ZOI of POL-CAR-
226 DL and POL-SA-DL wafers was respectively $4.5\pm 0.1\text{mm}$ and $4.6\pm 0.3\text{mm}$ for *E. coli*.

227

228 **3.6 Antimicrobial efficacy of marketed wound dressings (2×10^5 CFU/mL)**

229 Figures 2 (D) and 6 (K, L and M) show the ZOI of silver loaded marketed dressings
230 (Table 5) (Aquacel[®] Ag, Melgisorb[®] Ag and Allevyn[®] Ag) for *S. aureus*, *P. aeruginosa* and
231 *E. coli*. There were very small ZOIs observed for all three different strains of microorganisms
232 in the presence of these marketed silver based dressings, though these bacteria were
233 completely absent in the area directly underneath the dressing as shown in figure 6 inset (M,
234 *S. aureus*). The ZOI for *S. aureus* was increased for Allevyn[®] Ag foam dressing ($2.3 \pm$
235 0.1mm) while all three marketed dressings showed a ZOI of $2.0\pm 0.1\text{mm}$ for *P. aeruginosa*.
236 The ZOI for *E. coli* was higher for Allevyn[®] Ag foam dressing ($2.9\pm 0.0\text{mm}$) compared to
237 Aquacel[®] Ag and Melgisorb[®] Ag ($2.0\pm 0.0\text{mm}$).

238 **4. Discussion**

239 One of the overall objectives of the broad study was to compare the properties of dense
240 dressings such as films to corresponding porous formulations such as freeze-dried wafers
241 relative to commercial silver based dressings. Drying in an oven only yields non porous
242 films and therefore it was important to freeze-dry other gels in a freeze-dryer. The reason
243 for plasticising the films, was purely to improve the flexibility and ease of handling, to
244 fulfil one of the key functional performance requirements for film dressings. The
245 hypothesis for the comparison, was that the differences in physical properties (porosity),
246 which are known to significantly affect rate of hydration and swelling, will also
247 significantly affect the rate of drug diffusion out of the swollen gels and subsequently
248 affect the degree of antibacterial efficacy. The hypothesis for the comparison, was that the
249 differences in physical properties (porosity), which are known to significantly affect rate of
250 hydration and swelling, will also significantly affect the rate of drug diffusion out of the
251 swollen gels and subsequently affect the degree of antibacterial efficacy.

252 Ineffective control of wound infections caused by antibiotic resistant strains of
253 pathogens has intensified the need to consider modifying current approaches including use
254 of medicated dressings which can overcome resistance and reduce bacterial biofilm
255 formation. This study assessed the *in vitro* antibacterial activity of composite films and
256 wafers combining antibacterial (STP) and anti-inflammatory (DLF) drugs for targeting two
257 phases of wound healing. The two drugs were also selected based on their reported
258 synergistic antibacterial effect when administered systemically [14]. Many texts refer to
259 bacterial bio-burden greater than 10^5 CFU/mL organisms per gram of tissue as a criterion
260 for infection [3,6]. In this study we used 2×10^5 CFU/mL of *S. aureus*, *P. aeruginosa* and *E.*
261 *coli* to evaluate antimicrobial efficacy of DL film and wafer dressings and compared their
262 performance against marketed silver dressings.

263 POL-CAR-BLK films did not show any zone against all three different
264 microorganisms (Figure 3 A) implying that the observed antibacterial effect was solely due
265 to the presence of STP and DLF. The formulated films, wafers and marketed dressings
266 showed antibacterial efficacy against bacterial bio-burden 2×10^5 CFU/mL of *S. aureus*, *P.*
267 *aeruginosa* and *E. coli*. Both *P. aeruginosa* and *E. coli* are Gram-negative microorganisms
268 and required a higher MIC of STP compared to *S. aureus*. This means STP is more effective
269 against the Gram-positive microorganism *S. aureus* than the Gram-negative *E. coli* and *P.*
270 *aeruginosa* which is interesting, given the fact the *S. aureus* and related species are a major
271 cause of antibiotic resistance [14].

272 During the antibacterial study, the films and wafers swelled when placed on the
273 highly water saturated agar gel under incubation, simulating a broken skin (wound) surface
274 and this is to be expected. The swelling of the drug loaded polymeric dressings is an
275 important characteristic as that is important to ensure ease of drug dissolution, diffusion out
276 of the swollen gel and eventually release to reach the target bacterial organisms.

277 To kill the bacteria, STP and DLF must interact with the binding site, occupy a
278 critical number of sites of the bacteria and remain there long enough to inhibit normal
279 biochemical reactions [20]. It's been reported that antimicrobial activity is either
280 concentration or time dependent [21,22]. Concentration dependent drugs include
281 aminoglycosides (e.g. STP), whose ability to kill bacteria is dependent on the presence of
282 high concentrations at the site of infection. At least a ratio of 10:1 is required for such
283 concentration dependent antibiotics to effectively kill bacteria and prevent development of
284 resistance [21,23,24,25]. On the other hand, drug concentrations above the MIC should
285 remain for long periods of time at the site of infection in order to achieve antibacterial
286 action [21,22].

287 In previous studies [13,14,15], it has been demonstrated that the concentrations
288 required to kill *S. aureus* is higher than *P. aeruginosa* which is time dependent. DLF required
289 higher concentrations to kill the bacteria that are beyond those clinically achievable with
290 antibiotics, implying that DLF on its own could not effectively inhibit *P. aeruginosa* based
291 infections. Dutta *et al.*, [14] previously demonstrated that when DLF is used *in vitro*, it
292 showed higher MIC values compared to conventional antibiotic drugs such as STP but *in*
293 *vivo*, the amount of DLF required to protect an animal from *Mycobacterium spp* was much
294 lower. This suggests that DLF might be used as adjuvant to current to manage bacterial
295 infections [13,14,15] as was done in this study.

296 For *S. aureus*, different ZOI were observed attributed to the rate of diffusion of STP
297 and DLF (films and wafers) and silver (commercial) from the dressings. Both POL-CAR;
298 POL-SA films had significantly higher ZOI suggesting a synergistic action between both
299 drugs compared to each individual drug (refer to figure1). ZOI was ellipsoidal for POL-SA
300 films due to the rapid initial swelling and disintegration of the polymer matrix and rapid
301 diffusion of STP and DLF through the free flowing swollen gels (figure 4 E&F). Bajpai &
302 Sharma [26] explained that the more rapid swelling of SA is due to the mannuronate block
303 where Ca^{2+} binds to the poly gluconate units which starts to disintegrate the swollen matrix
304 [26]. Differences in the ZOI of POL-CAR-DL and POL-SA-DL formulation could be related
305 to the two different polymers (CAR and SA), their percentage ratios used and their different
306 swelling mechanisms (surface wetting, hydration, hydrogel formation and erosion) [17]
307 which subsequently affects rate of drug diffusion through the matrix and onto the bacterial
308 colonies.

309 Maximum ZOI was observed for POL-SA-DL and POL-SA-DL-9%GLY films due
310 to rapid swelling and subsequently rapid diffusion of both STP and DLF from the swollen
311 matrix. This supports the swelling and drug release data from previous studies [16,17].

312 All the DL films showed greater antibacterial activity compared to wafers which was
313 interesting. Wafers generally have a higher loading capacity, faster hydration and cumulative
314 percent drug release compared to films due to their generally more porous nature [27].
315 However, it was observed that higher drug loading in the wafers resulted in the formation of
316 greater amounts of sodium sulphate which decreased the hydration capacity [16,17] of DL
317 wafers subsequently affecting drug diffusion with a consequent decrease in ZOI compared
318 to films but greater than the marketed dressings.

319 From a pharmaceutical perspective, these differences could be associated with the
320 total amounts of polymer present in films and wafers which resulted in the different
321 hydration rates and eventually different ZOIs. For example, the weights ranged from 22.1
322 mg and 30.3 mg for POL-CAR DL films and wafers respectively. This was also true for the
323 POL-SA DL films and wafers (17.9 mg and 24.6 mg for films and wafers respectively). It
324 should be noted that though both formulations had similar diameters, their contents were
325 different as the films were cut out directly from a bigger sheet due to difficulty of removing
326 a film with small diameter whilst the wafers were cast directly into 2 cm diameter moulds
327 due to ease of removal. It is very difficult to effectively cut a relatively thick wafer into
328 circular discs without damaging the structure due to their soft and porous nature.

329 More interestingly, the formulated film and wafer dressings, showed greater
330 antibacterial efficacy than marketed silver based antibacterial dressing which showed either
331 lower or absence of ZOIs for all three different microorganisms even though the area directly
332 under the discs showed no microbial growth. This may be due to two reasons: (i) the lower
333 amounts of silver present in these dressings (Figure 6) relative to the combined
334 concentrations of STP and DLF present in the composite films and wafers and (ii) most
335 likely due to STP and DLF present in both films and wafers acting synergistically to kill the
336 bacteria and potentially inhibiting biofilm formation and resistance of the bacteria. DLF

337 consists of a secondary amino group and a phenyl ring, both ortho positions of which are
338 occupied by chlorine atoms. This causes an angle of torsion between the two aromatic rings,
339 which presents structural similarities with phenothiazine and this is responsible for its
340 antibacterial activity against microorganisms such as *E. coli*, *S. aureus* and *P. aeruginosa*
341 [14,15]. DLF's antibacterial activity involves the inhibition of bacterial DNA synthesis
342 whereas STP acts by binding to 30S ribosomal subunits in the microorganisms and
343 disrupting the initiation and elongation steps in protein synthesis. On the other hand, silver
344 in the presence of moisture, such as wound exudate, readily ionises to release silver ions
345 (Ag^+) which is involved in oxidation reactions by catalysing reactions between oxygen
346 present in the cell and hydrogen from thiol groups. This results in disulphide bond formation,
347 ultimately inhibiting cell function due to changes in protein structure, resulting in protein
348 denaturation and enzyme inhibition [28]. The increased antibacterial activity of the film and
349 wafer dressings suggests a potential application in chronic wound management.

350 Formulations administered for systemic use usually have to overcome the challenges
351 to drug absorption, metabolism, distribution and elimination before the drug reaches the
352 target sites for activity, hence such systemic formulations tend not to always have direct *in*
353 *vitro-in vivo* correlations. For formulations such as wound dressings, intended for direct
354 application, where the drug(s) are in direct contact with the target tissues, a high positive *in*
355 *vitro – in vivo* correlation tends to exist due to minimal pharmacokinetic barriers.

356 Silver is a widely used anti-microbial agent effective against infection causative
357 wound pathogens which are responsible for delayed wound healing and can be added to a
358 range of composite dressings [29]. Silver containing wound dressings release silver ions
359 which vary due to the different forms (silver sulfadiazine, ionic silver nanoparticles
360 containing scaffolds, nanofiber containing silver nanoparticles, silver-containing activated
361 carbon and fibres) and the amount of the silver present [1,30]. Although there are important

362 questions raised by Modak *et al* [31] in regards to the use of silver in infected wounds and
363 formation of biofilms by the microorganisms, the versatile effect of silver carries a low risk
364 of resistance even though some studies in burn wounds have shown bacterial resistance to
365 silver sulfadiazine and silver nitrate by *Pseudomonas spp* [31]. Moreover, the antimicrobial
366 effect of silver incorporated in a number of dressings depends on the release rate of silver
367 ions which influences the overall antimicrobial effect [32]. In comparative antimicrobial
368 efficacy studies, it was reported that certain types of methicillin resistant strains among *S.*
369 *aureus*, *P. aeruginosa*, and *E. coli* were less sensitive to Urgotul SSD[®], Bactigras[®],
370 Acticoat[®], Askina Calgitrol Ag[®] and Aquacel Ag[®] antimicrobial dressings [33].
371 Furthermore, *in vivo* silver can bind to proteins present in biofilms instead of binding to the
372 bacterial cell walls, resulting in reduced antimicrobial effect against the bacteria [34].
373 Another potential concern is that silver does not act specifically against bacteria but also acts
374 on any host proteins. Therefore, if very few bacteria counts are present at the wound site,
375 then the effect on host tissue is greater which could slow down healing [35]. Concentrations
376 above 1mg/L (1 part per million) of silver reacts with wound exudate and could cause
377 transient skin staining [36]. Li and co-authors suggested that bacterial resistance could be
378 induced when low concentrations of silver were used [37]. There is therefore the possibility
379 of these silver containing dressings inducing resistance from *S. aureus* and *P. aeruginosa*
380 which are known to be able to form biofilms in an infected chronic wound environment [38].
381 However, because there was absence of bacteria in the immediate application area beneath
382 the marketed dressings, it implies the silvers dressing were effective to kill the bacteria in
383 only the applied area of a wound and could also potentially limit or completely prevent
384 infection from external sources.

385

386 **5 Conclusion**

387 Composite polymer based dressings containing STP and DLF appear to show
388 significantly higher inhibition of the three bacterial strains compared to silver containing
389 commercial dressings. STP can help to reduce bacterial infection by its known antimicrobial
390 action and potentially in synergy with DLF while the latter can also help to reduce the
391 swelling and pain associated with injury due to its anti-inflammatory action. However, these
392 will, require further investigations in an *in vitro* cell culture (for cell viability and cell
393 migration/proliferation) and *in vivo* animal study.

394

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398 **6** **References**

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521 **FIGURE LEGENDS**

522 **Figure 1:** ZOI of control STP (N) and control DLF (O) for *S. aureus*, *P. aeruginosa* and *E.*
523 *coli*. The inset of control DLF shows the absence of bacteria around the applied area of the
524 disk (mean \pm SD, $n = 3$).

525 **Figure 2:** Extracted data comparing the measured ZOI data (mm) of *S. aureus*, *P.*
526 *aeruginosa* and *E. coli* for the various formulations and marketed dressings tested.(A)
527 POL-CAR (DL and DL-20%GLY) films and STP and DLF (mean \pm SD, $n = 3$). (B) POL-
528 SA-DL and POL-SA-DL-9%GLY films and control STP and DLF (mean \pm SD, $n = 3$). (C)
529 POL-CAR-DL-An and POL-SA-DL-An wafers and control STP and DLF (mean \pm SD, $n =$
530 3). (D) The marketed dressings (Aquacel[®] Ag, Melgisorb[®] Ag, Allevyn[®] Ag (mean \pm SD,
531 $n=3$).

532 **Figure 3:** The digital images of ZOI of (A) POL-CAR-BLK, (B) POL-CAR-DL, (C) POL-
533 CAR-DL-20%GLY films observed for *S. aureus*, *P. aeruginosa* and *E. coli*(mean \pm SD,
534 $n=3$).

535 **Figure 4:** The digital images of ZOI of (D) POL-SA-BLK, (B) POL-SA-DL, (C) POL-SA-
536 DL-9%GLY observed for *S. aureus*, *P. aeruginosa* and *E. coli*(mean \pm SD, $n = 3$).

537 **Figure 5:** Digital images of ZOI of (G) POL-CAR-BLK-An, (H) POL-CAR-DL-An, (I)
538 POL-SA-BLK-An, (J) POL-SA-DL-An, observed for *S. aureus*, *P. aeruginosa* and *E. coli*
539 (mean \pm SD, $n = 3$).

540 **Figure 6:** Digital images of ZOI observed for *S. aureus*, *P. aeruginosa* and *E. coli* by (K)
541 Aquacel[®] Ag; (L) Melgisorb[®] Ag; and (M) Allevyn[®] Ag. Inset shows the absence of bacteria
542 in the immediate applied area of the dressing (mean \pm SD, $n = 3$).

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Table 1: Formulations used to evaluate antimicrobial efficacy against *S. aureus*, *P. aeruginosa* and *E. coli*.

Formulation	CODE
POL-CAR-BLK	A
POL-CAR-DL	B
POL-CAR-DL-20%GLY	C
POL-SA-BLK	D
POL-SA-DL	E
POL-SA-DL-9%GLY	F
POL-CAR-BLK-An	G
POL-CAR-DL-An	H
POL-SA-BLK-An	I
POL-SA-DL-An	J
Aquacel® Ag	K
Melgisorb® Ag	L
Allevyn® Ag	M
STP	N
DLF	O

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554 Table 2: Quantities of the polymers, drugs and GLY (varying amounts based on total solid
 555 weight) within composite polymer gels used for formulation of POL-CAR and POL-SA
 556 (BLK and DL) films.

Formulation	POL (gm)	CAR (gm)	SA (gm)	GLY (gm)	DLF (gm)	STP (gm)	Total weight (gm)	% GLY Content
POL-CAR-BLK	0.75	0.25	-	0.00	-	-	1.00	0.00
POL-CAR-BLK	0.75	0.25	-	0.10	-	-	1.10	9.09
POL-CAR-BLK	0.75	0.25	-	0.25	-	-	1.25	20.00
POL-CAR-BLK	0.75	0.25	-	0.50	-	-	1.50	33.33
POL-CAR-BLK	0.75	0.25	-	0.75	-	-	1.75	42.86
POL-CAR-BLK	0.75	0.25	-	1.00	-	-	2.00	50.00
POL-CAR-DL	0.75	0.25	-	0.00	0.10	0.30	1.40	0.00
POL-CAR-DL	0.75	0.25	-	0.10	0.10	0.30	1.50	6.67
POL-CAR-DL	0.75	0.25	-	0.25	0.10	0.30	1.65	15.15
POL-CAR-DL	0.75	0.25	-	0.50	0.10	0.30	1.90	26.32
POL-CAR-DL	0.75	0.25	-	0.75	0.10	0.30	2.15	34.88
POL-CAR-DL	0.75	0.25	-	1.00	0.10	0.30	2.40	41.67
POL-SA-BLK	0.50	-	0.50	0.00	-	-	1.00	0.00
POL-SA-BLK	0.50	-	0.50	0.10	-	-	1.10	9.09
POL-SA-BLK	0.50	-	0.50	0.25	-	-	1.25	20.00
POL-SA-BLK	0.50	-	0.50	0.50	-	-	1.50	33.33
POL-SA-DL	0.50	-	0.50	0.00	0.05	0.15	1.20	0.00
POL-SA-DL	0.50	-	0.50	0.10	0.05	0.15	1.30	7.69
POL-SA-DL	0.50	-	0.50	0.25	0.05	0.15	1.45	17.24
POL-SA-DL	0.50	-	0.50	0.50	0.05	0.15	1.70	34.48

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567 Table 3: Composition of polymers and drugs (varying quantity) present in composite
568 polymer gels used to produce composite freeze dried POL-CAR and POL-SA (BLK and
569 DL) wafers.

Pure material	POL-CAR-BLK	POL-CAR-DL	POL-SA-BLK	POL-SA-DL
	(weight in gm)			
POL	0.75	0.75	0.50	0.50
CAR	0.25	0.25	-	-
SA	-	-	0.50	0.50
STP	-	0.30	-	0.25
DLF	-	0.25	-	0.10
Total weight	1.00	1.55	1.00	1.35

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577 **Table 4:** Stock solutions of STP and DLF used to evaluate MIC of *S. aureus*, *E. coli* and *P.*
578 *aeruginosa* (mean \pm SD, $n = 3$).

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	Stock solution 1	Stock solution 2	Stock solution 3
STP	10000 mg/L (254 mg of STP + 20 ml of distilled water)	1000 mg/L (1 ml of stock solution 1 + 9 ml of distilled water)	100 mg/L (1 ml of stock solution 2 + 9 ml of distilled water)
DLF	10000 mg/L (200 mg of DLF + 20 ml of distilled water)	1000 mg/L (1 ml of stock solution 1 + 9 ml of distilled water)	100 mg/L (1 ml of stock solution 2 + 9 ml of distilled water)

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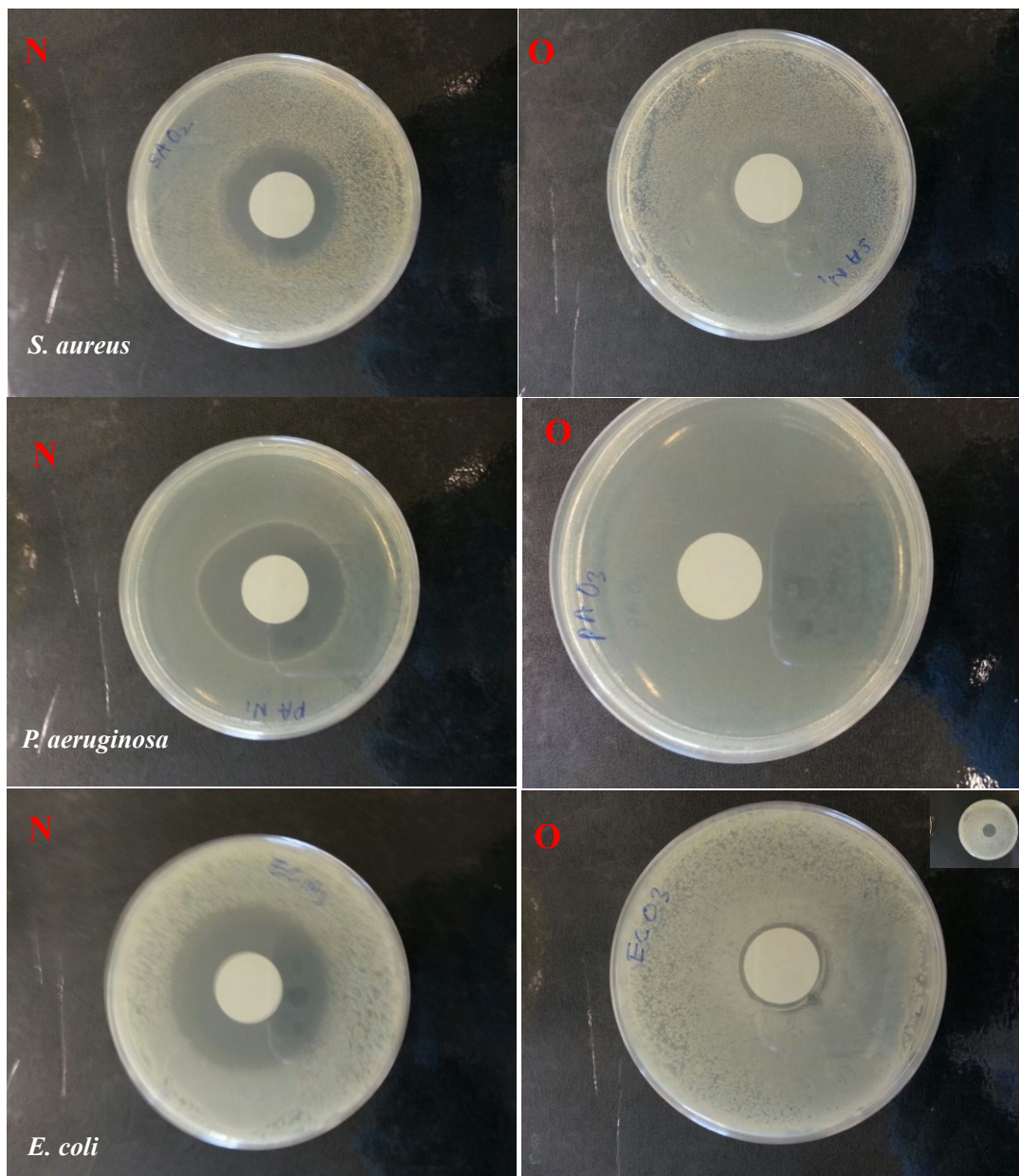
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Table 5: Description of the silver containing dressings used for antimicrobial study (Hamberg *et al.*, 2012) (mean \pm SD, $n = 3$).

Product	Formulation details	Silver content (mg/cm ²)
Aquacel [®] Ag	Sodium carboxymethylcellulose with ionic silver	0.08-0.09
Melgisorb [®] Ag	Alginate dressing with silver sodium hydrogen zirconium phosphate	0.08
Allevyn [®] Ag	Polyurethane foam dressing with soft gel adhesive and silver sulphadiazine	0.90

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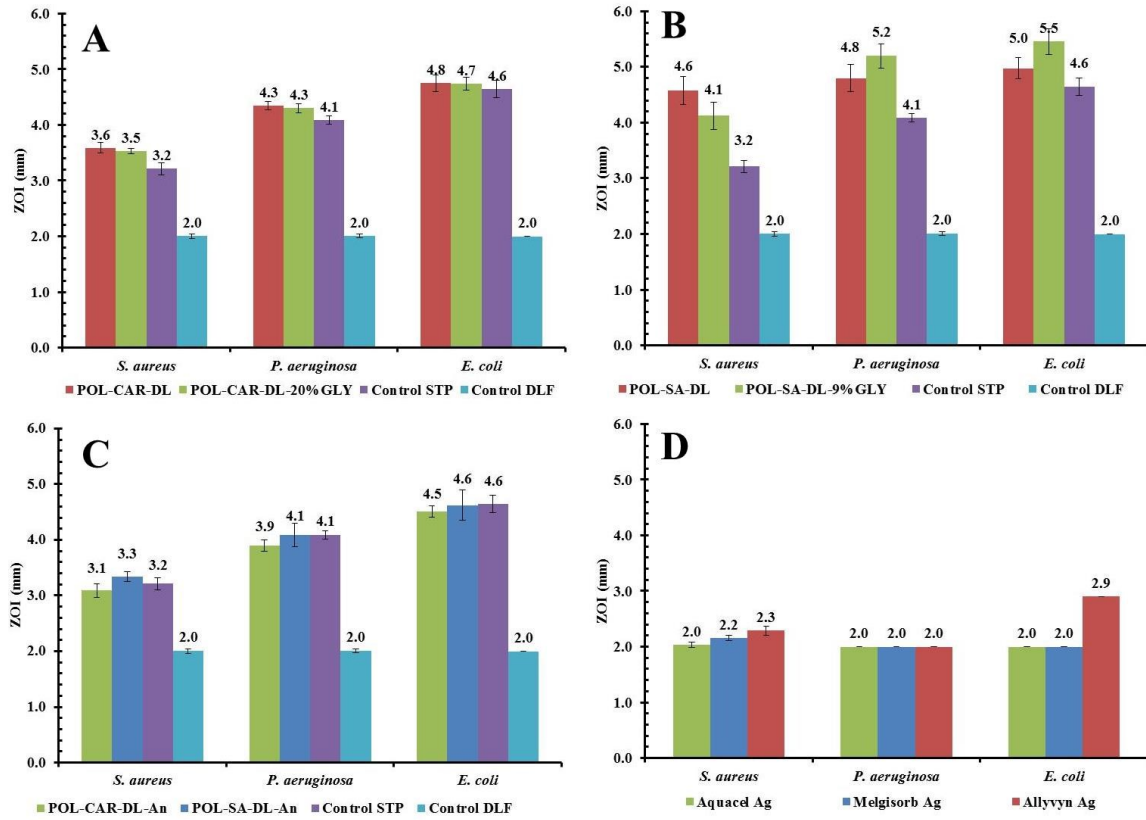


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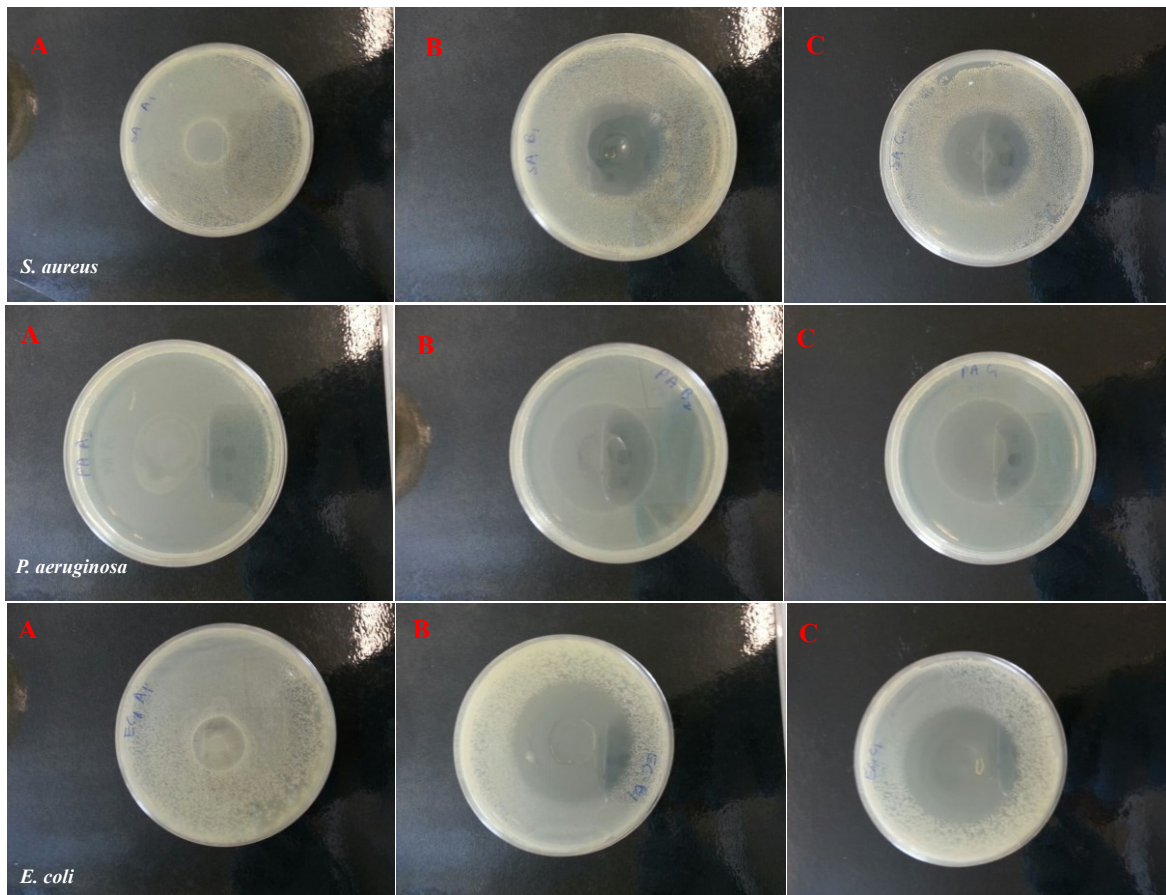
599 **Figure 1**

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Figure 2

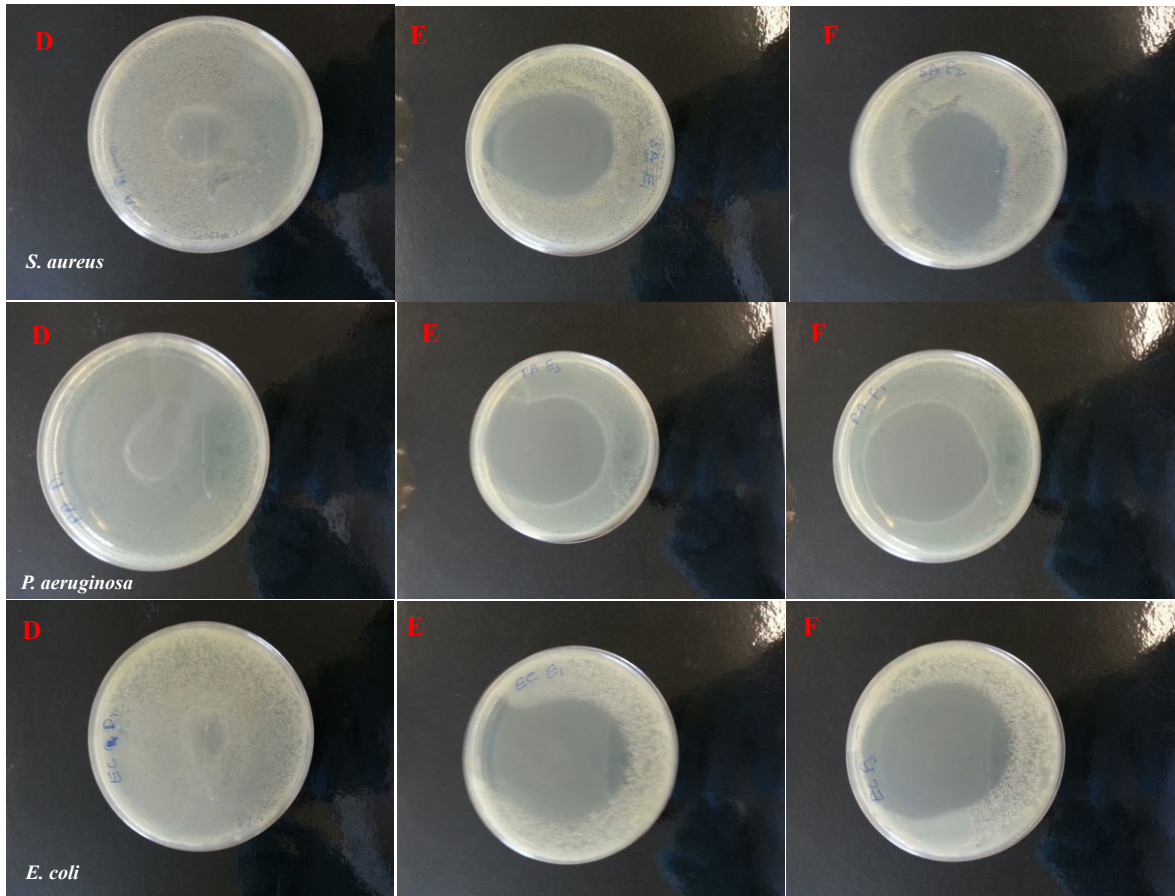


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618 **Figure 3**

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622 **Figure 4**

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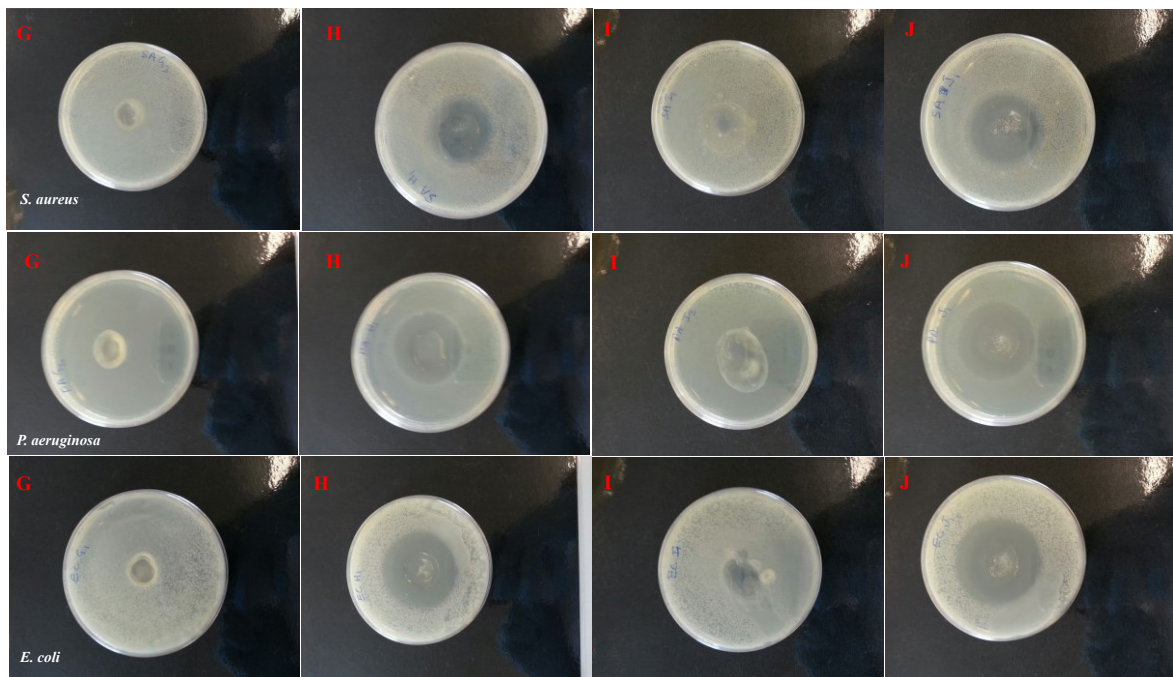
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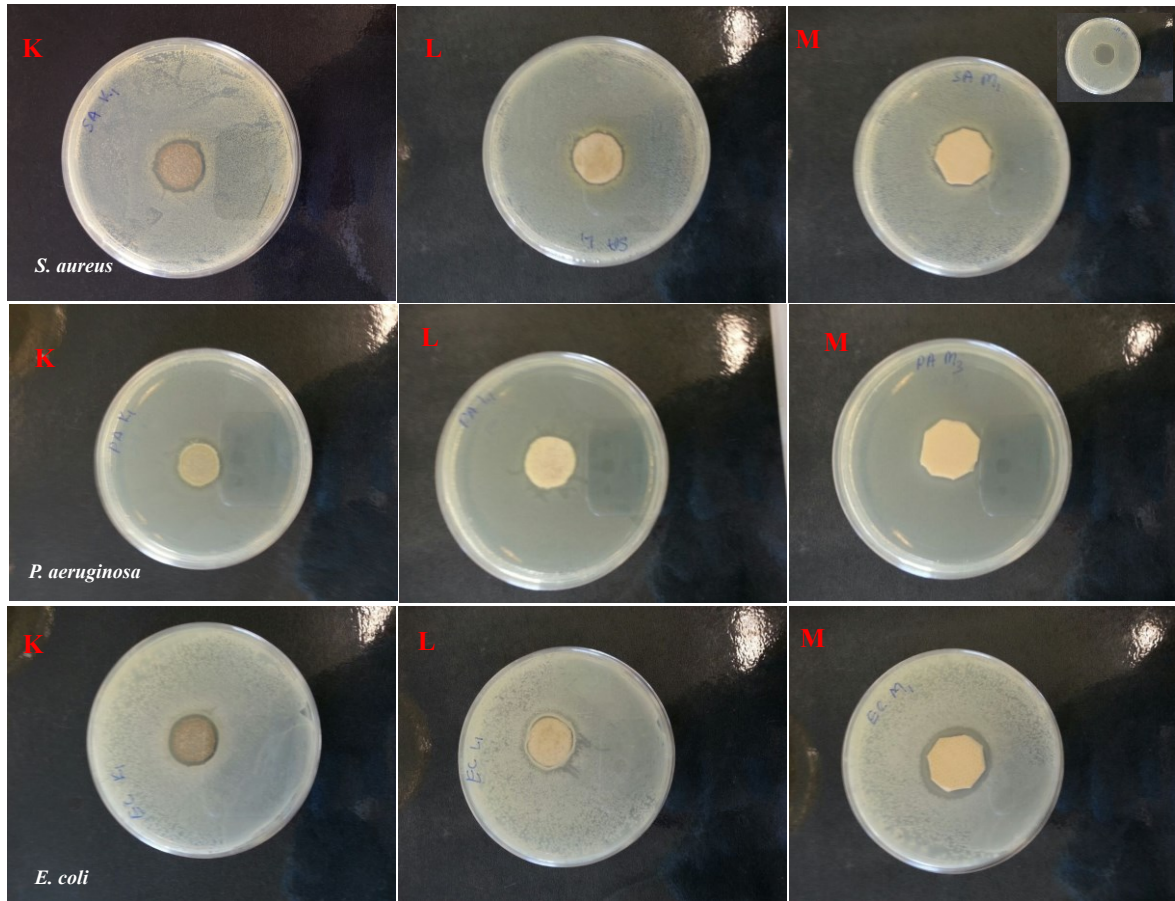
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639 **Figure 5**

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645 **Figure 6**

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