Original Article

Evaluation Of Silver Nanogel Loaded With Micronised Activated Charcoal For Wound Healing Applications

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Abstract

This study reports experiments that aims to combine silver and activated charcoal in a gel formulation to potentially create a gel that is suitable for use in treating infected wounds and prevent wounds from being infected; Method: Gels that were created were characterised via pH, moisture content, and spreadability analysis. Fourier Transformed Infrared (FTIR) spectrometry was used to check for the functional groups present in the formulation to ensure that the components of the gels were properly incorporated. Stability testing was tested by storing gels in different temperatures and rechecking the physical characteristics. Finally, for its antibacterial activity assessment, staphylococcus aureus ATCC 25923 strain was used to perform antibacterial studies via well diffusion assay and Minimum Bactericidal Concentration (MBC) measurement described by a previous paper. Results: Stability studies showed that all physical properties of the gel generally maintained, while antibacterial effect in disc diffusion testing and minimum bactericidal concentration testing was inconclusive, with one formulation showing a significantly smaller zone of inhibition; Conclusions: The gels made in this experiment seemed to not show antibacterial effect that was consistent, future studies should replace silver nitrate with silver sulfadiazine in formulation and in vivo testing should be considered.

Keywords: silver nitrate, silver sulfadiazine, nanogel, antibacterial, activated charcoal, wound infection.

INTRODUCTION

With regards to wounds, numerous topical wound dressings and formulations containing silver are now available for use in infected wounds, with examples being stated in the British National Formulary [1]. Silver can also be made into nanoparticles which were first made into nanoparticles as far back as 120 years ago, where particles as small as 7 to 9nm were reported to have been made [2]. Before, these so called 'nanosilver' formulations were also used by doctors to treat bacterial infections such as syphilis and were even sold over-the-counter [3], where prescriptions are not needed to get these products [4]. In addition, this therapeutic effect of silver can be achieved by only using a small amount of silver, with one article claiming that an ideal dressing with silver would be one that has a concentration of as little as 30 parts per million (ppm) and above of silver ions [5]. However, as with all medicine, silver dressings and formulations can occasionally give side effects when used, with notable examples including silver sulfadiazine causing a burning sensation, itching, rashes and argyria [5,6], where an accumulation of silver in the body occurs and manifests itself as the skin being discoloured to either a blue or a black colour [7]. Argyria itself may not be life threatening, but many articles have discussed how having the skin discoloured is considered by many people to be negative [3,7,8]. Nevertheless, silver is still used in infected wounds despite these side effects, and is also used as an adjunct in treating and preventing infections of wounds, from leg sores to even where skin grafts are made [1]. With regards to silver and antibacterial activity, cell wall puncturing [9], ribosome denaturation [10], adenosine triphosphate (ATP) production interference [11], and deoxyribonucleic acid (DNA) replication interruption [12] are some of the proposed mechanisms of actions by which antibacterial effects are achieved. Additionally, silver is antibacterial as they can cause membrane denaturation, being achieved by the accumulation of silver nanoparticles [13]. In addition to these mechanisms, reactive oxygen species that are produced from the damage that is already present when silver targets the electron transport chain [11] in the bacteria can cause further damage, with the reactive oxygen species disturbing the cell membrane and binding to DNA to prevent DNA replication [14]. Other than antibacterial effects, silver is also used in other aspects of medicine and health, such as diagnosis of cancer and neuropathy [15,16]. Other than antibacterial effects, silver is also used in other aspects of medicine and health, such as diagnosis of cancer and neuropathy [15,16].

Derived from acrylic acid, Carbopol® is a polyacrylic acid polymer that is also mucoadhesive which means that it can extend the time in which medicine that is found within a formulation is in contact with a membrane [17]. Carbopol can be made into a gel when it is neutralised, including Carbopol® 940 in this experiment, and this is due to the fact that when it is neutralised, cross bonds would be formed and therefore, be able to absorb and hold water [18,19]. In medicine, Carbomer is utilised in many ophthalmic gel formulations, with some of the products using Carbomer as a vehicle for medication such as pilocarpine and ganiciclovir, or using Carbomer as the therapeutic agent itself to be applied to dry eyes [20–22]. While the polyacrylic acid polymer used in this experiment is Carbopol® 940, there are other types of the same polymer under different names such as Carbopol® 910, 934, 934P, 941 and 962[23]. These codes refer to the different molecular weights and components that are found within each type [24,25]. There are also other forms of Carbopol® where the compounds are modified, one example being the Carbopol® Ultrez 20 [26]. In addition to Carbopol® 940 being non-toxic and non-irritating, Carbopol® 940 gels have shown to increase contact time of drugs with the applied area as described by a systematic review that concluded that Carbopol 940 in eye drops achieved this effect [27]. In terms of wounds, meanwhile, one study has shown that improved fluid flow to the tissue is achieved when carbomer 940 was used in the experiment treating on burn wounds [28] and even lowers the extent to which tissues that are dead that burn wounds usually cause [28].

PVA is another polymer that is used in many instances from making paper [29] to all the way to 3D printing, with one study using 3D printing to form filaments of PVA to contain either paracetamol or caffeine [30]. Wound care also sees benefit from PVA use, as many PVA wound dressing hydrogels are available, though to compensate for its drawback for not being elastic enough, many of its examples would include dressings that contain chitosan [31], alginate [32], starch [33], and carrageenan [34,35].

Sangelose® is the brand name given to the Hydroxypropyl methylcellulose stearoxy ether that is produced by Daido Chemical Corporation, and it is also called hydrophobically-modified hydroxypropyl methylcellulose (HPMC) [36]. In terms of its use in research, it has seen use in creating glimepiride tablets that disintegrate when taken orally [37], creating a metronidazole gel [38], and also a diclofenac potassium gel, the third of which also looked into the different types of sangelose, being the 60L, 60M, 90M for the hydrophobically-modified HPMC while comparing it to the conventional and hydrophilic 50cPs HPMC [39].

Activated charcoal is called as such due to its processing, where the carbon material is heated with a hydroxide of an alkali metal which then produces carbon that is said to be “active” [40]. The carbon material can come from coconut shells [41] such as the ones used in this experiment, but it can be made from other sources, such as rice husks [42] and olive waste [43]. In addition to its high density, and purity [44], coconut shell as a waste product is abundant and is often used in other areas, one of which being construction [45]. As it can adsorb many substances that are harmful and noxious, activated charcoal has seen many uses in terms of poisoning and overdoses due to it preventing the absorption of the noxious substances into the bloodstream [46]. With regards to wounds, activated charcoal or activated carbon is used in some wound dressings, with two examples being the Actisorb [47] and Clinisorb, the former stating it is able to adsorb bacterial toxins [47] and the latter stating it is able to control wound odour respectively [48,49].

A recent study in 2021 that looked back at three years of bacteria that was taken from infected wounds and the study recorded down the frequency that each pathogen was observed [50]. The study revealed that most common species were Gram-negative, with Pseudomonas aeruginosa being the most frequent bacteria from the Gram-negative groups [50]. However, despite that the Gram-positive group making the smaller proportion of the overall percentage, Staphylococcus Aureus ended up being the pathogen that was observed the most, with 90 cases being reported as opposed to even Pseudomonas aeruginosa with 72 cases [50]. Other studies have shown similar results, with Staphylococcus Aureus dominating the cases of Gram-positive bacteria infecting wounds [51–53]. It is also because of this prevalence of Staphylococcus Aureus in wound infection that it was selected as the pathogen to be studied in the antibacterial testing.

The four overlapping phases of wound healing, haemostasis, inflammation, proliferation, and remodelling [54] can be affected by infections, particularly extending the inflammation phase as levels of inflammatory cytokines are elevated due to the bacteria and endotoxins [55]. With reference to silver sulfadiazine, meanwhile, silver sulfadiazine has been reported to delay wound healing, with one review showing multiple references that has shown to slower epithelialisation [5]. One article has also observed that silver is highly toxic to fibroblast [56], of which is important for wound healing [57]. A 2004 article showed that to maintain antibacterial effect without adversely affecting epidermal cells is a 0.5% silver nitrate solution [58].

MATERIALS AND METHODS

Materials

Silver Nitrate, and Pluronic F127 originated from Sigma Aldrich, Darmstadt, Germany. Meanwhile, activated coconut shell charcoal (Micronized activated charcoal powder) will be procured from Take It Global Sdn Bhd, Penang, Malaysia. Polyvinyl alcohol (PVA) was purchased from Merck KGaA, Darmstadt, Germany. The Carbopol® was from Otto Chemi Private Limited, Mumbai, India. Sodium Hydrogen Carbonate was from Glentham Life Science, Corsham, United Kingdom. Sangellose 60M will be from Daido Chemical Corporation, Osaka, Japan. Distilled water was obtained from the school laboratory.

Table 1: Table of composition of each formulation

Formulation	F1	F2	F3	F4	F5	F6	F7	F8
$AgNO_3$ (mg)	20	20	20	20	48	48	48	48
Water (ml)	80	80	80	80	80	80	80	80
PVA (mg)	800	2000	0	0	800	2000	0	0
Carbopol 940 (mg)	0	0	200	400	0	0	200	400
Pluronic F127 (mg)	2000	2000	0	0	2000	2000	0	0
$NaHCO_3$ (mg)	0	0	40	80	0	0	40	80
Sangelose® 60M (mg)	3200	3200	0	0	3200	3200	0	0
Activated charcoal (mg)	1000	1000	1000	1000	1000	1000	1000	1000

Preparation of Silver (ag) nanogel loaded with activated charcoal

PVA gels was made by dissolving distilled water with PVA with a magnetic stirrer until the added polymer has completely dissolved with a heated magnetic stirring board. From here, silver nitrate and Pluronic F127 was added and mixed for one hour. Afterwards, activated charcoal was added in and stirred for five minutes.

Meanwhile, the Carbopol formulations had the silver nitrate dissolved in the water first before having the Carbopol added. Once the Carbopol was added, the mixture was left to be stirred for one hour. Activated charcoal was then added and mixed for a further five minutes. Sodium Hydrogen Carbonate was then used to gel the Carbopol formulations.

Once formulations were made, glass amber jars were used to store them, with each glass amber jar holding 40g. Once made, jars of each formulation were either used for testing or placed into different conditions for stability testing, detailed in the stability testing section.

Composition of formulations

Table 1 shows the amount of each ingredient that is used in each formulation. Activated charcoal and water remains constant for all formulations.

Measurement of pH

pH was measured by the Fisher Scientific Accumet AE150 (Thermo Fisher Scientific, Massachusetts, United States of America). Before the machine was used, the machine was calibrated by measuring Thermo Scientific Orion Application Solution 910107 pH 7.00 Buffer (Thermo Fisher Scientific, Massachusetts, United States of America). The probe provided was then placed into the gel directly and the pH that it recorded was used. Four measures were taken and an average was calculated and reported. This was also done for the stability testing at 37 days.

Moisture content measurement

Moisture content was measured by using the Shimadzu Corporation Model MOC63u Moisture Analyser (Shimadzu, Kyoto, Japan). Each time a formulation was measured, an aluminium foil is folded and placed onto the plate. Once the mass of the built-in weighing machine was zeroed with the foil on, approximately 1g of formulation was weighed and then spread out to ensure even drying. This measurement was done four times for each formulation and an average was taken to record the results.

Spreadability testing

The method used is modified from Chaudhary and Verma in a 2014 article [59].After measuring 1g of one formulation, the formulation is placed onto a white tile. It was then pressed on with a petri dish and subsequently weighted down by a 1L glass conical flask filled with approximately 400g of water for 15 minutes. Once that was complete, the diameter of the formulation was measured. As the spread of the formulations did not show a perfect circle, the diameter of each formulation was measured crosswise and diagonally, resulting 4 diameters being measured from one spreadability test. An average was then calculated to give the average diameter from that one test. Each formulation then had this repeated 3 more times, giving 4 average diameters that was then used to calculate another average diameter.

Toxicity studies

A toxicity study was performed with mung bean seeds (vigna radiata) [60], with reference to an article in 1996, with some modifications [61]. A container of green bean seeds was soaked overnight in tap water, and then rinsed with distilled water. To new dry containers, cotton balls, instead of petri dishes and Whatman filter papers, were placed compactly and 20 green bean seeds were planted. 10g of each formulation was then dissolved in 100ml of water, from which then approximately 10g of the solution was used to water the beans. The watering was done twice a day, for five days. From there, the number of seeds germinated and the radicle length of 6 sprouts with the longest radicle length from each container was measured. The number of seeds germinated, instead of being defined as a seed attaining a radicle length of at least 5mm, is defined in this paper as the presence of the radicle protruding the testa as counting radicles that have grown at least 5mm are counted as post-germination [62]. The average and standard deviation was then calculated. From there, the percentage of seeds germinated and the relative percentage root growth were then calculated with respect to the control using equations 1 and 2 respectively.

The equation that is given in the 1996 paper for Equation 2 includes a square root in the equation for GI. However, the reference that was used for the equation that was used in that paper referenced a paper from 1994, of which the percentage root growth equation shows no square root [63]. Because of that, the 1994 version of the equation will be used instead of the one reported in the 1996 paper. The two values were then used to calculated germination index (GI), given with Equation 3.

$$\%\ \text{seed germination} = \frac{\text{germination \% in formulation}}{\text{germination \% in control}} \times 100\% \quad (1)$$

Equation 1: Percentage seed germination equation

$$\%\ \text{root growth} = \frac{\text{mean root length in formulation}}{\text{mean root length in control}} \times 100\% \quad (2)$$

Equation 2:Percentage root growth equation

$$\text{Germination index (\%)} = \frac{\%\ \text{seed germination} \times \%\ \text{root growth}}{100\%} \quad (3)$$

Equation 3:Germination index equation

Fourier Transformed Infrared (FTIR) spectroscopy

FTIR spectroscopy was performed using the Shimadzu IRSpirit QATR-S spectrophotometer (Shimadzu, Kyoto, Japan). The spectrophotometer was run for 30 scans and was read at 100% transmittance cut off. As F3 and F8 were the optimized formulations, the PVA, Pluronic F127, and Sangelose 60M were not read as those are not present in the F3 and F8 formulations. As multiple components are to be included in one graph, the actual absorbance of the components and formulations had to be adjusted by adding a 100% transmittance gap to create the transmittance graph.

Stability

Originally, the stability testing was to be performed on days 7, 14, and 28. However, due to time constraints with the COVID-19 situation at the time of writing, the stability testing days were changed to just day 37.

Amber glass jars of the formulations would be kept under 4˚C, 40˚C and 25˚C (room temperature) for 37 days. The temperatures at which the formulations are stored are based on a study performed in 2015 [64]. At the 37th day, the pH, moisture content, and spreadability will be checked as per the protocol mentioned in each subsection respectively.

For jars to be kept under 40˚C (±2˚C), the jars with formulations are kept in a Memmert CO_2 incubator under 75% humidity. Meanwhile, jars to be kept under 4˚C (±2˚C) are placed in a cold room that is available in the campus' laboratories. Finally, for the jars to be kept under room temperature, they are simply kept in a drawer that is found on the lab benches.

Bacterial studies (Bacteria growing, Well diffusion zone of inhibition, Minimum Bactericidal Concentration)

Staphylococcus aureus bacteria were chosen as per literature review over its prevalence of occurrence in wounds [50]. The ATCC 25923 strain was chosen to perform this experiment. For growing, mannitol salt agar was used to grow the ATCC 25923 staphylococcus aureus as it is a selective medium[65]. After forming a 4mm deep mannitol salt agar in an 8.5cm petri dish, a sterilized inoculating loop was used to inoculate thawed ATCC 25923 staphylococcus aureus onto the mannitol salt agar. Once inoculated, the petri dish was then placed in a Thermo Scientific MaxQ SHKE 6000 shaking incubator at 37˚C, while the shaker is kept stationary. The bacteria were confirmed to be Staphylococcus aureus through light microscopy and showing a positive catalase test [65]. This growing procedure was performed each time when bacteria colonies are required should a previous batch has been kept for over a week since being made. Bacterial suspension by adding bacterial colonies to Mueller-Hinton Broth was then made by referring to a 0.5 McFarland Standard for well diffusion assay and 1.0 McFarland Standard for the MBC and comparing the turbidity of both suspensions visually [66].

For the well diffusion test, the CLSI guidelines were used to develop this protocol [67]. Mueller Hinton Agar (MHA) (Oxoid CM0337 Muller-Hinton Agar IVD) was first made according to the ratios indicated on the package. Once autoclaved and poured in petri dishes, the agar was kept under 4˚C overnight to allow to harden and set. To each petri dish of agar, 100µL of 0.5 McFarland Standard adjusted bacteria suspension was added and spread using a plastic Biologix cell spreader (Cat65-1001). After allowing it to dry, 6mm diameter holes were then made using metal borers, with each petri dish having 4 holes at least 24mm apart from each other. F3 and F8 gels are dissolved in Mueller-Hinton Broth (#69444, Bio-Rad, Hercules, California, United States of America) by using a Genie Vortex 2 machine so that dilutions of 50% of each gel are made. This was made by using 5g of gel and 5ml of Muellor Hinton Broth. 10% w/v of silver sulfadiazine (SSD) cream was also made as a positive control whereas Muller-Hinton Broth itself was use as a negative control. To properly suspend the gels and creams, they were placed in a sonicator (Branson Ultrasonic Sonicator, Brookfield, Connecticut, United States of America) for 15 minutes. Afterwards, 25% and 10% dilutions for F3 and F8 were made and were also used for testing. 50µL of each formulation was then pipetted into each well that was made. The petri dishes were then left to incubate in the incubator at 37˚C for 24 hours, where the diameters of the zones of inhibition are measured for each formulation.

For minimum bactericidal concentration, the method described by Klein, Lentsch, and Christoffersen is employed with some modifications [68]. The formulations were prepared the same way as the formulations made for the well diffusion test. 20µl of 1.0 McFarland Standard adjusted bacteria suspension was then added to 2ml of each formulation, including the SSD cream and broth, and also to 2g of F3 and F8 gel in bijou bottles. Once the bacterial suspension was added to the formulations, all bottles were vortexed and left in an incubator at 37˚C for 24 hours. Once complete, the bottles were then revortexed and a loopful of each formulation was then spread onto Muller Hinton Agar to inoculate the agar, after which a further 48 hours of incubation under 37˚C was performed.

At the end of the incubation, the agar plates were checked for any bacterial growth, where the absence of growth is deemed to be bactericidal.

Statistical analysis

Statistical analysis for the physical properties, that is the pH, moisture content, and spreadability of the gel included the calculation of average and standard

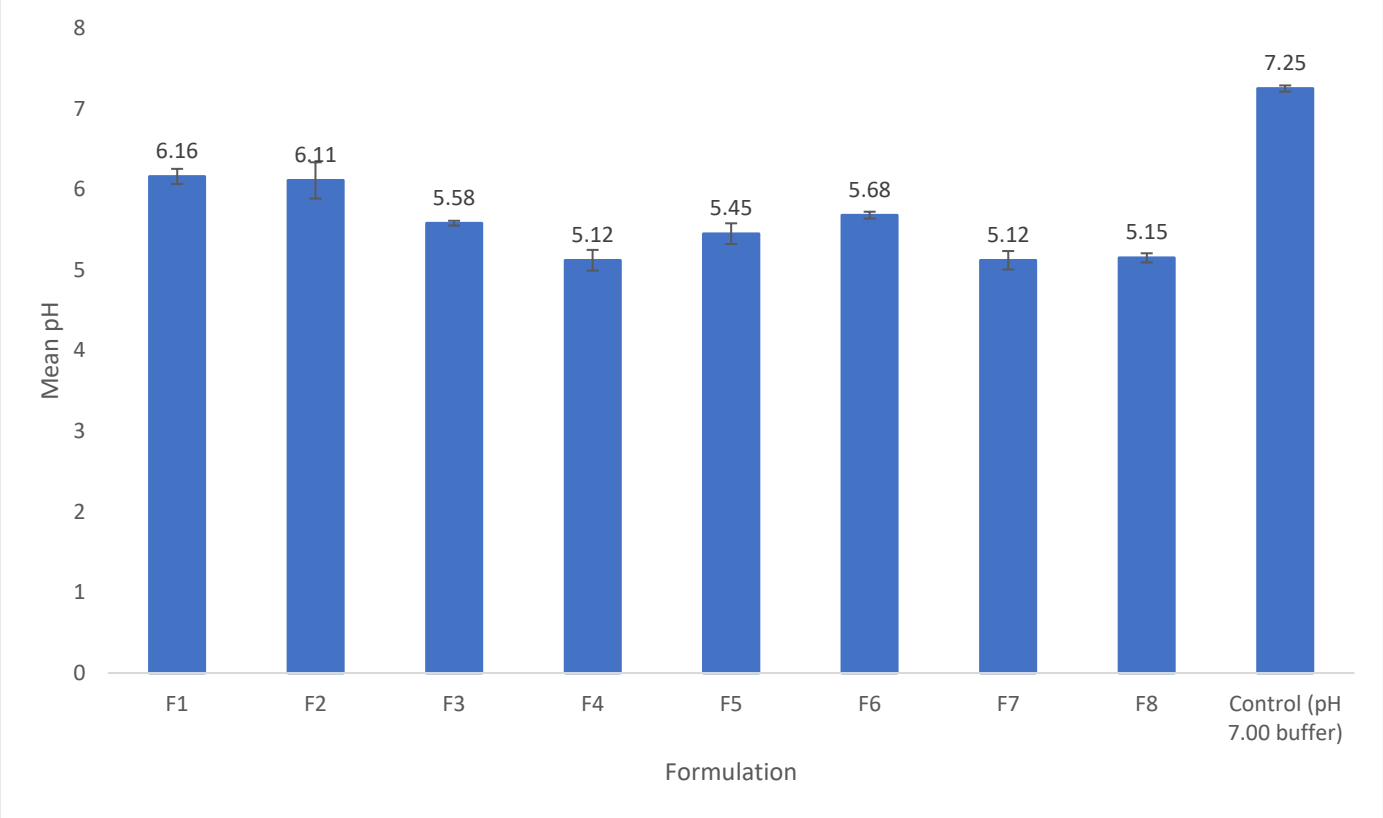


Figure 1:Bar graph of mean pH for each formulation.

Toxicity studies had means and standard deviations calculated for radicle length. Kruskal Wallis test was used for the statistical analysis for germination index, as normality testing showed a skewed data [69]. As no statistically significant result was produced, no post hoc testing was performed.

Meanwhile, antibacterial studies had averages and standard deviations calculated for the zone of inhibition [70]. One-way ANOVA with post hoc testing was used for statistical testing, with post hoc testing performed using the Tukey's test [70].

Both the Kruskal Wallis and one-way ANOVA testing will be performed using the R program application.

Results & Discussion

pH

Acidic pH was observed with all formulations measured. Using the pH 5.5 as the target, F1 showed the highest amount of deviation from 5.5, as with a pH of 6.16, it is 0.66 higher than 5.5. This is followed by F2 with 0.61 higher from 5.5,

F4 and F7 both being 0.38 lower from 5.5, F8 being 0.35 lower than 5.5, F6 being 0.18 higher than 5.5, F3 being 0.08 higher than 5.5, and finally F5 being 0.05 lower than 5.5. The error bars for each formulation showed the standard deviation from the mean in the data obtained, with F2 showing the highest standard deviation, while F3 showing the least. However, the standard deviation seems to be small for all formulations, with F2 showing a standard deviation of ±0.225mm, rounded to three significant figures. Figure 1 shows the mean of the pH of each of the formulations.

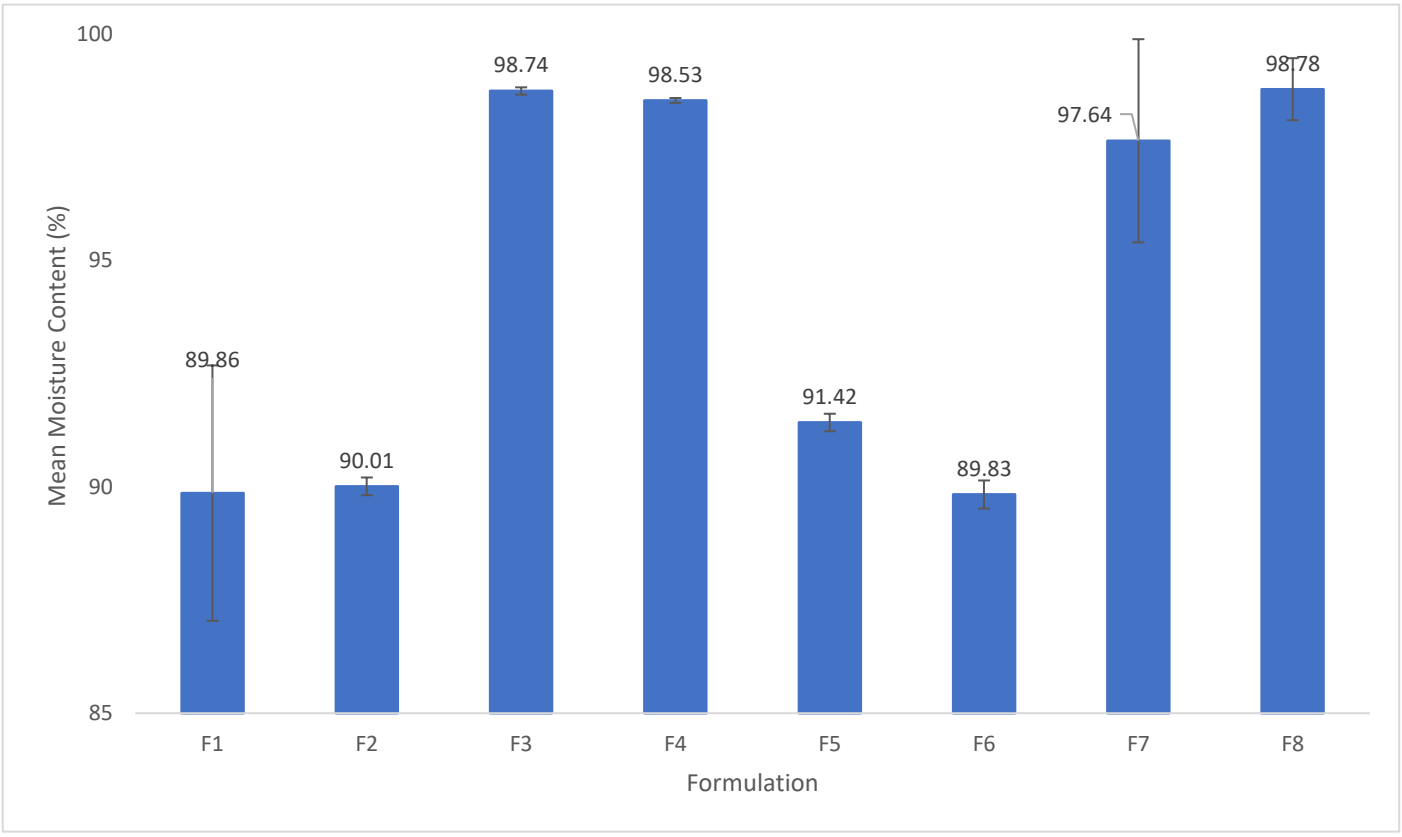


Figure 2: Bar graph of mean moisture content measured in %.

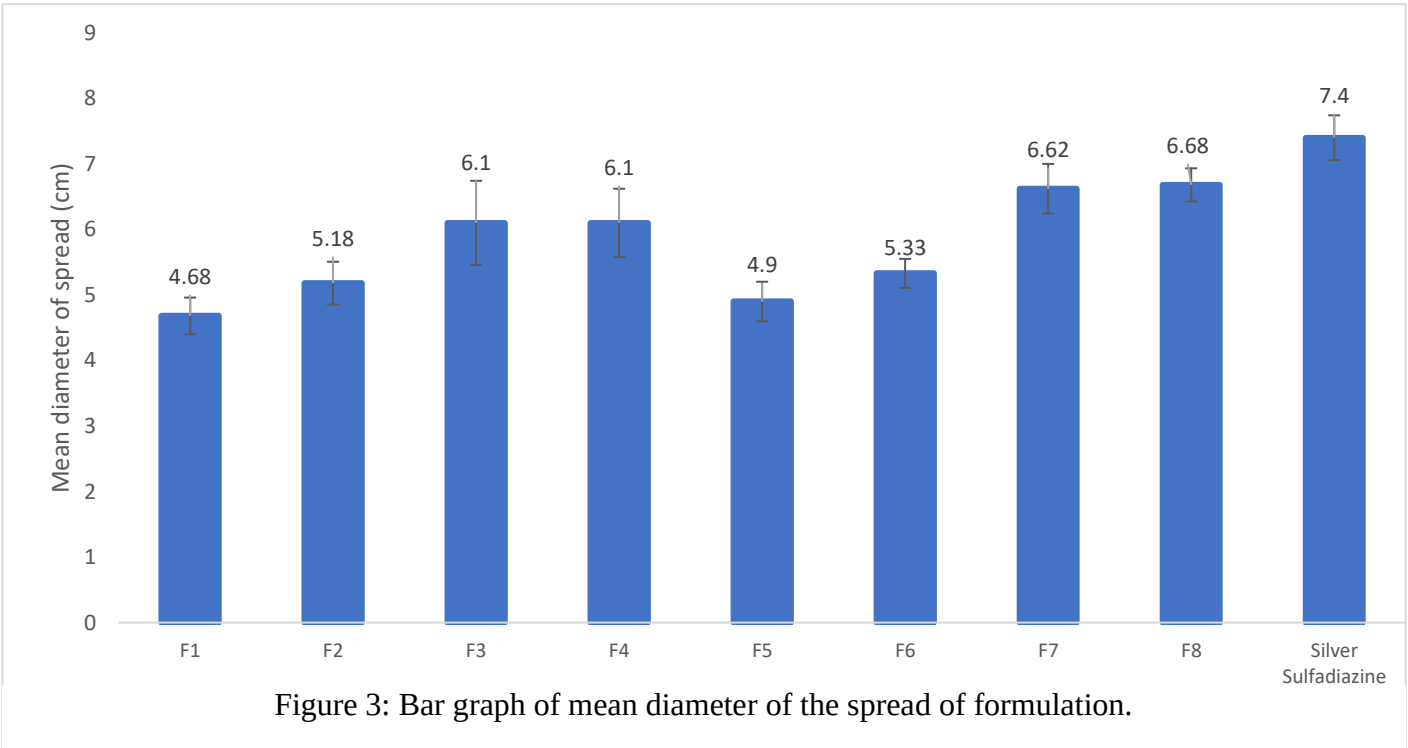


Figure 3: Bar graph of mean diameter of the spread of formulation.

Moisture Content

All formulations showed a mean moisture content higher than 89%, with F8 showing the highest amount of moisture of 98.78%, only marginally higher than F3 with 98.74%. Overall, formulations F3, F4, F7, and F8 that are made with Carbopol® generally have higher moisture content than the Sangelose® formulations F1, F2, F5 and F6. Figure 2 shows the measurement of moisture content.

Spreadability Measurement

Figure 3 shows the mean of the diameters of the formulations when spread onto a white tile after being pressed for 15 minutes. Generally, the higher amount of polymer and silver, the higher the apparent spreadability, with the exception of F3 and F4, where the increased amount of polymer seemed to produce a similar mean diameter. Additionally, Carbopol® formulations seemed to show a higher amount of spreadability relative to the Sangelose® formulations. When compared to silver sulfadiazine cream, however, all formulations showed a lower amount of spreadability.

FTIR

Figure 4 shows the results obtained from the FTIR measurement. The sharp but weak peak at 1635cm^{-1} wavelength for both F3 and F8 could represent either the Alkenyl C=C stretch or Aryl-substituted C=C as the peaks that these stretching are found are at 16 80

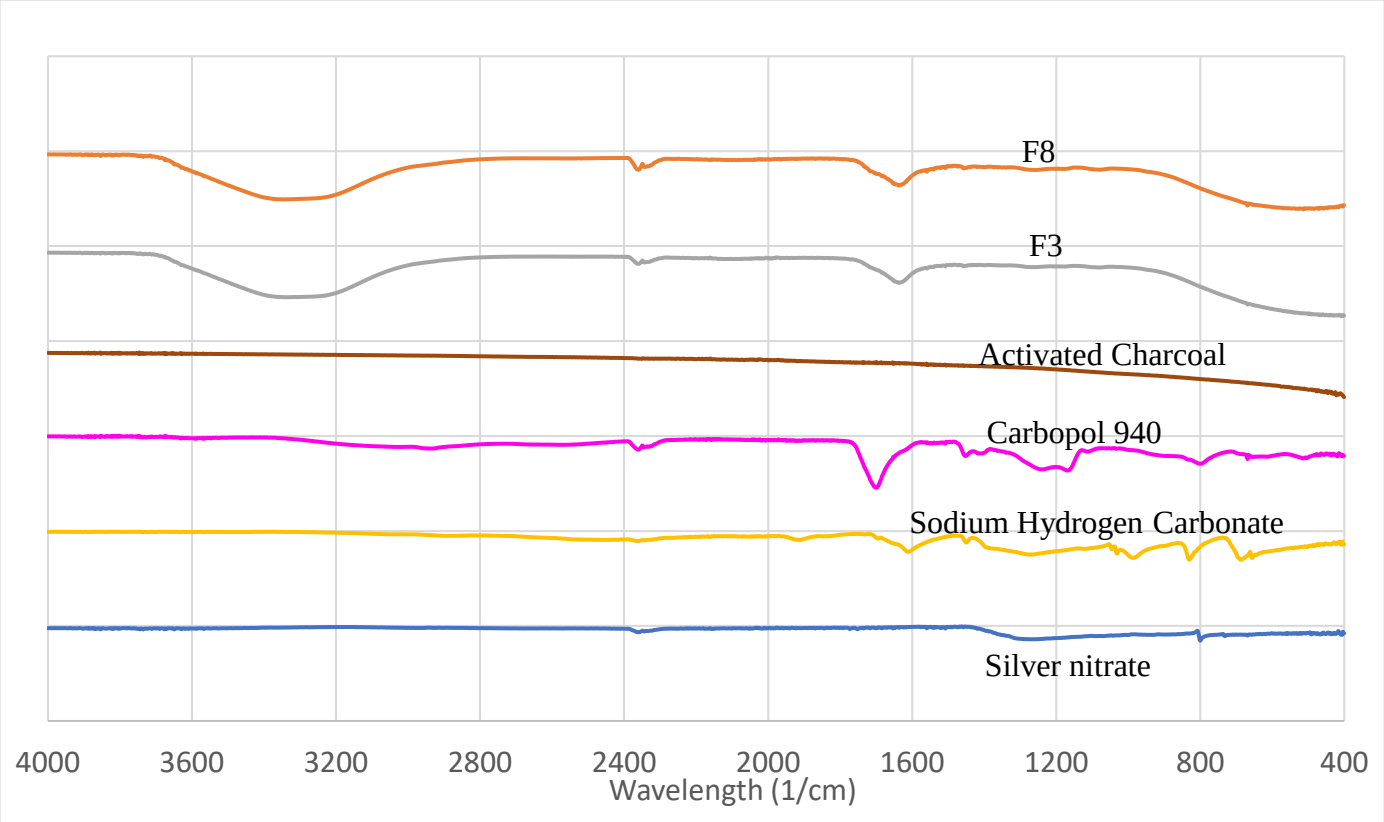


Figure 4:FTIR absorbance for each material tested by wavelength (1/cm)

to 1620 cm^{-1} and 1625cm^{-1} [71]. As Carbopol 940 is a polymer made up of polyacrylic acid [17] which does not have an aryl structure, these peaks must represent the alkenyl C=C stretching. Carbopol 940, meanwhile showed a strong and sharp peak at the 1701 cm^{-1} wavelength which corresponds to the carboxylic acid peak [71]. The peak at 1448 cm^{-1} for sodium hydrogen carbonate is also seen in other

carbonate ions, as well as the sharp peak that is at 831 cm^{-1}, although usually in carbonate salts, peaks appear between 880 to 860 cm^{-1}. The silver nitrate showed two standout peaks, which were the broad and intense peak that is seen at the 1380 to 1350 cm^{-1}

region, and the narrow peak at approximately 800 cm-1, although usually the latter peak generally appears at the 840 to 815 cm^{-1} wavelengths [71]. Activated charcoal showed only one peak that is weak at 1652 cm^{-1}, which corresponds to the Alkenyl C=C stretch [71]. The formulations F3 and F8 seems to show a similar peak pattern to all of the other ingredients combined, most notably the peaks for Carbopol 940 and silver nitrate.

Table 2: Table of results for toxicity testing

Formulation	Number of Seeds Germinated	Mean Radicle length ± SD (cm)	Percentage Seed germination (%)	Percentage root growth (%)	Germination index(%)
F1	20	3.53 ± 0.37	111.11	93.63	104.04
F2	20	3.02 ± 0.96	111.11	80.11	89.01
F3	18	1.72 ± 0.34	100.00	45.62	45.62
F4	16	1.27 ± 0.16	88.89	33.69	29.94
F5	20	2.35 ± 0.59	111.11	62.33	69.26
F6	19	1.73 ± 0.84	105.56	45.89	48.44
F7	17	2.15 ± 0.58	94.44	57.03	53.86
F8	18	1.62 ± 0.53	100.00	42.97	42.97
Distilled water	18	3.77 ± 0.89	100.00	100.00	100.00

Toxicity

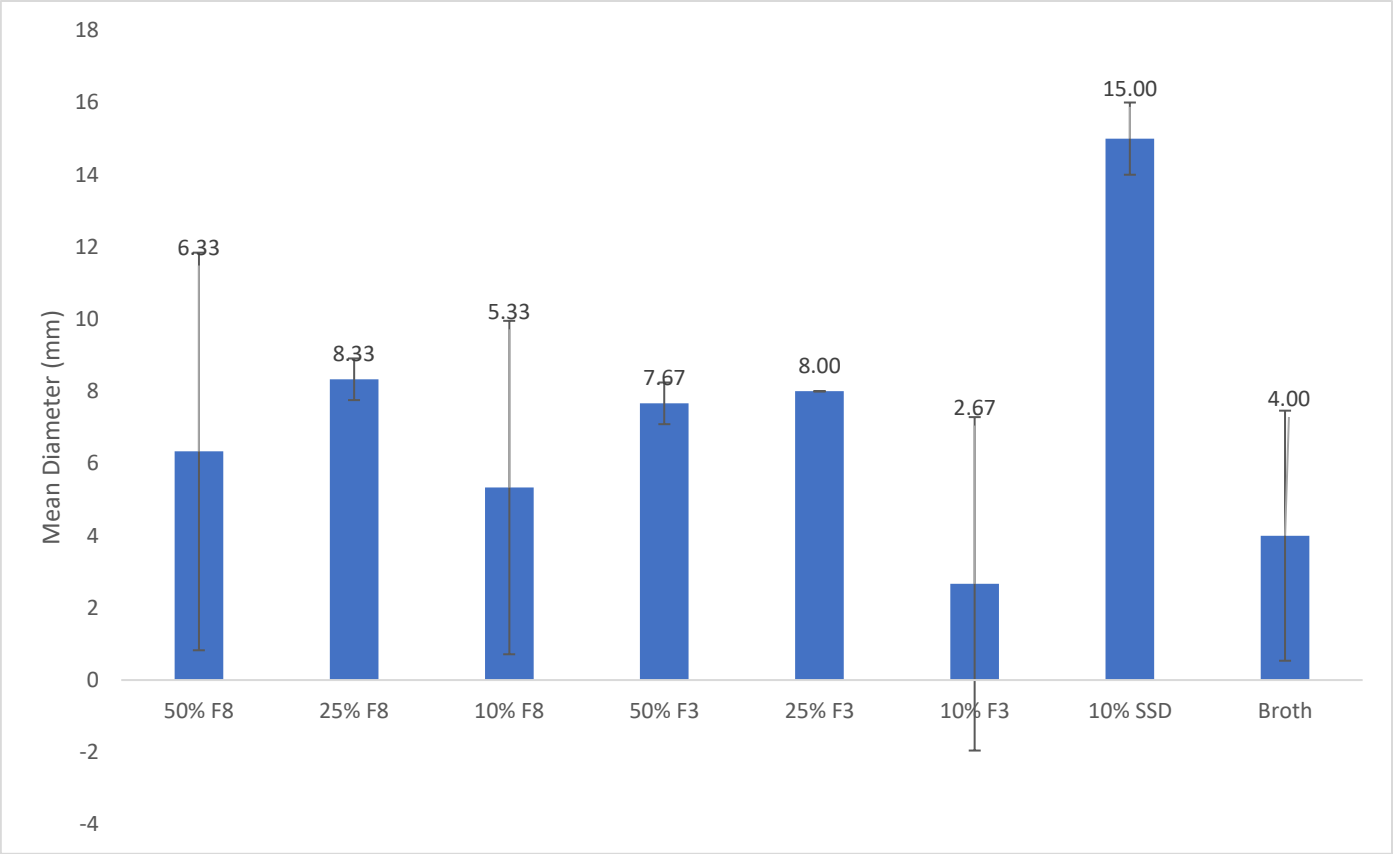
Toxicity results are presented in table 1 as shown. All formulations showed seeds that have germinated, with F4 showing the lowest number of seeds germinating and F1, F2, and F5 all showed all seeds germinating. It is worth noting that F7 in the beginning had 20 seeds, however upon recounting at the end of the 5 days, one seed could not be found and was counted as not germinated. Overall, the Carbopol® formulations all showed a lower number of seeds germinating when compared to the formulations made with PVA and Sangelose® formulations. Meanwhile, germination index scores show that only F1 surpassed a germination index of 100% with 104.04%, whereas all the other formulations have germination indices that are below 100% when compared to the distilled water control group.Kruskal-Wallis test for statistical significance showed no statistically significant different results, despite some results showing a large difference of germination index when compared to others, particularly F4 with a low germination index of 29.94, compared to F1 with a high 104.04% germination index.

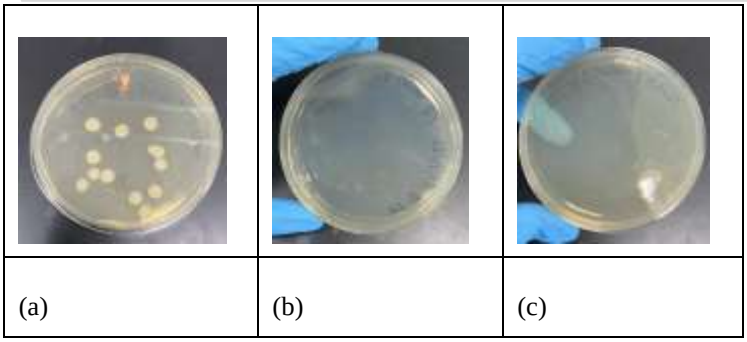
Zone of inhibition

Measurement of zone of inhibition showed mixed results, as 25% dilution for both F3 and F8 showed the largest zone of inhibition within each of their own dilutions, whereas 10% F3 and F8 showed the smallest, suggesting that the lower concentration of silver nitrate, the higher the antibacterial effect, however only up to a certain point, in which decreasing past that particular concentration will instead decrease the antibacterial effect. Meanwhile, 10% SSD cream consistently showed the largest zone of inhibition whereas 10% F3 showed the smallest zone of inhibition. Figure 5 shows the results. One-way ANOVA showed a significant result among the different dilutions in that 10% F3 and the broth when compared to the 10% SSD cream with a p value of <0.05 between the broth and the 10% SSD, <0.05 between the 10% SSD and 10% F8, and <0.01 for the 10% F3 and 10% SSD cream. The other formulations, meanwhile, showed no statistically significant results.

Minimum bactericidal concentration (MBC) studies

Minimum bactericidal concentration studies showed inconsistent results. For example, the assigned positive control SSD was shown to have growth after applying an incubated sample suspended with bacteria in one run of the experiment when it was absent for the other two runs. Figure 4 shows evidence of this.





(a) (b) (c)

Figure 4: (a) 10% SSD cream sample for 48 hours from 21/4/22, colonies were seen to be forming;(b) Agar inoculated and incubated the same way as the agar in (a) but read 48 hours from 23/4/22; (c) Agar inoculated and incubated the same way as the agar in (a) but read 48 hours from 26/4/22

However, there are noteworthy difference in the observed colonies that were seen between the agar plates. For example, the agar for the F8 50% performed on 26th, April showed colonies that were larger in comparison to the colonies that are present in the broth negative control that shows a growth that has formed as a streak pattern with small, circular colonies present on one side. Additionally, there were some agar plates that has shown colonies that were yellow in colour that were absent in other plates. Figure 7 shows some of these noteworthy differences. Note that all three plates shown are from the same run that was performed on the 26th, April 2022.

Figure 3: Mean diameter of zone of inhibition; Note: * p<0.05 between broth and 10% SSD, **p<0.05 between 10% SSD and 10% F8, *** p<0.01 between 10% SSD and 10% F3

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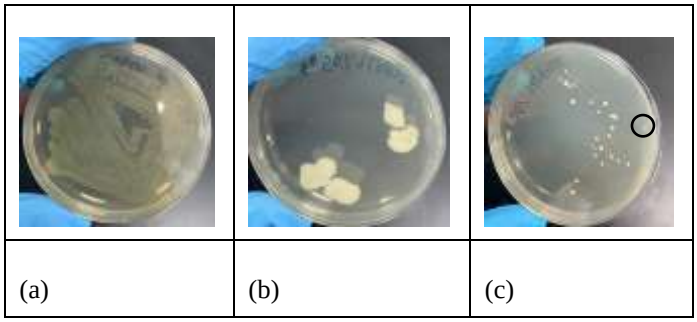

(a) (b) (c)

Figure 1: Agar inoculated with staphylococcus aureus suspended in (a) broth; (b) suspended in 50% F8; (c) suspended in 50% F3. The circled colony shows a yellow colour that is different from the other colonies present.

Table 3: Table of mean pH, moisture content, and diameter of spread for formulations F3 and F8 stored under different temperatures.

	Mean pH in different conditions				Mean moisture content (%) in different conditions				Mean diameter of spread (cm) in different conditions			
		25˚C	4 ± 2˚C	40 ± 2˚C		25˚C	4 ± 2˚C	40 ± 2˚C		25˚C	4 ± 2˚C	40 ± 2˚C
	Day 0	Day 37	Day 37	Day 37	Day 0	Day 37	Day 37	Day 37	Day 0	Day 37	Day 37	Day 37
F3	5.58 ± 0.03	5.51 ± 0.07	5.57 ± 0.03	5.48 ± 0.06	98.74 ± 0.08	98.90 ± 0.13	98.96 ± 0.18	98.83 ± 0.07	6.10 ± 0.64	6.49 ± 0.45	6.22 ± 0.37	5.91 ± 0.05
F8	5.15 ± 0.06	5.22 ± 0.06	5.30 ± 0.08	5.08 ± 0.05	98.78 ± 0.68	98.82 ± 0.18	98.62 ± 0.20	98.53 ± 0.04	6.68 ± 0.25	6.04 ± 0.71	5.76 ± 0.40	5.11 ± 0.32

With the exception of the SSD cream, F8 gel, F3 gel, and F8 50%, all other formulations showed growth on all three runs of the experiments in terms of general colony growth, regardless of shape, size, colour and number.

Stability

Stability studies were performed to look at the pH, moisture content, and spreadability after 37 days. For the pH, all formulations showed a pH that is above 5. Meanwhile, measurements for F3 showed a small increase in moisture content under all temperature storage conditions employed whereas F8 showed an increase when stored under 25˚C whereas decreased under 4˚C and 40˚C. Spreadability studies showed mixed results for F3, whereas all the results for F8 showed an apparent decrease in the ability to spread for all temperature conditions. Table 3 shows the results for both F3 and F8 being the optimised formulations.

Optimisation of formulation was carried out with supervision by Dr. Rajan Rajabalaya. At the end, F3 and F8 were chosen for further study. F3 and F8 were chosen as the optimised formulations as comparatively, the Carbopol formulations overall showed a lower deviation from the pH of skin of

5.5 which protects the skin from microorganisms [72], and also showed the highest spreadability. While toxicity studies have shown that the radicle lengths for the sprouts grown with F3 and F8 are considerably lower and have much lower germination index, most of the bean sprouts still have germinated.

The stability studies have shown that the formulations, while showing some changes of pH throughout the period of stability testing, has remained relatively consistent for 37 days. For the pH, acidic pH not only helps to protect from microorganisms, but also can have synergistic effect in terms of the antimicrobial effect with formulations that have microbicide incorporated, provided that this

acidic pH is maintained [73]. Meanwhile, moisture content is measured to ensure that moisture is provided to the wound to ensure that wound healing is promoted. As for infection, meanwhile, the idea that moisture contributing to wound infection is unfounded as early as from 1994 [74], thus the moisture content that is higher than 90% found in both formulations are still considered to be suitable for use.

The zone of inhibition testing generally showed unfavourable results to all the formulations when compared to the marketed product of silver sulfadiazine cream, which suggests that all formulations have lower antibacterial effect when compared to the silver sulfadiazine. However, zone of inhibtion, while being able to show a formulation or antibiotic's antimicrobial efficacy against a bacterium, does not always equate a larger zone of inhibition to a better antimicrobial efficacy. For example, according to the Clinical and Laboratory Standards Institute (CLSI) M100 Performance Standards for Antimicrobial Susceptibility Testing guidelines show that to deem a staphylococcus species to be susceptible to penicillin needs to be higher than 29mm for all staphylococcus species, whereas oxacillin requires to show a different minimum zone diameter for different species of staphylococcus aureus, two examples of that being more than 22mm for staphylococcus aureus, and more than 18mm for staphylococcus schleiferi [67], both of which is lower than the 29mm that is needed for penicillin. Not only that, the diffusion test does not differentiate bactericidal effects and bacteriostatic effects [75] and so a larger zone of inhibition does not necessarily mean bacteria is killed. Furthermore, while multiple articles do use silver in diffusion testing [76,77], two articles, one in 2018 [78], and another in 2019 [79] have both stated that there is no standard for testing dressings and silver nanoparticles respectively, making comparisons for silver difficult as there is no standard for the produced formulations to match against. Finally, it is worth noting that the ability to travel through agar should be taken into account as the principle behind the disk diffusion and well diffusion tests are that the antimicrobial agent needs to diffuse through the agar medium to exhibit antimicrobial effect [75]. However, as the formulation has charcoal combined with silver, charcoal's ability to adsorb materials can prevent the silver from travelling through the agar, as one study notes that charcoal was able adsorb silver nanoparticles from water[80].

Meanwhile, the growth that were seen on the Minimum Bacterial Concentration (MBC) experiments were inconsistent, with some plates showing different growths when compared to one another. This is important to note as the different growth patterns can be a result of contamination from other bacteria or fungi [81,82], and since the gel is not meant for antifungal purposes, the colony growth in some of the plates could possibly be counted as the formulation not being able to show antibacterial effect against staphylococcus aureus.

Further studies should focus on in vivo studies on animals to assess toxicity, wound healing and infection prevention. One improvement that can be made is that the formulation can be made with silver sulfadiazine as silver nitrate and silver sulfadiazine offers a different concentration of silver ions by weight [83]. Not only that, the sulfadiazine component in SSD also possess antibacterial properties as it itself is an antibiotic [84,85], which also may have been why the SSD cream showed larger zone of inhibitions and also had more success in not having colony growth after being incubated with bacteria inoculated.

Statistical analysis

All the experiments were conducted in triplicates and results are represented as the means and standard errors of the means. For antibacterial data, student 't' was performed.

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ABBREVIATIONS

[FTIR] = Fourier Transformed Infrared
[MIC] = Minimum Inhibitory Concentration
[PVA] = Polyvinvyl Alcohol
[AC] = Activated charcoal
[MHA0 = Mueller Hinton Agar
[SSD] = Silver sulfadiazine
[WH] = Wound Healing

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