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The interactive effects of medicinal dyes with conventional antimicrobials against skin pathogens

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Abstract

Aims: This study aimed to explore potential synergistic effects of medicinal dyes with antimicrobials against pathogens responsible for skin infections.

Methods and results: Antimicrobial testing was conducted using minimum inhibitory concentrations and minimum bactericidal/fungicidal concentration assays. The fractional inhibitory index (ΣFIC) of combinations was calculated, and isobolograms were constructed on selected combinations. Toxicity studies were conducted using the brine-shrimp lethality assay. Combination (1:1 ratio) studies noted that 26% of dye-antibiotic combinations were synergistic against the Gram-positive strains, 15% against the Gram-negative strains, and 14% against the yeasts. The Mercurochrome: Betadine® combination noted synergy at ratios against all the *Staphylococcus aureus* strains with ΣFIC values ranging from 0.05 to 0.48. The combination of Gentian violet with Gentamycin noted a 15-fold decrease in toxicity, and a selectivity index of 977.50 against the *Escherichia coli* (DSM 22314) strain. Time-kill studies were conducted on the combinations with the highest safe selectivity index (SI) value and lowest safe SI value i.e. Gentian violet with Gentamycin and Malachite green with Neomycin. Both combinations demonstrated better antimicrobial activity in comparison to the independent values and the controls.

Conclusion: This study highlights the potential for medicinal dye combinations as a treatment for skin infections.

Impact Statement

Many antibiotics have become ineffective in treating the simplest of infections thereby sparking new interest in combination therapy. Research into medicinal dyes has all been forgotten in the shadow of antibiotic efficacy. The current study provides a reasonable option for combatting antimicrobial resistance by examining the potential of combination therapy of medicinal dyes with antimicrobials. Not only do these combinations provide feasible synergistic options, but also target clinically resistant pathogens.

Keywords: medicinal dyes; commercial antimicrobials; skin infections; antimicrobial resistance; combinations; synergy; toxicity

Introduction

The skin is one of the first line defences against microbial invasion (Williamson et al. 2017). Infections caused by antibiotic-resistant bacteria, especially skin and soft tissue infections, have become prevalent in hospital and community settings. This has led to a change in the approach to empiric antibiotic use (Lushniak 2014). Antibiotic resistance is now a worldwide crisis, as difficulty in treating microbial pathogens has led to a high rate of mortality (Frieri et al. 2017). Many antibiotics have become ineffective in treating the simplest of infections, and this has led to a high number of hospitalizations, failures in treatment, and the development of more drug-resistant pathogens (Martens and Demain 2017).

Therefore, treatment plans have changed from monotherapy to combination therapy (Poustchi et al. 2021). Combination therapy can reduce resistance, lower treatment costs, and even reduce the therapeutic dose needed, and this has been found to be an effective strategy (Sullivan et al. 2020, Poustchi et al. 2021). One such strategy is the possible combination with alterative antimicrobials such as dyes.

Dyes were introduced into medicine in the 19th century by Koch and Ehrlich and have been used for centuries to treat various medical conditions (Wainwright 2014). The popularity decreased due to the discovery of antibiotics as well as their adverse side effects, ranging from tissue colouration to toxicity and mutagenicity (Wainwright 2014). Research into dyes as an antiseptic has been largely lacking in recent years and most clinical work involving dyes has been carried out before 1945, after which, antibiotics took the spotlight (Wainwright 2014).

Commonly used over-the-counter dyes such as Gentian violet, Iodine tincture, and Mercurochrome are commercially sold in various grocery stores and pharmacies. As described by Wainwright (2008), there are two major factors as to why medicinal dyes are regularly used; firstly, is the ability to discolour the skin, and secondly is that dyes have been shown to cause mutation *in vivo*, which can be a cause for concern. As effective as dyes are, they are not without risk as it is the case with most medicines and, indeed, antibiotics. Dyes are known to have toxicity (Wainwright 2008). It is therefore critical to assess the toxicity of the individual dyes and combinations at varied concentrations to determine the safety.

The purpose of this study was to determine the interactive antimicrobial and toxicity effects of seven medical dyes (Fuchsine, Gentian violet, Iodine tincture, Malachite green, Methylene blue, Mercurochrome, and Potassium permanganate) with seven conventional antimicrobials (Chlorohexidine, Fusidic acid, Nystatin, Ketoconazole, Miconazole, Povidone io-

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dine, and Mupirocin) associated with skin infection treatment. The impact of toxicity was also considered.

Materials and methods

Dye and antibiotic preparation

A selection of dyes for consideration was based on a review of the literature where antimicrobial activity has been observed (Owen 1913, Smith 1915, Gupta et al. 2008, Berrios and Arbiser 2011, Wainwright 2014, Rosa et al. 2015, Korkmaz et al. 2019). The dyes (indicated for topical use only) were Fuchsine (Sigma-Aldrich), Methylene blue (Sigma-Aldrich), Malachite green (Saarchem), Potassium permanganate (ACE), Gentian violet, Iodine tincture, and Mercurochrome (Dis-Chem Pharmacies). The dyes were initially prepared to a concentration of 32 mg/ml^{-1} in sterile water, except Gentian violet (5.00 μg/ml^{-1}), Iodine tincture (25.00 μg/ml^{-1}), and Mercurochrome (20.00 μg/ml^{-1}) as they were procured at the prepared concentrations.

The antibiotics Fusidic acid (±100%), Mupirocin (≥92%), Miconazole (99.50%), Gentamycin (±100%), Neomycin (90%–100%), Nystatin (≥4400 USP units per mg), and Ketoconazole (±100%) (all obtained from Sigma-Aldrich), as well as Betadine® and Steriscrub (Dis-Chem, South Africa) were selected based on use to treat skin infections. The antibiotics and antifungals were prepared at a starting concentration of 10.00 μg/ml^{-1}, while Betadine® (100.00 μg/ml^{-1}) was procured at a prepared concentration, and Steriscrub was prepared to a concentration of 32 000.00 μg/ml^{-1} diluted in 1:1 ratio of water to acetone. A preliminary test was conducted to determine the viable concentrations (32 000.00 μg/ml^{-1} for medicinal dyes and 10.00 μg/ml^{-1} for antibiotics and antifungals) required for further dilution that still note antimicrobial activity.

Antimicrobial analysis

The pathogens were selected based on their role in causing dermatological infections. The strains included reference strains (American Type Culture Collection ATCC; Davies Diagnostics, Johannesburg, South Africa). Clinical strains were received from the NHLS Infection Control and Microbiology Laboratory (National Health Laboratory Services), and selected resistant strains (Deutsche Sammlung von Mikroorganismen; DSM) were obtained from the German culture collection (The Leibniz Institute). The bacterial strains included: *Staphylococcus aureus* (ATCC 46644), *S. aureus* clinical strain, methicillin resistant *Staphylococcus aureus* (MRSA) (ATCC 43300), Gentamycin–methicillin resistant *Staphylococcus aureus* (GMRSA) (ATCC 33592), *Staphylococcus epidermidis* (ATCC 12228), *Staphylococcus epidermidis* clinical strain, *Staphylococcus epidermidis* resistant strain (ATCC 51625), *Pseudomonas aeruginosa* (ATCC 27858), *P. aeruginosa* clinical strain, *P. aeruginosa* resistant strain (ATCC 46316), *Escherichia coli* (ATCC 8379), *E. coli* clinical strain, and *E. coli* resistant strain (DSM 22314). The yeast strains included *Candida albicans* (ATCC 10231), *C. albicans* clinical strain and *C. albicans* resistant strain (ATCC 90028). All microorganisms were cultured in Tryptone Soya broth (TSB) (Oxoid). Bacterial strains were incubated at 37°C for 24 h and the yeasts at 37°C for 48 h.

Minimum inhibitory concentration and minimum bactericidal/fungicidal assay

To evaluate the antimicrobial activity of samples independently and in combination, the Clinical and Laboratory Standards Institute (CLSI) guidelines (2020) was followed. In a 96-well micro-titre plate, 100.00 μl of TSB was added. The medicinal dyes, commercial antimicrobials and/or combinations were added into the first row of wells according to the following ratios: 100.00 μl of selected dye and/or commercial antimicrobial and for combinations, 50.00 μl of dye and 50.00 μl antimicrobial (1:1 ratio). A positive control of a broad-spectrum commercial antimicrobial (Ciprofloxacin at 0.01 mg/ml^{-1} for bacteria and Amphotericin B at 0.10 mg/ml^{-1} for the yeasts), a negative solvent control of 32.00 mg/ml^{-1} water to acetone and a negative broth control of sterile TSB was added to each micro-titre plate. The positive control was added to ensure antimicrobial susceptibility of pathogens. The TSB culture control was used to ensure that the media will support the growth of the pathogen. The negative control was added to ensure the solvent does not exhibit antimicrobial activity. Thereafter, a two-fold serial dilution was carried out. Pathogens were added into each well (100.00 µl) with the approximate inoculum size of 1×10^8 colony forming units (CFU)/ml^{-1}. Microtitre plates were then sealed with a sterile adhesive seal (Macherey-Nagel GmbH & Co KG) and incubated as per respective incubation conditions. After the appropriate incubation period, 40 μl of the indicator, 0.4 mg/ml^{-1} *p*-iodonitrotetrazolium violet (Sigma-Aldrich), was added to each well. The lowest concentration that inhibits growth was the minimum inhibitory concentration (MIC) end-point value and was shown with the absence of purple-pink colour in the well (Hübsch et al. 2014). The MIC assays are largely dependent on a colorimetric change; therefore, interpretation of results can be difficult when dyes or dye combinations are used, due to the staining properties. To overcome this problem with staining due to the dyes minimum bactericidal (MBC) assays were conducted, whereby a loop of culture was taken from each well from the MIC assays and streaked onto subdivided Tryptone Soya agar (TSA) plates. The plates were then incubated under the optimal incubation conditions. The absence of growth on the agar plates indicated the bactericidal or fungicidal activity, respectively. The study was done in triplicate and comparisons were made between how the clinical and reference strains responded to the test inhibitors.

Fractional inhibitory concentration index and varied ratio concentrations

Interactions between the combinations of dyes and antimicrobials were studied using the sum of the fractional inhibitory concentration (ΣFIC). The ΣFIC was calculated where (a) represents the dye sample and (b) represents the commercial antimicrobial sample (van Vuuren and Viljoen 2011) (Equation 1):

$$\mathrm{FIC}^{(\mathrm{i})} = \frac{\text{MIC (a) in combination with (b)}}{\text{MIC (a) independently}}$$
$$\mathrm{FIC}^{(\mathrm{ii})} = \frac{\text{MIC (b) in combination with (a)}}{\text{MIC (b) independently}} \quad (1)$$

The FIC index is then calculated using the following equation: ΣFIC = FIC (i) + FIC (ii). The interactions were

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classified as being synergistic (ΣFIC is ≤ 0.5), additive (>0.5–1.0), indifferent (>1.0–≤ 4.0) or antagonistic (>4.0) (van Vuuren and Viljoen 2011).

If a pattern was observed i.e. majority of the combinations noted synergy or antagonism when tested against a specific pathogen group (Gram-positive, Gram-negative strains, and yeasts) then that combination was chosen for further varied ratio testing. The dye samples and commercial antimicrobials in combination were prepared in nine different ratios (9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8, and 1:9) and the MIC values determined as per the method described in the 'Minimum inhibitory concentration and minimum bactericidal/fungicidal assay' section.

Data points for each ratio were plotted first on an excel spreadsheet and then transferred to GraphPad Prism® (Version 5) software where an isobologram was constructed. The ΣFIC and varied ratio concentrations were conducted in triplicate.

Toxicity studies

To determine the toxicity of the medicinal dyes and commercial antimicrobials the brine shrimp lethality assay (BLSA) was carried out (Hübsch et al. 2014). The medicinal dyes were tested at concentration of 500.00 $\mu g/ml^{-1}$ except Gentian violet, Iodine tincture, and Mercurochrome, which were tested at the predetermined procured concentration as a starting point. The antibiotics and antifungals were also tested at 500.00 $\mu g/ml^{-1}$ except Betadine®, which was already at a predetermined concentration. Combinations tested in the toxicity studies were based on their antimicrobial activity (synergistic and antagonistic interactions), varied ratio testing and the individual toxicity studies of the medicinal dyes and commercial antimicrobials (non-toxic values). Both synergistic and antagonistic interactions were considered equally important.

For the BSLA, Tropical sea salt weighing 32.00 g was dissolved in 1.00 l of distilled water. Dried, brine-shrimp (*Artemia franciscana*) eggs (Ocean Nutrition™) weighing 0.50 g was added to the artificial sea water. To achieve a high hatch rate, a rotary pump (Kiho) was used to aerate the water and disperse the eggs. The eggs were incubated for 18–24 h at ambient temperature. A 220–240 V lamp was used as a source of light and warmth to help the hatching process. Once hatched, 400.00 μl of saltwater containing live brine-shrimp (40–60 brine-shrimp) was added to each micro-titre plate well. In each well, 400.00 μl of the sample (antimicrobials, dyes, or combinations) was added. The negative control used was salt water with a concentration of 32.00 g/l. The positive control that was used is 1.60 mg/ml^{-1} potassium dichromate (Sigma-Aldrich). After 24 and 48 h, the plates were observed under a light microscope (Olympus) and the dead shrimp were counted. Lastly, a lethal dose of 50 μl acetic acid (100% v/v) (SAAR Chem-Trade Pvt. Ltd) was added in each well and a total count of dead shrimp were taken; thereafter, the percentage of mortality calculated (Equation) A percentage mortality of 50% or greater is considered toxic (Bussmann et al. 2011). The tests were conducted in triplicate.

$$\text{Percentage mortality (\%)} = \frac{\text{Total number of dead shrimp at 24/48 h}}{\text{Total number of shrimp}} \times 100 \quad (2)$$

If the sample (dye, commercial antimicrobial, or combination) was determined to be toxic, the concentration of the sample was lowered whereby a dose-response was determined from a series of concentrations. An LC_{50} value was then determined and classified as highly toxic (LC_{50} value <249 $\mu g/ml^{-1}$), moderate toxicity (LC_{50} value of 250–499 $\mu g/ml^{-1}$) and low toxicity (LC_{50} value of 500–1000 $\mu g/ml^{-1}$) (Bussmann et al. 2011). The selectivity index (SI), which is defined as the ratio of toxicity to antimicrobial activity (MIC) was calculated (Equation 3) (Elisha et al. 2017).

$$SI = \frac{LC_{50}}{MIC} \quad (3)$$

Time-kill studies

Time-kill studies were conducted on selected combinations (including individual samples) that demonstrated the best antimicrobial activity with the least toxicity when combined, i.e. the highest SI index. In this study, the highest SI index and lowest SI index where a non-toxic relationship was observed was selected to determine the bactericidal or bacteriostatic effect over time. The time-kill assay method was adapted from Kurrimboccus et al. (2021). A quantity of 100.00 μl of TSB was added into the first row of a 48-well micro-titre plate. The remaining rows were filled with 900.00 μl of sterile 0.90% sodium chloride (NaCl) solution. A 0.5 McFarland's turbidity standard of culture (approximate inoculum concentration of 1×10^6 CFU/ml^{-1}) was prepared, and 100.00 μl thereof was transferred into the first row of the microtitre plate. A volume of 100.00 μl of the individual medicinal dye, commercial antimicrobial, and each combination (medicinal dye: commercial antimicrobial) was added into the first well of each column. Thereafter 100.00 μl was transferred to the subsequent well containing 0.90% NaCl. Five successive dilutions were then performed and then incubated at 37°C for 24 h. A 50.00 μl sample was withdrawn at 0, 3, 6, and 24 h and spread onto TSA plates and incubated at 37°C for 24 h, after which the number of colonies were counted. The logarithm of CFU/ml^{-1} present in the original well with time were calculated (Equation 4).

$$\text{Log}(CFU/ml^{-1}) = \log_{10}\text{Colony count} \times 1 \times 10^9 \quad (4)$$

A positive control of ciprofloxacin (0.01 mg/ml^{-1}) was used to ensure the pathogen was susceptible. A culture control was also included, and the assay was conducted in triplicate.

Results

Antimicrobial activity of the commercial antimicrobials

To study the susceptibilities for the combinations—it was necessary to initially determine the MIC/MBC breakpoint ranges for the commercial antimicrobials (Supplementary Tables S1–S3). The EUCAST and CLSI breakpoint ranges have been used as a susceptibility reference with most of the commercial antimicrobials falling within the determined ranges. Fusidic acid noted low MIC/MBC values within the range of the EUCAST criteria against all *S. epidermidis* strains. The Gram-negative pathogens demonstrated the most susceptibility to Betadine® with MBC values ranging from 1.56 to 2.60 $\mu g/ml^{-1}$. Overall, the pathogens demonstrated the most resilience against Steriscrub. Ketoconazole, Miconazole, and Nystatin. When tested against *C. albicans* A100, Nystatin demonstrated the most potent antimicrobial activity

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with an MIC/MBC value of 0.20 μg/ml^{-1}. *Candida albicans* ATCC 90028 demonstrated the most resistance to all three antifungals—Ketoconazole, Miconazole, and Nystatin with MBC values of >25.00 μg/ml^{-1}. The Gram-positive strains demonstrated better susceptibility to the commercial antimicrobials; however, no patten was observed among the reference, clinical and resistant strains where one strain was consistently susceptible in comparison to the other.

Antimicrobial activity of the medicinal dyes

The MBC values of the selected medicinal dyes are given in Table 1 . Most of the pathogens demonstrated high susceptibility to the medicinal dyes. Values ranged from the lowest concentration of 0.00003 to >8000.00 μg/ml^{-1}. The best antimicrobial activity was attributed to Gentian violet (0.00003 µg/ml^{-1} against *S. epidermidis* clinical strain), Iodine tincture (0.002 µg/ml^{-1} against *S. epidermidis* clinical strain and Malachite green (0.0009 µg/ml^{-1} against *S. epidermidis* ATCC 12228) in comparison to the other medicinal dyes tested.

Relatively high MBC values (ranging from 1.56 to 8000.00 μg/ml^{-1}) were observed against Fuchsine for the Gram-positive strains, Gram-negative strains, and the yeasts. Gentian violet demonstrated the lowest MBC values among the dyes (0.00003–0.52 µg/ml^{-1}). Iodine tincture demonstrated the lowest MBC value of 0.002 μg/ml^{-1} against the *S. epidermidis* clinical strain. Furthermore, the Iodine tincture demonstrated the same MBC value among all the strains—reference, clinical and resistant of *P. aeruginosa* and *E. coli*. The *S. epidermidis* strains demonstrated higher susceptibility to Malachite green in comparison to the other pathogens. The lowest MBC value of 0.0009 μg/ml^{-1} was observed against *S. epidermidis* ATCC 12228.

Mercurochrome had the second lowest MBC values among the *S. aureus* strains after Gentian violet. The MBC values ranged from 0.13 to 0.44 μg/ml^{-1}. The highest susceptibility was noted by *S. epidermidis* (BA 16), the clinical strain with an MBC value of 0.016 μg/ml^{-1}. Methylene blue noted the second highest MBC values among the dyes. The *P. aeruginosa* strains (ATCC 27853 and ATCC 46316) demonstrated resilience against Methylene blue and Mercurochrome.

All pathogens demonstrated a high resilience to Potassium permanganate with MBC values ranging from 4000.00 to >8000.00 μg/ml $^{-1}$. All strains behaved as expected against all controls. The Gram-positive and Gram-negative bacteria demonstrated susceptibility to Ciprofloxacin and the yeasts demonstrated susceptibility to Amphotericin B with values ranging from 1.25 to 2.50 μg/ml^{-1}. Growth of all microorganisms was noted against the culture control and the negative control.

1:1 Combinations

A total of 567 combinations were tested against the Gram-positive strains, Gram-negative strains, and yeasts, and the MBC/ΣFIC values were determined (Ramfol 2023). When tested, predominantly indifference (38.81%) was noted, followed by additive interactions (27.87%), synergy (20.46%), and lastly antagonism with 12.87%. A summary of all interactions is presented in Fig. 1. The synergistic and antagonistic interactions were the primary focus of this study, and hence these are elaborated on in Tables 2 and 3.

Table 1. The antimicrobial activity (MBC values in µg/ml^{-1}) of the medicinal dyes

Dyes	*Staphylococcus aureus*				*Staphylococcus epidermidis*			*Pseudomonas aeruginosa*			*Escherichia coli*			*Candida albicans*		
	Reference strain (ATCC 64466)	Clinical strain (clinical 6437938)	Resistant strain		Reference strain (ATCC 12228)	Clinical strain (clinical BA16)	Resistant strain (ATCC 51625)	Reference strain (ATCC 27853)	Clinical strain (DSM 46316)	Resistant strain (ATCC 46316)	Reference strain (ATCC 8379)	Clinical strain (clinical 66 437 873)	Resistant strain (DSM 22314)	Reference strain (ATCC 10231)	Clinical strain (A100)	Resistant strain (ATCC 90028)
			MRSA (ATCC 43300)	GMRSA (ATCC 33592)												
Fuchsine	312.50	500.00	500.00	25.00	6.25	1.56	100.00	8000.00	200.00	8000.00	166.67	2000.00	2666.67	250.00	200.00	208.33
Gentian violet	0.03	0.03	0.03	0.00025	0.002	0.00003	0.01	0.01	0.01	0.52	0.01	0.03	0.02	0.00025	0.00025	0.001
Iodine tincture	1.88	1.56	1.56	0.78	0.16	0.002	0.39	0.78	0.78	0.78	0.78	0.78	0.78	0.78	1.56	0.78
Malachite green	1.00	2.50	1.00	0.03	0.0009	0.002	1.00	400.00	0.63	800.00	1.67	10.00	10.00	0.32	0.63	5.00
Mercurochrome	0.44	0.27	0.13	0.42	0.14	0.016	0.06	>5.00	0.08	>5.00	0.31	0.08	2.50	0.42	0.08	0.08
Methylene Blue	20.00	50.00	208.33	50.00	12.50	20.00	40.00	>8000.00	133.33	>8000.00	40.00	800.00	800.00	12.50	26.67	26.67
Potassium Permanganate	7000.00	>8000.00	>8000.00	>8000.00	>8000.00	8000.00	8000.00	>8000.00	>8000.00	>8000.00	>8000.00	>8000.00	>8000.00	8000.00	4000.00	>8000.00

MRSA: methicillin-resistant *Staphylococcus aureus*, GMRSA: gentamycin-methicillin resistant *Staphylococcus aureus*.

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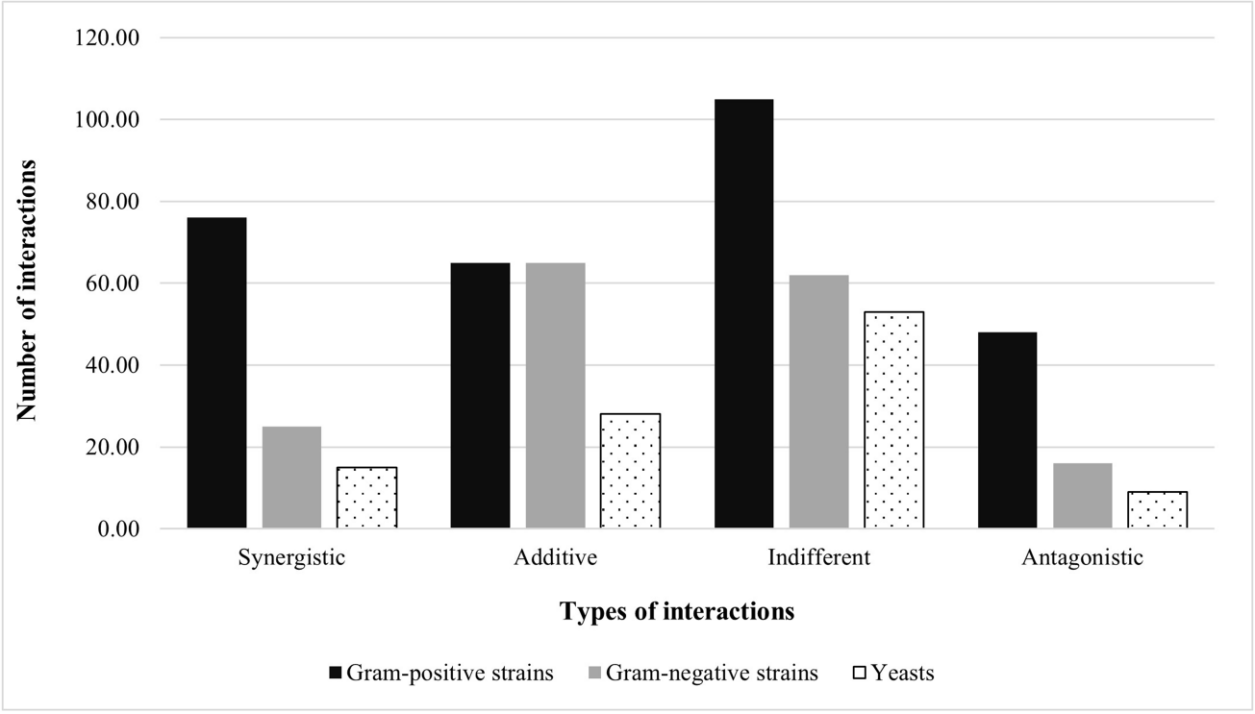

Figure 1. Summary of the 1:1 dye: commercial antimicrobial interactions. The most synergistic and antagonistic interactions were observed against the Gram-positive strains.

Mercurochrome with Betadine® was the combination to yield the most (eight interactions) synergy with ΣFIC values ranging from 0.05 to 0.48 (Table 2). This was followed by the combination of Fuchsine with Gentamycin (seven interactions) with ΣFIC values ranging from 0.001 to 0.50. The most synergistic interaction (ΣFIC value of 0.001) was observed for the combination of Fuchsine with Gentamycin when tested against *P. aeruginosa* DSM 46316.

The highest number (six interactions) of antagonistic interactions was observed with Fuchsine with Steriscrub, followed by Malachite green in combination with Betadine (five interactions). The most antagonistic combination (Malachite green with Betadine) was observed against MRSA ATCC 43300 with an ΣFIC value of 1000.50 (Table 3). Malachite green in combination with Fusidic acid also noted a high ΣFIC value of 1000.20 against MRSA ATCC 43300.

Further attention was paid to a selection of combinations that noted synergy and antagonism (bold in Tables 2 and 3). Toxicity as well as varied ratio studies were conducted on these combinations. Furthermore, the two combinations demonstrating potent antimicrobial activity (Table 2, bold and italics) were further investigated in different ratios to determine if synergy was apparent at varied concentrations.

Toxicity analysis

Toxicity analysis of commercial antimicrobials

The commercial antimicrobials predominantly demonstrated low percentage mortalities and were deemed non-toxic (Table 4). Betadine®, however, demonstrated the highest percentage mortality of 100% at 24 and 48 h with an LC_{50} value of 0.20 $\mu g/ml^{-1}$ at both 24 and 48 h. Gentamycin demonstrated the lowest percentage mortality of 0% at 24 h 1.11% at 48 h. The antifungals tested also noted low percentage mortalities.

Toxicity analysis of medicinal dyes

The various toxicity profiles of the medicinal dyes are noted in Table 5. At 24 h, Fuchsine, Malachite green, and Methylene blue were observed to be non-toxic. At 48 h, all medicinal dyes demonstrated high percentage mortalities. Four medicinal dyes (Gentian violet, Iodine tincture, Malachite green, and Mercurochrome) noted a percentage mortality of 100%. Iodine tincture demonstrated the greatest toxicity of all dyes tested with the percentage mortality rate of 100% at 24 and 48 h even when the dye concentrations were decreased. It also noted the lowest LC_{50} value of 0.0001 $\mu g/ml^{-1}$ at 24 and 48 h confirming the toxicity of the dye. Malachite green demonstrated a percentage mortality of 38.20% at 24 h and 100% at 48 h with a low LC_{50} value of 3.91 $\mu g/ml^{-1}$ noted at both 24 and 48 h. Methylene blue demonstrated the lowest percentage mortality of 33.33% at 24 h and 84.95% at 48 h. The dye demonstrated an LC50 value of 250.00 $\mu g/ml^{-1}$ at 24 h and 125.00 $\mu g/ml^{-1}$ at 48 h. The LC_{50} values of 125 $\mu g/ml^{-1}$ at 24 h and 15.63 $\mu g/ml^{-1}$ at 48 h were observed for Potassium permanganate.

Toxicity analysis of combinations

The medicinal dyes exhibited very high toxicity and therefore were combined with various commercial antimicrobials to determine if the toxicity could be reduced. Selected combinations (synergy and antagonism) (Tables 2 and 3, bold) were selected for the brine shrimp lethality assay, and the toxicities are shown in Table 6. The dyes independent toxicities are shown in the table for comparison. The percentage mortalities at the LC_{50} values (the non-toxic values) are also shown in the table as a reference only.

The combination of Gentian violet with Gentamycin demonstrated the greatest decrease in dye toxicity. Hence, this combination was the most synergistic combination. An

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Table 2. Summary of synergistic combinations grouped by dye combinations.

Combinations	ΣFIC value	Pathogen/strain
Fuchsine + Betadine®	0.46	*S. aureus* ATCC 64466
Fuchsine + Fusidic acid	0.16	*S. aureus* Clinical 6437938
Fuchsine + Gentamycin	0.10	*S. aureus* ATCC 64466
	0.50	*S. aureus* Clinical 6437938
	0.50	MRSA ATCC 43300
	0.28	*S. epidermidis* ATCC 12228
	0.12	*S. epidermidis* ATCC 51625
	0.001	*P. aeruginosa* DSM 46316
	0.50	***P. aeruginosa* ATCC 46316**
Fuchsine + Ketoconazole	0.04	*C. albicans* Clinical A100
Fuchsine + Neomycin	0.10	*S. aureus* ATCC 64466
	0.50	*S. aureus* Clinical 6437938
	0.21	MRSA ATCC 43300
	0.31	*S. epidermidis* ATCC 12228
	0.002	*P. aeruginosa* DSM 46316
Fuchsine + Steriscrub	0.38	*S. epidermidis* ATCC 51625
	0.44	***P. aeruginosa* DSM 46316**
	0.10	*E. coli* Clinical 66437873
	0.42	***E. coli* DSM 22314**
Gentian violet + Betadine®	0.25	*S. aureus* ATCC 64466
	0.09	*S. epidermidis* ATCC 12228
	0.30	*P. aeruginosa* ATCC 46316
	0.44	*E. coli* Clinical 66437873
Gentian violet + Fusidic acid	0.33	*S. aureus* ATCC 64466
Gentian violet + Gentamycin	0.26	*S. aureus* ATCC 64466
	0.15	MRSA ATCC 43300
	0.36	*P. aeruginosa* ATCC 46316
	0.50	***E. coli* DSM 22314**
Gentian violet + Mupirocin	0.27	*S. aureus* ATCC 64466
	0.15	MRSA ATCC 43300
	0.02	*S. epidermidis* ATCC 12228
Gentian violet + Neomycin	0.27	*S. aureus* ATCC 64466
	0.16	MRSA ATCC 43300
	0.04	*S. epidermidis* ATCC 12228
	0.42	*E. coli* DSM 22314
Gentian violet + Steriscrub	0.25	*S. aureus* ATCC 64466
	0.06	*S. epidermidis* ATCC 12228
	0.31	*S. epidermidis* ATCC 12228
	0.33	*P. aeruginosa* ATCC 46316
Iodine tincture + Betadine®	0.46	*S. aureus* ATCC 64466
	0.37	MRSA ATCC 43300
	0.15	*S. epidermidis* ATCC 12228
Iodine tincture + Gentamycin	0.42	*S. aureus* ATCC 64466
Iodine tincture + Mupirocin	0.43	*S. aureus* ATCC 64466
	0.16	*S. epidermidis* ATCC 12228
Iodine tincture + Neomycin	0.44	*S. aureus* ATCC 64466
	0.34	*S. aureus* Clinical 6437938
	0.26	MRSA ATCC 43300
Iodine tincture + Steriscrub	0.40	*S. aureus* ATCC 64466
	0.37	*P. aeruginosa* ATCC 27853
	0.25	*P. aeruginosa* ATCC 46316
	0.22	*E. coli* Clinical 66437873
	0.14	*C. albicans* ATCC 10231
	0.28	*C. albicans* ATCC 90028
Malachite green + Miconazole	**0.46**	***C. albicans* Clinical A100**
Malachite green + Mupirocin	0.10	*S. aureus* Clinical 6437938
	0.13	*S. epidermidis* Clinical BA16
Malachite green + Neomycin	0.02	*S. aureus* Clinical 6437938
	0.02	*S. epidermidis* Clinical BA16
	0.38	*E. coli* ATCC 8379
	0.50	***E. coli DSM 22 314***
Malachite green + Steriscrub	0.38	*S. epidermidis* ATCC 51625
Mercurochrome + Betadine®	**0.27**	***S. aureus* ATCC 64466**
	0.40	***S. aureus* Clinical 6437938**
	0.45	**MRSA ATCC 43300**
	0.05	GMRSA ATCC 33592
	0.48	***S. epidermidis* ATCC 12228**
	0.40	***S. epidermidis* Clinical BA16**
	0.38	*E. coli* ATCC 8379
	0.07	*C. albicans* ATCC 90028
Mercurochrome + Fusidic acid	0.39	*S. aureus* ATCC 64466
Mercurochrome + Ketoconazole	0.09	*C. albicans* ATCC 10231
Mercurochrome + Mupirocin	0.36	*S. aureus* ATCC 64466
	0.44	GMRSA ATCC 33592
Mercurochrome + Neomycin	0.30	MRSA ATCC 43300
	0.50	GMRSA ATCC 33592
Mercurochrome + Steriscrub	0.41	*S. epidermidis* ATCC 12228
Methylene blue + Betadine®	0.50	*S. aureus* ATCC 64466
	0.50	*S. epidermidis* ATCC 12228
Methylene blue + Gentamycin	0.30	MRSA ATCC 43300
	0.50	GMRSA ATCC 33592
	0.15	*P. aeruginosa* DSM 46316
	U	*E. coli* Clinical 66437873
Methylene blue + Ketoconazole	0.01	*C. albicans* Clinical A100
Methylene blue + Neomycin	0.30	MRSA ATCC 43300
	0.41	GMRSA ATCC 33592
	0.18	*P. aerugi nosa* DSM 46316
	0.06	*E. coli* Clinical 66 437873
Methylene blue + Nystatin	0.06	*C. albicans* Clinical A100
Methylene blue + Steriscrub	0.50	*S. epidermidis* ATCC 12228
	0.07	*S. epidermidis* Clinical BA16
	0.38	*S. epidermidis* ATCC 51625
	0.10	*E. coli* Clinical 66 437873
Potassium Permanganate + Betadine®	0.40	*S. aureus* ATCC 64466
	0.28	*S. epidermidis* Clinical BA16
	0.08	*S. epidermidis* ATCC 51625
Potassium Permanganate + Gentamycin	0.33	*S. epidermidis* Clinical BA16
Potassium Permanganate + Steriscrub	0.09	*S. epidermidis* Clinical BA16

Bold: combinations chosen for further testing. U: the mean MBC values were not absolute values (either >8000.00 μg/ml or <0.01 μg/ml), and hence tentative FIC values were established.

over 15-fold decrease is observed with Gentian violet when combined with the commercial antimicrobial. Three other combinations demonstrated a decrease in toxicity and were as follow; Fuchsine with Gentamycin, Malachite green with Neomycin, and Malachite green with Miconazole. Mercurochrome combined with Betadine® demonstrated a five-fold increase in toxicity when combined. Interestingly, this combination noted the best activity against the Gram-positive pathogens. Fuchsine with Steriscrub also noted a decrease in toxicity when combined. Out of the synergistic combinations tested, 66.67% of these combinations demonstrated a decrease in toxicity when tested against the brine shrimp.

As expected, most of the antagonistic combinations noted increased toxicity (i.e. antagonism); however, there were a few combinations that demonstrated decreased toxicity (synergism) while not having potent antimicrobial activity. Out of the antagonistic combinations tested, 50% of these combinations demonstrated a decrease in toxicity when tested against the brine shrimp.

While Gentian violet demonstrated decreased toxicity previously (when combined in a synergistic 1:1 combination), it did not demonstrate a decrease in toxicity in the antagonistic (1:1) combinations, which may be attributed to the

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Table 3. Summary of antagonistic combinations grouped by dye combinations.

Combinations	ΣFIC value	Pathogen/strain
Fuchsine + Betadine®	4.04	*S. epidermidis* Clinical BA16
	29.87	*S. epidermidis* ATCC 51625
	4.08	*E. coli* DSM 22 314
	10.67	*C. albica ns* ATCC 10231
Fuchsine + Fusidic acid	68.01	*S. epidermidis* Clinical BA16
	5.81	*S. epidermidis* ATCC 51625
Fuchsine + Gentamycin	68.01	*S. epidermidis* Clinical BA16
Fuchsine + Ketoconazole	U	*C. albicans* ATCC 90028
Fuchsine + Miconazole	U	*C. albicans* ATCC 90028
Fuchsine + Mupirocin	72.01	*S. epidermidis* Clinical BA16
	5.01	*S. epidermidis* ATCC 51625
Fuchsine + Neomycin	4.01	GMRSA ATCC 33592
	65.00	*S. epidermidis* Clinical BA16
Fuchsine + Nystatin	**4.08**	***C. albicans* ATCC 10231**
	U	*C. albicans* ATCC 90028
Fuchsine + Steriscrub	5.94	GMRSA ATCC 33592
	106.89	*S. epidermidis* Clinical BA16
	U	*P. aeruginosa* ATCC 27853
	U	*P. aeruginosa* ATCC 46316
	U	*E. coli* Clinical 66 437873
	5.13	*C. albicans* Clinical A100
Gentian violet + Betadine®	6.79	*S. aureus* Clinical 6437938
	5.99	***P. aeruginosa* ATCC 27853**
Gentian violet + Fusidic acid	5.04	*S. aureus* Clinical 6437938
Gentian violet + Gentamycin	8.02	*S. epidermidis* ATCC 12228
	4.01	*P. aeruginosa* DSM 46316
Gentian violet + Mupirocin	107.78	*S. aureus* Clinical 6437938
Gentian violet + Neomycin	**4.02**	***P. aeruginosa* DSM 46316**
Gentian violet + Steriscrub	**5.09**	**GMRSA ATCC 33592**
Iodine tincture + Betadine®	62.63	*S. epidermidis* Clinical BA16
Iodine tincture + Fusidic acid	**4.91**	**GMRSA ATCC 33592**
	8.81	*S. epidermidis* ATCC 12228
	4.50	***S. epidermidis* Clinical BA16**
Iodine tincture + Gentamycin	126.00	*S. epidermidis* Clinical BA16
Iodine tincture + Mupirocin	31.75	*S. epidermidis* Clinical BA16
	8.01	*S. epidermidis* ATCC 51625
Iodine tincture + Neomycin	125.25	*S. epidermidis* Clinical BA16
Iodine tincture + Steriscrub	41.72	*S. epidermidis* Clinical BA16
Malachite green + Betadine®	1000.50	MRSA ATCC 43300
	4.43	***S. epidermidis* ATCC 12228**
	5.33	***S. epidermidis* Clinical BA16**
	5.60	*P. aeruginosa* clinical strain
	4.27	***E. coli* DSM 22314**
Malachite green + Fusidic acid	1000.20	MRSA ATCC 43300
	7.88	*S. epidermidis* Clinical BA16
	5.81	*S. epidermidis* ATCC 51625
Malachite green + Mupirocin	13.02	MRSA ATCC 43300
	5.01	*S. epidermidis* ATCC 51625
Malachite green + Neomycin	5.31	*P. aeruginosa* ATCC 46316
Malachite green + Steriscrub	17.42	*S. epidermidis* ATCC 12228
	10.42	*S. epidermidis* Clinical BA16
Mercurochrome + Gentamycin	5.50	*S. epidermidis* ATCC 51 625
Mercurochrome + Mupirocin	7.00	*S. epidermidis* ATCC 51625
Mercurochrome + Neomycin	10.48	*S. epidermidis* ATCC 51625
Mercurochrome + Steriscrub	5.15	*S. epidermidis* Clinical BA16
	4.37	*S. epidermidis* ATCC 51625
	10.60	***E. coli* DSM 22 314**
Methylene blue + Betadine®	4.03	*E. coli* DSM 22314
	6.41	*C. albicans* ATCC 10231
Methylene blue + Fusidic acid	20.23	*S. epidermidis* ATCC 51625
Methylene blue + Gentamycin	**4.09**	***P. aeruginosa* ATCC 46316**
Methylene blue + Miconazole	U	*C. albicans* ATCC 90028
Methylene blue + Mupirocin	4.59	MRSA ATCC 43300
	17.03	*S. epidermidis* ATCC 51625
Potassium Permanganate + Fusidic acid	U	*S. aureus* ATCC 64466
Potassium Permanganate + Gentamycin	U	*S. aureus* ATCC 64466

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Table 3. Continued

Combinations	ΣFIC value	Pathogen/strain
	U	*P. aeruginosa* ATCC 27853
Potassium Permanganate + Mupirocin	U	*S. aureus* ATCC 64466
	16.28	*S. epidermidis* Clinical BA16
	16.53	*S. epidermidis* ATCC 51625
Potassium Permanganate + Neomycin	U	*S. aureus* ATCC 64466
	U	*P. aeruginosa* ATCC 27853
	U	*E. coli* ATCC 8379
Potassium Permanganate + Nystatin	7.25	*C. albicans* Clinical A100

Bold: combinations chosen for further testing. U: the mean MBC values were not absolute values (either >8000.00 μg/ml or <0.01 μg/ml), and hence tentative FIC values were established.

Table 4. The toxicity analysis of the commercial antimicrobials.

Conventional antimicrobial	Percentage mortality (%)		LC_{50} (μg/ml)	
	24 h	48 h	24 h	48 h
Betadine®	**100.00**	**100.00**	**0.20**	**0.20**
Fusidic acid	9.78	46.79	500.00	500.00
Gentamycin	0.00	1.11	500.00	500.00
Ketoconazole	8.31	29.58	500.00	500.00
Miconazole	0.72	6.62	500.00	500.00
Mupirocin	35.34	**72.22**	500.00	**125.00**
Neomycin	4.10	5.84	500.00	500.00
Nystatin	0.00	40.17	500.00	500.00
Steriscrub	46.61	**92.84**	500.00	**250.00**
Controls				
Negative control (Salt water 32 mg/ml)	1.86	6.38	N/A	N/A
Positive control (Potassium dichromate)	100.00	100.00	N/A	N/A

Bold: toxic values.

Table 5. The toxicity analysis of the medicinal dyes.

Medicinal dye	Percentage mortality (%)		LC_{50} (μg/ml)	
	24 h	48 h	24 h	48 h
Fuchsine	39.38	**90.36**	125.00	31.25
Gentian violet	**100.00**	**100.00**	**0.01**	**0.001**
Iodine Tincture	**100.00**	**100.00**	**0.0001**	**0.0001**
Malachite green	38.20	**100.00**	**3.91**	**3.91**
Mercurochrome	**100.00**	**100.00**	**0.02**	**0.01**
Methylene blue	33.33	**84.95**	250.00	125.00
Potassium permanganate	**71.42**	**94.30**	125.00	15.63
Controls				
Negative control (Salt water 32 mg/ml)	1.86	6.38	N/A	N/A
Positive control (Potassium dichromate)	100.00	100.00	N/A	N/A

Bold: toxic values.

weak antimicrobial activity. Iodine tincture with Fusidic acid demonstrated the greatest decrease in toxicity, while Fuchsine with Nystatin demonstrated a much smaller decrease. Two other combinations demonstrated a decrease in toxicity of the medicinal dyes; Mercurochrome with Steriscrub and Methylene blue with Gentamycin. Interestingly, two medicinal dyes, Fuchsine and Methylene blue when in combination with the commercial antimicrobials, respectively, demonstrated the lowest decrease in toxicity which could be due to the fact the dyes were not highly toxic initially.

While understanding which combinations are antagonistic is important, the synergistic interactions were further focused on. Two combinations: Gentian violet with Gentamycin and Malachite green with Neomycin, demonstrated potent antimicrobial activity and reduced toxicity, hence, were chosen for further in-depth testing.

The selectivity indexes

Most of the medicinal dyes were considered toxic, while the opposite is true for the commercial antimicrobials. The 1:1 combinations have shown potential in decreased toxicity once combined. It is therefore important to determine the SI of the dyes, commercial antimicrobials, and combinations to ensure a non-toxic relationship to the host while maintaining potent antimicrobial activity. The SI also helps ensure the dye's antimicrobial activity is not due to a toxic metabolite and rather the dye itself.

The SIs of the Gram-positive pathogens

Table 7 demonstrates the SI of the individual dyes against the Gram-positive pathogens. Most of the medicinal dyes demonstrated low SI values. The LC_{50} values that are added in the table are mainly just a reference of the toxicity of each sample.

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Table 6. The toxicity of the 1:1 combinations.

Combinations		Concentration (μg/ml)		Percentage mortality (%) at LC_{50} value	LC_{50} value of combination (μg/ml)	Change in toxicity of dye when combined with commercial antimicrobials
		Dye tested independently	Dye in combination with commercial antimicrobial			
Synergistic combinations	Fuchsine + Gentamycin	31.25	62.50	41.04	125.00	Decrease
	Fuchsine + Steriscrub	31.25	3.91	40.21	7.81	Increase
	Gentian violet + Gentamycin	0.001	0.01	30.21	15.64	Decrease
	Malachite green + Neomycin	3.91	7.81	34.59	15.62	Decrease
	Malachite green + Miconazole	3.91	7.81	46.39	15.62	Decrease
	Mercurochrome + Betadine®	0.01	0.004	45.33	0.20	Increase
Antagonistic combinations	Fuchsine + Nystatin	31.25	15.63	42.50	31.26	Decrease
	Gentian violet + Betadine®	0.001	0.001	35.36	0.20	No change
	Gentian violet + Neomycin	0.001	0.0001	2.91	0.24	Increase
	Gentian violet + Steriscrub	0.001	0.001	43.33	1.95	No change
	Iodine tincture + Fusidic acid	0.0001	0.05	40.05	2.00	Decrease
	Malachite green + Betadine®	3.91	1.95	12.67	2.15	Increase
	Mercurochrome + Steriscrub	0.01	0.02	23.61	7.83	Decrease
	Methylene blue + Gentamycin	125	250.00	34.41	500.00	Decrease

Table 7. The SI values of the dyes and commercial antimicrobials against the Gram-positive pathogens.

LC_{50}		SI						
		Staphylococcus aureus				*Staphylococcus epidermidis*		
		Reference strain (ATCC 64466)	Clinical strain (clinical 6437938)	MRSA (ATCC 43300)	GMRSA (ATCC 33592)	Reference strain (ATCC 12228)	Clinical strain (clinical BA16)	Resistant strain (ATCC 51625)
Dyes								
Fuchsine	31.25	0.10	0.06	0.06	1.25	5.00	**20.03**	0.31
Gentian violet	0.001	0.03	0.03	0.03	4.00	0.64	**32.26**	0.10
Iodine tincture	0.0001	0.0001	0.0001	0.0001	0.0001	0.001	0.06	0.0003
Malachite green	3.91	3.91	1.56	3.91	**130.33**	**4344.44**	U	3.91
Mercurochrome	0.01	0.02	0.04	0.08	0.02	0.07	0.64	0.17
Methylene blue	125.00	6.25	2.50	0.60	2.50	**10.00**	6.25	3.13
Potassium permanganate	15.63	0.002	U	U	U	U	0.002	0.002
Antimicrobials								
Betadine® (Povidone-Iodine)	0.20	0.03	0.11	0.03	0.02	0.20	0.08	0.03
Fusidic acid	500.00	**200.00**	**200.00**	**200.00**	**6250.00**	**12 500.00**	**6250.00**	**7142.86**
Gentamycin	500.00	4.00	**20.00**	**20.00**	**80.00**	**3125.00**	**6250.00**	**1612.90**
Mupirocin	125.00	5.00	5.00	3.99	**781.25**	**1562.50**	**3125.00**	**1562.50**
Neomycin	500.00	**40.00**	**20.00**	**40.00**	**12.00**	**793.65**	**1612.90**	**793.65**
Steriscrub (Chlorohexidine)	250.00	1.18	1.00	2.00	1.88	**10.00**	1.50	1.25

U: the mean MBC values were not absolute values (either >8000.00 μg/ml or <0.01 μg/ml), and hence the SI was unable to be calculated. Bold: non-toxic values; LC_{50} values: added as a reference.

Malachite green demonstrated the highest SI values of 130.33 against GMRSA ATCC 33952 and 4344.44 against *S. epidermidis* ATCC 12228. Fuchsine and Gentian violet noted a high SI value of 20.03 and 32.26 against *S. epidermidis* Clinical BA16, respectively. Iodine tincture demonstrated the lowest SI value of 0.0003 against the *S. epidermidis* ATCC 51625. Iodine tincture also noted the lowest SI values of 0.0001–0.06 against the other Gram-positive strains.

Unlike the medicinal dyes, most of the commercial antimicrobials demonstrated high SI values (a good SI-which is a non-toxic relationship with the sample and the host while having potent antimicrobial activity). Betadine® was the only commercial antimicrobial that noted low SI values of 0.03–0.20 for all Gram-positive pathogens. While it did demonstrate good antimicrobial efficacy among the *S. aureus* and *S. epidermidis* strains, it noted highly toxic values, hence the low SI values. Fusidic acid and Neomycin demonstrated high SI values against all *S. aureus* and *S. epidermidis* strains. Fusidic acid demonstrated the highest SI value of 12500.00 against *S. epidermidis* ATCC 12228. This means that Fusidic acid

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Table 8. The SI values of the dyes and commercial antimicrobials against the Gram-negative pathogens.

Sample	LC_{50}	SI					
		Pseudomonas aeruginosa			*Escherichia coli*		
		Reference strain (ATCC 27853)	Clinical strain (DSM 46316)	Resistant strain (ATCC 46316)	Reference strain (ATCC 8379)	Clinical strain (clinical 66437873)	Resistant strain (DSM 22314)
Dyes							
Fuchsine	31.25	0.004	0.16	0.004	0.19	0.02	0.01
Gentian violet	0.001	0.10	0.10	0.002	0.10	0.03	0.05
Iodine tincture	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001
Malachite green	3.91	0.01	6.21	0.005	2.34	0.39	0.39
Mercurochrome	0.01	U	0.13	U	0.03	0.13	0.004
Methylene blue	125.00	U	0.94	U	3.13	0.16	0.16
Potassium permanganate	15.63	U	U	U	U	U	U
Antimicrobials							
Betadine® (Povidone-Iodine)	0.20	0.10	0.08	0.09	0.13	0.08	0.13
Gentamycin	500.00	**200.00**	2.00	**200.00**	**20.00**	U	**47.98**
Neomycin	500.00	U	**20.00**	**125.00**	2.00	2.00	3.00
Steriscrub (Chlorohexidine)	250.00	1.25	0.63	1.25	3.75	0.13	6.00

U: the mean MBC values were not absolute values (either >8000.00 μg/ml or <0.01 μg/ml), and hence the SI was unable to be calculated. Bold: non-toxic values; LC_{50} values: added as a reference.

proved to have good antimicrobial activity against the strains and is also safe to use based on the BSLA studies.

The SIs for the Gram-negative pathogens

All medicinal dyes noted low SI values ranging from 0.0001 to 6.21 (Table 8). Iodine tincture demonstrated the lowest SI values of 0.0001 among all the *P. aeruginosa* and *E. coli* strains. Unlike the Gram-positive strains, most of the commercial antimicrobials noted low SI values (Table 8). Overall, Betadine® demonstrated the lowest SI values (as with the Gram-positive strains) with SI values ranging from 0.08 to 0.13. Steriscrub also noted low SI values against the *P. aeruginosa* and *E. coli* strains. Gentamycin demonstrated the highest SI value of 200.00 against the *P. aeruginosa* ATCC 27853 and ATCC 46316.

The SIs for the yeasts

Most of the medicinal dyes demonstrated low SI values (Table 9) and, hence, were toxic even though they demonstrated good antimicrobial activity. Iodine tincture noted the lowest SI value of 0.0001. Only two medicinal dyes demonstrated high SI values-both against *C. albicans* ATCC 10231. Malachite green noted an SI value of 12.22, and Methylene blue demonstrated an SI value of 10.00. Betadine® noted low SI values of 0.05–0.15 against the *C. albicans* strains. All three antifungals demonstrated high SI values against the *C. albicans* ATCC 10231 and A100 strain. Nystatin noted the highest SI value of 2500.00 against *C. albicans* A100.

The SIs of the combinations

Table 10 provides the SI values of the combinations tested. The synergistic and antagonistic combinations were selected for further study based on the MIC/MBC and toxicity values. Synergistic combinations were chosen as they have the potential to become a treatment option, while the antagonistic combinations show which combinations have the potential to cause adverse effects and should not be used. Fuchsine in combination with Steriscrub demonstrated the lowest SI value of 0.17 against the *E. coli* strain. Four combinations noted high SI value; Fuchsine and Gentamycin (SI = 100.03), Gentian violet and Gentamycin (SI = 977.50), Malachite green with Miconazole (SI = 128.87), and Malachite green with Neomycin (SI = 50.37). As expected, 90% of the antagonistic combinations demonstrated a toxic relationship; therefore, the combinations cannot be used due to the high toxicity. Malachite green and Betadine® noted the lowest SI value of 0.12 against *E. coli* DSM 22314. Only one combination demonstrated a high SI value of 320.61, which was Methylene blue with Gentamycin against *P. aeruginosa* ATCC 46316.

Further in-depth studies

Varied ratio studies

Synergy was observed only in the 1:1 ratio of Gentian violet with Gentamycin when tested against the *E. coli* resistant strain (Fig. 2). Ratios with Gentian violet in a higher concentration mainly demonstrated synergy with additive effects in selected ratios as detailed in methods.

The varied ratios were determined using the MIC/MBC method mentioned in Antimicrobial analysis. The ΣFIC value was calculated and interactions were determined, which were then plotted on a graph using the GraphPad Prism® (Version 5) software, and an isobologram was created. The majority of the points plotted are on the line or below, which demonstrates synergy. The red point in the isobologram is the 1:1 interaction. The combination noted synergy and some additive effects therefore it was chosen for further analysis. The combination noted potent antimicrobial activity in comparison to other combinations tested (Ramfol 2023).

Malachite green with Neomycin tested against the *E. coli* resistant strain also demonstrated a synergistic interaction only in a 1:1 ratio, and additive interactions were predominantly noted for the other ratios (Fig. 3).

The varied ratios were determined using the MIC/MBC method previously mentioned. The combination was chosen for further analysis as it noted one of the best antimicrobial activities in comparison to other combinations (Ramfol 2023).

Time-kill studies

Time-kill studies were then conducted on these two combinations based on the non-toxic SI. While both combinations

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Table 9. The SI values of the dyes and commercial antimicrobials against the yeasts.

Sample	LC_{50}	SI		
		Candida albicans		
		Reference strain (ATCC 10231)	Clinical strain (A100)	Resistant strain (ATCC 90028)
Dyes				
Fuchsine	31.25	0.13	0.16	0.15
Gentian violet	0.001	4.00	4.00	1.00
Iodine tincture	0.0001	0.0001	0.0001	0.0001
Malachite green	3.91	**12.22**	6.21	0.78
Mercurochrome	0.01	0.02	0.13	0.13
Methylene blue	125.00	**10.00**	4.69	4.69
Potassium permanganate	15.63	0.002	0.004	U
Antimicrobials				
Betadine® (Povidone-Iodine)	0.20	0.15	0.08	0.05
Ketoconazole	500.00	**26.67**	**159.74**	U
Miconazole	500.00	**80.00**	**400.00**	U
Nystatin	500.00	**80.00**	**2500.00**	U
Steriscrub (Chlorohexidine)	250.00	0.31	0.31	0.31

U: the mean MBC values were not absolute values (either >8000.00 μg/ml or <0.01 μg/ml), and hence the SI was unable to be calculated. Bold: non-toxic values; LC_{50} values: added as a reference.

Table 10. SI of synergistic and antagonistic combinations.

Combinations		Corresponding pathogens	LC_{50}	SI
Synergistic combinations	Fuchsine + Gentamycin	*P. aeruginosa* ATCC 46316	**125.00**	**100.03**
	Fuchsine + Steriscrub	*E. coli* DSM 22314	7.81	0.17
	Fuchsine + Steriscrub	*P. aeruginosa* DSM 46316	7.81	0.52
	Gentian violet + Gentamycin	*E. coli* DSM 22314	15.64	**977.50**
	Malachite green + Miconazole	*C. albicans* A100	15.62	**128.87**
	Malachite green + Neomycin	*E. coli* DSM 22314	15.62	**50.37**
	Mercurochrome + Betadine®	*S. aureus* Clinical 6437938	0.20	5.11
	Mercurochrome + Betadine®	MRSA ATCC 43300	0.20	2.25
	Mercurochrome + Betadine®	*S. epidermidis* ATCC 12228	0.20	3.00
	Mercurochrome + Betadine®	*S. epidermidis* Clinical BA16	0.20	5.11
	Mercurochrome + Betadine®	*S. aureus* ATCC 64466	0.20	1.55
	Mercurochrome + Betadine®			
Antagonistic combinations	Fuchsine + Nystatin	*C. albicans* ATCC 10231	31.26	1.78
	Gentian violet + Betadine®	*P. aeruginosa* ATCC 27853	0.20	1.68
	Gentian violet + Neomycin	*P. aeruginosa* DSM 46316	0.24	4.35
	Iodine tincture + Fusidic acid	GMRSA ATCC 33592	2.00	4.51
	Iodine tincture + Fusidic acid	*S. epidermidis* Clinical BA16	2.00	2.23
	Malachite green + Betadine®	*S. epidermidis* ATCC 12228	2.15	0.90
	Malachite green + Betadine®	*S. epidermidis* Clinical BA16	2.15	0.23
	Malachite green + Betadine®	*E. coli* DSM 22314	2.15	0.12
	Mercurochrome + Steriscrub	*E. coli* DSM 22314	7.83	1.58
	Methylene blue + Gentamycin	*P. aeruginosa* ATCC 46316	**500.00**	**320.61**

LC_{50} values: added as a reference; bold: non-toxic values.

have noted potent antimicrobial activity and a non-toxic relationship, it is also important to determine the bactericidal/bacteriostatic ability over time. Both combinations were tested against the *E. coli* resistant strain.

The combination of Gentian violet and Gentamycin

When a time-kill analysis was undertaken on Gentian violet with Gentamycin, a slower killing action against the *E. coli* resistant strain was observed in comparison to the positive

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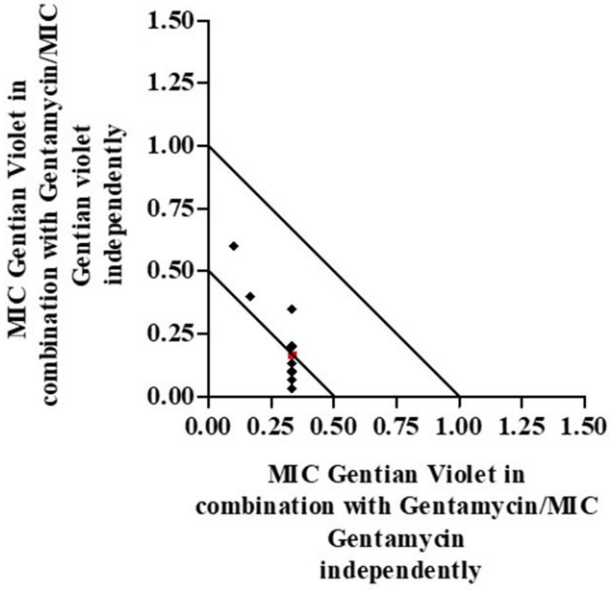

Figure 2. Isobologram of combinations of Gentian violet with Gentamycin against *E. coli* DSM 22314. Six synergistic interactions were observed. No antagonism was evident.

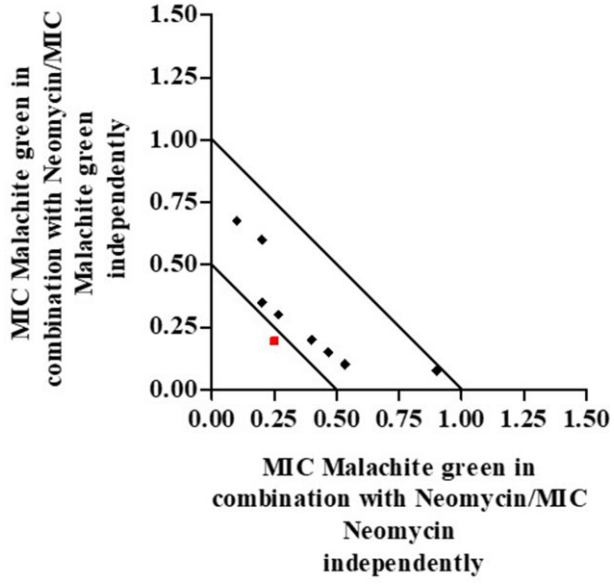

Figure 3. Isobologram of combinations of Malachite green with Neomycin against *E. coli* DSM 22314. Only one synergistic (1:1 ratio) was observed.

control (Fig. 4). Gentamycin demonstrated a similar pattern to Gentian violet, however, at 6–24 h, Gentamycin demonstrates a more bactericidal effect, which is indicated by the change in steepness of the curve. Gentian violet demonstrated a bacteriostatic effect on the *E. coli* resistant strain as not all bacteria were killed after 24 h. The current study noted bacteriostatic activity when Gentamycin was tested. (interrupted black line). The combination of Gentian violet and Gentamycin demonstrated better activity (the green line) in comparison to the samples when tested individually. This shows that Gentamycin with Gentian violet acts synergistically and demonstrates bacteriostatic activity better than the Ciprofloxacin control (Fig. 4).

The combination of Malachite green and Neomycin

As with the previous combination, Malachite green and Neomycin demonstrated a slow killing action (Fig. 5). From 0 to 24 h, Malachite green demonstrates a bacteriostatic effect on the pathogen. Neomycin demonstrates bacteriostatic activity similar to Malachite green; however, at 6–24 h, Neomycin noted better activity in comparison to Malachite green as indicated by the slightly steeper curve.

The combination of both Malachite green and Neomycin noted synergy as seen by the green line. From 0 to 6 h, the combination notes a bacteriostatic effect with a steady decline. Thereafter, a steep decline of pathogen viability was noted. At 24 h, the pathogen is completely killed.

Interestingly, the combination of Malachite green and Neomycin demonstrated better activity in comparison to the combination of Gentian violet and Gentamycin. Gentian violet with Gentamycin demonstrated a higher SI value, hence it was the most non-toxic combination and noted a high antimicrobial activity, however, Malachite green with Neomycin proved to be a better combination with bactericidal activity and even with a lower SI value. It is also important to note that both combinations demonstrated better activity to the antibiotic controls in this study.

Discussion

While there are many other notable uses of medicinal dyes, the focus of this study is on skin infections. Fuchsine is known to possess anaesthetic, bactericidal (Gram-positive), and fungicidal properties. It has been used to treat burns, eczema, and pyodermas (Gupta et al. 2008, Berrios and Arbiser 2011). Fuchsine dye in a 2%–5% solution has also been used for the treatment of burns (Balabanova et al. 2003). Gentian violet (also known as crystal violet) has been known to exhibit antitrypanosomal, antifungal, anthelminthic, and antibacterial properties (Berrios and Arbiser 2011). Other than dermatological treatments, Gentian violet has been used in a cream to treat vaginal infections and has been effective in a 0.5% aqueous solution used to treat oropharyngeal candidiasis (Balabanova et al. 2003). Iodine tincture has historically been used as a first line treatment for cuts and abrasions as it exhibits antifungal and antiseptic properties (Owen 1913, Smith 1915). In the early 1900s, Iodine was used as a preoperative preparation as it was found to have antimicrobial activity against Gram-positive, Gram-negative bacteria, fungi, and viruses (Morgan et al. 1996). The popularity decreased as it tends to burn the skin after prolonged use. Povidone-iodine has since replaced Iodine tincture as an antimicrobial (Morgan et al. 1996). Malachite green was previously known as Brilliant green; however, current preparations of Brilliant green do not contain malachite, a term used for the green colour of the leaves of the Malvaceae family of plants. Malachite green has primarily been used against mycobacterial infections; however, it has also been used as an antiseptic and antifungal to treat superficial pyodermas. The dye is also used as a secondary treatment of topical skin conditions (Berrios and Arbiser 2011, Rosa et al. 2015). Mercurochrome has been used as a long-acting antibacterial and antifungal agent to prevent wound sepsis while also aiding in wound healing (Wainwright

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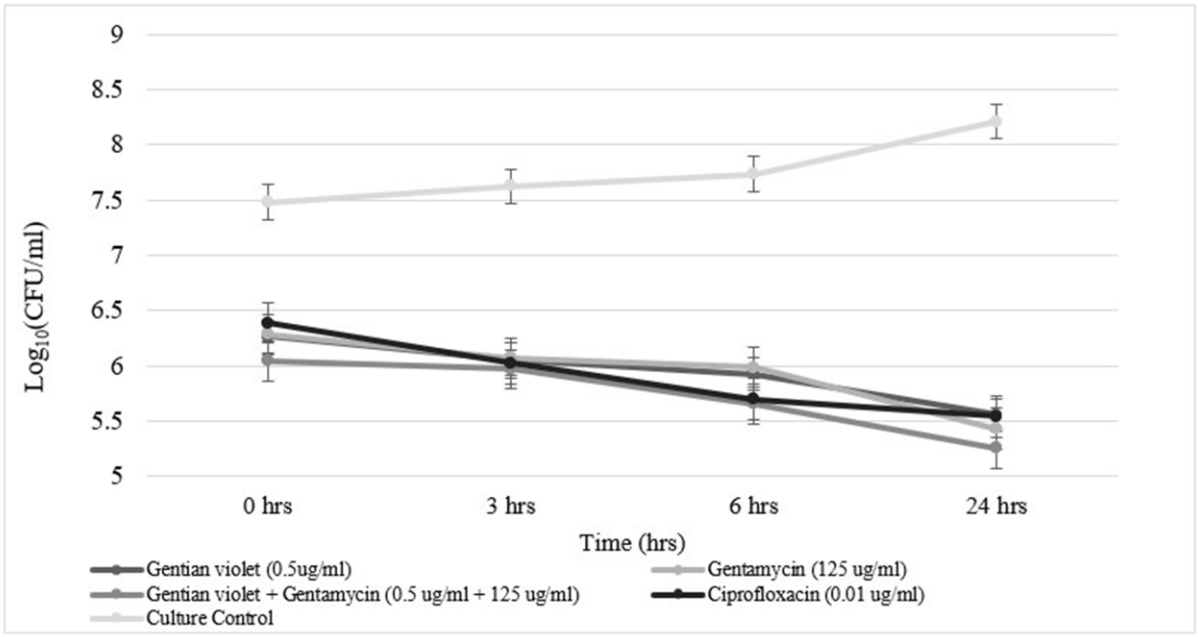

Figure 4. Time kill of Gentian violet and Gentamycin. The combination noted better activity when compared to individual samples and the controls tested.

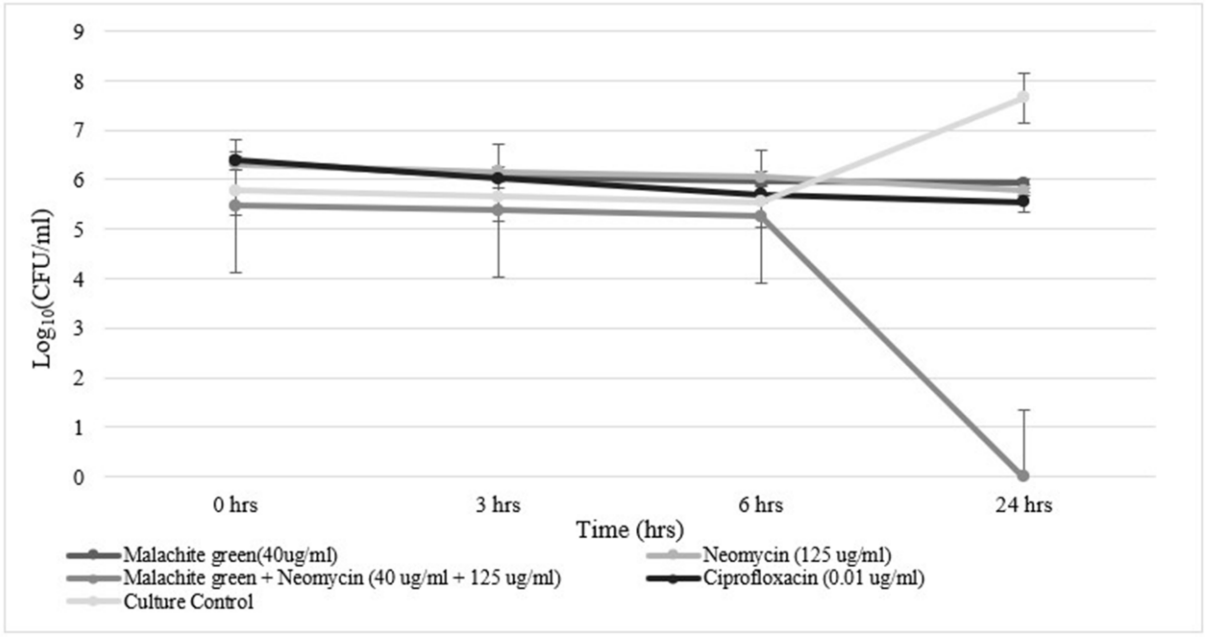

Figure 5. Time-kill studies of Malachite green and Neomycin. The combination of Malachite green and Neomycin demonstrated potent bactericidal activity in comparison to individual samples and controls.

2008, Korkmaz et al. 2019). Mercurochrome was previously used intravenously and was known as a 'true bloodstream antiseptic' (Cleary 1925). Ehrlich had used Methylene blue to treat malaria. Later, Methylene blue was found to have activity against onychomycosis (Wainwright 2014, Souza et al. 2017). Potassium permanganate solution was previously used in the treatment of impetigo. It was also used to treat tinea pedis and ringworm (Ive 1973), and is known to have disinfectant, deodorizing, and astringent properties. It may be used as a cleansing agent for wounds when in a weaker solution (SAMF 2020). While these aspects provide a good grounding for dyes to be used as antimicrobials, the use in combination with conventional antibiotics has not been given adequate attention. This study focuses on this in depth.

When examining the antibiotic breakpoints (Supplementary Tables S1–S3) the EUCAST (2020) guidelines for breakpoint MIC values were used for comparison. Fusidic acid noted low MIC/MBC values within the range of the

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EUCAST criteria against all *S. epidermidis* strains. A study testing the efficacy of Betadine® against various organisms noted an MIC range of 4.00–1024.00 $\mu g/ml^{-1}$ against *E. coli* (Gmur and Karpiński 2020). The current study notes MIC values lower than 4.00 $\mu g/ml^{-1}$, demonstrating better susceptibility. In 2016, a study noted the MIC value of Steriscrub to be 16.00 $\mu g/ml^{-1}$ and the MBC value of 32.00 $\mu g/ml^{-1}$, a much lower value to the current study (Akca et al. 2016). Steriscrub has been used repeatedly in a clinical settings and bacteria repeatedly exposed to Steriscrub may have resulted in resistance (Hashemi et al. 2019).

There have been various older studies noting the uses of medicinal dyes for the treatment of skin infections. An older study on the antibacterial activity of aniline dyes noted that Fuchsine can inhibit the growth of Gram-positive bacteria, including *S. aureus* (Krumwiede and Pratt 1914). In a later study, it was noted that Fuchsine had a bacteriostatic effect on Gram-positive bacteria (Rogosa 1934). While these are much older studies, the results of the current study are in line with the literature. Dumisa et al. (2020), noted the MBC values of GMRSA ATCC 33592 to be 1000.00 $\mu g/ml^{-1}$, vastly higher than the current study. The expiry date and storage of the sample in the previous study are unknown, and this could have attributed to the vast difference in the MBC values. Potassium permanganate has been on the World Health Organization's (WHO) essential drug list due to the antiseptic properties and has been broadly used in wound care (Agnihotri et al. 2019). When tested against the pathogens in the current study, a high resistance rate was noted. With the dye being used so frequently used for skin infections, resistant mechanisms may possibly have developed. A study of general antiseptics was conducted against *C. albicans*, which demonstrated susceptibility to Potassium permanganate at 10 000.00 $\mu g/ml^{-1}$ (Abed and Hussein 2016). In this study, the highest concentrations tested were 8000.00 $\mu g/ml^{-1}$.

When the 1:1 combinations of medicinal dyes with commercial antimicrobials were tested comparisons with literature were virtually non-existent and only other combinations were noted. Other combination studies demonstrating synergy included a study on photodynamic therapy (PDT) with Methylene blue in combination with four different surfactants [SDS (anionic), CTAC (cationic), HPS (zwitterionic), and Triton X-100 (non-ionic)] were tested against *C. albicans* (reference strain) (Lyon et al. 2012). The combination of both Methylene blue PDT and surfactants proved to have better activity than when tested individually (Lyon et al. 2012). This earlier study affirms that the use of Methylene blue in combination has the potential to yield synergistic effects, which is confirmed in the current study, but with alternate antimicrobials. Dumisa et al. (2020), noted a few antagonistic combinations when Methylene blue and Malachite green were tested in combination with various medicinal plants against the *S. aureus* and *C. albicans* strains. While this study cannot be compared to the current study as medicinal plants were used, it does demonstrate that antagonism may occur with medicinal dyes.

Except for Betadine, Mupirocin, and Steriscrub, all commercial antimicrobials were found to be non-toxic. Fusidic acid topically is well tolerated and is currently used in a cream and ointment called Fucidin (SAMF 2020). When used topically, Ketoconazole may cause itchiness, dryness, and stinging at the site of application. Hypersensitivity reactions such as anaphylaxis and urticaria may also occur (Sinawe and Casadesus 2024). While the antifungal application has been noted to have some serious toxic effects (Mourad and Perfect 2018, Sinawe and Casadesus 2020), demonstrated a non-toxic concentration of 500 $\mu g/ml^{-1}$. At high concentrations, Betadine® causes necrosis, while at low concentrations, it causes apoptosis (Punjataewakupt et al. 2019). The use of Betadine® may delay wound healing, which may be caused by epithelial cell death (Punjataewakupt et al. 2019). Betadine® has also been noted to cause acute kidney injury when used topically (Punjataewakupt et al. 2019). Betadine® has been found to have the most toxicity in the current study, substantiating the previous literature found. Gentamycin has been known to cause otoxicity, hearing loss, and vestibular disorders (Noack et al. 2017). Nephrotoxicity has also been noted with the use of Gentamycin, in patients who have had extended treatment (Pasmooij 2018). These effects have been frequently reported when Gentamycin was used parentally and with multiple dose treatment regimens. When used as a single dose treatment regimen, however, a lower toxicity profile was noted (Hayward et al. 2018). In this study, the brine shrimp lethality assay was conducted, and a single dose was used, and effects were tested at 24 and 48 h, possibly leading to the low toxicity value observed. Not many adverse effects of Miconazole are noted. However, it can cause stinging, burning, pruritus, and contact dermatitis when used topically (McKeny *et al*. 2021). Hübsch et al. (2014) tested the toxicity of Nystatin in the brine shrimp lethality assay and noted that Nystatin demonstrated no toxicity at 24 and 48 h. The current study supports these results. Intravenously, Nystatin is highly toxic; hence, topicay use is only recommended (Scheibler *et al*. 2017). Dumisa et al. (2020) determined the toxicity of the medicinal dyes; however, not many other studies have been conducted over the years. Brine-shrimp toxicity assays conducted on dyes and combinations with plant extracts (Dumisa et al. 2020), showed that the dyes tested were toxic. Debate has existed on the safety and oncogenic properties of Gentian violet, as previous studies have shown that Gentian violet can interact with deoxyribonucleic acid (DNA) cells (Maley and Arbiser 2013). Earlier studies on mice have noted an increase in hepatocellular carcinomas as well as an increase in thyroid cancerswhen fed large quantities of Gentian violet (Maley and Arbiser 2013). Other studies believed that Gentian violet was safe to use and did not have major contra-indications (Maley and Arbiser 2013). Gentian violet is currently sold in major grocery stores and pharmacy retail stores with minimal side effects noted (Prabha et al. 2020), making the earlier findings by Maley and Arbiser (2013) substantiated. Iodine tincture was noted as being the most toxic dye in the current study with the lowest LC_{50} values. Iodine can cause serious corrosive damage to the gastrointestinal tract and mucous membranes (Owen 2018). Exposure to the skin and eyes may cause severe burns, and lethal doses have varied in concentration from 200 mg to 20 g (Owen 2018). Iodine tincture as with Gentian violet is sold in retail stores without reservation. Malachite green was also noted to be one of the most toxic dyes in the study by Dumisa et al. (2020). Malachite green has been reported to cause reproductive abnormalities as well as being a carcinogen and has teratogenic properties. This has led the dye to being banned in the USA, Canada, and Europe. As it is a potent antimicrobial, it is still steadily used in various other countries such as South Korea and China (Gopinathan et al. 2015). Previous studies have noted a dose-dependent toxicity with Methylene blue (Clifton and Leikin 2003).

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Relatively lower toxicity was observed in comparison to the other medicinal dyes. An old study reported that the ingestion of Potassium permanganate crystals resulted in corrosive burns to the mouth, esophagus, and trachea (Southwood et al. 1987).

Once the toxicity of the individual dyes was tested, the medicinal dyes were then tested in combination. Little to no research on the toxicity of dye combinations was found. Linezolid is used to treat tuberculosis; however, it is a toxic drug to the host. A combination of bedaquiline, pretomanid, and linezolid has been used to treat drug resistant tuberculosis; preserving the drug's efficacy as well as reducing the toxicity of linezolid (Bigelow et al. 2020). While this study does not relate to the current medicinal dye study, it does demonstrate that combinations of antimicrobials are able to have better efficacy as well as reduced toxicity to the host.

The SIs also help ensure the dye's antimicrobial activity is not due to a toxic metabolite and rather the dye itself. Dzoyem et al. (2016) suggested that the good selectivity index (high SI values) is due to the sample (the medicinal dye or commercial antimicrobial) antimicrobial activity rather than a toxin associated with a metabolite of the sample. The SI values of the Gram-positive pathogens overall were much lower in comparison to the commercial antimicrobials. The high SI values (>10.00) mean that Malachite green, Fuchsine, and Gentian violet are safe to use with low toxic effects. Iodine tincture noted the lowest SI values among all dyes, which means that while the dye has a potent antimicrobial activity, it is still highly toxic against the host as well.

While the medicinal dyes have proved to have potent efficacy against the Gram-negative pathogens, their efficacy could possibly be due to a toxic metabolite and is unsafe to use on the host. The low SI values means that it does demonstrate potent antimicrobial activity and low toxic effects; hence, it may be considered safe. While this Iodine tincture did demonstrate potent antimicrobial activity against the yeasts, it also proved to be highly toxic hence, is not an ideal choice or safe to use. Malachite green and Methylene blue noted an SI value >10.00 demonstrating good antimicrobial efficacy while having minimal adverse effects to the host. The high SI value noted for Nystatin indicates that a good antimicrobial activity is shown, and the antimicrobial noted fewer toxic effects. The four combinations noted potent antimicrobial activity against each of the pathogens, which is due to the samples and not a toxic metabolite as well as a non-toxic relationship with the host making them ideal combinations for use.

Further in-depth (various ratio studies) were presented on only the two best overall combinations. Gentamycin and Neomycin both come from the same class of antibiotics (aminoglycosides), yet, in combination, Neomycin demonstrates more additive effects. Dumisa et al. (2020) conducted varied ratio studies on crystal violet in combination with various plant extracts and noted synergy among all ratios, which demonstrated the potential of varied ratio testing among medicinal dye combinations.

Time-kill studies were conducted on two combinations (Gentian violet in combination with Gentamycin and Malachite green in combination with Neomycin) based on the positive SI observed. Gentian violet has been observed to have a bacteriostatic effect on various bacteria (Ingraham 1933, Rogosa 1934). This has been demonstrated in the current study as well. While Gentamycin is known for its bactericidal activity, a previous study conducting time-kill studies on Gentamycin observed bactericidal activity only after 6 and 48 h (Bortolin et al. 2017).

Gentamycin has also been used in combination with other antimicrobials for an enhanced synergistic effect (Krause et al. 2016). One example to note would be the combination of Gentamycin with β-lactams (Krause et al. 2016).

The second combination chosen was Malachite green and Neomycin, which noted bactericidal activity better than the controls tested. The medical dye used noted a bacteriostatic effect in the current study which has been observed previously (Chaudhary et al. 2017). Neomycin comes from the same group of aminoglycosides as Gentamycin (Krause et al. 2016). As with Gentamycin, Neomycin is also most often combined with β-lactams due to the synergy the combination provides (Krause et al. 2016). As proven in the current study, Neomycin does provide a synergistic effect when combined with the dye.

Conclusion

The medicinal dyes have demonstrated potent antimicrobial activity, much more than the commercial antimicrobials but determining the toxicity of medicinal dyes is important considering reports of adverse effects. This study has shown that when dyes are combined with a selection of commercial antibiotics there is the potential to decrease not only the toxic effects but enhance antimicrobial efficacy. This was particularly seen for the combination of Malachite green with Neomycin. This study has successfully shown the potential to use combinations of medicinal dyes and commercial antimicrobials against pathogens that have been known to cause skin infections. Future studies should examine a more clinical approach to dye: antibiotic therapy with the aim of lessening the burden of currently prescribed antibiotics.

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Supplementary data

Supplementary data is available at *JAMBIO Journal* online.

Conflict of interest: The authors declare that there are no competing interests.

Author contributions

Rhea Ramfol (Data curation, Formal analysis, Investigation, Methodology, Writing – original draft), and Sandy van Vuuren (Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Writing – review & editing)

Data availability

The data underlying this article are available in the article and in the online supplementary material.

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