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[Bahgat Gerges](#)*, [Joel Rosenblatt](#), [Y-Lan Truong](#), [Ying Jiang](#), [Issam Raad](#)

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Article

Elevated Antibacterial Activity with Decreased Cytotoxicity of a Polygalacturonic + Caprylic Acid Wound Ointment Compared with Hypochlorous Acid in a Three-Dimensional Wound Biofilm Model

Bahgat Gerges *, Joel Rosenblatt, Y-Lan Truong, Ying Jiang and Issam Raad

Department of Infectious Diseases, Infection Control and Employee Health, The University of Texas MD Anderson Cancer Center, Houston, Texas, USA

* Corresponding: bzgerges@mdanderson.org

Abstract

Introduction: Natural, plant-based agents are alternatives to antibiotics and antiseptics in treating bacterially colonized wounds and potentially offer an antimicrobial activity without cytotoxicity. In this study, we used a three-dimensional fibrin-gel wound biofilm (FGWB) model to be more representative of the wound biofilm environment. We compared a common antiseptic wound care agent, hypochlorous acid (HOCl), with a combination of two plant-based agents; polygalacturonic acid (PG) and caprylic acid (CAP) for bacterial biofilm eradication and assessed their cytotoxicities towards fibroblast cells. **Material and Methods:** The efficacy PG+CAP ointment was compared to HOCl irrigant solution in biofilm eradication using the FGWB against clinical resistant bacterial isolates of MRSA, MDR *Pseudomonas aeruginosa*, CRE *Escherichia coli*, and *Streptococcus pyogenes*. Trypan blue exclusion and Alamar blue conversion assays were used for cytotoxicity assays. **Results:** PG+CAP produced a significantly greater reduction of viable organisms than HOCl for all tested bacterial isolates in the FGWB model ($P \leq 0.05$). Also, cytotoxicity tests showed that, PG+CAP was comparable to the non-antimicrobial negative control and was less cytotoxic than HOCl ($P \leq 0.05$). **Conclusion:** PG+CAP was highly effective against biofilms of highly resistant bacterial isolates in the FGWB model, and less cytotoxic than HOCl. PG+CAP merits further *in vivo* study.

Keywords: Polygalacturonic + caprylic acids (PG+CAP); Hypochlorous acid (HOCl); fibrin gel wound biofilm (FGWB) model; trypan blue exclusion assay; Alamar blue conversion assay; methicillin resistant *Staphylococcus aureus* (MRSA); multi-drug resistant (MDR) *P. aeruginosa*; carbapenem resistance *E. coli*; *Streptococcus pyogenes*

1. Introduction

Prolonged inflammation due to bacterial infection plays a significant role in the development of chronic wound biofilms due to delayed healing [1]. Treating bacterial chronic wound biofilms is a significant problem and is one of the main reasons for development of resistant bacterial isolates when antibiotic ointments are used [2]. In contrast to antibiotics, antiseptics which can be bactericidal or bacteriostatic, are used to reduce the presence of bacteria in wounds without inducing antibiotic-resistant bacteria [2–4]. However, the use of antiseptics during wound-healing has been associated with cytotoxicity [4]. Cytotoxicity also produces inflammation in wounds and contributes to delayed wound healing by degrading newly deposited granulation tissues [4–6]. Natural, plant-based agents, which can eradicate bacterial biofilms without leading to antibiotic resistance or cytotoxicity, are alternatives to antiseptics in the treatment of bacterially contaminated wounds that potentially offer an improved balance of antimicrobial activity without cytotoxicity [7].

Biofilm-producing bacteria are of great importance from public health perspective because they cause 80% of the infections that occur in the human body [8]. Chronic wounds represent a significant economic burden, including \$25 billion dollars annually in the United States alone [9,10]. Bacterial biofilms comprise microbial communities surrounded by extracellular matrices of polymeric macromolecules [2]. Bacteria within the biofilm become less susceptible to antimicrobial agents through physical barriers to access created by the biofilm extracellular matrix as well as by phenotypic adaptations including altering metabolism, genetic regulation, and protein expression [3,11]. Many traditional biofilm models have been used to study the efficacy of antimicrobial agents against pathogenic bacterial biofilms [12], but these traditional models are not representative of the three-dimensional matrix of microbes found in wound biofilms. Therefore, In the current study, we used a three-dimensional fibrin-gel wound biofilm (FGWB) model to be more representative of the three-dimensional extracellular matrix in which bacterial wound biofilms actually are enmeshed.

In vitro cytotoxicity assays are indicators of inflammatory reactions by damaged cells in wound beds [13,14]. Many *in vitro* methods can be used to investigate the cytotoxic effects of antimicrobial agents on tissue cells. We used the Trypan blue assay to distinguish living cells having intact viable membranes from lysed or disrupted cells that release inflammatory cytokines [15]. This is a coarse measure of whether contact with chemical agents kills or severely damages fibroblast membrane integrity causing release of inflammatory cytokines that recruit inflammatory cells to wound beds [15]. We also used the Alamar blue conversion assay was used to assess whether contact with chemical agents alters cellular metabolic activity [16–18]. Altered metabolic activity is more sensitive than trypan blue assay indicates whether contact with chemical agents impairs the ability of fibroblasts to perform their wound repair functions.

In the current study, we compared a widely used antiseptic agent in wound care, hypochlorous acid (HOCl), to a novel combination of two natural plant-based agents; polygalacturonic acid (PG) and caprylic acid (CAP). Previous studies have shown that PG+CAP is highly effective against pathogenic bacterial isolates in biofilms both *in vitro* and *in vivo* [19,20]. Hypochlorous acid, a widely used, commercially available, antimicrobial wound irrigant, has demonstrated fast acting inhibition of bacterial wound infection through disruptive reactions with different bacterial lipids, nucleotides, and proteins [21, 22). Although the mechanism of action of HOCl against microorganisms is not completely understood, many studies have reported that HOCl rapidly kills microbes through these oxidative chemical reactions [23–28]. Here we report studies of the comparative efficacies of PG+CAP and HOCl in eradicating bacterial biofilms using three-dimensional FGWB as well as their relative cytotoxicities towards fibroblasts cells which are essential for dermal wound repair and healing.

2. Materials and Methods

Antibacterial Agents:

We compared the efficacy of PG+CAP ointment with that of HOCl (PG: Sigma-Aldrich Inc, St. Louis, MO, USA, catalog #P3889-100G, CAS #25990-10-7; CAP: Sigma-Aldrich, catalog #03907, CAS #124-07-2; HOCl: Aqua Science Inc, Columbus, OH, USA, catalog #01S.06E, CAS #7790-92-3). PG+CAP ointment was prepared in a laboratory as previously described by Gerges *et al* [19], and the HOCl wound solution was used at a concentration of 400 ppm as directed by the manufacturer. An aqueous gel containing 2-hydroxyethylcellulose (Fisher Scientific/Janssen Pharmaceuticals [now Johnson & Johnson Innovative Medicine], Belgium, catalog #G33-500, CAS #56-81-5) was used as the ointment base for PG+CAP ointment.

Three-Dimensional FGWB Model:

A quantitative, *in vitro*, three-dimensional FGWB model adapted from the model of Besser and Stuermer [29] and prepared according to the specifications of Truong *et al* [30] and Gerges *et al* [31] was used in the current study. Briefly, 20 mg/mL fibrinogen (Fisher Scientific, catalog #34-157-61GM) was slowly dissolved in phosphate-buffered saline (PBS) at 37° C; 5 units/mL thrombin (Sigma-Aldrich, catalog #T7009-100UN; CAS #9002-04-4) was dissolved in PBS; and 125mM calcium chloride (Sigma-Aldrich, catalog #C3306-100G, CAS #10035-04-8) was dissolved in deionized water.

Biofilm Eradication Assay:

Biofilm eradication testing was conducted using highly virulent clinical bacterial isolates of MRSA (MDA #120), multidrug-resistant (MDR) *Pseudomonas aeruginosa* (MDA #118), carbapenem-resistant Enterobacterales (CRE) *Escherichia coli* (MB #9245), and *Streptococcus pyogenes* (MB #3175) as representative hospital-acquired infection pathogens from cancer patients. The organisms were grown from glycerol stock on trypticase soy agar + 5% sheep blood (Remel, Lenexa, Kansas, USA, reference #R01202). Each organism was inoculated into Muller Hinton broth (Fisher Scientific, BBL Mueller Hinton II Broth Cation Adjusted, Sparks, MD, USA, catalog #B12322) and diluted to 0.5 McFarland. Additional dilutions were made to obtain $\sim 10^4$ colony-forming units/mL (CFUs/mL). The efficacy of PG+CAP versus HOCl was determined using the FGWB model as described by Gerges *et al* [31]. Six replicates of FGWB were used for each of PG+CAP, HOCl, and negative control against each organism. Regrowth experiments were conducted as described by Gerges *et al* 2025 [31] to ensure that eradication was complete on FGWB disks from which no viable colonies were recovered following exposure to PG+CAP or HOCl.

Cytotoxicity Tests:

1. Culturing of Fibroblasts cells:

Fibroblast NCTC clone 929 (L cell, L-929, derivative of Strain L) was used in the current study. Cell culture was performed according to fibroblast protocols as described by Gomes *et al* 2011 [32]. and as follows; the vial of fibroblasts was thawed by gentle agitation in a 37° C water bath for approximately 2 minutes, under strict aseptic conditions. The vial content was transferred to a centrifuge tube containing 9.0 mL complete culture medium and spun at $400 \times g$ for 10 minutes. The complete medium consisted of ATCC-formulated Eagle Minimum Essential Medium (Sigma-Aldrich Inc, Millipore Sigma, USA, catalog #M4655) plus horse serum (ATCC 30-2040) at a final concentration of 10%. The cells pellet was resuspended with 4 mL of complete medium, then 1 mL of this solution was added to 10 mL of complete medium in a T-25-cm² tissue culture flask and incubated at 37° C + 5% CO₂. The fibroblasts were refreshed every 48 hours by replacing the old medium with fresh medium, and fibroblasts were observed daily under inverted microscope for 60-70% confluence.

2. Subculturing procedure:

Briefly, the old culture medium was removed and discarded, and the cell layer was rinsed with Hanks balanced salt solution (Sigma-Aldrich Inc, Millipore Sigma, USA, catalog #H6648) to remove all traces of serum that contained trypsin inhibitor. Two milliliters of trypsin-EDTA solution 0.25% (gibco reference #2500-056) were added to the flask, and the cells were observed under an inverted microscope until the cell layer was dispersed. Then, 8.0 mL of complete growth medium was added, and cells were aspirated by gently pipetting. Appropriate aliquots of the cell suspension were added to a new culture T-flask and incubated at 37° C + 5% CO₂.

3. Cytotoxicity techniques:

PG+CAP ointment was compared with HOCl irrigant solution for cytotoxicities using the Trypan blue exclusion assay as described by Strober [15] and the Alamar blue conversion assay as described by O'Brien *et al* [16]. Briefly, for the Trypan blue exclusion assay; 1 mL of culture medium containing 20,000 cells/mL was added in each well of a 24-well tissue culture plate, then an equal quantity of tested antimicrobial agent was added to corresponding wells and incubated for 24 hours at 37° C + 5% CO₂. All content of each well was collected in small tubes and centrifuged at 1160 rpm for 7 minutes. Supernatant was discarded, and 1 mL of medium (without serum) was added to each tube and mixed gently, and then 50 μ L of the cell suspension was added in a cryovial, with an equal quantity of Trypan blue dye 0.4% (gibco reference #1520-061), mixed gently up and down, and incubated for 1-2 minutes at room temperature as recommended [32]. Then, 10 μ L was added into a hemocytometer to count unstained cells (live cells) and stained blue cells (dead cells) separately. The percentage of viable cells were calculated as follow:

% of viable cells = total number of viable cells $\times$ 100/total number of viable and dead cells.

For the Alamar blue conversion assay; every well of 96-well tissue culture plates was seeded with 100 μ L of the suspension containing 3,200 cells/well, incubated at 37° C + 5% CO₂, and observed daily for 60-70% confluence (~72 hours). The old medium was then aspirated and washed with Hanks balanced salt solution. Antimicrobial agent was added to each corresponding well and incubated for 24 hours at 37° C + 5% CO₂. The medium containing ointments was replaced with only Hanks balanced salt solution, and then 10% volume (10 μ L) of Alamar blue dye (Alamar blue cell viability reagent, Thermo Fisher Scientific/Invitrogen, reference #DAL 1025) was added and incubated for 4 hours at 37° C + 5% CO₂. The plate was read at 570 nm using a spectrophotometer, and the absorbance for each tested agent was compared with that of cells treated with phosphate-buffered saline (negative control). PG+CAP ointment and HOCl irrigant were used at 2% concentrations.

Statistical Analysis:

To compare the efficacies of the treatments in biofilm eradication, colony-forming unit/milliliter (CFU/mL) values for each bacterial isolate were analyzed across the three groups; PG+CAP, HOCl, and negative control, using the Kruskal–Wallis test. When this test detected a significant difference, pairwise comparisons were carried out using the Wilcoxon rank-sum test. To control the overall Type I error from multiple comparisons, p-values were adjusted using the Holm-Bonferroni method. The reduction in CFU/mL relative to the negative control, expressed as log₁₀, was also computed for both the PG+CAP and HOCl treatment groups. For cytotoxicities evaluation, viable cell counts following treatment with PG+CAP or HOCl were compared individually against the negative control using the Wilcoxon rank-sum test. All tests were two-sided, with a significance level set as $P \leq 0.05$. Data analyses were conducted using SAS version 9.4 (SAS Institute Inc., Cary, NC, USA).

3. Results

Figure 1 presents the median recovered viable colonies (CFUs/mL.) of tested infectious bacterial biofilms of MRSA, MDR *P. aeruginosa*, CRE *E. coli*, and *St. pyogenes* after three-hours of exposure to PG+CAP or HOCl in a FGWB model. **Table 1** presents the log₁₀ reductions in CFUs/mL. After three-hours of exposure, relative to the negative control, PG+CAP reduced viable MRSA CFUs/mL by 7.9 log₁₀, and HOCl reduced MRSA CFUs/mL by 4.2 log₁₀. PG+CAP and HOCl reduced viable MDR *P. aeruginosa* CFUs/mL by 8.28 log₁₀ and 2.32 log₁₀, respectively, CRE *E. coli* CFUs/mL by 4.26 log₁₀ and 1.69 log₁₀, respectively, and *St. pyogenes* by 5.12 log₁₀ and 3.0 log₁₀, respectively. The antibacterial superiority of PG+CAP relative to HOCl was statistically significant ($P \leq 0.05$) against all tested bacterial isolates.

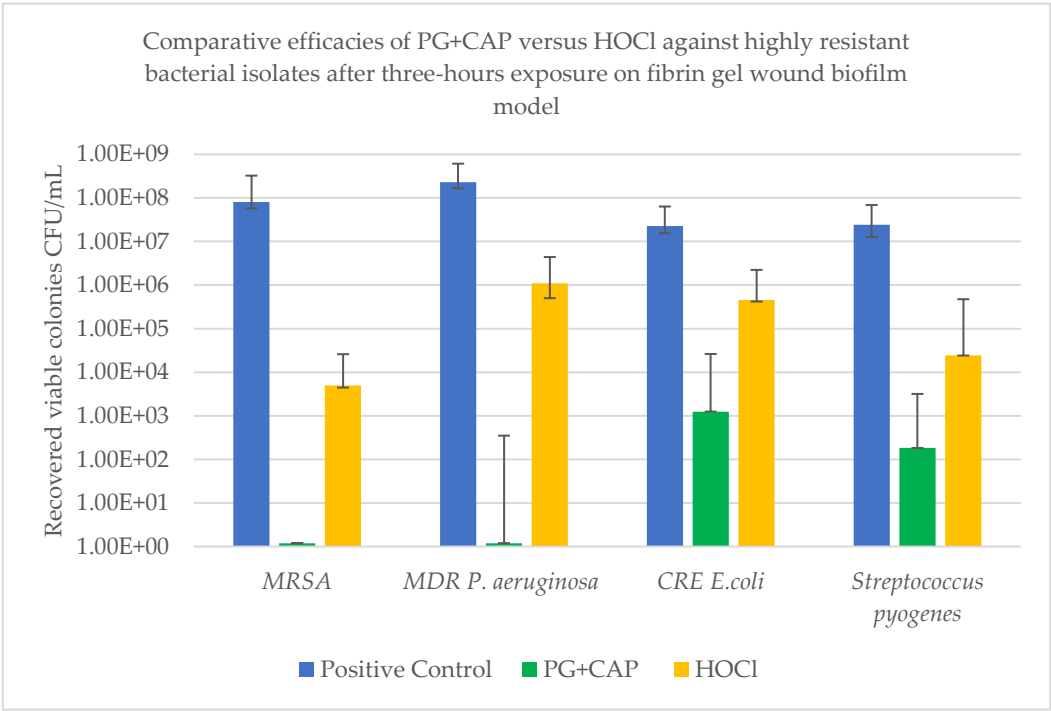


Figure 1. Efficacies of PG+CAP and HOCl against tested bacterial biofilms after three-hours exposure using fibrin gel wound biofilm model. Values are presented as the median number of recovered viable colony-forming units/mL (CFUs/mL) for six replicates. Bars indicate the median numbers of recovered viable colonies, and error bars indicate the ranges. Abbreviations: MRSA, methicillin resistant *Staphylococcus aureus*; MDR, multi-drug resistant; CRE, carbapenem-resistant Enterobacterales.

Table 1. Log₁₀ reduction of colony-forming units/mL (CFUs/mL) for tested bacterial isolates after three-hours of exposure to polygalacturonic acid + caprylic acid or hypochlorous acid in a fibrin-gel wound biofilm model.

Efficacy variable	Tested organism			
	MDR		CRE	
	MRSA	<i>Pseudomonas aeruginosa</i>	<i>Escherichia coli</i>	<i>Streptococcus pyogenes</i>
Log ₁₀ median viable colonies for negative control	8.00E+07	2.31E+08	2.25E+07	2.43E+07
Log ₁₀ reduction of CFU with PG+CAP relative to negative control	7.9	8.28	4.26	5.12
Log ₁₀ reduction of CFU with HOCl relative to negative control	4.2	2.32	1.69	3.0
P value for PG+CAP versus HOCl	0.0028	0.0043	0.005	0.019

Abbreviations: MRSA, methicillin resistant *Staphylococcus aureus*; MDR, multi-drug resistant; CRE, carbapenem-resistant Enterobacterales. Statistical comparisons between PG+CAP and negative control, as well as between HOCl and negative control, were statistically significant for all tested bacterial isolates ($P \leq 0.05$).

Table 2 shows results of the Trypan blue exclusion cytotoxicity assay. The mean number of viable fibroblast cells after treatment with PG+CAP was statistically similar to that of the negative control cells after treatment with phosphate-buffered saline ($P = 0.26$). Treatment with HOCl led to significantly reduced cell viability of fibroblasts cells compared with PG+CAP and negative control ($P \leq 0.05$).

Table 2. *In vitro* cytotoxicity assessment of cell viability of L-929 fibroblasts after exposure to 2% polygalacturonic acid + caprylic acid (PG+CAP) ointment and 2% hypochlorous acid (HOCl) irrigant for 24 hours, using the Trypan blue exclusion method.

Treatment	Mean $\pm$ SD cells		% Viable	P value*	P value**
	Live cells	Dead cells			
Negative control	$2.69 \times 10^6 \pm 7.59 \times 10^4$	$6.0 \times 10^4 \pm 8.16 \times 10^3$	97.82	0.26	-
2% PG+CAP	$2.65 \times 10^6 \pm 3.77 \times 10^4$	$6.5 \times 10^4 \pm 5.77 \times 10^3$	97.61	-	0.26
2% HOCl	$2.46 \times 10^6 \pm 4.83 \times 10^4$	$4.43 \times 10^5 \pm 1.50 \times 10^4$	84.76	<0.001	<0.001

Figure 2 and Table 3 present the results of the Alamar blue conversion metabolic activity assay. Fibroblasts cells treated with PG+CAP had similar metabolic activity to that observed in the negative control that treated with phosphate-buffered saline ($P = 0.083$), whereas the metabolic activity of fibroblasts cells was significantly reduced after treatment with HOCl compared with negative control fibroblasts cells and fibroblasts cells treated with PG+CAP ($P \leq 0.05$).

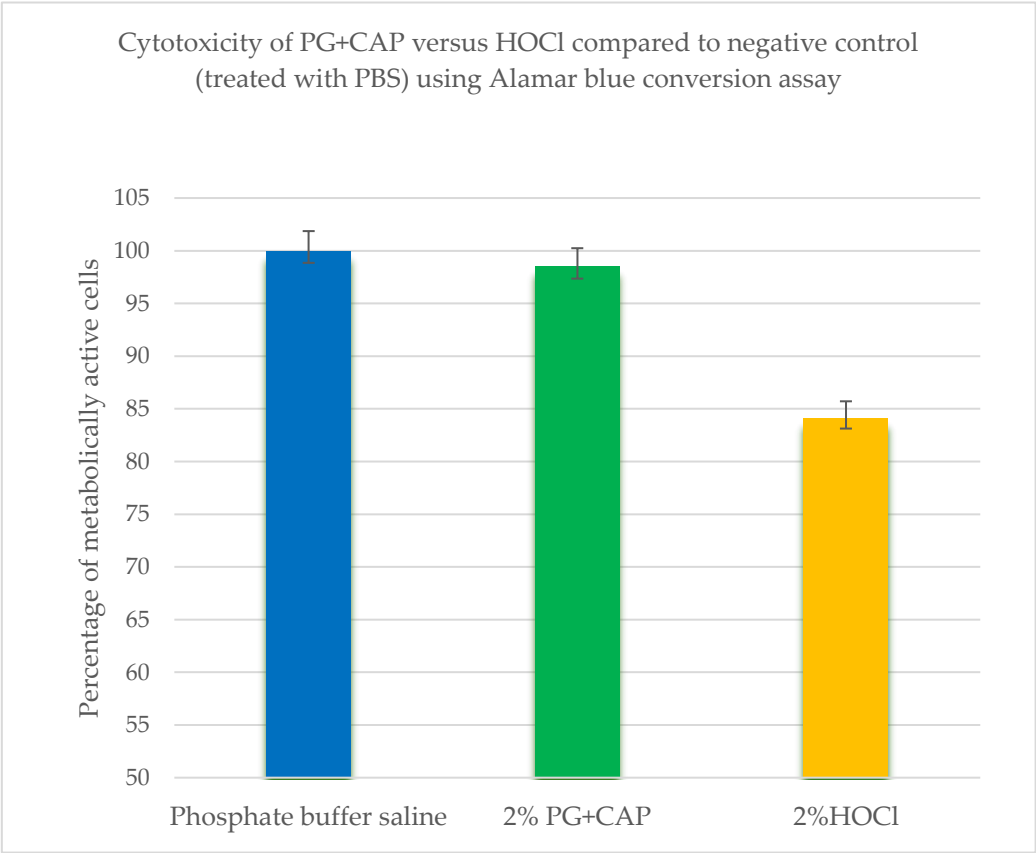


Figure 2. *In vitro* cytotoxicity assessment of cell metabolic viability of L-929 fibroblasts after exposure to 2% PG+CAP ointment and 2% of HOCl irrigant for 24 hours, using the Alamar blue conversion metabolic assay. Treated cells with phosphate buffer saline were used as negative control. Results are expressed as a percentage

of absorbance relative to negative control as measured by a spectrophotometer at 570 nm. Higher metabolic activity indicates lower cytotoxicity.

Table 3. *In vitro* cytotoxicity assessment of cell metabolic activity of L-929 fibroblasts after exposure to 2% polygalacturonic acid and caprylic acid (PG+CAP) ointment or 2% hypochlorous acid (HOCl) irrigant for 24 hours, using the Alamar blue conversion metabolic assay.

Treatment	Median reading	Interquartile range	P value*	P value**
Negative control	1.124	1.121 - 1.129	0.083	-
2% PG+CAP	1.111	1.108 - 1.131	-	0.083
2% HOCl	0.942	0.848 - 1.00	0.014	0.014

Treated cells with phosphate buffer saline (PBS) were used as negative control. *P-value is from the Wilcoxon rank-sum test comparing the cells viability between the corresponding treatment and PG+CAP. **P-value is from the Wilcoxon rank-sum test comparing the cells viability between the corresponding treatment and negative control.

Results are expressed as the absorbance relative to control as measured by a spectrophotometer at 570 nm. Higher metabolic activity indicates lower cytotoxicity. Treated cells with phosphate buffer saline were used as negative control. *P-value is from the Wilcoxon rank-sum test comparing the metabolic activity between the corresponding treatment and PG+CAP. **P-value is from the Wilcoxon rank-sum test comparing the metabolic activity between the corresponding treatment and control.

4. Discussion

Acute inflammation plays an important role in normal wound healing. The acute response to dermal wounds includes recruitment of neutrophils, macrophages, and lymphocytes which clear damaged tissue and colonizing bacteria through phagocytosis, secretion of reactive oxidative moieties (such as HOCl) and secretion of proteases [33,34]. Subsequently a key part of normal wound healing as inflammation subsides is the transition to the proliferation and remodeling phases in which cytokine-recruited fibroblasts and keratinocytes take over key roles [35]. Fibroblasts play key roles in generating new granulation tissue over which keratinocytes can proliferate to re-epithelialize the wound [36].

Chronic non-healing wounds occur when the inflammatory phase becomes entrenched preventing transition to the proliferation phase [37]. Aberrant inflammation associated with chronic non-healing wounds frequently contains bacteria that are able to survive and evade immune reactions or antibiotics by forming protective biofilms [38]. Furthermore, sustained tissue damage by chemotherapeutic agents can further impede successful transition [1]. Hence, therapies to restore normal healing of chronic wounds need to be able to eradicate bacteria in wound biofilms. In the current study, we allowed bacteria to form mature protective biofilms enmeshing in both bacterial extracellular matrix as well as fibrin, an abundant protein present in inflammatory wound exudate [39]. Our experiments showed a single treatment of PG+CAP for three hours was highly effective in eradicating bacteria in the mature fibrin gel biofilms but did not fully eradicate all bacteria. MRSA and MDR *P. aeruginosa* biofilms were fully eradicated; however, CRE *E. coli* and *St. Pyogenes* had reduced viable bacterial concentrations (over 4 Log₁₀ reductions) but were not fully eradicated in a single treatment. The three-hours exposure was selected as a representative duration over which the applied ointment would not be expected to become excessively diluted by diffusion from the wound bed or dilution by wound exudate. The tested bacteria are common resistant dermal wound pathogens associated with chronic non-healing wounds [40,41]. The PG+CAP results suggest multiple continued applications of PG+CAP would be required to sustain minimal bacterial bioburden in chronic wounds allowing granulation tissue formation to proceed without excessive inflammatory disruption. HOCl was significantly less effective than PG+CAP in reducing bacterial

bioburden in fibrin gel biofilms against all tested bacteria. This suggests once biofilm matures, a robust bacterial population could survive HOCl irrigation and continue inducing inflammatory responses.

Effective chronic wound therapies further need to optimize eradication of bacterial biofilms with minimizing cytotoxic effects on proliferative cells so as not to produce inflammation that is counterproductive to chronic wound healing and closure. The results of our experiments show that PG+CAP was comparable to control and significantly superior to HOCl in minimizing cytotoxic reaction against fibroblasts. Fibroblasts play a key role in wound healing proliferative and remodeling phases and especially in production of new granulation tissue. Thus, repetitive applications of HOCl, although not necessarily damaging to tissue, might impair critical healing activities of fibroblasts.

HOCl exerts its antibacterial activity primarily by oxidative chemical reactions [42]. It may therefore be limited in the three-dimensional fibrin gel wound biofilms by being consumed through non-specific oxidative reactions with extracellular matrix molecules. Bacterial extracellular matrix and fibrin are rich in oxidizable primary and side chains that can rapidly consume the HOCl before is able to react with resident bacteria [43]. Our finding of limited HOCl antibacterial efficacy against bacteria embedded in complex matrices like wound biofilms has similarly been reported by other authors [42,44]. PG+CAP synergistically works by CAP disrupting bacterial membranes with PG providing optimal pH and emulsifying CAP for optimal bioavailability [7,45]. CAP has been shown to provide an optimal lipid chain length for selectively minimizing disruption of human cell membranes while killing bacteria. Longer chain fatty acids produce progressively increase human cell membrane disruption [46]. In addition, PG has been shown to inhibit destructive wound matrix metalloproteases (MMP-2 and MMP-9) produced during inflammation responses while maintaining wound hydration [47]. PG is derived from pectin in tree fruits such as apple and is readily available at low cost [48]. CAP can be extracted from coconut and is also readily available at low cost [49,50]. Thus PG+CAP is expected to be a practical plant-based wound treatment technology.

Our study has several limitations. It was entirely *in vitro*; thus *in vivo* verification is required. A pilot six-weeks clinical trial did demonstrate safety and effectiveness of PG+CAP in improving chronic wounds in humans [20], but additional *in vivo* study is required. The models used in this study attempted to replicate many real complexities of chronic wound biofilm extracellular matrixes; however, the full complexity of biofilms in wound beds may not have been entirely replicated. Also, wounds frequently are colonized by multiple bacterial species while our models were single species. Nevertheless, our results showed that HOCl may have limited effectiveness in chronic wounds heavily colonized with mature bacterial biofilms and that PG+CAP is a promising alternative with superior antibiofilm and safety profiles that merits further study.

Author Contributions: BG, JR, and IR designed the study. BG, Y-LT performed bacterial biofilm eradication and cytotoxicity tests. YJ performed the statistical analysis. BG, JR, and IR reviewed and analyzed data and contributed conclusions. BG, and JR wrote the manuscript. All authors read, commented on, and approved it. IR reviewed the final manuscript and approved it.

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Data Availability Statement: Data is available on request.

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Institutional Review Board Statement: Not applicable, because this study is not a clinical study.

Informed Consent Statement: Not applicable, because this study is not a clinical study.

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Conflicts of Interest: Issam Raad and Joel Rosenblatt are co-inventors of the polygalacturonic acid + caprylic acid technology, which is owned by The University of Texas MD Anderson Cancer Center. The other authors have no competing interests. All authors approve the submission.

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