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Ramfol, Rhea

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THE COMBINATION OF MEDICINAL DYES WITH CONVENTIONAL ANTIMICROBIALS: POTENTIAL FOR SYNERGY IN TOPICAL SKIN INFECTIONS



UNIVERSITY OF THE
WITWATERSRAND,
JOHANNESBURG

Rhea Ramfol

A dissertation submitted to the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, in fulfilment of the requirements for the degree of Master of Pharmacy

May 2023

DECLARATION

I, Rhea Ramfol, hereby declare that this dissertation is my own work, with all other sources of information acknowledged by means of a complete reference list. This dissertation is being submitted in fulfilment of the degree of Master of Pharmacy, at the University of the Witwatersrand, Johannesburg. This work has not been submitted before for any degree or examination to this or any other university.



Rhea Ramfol

Date: 16 May 2023

ABSTRACT

Medicinal dyes have been used for centuries to treat ailments, however, research into their use as an antimicrobial has been lacking in recent years and most clinical work predates to 1945 and has now been overshadowed by antibiotics. Many antibiotics have become ineffective in treating the simplest of infections thereby sparking a new interest in combination therapy. This study aimed to explore potential synergistic effects of medicinal dyes and antimicrobials against pathogens (reference, resistant and clinical strains) responsible for skin infections.

To test the activity of the medicinal dyes and commercial antimicrobials, minimum inhibitory concentrations (MIC) and minimum bactericidal concentration (MBC) assays were conducted against Gram-positive and Gram-negative bacteria, and yeasts. Combination studies were conducted in equal ratios (1:1) and the fractional inhibitory index (ΣFIC) was calculated. Varied ratio combinations were conducted on selected combinations and represented visually as isobolograms. Gentian violet demonstrated the strongest antimicrobial activity against all pathogens with a notably strong MBC value of 0.000031 µg/ml against *Staphylococcus epidermidis* (Clinical BA16), 0.01µg/ml against *Pseudomonas aeruginosa* (ATCC 27858 and DSM 46316) and *Escherichia coli* (ATCC 8379). It also demonstrated the strongest activity against *Candida albicans* with an MIC value of 0.00025 µg/ml.

Among the antibiotics, Mupirocin and Fusidic acid noted the strongest antimicrobial activity with an MIC/MBC of 0.04 µg/ml against *S. epidermidis* (Clinical BA16 and ATCC 51625), followed by Betadine® with an MIC/MBC of 1.56 µg/ml against *E. coli* ATCC 8379 and DSM 22314) and an MIC/MBC of 1.30 µg/ml against *C. albicans* (ATCC 10231). Individually, medicinal dyes noted stronger antimicrobial activity compared to conventional antimicrobials. Combination studies (1:1 ratio) noted 26% of dye-antibiotic combinations were synergistic against the Gram-positive strains, 15% against the Gram-negative strains and 14% against the yeasts. In the varied ratio studies, the Mercurochrome: Betadine® combination noted synergy among all the *Staphylococcus aureus* strains with the 1:1 ΣFIC values ranging from 0.05 to 0.48. Fuchsine with Gentamycin noted the most synergistic ΣFIC value of 0.001 against *P. aeruginosa* (DSM 46316)

strain. Methylene blue with Ketoconazole demonstrated the best synergistic ΣFIC value of 0.01 among the yeasts.

The brine shrimp lethality assay was then undertaken to determine the toxicity of the medicinal dyes, commercial antimicrobials, and selected combinations (used in varied ratio studies). Four dyes (Iodine tincture, Gentian violet, Malachite green and Mercurochrome) demonstrated high toxicity at 24 and 48 hrs. All four dyes demonstrated a percentage mortality of 100% at 48 hrs. The majority of the commercial antibiotics were non-toxic at both 24 and 48 hrs. Combination studies (1:1) on selected (50%) synergistic combinations observed a decrease in toxicity. The combination of Gentian violet with Gentamycin noted a fifteen-fold decrease in toxicity – the most synergistic combination. Malachite green in combination with Neomycin noted the second highest decrease in toxicity.

The selectivity index (SI) of the medicinal dyes, commercial antimicrobials and selected combinations were calculated based on the brine shrimp lethality assay together with the MIC. A low SI (<10.00) was observed amongst the majority of the dyes tested. The lowest SI was demonstrated by Iodine tincture with a SI value of 0.0001 against all *S. aureus* strains, Gram-negative strains, and yeasts. The commercial antimicrobials noted higher SI values in comparison with the medicinal dyes. The highest SI value observed was 12500.00 against *S. epidermidis* (ATCC 12228) when calculated against Fusidic acid. Gentamycin demonstrated high SI values among majority of the Gram-negative strains. All three antifungals demonstrated high SI ratios among *C. albicans* (ATCC 10231 and A100). For the combinations tested, Gentian violet with Gentamycin demonstrated the highest SI of 977.50 against the *E. coli* (DSM 22314) strain.

Time-kill studies were conducted on the combinations with the highest safe SI value and lowest safe SI value: Gentian violet with Gentamycin and Malachite green with Neomycin respectively. Both medicinal dyes demonstrated bacteriostatic activity over a period of 24 hrs. When combined, Malachite green with Neomycin demonstrated bactericidal activity.

This study has demonstrated that selected dye: antimicrobial combinations (20.46%) potentially act synergistically, however, when the antimicrobial activity and toxicity of these combinations

have been investigated, two combinations demonstrate more promise in comparison to the others: Gentian violet with Gentamycin and Malachite green with Neomycin. These combinations have the potential for further clinical studies as a future treatment for skin infections. This study also highlights the importance of further evaluation of the antimicrobial properties of medicinal dyes in combination with commercial antimicrobials in order to determine viable options to treat skin infections.

DEDICATION

To my parents, Ojen and Naina, without your endless love and support I would have not made it this far. I truly appreciate all the encouragement, sacrifices and prayers you have made which has allowed to me come this far. Thank you for everything.

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Publications – Appendix B (Abstract)

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LIST OF ABBREVIATIONS

°C	Degrees Celsius
%	Percentage
<	Less than
=	Equal to
>	Greater than
≤	Less than or equal to
≥	Greater than or equal to
ATCC	American type culture collection
AIDS	Acquired immune deficiency syndrome
BSLA	Brine-shrimp lethality assay
CC	Culture control
CFU	Colony forming units
CSLI	Clinical and Laboratory Standards Institute
DNA	Deoxyribonucleic acid
EUCAST	European Committee on Antimicrobial Susceptibility Testing
FIC	Fractional inhibitory concentration
ΣFIC	Sum of the fractional inhibitory concentration
g	Gram
GMRSA	Gentamycin-Methicillin-Resistant *Staphylococcus aureus*
hrs	Hours
INT	*p*-iodonitrotetrazolium violet
LC_{50}	Lethal dose to kill 50.00%
MRSA	Methicillin-resistant *Staphylococcus aureus*
mg	Milligram
mg/L	Milligram per litre
mg/mL	Milligram per millilitre
µg	Microgram
µg/mL	Microgram per millilitre

µL	Microlilitre
MIC	Minimum inhibitory concentration
min	Minutes
mL	Millilitre
pH	Potential hydrogen
SI	Selectivity index
SSTI	Skin and soft tissue infections
Spp.	Species
TSB	Tryptone Soya broth
TMP-SMX	Trimethoprim- sulfamethoxazole
WHO	World Health Organisation

CHAPTER 1

Introduction and literature review

1.1. Skin and soft tissue infections

One of the first line defences against microbial invasion is the skin (Williamson *et al*., 2017). The skin serves as a protective barrier by secreting low pH, sebaceous fluid and fatty acid that inhibits the growth of pathogens (Ki and Rotstein, 2008). Healthy skin carries a wide range of bacteria and depending on the environment, the type of bacteria and the host, the bacteria can be protective or harmful (Williamson *et al.,* 2017). Broken skin such as surgical incisions, insect bites or even trauma to the skin can cause the spread of bacteria leading to skin and soft tissue infections (Williamson *et al*., 2017).

Skin and soft tissue infections (SSTI) develop in three steps: bacterial adherence to host cells, invasion of tissue with evasion of host defenses and elaboration of toxins (Ki and Rotstein, 2008). Most SSTI's are caused by *Staphylococcus* and *Streptococcus* species especially *Staphylococcus aureus* and methicillin-resistant *Staphylococcus aureus* (MRSA) (Song *et al*., 2017). Skin and soft tissue infections are extremely common and lead to high rates of hospitalization especially in children (Keren *et al*., 2012; Williamson *et al*., 2017). Due to the diversity of SSTI's, each infection's clinical manifestation differs; however, most infections present with non-specific, typical features such as erythema, edema, pain and warmth.

Severe infections often present with more severe systemic symptoms such as extremes in body temperature, tachycardia, and extreme pain (Ki and Rotstein, 2008). To determine the treatment required for SSTI's, one would need to determine the severity of the infection. The size of the lesion is vital in determining severity, as most often more severe infections involve a higher proportion of soft tissue and skin (Ki and Rotstein, 2008). The site of the infection is just as important as most SSTI's tend to involve the lower extremities (Ki and Rotstein, 2008;

Ramakrishnan *et al*., 2015). Infections to the lower extremities remain a significant cause of morbidity and mortality in hospitalized and non-hospitalized patients (Sharpe *et al*., 2005).

While most infections are caused by bacterial pathogens, there are various fungi which can cause mild to severe SSTI's. Fungal infections of the skin and nails are the most common infections and are seen worldwide (Havlickova *et al*., 2008). Cultural, environmental factors as well as the geographical location play a role in the type of fungal infection and well as their causative agents (Havlickova *et al*., 2008). Even when people have similar risk factors, they are not equally susceptible to fungal infections (Sahoo and Mahajan, 2016).

Fungal soft tissue infections normally occur in severely immunocompromised patients such as patients who are receiving chemotherapy, patients who are on immunosuppressive medications, patients who have severe metabolic disease or those with Acquired Immune Deficiency Syndrome (AIDS) (Sachse, 2001). Fungal infections flourish at surface temperatures ranging from 25 to 28 degrees Celsius (°C). The warm and humid conditions support the growth of these infections (Havlickova *et al*., 2008). Fungal infections are prevalent in sub-tropical and topical areas (Sahoo and Mahajan, 2016).

1.2. Overuse of hand sanitizers during Covid-19

Currently, the novel coronavirus known as "Covid-19" has posed a global challenge since is discovery in December 2019 (Zhai *et al.,* 2020). A respiratory infection that is transmitted via human-to-human transmission by droplets, contaminated hands, or surfaces (Zhai *et al*., 2020). The World Health Organization (WHO) recommended frequent and correct washing of hands with suitable disinfectants as a means to reduce transmission (Beiu *et al*., 2020). Frequent hand washing and sanitization can cause adverse effects (Beiu *et al*., 2020). The repeated use of sanitizer or hand washing can lead to a disruption in the skin barrier (Patruno *et al*., 2020).

Skin reactions from excessive dryness to irritant contact dermatitis and even allergic contact dermatitis have been noted (Beiu *et al*., 2020). The maintenance of the skin flora is vital for healthy skin (Breidablik *et al*., 2020). The micro-organisms on normal skin are not pathogenic and are able to replicate locally are known as "resident microbes" and able to be controlled by disinfections

(Breidablik *et al*., 2020). “Transient microbes” are bacteria that are temporarily found on the skin and are not viable on the skin for an extended period, however, if they do become pathogenic, they may easily transmit to wounds or dermatitis (Breidablik *et al*., 2020). Health care workers wearing personal protection equipment experience adverse effects such as hyperhydration to their skin as well as epidermal barrier breakdown, friction and contact reactions which has manifested as Erythema, papules, maceration, and scaling causing burning, itching, and stinging (Darlenski and Tsankov, 2020).

The use of sanitizers being either ethanol based or those containing isopropyl alcohol is recommended due to the broad antimicrobial activity of the sanitizer (Mahmood *et al*., 2020). Most commercial sanitizers contain between 60% to 95% ethanol or isopropyl alcohol (Mahmood *et al*., 2020). Currently, sanitizers are being used frequently for better hand hygiene, however, excessive use or misuse of sanitizers increase skin penetrability and reduces the oil and water from human skin which may lead to skin irritation and coarseness (Mahmood *et al*., 2020). This greatly reduces the skin’s ability to provide an effective skin barrier which protects against other harmful bacteria and viruses causing an increased risk of entry of other micro-organisms into the skin. In addition, the overuse or misuse of alcohol-based sanitizers pose a risk of increase antimicrobial resistance (Mahmood *et al*., 2020). Micro-organisms are able to mutate through a natural process with repeated use of sanitizers allowing them to survive. Mahmood *et al*. (2020) demonstrated that *Escherichia coli* and *Pseudomonas aeruginosa* had a 48% and 68% respectively resistant effect to all hand sanitizers on the market (Mahmood *et al*., 2020). The increased use of hand sanitizers causes an imbalance with the skin and residing bacteria leading to a possible increased resistance rate. This leads to a reduction in topical treatment options such as antibacterial creams or ointments.

1.3. Types of skin and soft tissue infections and their treatment

Skin and soft tissue infections can range from superficial to severe infections affecting any part of the body and may affect subcutaneous tissue, muscle and even fascia (Sartelli *et al*., 2018). Table 1.1 shows the most common skin and soft issue infections.

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). Some of the most prescribed antimicrobial agents are topical antibiotics and even though they are widely used, prescriptions of topical antibiotics are only indicated for a limited number of skin pathogens (Williamson *et al*., 2017). Table 1.2 shows the most common antibiotics used topically in skin and soft tissue infections as well as the antimicrobial properties. When referring to the antibiotics, antifungals and antiseptics listed in Table 1.2, an overall term "commercial antimicrobial" will be used for all samples.

Depending on the severity of disease, treatment normally requires dual therapy of topical agents and oral agents. For milder cases, the most common topical agent employed is Mupirocin (Bactroban©), however, emerging resistance has been shown (Stevens *et al*., 2005; Ramakrishnan *et al*., 2015).

1.3.1. Impetigo

Impetigo is one of the most common bacterial skin infections. It normally occurs in children between the ages of two to five, however, it can occur in adults (Clebak and Malone, 2018). It is normally caused by *S. aureus* and *Streptococcus. pyogenes*. Impetigo can be caused due to an infection from damaged skin or normal skin being directly invaded by micro-organisms (Sukumaran and Senanayake, 2016).

In children, impetigo is the third most common infection found in children and overall, accounts for 50% to 60% of all bacterial infections (Clebak and Malone, 2018). Impetigo in children is highly contagious and has two types: bullous and non-bullous impetigo (Sukumaran and Senanayake, 2016). Non-bullous impetigo first presents as a maculopapular lesion which later turns into a blister (Clebak and Malone, 2018). Once the blister breaks, the lesion dries and forms a yellow crust that may cause itchiness (Sukumaran and Senanayake, 2016). Bullous impetigo is mainly caused by *S. aureus* and forms a brown crust once the blister breaks (Sukumaran and Senanayake, 2016).

Impetigo may be treated in various ways including topical antibiotics such as Fusidic acid and Mupirocin if a mild infection occurs (Loadsman *et al*., 2019; Gorges *et al*., 2021). If there are a number of lesions or more severe then systemic antibiotics may be used namely, Penicillin's, Cephalosporins and Macrolides (Loadsman *et al*., 2019; Gorges *et al*., 2021). Topical antiseptics (Chlorohexidine and Povidone-iodine) may be applied (Loadsman *et al*., 2019).

Non-bullous impetigo is usually treated using two antibiotic creams: Fusidic acid and Mupirocin and are just as effective as oral antibiotics when the infection is not extensive (Clebak and Malone, 2018). If there are a number of lesions or it affects several areas then systemic antibiotics may be prescribed (Clebak and Malone, 2018). Bullous impetigo may also be treated with Fusidic acid and Mupirocin however, if extensive, oral agents such as first- generation Cephalosporins, Clindamycin and Penicillin's may be used (Johnson, 2020; Robinson *et al*., 2021) Treatment for folliculitis would also include topical antibiotics Fusidic acid and Mupirocin and is considered the first line treatment (Clebak and Malone, 2018). If patients do not respond to topical agents, then therapy is escalated with oral antibiotics (Stevens *et al*., 2005). Penicillin would routinely be given orally; however, MRSA is highly resistant to beta-lactams therefore Penicillin may prove ineffective. (Stevens *et al*., 2005; Sartelli *et al*., 2018).

1.3.2. Cellulitis and erysipelas

Cellulitis and erysipelas both present with warmth and redness of the skin, however, erysipelas is more a superficial infection and mainly caused by *S. aureus* (Clebak and Malone, 2018). Cellulitis, in contrast, affects deeper layers of the skin as well as subcutaneous fat (Clebak and Malone, 2018). Cellulitis affects lower limbs unilaterally, rapidly spreading and the lesion does not contain distinct, sharp borders which are normally seen with erysipelas (Sukumaran and Senanayake, 2016; Clebak and Malone, 2018). Cellulitis is caused by *S. aureus;* however, methicillin-resistant *S. aureus* (MRSA) has become an increasingly common cause of skin infections (Clebak and Malone, 2018).

Trimethoprim-sulfamethoxazole with Cephalexin may be used to treat cellulitis, however, if there is an allergy to Trimethoprim-sulfamethoxazole then Clindamycin may be substituted. Treatment is normally five days but can be extended depending on the severity (Brown and Watson, 2021).

First-line treatment of erysipelas is an approximately five-day treatment with Penicillin. Other supportive treatments may be used such as elevating the limb, cold compresses, and paracetamol for fever (Michael and Shaukat, 2021).

1.3.3. Folliculitis

Folliculitis is the inflammation of hair follicles (Clebak and Malone, 2018). It presents with redness and pustules affecting moist skin and hair (Sukumaran and Senanayake, 2016; Clebak and Malone, 2018). Folliculitis (especially superficial folliculitis) is mainly caused by *S. aureus;* however, it can be caused *P. aeruginosa* as well (Winters and Mitchell, 2022). *Pseudomonas aeruginosa* associated folliculitis may be prevalent due to exposure to water and hot tubs (Clebak and Malone, 2018; Winters and Mitchell, 2022). Folliculitis caused by the *Staphylococcal* spp. and Gram-negative organisms will normally resolve by itself in 7 to 10 days if they are simple infections, however, if the case is more severe then topical antibiotics such as Mupirocin and Clindamycin may be used (Winters and Mitchell, 2022). Oral antibiotics may be used such as Ampicillin, Trimethoprim-sulfamethoxazole, and Ciprofloxacin (Winters and Mitchell, 2022).

1.3.4. Furuncle and carbuncles

A furuncle which is also known as a boil, is an infection of the hair follicle with a collection of pus that moves into the subcutaneous tissue forming an abscess (Clebak and Malone, 2018). A carbuncle is a collection of two or more furuncles which converge and drain through a follicular opening (Clebak and Malone, 2018; Troxell and Hall, 2020). There is typically redness at the site, tenderness, and inflammation (Troxell and Hall, 2020). Carbuncles occur in young to middle-aged adults and more frequently in men than women (Troxell and Hall, 2020). A skin abscess is a collection of pus that affects the dermis and deeper skin tissue (Clebak and Malone, 2018). Furuncles, carbuncles and skin abscesses are caused by *S. aureus* (Clebak and Malone, 2018; Troxell and Hall, 2020).

Carbuncles are incised and drained after which a course of oral antibiotics are given such as Cephalosporins (Troxell and Hall, 2020). Topical antibiotics such as Mupirocin is also used in conjunction with the oral antibiotics (Troxell and Hall, 2020). In cases where MRSA is the cause

of the infection, Linezolid, Trimethoprim- sulfamethoxazole (TMP-SMX) or a Tetracycline would be the antibiotic of choice (Sartelli *et al*., 2018). Deep tissue infections often require in-patient care and are treated with intravenous antibiotics (Ramakrishnan *et al*., 2015).

1.3.5. Candidal infections

The most common infection caused by *Candida* spp. is Candidal intertrigo (Clebak and Malone, 2018). Frequent antibiotic use, diabetes mellitus, obesity and even repetitive skin friction increase the likelihood of intertrigo (Clebak and Malone, 2018). Intertrigo is an inflammatory condition that may cause itchiness and redness (Clebak and Malone, 2018).

Diaper dermatitis caused by *Candida* occurs when the skin is in contact with a wet diaper (Clebak and Malone, 2018). It is one of the most common conditions affecting babies and usually affects children in the first two years (Petek *et al*., 2022). It mainly occurs on the buttocks, lower abdomen, genitalia, and upper thighs and presents as pustules and redness (Clebak and Malone, 2018). Treatment of candidal diaper dermatitis would require a topical barrier cream and tropical antifungals such as Nystatin, Clotrimazole, Miconazole, Ketoconazole, and Sertaconazole (Clebak and Malone, 2018). Nystatin is normally first line treatment in most cases and if no improvement is seen after use, then switching may occur to Miconazole or Ketoconazole (Benitez Ojeda and Mendez, 2022)

Infections such as tinea corporis, tinea cruris, and tinea pedis require topical antifungals such as Terbinafine and are the first choice in therapy (Clebak and Malone, 2018). If the fungal infection persists, oral antifungals such as Itraconazole and Terbinafine would need to be prescribed (Clebak and Malone, 2018). Topical antifungals are the best choice of therapy for Candidal intertrigo and topical preparations of Nystatin, Clotrimazole, Ketoconazole, Miconazole, or Econazole are considered the first-line therapy (Clebak and Malone, 2018). If severe and resistant fungal infections occur, oral Fluconazole and Itraconazole can be used (Clebak and Malone, 2018).

Table 1.1 : Common skin and soft tissue infections

Infection	Clinical features	Responsible pathogen	Reference
Impetigo	Common infection of the skin epidermis, which affects nose, mouth or limbs. It can occur at any age, however, most common in children aged two to five. Can cause mild soreness, redness and appears as a maculopapular lesion.	*S. aureus*	Ramakrishnan *et al*; 2015; Clebak and Malone, 2018
Erysipelas, cellulitis	Erysipelas: a superficial infection affecting face, ears, or lower limbs. Seen as raised inflamed skin. Cellulitis: Involves deeper skin layers and subcutaneous fat when compared to erysipelas and occurs after skin layers are already compromised. Appears as rapidly spreading, painfully hardened area of subcutaneous tissue with overlying warmth and erythema.	Erysipelas and cellulitis are caused by *S. aureus* with MRSA becoming more common. It may also be caused by *S. epidermidis* Cellulitis restricted to the lower or upper limbs may also be caused by *E. coli*.	Ramakrishnan *et al*, 2015; Clebak and Malone, 2018; Natsis and Cohen, 2018; Petkovšek *et al*., 2009
Folliculitis	Infection or inflammation of hair follicles. Presents with redness and pustules.	Mainly caused by *S. aureus* and *S. epidermidis*, however, can also be caused by *P. aeruginosa* and *Candida* spp. in immunocompromised patients.	Ramakrishnan *et al*; 2015; Clebak and Malone, 2018, Natsis and Cohen, 2018
Furuncles, carbuncles (deep folliculitis)	Furuncle: Also known as a boil, is the infection of the hair follicle and contains pus, redness, swelling. Carbuncle: Is a collection of furuncles involving deeper underlying tissue and drain through one opening.	*S. aureus* and *S. epidermidis*	Ramakrishnan *et al*; 2015; Clebak and Malone, 2018, Natsis and Cohen, 2018
Skin abscess	A collection of pus within the dermis and deeper skin tissues	*S. aureus* and *S. epidermidis*	Ramakrishnan *et al*; 2015; Clebak and Malone, 2018, Natsis and Cohen, 2018
Intertrigo	An inflammatory condition of the skin folds that has developed due to broken skin e.g., chaffing of skin	*C. albicans*	Clebak and Malone, 2018
Tinea unguium (onychomycosis)	Fungal infection that mainly occurs on the toenails rather than the fingernails. Requires long term therapy.	*C. albicans*	Havlickova *et al*., 2008
Candidal diaper dermatitis	Occurs when skin is contact with a wet diaper. Presents with redness and pustules.	*C. albicans*	Clebak and Malone, 2018

Table 1.2: Antimicrobials and properties

Antimicrobial	Antimicrobial properties/activity	Activity against pathogens	References
Chlorohexidine (Steriscrub)	Antiseptic used in skin disinfections. It is used in preoperative bathing to reduce surgical skin infections. Used in the treatment of Gram-positive and Gram-negative bacteria.	Gram-positive and Gram-negative bacteria.	Rossiter and Blackman, 2012; Supple *et al*, 2015: George *et al*., 2016
Fusidic acid	Limited spectrum of activity and used mainly against Gram-positive bacteria. Recommended as first line treatment of primary and secondary infections as it can easily penetrate damaged and intact skin.	Limited spectrum of activity used mainly against Gram-positive bacteria and *Staphylococcal* infections.	Verbist,1990; Rossiter and Blackman, 2012; Bonamonte *et al*, 2014
Gentamycin	Treats a variety of skin infections such as impetigo, folliculitis, and dermatitis. Other *streptococci* and *enterococci* are resistant.	Used to treat many Gram-positive and Gram-negative infections such as *S. aureus, E. coli* and even some strains of *S. epidermidis* and *P. aeruginosa*	Rossiter and Blackman, 2012; Oesterreicher *et al*., 2018
Ketoconazole	Broad-spectrum antifungal. Used against seborrheic dermatitis due to its anti-inflammatory properties. An imidazole effective in treating cutaneous candidiasis and dermophytes	Effective in treating cutaneous candidiasis and dermophytes. Bacteriostatic properties against *Staphylococcus aureus* have been reported	Borgers and Degreef, 2007; Rossiter and Blackman, 2012
Neomycin	A broad-spectrum aminoglycoside like Gentamycin.	Used to treat Gram-negative and some Gram-positive bacteria.	Rossiter and Blackman, 2012; Bohbot *et al*., 2019
Miconazole	Used in the treatment of local and systemic fungal infections.	Effective in treating cutaneous candidiasis and dermophytes	Rossiter and Blackman, 2012; Parashar *et al*, 2013
Mupirocin	Used to treat primary and secondary skin infections	Used in *Staphylococcal* infections.	Gisby and Bryant, 2000; Rossiter and Blackman 2012
Nystatin	Used as a potent broad-spectrum antifungal. It is not effective against skin infections caused by dermophytes.	It is used to treat *Candida* infections of the skin and mouth.	Rossiter and Blackman 2012; Elosaily *et al*, 2014
Povidone-iodine (Betadine®)	Broad-spectrum antimicrobial. Promotes wound healing with its anti-inflammatory effects.	Used to treat Gram-negative and Gram-positive bacteria and antibiotic resistant strains. Some bacteria it's active against are MRSA and *E. coli.*	Rossiter and Blackman, 2012; Hoekstra *et al*,2017

1.4. Types of pathogens in skin and soft tissue infections and resistance patterns

Antibiotic resistance is now a worldwide crisis affecting the health of humans, as difficulty in treating microbial pathogens has led to a high rate of mortality (Frieri *et al*., 2017; Yadav and Kapley, 2021). Many antibiotics have become ineffective in treating the simplest of infections (as a result of antimicrobial resistance) 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; Lai *et al*., 2022). The most common pathogens isolated from the skin are *S. aureus*, *E. coli*, and *P. aeruginosa* (Smith *et al*., 2020). In a hospital setting, *E. coli* and MRSA have led nosocomial infections. Fungi especially, *Candida* have been recognized to have caused infections, however, they have not been adequately studied (Smith *et al*., 2020).

1.4.1. *Staphylococcus aureus*

Staphylococcus aureus is normally found on the skin and mucosa and is a Gram-positive pathogen (Leung, 2014). It enters the body though broken skin and can cause mild to severe sepsis especially in an immunocompromised patient (Leung, 2014). *Staphylococcus aureus* is noted to be a major cause of SSTI's worldwide. *Staphylococcus aureus* an opportunistic pathogen that can cause minor skin infections to severe sepsis (Abdelbary *et al*., 2017). It is a well-adapted human pathogen and capable of living freely, spreading from person to person (Stefani and Goglio, 2010). It has become imperative to find new antimicrobials therapies due to the rise of resistant strains of *S. aureus* (Lalaouna *et al*., 2018). An older study (Stefani and Goglio, 2010), indicated that the number of antibiotic-resistant strains had reached epidemic proportions globally and recent statistics have echoed that little has changed since 2010 (Guo *et al*., 2020; Mlynarczyk-Bonikowska *et al*., 2022).

Lim *et al*., (2018) conducted a five-year study on antimicrobial susceptibility and skin infections. Skin swabs were taken from 197 patients with varying degrees of skin infections, and it was noted that most infections were caused *by S. aureus*. From all the swabs taken, 77% were found to be penicillin-resistant and 28% were found to be methicillin-resistant. A small percentage of samples were resistant to three or more antibiotics. It was noted that when compared to secondary infections, primary infections had a higher proportion of methicillin-resistant species as well as a

higher number of pathogens resistant to antibiotics. A primary infection is the initial infection and the first exposure to that pathogen while the secondary infection normally occurs during or after treatment due to changes in the immune system (Dockrell *et al*., 2018; Boskey, 2020) The higher proportion of MRSA could be because an infection of the intact skin barrier may have greater virulence than an infection in an already damaged skin barrier (Lim *et al*., 2018). Even though the majority of the *S. aureus* strains proved to be Penicillin resistant, some have shown added resistance to Erythromycin, Clindamycin and Gentamycin (Lim *et al*., 2018). The rise in resistance among the *S. aureus* strains makes treatment problematic as these strains are common causes of skin and soft tissue infections (Stevens *et al*., 2005). The high dispensing rates of Mupirocin and Fusidic acid has also led to an increase in resistance in *S. aureus* strains in New Zealand (Williamson *et al*., 2017). A literature review conducted in Africa; observed that 14% of *S. aureus* strains were Mupirocin-resistant *S. aureus* strains which are prevalent in South Africa (Shittu *et al*., 2018) Another literature review observed 92.6% of the *S. aureus* strains contain Gentamycin resistant genes (Ekwanzala *et al*., 2018).

1.4.2. *Staphylococcus epidermidis*

Hospital infections have been a cause for concern worldwide (Chabi and Momtaz, 2019). *Staphylococcus epidermidis* has been the most underestimated pathogen in hospital infections and is a coagulase-negative staphylococci (Galkowska *et al*., 2009). *Staphylococcus epidermidis* has been isolated from various wound infections, soft tissue infections, respiratory tract, and urinary tract infections as well as various medical devices (Chabi and Momtaz, 2019). It has a pool of antibiotic resistant genes that can be transferred by "horizontal gene transfer" to other Gram-positive pathogens such as methicillin susceptible *Staphylococcus aureus* (MSSA) (Galkowska *et al*., 2009). *Staphylococcus epidermidis* is a member of the group of coagulase-negative *Staphylococci* and compared to *S. aureus* is unable to produce coagulase (Vuong and Otto, 2002). The pathogen is found on the skin and mucous membranes and are part of the normal bacterial flora (Vuong and Otto, 2002). A study conducted by Al Laham *et al*. (2017), noted that *S. epidermidis* showed a high resistance rate to various antibiotics tested. Complete resistance was noted to the antibiotic's Ampicillin and Benzylpenicillin (Al Laham *et al*., 2017). High rates of resistance were noted towards Amoxicillin/ Clavulanic acid (Augmentin©), Carbapenems and

Cephalosporins (Al Laham *et al*., 2017). Chabi and Momtaz (2019) conducted a study in which, antibiotic resistance profiles were determined for *S. epidermidis*. The results noted the highest resistance rate to the following antibiotics: Cephalosporins, Penicillin, Tetracycline, Macrolides, Aminoglycosides, and Fluoroquinolones (Chabi and Momtaz, 2019). A study conducted on catheter related infections in a hospital in Pretoria, South Africa, observed multidrug resistant *S. epidermidis* strains (Ehlers *et al*., 2018). These *S. epidermidis* strains have shown high resistance to three antibiotic groups: β-lactams, Aminoglycosides, and Macrolides (Ehler *et al*., 2018).

1.4.3. *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is a Gram-negative opportunistic pathogen found on the skin and is responsible for many community-acquired infections including SSTI's (Wu *et al.*, 2011; Andonova and Urumova, 2013). *Pseudomonas aeruginosa* can cause skin infections ranging from superficial to deep. The pathogen mainly causes mild infections; however, can cause fatal, acute, and chronic conditions in patients who are immunocompromised (Wu *et al.*, 2011; Thi *et al*., 2020). The pathogen has a high resistance to many antimicrobials and a strong ability to form biofilms making it very difficult to eradicate (Nagoba *et al*., 2017). One of the reasons of the high resistance of *P. aeruginosa* to many antimicrobials is due to low permeability of the outer membrane (Nagoba *et al*., 2017). *Pseudomonas aeruginosa* can cause a variety of infections especially in hospitals which can range from superficial infections in wounds, burns and abscesses (Nagoba *et al*., 2017). *Pseudomonas aeruginosa* strains have shown resistance to the beta-lactams such as ampicillin and ceftriaxone and have also known to acquire resistance to other antibiotics during treatment such as Carbapenems (Dame *et al*., 2020).

Studies over the past decade have shown that infections caused by *P. aeruginosa* are becoming increasingly difficult to treat due to increased resistance occurring (Wu *et al*., 2011). In an African study 1000 clinical strains were obtained from various hospitals in Calabar, Nigeria and the results noted that 70% of *P. aeruginosa* isolates were resistant to Cephalosporins (Eyo *et al*., 2015). Alhlale *et al*. (2020) conducted a study to determine the effectiveness of antibiotics to surgical wounds in government hospitals in Yemen. The study showed that *P. aeruginosa* was completely resistant to Amoxicillin and Vancomycin (Alhlale *et al*., 2020). The study also noted resistance

towards Tetracyclines at 92.3%, Erythromycin at 84.6%, Nalidixic acid, Nitrofurantoin at 76.9%, and Ciprofloxacin at 69.2% (Alhlale *et al.*, 2020). A study conducted in a private hospital in Durban, South Africa conducted susceptibility tests on multidrug resistant strains of *P. aeruginosa* (Adjei *et al*., 2018). The highest resistance was observed against Ciprofloxacin and Amikacin (Adjei *et al*., 2018). Resistance to Piperacillin, Aztreonam and Meropenem was also observed (Adjei *et al*., 2018). A more recent study conducted in a rural hospital in Eastern Cape, South Africa preformed antibiotic susceptibility tests on various *P. aeruginosa* strains (Hosu *et al*., 2021). Results from this study have shown that out of 204 isolates tested, the highest resistance demonstrated was to Piperacillin followed by Aztreonam strains (Hosu *et al*., 2021). These strains, also noted resistance to Gentamycin, Meropenem, Amikacin and various other antibiotics (Hosu *et al*., 2021).

1.4.4. *Escherichia coli*

Escherichia coli is a "rod-shaped", Gram-negative pathogen which is normally found in the lower intestine (Safardoust-Hojaghan *et al*., 2017). Most strains of *E. coli* are harmless (Safardoust-Hojaghan *et al*., 2017). A literature review noted *E. coli* as one of the causative pathogens in skin and soft tissue infections. Various infections such as cellulitis in the upper and lower limbs, neonatal omphalitis, burn injury infections and surgical site infections have been known to be caused by *E. coli* (Petkovšek *et al*., 2009). The rate at which *E. coli* is becoming resistant to the majority of the antibiotics has been increasing (Lee *et al*., 2018). Enzymes called beta-lactamases produced by *E. coli* hydrolyze the beta-lactam ring (Walther *et al*., 2017). Due to this, *E. coli* has shown resistance to Penicillin, Cephalosporins (even the newer generations) and Monobactams (Walther *et al*., 2017). A study observing the resistance patterns of various *E. coli* strains in urban and rural areas of South Africa found the strains to be highly resistant to Ampicillin, Trimethoprim-sulfamethoxazole, and Ciprofloxacin (Kubone *et al*., 2020). A low resistance rate was observed against Gentamycin and Amikacin (Kubone *et al*., 2020).

1.4.5. *Candida albicans*

Candida albicans is a "dimorphic commensal fungus" that is found on the human skin (Kashem and Kaplan, 2016). It is an opportunistic human yeast pathogen responsible for most of the

Candida infections encountered (Yang, 2003). *Candida albicans* is generally a harmless pathogen that turns into an opportunistic pathogen in immunocompromised patients (Dadar *et al.*, 2018). An abnormal growth in *C. albicans* such as change in pH leads to candidiasis (Dadar *et al*., 2018). *C. albicans* are most often responsible for fungal skin infections such as oropharyngeal thrush, vaginitis, diaper dermatitis, and intertrigo (Davis, 1995). Symptoms include thickening of the skin, hyperkeratosis, and erythema (Kühbacher *et al*., 2017). Resistance has been noted towards a group of antifungals called the azoles consisting of two subgroups: the imidazoles and triazoles (Casalinuovo *et al*., 2004). It has also been noted that *C. albicans* has a high rate of resistance to fluconazole a triazole due it being the most prescribed antifungal (Casalinuovo *et al*., 2004). A more recent study noted resistance to Caspofungin and Anidulafungin with various *C. albicans* strains (dos Santos Abrantes *et al*., 2014). More than 50% of the *C. albicans* strains, exhibited resistance to all azoles tested (dos Santos Abrantes *et al*., 2014).

1.5. Combination therapy with antimicrobials

Multidrug resistant bacteria causing infections have been a growing cause for concern and have become a threat globally (Namivandi-Zangeneh *et al*., 2021). The inappropriate use of antibiotics has been a major driver of antimicrobial resistance and have made treating these infectious diseases a problem (Namivandi-Zangeneh *et al*., 2021). 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 has been found to be an effective strategy (Sullivan *et al*., 2020; Poustchi *et al*., 2021). The concept of antimicrobial combination therapy helps improve different mechanisms of action and the toxicity of antimicrobials (Rybak and McGrath, 2012). Combination therapy has the potential to lead to synergistic effects and has the potential to slow antimicrobial resistance (Rybak and McGrath, 2012).

When dealing with the concept of antimicrobial therapy, an understanding of the synergistic, additive and antagonist effects is important. The concept of a synergistic effect is combining two or more antimicrobials to produce a greater effect that exceeds the sum of the individual effects hence, increased antimicrobial activity (Berthoud, 2013; Caesar and Cech, 2019). Synergistic combinations not only allow for better antimicrobial activity but may also reduce the toxic effects

caused by one or both of the samples (medicinal dyes and commercial antimicrobials) (Caesar and Cech, 2019). An additive effect is simply combining two or more antimicrobials to produce the sum of the individual antimicrobials (Roell *et al*., 2017). The indifferent or non-interactive effect is when both antimicrobials are not active or one of them has no antimicrobial activity (Greco 1995; Johnson *et al*., 2004). An antagonistic effect is the opposite of synergy, where the combined effect of the antimicrobials is less than what would have been expected if tested independently hence, decreased antimicrobial activity (Roell *et al*., 2017). Various studies have been conducted demonstrating the synergistic effect of combination therapy of antimicrobials.

Acute skin infections have been a major cause of hospital admissions over the years (Wargo *et al.,* 2015). A study conducted observed the synergistic effect of Vancomycin and Clindamycin against skin infections (Wargo *et al.,* 2015). Treatment with combination therapy of Vancomycin and Clindamycin did not largely reduce the length of hospital stay in comparison to the individual therapies (Vancomycin or Clindamycin individually), but it did significantly reduce the readmission rate of skin infections when treatment of combination therapy was given (Wargo *et al.,* 2015).

Phee *et al*. (2015), conducted antimicrobial susceptibility and interaction studies on a combination of two antibiotics, Fusidic acid and Colistin against a resistant strain of *Acinetobacter baumannii*, a Gram-negative bacterium that is responsible for various skin and soft tissue, bloodstream, respiratory and device-related infections. The disc diffusion assay was used to determine whether synergy occurred. Time-kill studies were performed on the combination as well. Synergy was noted on all strains of *A. baumannii* that were tested. The results from the time-kill studies showed that the combination of both antibiotics was rapidly bactericidal.

A study to determine the synergism of Mupirocin with various antimicrobials against MRSA was conducted (Khoshnood *et al*., 2019). It was noted that Mupirocin in combination with Amoxicillin-clavulanate showed synergism (Khoshnood *et al*., 2019). Clark *et al*. (2015) conducted a study to determine the susceptibility of Fusidic acid, chlorohexidine, and Miconazole on canine methicillin-resistant and susceptible staphylococcal isolates. Each antimicrobial was tested individually as well as in combinations. A notable combination of Chlorohexidine and Miconazole showed a synergistic interaction (Clark *et al*., 2015). A study conducted by Bhardwaj *et al*. (2021),

determined the synergistic effect of Vancomycin and Chlorohexidine. It was noted that *Enterococcus faecium* was resistant to Vancomycin due to resistant genes in VanA-type *E. faecium*, however, when both antimicrobials were combined, susceptibility to Vancomycin was noted in the presence of Chlorohexidine (Bhardwaj *et al*., 2021). Over the years, various studies have provided insight into combination therapy and the potential synergistic effect that combinations have therapeutically especially in skin infections. The rise in resistance has led to search for alternative combination therapies. One such option is the use of medicinal dyes in combination with commercial antimicrobials.

1.6. Medicinal dyes

Medicinal dyes have been used for centuries to treat various ailments (Wainwright, 2014). Its popularity has decreased due to the discovery of antibiotics as well as their adverse side effects, ranging from tissue colouration to toxicity and mutagenicity (Wainwright, 2014). Medicinal dyes are easily accessible and relatively inexpensive, which raises the question as to why its popularity has decreased over the years.

Dyes were introduced into medicine in the 19th century by two scientists, Koch, and Ehrlich (Wainwright, 2014). Ehrlich's main contribution was to treat infectious diseases. He used Methylene blue, a synthetic dye, to cure malaria. Browning, a scientist introduced dyes such as Gentian violet and Brilliant green, during World War I, to treat infections in the battlefield. This was to achieve antisepsis and healthy wound closure (Wainwright, 2014).

Research into dyes as an antiseptic has been largely lacking in recent years and most clinical work involving dyes were carried out before 1945 after which, antibiotics took the spotlight (Wainwright, 2014). While the research into medicinal dyes has been lacking, dyes have been used for various other reasons such as aquaculture and textiles.

In the early 1900's, 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). Its popularity decreased as it tends to burn the skin after prolonged use and over time Povidone-iodine replaced Iodine tincture as an antimicrobial (Morgan *et al*., 1996). In the year

1925, Mercurochrome was used intravenously as an antimicrobial and at the time, it was said to be a “true bloodstream antiseptic” (Cleary, 1925). Mainly used in animals, it rendered the blood sterile after a few hours of the animal being infected with artificial septicemia (Cleary, 1925). The article written by Cleary, (1925) also noted two cases of Mercurochrome being used intravenously to treat typhoid fever. Over a course of a few days, Mercurochrome in a 1% solution was injected into two children who were infected with typhoid fever. The results noted that both children recovered, with the severity and duration of the fever reduced (Cleary, 1925).

Potassium permanganate solution was previously used in the treatment of impetigo. It was also used to treat tinea pedis and ringworm (Ive, 1973). In addition, Potassium permanganate was noted to treat *Candida* infections (Miller, 1931). It was applied topically to the intertriginous areas between the toes and under the breasts (Miller, 1931).

Fuchsine is noted to have antiseptic activity against Gram-positive bacteria (Balabanova *et al*., 2003). It has been used to treat various skin conditions such as pyodermas, dermatitis, intertrigo and eczema (Balabanova *et al*., 2003). Fuchsine dye in a 2% to 5% solution has been used for the treatment of burns (Balabanova *et al*., 2003).

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). Gentian violet has been found to be more effective against bacteria at high pH values (Balabanova *et al*., 2003). It has been reported that Gentian violet inhibits DNA replication in *Clostridium perfringens, Micrococcus luteus* and *E. coli* (Balabanova *et al*., 2003).

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. Bactericidal activity was previously noted against Gram-positive bacteria (Susarla *et al*., 2017). It can be used in a diluted solution as a local antiseptic for the treatment for burns (Balabanova *et al*., 2003; Susarla *et al*., 2017).

Methylene blue was used as an antimalarial. It is also used by several European transfusion services in the photodecontamination of blood plasma and has shown activity against enveloped viruses (Wainwright, 2000). Methylene blue has many uses in medicine due to it being an inhibitor of nitric oxide synthase and guanylate cyclase (Ginimuge and Jyothi, 2010). It has been reportedly used in refractory vasoplegia; by inhibiting guanylate cyclase. It decreases cyclic guanosine monophosphate (C-GMP) and allows for vascular smooth muscle relaxation (Ginimuge and Jyothi, 2010). It has been used to treat septic shock, also by being a potent guanylate cyclase inhibitor (Ginimuge and Jyothi, 2010).

While there are many other notable uses of medicinal dyes, the focus of this study is on skin infections, antimicrobial activity and potential synergistic combinations. A selection of dyes (Table 1.3), based on the review of various literature where antimicrobial activity has been observed was selected for this study. These particular dyes are all indicated for topical use only.

Table 1.3: Types of dyes and antimicrobial properties

Dyes	Antimicrobial properties	References
Fuchsine	Known to possess anesthetic, bactericidal (Gram-positive), and fungicidal properties. It is used to treat burns, eczema, and pyodermas.	Gupta *et al*., 2008; Berrios and Arbiser, 2011
Gentian violet	Exhibits anti-trypanosomal, anti-fungal, anti-helminthic and antibacterial properties. Also known as crystal violet	Berrios and Arbiser, 2011
Iodine tincture	Has historically been used as a first line treatment for cuts and abrasions. Exhibits antifungal and antiseptic properties.	Owen, 1913; Smith, 1915
Malachite green	Primarily used against mycobacterial infections. Has been used as an antiseptic and antifungal to treat superficial pyodermas. Also used as a secondary treatment of topical skin conditions.	Junqueira *et al*., 2010; Berrios and Arbiser, 2011; Rosa *et al*., 2015
Mercurochrome	Used as a long-acting antibacterial and antifungal agent to prevent wound sepsis. Aids in wound healing.	Wainwright, 2003; Korkmaz *et al*., 2019

Dyes	Antimicrobial properties	References
Methylene blue	Found to have activity against toenail onychomycosis.	Souza *et al*., 2017
Potassium permanganate	Known to have disinfectant, deodorizing and astringent properties. In a weak solution it may be used as a cleansing agent for wounds.	Rossiter and Blackman, 2012

1.7. Combinations of medicinal dyes with antimicrobials

Medicinal dyes have been noted for the effectiveness over the years, however, due to their toxicity, their therapeutic effectiveness is decreased (Wainwright, 2008; Rosa *et al*., 2015). Combination therapy with medicinal dyes has the potential to reduce the toxicity, increase effectiveness while being a cost-effective therapeutic option. Dyes when considered both independently and when used in combination in medicinal therapy is largely an untouched area, however, synergy has been noted with various combinations of other antimicrobials.

In 1939 (the pre-antibiotic era), a combination study on Gentian violet and Brilliant green was conducted on wound infections (Maplestone and Dey, 1939). The combination showed that the enhanced fungicidal and bactericidal effect was greater than the effect of Gentian violet alone (Maplestone and Dey, 1939). In another study, the effect of a triple dye combination consisting of Crystal violet, Brilliant green and Proflavin hemisulfate was tested to prevent the colonization of MRSA in neonates. The triple dye therapy was applied to the umbilical cord and surrounding abdomen, after which swabs were taken (Rosenfeld *et al*., 1990). The results showed that the triple dye therapy had reduced the number of MRSA-colonies in neonates (Rosenfeld *et al*., 1990). Arbiser *et al.* (2012) conducted a study combining Gentian violet and Imiquimod in a topical preparation to treat cutaneous melanoma metastases. Imiquimod is an immune response modifier and is used externally for the treatment of genital and perianal warts (Saunder, 2000). The study was performed on a 92-year-old white male who had marginal zone lymphoma. After four months of daily application of both drugs, the lesions were re-examined which showed that the largest lesion was replaced by a depressed scar and no re-occurrence was noted. A study conducted by Rosa *et al*., (2015), tested the effectiveness of photodynamic inactivation of a combination of

Methylene blue, Toluidine blue and Malachite green against *S. aureus* biofilms in bone specimens. Eight specimens of compact bone and cancellous bone were inoculated with *S. aureus* and incubated at appropriate temperatures for 14 days to allow for biofilm formation. The bones were then separated within the groups according to treatment and controls. It was noted that the combined therapy with 660 nm of laser light proved to be effective in the inactivation of *S. aureus* biofilms (Rosa *et al*., 2015). In a more recent study by Dumisa (2020), combination studies were conducted with medicinal dyes and medicinal plant combinations against various pathogens which has yielded positive results. Gentian violet in combination with various plant extracts yielded synergistic effects against *S. aureus*. A notable combination of Gentian violet with *Gunnera perpensa* yielded synergistic effects against a clinical strain tested. A combination of Gentian violet with *Calodendrum capense* and the root of *Aristea ecklonii* also yielded synergistic results against a methicillin-resistant strain. When combined with the plant extract *Melianthus comosus*, Gentian violet provided a notable combination for the clinical and Fluconazole-resistant strain of *C. albicans*. It was noted that 84% of combinations with Gentian violet were synergistic. Brilliant green in combination has shown activity against resistant *S. aureus* stains. When combined with *G. perpensa,* synergistic effects were noted for the reference strain and Gentamicin-methicillin-resistant strain of *S. aureus*. Brilliant green combined with *Brachylaena discolour* showed synergism against *P. aeruginosa*. Overall, it was noted that 60% of combinations were synergistic. Combinations with Malachite green mostly (82%) yielded synergistic results.

Raw data obtained from a pilot study (Laher and Makra, 2016) undertaken within the pharmaceutical microbiology research group has shown synergistic activity of various medicinal dyes when in combination with antimicrobials. Iodine tincture in combination with Amphotericin B and Nystatin showed synergistic effects against *Candida albicans*. Methylene blue in combination with Ciprofloxacin had shown synergistic effects against *S. aureus*. Methylene blue in combination with Erythromycin showed an additive effect and in combination with Penicillin G showed synergism against *S. epidermidis*. Malachite green in combination with Tetracycline, Erythromycin and Ciprofloxacin showed a synergistic effect. A combination of Gentian violet with Tetracycline, Erythromycin and Ciprofloxacin also demonstrated synergistic effects. While that study demonstrated some potential, it was somewhat limited as no consideration was given for topical antimicrobials. Clearly, combinations of dyes, albeit promising, remain largely unexplored

and may provide effective antimicrobial alternatives. Undertaking research into medicinal dyes and antimicrobials could produce beneficial outcomes and lead to a positive impact on reducing antibiotic resistance.

1.8. Cidal effects

1.8.1. Minimum bactericidal or fungicidal concentration (MBC/MFC)

In order to determine if a sample has a bactericidal effect, the minimum bactericidal concentration assay is conducted. The lowest concentration of a sample to kill a particular organism is known as the minimum bactericidal/fungicidal concentration (MBC/MFC) (Yilmaz, 2012). The bactericidal concentration is normally determined after the minimum inhibitory concentration assay (MIC) is conducted (Yilmaz, 2012). This is undertaken on concentrations that have shown an inhibitory effect, whereby samples are transferred onto un-inoculated media and the lowest dilution that prevents growth on an agar plate is classified as the MBC value (Sykes and Rankin, 2013). The MBC assay has been widely used as a guideline to determine the antimicrobial effects of various antibiotics and new antimicrobials (Santos *et al*., 2020). There is no one method for MBC testing, however it is most often always conducted after the MIC assay is completed (Santos *et al*., 2020).

Minimum bactericidal concentration assays have been used to test many antibiotics however, research into MBC testing into dyes is minimal. The MBC assay was used to test twelve antimicrobials against various strains of *Listeria monocytogenes* (Rodríguez-Melcón *et al*., 2021). The results of the MBC were determined in "parts per million" (ppm) and were then compared to the breakpoint values of the European Committee on Antimicrobial Susceptibility Testing (EUCAST) and the Clinical and Laboratory Standards Institute (CLSI) guidelines (Rodríguez-Melcón *et al*., 2021). The MBC values noted that resistance was observed for Gentamicin, Cefoxitin, Fosfomycin, and Cephalothin (Rodríguez-Melcón *et al*., 2021).

The MBC assay was used to determine the antimicrobial activity of Iodine in a gas-reaction using cyclodextrin metal-organic frameworks to treat wound infections (Chen *et al*., 2022). The MIC and MBC of the framework against *E. coli* and *S. aureus* was 750.00 µg/ml (Chen *et al*., 2022).

One study that conducted MBC/MFC testing against dyes is Dumisa (2020). As the dyes have strong staining abilities, the MBC/MFC assay is necessary to determine the killing ability of the medicinal dyes. Dumisa (2020), noted a majority of the MBC/MFC values to be higher than the MIC values for the medicinal dyes with values >8000.00 µg/ml.

1.8.2. Time-kill studies

The most appropriate method to determine a bactericidal or fungicidal effect would be to use a time-kill test (Balouiri *et al*., 2016). To determine the killing of a bacterial strain over time by one or more bacterial agents in a carefully controlled environment is known as the time-kill method (Pfaller *et al*., 2004). The time-kill method allows one to determine the interaction between an antimicrobial and the microbial strain and reveals a time-dependent and/or concentration-dependent effect (Balouiri *et al*., 2016). Studying varying concentrations of antimicrobials will help assess the bactericidal or fungicidal rate as well as assess combinations of antimicrobials for potential synergy (Sendi and Ruppen, 2015).

Time-kill studies have been conducted on various antimicrobials such as chlorohexidine for the treatment of oral infections caused by various *Candida* species. At a concentration of 0.2%, Chlorohexidine is able to kill strains of *C. albicans* and *Candida tropicalis* (Shrestha *et al.,* 2011). Studies have been conducted on Nystatin, Ketoconazole and Miconazole which show possible fungicidal activity against *Candida* strains (Lefler and Stevens, 1984; Shin and Pyun, 2004). Time-kill studies were conducted on a Povidone iodine- alcohol gel to determine its activity against 25 pathogens (Jeng and Severin, 1998). The test was conducted at 15 seconds and 30 seconds of exposure (Jeng and Severin, 1998). The results showed bactericidal activity against *S. aureus* resistant strains, as well as methicillin resistant *S. epidermidis*, *S. pyogene*s and *P. aeruginosa* (Jeng and Severin, 1998). Time-kill studies conducted with 0.4% Povidone iodine and 0.4% Dexamethasone against ocular pathogens such as MRSA, *P. aeruginosa* and *C. albicans* were taken at 15, 30 and 60 seconds of exposure (Pelletier *et al*., 2011). Results showed that bacterial and fungal isolates were killed at 15 seconds of exposure and the percentage kill was 99.9% (Pelletier *et al*., 2011).

Dumisa (2020), conducted time-kill studies on a notable combination of Gentian violet and *Gacinia livingstonei* against an *S. aureus* (ATCC 25923) strain. The combination of Gentian violet and *G. livingstonei*, both at a concentration of 0.75 mg/ml have proven to be bactericidal with all micro-organisms killed in three hours. The combination of Gentian violet and *G. livingstonei* at concentrations of 0.38 mg/ml and 0.19 mg/ml also proved to have a bactericidal effect, however, the time taken to kill the micro-organisms was double the time i.e., six hours.

While there are various time-kill studies on antimicrobials, there is a lack of data in terms of time-kill studies of medicinal dyes and there is no evidence of time-kill efficacy of dye combinations with existing antimicrobials.

1.9. Toxicity studies of medicinal dyes and combinations

There is a common misunderstanding that over-the-counter dyes such as Mercurochrome are not toxic, however, this is not always true. This misconception has led to dyes such as Gentian violet, Iodine tincture and Mercurochrome being sold over the counter in various grocery stores and pharmacies. As described by Wainwright (2008), there are two major factors as to why medicinal dyes are not 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 at varying degrees (Wainwright, 2008). It is therefore critical to assess the toxicity of the individual dyes and combinations at varied concentrations in order to determine the safety.

Brine-shrimp toxicity assays conducted on dyes and combinations with plant extracts by Dumisa (2020), showed that the dyes tested were toxic. This was noted by the LC_{50} values i.e., the concentration of a sample that will kill 50% of the brine-shrimp population. Gentian violet showed to be a toxic dye with a high percentage mortality and low LC_{50} values of 46 µg/mL and 75 µg/mL at 24 and 48 hrs. However, it was noted that Gentian violet demonstrated dose-dependent toxicity therefore, at lower concentrations, Gentian violet was non-toxic. Malachite green and Mercurochrome were among the toxic dyes with LC_{50} values of 17 µg/ml at 48 hrs and 8 µg/ml at 48 hrs respectively. It was noted that Mercurochrome also exhibits a dose-dependent toxicity similar to Gentian violet. Brilliant green was found to be the most toxic dye with LC_{50} values of

45 µg/ml at 24 hrs and 7 µg/ml at 48 hrs. It was noted that the percentage mortality in brine shrimp was 100% at 48 hrs. The least toxic dye was Fuchsine. The LC_{50} values of Fuchsine were 746 µg/ml at 24 hrs and 355 µg/ml at 48 hrs, making it a moderately toxic dye. Fuchsine has recorded a less than 50% mortality at 48 hrs at a concentration of 125 µg/ml. Dumisa (2020), also tested the toxicity of plant: dye samples that exhibited antimicrobial synergistic effects. Plants in combination with Gentian violet lowered the toxicity of Gentian violet. Malachite green in combination with various plant extracts also showed a reduction in toxicity especially when combined with *Gunnera perpensa*. Brilliant green was noted to be one of the most toxic dyes, however, when combined with plant extracts, the toxicity significantly reduced. The study has shown that when in combination, the toxicity of dyes can be reduced, and this type of toxicity study has potential for further investigation with conventional antibiotics.

1.10. Selectivity index (SI)

The selectivity index (SI) is known as the relationship of a sample's antimicrobial activity (MIC value) to its toxicity (Adamu *et al*., 2014). Higher SI values are known as a good selectivity index, these values demonstrate a non-toxic relationship, and the sample is deemed safe to use (Dzoyem *et al*., 2016). The SI value is used to calculate the efficacy and safety of a sample against a particular organism. The SI has been used to calculate toxicity: antimicrobial relationship on numerous medicinal plants (Adamu *et al*., 2014; Bagla *et al*., 2014; Memvanga *et al*., 2015; Dzoyem *et al*., 2016; Adekunle *et al*., 2022). Very little studies have been observed for SI values of the medicinal dyes. Dumisa (2020), calculated the SI values of selected dyes and combinations. The dyes were observed to have very low SI values demonstrating that those dyes have a antimicrobial: toxicity relationship and should not be used. It is, therefore, important to determine the various SI's of the medicinal dyes in combination with antibiotics as the safety index is an important tool in determining the therapeutic potential.

1.11. Aim and objectives

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 iodine and Mupirocin).

In order to achieve this, the following objectives were designed:

- To determine the antimicrobial inhibitory properties of selected conventional antibiotics and dyes against pathogens associated with skin infections by determining MIC's and determining the minimum bactericidal/fungicidal activity (MBC/MFC).

- To assess the interactions of commercial antimicrobials and dyes by determining the sum of the fractional inhibitory concentration (ΣFIC). Where synergy or antagonism is observed in the ΣFIC assessment, various ratios of medicinal dyes and conventional antimicrobial will be combined and evaluated for interactive antimicrobial effects (isobologram analysis).

- To perform toxicity studies on the medicinal dyes, antimicrobial agents and notable combinations using the brine-shrimp lethality assay.

- To determine the selectivity index of the medicinal dyes, commercial antimicrobials, and selected combinations.

- To conduct time-kill studies on medicinal dyes, commercial antimicrobials, and selected combinations to determine the cidal effects over a period of time.

CHAPTER 2

Materials and methods

2.1. Introduction

Antimicrobial testing procedures that are reliable and consistent, provide valuable information that could lead to outcomes that positively change healthcare (Kuper *et al*., 2009). The objective of this chapter is to outline the methods conducted in this study in order to determine the efficacy of the medicinal dyes and commercial antimicrobials in combination. An overview of each process carried out is given in Figure 2.1.

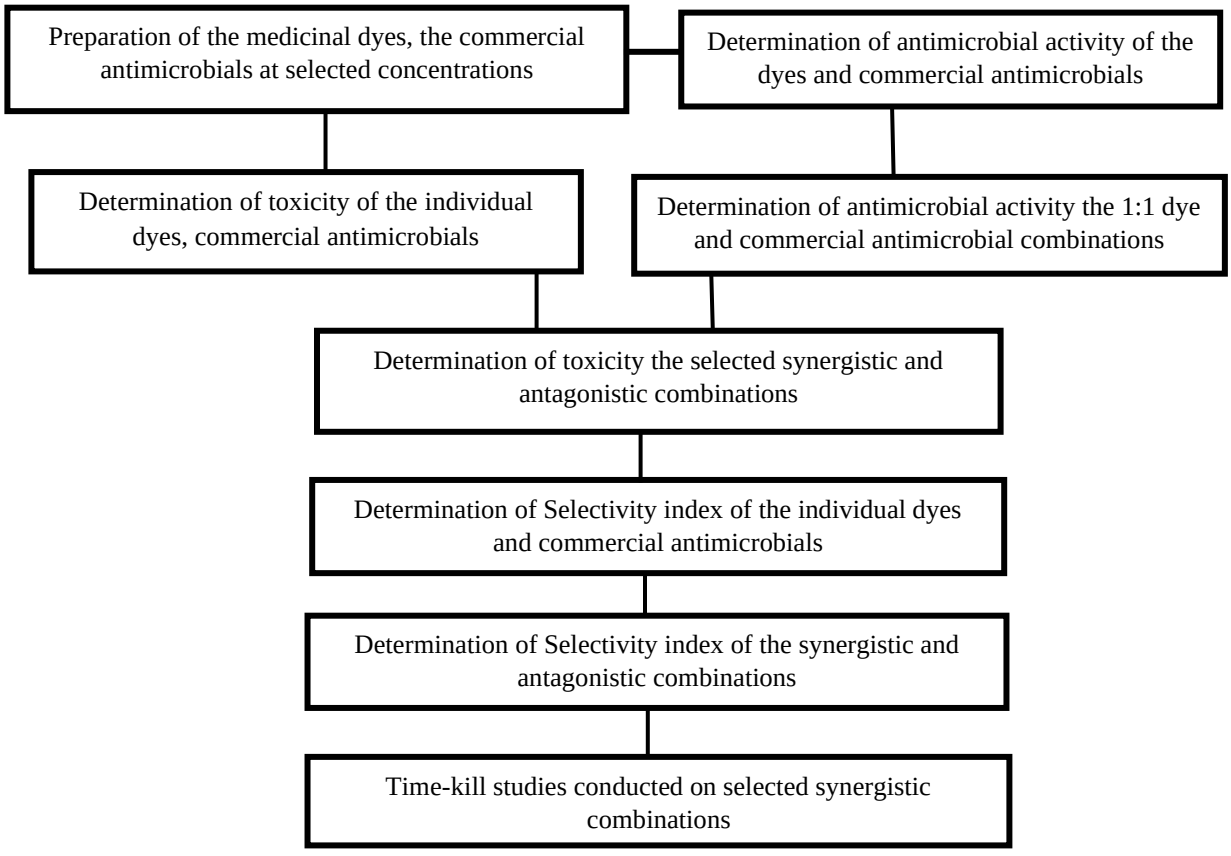


Figure 2.1: Overview of methodology

2.2. Dye and antibiotic preparation

The dyes were selected based on their medicinal properties, their prior use as primary antimicrobials in the pre-antibiotic era, and their abilities to be used topically (Table 2.1). The antimicrobials selected are based on their use in the treatment of skin infections (Table 2.2).

Table 2.1: Medicinal dyes and concentrations as procured from suppliers.

Medicinal dye	Supplier (Batch number)	Purity	State
Fuchsine	Sigma-Aldrich (MKBX6977V)	>85%	Solid
Gentian violet	Dis-chem (BN 62)	N/A	Liquid
Iodine tincture	Dis-chem (045141)	N/A	Liquid
Malachite green	Saarchem (8568)	N/A	Solid
Mercurochrome	Dis-chem (040618)	N/A	Liquid
Methylene blue	Sigma-Aldrich (MKCC3313)	95%	Solid
Potassium permanganate	ACE (Associated Chemical Enterprises) (10145)	99.50%	Solid

N/A: Information on purity of substance not available

Table 2.2: Commercial antimicrobials and concentrations as procured from suppliers.

Commercial antimicrobials	Supplier (Batch number)	Purity	State
Betadine®	Dis-chem (18J56N37)	N/A	Liquid
Fusidic acid	Sigma-Aldrich (F0756-1G)	±100%	Solid
Gentamycin	Sigma-Aldrich (345814-1GM)	±100%	Solid
Ketoconazole	Sigma-Aldrich (LRAA1641)	±100%	Solid

Commercial antimicrobials	Supplier (Batch number)	Purity	State
Miconazole	Sigma-Aldrich (LRAB5528)	99.50%	Solid
Mupirocin	Sigma-Aldrich (M7694-50MG)	≥92%	Solid
Neomycin	Sigma-Aldrich (WXBD2162V)	90% - 100%	Solid
Nystatin	Sigma-Aldrich (SLBC7148V)	≥4 400 USP units per mg	Solid
Steriscrub	Dis-chem (043649)	N/A	Liquid

N/A: Information on purity of substance not available

2.3. Antimicrobial analysis

The pathogens were selected on the basis of their role in causing dermatological infections. The strains included were reference strains (American Type Culture Collection, ATCC; procured from Davies Diagnostics (Pty) Ltd. (Johannesburg, South Africa), clinical strains received from NHLS Infection Control and Microbiology Laboratory (University of Witwatersrand), and resistant DSM strains procured from Deutsche Sammlung von Mikroorganismen (DSM) – German culture Collection (The Leibniz Institute). The bacterial strains included: *Staphylococcus aureus* (ATCC 46644), *Staphylococcus aureus* clinical strain (Clinical 6437938), methicillin resistant *Staphylococcus aureus* (MRSA) (ATCC 43300), Gentamycin-methicillin resistant *Staphylococcus aureus* (GMRSA) (ATCC 33592), *Staphylococcus epidermidis* (ATCC 12228), *Staphylococcus epidermidis* clinical strain (Clinical BA16), *Staphylococcus epidermidis* resistant strain (ATCC 51625), *Pseudomonas aeruginosa* (ATCC 27858), *Pseudomonas aeruginosa* clinical strain (DSM 46316), *Pseudomonas aeruginosa* resistant strain (ATCC 46316), *Escherichia coli* (ATCC 8379), *Escherichia coli* clinical strain (Clinical 6437873) and *Escherichia coli* resistant strain (DSM 22314). The yeast strains included *Candida albicans* (ATCC 10231), *Candida albicans* clinical strain (A100) and *Candida albicans* resistant strain (ATCC 90028). All micro-organisms were cultured in Tryptone Soya broth (TSB) (Oxoid). Bacterial strains were incubated (Memmert Incubator IN260) at 37 ºC for 24 hrs and yeasts at 37 ºC for 48 hrs. An ethics waiver for the use of

the pathogens was obtained from the Human Ethic Committee at the University of Witwatersrand (Reference Number W-CBP-200814-01) (Appendix C).

2.4. Minimum inhibitory concentration (MIC)

The MIC value is the minimum concentration that prevents visible growth of the pathogen (Q Laboratories, 2021). Minimum inhibitory concentration assays were used to evaluate the antimicrobial activity of samples independently and in combination. The Clinical and Laboratory Standards Institute (CLSI) guidelines (2020) was followed to ensure accuracy of microbiological assays and transfer techniques. The medicinal dyes were initially prepared to a concentration of 32 mg/ml (32000.00 µg/ml) in sterile water, except Gentian violet (5.00 µg/ml), Iodine tincture (25.00 µg/ml) and Mercurochrome (20.00 µg/ml) as they were procured at the prepared concentration. Among the commercial antimicrobials, the antibiotics and antifungals were prepared at a starting concentration of 10.00 µg/ml while Betadine® (100.00 µg/ml) was procured at a prepared concentration, and Steriscrub was prepared to a concentration of 32000.00 µg/ml diluted in 1:1 ratio of water to acetone. A preliminary test was conducted to determine the concentrations (32000.00 µg/ml for medicinal dyes and 10.00 µg/ml for antibiotics and antifungals) required for further dilution that still note antimicrobial activity.

In a 96-well micro-titre plate, 100.00 µl of Tryptone Soya broth (TSB) was added. The medicinal dyes, commercial antimicrobials and 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), a negative solvent control of 32.00 mg/ml water/acetone and a negative broth control of sterile TSB was added to each micro-titre plate. The positive control (Ciprofloxacin at 0.01 mg/ml and Amphotericin B at 0.10 mg/ml) 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. Microtitre plates were then be sealed with a sterile adhesive sealer (Macherey-Nagel GmbH & Co KG) and incubated as per respective incubation conditions.

After the appropriate incubation period of 24 hrs for bacteria and 48 hrs for the yeasts, 40 µl of the indicator, 0.4 mg/ml *p*- iodonitrotetrazolium violet (INT) (Sigma-Aldrich), was added to each well. The INT acted as an indicator of microbial grown and turned a purple-pink colour. The lowest concentration that inhibits growth was the 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 their staining properties. Hence, in some cases it was difficult to visualize the purple-pink colour due to the colour of the medicinal dyes (Figure 2.2). In order to overcome this problem with staining MBC assays were conducted. The study was done in triplicate and comparisons were made between how the clinical and reference strains respond to the test inhibitors.

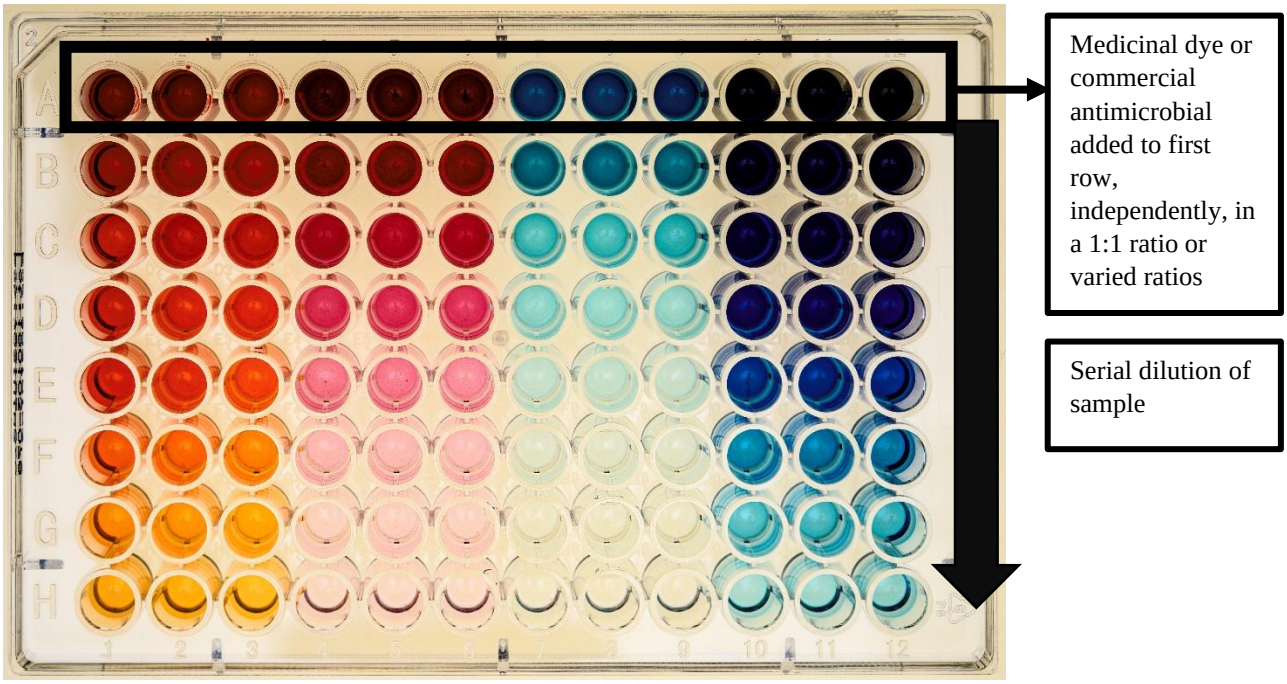


Figure 2.2: Representation of the MIC plate before culture is added

2.5. Minimum bactericidal/fungicidal concentration assay (MBC/MFC)

To further verify the activity of dyes and dye combinations the MBC assay was used. Immediately after the MIC assays are carried out to completion, the MBC assays followed. The MBC is the

lowest concentration required to kill a pathogen (Q Laboratories, 2021). A loop of culture was taken from each well and streaked onto a subdivided agar plate (Figure 2.3). The plate was then incubated under the appropriate incubation conditions. The absence of growth on the agar plates indicated the bactericidal or fungicidal activity respectively (Figure 2.3).

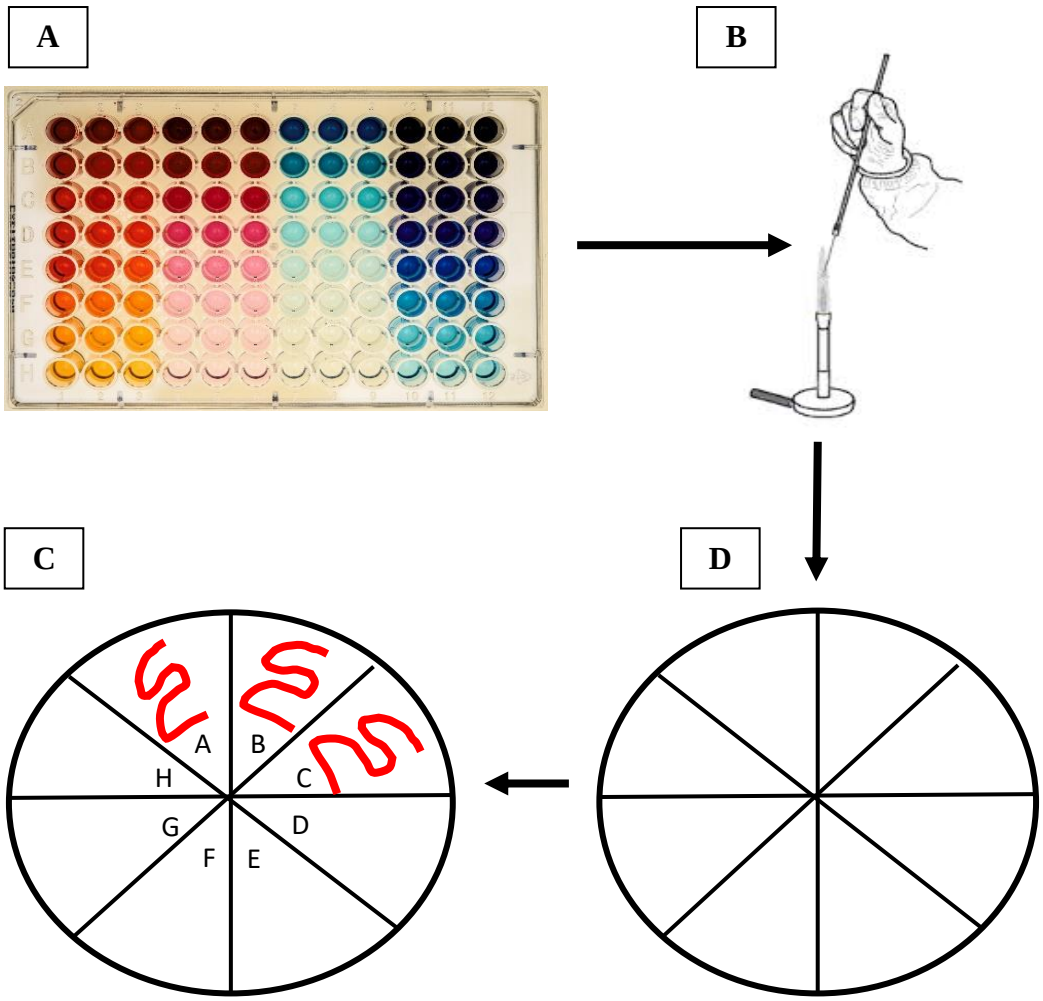


Figure 2.3: Representation of the MBC assay: A.) The incubated MIC plate, B.) Loop of culture, C.) Subdivided plate used to streak culture, D.) Incubated agar plate with red streaks demonstrating the growth of culture.

2.6. Fractional inhibitory concentration (FIC) assessment

Interactions between the combinations of dyes and antimicrobials was studied using the sum of the fractional inhibitory concentration (ΣFIC). The ΣFIC was calculated using Equation 2.1, where (a) represents the dye sample and (b) represents the commercial antimicrobial sample (van Vuuren and Viljoen, 2011):

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

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

Equation 2.1

The FIC index is then calculated as follows; ΣFIC = FIC (i) + FIC (ii). The interactions were 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).

The results from the MIC assays were recorded on a template which was transferred onto an excel sheet (Microsoft 365, 2017). The ΣFIC values were then calculated and classified as synergistic, additive, indifferent or antagonistic. The data was analyzed, and the two most important interactions (synergistic and antagonistic) were considered. The combinations amongst both interactions were chosen for varied ratio concentration assays based on combinations that noted similar trends among the pathogen groups (Gram-positive, Gram-negative strains and yeasts). For example, when a combination demonstrated mostly synergy and no antagonism was noted, then that combination was selected for further ratio testing. The same was considered for the antagonistic combinations, if the majority of the strains noted antagonism and no synergy was noted then that combination was also chosen for varied ratio testing. Varied ratio testing was undertaken to determine if other variations of the concentration of the two samples would produce a similar or different effect.

2.7. Varied ratio concentrations

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 was prepared in nine different ratios (9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8, 1:9) and the MIC values determined as per the method described in Section 2.4.

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. An isobologram is a visual representation of the interactions of both samples. Figure 2.4 demonstrates an example of an isobologram.

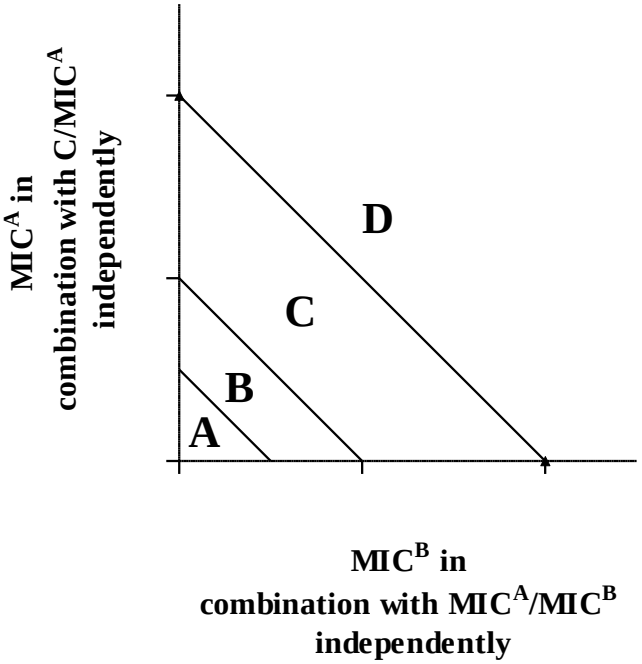


Figure 2.4: Visual representation of an isobologram: Data points falling in quadrant **A** (below the 0.5 line) show a synergistic interaction, quadrant **B** (between the 0.5 line and 1.0 line) shows an additive interaction, quadrant **C** (between the 1.0 line and including the 4.0 line) notes indifferent interactions and quadrant **D** (above the 4.0 line) demonstrates the antagonistic interactions.

2.8. Toxicology studies

The brine shrimp lethality assay is used as a preliminary assessment of the toxicity of samples. This method is preferred as it is simple and inexpensive (Suryawanshi *et al.*, 2020). To determine the toxicity of the medicinal dyes and commercial antimicrobials the brine shrimp lethality assay was carried out as outlined in Hübsch *et al*., (2014). For this assay, the medicinal dyes were tested at a concentration of 500.00 µg/ml except for Gentian violet, Iodine tincture and Mercurochrome which were tested at the predetermined concentration (as it was procured ready-made). The antibiotics and antifungals were also tested at 500.00 µg/ml except for Betadine® which was already at a predetermined concentration as procured from the supplier. The 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.

Topical 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 hrs at ambient temperature. A 220-240V 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 well of a micro-titre plate. In each well, 400.00 µl of the sample (antimicrobials, dyes, or combinations) was added (Figure 2.5). The tests were done in triplicate. The negative control used was salt water with a concentration of 32.00 g/L as it simulates natural sea water. This is to ensure the saltwater supports the growth and survival of the brine shrimp. The positive control potassium dichromate (Sigma-Aldrich) at 1.60 mg/ml was used. This ensures the death of the brine-shrimp. After 24 and 48 hrs, 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 2.2). A percentage mortality of 50% or greater is considered toxic (Bussmann *et al*., 2011).

$$\text{Percentage mortality (\%)} = \frac{\text{Total number of dead shrimp at 24/48 hrs}}{\text{Total number of shrimp}} \text{ x } 100$$

Equation 2.2

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. The LC_{50} value was determined to be the concentration of a sample that kills 50% of the brine shrimp population. Dumisa (2020) used the following parameters created by Bussmann *et al*., 2011: Highly toxic (LC_{50} value <249 µg/ml), moderate toxicity (LC_{50} value of 250-499 µg/ml) and low toxicity (LC_{50} value of 500-1000 µg/ml). These parameters were used to determine the toxicity of the samples (medicinal dyes, commercial antimicrobials, and combinations) in this study. For the combinations, a decrease in toxicity (in comparison to the independent medicinal dye value) would note a synergistic interaction and an increase in toxicity would note an antagonistic interaction.

The number of brine-shrimp was recorded after 0, 24 and 48 hrs. This was then transferred to an excel spreadsheet where the percentage mortalities were calculated (Microsoft 365, 2017).

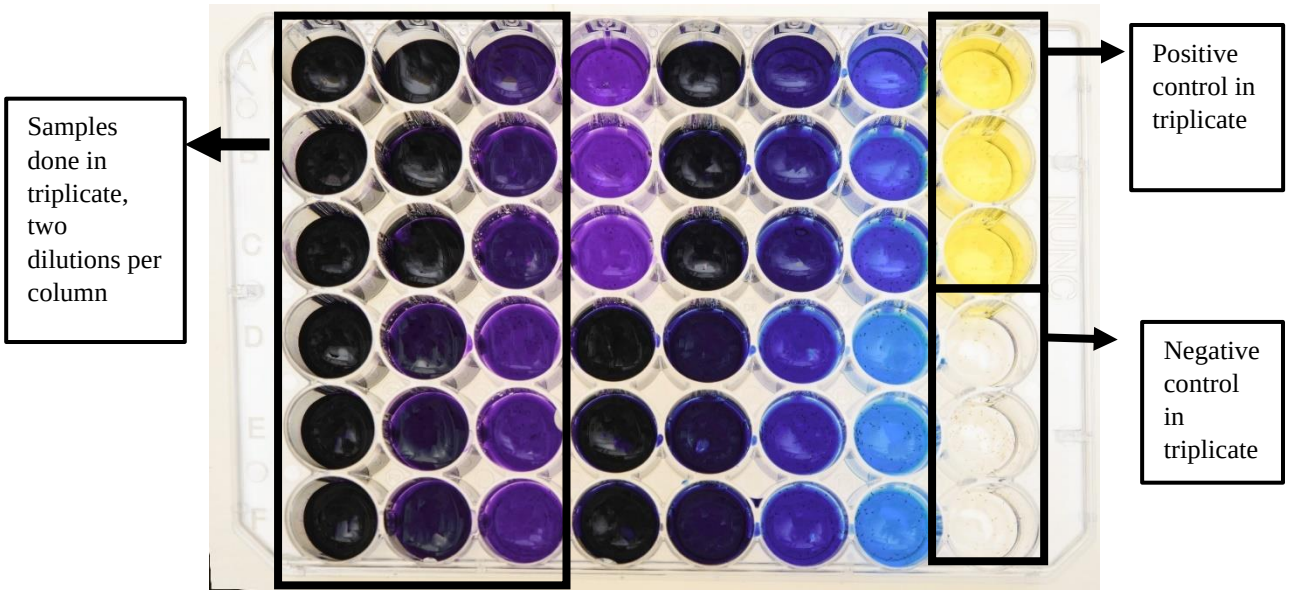


Figure 2.5: A 48-well micro-titre plate used in the brine-shrimp lethality assay

2.9. Selectivity index (SI)

The ratio of toxicity to antimicrobial activity (MIC) is defined as the selectivity index (SI) and calculated on an excel sheet using Equation 2.3 (Microsoft 365, 2017) (Elisha *et al*., 2017).

$$\mathrm{SI} = \frac{\mathrm{LC}_{50}}{\mathrm{MIC}}$$

Equation 2.3

There is a wide range of classifications used for the SI of samples, however, the general rule has been that the higher the SI, the lower the toxicity of the sample against the host (Bagla *et al*., 2014; Lyu *et al*., 2016). It was proposed that a SI value that is <1.0 would be toxic and >10.0 would be non-toxic (selectively toxic to the pathogen) (Sakong, 2012). Another study by Dzoyem *et al*., (2016) observed the selectivity index as a measure of potential efficacy between the antimicrobial activity of a sample and it toxic effects. Therefore, the higher the SI means the difference between concentration of a sample able to kill bacteria and its toxicity to its host is within a safe range (Dzoyem *et al*., 2016). The SI was analyzed for the dyes, commercial antimicrobials, and combinations.

2.10. Time-kill studies

Time-kill studies were conducted on combinations of interest where increased antimicrobial activity was observed (synergy) and reduced toxicity was also noted. These combinations were further tested 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 standard of culture (approximate inoculum concentration of 1 x 10^6 CFU/mL) 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, the contents of each well of the first row (containing broth, dye: commercial antimicrobial and inoculum) were thoroughly mixed and 100.00 µL was transferred to the subsequent well containing 0.90% NaCl (Figure 2.6).

Five successive dilutions were then performed into 0.90% NaCl. The microtitre plates were sealed to prevent evaporation and then incubated at 37 °C for 0, 3, 6 and 24 hrs. A 50.00 µL sample was withdrawn from the last three wells at 0, 3, 6 and 24 hrs. Samples were spread onto previously prepared Tryptone Soya agar plates and incubated at 37 ºC for 24 hrs after which the number of colonies were counted. The logarithm of CFU/mL present in the original well with time were calculated using Equation 2.4 and were plotted onto an Excel spreadsheet (Microsoft 365, 2017)

$$\text{Log (CFU/mL)} = \log_{10} \text{Colony count} \times 1 \times 10^{9}$$

Equation 2.4

A positive control of Ciprofloxacin (0.01 mg/ml) was used to ensure the pathogen was susceptible. A culture control was also included of each respective pathogen. The assay was conducted in triplicate. The time-kill results were recorded and transferred onto an excel spreadsheet (Microsoft 365, 2017). The logarithms of the values were calculated and plotted into line graphs (Microsoft 365, 2017)

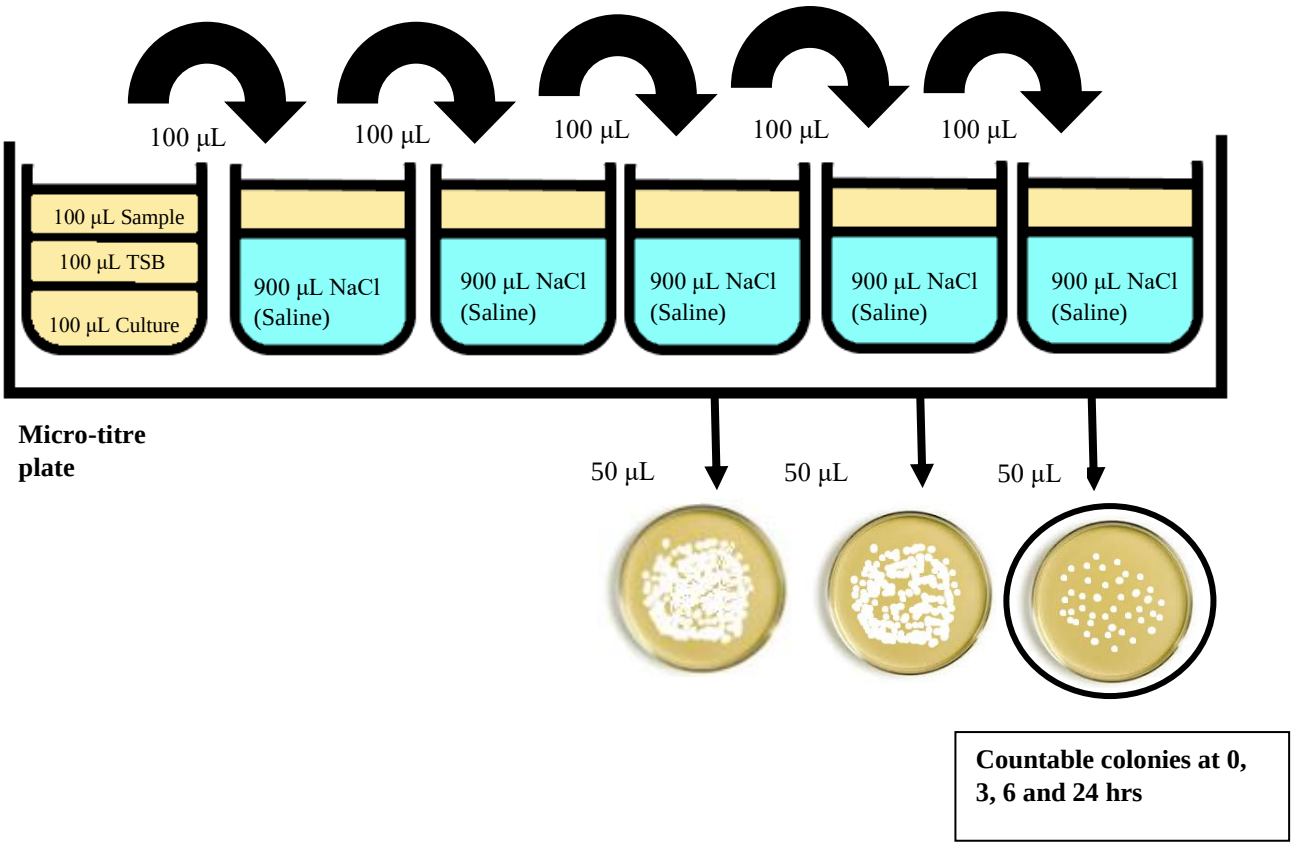


Figure 2.6: Adapted time-kill method

CHAPTER 3

Antimicrobial activity: Gram-positive pathogens

3.1. Introduction

The objective of this chapter was to determine the antimicrobial activity of the dyes and the commercial antimicrobials as well as the combinations against *S. aureus* and *S. epidermidis*. Figure 3.1 demonstrates the flow in which the antimicrobial testing was carried out.

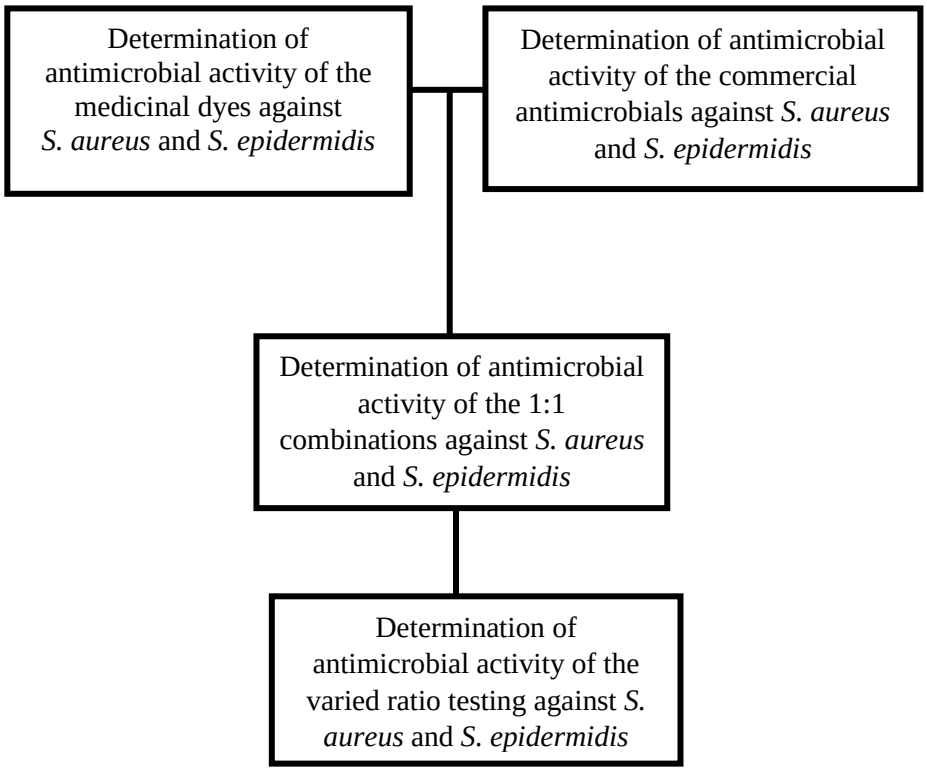


Figure 3.1 : The process whereby the antimicrobial testing was carried out.

3.2. Results and discussion

3.2.1. Antimicrobial activity (MIC) of medicinal dyes against Gram-positive pathogens

All medicinal dyes were tested against various strains of *S. aureus* and *S. epidermidis* (Table 3.1). Fuchsine noted MBC values ranging from 25.00-500.00 μg/ml for *S. aureus* strains. Despite being a resistant strain, GMRSA demonstrated the most susceptibility (MBC 25.00 μg/ml) to Fuchsine compared to the other *S. aureus* strains. The *S. epidermidis* strains noted MBC values of 1.56-100.00 μg/ml. The clinical strain (Clinical BA16) noted the highest susceptibility compared to the other *S. epidermidis* and *S. aureus* strains. The resistant strain of *S. epidermidis* (ATCC 51625) noted the lowest susceptibility among the *S. epidermidis* strains which is to be expected from a resistant strain. An older study into 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 as Fuchsine has noted susceptibility to the *S. aureus* and *S. epidermidis* strains. Dumisa (2020), noted MBC values of >8000.00 – 9375.00 μg/ml for Fuchsine when tested against the *S. aureus* strains. While these values are much higher in comparison to the current study, it must be noted that different *S. aureus* strains were studied.

The *S. aureus* and *S. epidermidis* strains demonstrated the most susceptibility towards Gentian violet in comparison to the other dyes tested. The MBC values noted for the *S. aureus* strains ranged from 0.00025-0.03 μg/ml. It was observed that GMRSA demonstrated the highest susceptibility to Gentian violet. The reference strain, clinical strain and MRSA noted the same values for Gentian violet which could be attributed to very little resistance developing against Gentian violet over time. The reference strain of *S. epidermidis* noted better susceptibility to Gentian violet (MBC of 0.002 μg/ml) compared to the reference strain of *S. aureus*. The clinical strain of *S. epidermidis* (MBC of 0.00003 μg/ml) noted the highest susceptibility to Gentian violet compared to all Gram-positive strains tested. An older study found Gentian violet also had low MBC values (0.0025 to 0.08 μg/ml) against MRSA (Saji, 1992). The current study noted an MBC value of 0.03 μg/ml against MRSA and this falls within the range noted by Saji (1992). This demonstrates that not much resistance has occurred in the past years. A later study found that

Gentian violet demonstrated low MIC values against MRSA (Maley and Arbiser, 2013). The current study noted similar higher susceptibility for Gentian violet with low MBC values. Dumisa (2020), tested the antimicrobial activity of Gentian violet and noted MBC values of 1500.00 µg/ml against the *S. aureus* reference (ATCC 25923) strain. This value is vastly higher than that found in the current study. The *S. aureus* clinical strain tested noted an MBC value of 688.00 µg/ml (Dumisa, 2020). These values are also different to the current study. This could be due to the current study using a prepared solution of Gentian violet at 0.5% while Dumisa (2020), prepared a concentration of 32.00 µg/ml from pure crystal violet powder. The expiry date of the powder is unknown.

The *S. aureus* strains noted poorer susceptibility to Iodine tincture compared to the *S. epidermidis* strains. Amongst the *S. aureus* strains, GMRSA demonstrated the highest susceptibility against Iodine tincture with an MBC value of 0.78 µg/ml. The reference strain of *S. aureus* noted the poorest susceptibility (MBC of 1.88 µg/ml) compared to the clinical and the resistant strains, demonstrating higher resistance. As with the other two dyes (Fuchsine and Gentian violet), Iodine tincture demonstrated the lowest MBC value of 0.002 µg/ml against the *S. epidermidis* clinical strain. Literature has noted that in 150 years of being on the market, no resistance or cross-resistance has been reported for Iodine (Bigliardi *et al*., 2017). In comparison to Dumisa *et al*., (2020) the current study noted lower MBC values for Iodine tincture.

Malachite green demonstrated the same MBC value of 1.00 µg/ml against the reference strain of *S. aureus* and MRSA (a resistant strain). The reference strain of *S. aureus* could have developed resilience to Malachite green hence, the MBC value is the same. The clinical strain of *S. aureus* noted the most resilience among the Gram-positive strains tested. When testing against the *S. epidermidis* strains, the reference strain noted the highest susceptibility (MBC value of 0.0009 µg/ml), and the resistant strain noted the lowest MBC (1.00 µg/ml) which is to be expected from both strains. Malachite green has been used in photodynamic therapy against *S. aureus*. The dye has promoted microbial reduction in biofilms and reduced the CFU. This is due to Malachite green being able to easily penetrate the cell membrane in Gram-positive bacteria (Vilela *et al*., 2012). This mechanism may possibly be the way Malachite green disrupts planktonic cells as well, leading to the high susceptibility noted in the current study. Dumisa (2020) observed that

Malachite green demonstrated MBC values of 500.00 to >8000.00 µg/ml for the *S. aureus* strains while the MIC values noted much lower values for the same strains. Though MICs cannot be compared to MBCs, the current study noted low MBC values in a similar pattern with the MIC values in the previous study, where the resistant strains of *S. aureus* demonstrated better susceptibility to Malachite green.

Mercurochrome noted the second lowest MBC values among the *S. aureus* strains after Gentian violet. The MBC values ranged from 0.13-0.44 µg/ml. The highest susceptibility was noted by MRSA, a resistant strain. Mechanisms of resistance by *S. aureus* to various antibiotics (Methicillin, Penicillin etc.) has been well documented (SCENIHR, 2009; Stefani and Goglio, 2010; Lalaouna *et al*., 2018; Lim *et al*., 2018). The low MBC value could then be attributed to low resistance demonstrated by the *S. aureus* strains. A very early study by Clapp and Martin (1920), demonstrated the effectiveness of Mercurochrome against Gram-negative organisms in neonatal eye infections by using a 2% solution in the affected eye. The current study noted lower concentrations of Mercurochrome used to inhibit the Gram-positive strains. Dumisa *et al*. (2020), noted relatively higher MBC values for Mercurochrome compared to the current study. It should be noted, however, that different strains were used in both studies.

The *S. aureus* strains demonstrated poorer susceptibility to Methylene blue when compared to the *S. epidermidis* strains. The reference strain of *S. aureus* noted the highest susceptibility (MBC value of 20.00 µg/ml) among the *S. aureus* strains. The most resistance was demonstrated by MRSA with an MBC value of 208.33 µg/ml. Being a resistant strain, this was expected. The reference strain of *S. epidermidis* noted the highest susceptibility to Methylene blue with an MBC value of 12.50 µg/ml and the resistant strain noted the poorest susceptibility. A more recent study noted the MIC value of Methylene blue against *S. aureus* to be 16.00 µg/ml, and against *S. epidermidis* to be 8.00 µg/ml (Thesnaar *et al*., 2021). While the MIC values cannot be compared to the MBC values, Methylene blue demonstrated better activity against *S. epidermidis* than *S. aureus* which is a pattern also noted in the current study.

Potassium permanganate demonstrated the lowest efficacy among all the dyes. Among the *S. aureus* strains, only the reference strain was marginally susceptible with an MBC value of 7000.00 µg/ml while the other strains noted resistance with MBC values of >8000.00 µg/ml. The *S.*

epidermidis strains demonstrated similar activity to the *S. aureus* strains, however, the clinical strain of *S. epidermidis* was the only strain (again marginally) susceptible to Potassium permanganate with an MBC value of 8000.00 μg/ml while the reference and resistant strain noted values of >8000.00 μg/ml. Potassium permanganate has been on the World Health Organization's (WHO) essential list due to it having antiseptic properties and has been broadly used in wound care (Agnihotri *et al*., 2019) The current study, however, did not show Potassium permanganate having any efficacy against *S. aureus* and *S. epidermidis.* With the dye being used so frequently for skin infections, resistant mechanisms may possibly have developed.

Table 3.1: The minimum bactericidal value (MBC) of the medicinal dyes against Gram-positive pathogens (μg/ml).

Dyes	***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)**
Fuchsine	312.50	500.00	500.00	25.00	6.25	1.56	100.00
Gentian violet	0.03	0.03	0.03	0.00025	0.002	0.00003	0.01
Iodine tincture	1.88	1.56	1.56	0.78	0.16	0.002	0.39
Malachite green	1.00	2.50	1.00	0.03	0.0009	0.002	1.00
Mercurochrome	0.44	0.27	0.13	0.42	0.14	0.016	0.06
Methylene blue	20.00	50.00	208.33	50.00	12.50	20.00	40.00
Potassium permanganate	7000.00	>8000.00	>8000.00	>8000.00	>8000.00	8000.00	8000.00
Controls							
Culture control	>8000.00	>8000.00	>8000.00	>8000.00	>8000.00	>8000.00	>8000.00
Negative control (water/acetone)	>8000.00	>8000.00	>8000.00	>8000.00	>8000.00	>8000.00	>8000.00
Positive control (Ciprofloxacin)	2.50	1.25	2.50	1.25	1.25	1.25	1.25

3.2.2. Antimicrobial activity (MIC/MBC) of commercial antimicrobials against Gram-positive pathogens

The MIC and MBC were determined for the selected commercial antimicrobials associated with skin and soft tissue infections. The commercial antimicrobials were tested against Gram-positive *S. aureus* and *S. epidermidis* strains (Table 3.2). When tested against the *S. aureus* clinical strain, Betadine® noted an MIC of 1.87 µg/ml and MBC of 6.25 µg/ml. The clinical strain *S. aureus* demonstrated the highest susceptibility (MIC value of 1.87 µg/ml) to Betadine® among the *S. aureus* strains. The GMRSA strain of *S. aureus* noted the poorest susceptibility to Betadine® compared to all the Gram-positive strains tested. The *S. epidermidis* reference strain demonstrated the highest susceptibility among all the Gram-positive strains (MIC/MBC value of 0.98 µg/ml). Betadine® tested against the clinical and resistant strains noted higher MIC/MBC values compared to the reference strain; an MIC value of 2.50 µg/ml and an MBC value of 3.12 µg/ml against the clinical strain while an MIC/MBC value of 6.25 µg/ml was noted against the resistant strain. Betadine® (concentration 7.5 – 10%) tested against *S. aureus* and *S. epidermidis* observed an MBC value of ≤14000.00 µg/ml (Kampf, 2018). The current study tested Betadine® at a concentration of 10% with MIC/MBC values much lower than that observed by Kampf (2018).

Compared to Betadine®, Fusidic acid noted much better susceptibility among the *S. aureus* and *S. epidermidis* strains. When tested against the *S. aureus* strains, Fusidic acid noted the lowest MIC/MBC against GMRSA compared to the other *S. aureus* strains (0.08 and 0.63 µg/ml respectively) which was unexpected, as it is a resistant strain. The *S. epidermidis* strains noted better susceptibility to Fusidic acid compared to the *S. aureus* strains with MIC/MBC values of 0.04-0.08 µg/ml. According to EUCAST (2020) guidelines for breakpoint MIC values, Fusidic acid has an MIC range of 0.02-0.50 µg/ml. Fusidic acid noted low MIC/MBC values within the range of the EUCAST criteria against all *S. epidermidis* strains. *Staphylococcus aureus* isolates have developed increased resilience (by 4%) to Fusidic acid between 2010-2020 (Hajikhani *et al*., 2021). The increase in resistance observed by Hajikhani *et al*. (2021), could explain the higher MIC/MBC value of Fusidic acid when tested against the *S. aureus* strains.

When tested against Gentamycin, the *S. aureus* strains demonstrated lower susceptibility in comparison to the *S. epidermidis* strains. Unexpectedly, the reference strain of *S. aureus* noted the

most resistance against Fusidic acid with an MIC/MBC value of 125.00 µg/ml, a higher concentration compared to the resistant strains. Resistance to antibiotics has been gradually increasing and would account for the high MIC/MBC values of Gentamycin (Safdar *et al*., 2004; Ahmed *et al*., 2014; Liu *et al*., 2020). The clinical strain of *S. epidermidis* noted the most susceptibility towards Gentamycin compared to the other Gram-positive strains with an MIC/MBC value of 0.08 µg/ml. According to the CLSI guidelines, the breakpoint values for Gentamycin is ≤ 4.00 µg/ml for susceptible *Staphylococci.* The CLSI (2020) guidelines proved a value of 8.00 µg/ml is still susceptible but increased exposure over a period and ≥ 16.00 µg/ml for resistance. According to the CLSI criteria, the *S. aureus* strains in the current study noted resistance against Gentamycin, except for the GMRSA strain which notes increased exposure. The *S. epidermidis* strains all have MIC/MBC values lower than 4.00 µg/ml demonstrating susceptibility to Gentamycin.

Mupirocin demonstrated better activity to the *S. aureus* and *S. epidermidis* strains with MIC/MBC values of 0.16-31.30 µg/ml noted against the *S. aureus* strains. Mupirocin noted the lowest MIC/MBC (higher susceptibility) value of 0.16 µg/ml against GMRSA, the resistant strain compared to the other *S. aureus* strains. The *S. epidermidis* clinical strain noted the highest susceptibility (MIC/MBC value of 0.04 µg/ml) to Mupirocin among the Gram-positive strains. The *S. epidermidis* strains demonstrated better susceptibility to the commercial antimicrobials compared to the *S. aureus* strains. Minimum inhibitory concentration values for *S. aureus* isolates showed values of ≤ 4.00 µg/ml for susceptibility and values ranged from 8.00-64.00 µg/ml show low-level resistance (Khoshnood *et al*., 2019). According to the Mupirocin MIC/MBC values, all *S. aureus* strains demonstrated low-level resistance in this study, except for GMRSA which fell under the susceptible range indicated by Khoshnood *et al*., (2019).

The extensive use of Mupirocin ointment has led to increased resistance and failures in treatment (Lepelletier *et al*., 2020). The current study demonstrated much higher MIC/MBC values of Mupirocin when tested against the *S. aureus* reference strain, clinical strain, and MRSA, which could be attributed the increased resistance developed by these strains as indicated by Lepelletier *et al*. (2020).

Neomycin comes from the same family of aminoglycosides as Gentamycin (Rossiter *et al*., 2020) and a similar patten of efficacy would be expected from the antibiotic, however, susceptibility patterns were different to Gentamycin. In comparison Neomycin noted lower MIC/MBC values of 12.50 µg/ml against the *S. aureus* reference strain. An MIC/MBC value of 41.67 µg/ml against GMRSA was noted by Neomycin.

Neomycin also noted slightly higher MIC/MBC values of 0.31-0.63 µg/ml against the *S. epidermidis* strains. An older study tested the MIC activity of Neomycin demonstrated low MIC values of 5.00 µg/ml against *S. aureus* (Mehta and Champney, 2003). The current study noted much higher MIC values which may be accredited to the increasing resistant patterns of *S. aureus*. Bessa *et al*. (2016), investigated resistance among 100 *S. aureus* isolates and observed that 42.60% of the strains were resistant to Neomycin.

Steriscrub noted high MIC/MBC values of 125.00-250.00 µg/ml against the *S. aureus* strains and MIC/MBC values of 25.00-200.00 µg/ml against the *S. epidermidis* strains. In comparison to the various other antimicrobials tested, Steriscrub noted much higher MIC/MBC values against the *S. epidermidis* strains. In 2016, a study noted the MIC value of Steriscrub to be 16.00 µg/ml and the MBC value of 32.00 µg/ml, a much lower value to the current study (Akca *et al*., 2016). Steriscrub has been used repeatedly in a clinical setting and bacteria would be repeatedly exposed to Steriscrub which may have resulted in resistance (Hashemi *et al*., 2019).

3.2.3. Overview of activity of the commercial antimicrobials against the Gram-positive pathogens

In summary, the majority of the commercial antimicrobials (66.67%) noted better efficacy against the resistant strains of *S. aureus* – GMRSA compared to the other *S. aureus* strains. All commercial antimicrobials noted better overall efficacy to the *S. epidermidis* strains compared to the *S. aureus* strains. Fusidic acid demonstrated the best efficacy against the *S. aureus* strains while Fusidic acid and Mupirocin noted stronger efficacy against the *S. epidermidis* strains. Steriscrub demonstrated the weakest efficacy overall.

Table 3.2: The minimum inhibitory and minimum bactericidal concentrations (MIC/MBC) of commercial antimicrobials against the Gram-positive strains (µg/ml)

Antimicrobials	*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)	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Betadine® (Povidone-Iodine)	6.25	6.25	1.87	6.25	6.25	6.25	12.50	12.50	0.98	0.98	2.50	3.12	6.25	6.25
Fusidic acid	2.50	2.50	2.50	2.50	2.50	2.50	0.08	0.63	0.04	0.04	0.08	0.08	0.07	0.07
Gentamycin	125.00	125.00	25.00	25.00	25.00	25.00	6.25	6.25	0.16	0.16	0.08	0.08	0.31	0.31
Mupirocin	25.00	25.00	25.00	25.00	31.30	31.30	0.16	0.16	0.08	0.08	0.04	0.04	0.08	0.08
Neomycin	12.50	12.50	25.00	25.00	12.50	12.50	41.67	41.67	0.63	0.63	0.31	0.31	0.63	0.63
Steriscrub (Chlorohexidine)	212.50	220.00	250.00	250.00	125.00	125.00	133.33	133.33	25.00	25.00	166.67	166.67	200.00	200.00
Controls														
Culture control	>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	>8000.00
Negative control (water/acetone)	>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	>8000.00
Positive control (Ciprofloxacin)	2.50	1.25	2.50	1.25	1.25	1.25	1.25	2.50	1.25	1.25	2.50	1.25	1.25	1.25

MRSA: Methicillin-resistant *Staphylococcus aureus*, GMRSA: Gentamycin-Methicillin resistant *Staphylococcus aureus*

3.3. Antimicrobial activity of 1:1 combinations

Tables 3.3 to 3.16 presents the mean combined MBC's and ΣFIC of the medicinal dyes with antimicrobials. The values in bold note better efficacy of the combination compared to the individual values. The calculation of the ΣFIC values enable the interaction of the combination to be determined (synergistic, additive, indifferent or antagonistic). As with the independent values, all controls responded as expected. All pathogens were susceptible to ciprofloxacin (positive control) The negative control and culture control demonstrated microbial growth, as expected. The ΣFIC value of medicinal dyes in combination with various commercial antimicrobials tested against the *S aureus* and *S. epidermidis* strains could not be determined as no endpoint MBC value was observed and hence are depicted as "U" within Table 3.7 to 3.20, however, a tentative interpretation of synergy, additive, indifferent or antagonism was observed based on the comparison to individual MBC values of the individual medicinal dye and commercial antimicrobial respectively.

3.3.1. Combinations with Fuchsine

Out of the combinations tested with Fuchsine, 66.67% of those combinations noted enhanced/better MBC values compared to when the samples were evaluated independently (Table 3.3 and 3.4). Fuchsine in combination with Gentamycin, Mupirocin and Neomycin noted the lowest MBC values when tested against the *S. aureus* reference strain with MBC values ranging from 31.51 to 41.85 μg/ml. Fuchsine in combination with Gentamycin or Neomycin noted the lowest ΣFIC of 0.10 against the *S. aureus* reference strain. All combinations tested against the *S. aureus* clinical strain demonstrated lower MBC values when compared to the independent versions. Fuchsine in combination with Fusidic acid demonstrated the lowest MBC value of 50.16 μg/ml when tested against the *S. aureus* clinical strain. The majority of the Fuchsine combinations demonstrated lower MBC values in comparison to the independent versions when tested against MRSA. The lowest MBC value noted was 104.20 μg/ml with the combination of Fuchsine and Neomycin. This combination also demonstrated the lowest ΣFIC of 0.21 amongst the combinations tested against MRSA. Combinations with Fuchsine tested against GMRSA did not demonstrate any enhanced (lower) MBC values in comparison to individual MBC values. Antagonism was

noted only when combinations were tested against GMRSA. Fuchsine with Steriscrub noted the highest ΣFIC value of 5.94 when tested against GMRSA.

Table 3.3: The mean MBC values and ΣFIC values of Fuchsine and commercial antimicrobials in combination against the *S. aureus* strains (µg/ml)

Combinations	*Staphylococcus aureus*											
	Reference strain (ATCC 44664)			**Clinical strain (Clinical 6437938)**			**MRSA (ATCC 43300)**			**GMRSA (ATCC 33592)**		
	Mean MBC	**ΣFIC**	**Int.**	**Mean MBC**	**ΣFIC**	**Int.**	**Mean MBC**	**ΣFIC**	**Int.**	**Mean MBC**	**ΣFIC**	**Int.**
Fuchsine + Betadine®	**125.39**	**0.46**	**Syn.**	**250.78**	0.62	Add.	**208.98**	0.52	Add.	62.70	2.52	Ind.
Fuchsine + Fusidic acid	**208.4**	0.69	Add.	**50.16**	**0.16**	**Syn.**	583.52	1.24	Ind.	83.36	3.66	Ind.
Fuchsine + Gentamicin	**31.51**	**0.10**	**Syn.**	**250.08**	**0.50**	**Syn.**	**250.08**	**0.50**	**Syn.**	100.00	4.00	Ind.
Fuchsine + Mupirocin	**41.85**	**0.13**	**Syn.**	**333.44**	0.67	Add.	**125.04**	**0.25**	**Syn.**	25.08	1.50	Ind.
Fuchsine + Neomycin	**31.51**	**0.10**	**Syn.**	**250.08**	**0.50**	**Syn.**	**104.20**	**0.21**	**Syn.**	100.31	4.01	Ant.
Fuchsine + Steriscrub	**209.17**	0.83	Add.	**208.33**	0.63	Add.	**208.33**	0.76	Add.	250.00	5.94	Ant.

Mean MBC: The mean MBC for the combination, Int: Interaction, Syn: Synergistic interaction, Add: Additive interaction, Ant: Antagonistic interaction, Ind: Indifferent interactions, Bold MBC: Reduced MBC values in comparison to independent values; Bold ΣFIC and Int: Synergy

Much lower MBC values were observed for the *S. epidermidis* reference strain in comparison to the *S. aureus* reference strain (Table 3.4). The lowest MBC values noted were for Fuchsine in combination with Gentamycin or Neomycin with an MBC value of 1.57 µg/ml against the *S. epidermidis* reference strain. Fuchsine in combination with commercial antimicrobials also demonstrated the greatest number of antagonistic combinations (ΣFIC value of >4.00) against the *S. epidermidis* clinical strain which clearly demonstrates that results against a single test pathogen cannot be generalized against all strains. Fuchsine and Steriscrub noted the highest ΣFIC value of 106.89 against the *S. epidermidis* clinical strain. Fuchsine demonstrated the least synergistic interactions against the *S. epidermidis* strains compared to the *S. aureus* strains, with the lowest ΣFIC of 0.09 (Fuchsine in combination with Neomycin) against the *S. epidermidis* resistant strain (Table 3.8).

Table 3.4: The mean MBC values and ΣFIC values of Fuchsine and commercial antimicrobials in combination against the *S. epidermidis* strains (μg/ml)

Combinations	*Staphylococcus epidermidis*								
	Reference strain (ATCC 12228)			**Clinical strain (Clinical BA16)**			**Resistant strain (ATCC 51625)**		
	Mean MBC	**ΣFIC**	**Int.**	**Mean MBC**	**ΣFIC**	**Int.**	**Mean MBC**	**ΣFIC**	**Int.**
Fuchsine + Betadine®	**3.22**	0.60	Add.	**3.22**	4.04	Ant.	**51.56**	0.75	Add.
Fuchsine + Fusidic acid	**3.14**	0.76	Add.	U	68.01	Ant.	100.31	5.81	Ant.
Fuchsine + gentamicin	**1.57**	**0.28**	**Syn.**	U	68.01	Ant.	**6.27**	**0.12**	**Syn.**
Fuchsine + Mupirocin	**3.16**	**0.28**	**Syn.**	U	72.01	Ant.	100.31	5.01	Ant.
Fuchsine + Neomycin	**1.57**	**0.31**	**Syn.**	U	65.00	Ant.	**6.27**	**0.09**	**Syn.**
Fuchsine + Steriscrub	**6.25**	0.63	Add.	91.67	106.89	Ant.	**50.00**	**0.38**	**Syn.**

Mean MBC: The mean MBC for the combination, Int: Interaction, Syn: Synergistic interaction, Add: Additive interaction, Ant: Antagonistic interaction, Ind: Indifferent interactions, Bold MBC: Reduced MBC values in comparison to independent values; Bold ΣFIC and Int: Synergy, U: The mean MBC values were not absolute values (either >8000.00 μg/ml or less than 0.01 μg/ml) and hence tentative FIC values were established

A solution of chlorohexidine and Fuchsine has been proposed to treat ear, nose, and throat infections due to the antiseptic ability of chlorhexidine and Fuchsine's activity against Gram-positive bacteria and fungi. The increased benefit and low toxicity of these antimicrobials are ideal for treating conditions such as Otis media (Jungheim *et al*., 2006). In the current study Fuchsine combined with Steriscrub (chlorohexidine), mainly demonstrated additive interactions and only one synergistic interaction against the Gram-positive strains which confirms the susceptibility to the combination and the use of the combination as a treatment option.

3.3.2. Combinations with Gentian violet

When tested against the *S. aureus* strains, 75% of the combinations demonstrated lower (enhanced) MBC values compared to the medicinal dyes and commercial antimicrobials independently (Table 3.5). All combinations with Gentian violet demonstrated synergy against the *S. aureus* reference strain. Gentian violet with Fusidic acid noted the lowest MBC value against the *S. aureus* clinical strain. The highest, most antagonistic ΣFIC value was 107.78 which was the combination of Gentian violet and Mupirocin against the clinical strain of *S. aureus.* Combinations of Gentian violet against MRSA observed additive and synergistic interactions. The lowest and most

synergistic ΣFIC value of 0.15 which was Gentian violet combined with Mupirocin and Gentamycin, respectively. The same MBC value of 0.01 µg/ml was noted for the Gentian violet combinations (Fusidic acid, Gentamycin, Mupirocin and Neomycin) when tested against GMRSA. When combined with Fusidic acid, Gentian violet also noted lower MBC values (cidal activity) against all other *S. aureus* strains. No synergy was observed against GMRSA.

Dumisa (2020), noted greatly reduced MIC values (better activity) of Gentian violet when combined with plant extracts against *S. aureus* strains. The current study demonstrates promising efficacy for Gentian violet when combined with selected commercial antimicrobials making this a versatile antimicrobial.

Table 3.5: The mean MBC values and ΣFIC values of Gentian violet and commercial antimicrobials in combination against the *S. aureus* strains (µg/ml)

Combinations	***Staphylococcus aureus***											
	Reference strain (ATCC 44664)			**Clinical strain (Clinical 6437938)**			**MRSA (ATCC 43300)**			**GMRSA (ATCC 33592)**		
	Mean MBC	**ΣFIC**	**Int.**	**Mean MBC**	**ΣFIC**	**Int.**	**Mean MBC**	**ΣFIC**	**Int.**	**Mean MBC**	**ΣFIC**	**Int.**
Gentian violet + Betadine®	**0.78**	**0.25**	**Syn.**	**0.41**	6.79	Ant.	**0.27**	0.54	Add	**0.05**	1.00	Add.
Gentian violet + Fusidic acid	**0.03**	**0.33**	**Syn.**	**0.01**	5.04	Ant.	**0.04**	0.51	Add	**0.01**	1.06	Ind.
Gentian violet + Gentamicin	U	**0.26**	**Syn.**	33.34	3.13	Ind.	**0.08**	**0.15**	**Syn.**	**0.01**	1.00	Add.
Gentian violet + Mupirocin	U	**0.27**	**Syn.**	**0.94**	107.78	Ant.	**0.08**	**0.15**	**Syn.**	**0.01**	1.03	Ind.
Gentian violet + Neomycin	U	**0.27**	**Syn.**	25.00	2.34	Ind.	**0.08**	**0.16**	**Syn.**	**0.01**	1.00	Add.
Gentian violet + Steriscrub	**25.00**	**0.25**	**Syn.**	**25.00**	1.44	Ind.	**31.51**	0.54	Add	500.02	5.09	Ant.

Mean MBC: The mean MBC for the combination, Int: Interaction, Syn: Synergistic interaction, Add: Additive interaction, Ant: Antagonistic interaction, Ind: Indifferent interactions, Bold MBC: Reduced MBC values in comparison to independent values; Bold ΣFIC and Int: Synergy, U: The mean MBC values were not absolute values (either >8000.00 µg/ml or less than 0.01 µg/ml) and hence tentative FIC values were established

Gentian violet in combination with various commercial antimicrobials demonstrated synergy against the *S. epidermidis* reference strain. A lower MBC value was observed of 0.001 µg/ml

among various combinations of Gentian violet (Table 3.6). The most synergistic interactions were noted against the reference strain of *S. epidermidis*. The lowest ΣFIC value of 0.02 was noted with the combination of Gentian violet and Mupirocin against the *S. epidermidis* reference strain. Among the *S. epidermidis* strains, only one antagonistic interaction was noted: Gentian violet in combination with Gentamycin with an ΣFIC value of 8.02. This value is tentatively based on visual representation as the MBC for Gentian violet was <0.01 and Gentamycin was <0.02 thus the ΣFIC could not be calculated. Indifferent interactions were observed by all Gentian violet combinations against the clinical strain of *S. epidermidis*. Only one combination (Gentian violet with Steriscrub) demonstrated synergy against the *S. epidermidis* resistant strain with an ΣFIC value of 0.31.

Table 3.6: The mean MBC values and ΣFIC values of Gentian violet and commercial antimicrobials in combination against the *S. epidermidis* strains (μg/ml)

Combinations	***Staphylococcus epidermidis***								
	Reference strain (ATCC 12228)			**Clinical strain (Clinical BA16)**			**Resistant strain (ATCC 51625)**		
	Mean MBC	**ΣFIC**	**Int.**	**Mean MBC**	**ΣFIC**	**Int.**	**Mean MBC**	**ΣFIC**	**Int.**
Gentian violet + Betadine®	**0.01**	**0.09**	**Syn.**	**0.01**	2.00	Ind.	**0.78**	0.62	Add.
Gentian violet + Fusidic acid	**0.03**	1.50	Ind.	**0.001**	2.02	Ind.	0.16	3.41	Ind.
Gentian violet + Gentamicin	U	8.02	Ant.	**0.001**	1.01	Ind.	**0.16**	1.50	Ind.
Gentian violet + Mupirocin	**0.001**	**0.02**	**Syn.**	**0.002**	2.71	Ind.	0.16	3.01	Ind.
Gentian violet + Neomycin	**0.001**	**0.04**	**Syn.**	**0.001**	1.34	Ind.	**0.16**	1.25	Ind.
Gentian violet + Steriscrub	**0.39**	**0.06**	**Syn.**	**0.39**	2.00	Ind.	**12.5**	**0.31**	**Syn.**

Mean MBC: The mean MBC for the combination, Int: Interaction, Syn: Synergistic interaction, Add: Additive interaction, Ant: Antagonistic interaction, Ind: Indifferent interactions, Bold MBC: Reduced MBC values in comparison to independent values; Bold ΣFIC and Int: Synergy, U: The mean MBC values were not absolute values (either >8000.00 μg/ml or less than 0.01 μg/ml) and hence tentative FIC values were established

A previous study tested the combination of Gentian violet with medical grade polyvinyl chloride to determine the antibacterial activities of the combination (Sowe *et al*., 2009). It was observed that the zone of inhibitions (efficacy) increased with the addition of Gentian violet (concentration of 1%) (Sowe *et al*., 2009). The current study observed a similar trend with combinations of Gentian violet demonstrating lower MBC values (cidal activity).

3.3.3. Combinations with Iodine tincture

Iodine tincture in combination with all commercial antimicrobials except Steriscrub, demonstrated reduced MBC values (better activity) in combination compared to when Steriscrub was investigated independently (Table 3.7 and 3.8). Iodine tincture in combination with Gentamycin noted the same MBC value of 1.09 μg/ml among all the *S. aureus* strains (Table 3.7). Iodine tincture in combination with Neomycin demonstrated the lowest MBC value of 0.55 μg/ml when tested against MRSA, the lowest MBC among all *S. aureus* strains.

The combinations of Iodine tincture and the commercial antimicrobials predominantly demonstrated synergistic interactions against the *S. aureus* reference strain (Table 3.11); except one combination (Iodine tincture and Fusidic acid) which noted an additive interaction. Iodine tincture and Fusidic acid demonstrated the most antagonistic interaction with an ΣFIC value of 4.91 against GMRSA.

Table 3.7: The mean MBC values and ΣFIC values of Iodine tincture and commercial antimicrobials in combination against the *S. aureus* strains (μg/ml)

Combinations	***Staphylococcus aureus***											
	Reference strain (ATCC 44664)			**Clinical strain (Clinical 6437938)**			**MRSA (ATCC 43300)**			**GMRSA (ATCC 33592)**		
	Mean MBC	**ΣFIC**	**Int.**	**Mean MBC**	**ΣFIC**	**Int.**	**Mean MBC**	**ΣFIC**	**Int.**	**Mean MBC**	**ΣFIC**	**Int.**
Iodine tincture + Betadine®	**1.95**	**0.46**	**Syn.**	**3.91**	1.00	Add.	**1.56**	**0.37**	**Syn.**	**0.55**	0.51	Add.
Iodine tincture + Fusidic acid	**1.57**	0.83	Add.	**1.88**	1.40	Ind.	**2.19**	1.25	Ind.	**1.09**	4.91	Ant.
Iodine tincture + Gentamicin	**1.09**	**0.42**	**Syn.**	**1.09**	0.51	Add.	**1.09**	0.51	Add.	**1.09**	1.05	Ind.
Iodine tincture + Mupirocin	**1.09**	**0.43**	**Syn.**	**1.46**	0.68	Add.	**1.09**	0.51	Add.	**0.27**	0.75	Add.
Iodine tincture + Neomycin	**1.09**	**0.44**	**Syn.**	**0.73**	**0.34**	**Syn.**	**0.55**	**0.26**	**Syn.**	**1.09**	1.01	Ind.
Iodine tincture + Steriscrub	**62.70**	**0.40**	**Syn.**	500.39	2.25	Ind.	333.59	1.94	Ind.	**62.55**	0.53	Add.

Mean MBC: The mean MBC for the combination, Int: Interaction, Syn: Synergistic interaction, Add: Additive interaction, Ant: Antagonistic interaction, Ind: Indifferent interactions, Bold MBC: Reduced MBC values in comparison to independent values; Bold ΣFIC and Int: Synergy

The combinations against the *S. epidermidis* strains have no pattern and the lower MBC values are more random (Table 3.8). Iodine tincture with Betadine® demonstrated the most synergy against the *S. epidermidis* reference strain with a ΣFIC value of 0.15. The most antagonistic interactions were observed against the *S. epidermidis* clinical strain. The highest and most antagonistic ΣFIC value of 126.00 was observed with the combination of Iodine tincture with Gentamycin against the *S. epidermidis* clinical strain. No synergy was observed against the *S. epidermidis* resistant strain.

Table 3.8: The mean MBC values and ΣFIC values of Iodine tincture and commercial antimicrobials in combination against the *S. epidermidis* strains (µg/ml)

Combinations	***Staphylococcus epidermidis***								
	Reference strain (ATCC 12228)			**Clinical strain (Clinical BA16)**			**Resistant strain (ATCC 51625)**		
	Mean MBC	**ΣFIC**	**Int.**	**Mean MBC**	**ΣFIC**	**Int.**	**Mean MBC**	**ΣFIC**	**Int.**
Iodine tincture + Betadine®	**0.15**	**0.15**	**Syn.**	**0.49**	62.63	Ant.	**0.98**	0.62	Add.
Iodine tincture + Fusidic acid	1.09	8.81	Ant.	0.46	4.50	Ant.	0.55	3.41	Ind.
Iodine tincture + Gentamicin	**0.16**	0.58	Add.	0.27	126.00	Ant.	**0.55**	1.50	Ind.
Iodine tincture + Mupirocin	**0.14**	**0.16**	**Syn.**	**0.07**	31.75	Ant.	1.46	8.01	Ant.
Iodine tincture + Neomycin	**0.55**	2.41	Ind.	0.27	125.25	Ant.	**0.55**	1.25	Ind.
Iodine tincture + Steriscrub	**12.60**	0.60	Add.	**8.40**	41.72	Ant.	**41.99**	1.04	Ind.

Mean MBC: The mean MBC for the combination, Int: Interaction, Syn: Synergistic interaction, Add: Additive interaction, Ant: Antagonistic interaction, Ind: Indifferent interactions, Bold MBC: Reduced MBC values in comparison to independent values; Bold ΣFIC and Int: Synergy

Iodine has been previously combined in a Zeolitic imidazolate framework-8 to determine the antimicrobial activity against *S. aureus* and *S. epidermidis.* A 100% killing rate was observed in two minutes (Au-Duong and Lee, 2017). This demonstrates the susceptibility of selected Gram-positive pathogens to iodine tincture in the framework. Research into other combinations with Iodine is lacking, however, a more recent study by Dumisa (2020), did observe that there weren't any noteworthy interactions with Iodine tincture when tested in combination with medicinal plants.

3.3.4. Combinations with Malachite green

For the majority of the Malachite green combinations (90.48%), a lower MBC value was observed in comparison to when tested independently (Table 3.9). Malachite green combinations tested against the *S. aureus* reference strain noted reduced MBC values in comparison to independent values, however, with the majority of the combinations, indifferent interactions were observed. The same MBC values were noted when Malachite green was combined with Gentamycin, Mupirocin and Neomycin respectively. The combinations noted an MBC value of 2.63 µg/ml against the *S. aureus* reference strain; 0.33 µg/ml against the *S. aureus* clinical strain and 1.31 µg/ml against MRSA. Malachite green in combination with Fusidic acid, Gentamycin, Mupirocin or Neomycin noted the lowest MBC value of 0.08 µg/ml when tested against GMRSA.

Although lower MBC values was observed with the majority of the combinations tested against the *S. aureus* strains, synergy was only observed when Malachite green in combination with Gentamycin, Mupirocin or Neomycin was tested against the *S. aureus* clinical strain. The lowest ΣFIC value of 0.02 was noted with the combination of Gentamycin and Neomycin against the *S. aureus* clinical strain. The highest and most antagonistic ΣFIC value of 1000.50 was noted with the combination of Malachite green with Betadine® against MRSA. Indifferent interactions were observed with the Malachite green combinations among the *S. aureus* reference strain and GMRSA.

Table 3.9: The mean MBC values and ΣFIC values of Malachite green and commercial antimicrobials in combination against the *S. aureus* strains (µg/ml)

Combinations	***Staphylococcus aureus***											
	Reference strain (ATCC 44664)			**Clinical (Clinical 6437938)**			**MRSA (ATCC 43300)**			**GMRSA (ATCC 33592)**		
	Mean MBC	**ΣFIC**	**Int.**	**Mean MBC**	**ΣFIC**	**Int.**	**Mean MBC**	**ΣFIC**	**Int.**	**Mean MBC**	**ΣFIC**	**Int.**
Malachite green + Betadine®	**4.13**	1.50	Ind.	**1.64**	0.56	Add.	1003.13	1000.50	Ant.	**0.26**	2.02	Ind.
Malachite green + Fusidic acid	**1.29**	1.27	Ind.	**1.29**	0.52	Add.	1000.31	1000.20	Ant.	**0.08**	2.24	Ind.
Malachite green + Gentamicin	**2.63**	2.01	Ind.	**0.33**	**0.02**	**Syn.**	**1.31**	1.01	Ind.	**0.08**	2.00	Ind.

Combinations	*Staphylococcus aureus*											
	Reference strain (ATCC 44664)			**Clinical (Clinical 6437938)**			**MRSA (ATCC 43300)**			**GMRSA (ATCC 33592)**		
	Mean MBC	**ΣFIC**	**Int.**	**Mean MBC**	**ΣFIC**	**Int.**	**Mean MBC**	**ΣFIC**	**Int.**	**Mean MBC**	**ΣFIC**	**Int.**
Malachite green + Mupirocin	**2.63**	2.03	Ind.	**0.33**	0.10	**Syn.**	**1.31**	13.02	Ant.	**0.08**	2.13	Ind.
Malachite green + Neomycin	**2.63**	2.05	Ind.	**0.33**	**0.02**	**Syn.**	**1.31**	3.4	Ind.	**0.08**	2.00	Ind.
Malachite green + Steriscrub	**100.31**	0.78	Add.	**105.21**	0.83	Add.	**101**	1.53	Ind.	**5.26**	1.71	Ind.

Mean MBC: The mean MBC for the combination, Int: Interaction, Syn: Synergistic interaction, Add: Additive interaction, Ant: Antagonistic interaction, Ind: Indifferent interactions, Bold MBC: Reduced MBC values in comparison to independent values; Bold ΣFIC and Int: Synergy

The majority of the combinations tested against the *S. epidermidis* strains demonstrated lower MBC values compared to independent values, mostly indifferent and antagonistic interactions were observed (Table 3.10). Malachite green in combination with Gentamycin, Mupirocin and Neomycin noted the same low MBC value of 0.02 µg/ml when tested against the *S. epidermidis* clinical strain (Table 3.10). Malachite green combinations tested against the *S. epidermidis* reference and clinical strains, noted lower MBC values in comparison to the independent values.

The most antagonistic interaction against the *S. epidermidis* strains was observed for the combination of Malachite green with Steriscrub against the reference strain with an ΣFIC value of 17.42. The lowest ΣFIC of 0.02 was with Malachite green combined with Neomycin against the *S. epidermidis* clinical strain.

While no previous studies examined the combination of Malachite green with conventional antimicrobials, various studies have been conducted on the antimicrobial activity of Malachite green against *S. aureus* independently (Fung and Miller, 1973; Golding *et al*., 1998; Vilela *et al*., 2012; Rosa *et al*., 2015). Dumisa (2020), observed enhanced values with Malachite green in combination with plant extracts. A similar pattern was noted with the combination of Malachite green and commercial antimicrobials.

Table 3.10: The mean MBC values and ΣFIC values of Malachite green and commercial antimicrobials in combination against the *S. epidermidis* strains (µg/ml)

Combinations	*Staphylococcus epidermidis*								
	Reference strain (ATCC 12228)			Clinical strain (Clinical BA16)			Resistant strain (ATCC 51625)		
	Mean MBC	ΣFIC	Int.	Mean MBC	ΣFIC	Int.	Mean MBC	ΣFIC	Int.
Malachite green + Betadine®	**0.09**	4.43	Ant.	**0.13**	5.33	Ant.	**2.06**	0.75	Add.
Malachite green + Fusidic acid	**0.004**	3.64	Ind.	**0.02**	7.88	Ant.	**1.31**	5.81	Ant.
Malachite green + Gentamicin	**0.003**	2.17	Ind.	**0.02**	**0.06**	**Syn.**	1.31	2.00	Ind.
Malachite green + Mupirocin	**0.002**	1.80	Ind.	**0.02**	**0.13**	**Syn.**	1.31	5.01	Ant.
Malachite green + Neomycin	**0.002**	1.27	Ind.	**0.02**	**0.02**	**Syn.**	**1.31**	1.50	Ind.
Malachite green + Steriscrub	**1.58**	17.42	Ant.	**2.10**	10.42	Ant.	**25.25**	**0.38**	**Syn.**

Mean MBC: The mean MBC for the combination, Int: Interaction, Syn: Synergistic interaction, Add: Additive interaction, Ant: Antagonistic interaction, Ind: Indifferent interactions, Bold MBC: Reduced MBC values in comparison to independent values; Bold ΣFIC and Int: Synergy,

3.3.5. Combinations with Mercurochrome

Mercurochrome in combination with Betadine® noted lower (enhanced) MIC values in comparison to when studied independently. Minimum bactericidal concentration values for this combination against all *S. aureus* strains ranged from 0.31 – 0.85 µg/ml (Table 3.11). Mercurochrome combined with Betadine® noted synergy among all *S. aureus* combinations. Combinations with Mercurochrome demonstrated the most synergy against the *S. aureus* reference strain. While the combinations did demonstrate lower MBC values in comparison to the independent values against *S. aureus* clinical strain, mostly additive interactions were noted. The lowest MBC value observed (among the *S. aureus* strains) of 0.04 µg/ml was noted against MRSA with the combination of Mercurochrome and Gentamycin (Table 3.11). The lowest ΣFIC value of 0.05 was noted with the combination Mercurochrome and Betadine® against GMRSA.

Table 3.11: The mean MBC values and ΣFIC values of Mercurochrome and commercial antimicrobials in combination against the *S. aureus* strains (µg/ml)

Combinations	*Staphylococcus aureus*											
	Reference strain (ATCC 44664)			**Clinical strain (Clinical 6437938)**			**MRSA (ATCC 43300)**			**GMRSA (ATCC 33592)**		
	Mean MBC	**ΣFIC**	**Int.**	**Mean MBC**	**ΣFIC**	**Int.**	**Mean MBC**	**ΣFIC**	**Int.**	**Mean MBC**	**ΣFIC**	**Int.**
Mercurochrome + Betadine®	**0.85**	**0.27**	**Syn.**	**0.23**	**0.40**	**Syn.**	**0.31**	**0.45**	**Syn.**	**0.40**	**0.05**	**Syn.**
Mercurochrome + Fusidic acid	**0.23**	**0.39**	**Syn.**	**0.39**	1.01	Ind.	**0.20**	1.03	Ind.	**0.12**	0.67	Add.
Mercurochrome + Gentamicin	**0.23**	**0.36**	**Syn.**	**0.27**	0.72	Add.	**0.04**	**0.30**	**Syn.**	**0.47**	0.78	Add.
Mercurochrome + Mupirocin	**0.23**	**0.36**	**Syn.**	**0.27**	0.55	Add.	**0.12**	0.60	Add.	**0.12**	**0.44**	**Syn.**
Mercurochrome + Neomycin	**0.47**	0.72	Add.	**0.23**	0.57	Add.	**0.06**	**0.30**	**Syn.**	**0.31**	**0.50**	**Syn.**
Mercurochrome + Steriscrub	542.01	3.32	Ind.	**125.08**	0.79	Add.	125.08	1.27	Ind.	**62.54**	0.56	Add.

Mean MBC: The mean MBC for the combination, Int: Interaction, Syn: Synergistic interaction, Add: Additive interaction, Ant: Antagonistic interaction, Ind: Indifferent interactions, Bold MBC: Reduced MBC values in comparison to independent values; Bold ΣFIC and Int: Synergy

Mercurochrome in combination with all commercial antimicrobials demonstrated lower MBC values against the *S. epidermidis* reference strain compared to when tested independently (Table 3.12). Mercurochrome combinations demonstrated better activity against the *S. aureus* strains in comparison to the *S. epidermidis* strains with more combinations noting lower (enhanced) MBC values.

Mercurochrome with Fusidic acid demonstrated the best activity with an MBC value or 0.03 µg/ml against the *S. epidermidis* clinical strain. As with the *S. aureus* strains, Mercurochrome with Betadine® demonstrated synergy among all the strains except for one- the *S. epidermidis* resistant strain. The Mercurochrome combinations noted only three synergistic interactions among the *S. epidermidis* strains. The most synergistic interaction observed was Mercurochrome with Betadine® with a ΣFIC value of 0.40 against the clinical strain. Mercurochrome in combination with the commercial antimicrobials demonstrated indifferent and antagonistic interactions against the *S. epidermidis* resistant strain.

Table 3.12: The mean MBC values and ΣFIC values of Mercurochrome and commercial antimicrobials in combination against the *S. epidermidis* strains (μg/ml)

Combinations	*Staphylococcus epidermidis*								
	Reference strain (ATCC 12228)			**Clinical strain (Clinical BA16)**			**Resistant strain (ATCC 51625)**		
	Mean MBC	**ΣFIC**	**Int.**	**Mean MBC**	**ΣFIC**	**Int.**	**Mean MBC**	**ΣFIC**	**Int.**
Mercurochrome + Betadine®	**0.23**	**0.48**	**Syn.**	**0.23**	**0.40**	**Syn.**	**0.39**	1.09	Ind.
Mercurochrome + Fusidic acid	**0.12**	1.57	Ind.	**0.03**	1.41	Ind.	**0.06**	0.92	Add.
Mercurochrome + Gentamycin	**0.12**	0.82	Add.	0.09	2.00	Add.	0.47	5.50	Ant.
Mercurochrome + Mupirocin	**0.12**	0.63	Add.	0.06	3.00	Ind.	0.47	7.00	Ant.
Mercurochrome + Neomycin	**0.12**	1.07	Ind.	**0.09**	1.25	Ind.	0.94	10.48	Ant.
Mercurochrome + Steriscrub	**3.16**	**0.41**	**Syn.**	**25.08**	5.15	Ant.	**41.93**	4.37	Ant.

Mean MBC: The mean MBC for the combination, Int: Interaction, Syn: Synergistic interaction, Add: Additive interaction, Ant: Antagonistic interaction, Ind: Indifferent interactions, Bold MBC: Reduced MBC values in comparison to independent values; Bold ΣFIC and Int: Synergy

While no previous studies examined the combination of Mercurochrome with conventional antimicrobials, Mercurochrome in combination with chloromycetin has been tested for the treatment of dry socket (alveolar osteitis), a complication in tooth extraction. This combination reduced the pain and demonstrated strong antimicrobial activity which yielded effective results in a single dressing (Bansal *et al*., 2012). Dumisa (2020), observed very few synergistic interactions among the combinations with mercurochrome and medicinal plants. The current study observed the same pattern – fewer synergistic interactions. A comparison of the two studies notes that when Mercurochrome is combined with commercial antimicrobials, better activity is observed overall, compared to when combined with plant extracts.

3.3.6. Combinations with Methylene blue

Methylene blue in combination with the commercial antimicrobials demonstrated little improvement in the MBC values for the *S. aureus* clinical strain (Table 3.13). All combinations tested against the *S. aureus* clinical strain demonstrated lower (enhanced) MBC values, however, no synergy was noted. Methylene blue in combination with Gentamycin and Neomycin,

respectively, demonstrated the lowest ΣFIC value of 0.30 against MRSA. Only one antagonistic interaction (Methylene blue combined with Mupirocin) was noted among the *S. aureus* strains which demonstrated the highest ΣFIC value of 4.59 against MRSA.

Table 3.13: The mean MBC values and ΣFIC values of Methylene blue and commercial antimicrobials in combination against the *S. aureus* strains (µg/ml)

Combinations	*Staphylococcus aureus*											
	Reference strain (ATCC 44664)			**Clinical strain (Clinical 6437938)**			**MRSA (ATCC 43300)**			**GMRSA (ATCC 33592)**		
	Mean MBC	**ΣFIC**	**Int.**	**Mean MBC**	**ΣFIC**	**Int.**	**Mean MBC**	**ΣFIC**	**Int.**	**Mean MBC**	**ΣFIC**	**Int.**
Methylene blue + Betadine®	**6.56**	**0.50**	**Syn.**	**26.25**	1.40	Ind.	271.68	1.44	Ind.	**51.56**	1.12	Ind.
Methylene blue + Fusidic acid	**20.63**	1.25	Ind.	**20.63**	0.65	Add.	**208.4**	1.03	Ind.	**12.58**	1.23	Ind.
Methylene blue + Gentamycin	**66.88**	3.34	Ind.	**50.16**	1.01	Ind.	**62.52**	**0.30**	**Syn.**	**20.63**	**0.50**	**Syn.**
Methylene blue + Mupirocin	50.16	2.51	Ind.	**66.88**	1.34	Ind.	**125.04**	4.59	Ant.	**2.58**	0.55	Add.
Methylene blue + Neomycin	50.16	2.51	Ind.	**50.16**	1.01	Ind.	**62.52**	**0.30**	**Syn.**	**20.63**	**0.41**	**Syn.**
Methylene blue + Steriscrub	505.00	2.60	Ind.	**220.00**	1.20	Ind.	416.67	2.11	Ind.	229.17	1.98	Ind.

Mean MBC: The mean MBC for the combination, Int: Interaction, Syn: Synergistic interaction, Add: Additive interaction, Ant: Antagonistic interaction, Ind: Indifferent interactions, Bold MBC: Reduced MBC values in comparison to independent values; Bold ΣFIC and Int: Synergy.

Combinations with Methylene blue demonstrated lower MBC values (enhanced activity) against the reference and clinical strain of *S. epidermidis* in comparison to when tested independently (Table 3.14). As with the *S. aureus* strains, not many synergistic interactions were noted among the *S. epidermidis* strains.

The combination of Methylene blue with Steriscrub demonstrated a low MBC value of 6.88 µg/ml against the *S. epidermidis* clinical strain, a much lower concentration than the dye and commercial antimicrobial when tested independently, thus, this combination noted the most synergistic interaction with a ΣFIC value of 0.07. One antagonistic interaction was also observed among the *S. epidermidis* strains. Methylene blue with Mupirocin demonstrated a high ΣFIC of 17.03 against the *S. epidermidis* resistant strain.

Table 3.14: The mean MBC values and ΣFIC values of Methylene blue and commercial antimicrobials in combination against the *S. epidermidis* strains (μg/ml)

Combinations	*Staphylococcus epidermidis*								
	Reference strain (ATCC 12228)			**Clinical strain (Clinical BA16)**			**Resistant strain (ATCC 51625)**		
	Mean MBC	**ΣFIC**	**Int.**	**Mean MBC**	**ΣFIC**	**Int.**	**Mean MBC**	**ΣFIC**	**Int.**
Methylene blue + Betadine®	**12.89**	**0.50**	**Syn.**	**13.13**	1.50	Ind.	**13.13**	0.75	Add.
Methylene blue + Fusidic acid	**10.45**	0.92	Add.	**2.58**	1.13	Ind.	41.25	20.23	Ant.
Methylene blue + Gentamycin	**12.54**	0.74	Add.	**5.16**	2.26	Ind.	**10.31**	1.25	Ind.
Methylene blue + Mupirocin	**8.36**	**0.17**	**Syn.**	**1.29**	1.06	Ind.	41.25	17.03	Ant.
Methylene blue + Neomycin	**8.36**	0.92	Add.	**20.63**	3.00	Ind.	**13.75**	1.00	Add.
Methylene blue + Steriscrub	**20.83**	0.50	**Syn.**	**6.88**	**0.07**	**Syn.**	**55.00**	**0.38**	**Syn.**

Mean MBC: The mean MBC for the combination, Int: Interaction, Syn: Synergistic interaction, Add: Additive interaction, Ant: Antagonistic interaction, Ind: Indifferent interactions, Bold MBC: Reduced MBC values in comparison to independent values; Bold ΣFIC and Int: Synergy.

Methylene blue in combination with silver nanoparticles was investigated against various pathogens including a *S. aureus* reference strain. The combination of both Methylene blue and silver demonstrated better cidal effects when combined compared to when tested individually (Belekov *et al*., 2020). In the current study, the majority of the combinations with Methylene blue demonstrated lower MBC values (enhanced effects) which affirms combinations of Methylene blue have potential for enhanced activity.

3.3.7. Combinations with Potassium permanganate

The majority of the Potassium permanganate combinations (54.76%) did not show much improvement in combination compared to the individual MBC values (Table 3.15 and 3.16). The MBC of Potassium permanganate was >8000.00 μg/ml, hence an endpoint value was unable to be determined and an ΣFIC value could not be calculated. This is represented by a "U" in the tables. Based on tentative interpretations when compared to the MBC values of individual samples, an interpretation is still provided.

Among the *S. aureus* strains, only combinations with Betadine® and Steriscrub demonstrated a reduced MBC value compared to the independent values tested (Table 3.15). Potassium permanganate in combination with Steriscrub noted an MBC value of 500.00 µg/ml against the reference strain of *S. aureus* and MRSA, this is the lowest among the *S. aureus* strains. Potassium permanganate was the only dye where antagonistic interactions were predominantly observed in combination against the *S. aureus* reference strain (66.67%). Only one synergistic interaction was observed (Potassium permanganate with Betadine® with an ΣFIC value of 0.40) when tested against the *S. aureus* reference strain.

Table 3.15: The mean MBC values and ΣFIC values of Potassium permanganate and commercial antimicrobials in combination, against the *S. aureus* strains (µg/ml)

Combinations	*Staphylococcus aureus*											
	Reference strain (ATCC 44664)			**Clinical strain (Clinical 6437938)**			**MRSA (ATCC 43300)**			**GMRSA (ATCC 33592)**		
	Mean MBC	**ΣFIC**	**Int**	**Mean MBC**	**ΣFIC**	**Int.**	**Mean MBC**	**ΣFIC**	**Int.**	**Mean MBC**	**ΣFIC**	**Int.**
Potassium permanganate + Betadine®	**626.95**	**0.40**	**Syn**	**2006.25**	U	Ind.	**1337.50**	U	Add.	**417.97**	U	Add.
Potassium permanganate + Fusidic acid	U	U	Ant.	U	U	Ind.	U	U	Ind.	U	U	Ind.
Potassium permanganate +Gentamycin	U	U	Ant.	U	U	Ind.	U	U	Ind.	U	U	Ind.
Potassium permanganate + Mupirocin	U	U	Ant.	U	U	Ind.	U	U	Ind.	U	U	Ind.
Potassium permanganate + Neomycin	U	U	Ant.	U	U	Ind.	U	U	Ind.	U	U	Ind.
Potassium permanganate + Steriscrub	**500.00**	1.21	Ind.	**1000.00**	U	Ind.	**500.00**	U	Add.	**1333.33**	U	Add.

Mean MBC: The mean MBC for the combination, Int: Interaction, Syn: Synergistic interaction, Add: Additive interaction, Ant: Antagonistic interaction, Ind: Indifferent interactions, Bold MBC: Reduced MBC values in comparison to independent values; Bold ΣFIC and Int: Synergy, U: The mean MBC values were not absolute values (either >8000.00 µg/ml. or less than 0.01 µg/ml) and hence tentative FIC values were established

While the majority of the combinations of Potassium permanganate noted reduced MBC values in comparison to when tested independently, only three synergistic interactions were observed. Among the *S. epidermidis* strains, Potassium permanganate with Betadine® demonstrated the

lowest MBC value of 125.39 µg/ml when tested against the *S. epidermidis* resistant strain (Table 3.16). The most synergistic interaction (ΣFIC value of 0.08) was with the combination of Potassium permanganate and Betadine® against the *S. epidermidis* resistant strain. Antagonistic interactions were noted with the combination of Potassium permanganate and Mupirocin against the *S. epidermidis* clinical strain (ΣFIC value of 16.28) and the *S. epidermidis* resistant strain (ΣFIC value of 16.53).

Table 3.16: The mean MBC values and ΣFIC values of Potassium permanganate and commercial antimicrobials in combination against the *S. epidermidis* strains (µg/ml)

Combinations	***Staphylococcus epidermidis***								
	Reference (ATCC 12228)			**Clinical (Clinical BA16)**			**Resistant (ATCC 51625)**		
	Mean MBC	**ΣFIC**	**Int.**	**Mean MBC**	**ΣFIC**	**Int.**	**Mean MBC**	**ΣFIC**	**Int.**
Potassium permanganate + Betadine®	**501.56**	U	Add.	**250.78**	**0.28**	**Syn.**	**125.39**	**0.08**	**Syn.**
Potassium permanganate + Fusidic acid	U	U	Ind.	U	U	Ind.	U	U	Ind.
Potassium permanganate + Gentamycin	**1000.31**	U	Add.	**2000.63**	0.33	Syn.	**1000.31**	1.12	Ind.
Potassium permanganate + Mupirocin	**2000.63**	U	Ind.	**2000.63**	16.28	Ant.	**4001.25**	16.53	Ant.
Potassium permanganate + Neomycin	**1000.31**	U	Add.	**2000.63**	2.25	Ind.	**1000.31**	0.63	Add.
Potassium permanganate + Steriscrub	**320.83**	U	Add.	**137.50**	**0.09**	**Syn.**	**1100.00**	0.63	Add.

Mean MBC: The mean MBC for the combination, Int: Interaction, Syn: Synergistic interaction, Add: Additive interaction, Ant: Antagonistic interaction, Ind: Indifferent interactions, Bold MBC: Reduced MBC values in comparison to independent values; Bold ΣFIC and Int: Synergy, U: The mean MBC values were not absolute values (either >8000.00 µg/ml or less than 0.01 µg/ml) and hence tentative FIC values were established

While no previous studies examined the combination of Potassium permanganate with conventional antimicrobials, a previous study tested the disinfecting activity of Potassium permanganate in combination with solution of sodium chloride that was electrochemically activated (Aliev *et al*., 2018). The study found that the combination at concentrations of 1:2000 to 1:3500 (Potassium permanganate: sodium chloride) was able to eradicate a strain of *S. aureus* completely in a period of 20 min (Aliev *et al*., 2018). While this study is not related to the current study, it does demonstrate the use of Potassium permanganate in combination may have potential against Gram-positive bacteria.

3.3.8. Overview of all combinations against the Gram-positive pathogens

Figure 3.2 shows the overall ΣFIC values of all medicinal dyes against the Gram-positive pathogens. The most synergistic interactions were observed against the S. *aureus* reference strain. Among the other *S. aureus* strains, the majority of the interactions were indifferent in comparison to synergy, additive or antagonistic. Among the Gram-positive pathogens, the highest number of indifferent interactions were noted against GMRSA. Among the *S. epidermidis* strains, the reference strain noted the highest number of synergistic interactions. The *S. epidermidis* clinical strain demonstrated the highest number of antagonistic interactions among the Gram-positive pathogens. The *S. epidermidis* noted the least number of additive interactions among all Gram-positive strains.

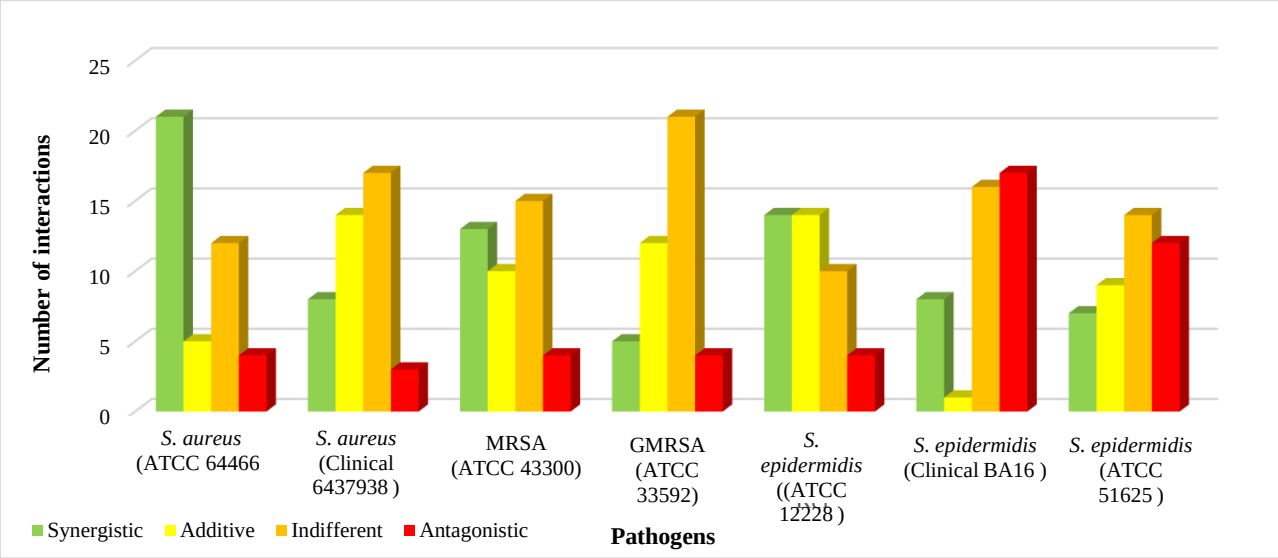


Figure 3.2: Summary of ΣFIC values for Gram-positive pathogens

3.4. Varied ratio combinations

Combinations were chosen based on their ΣFIC values and whether there was a pattern demonstrated by that combination against the Gram-positive strains. If the combinations demonstrated synergy among the majority of the pathogens tested, then varied ratio combination studies were conducted and if the combination noted antagonism against two or more pathogens, then varied ratio testing was also conducted. The combination was grouped into two groups: synergistic interactions and antagonistic interactions (Table 3.17). While synergistic ratios are

beneficial and highly effective therapeutically, antagonistic ratios are disadvantageous. This is helpful in finding combinations that may result in resistant mutations (Yin *et al*., 2014). Therefore, varied ratio testing was conducted on both synergistic and antagonistic combinations.

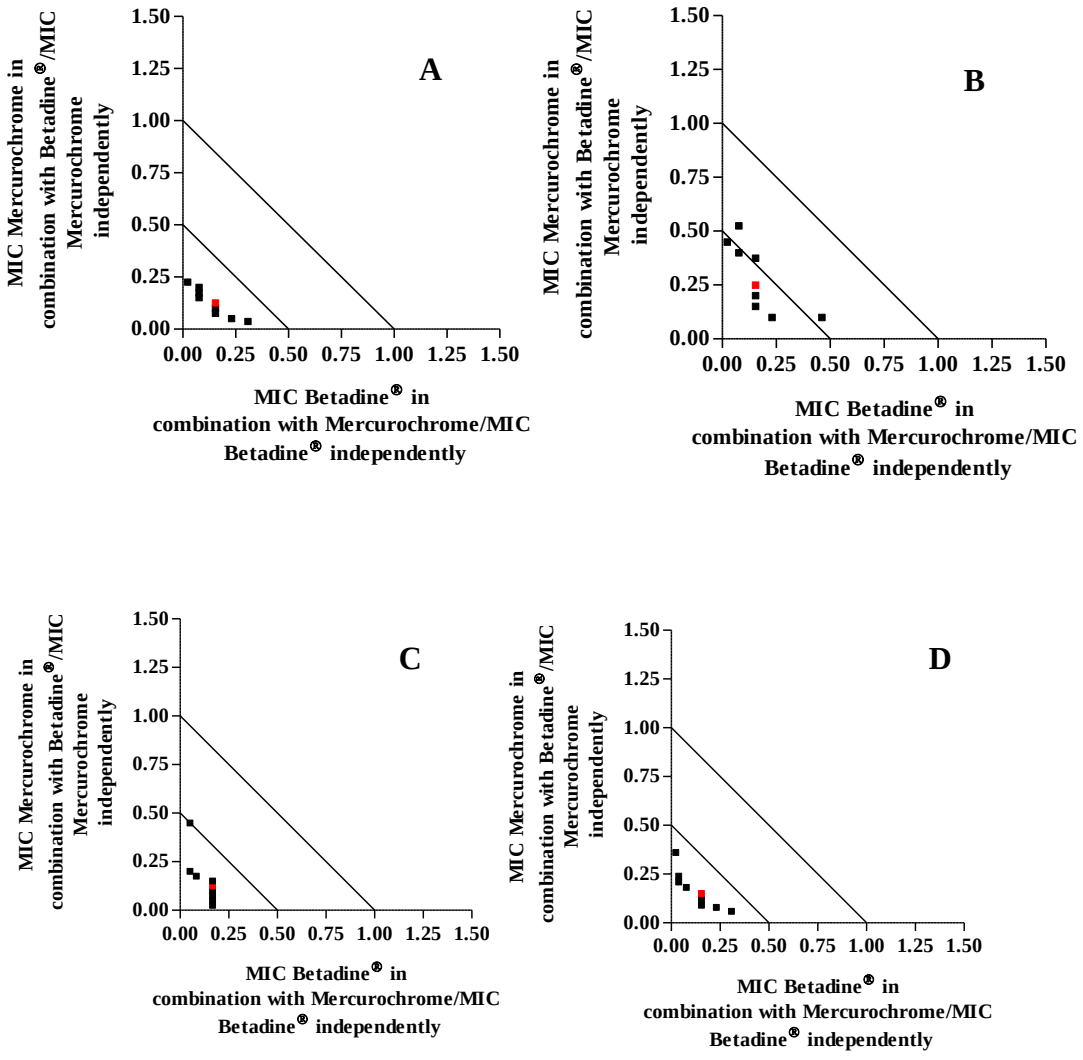
Table 3.17: Combinations chosen for varied ratio testing.

Combination		Corresponding Pathogen/s
Synergistic	Mercurochrome + Betadine®	*S. aureus* reference strain, *S. aureus* clinical strains, MRSA, *S. epidermidis* reference strain and *S. epidermidis* clinical strain
Antagonistic	Iodine tincture + Fusidic acid	GMRSA and *S. epidermidis* clinical strain
	Malachite green + Betadine®	*S. epidermidis* reference and clinical strain
	Gentian violet + Steriscrub	GMRSA

3.4.1. Synergistic varied ratio combinations of medicinal dyes with commercial antimicrobials against Gram-positive pathogens

Mercurochrome in combination with Betadine® was selected for further varied ratio testing as it demonstrated synergistic interactions amongst the majority of the Gram-positive pathogens tested. No antagonism was noted for the 1:1 ratio of this combination. The combination demonstrated synergy in all 1:1 ratios as indicated by the red square on each isobologram. Synergy was predominantly observed in all ratios of Mercurochrome and Betadine® against the Gram-positive pathogens tested (Figure 3.3), except for the *S. aureus* clinical strain (Figure 3.3.B) which noted additive effects in the following ratios: 9:1, 4:6 and 3:7 (Mercurochrome: Betadine®). To the best of my knowledge no research on the antimicrobial effects of this combination have been undertaken. Mercurochrome and Betadine® have been used separately as antiseptics with positive results (Clapp and Martin, 1920; Al-Azzawi and Abdullah, 2018; Kampf, 2018; Hoekstra *et al*,2017). Dumisa (2020), observed synergistic interactions of Gentian violet in varied ratio combinations with plant extracts against *S. aureus* strains. Dumisa (2020), attributed the synergistic effect to a dominant concentration of Gentian violet. While Gentian violet did

demonstrate synergy in the current study, Mercurochrome and Betadine® demonstrated better synergistic activity among the Gram-positive strains. While the results differ, it does demonstrate the potential of varied ratio testing among medicinal dye combinations. *Terminalia sericea* in combination with Gentamycin demonstrated synergistic effects against *Streptococcus pyogenes* (Nel *et al.*, 2020). While there is no relation to this study, this just demonstrates varied ratio testing has been conducted on antimicrobials to demonstrate the way in which the various dye combinations interact.



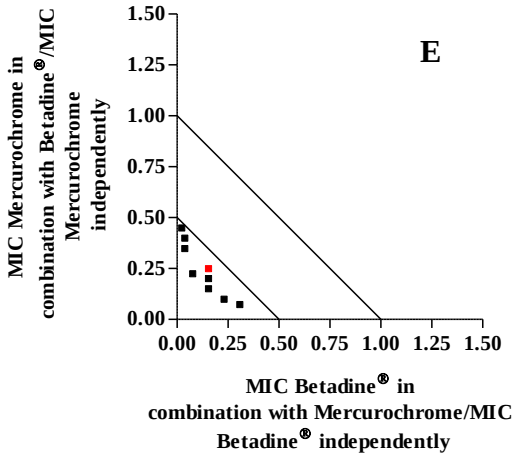


Figure 3.3: Isobologram of varied ratios of Mercurochrome with Betadine® against A.) *S. aureus* reference strain, B.) *S. aureus* clinical strain, C.) MRSA, D.) *S. epidermidis* reference strain, E.) *S. epidermidis* clinical strain. Red square = 1:1 combination

3.4.2. Antagonistic varied ratio combinations of medicinal dyes with commercial antimicrobials against Gram-positive pathogens

The antagonistic interactions did not demonstrate a clear pattern as the synergistic interactions did, the combinations were based on the number of antagonistic interactions observed against the Gram-positive pathogens tested. Potassium permanganate demonstrated several antagonistic interactions (Tables 3.15 and 3.16); however, the combination was not chosen for varied ratios as most of the interactions were tentative values where the ΣFIC value could not be calculated and an exact value could not be determined. Iodine tincture in combination with Fusidic acid noted three antagonistic interactions and no synergy (Tables 3.7 and 3.8). Malachite green with Betadine® also noted several antagonistic interactions with no synergy and the highest ΣFIC value was 1000.50. Hence, both combinations were chosen for varied ratio testing. Iodine tincture and Fusidic acid demonstrated antagonism when tested in a 1:1 ratio (indicated by the red square) against two pathogens - GMRSA and *S. epidermidis* clinical strain (Figure 3.4.A and 3.4.B respectively). Antagonism was predominantly observed against GMRSA with ratios where Fusidic acid was in higher concentrations. A slight variation (indifferent interactions) was noted with ratios where the Iodine tincture was dominant. When tested against the *S. epidermidis* clinical strain,

indifferent interactions were predominantly observed, with only the 1:1 and 8:2 ratios (where Iodine tincture is the dominant (higher) concentration) of the combination demonstrating antagonism. Malachite green and Betadine® also demonstrated antagonism when tested in a 1:1 ratio against two pathogens – *S. epidermidis* reference strain and *S. epidermidis* clinical strain (Figure 3.5.A and 3.5.B). An antagonistic interaction was observed against the *S. epidermidis* reference strain among the ratios where Malachite green was in higher concentrations and indifferent interactions were then noted for selected ratios (Figure 3.5.A). The combination largely noted antagonistic interactions against the *S. epidermidis* clinical strain with a slight deviation (indifferent interaction) of a few selected ratios (Figure 3.5.B). The combination of Gentian violet with Steriscrub observed predominantly indifferent interactions among the ratios with the 1:1 and 8:2 (Gentian violet being the dominant concentration) ratio demonstrating antagonism (Figure 3.6)

Limited studies are available with respect to varied ratios of medicinal dye interactions with other antimicrobials; however, data is available on antibiotics being tested for various interactions. In one study, varied ratio testing was conducted and isobolograms were plotted to determine how they interact, the combination of ciprofloxacin with bleomycin demonstrated antagonistic interactions against an *E. coli* strain (Adamowicz and Harcombe, 2020). While that study, does not relate to medicinal dyes, it does affirm the importance of varied ratio testing.

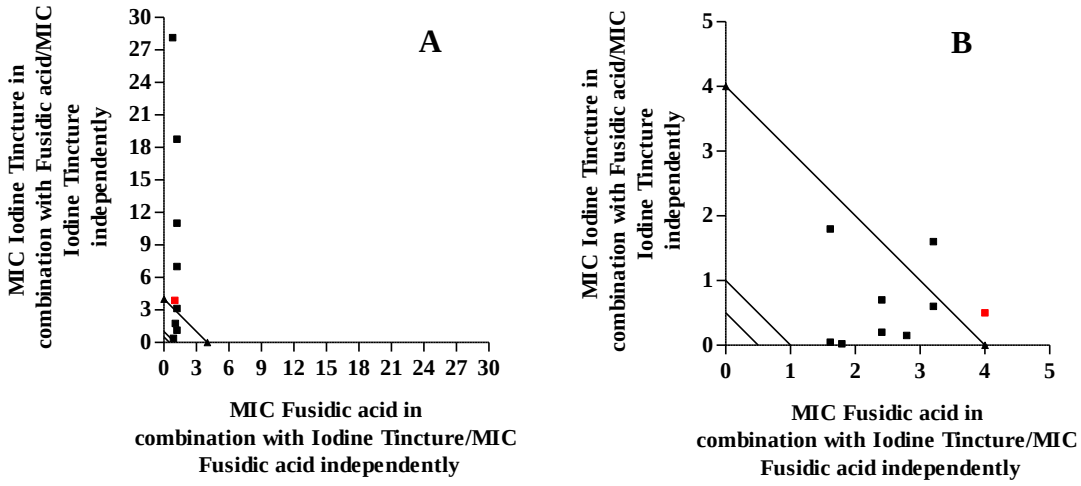


Figure 3.4: Isobologram of combinations of A.) Iodine tincture with Fusidic acid against GMRSA, B.) Iodine tincture with Fusidic acid against *S. epidermidis* clinical strain. Red square = 1:1 combination**.**

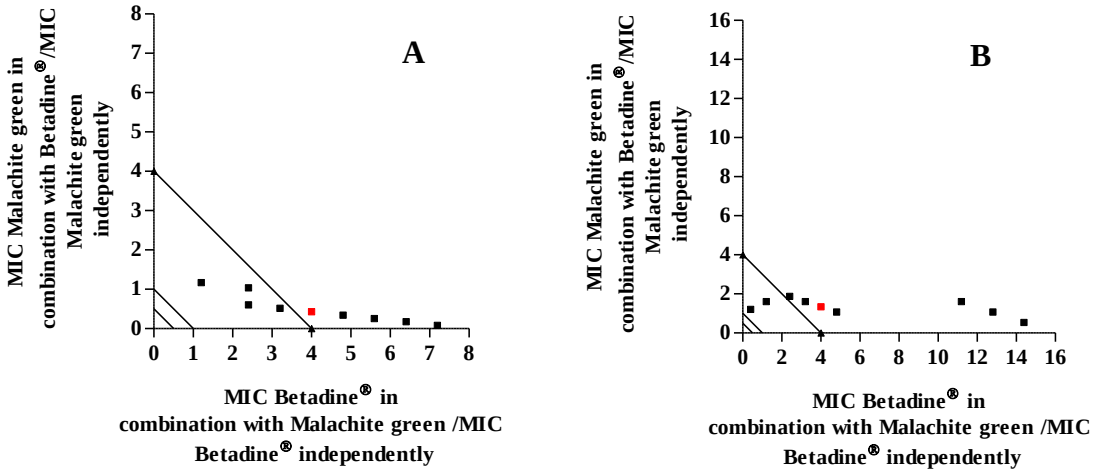


Figure 3.5: Isobologram of combinations of A.). Malachite green with Betadine® against *S. epidermidis* reference strain B.) Malachite green with Betadine® against *S. epidermidis* clinical strain

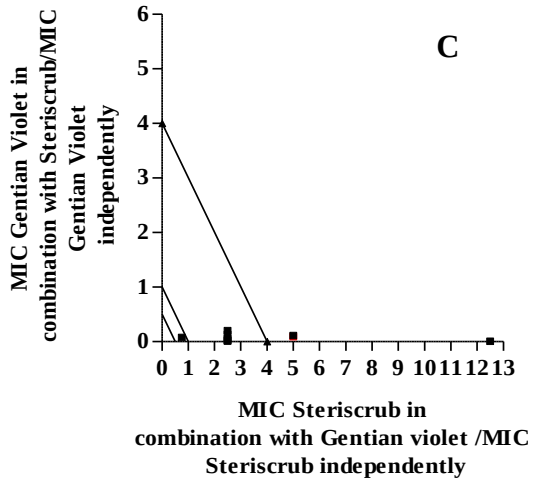


Figure 3.6: Isobologram of combinations of Gentian violet with Steriscrub against GMRSA

3.4.3. Overview of varied ratios

Mercurochrome and Betadine® demonstrated synergy (in the majority of the varied ratios) against the Gram-positive pathogens. This is the only combination to demonstrate a pattern among all the strains of *S. aureus* and *S. epidermidis*. The antagonistic combinations did not demonstrate a clear pattern. As observed with Mercurochrome with Betadine®, antagonism was only observed at the 1:1 ratio while varied ratios (of the synergistic combination) noted synergy when tested against all

strains (reference, clinical and resistant); the varied ratio testing conducted on the antagonistic combinations were mainly clinical and resistant strains. This could be attributed to the traits of a clinical or resistant strain.

3.5. Summary

- Gentian violet demonstrated the strongest antimicrobial activity against *S. epidermidis* (clinical strain) (MBC value of 0.000031 μg/ml) and GMRSA (MBC value of 0.00025 μg/ml).
- Fusidic acid demonstrated the strongest antimicrobial activity of all commercial antimicrobials tested, against *S. epidermidis* reference and clinical strain (MIC value of 0.04 μg/ml).
- Antagonism was observed among all Iodine tincture combinations against the *S. epidermidis* (clinical strain).
- The highest number of synergistic interactions (total of 21 interactions) were noted against the *S. aureus* (reference strain).
- Highest number of antagonistic interactions (total of 17 interactions) was noted against the *S. epidermidis* clinical strain.
- Mercurochrome and Betadine® in combination noted mostly synergy (lowest ΣFIC of 0.05 against GMRSA) when tested against the Gram-positive pathogens.
- Mercurochrome with Betadine® demonstrated synergy among all varied ratios tested against the Gram-positive pathogens.
- In the varied ratio testing, only the 1:1 combination's demonstrated antagonism among the combinations tested while the other ratios mostly demonstrated indifference.
- Mercurochrome with Betadine® demonstrated synergy amongst majority of ratios tested against the Gram-positive pathogens.

CHAPTER 4

Antimicrobial activity: Gram-negative pathogens

4.1. Introduction

The objective of this chapter was to determine the antimicrobial activity of the dyes and the commercial antimicrobials as well as the combinations against *P. aeruginosa* and *E. coli*. Figure 4.1 demonstrates the flow in which the antimicrobial testing was carried out.

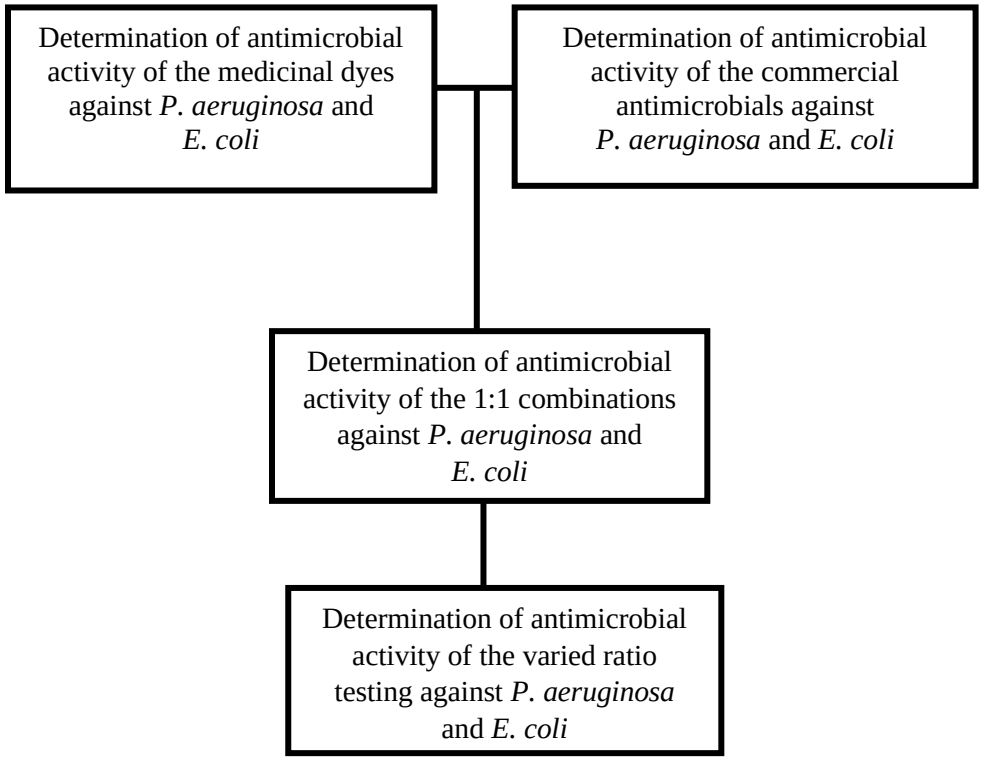


Figure 4.1: The flow of the study

4.2. Results and discussion

4.2.1. Antimicrobial activity of medicinal dyes against Gram-negative pathogens

The medicinal dyes were tested against the *P. aeruginosa* and *E. coli* strains and the MBC values are shown in Table 4.1.

Fuchsine noted relatively higher MBC values against *P. aeruginosa* compared to *E. coli*. Fuchsine demonstrated lower MBC values of 200.00 μg/ml against the clinical strain of *P. aeruginosa* (DSM 46316). When tested against the reference strain (ATCC 27853). Fuchsine demonstrated the same MBC value (8000.00 μg/ml) as the resistant strain. This could be due to the reference strain having gained resistance over time. The *E. coli* strains noted a slightly different pattern compared to *P. aeruginosa*. The reference strain of *E. coli* (ATCC 8379) noted the highest susceptibility while the resistant strain (DSM 22314) noted the lowest susceptibility, which is to be expected.

As with the *S. aureus* and *S. epidermidis* strains, Gentian violet noted the best MBC values against the *P. aeruginosa* and *E. coli* strains. Gentian violet noted MBC values in the range of 0.01-0.52 μg/ml against *P. aeruginosa* and MBC values of 0.01-0.03 μg/ml against the *E. coli* strains. Gentian violet noted better activity against the *E. coli* strains compared to the *P. aeruginosa* strains. For the *P. aeruginosa* strains, the resistant strain noted the poorest susceptibility due to the strain's resistant attributes, whereas the *E. coli* clinical strain (Clinical 66437873) noted the poorest susceptibility. Possibly the clinical strain has acquired some resistance. An old study noted that a concentration of 0.1% Gentian violet was able to inhibit the growth of *P. aeruginosa* (Fung and Miller, 1973). A later study conducted on the antimicrobial effect of Gentian violet demonstrated that *P. aeruginosa* was susceptible at a concentration between 0.01 to 0.02 ug/ml and a concentration of 0.5% was sufficient to be active against a wide range of organisms (Bakker *et al*., 1992). The current study noted concentrations lower than 0.5% were sufficient to inhibit the growth of P. *aeruginosa* and *E. coli* which is a lower concentration that that tested previously.

Interestingly, Iodine tincture demonstrated the same cidal effect among all the strains – reference, clinical and resistant of *P. aeruginosa* and *E. coli*. The MBC value noted for all strains was 0.78 μg/ml. According to McDonnell and Burke (2011), the Gram-negative pathogens such as *P.*

aeruginosa and *E. coli* note low resistance to Iodine tincture which could explain the same MBC value for all Gram-negative strains tested in the current study. Iodine tincture (concentration at 7.5%) demonstrated a killing effect against *E. coli* when tested against the pathogen (Al-Talib *et al*., 2019). While the method used in the study differs, the study does note that *E. coli* is sensitive to Iodine which is affirmed in the current study.

Malachite green was among one of the dyes that noted very low MBC values among the *S. aureus* and *S. epidermidis* strains, however, when tested against the *P. aeruginosa* and *E. coli* strains, it demonstrated much higher and more varied MBC values, demonstrating that the Gram-positive pathogens are more susceptible to the dye than the Gram-negatives pathogens. For the *P. aeruginosa* strains, the clinical strain demonstrated a much lower MBC value of 0.63 μg/ml compared to the reference and resistant strain. This could be attributed to low resistance patterns of the strain. In the *E. coli* strains, the reference strain noted the highest susceptibility (MBC of 1.67 μg/ml) which is expected. Malachite green noted a higher MBC value of 10.00 μg/ml against the clinical and resistant strains of *E. coli*; while the resistant strain of *E. coli* demonstrated more resilience than the reference strain, it still demonstrated better susceptibility to Malachite green compared to the *P. aeruginosa* reference and resistant strains. Tutuk and Gun (2012), noted that Malachite green tested against *P. aeruginosa* demonstrated an 88.9% reduction in the zone of inhibition against the pathogen. It was also noted that Malachite green demonstrated a dose dependent effect against *E. coli*. As the concentration of Malachite green increased, so did the zone of inhibition (Tutuk and Gun, 2012). Dumisa *et al*. (2020), noted MBC values of >8000.00 μg/ml for the *P. aeruginosa* strains tested. These values are higher than the ones in the current study, however, the strains tested in the previous study were different to the strains used in the current study which could have contributed to the difference in MBC values.

Overall, the *E. coli* strains noted higher susceptibility to Mercurochrome compared to the *P. aeruginosa* strains except for the clinical strain of *P. aeruginosa*. The clinical strain of *P. aeruginosa* and *E. coli* noted the same value of 0.08 μg/ml, the lowest MBC value in comparison to the other *P. aeruginosa* and *E. coli* strains. An older study noted the use of mercurochrome topically (1% solution) to treat gonorrhoea. It was used every day during treatment (average of 37 days) and promising results were noted (Statham, 1934). The frequency of re-infection was

reduced and very few complications were noted (Statham, 1934). While it is an old study, it does show that some Gram-negative pathogens are clinically susceptible to Mercurochrome which is affirmed in this *in vitro* study.

Dumisa (2020), noted high MBC values for Mercurochrome ranging from 703.00 to >5000.00 µg/ml. These values are higher than the current study, however, different clinical and resistant strains were also tested. Nakhara and Kozukue (1982), tested *P. aeruginosa* strains which have developed resilience to mercury containing products such as Mercurochrome found in hospitals. While the study was old, it could explain the difference in MBC values among the various strains tested and hence, could possibly explain the decreased susceptibility of the current *P. aeruginosa* strains compared to the *E. coli* strains.

The reference and resistant strain of *P. aeruginosa* noted the same pattern of antimicrobial activity against Methylene blue as with Mercurochrome, albeit much higher MBC values. Both strains noted the poorest susceptibility to Methylene blue with higher MBC values of >8000.00 µg/ml, while the clinical strain of *P. aeruginosa* noted the highest susceptibility among the *P. aeruginosa* strains.

The *E. coli* strains demonstrated better susceptibility to Methylene blue in comparison to the *P. aeruginosa* strains. When tested against the reference strain of *E. coli*, Methylene blue demonstrated the lowest MBC value of 40.00 µg/ml, against the clinical and resistant strain, methylene blue demonstrated higher MBC values compared to the reference strain. A very old study noted susceptibility to Methylene blue by *E. coli* mutant strains at concentrations of 100.00 -200.00 µg/ml (Sugino, 1966). These studies are very old, and resistance may well have developed over the years as seen by the values observed in the current study. All strains of *P. aeruginosa* and *E. coli* demonstrated the same MBC value of >8000.00 µg/ml.

Table 4.1: The minimum bactericidal value (MBC) of the medicinal dyes against Gram-negative pathogens (μg/ml)

Dyes	*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)**
Fuchsine	8000.00	200.00	8000.00	166.67	2000.00	2666.67
Gentian violet	0.01	0.01	0.52	0.01	0.03	0.02
Iodine tincture	0.78	0.78	0.78	0.78	0.78	0.78
Malachite green	400.00	0.63	800.00	1.67	10.00	10.00
Mercurochrome	>5.00	0.08	>5.00	0.31	0.08	2.50
Methylene blue	>8000.00	133.33	>8000.00	40.00	800.00	800.00
Potassium permanganate	>8000.00	>8000.00	>8000.00	>8000.00	>8000.00	>8000.00
Controls						
Culture control	>8000.00	>8000.00	>8000.00	>8000.00	>8000.00	>8000.00
Negative control (water/acetone)	>8000.00	>8000.00	>8000.00	>8000.00	>8000.00	>8000.00
Positive control (Ciprofloxacin)	2.50	1.25	2.50	1.25	1.25	1.25

4.2.2. Overview of activity against the Gram-negative pathogens

As with the Gram-positive pathogens, potassium permanganate demonstrated the weakest activity against the Gram-negative strains. Fuchsine noted higher MBC values overall, among the *P. aeruginosa* and *E. coli* strains demonstrating more resistance than the Gram-positive pathogens. Methylene blue demonstrated better efficacy against the *S. aureus* and *S. epidermidis* strains compared to the Gram-negative strains. Unlike the Gram-positive pathogens, *P. aeruginosa* and *E. coli* demonstrated more resistance to Malachite green and Mercurochrome especially the *P. aeruginosa* reference and resistant strain, which noted the most resistance to these strains. Gentian violet and Iodine tincture demonstrated the strongest efficacy among the *P. aeruginosa* and *E. coli* strains, however, as with the Gram-positive strains, Gentian violet demonstrated the lowest MBC values also noting its strong activity against the Gram-negative pathogens.

4.2.3. Antimicrobial activity of commercial antimicrobials against the Gram-negative pathogens

Fusidic acid was not tested against the Gram-negative bacteria as activity is mainly noted against Gram-positive bacteria with a limited activity against Gram-negative bacteria such as *Empedobacter brevis*, *Moraxella catarrhalis* and *Neisseria meningitidis* with no activity noted against Enterobacteriaceae (Biedenbach *et al*., 2010). Mupirocin was also not tested against the Gram-negative bacteria, as it is only used for the treatment of Staphylococcal skin and soft tissue infections (Rossiter *et al*., 2020). The average MIC/MBC values of the relevant antimicrobials are shown in Table 4.2.

Betadine® noted MIC/MBC values of 2.08-2.60 µg/ml against the *P. aeruginosa* strains, while when tested against the *E. coli* strains, MIC/MBC values of 1.56-2.60 µg/ml were observed. A study testing the resistance of various antimicrobials including Betadine® against various *P. aeruginosa* strains noted an MIC value of 32.00 µg/ml for Betadine® (Al-Azzawi and Abdullah, 2018). The MIC values in the current study noted much higher susceptibility to Betadine® compared to what was found in other studies. The vast difference could be due to different microbial strains being tested. Another study testing the efficacy of Betadine® against various organisms noted an MIC range of 4.00 – 1024.00 µg/ml against *E. coli*. The current study notes MIC values lower than 4.00 µg/ml demonstrating better susceptibility (Gmur and Karpiński, 2020).

Gentamycin noted better MIC/MBC values against the *P. aeruginosa* reference and resistant strain compared to the *S. aureus* strains, however, Gentamycin noted the highest MIC/MBC value of 250.00 µg/ml compared to the *P. aeruginosa* strains. Gentamycin demonstrated a low MIC/MBC value of 10.42 µg/ml against the *E. coli* resistant strain. The resistant strain noted the best susceptibility among the *E. coli* strains while the clinical strain of *E. coli* demonstrated the most resistance with Gentamycin noting an MIC/MBC value of >250.00 µg/ml. Gentamycin inhibits protein synthesis by binding to the 30S ribosomal subunit of bacteria (Abdelghany *et al*., 2012). Drug-resistance associated with Gentamycin is apparent. In previous studies and the current study, *P. aeruginosa* demonstrated susceptibility to Gentamycin regardless of resistance (Rukholm *et al*., 2006; Abdelghany *et al*., 2012). Antimicrobial resistance to *E. coli* has been identified as a global concern and the accumulation of "horizonal gene transfer" has led to the development of multidrug

resistant *E. coli* strains (Szmolka *et al*., 2012). This has been noted when observing the susceptibility patterns of the clinical strain of *E. coli*, demonstrating a high MIC/MBC of more than 250.00 µg/ml.

Neomycin demonstrated the lowest MIC/MBC value of 4.00 µg/ml against the *P. aeruginosa* resistant strain among the Gram-negative strains. The reference strain of *P. aeruginosa* noted the most resilience against Neomycin with an MIC/MBC value of >25.00 µg/ml. Khan *et al*. (2020) observed the MIC values noted for *P. aeruginosa* to be 16.00 µg/ml while the current study noted higher values for the *P. aeruginosa* reference and clinical strain demonstrating resistance. Among the *E. coli* strains, the resistance strain also noted the best susceptibility to Neomycin with an MIC/MBC value of 166.67 µg/ml. A study conducted on carbapenem-resistant Enterobacteriaceae which included strains of *E. coli* noted the MIC range of *E. coli* against Neomycin to be 1.00->256.00 µg/ml (Hu *et al*., 2017). This demonstrates a wide range of MIC values, however, the current study noted values in a similar range. The overused and misuse of aminoglycosides such as Neomycin over the years have led to increased resistance. This can be noted by the high MIC values of the *P. aeruginosa* and *E. coli* strains (Khan *et al*., 2020).

Steriscrub noted MIC/MBC values in the range of 200.00-400.00 µg/ml against the *P. aeruginosa* strains, with Steriscrub demonstrating the highest MIC/MBC value against the clinical strain. Steriscrub noted relatively lower MIC/MBC values against the *E. coli* reference (MIC/MBC of 66.67 µg/ml), and the *E. coli* resistant strain (MIC/MBC of 41.67 µg/ml). The clinical strain of *E. coli* noted the most resistance against Steriscrub, with an MIC/MBC value of 2000.00 µg/ml. An MIC value of 64.00-265.00 µg/ml for Chlorohexidine (Steriscrub) was noted in a study testing the activity of various antimicrobials against *P. aeruginosa* (Al-Azzawi and Abdullah, 2018).

4.2.4. Overview of activity against the Gram-negative strains

Among the Gram-positive and Gram-negative strains, *E. coli* noted the poorest susceptibility to Neomycin while *S. epidermidis* demonstrated high susceptibility among all strains. Compared to the other commercial antimicrobials tested against the various strains, Betadine® consistently demonstrated lower MIC/MBC's value against the individual reference, clinical and resistant strains of *P. aeruginosa* and *E. coli* strains. The Gram-negative pathogens demonstrated better susceptibility to Betadine® in comparison to the Gram-positive pathogens.

Table 4.2: The minimum inhibitory and minimum bactericidal concentrations (MIC/MBC) of commercial antimicrobials against the Gram-negative strains (μg/ml)

Antimicrobials	*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)	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Betadine® (Povidone-Iodine)	2.08	2.08	2.60	2.60	2.11	2.11	1.56	1.56	2.60	2.60	1.56	1.56
Gentamycin	2.50	2.50	250.00	250.00	2.50	2.50	25.00	25.00	>250.00	>250.00	10.42	10.42
Neomycin	>25.00	>25.00	25.00	25.00	4.00	4.00	250.00	250.00	250.00	250.00	166.67	166.67
Steriscrub (Chlorohexidine)	200.00	200.00	400.00	400.00	200.00	200.00	66.67	66.67	2000.00	2000.00	41.67	41.67
Controls												
Culture control	>8000.00	>8000.00	>8000.00	>8000.00	>8000.00	>8000.00	>8000.00	>8000.00	>8000.00	>8000.00	>8000.00	>8000.00
Negative control (water/acetone)	>8000.00	>8000.00	>8000.00	>8000.00	>8000.00	>8000.00	>8000.00	>8000.00	>8000.00	>8000.00	>8000.00	>8000.00
Positive control (Ciprofloxacin)	2.50	1.25	2.50	1.25	1.25	1.25	1.25	2.50	1.25	1.25	2.50	1.25

4.3. Antimicrobial activity of 1:1 combinations

As with the Gram-positive pathogens, the combinations were tested against the Gram-negative strains. MIC and ΣFIC values were calculated and discussed under the appropriate sub-sections. The controls responded as expected. The negative control (water/acetone) was used to ensure the sample and not the solvent demonstrated antimicrobial activity, and as expected, the negative control demonstrated microbial growth. Ciprofloxacin was used as the positive control to ensure the pathogens were susceptible. The culture control was used to ensure the TSB supports the growth of the pathogen, as expected, there was microbial growth. The ΣFIC value of medicinal dyes in combination with various commercial antimicrobials tested against the *E. coli* and *P. aeruginosa* strains could not be determined as no endpoint MBC value was observed and hence are depicted as "U" within Tables 4.3 to 4.16. Thus, a tentative interpretation of synergy, additive, indifferent or antagonism was observed in these cases based on the comparison to individual MBC values of the individual medicinal dye and commercial antimicrobials respectively.

4.3.1. Combinations with Fuchsine

Fuchsine combinations demonstrated much lower MBC values compared to the individual values. A total of 83.33% of the combinations demonstrated lower, enhanced MBC values (Table 4.3). While the combinations noted reduced values against the *P. aeruginosa* reference strain (except Steriscrub), no synergy was noted among the combinations. Fuchsine and Gentamycin notably demonstrated an enhanced MBC value of 0.12 µg/ml against the *P. aeruginosa* clinical strain, the lowest among all the Gram-negative strains. The most synergistic interaction observed was with the combinations of Fuchsine with Gentamycin with an ΣFIC value of 0.001 against the *P. aeruginosa* clinical strain. The highest number of synergistic interactions were observed against the *P. aeruginosa* clinical strain compared to the other Gram-negative pathogens (Table 4.3). Two tentative antagonistic interactions were noted with the combination of Fuchsine and Steriscrub against the *P. aeruginosa* reference and resistant strains.

Table 4.3: The mean MBC values (μg/ml) and ΣFIC values of Fuchsine and commercial antimicrobials in combination against *P. aeruginosa* strains

Combinations	*Pseudomonas aeruginosa*								
	Reference strain (ATCC 27853)			**Clinical strain (DSM 46316)**			**Resistant strain (ATCC 46316)**		
	Mean MBC	**ΣFIC**	**Int.**	**Mean MBC**	**ΣFIC**	**Int.**	**Mean MBC**	**ΣFIC**	**Int.**
Fuchsine + Betadine®	**1003.13**	1.63	Ind.	**125.39**	0.78	Add.	**2062.50**	3.21	Ind.
Fuchsine + Gentamycin	**4001.25**	1.00	Add.	**0.12**	**0.001**	**Syn.**	**2000.63**	**0.50**	**Syn.**
Fuchsine + Neomycin	**4001.25**	U	Add.	**125.04**	**0.002**	**Syn.**	**4001.25**	0.56	Add.
Fuchsine + Steriscrub	U	U	Ant.	**91.67**	**0.44**	**Syn.**	U	U	Ant.

Mean MBC: The mean MBC for the combination, Int: Interaction, Syn: Synergistic interaction, Add: Additive interaction, Ant: Antagonistic interaction, Ind: Indifferent interactions, Bold MBC: Reduced MBC values in comparison to independent values; Bold ΣFIC and Int: Synergy, U: The mean MBC values were not absolute values (either >8000.00 μg/ml or less than 0.01 μg/ml) and hence tentative FIC values were established

The *E. coli* strains demonstrated relatively higher MBC values in comparison to the *P. aeruginosa* strains (Table 4.4). No synergy was observed among the reference strain of *E. coli* with mainly additive interactions noted. A tentative antagonistic interaction (Fuchsine and Gentamycin) was noted against the *E. coli* clinical strain based on the comparison of the individual MBC values and the combination of Fuchsine with Gentamycin.

Among the *E. coli* strains, Fuchsine with Steriscrub demonstrated an ΣFIC value of 0.10 against the *E. coli* clinical strain - the most synergistic among the *E. coli* strains (Table 4.4). Fuchsine in combination with Steriscrub noted the lowest MBC value of 50.00 μg/ml against the *E. coli* resistant strain. Literature is lacking regarding the use of Fuchsine in combination for skin infections, therefore these studies demonstrate some novel findings where Fuchsine in combination may be beneficial when dealing with clinical strains.

Table 4.4: The mean MBC values (µg/ml) and ΣFIC values of Fuchsine and commercial antimicrobials in combination against *E. coli* strains

Combinations	*Escherichia coli*								
	Reference strain (ATCC 8379)			**Clinical strain (Clinical 66437873)**			**Resistant strain (DSM 22314)**		
	Mean MBC	**ΣFIC**	**Int.**	**Mean MBC**	**ΣFIC**	**Int.**	**Mean MBC**	**ΣFIC**	**Int.**
Fuchsine + Betadine®	**125.39**	1.00	Add.	**406.25**	2.70	Ind.	**206.25**	4.08	Ant.
Fuchsine + Gentamycin	**125.04**	0.75	Add.	**4001.25**	U	Ant.	**2000.63**	0.81	Add.
Fuchsine + Neomycin	**125.04**	0.75	Add.	4001.25	2.01	Ind.	**2000.63**	0.75	Add.
Fuchsine + Steriscrub	275.00	1.87	Add.	**200.00**	**0.10**	**Syn.**	**50.00**	**0.42**	**Syn.**

Mean MBC: The mean MBC for the combination, Int: Interaction, Syn: Synergistic interaction, Add: Additive interaction, Ant: Antagonistic interaction, Ind: Indifferent interactions, Bold MBC: Reduced MBC values in comparison to independent values; Bold ΣFIC and Int: Synergy, U: The mean MBC values were not absolute values (either >8000.00 µg/ml or less than 0.01 µg/ml) and hence tentative FIC values were established

4.3.2. Combinations with Gentian violet

Twenty out of the 22 combinations with Gentian violet noted lower (enhanced) MBC values when tested against the Gram-negative pathogens (Tables 4.5 and 4.6). Among the *P. aeruginosa* strains, Gentian violet in combination with Gentamycin demonstrated the lowest MBC value of 0.16 µg/ml against the *P. aeruginosa* reference strain (Table 4.5). While this combination noted the lowest MBC value, an additive interaction was observed. The most antagonistic combination with a ΣFIC of 5.99 was with the combination of Gentian violet with Betadine® against the *P. aeruginosa* reference strain. No synergy was noted among the *P. aeruginosa* clinical strain. Gentian violet with Betadine® demonstrated the most synergistic interaction (ΣFIC was 0.30) against the *P. aeruginosa* resistant strain. Among all the *P. aeruginosa* strains, synergy was only noted against the *P. aeruginosa* resistant strain.

Dumisa (2020), did not observe any synergistic interaction with combinations of Gentian violet when combined with plant extracts and tested against *P. aeruginosa*, however, when tested in combination with commercial antimicrobials, as in this study, Gentian violet demonstrates better potential for synergistic interactions.

Table 4.5: The mean MBC values (μg/ml) and ΣFIC values of Gentian violet and commercial antimicrobials in combination against *P. aeruginosa* strains

Combinations	*Pseudomonas aeruginosa*								
	Reference strain (ATCC 27853)			**Clinical strain (DSM 46316)**			**Resistant strain (ATCC 46316)**		
	Mean MBC	**ΣFIC**	**Int.**	**Mean MBC**	**ΣFIC**	**Int.**	**Mean MBC**	**ΣFIC**	**Int.**
Gentian violet + Betadine®	**1.09**	5.99	Ant.	3.14	3.21	Ind.	**0.55**	**0.30**	**Syn.**
Gentian violet + Gentamycin	**0.16**	0.79	Add.	**0.19**	4.01	Ant.	**0.31**	**0.36**	**Syn.**
Gentian violet + Neomycin	**0.66**	U	Ind.	**0.19**	4.02	Ant.	**0.94**	0.76	Add.
Gentian violet + Steriscrub	200.02	2.56	Ind.	100.02	2.25	Ind.	**41.73**	**0.33**	**Syn.**

Mean MBC: The mean MBC for the combination, Int: Interaction, Syn: Synergistic interaction, Add: Additive interaction, Ant: Antagonistic interaction, Ind: Indifferent interactions, Bold MBC: Reduced MBC values in comparison to independent values; Bold ΣFIC and Int: Synergy, U: The mean MBC values were not absolute values (either >8000.00 μg/ml or less than 0.01 μg/ml) and hence tentative FIC values were established

All combinations demonstrated lower MBC values in comparison to the individual values when tested against the *E. coli* reference strain, however, no synergy was noted among these combinations. Gentian violet in combination with Gentamycin demonstrated the lowest MBC value of 0.08 μg/ml against the *E. coli* clinical strain, however, the ΣFIC value could not be determined and a tentative interaction (additive was determined based on the individual values. The combination of Gentian violet and Neomycin also noted a low MBC value of 0.08 μg/ml against the *E. coli* clinical strain. The combination of Gentian violet and Gentamycin demonstrated the second lowest MBC value of 0.05 μg/ml against the *E. coli* resistant strain (Table 4.6). While it demonstrated a synergetic interaction when tested against the *E. coli* resistant strain, it did not demonstrate synergy against the *P. aeruginosa* reference strain. Gentian violet in combination with Neomycin demonstrated the lowest MBC value of 0.04 μg/ml against the *E. coli* resistant strain (Table 4.6).

Indifferent, additive, and synergistic interactions were noted among the *E. coli* strains; however, no antagonism was noted. Gentian violet in combination with both Gentamycin and Neomycin demonstrated synergy with a ΣFIC 0.50 and 0.42 respectively against the *E. coli* resistant strain (Table 4.6).

Orabase with Gentian violet was used to treat oral precancerous lesions. The combination yielded positive results when tested in the *in vivo* experiments on hamsters (Wang *et al*., 2020). This study while not related to antimicrobial activity demonstrates that Gentian violet combinations used in for other pharmacological conditions is effective.

Table 4.6: The mean MBC values (µg/ml) and ΣFIC values of Gentian violet and commercial antimicrobials in combination against *E. coli* strains

Combinations	*Escherichia coli*								
	Reference strain (ATCC 8379)			**Clinical strain (Clinical 66437873)**			**Resistant strain (DSM 22314)**		
	Mean MBC	**ΣFIC**	**Int.**	**Mean MBC**	**ΣFIC**	**Int.**	**Mean MBC**	**ΣFIC**	**Int.**
Gentian violet + Betadine®	**0.78**	1.00	Add.	**0.78**	**0.44**	**Syn.**	**0.41**	1.25	Ind.
Gentian violet + Gentamycin	**0.16**	1.01	Ind.	**0.08**	U	Add.	**0.05**	**0.50**	**Syn.**
Gentian violet + Neomycin	**0.16**	1.00	Add.	**0.08**	1.25	Ind.	**0.04**	**0.42**	**Syn.**
Gentian violet + Steriscrub	**50.01**	1.75	Ind.	**200.03**	1.10	Ind.	**12.52**	1.30	Ind.

Mean MBC: The mean MBC for the combination, Int: Interaction, Syn: Synergistic interaction, Add: Additive interaction, Ant: Antagonistic interaction, Ind: Indifferent interactions, Bold MBC: Reduced MBC values in comparison to independent values; Bold ΣFIC and Int: Synergy, U: The mean MBC values were not absolute values (either >8000.00 µg/ml or less than 0.01 µg/ml) and hence tentative FIC values were established

4.3.3. Combinations with Iodine tincture

Only 12.5% of the Iodine tincture combinations demonstrated synergy among the Gram-negative pathogens tested (Table 4.7 and 4.8). Synergy was observed with Iodine tincture combinations noting higher MBC values. Iodine tincture with Steriscrub was the only combination to demonstrated synergy against the *P. aeruginosa* reference strain (MBC value of 25.20 µg/ml and ΣFIC value of 0.37), *P. aeruginosa* resistant strain (MBC value of 16.80 µg/ml and ΣFIC value of 0.25) (Table 4.7). Indifferent and additive interactions were observed with the rest of the Iodine tincture combinations tested. The ΣFIC value of Iodine tincture and Neomycin could not be determined hence, a tentative interaction of indifference was determined.

Table 4.7: The mean MBC values (μg/ml) and ΣFIC values of Iodine tincture and commercial antimicrobials in combination against *P. aeruginosa* strains

Combinations	*Pseudomonas aeruginosa*								
	Reference strain (ATCC 27853)			**Clinical strain (DSM 46316)**			**Resistant strain (ATCC 46316)**		
	Mean MBC	**ΣFIC**	**Int.**	**Mean MBC**	**ΣFIC**	**Int.**	**Mean MBC**	**ΣFIC**	**Int.**
Iodine tincture + Betadine®	**1.63**	1.04	Ind.	**0.98**	0.55	Add.	**1.63**	1.03	Ind.
Iodine tincture + Gentamycin	**1.09**	1.12	Ind.	**1.09**	1.00	Add.	**1.09**	1.12	Ind.
Iodine tincture + Neomycin	**1.09**	U	Ind.	**0.55**	0.51	Add.	**1.09**	1.08	Ind.
Iodine tincture + Steriscrub	**25.20**	**0.37**	**Syn.**	**50.39**	0.63	Add.	**16.80**	**0.25**	**Syn.**

Mean MBC: The mean MBC for the combination, Int: Interaction, Syn: Synergistic interaction, Add: Additive interaction, Ant: Antagonistic interaction, Ind: Indifferent interactions, Bold MBC: Reduced MBC values in comparison to independent values; Bold ΣFIC and Int: Synergy, U: The mean MBC values were not absolute values (either >8000.00 μg/ml or less than 0.01 μg/ml) and hence tentative FIC values were established

Iodine tincture with Steriscrub was the only combination to demonstrate synergy against the *E. coli* clinical strain (MBC value of 21.00 μg/ml and ΣFIC value of 0.22) (Table 4.8). As with the *P. aeruginosa* strains, indifferent and additive interactions were observed with the rest of the Iodine tincture combinations tested. No antagonistic interactions were noted. Iodine tincture in combination with Gentamycin against *E. coli* clinical strain had an ΣFIC values that could not be determined hence, a tentative interaction ("U") of additive was determined.

Au-Duong and Lee (2017) tested the Zeolitic imidazolate framework-8 against *E. coli* to determine the antimicrobial activity; at a pH of 6.00, the strain of *E. coli* was completely eradicated in three minutes. This demonstrates the susceptibility of the Gram-negative pathogens to Iodine tincture within the framework.

Table 4.8: The mean MBC values (µg/ml) and ΣFIC values of Iodine tincture and commercial antimicrobials in combination against *E. coli* strains

Combinations	*Escherichia coli*								
	Reference strain (ATCC 8379)			**Clinical strain (Clinical 66437873)**			**Resistant strain (DSM 22314)**		
	Mean MBC	**ΣFIC**	**Int.**	**Mean MBC**	**ΣFIC**	**Int.**	**Mean MBC**	**ΣFIC**	**Int.**
Iodine tincture + Betadine®	**0.98**	0.75	Add.	**1.30**	0.75	Add.	**1.30**	1.00	Add.
Iodine tincture + Gentamycin	**1.09**	1.01	Ind.	**1.09**	U	Add.	**1.09**	1.03	Ind.
Iodine tincture + Neomycin	**1.09**	1.00	Add.	**1.09**	1.00	Add.	**2.19**	2.00	Ind.
Iodine tincture + Steriscrub	**41.99**	1.04	Ind.	**21.00**	**0.22**	**Syn.**	**21.00**	0.71	Add.

Mean MBC: The mean MBC for the combination, Int: Interaction, Syn: Synergistic interaction, Add: Additive interaction, Ant: Antagonistic interaction, Ind: Indifferent interactions, Bold MBC: Reduced MBC values in comparison to independent values; Bold ΣFIC and Int: Synergy, U: The mean MBC values were not absolute values (either >8000.00 µg/ml or less than 0.01 µg/ml) and hence tentative FIC values were established

4.3.4. Combinations with Malachite green

Malachite green in combination with Gentamycin noted a greatly reduced MBC value against the *P. aeruginosa* reference strain when compared to when tested independently (Table 4.9). A synergistic interaction (ΣFIC value of 0.15) was observed with Malachite green and Gentamycin against the *P. aeruginosa* reference strain. While this is the lowest ΣFIC value, the combination did not demonstrate the lowest MBC value. An MBC value of 0.64 µg/ml was observed for the combination of Malachite green and Neomycin against the *P. aeruginosa* clinical strain, the lowest MBC value among the *P. aeruginosa* strains (Table 4.9). The most antagonistic interactions were observed against the *P. aeruginosa* strains compared to the *E. coli* strains, with the most antagonistic ΣFIC value of 5.60 with the combination Malachite green and Betadine® against the *P. aeruginosa* clinical strain. No synergy was noted against the *P. aeruginosa* resistant strain.

Table 4.9: The mean MBC values (µg/ml) and ΣFIC values of Malachite green and commercial antimicrobials in combination against *P. aeruginosa* strains

Combinations	*Pseudomonas aeruginosa*								
	Reference strain (ATCC 27853)			**Clinical strain (DSM 46316)**			**Resistant strain (ATCC 46316)**		
	Mean MBC	**ΣFIC**	**Int.**	**Mean MBC**	**ΣFIC**	**Int.**	**Mean MBC**	**ΣFIC**	**Int.**
Malachite green + Betadine®	**26.25**	3.05	Ind.	8.25	5.60	Ant.	**206.25**	3.21	Ind.
Malachite green + Gentamycin	**10.31**	**0.15**	**Syn.**	**1.29**	2.00	Ind.	**500.16**	0.69	Add.
Malachite green + Neomycin	**10.31**	U	Add.	**0.64**	1.00	Add.	4001.25	5.31	Ant.
Malachite green + Steriscrub	U	U	Add.	**101**	1.85	Ind.	**666.67**	2.08	Ind.

Mean MBC: The mean MBC for the combination, Int: Interaction, Syn: Synergistic interaction, Add: Additive interaction, Ant: Antagonistic interaction, Ind: Indifferent interactions, Bold MBC: Reduced MBC values in comparison to independent values; Bold ΣFIC and Int: Synergy, U: The mean MBC values were not absolute values (either >8000.00 µg/ml or less than 0.01 µg/ml) and hence tentative FIC values were established

All Malachite green combinations demonstrated low MBC values against the *E. coli* reference strain in comparison to the independent values (Table 4.10). The lowest MBC value of 0.64 µg/ml was also observed for Malachite green in combination with both Gentamycin and Neomycin against the *E. coli* reference strain.

The Malachite green combinations demonstrated the highest number of synergistic interactions among the *E. coli* strains (Table 4.10). The most synergistic interactions (ΣFIC value of 0.38) were noted for Malachite green with both Gentamycin and Neomycin against the *E. coli* reference strain, both combinations had noted the lowest MBC values as well (Table 4.10). One other synergistic combination was noted against the *E. coli* resistant strain with the combination of Malachite green and Neomycin (ΣFIC value of 0.50).

Dumisa (2020), observed greatly reduced MBC values among combinations with Malachite green, which can be observed in the current study as well. This demonstrates Malachite green's antimicrobial activity does not diminish when combined with various other antimicrobials.

Table 4.10: The mean MBC values (µg/ml) and ΣFIC values of Malachite green and commercial antimicrobials in combination against *E. coli* strains

Combinations	*Escherichia coli*								
	Reference strain (ATCC 8379)			**Clinical strain (Clinical 66437873)**			**Resistant strain (DSM 22314)**		
	Mean MBC	**ΣFIC**	**Int.**	**Mean MBC**	**ΣFIC**	**Int.**	**Mean MBC**	**ΣFIC**	**Int.**
Malachite green + Betadine®	**1.64**	1.00	Add.	13.13	2.25	Ind.	**10.94**	4.27	Ant.
Malachite green + Gentamycin	**0.64**	**0.38**	**Syn.**	**10.31**	U	Add.	**5.16**	0.52	Add.
Malachite green + Neomycin	**0.64**	**0.38**	**Syn.**	**10.31**	1.00	Add.	**5.16**	**0.50**	**Syn.**
Malachite green + Steriscrub	**11.46**	0.78	Add.	**220.00**	2.10	Ind.	110.00	3.40	Ind.

Mean MBC: The mean MBC for the combination, Int: Interaction, Syn: Synergistic interaction, Add: Additive interaction, Ant: Antagonistic interaction, Ind: Indifferent interactions, Bold MBC: Reduced MBC values in comparison to independent values; Bold ΣFIC and Int: Synergy, U: The mean MBC values were not absolute values (either >8000.00 µg/ml or less than 0.01 µg/ml) and hence tentative FIC values were established

4.3.5. Combinations with Mercurochrome

Additive and indifferent interactions of the Mercurochrome combinations were observed against the *P. aeruginosa* strains (Table 4.11). In the *P. aeruginosa* strains, 41.66% of the combinations had MBC values of >8000.00 µg/ml, hence the ΣFIC value of these combinations could not be determined as no endpoint MBC values were observed and tentative values were determined based on the comparison of the individual MBC values. Among the *P. aeruginosa* strains, Mercurochrome in combination with both Gentamycin and Neomycin demonstrated the lowest MBC value of 0.23 µg/ml when tested against the *P. aeruginosa* clinical strain (Table 4.11). No antagonism was observed among the *P. aeruginosa* strains.

Table 4.11: The mean MBC values (μg/ml) and ΣFIC values of Mercurochrome and commercial antimicrobials in combination against *P. aeruginosa* strains

Combinations	*Pseudomonas aeruginosa*								
	Reference strain (ATCC 27853)			**Clinical strain (DSM 46316)**			**Resistant strain (ATCC 46316)**		
	Mean MBC	**ΣFIC**	**Int.**	**Mean MBC**	**ΣFIC**	**Int.**	**Mean MBC**	**ΣFIC**	**Int.**
Mercurochrome + Betadine®	**10.00**	U	Add.	**0.47**	1.13	Ind.	**7.50**	U	Add.
Mercurochrome + Gentamycin	U	U	Add.	**0.23**	1.95	Ind.	U	U	Add.
Mercurochrome + Neomycin	U	U	Add.	**0.23**	1.95	Ind.	U	U	Add.
Mercurochrome + Steriscrub	**402.50**	U	Add.	**25.16**	2.01	Ind.	U	U	Add.

Mean MBC: The mean MBC for the combination, Int: Interaction, Syn: Synergistic interaction, Add: Additive interaction, Ant: Antagonistic interaction, Ind: Indifferent interactions, Bold MBC: Reduced MBC values in comparison to independent values; Bold ΣFIC and Int: Synergy, U: The mean MBC values were not absolute values (either >8000.00 μg/ml or less than 0.01 μg/ml) and hence tentative FIC values were established

When tested against the *E. coli* strains, the dyes in combination with commercial antimicrobials did not demonstrate any better efficacy. All Mercurochrome combinations noted reduced MBC values in comparison to the individual values against the *E. coli* reference strain. However, only Mercurochrome in combination with Betadine® demonstrated synergy with an (ΣFIC value of 0.38 against the reference strain. The lowest MBC value of 0.06 μg/ml (Mercurochrome with Gentamycin) was observed against the *E. coli* clinical strain (Table 4.12). While both MBC values against the Gram-negative pathogens were low, they did not demonstrate a synergistic interaction when combined. The most synergistic interaction among the *E. coli* strains was observed against the resistant strain which was Mercurochrome in combination with Betadine® with an ΣFIC value of 0.28. One antagonistic combination was observed with the combination of Mercurochrome and Steriscrub against the *E. coli* resistant strain with a high ΣFIC value 10.60 (Table 4.12).

In a very old study, Mercurochrome combined with milk was used intravenously to treat gonorrheal urethritis in animals. While this very early study appears very controversial in current treatment practices as milk is not used in treatment of infections, the addition of milk to Mercurochrome was believed to activate the bacteriostatic activity of Mercurochrome (Potter *et*

al., 1926). While this study does not have quantitative data, nor does the study focus on human subjects, it does demonstrate the possibility of a clinical synergistic effect.

Table 4.12: The mean MBC values (μg/ml) and ΣFIC values of Mercurochrome and commercial antimicrobials in combination against *E. coli* strains

Combinations	*Escherichia coli*								
	Reference strain (ATCC 8379)			**Clinical strain (Clinical 66437873)**			**Resistant strain (DSM 22314)**		
	Mean MBC	**ΣFIC**	**Int.**	**Mean MBC**	**ΣFIC**	**Int.**	**Mean MBC**	**ΣFIC**	**Int.**
Mercurochrome + Betadine®	**0.38**	**0.38**	**Syn.**	**0.23**	0.58	Add.	**0.47**	**0.28**	**Syn.**
Mercurochrome + Gentamycin	**0.27**	0.63	Add.	**0.06**	U	Add.	**3.75**	1.12	Ind.
Mercurochrome + Neomycin	**0.27**	0.63	Add.	**0.23**	2.00	Ind.	**3.75**	1.01	Ind.
Mercurochrome + Steriscrub	**25.16**	0.87	Add.	**12.58**	1.01	Ind.	167.71	10.60	Ant.

Mean MBC: The mean MBC for the combination, Int: Interaction, Syn: Synergistic interaction, Add: Additive interaction, Ant: Antagonistic interaction, Ind: Indifferent interactions, Bold MBC: Reduced MBC values in comparison to independent values; Bold ΣFIC and Int: Synergy, U: The mean MBC values were not absolute values (either >8000.00 μg/ml or less than 0.01 μg/ml) and hence tentative FIC values were established

4.3.6. Combinations with Methylene blue

Methylene blue with Betadine® demonstrated reduced MBC values (better activity) when tested in combination among all *P. aeruginosa* and *E. coli* strains. Methylene blue combinations demonstrated relatively higher MBC values against the *P. aeruginosa* strains in comparison to the *E. coli* strains (Table 4.13 and 4.14). When tested against the *P. aeruginosa* reference strain, most Methylene blue combinations had ΣFIC values which could not be determined, and tentative additive interactions were found based on the comparison to the individual values (Table 4.13). As with Mercurochrome, Methylene blue in combination with both Gentamycin and Neomycin noted the lowest MBC value (MBC value of 20.63 μg/ml) against the *P. aeruginosa* clinical strain (Table 4.13).

The most synergistic combination noted among the *P. aeruginosa* strains was Methylene blue in combination with Gentamycin (ΣFIC value of 0.15) against the clinical strain. Only one

antagonistic combination was noted – Methylene blue with Gentamycin noted an ΣFIC value of 4.09 against the *P. aeruginosa* resistant strain.

A Photodynamic therapy (PDT) study of Methylene blue (5.00 μg/ml) in combination with Gentamycin (1.00 μg/ml) was tested against *P. aeruginosa* to determine the antimicrobial activity. The combination demonstrated a 6 $\log^{10}$ reduction in CFU (Pérez-Laguna *et al*., 2020). In the current study the combination (Methylene blue and Gentamycin) demonstrated reduced MBC values against *P. aeruginosa*.

Table 4.13: The mean MBC values (μg/ml) and ΣFIC values of Methylene blue and commercial antimicrobials in combination against *P. aeruginosa* strains

Combinations	***Pseudomonas aeruginosa***								
	Reference strain (ATCC 27853)			**Clinical strain (DSM 46316)**			**Resistant strain (ATCC 46316)**		
	Mean MBC	**ΣFIC**	**Int.**	**Mean MBC**	**ΣFIC**	**Int.**	**Mean MBC**	**ΣFIC**	**Int.**
Methylene blue + Betadine®	**26.25**	U	Add.	**51.56**	0.98	Add.	**206.25**	U	Add.
Methylene blue + Gentamycin	**200.63**	U	Add.	**20.63**	**0.15**	**Syn.**	**2000.63**	4.09	Ant.
Methylene blue + Neomycin	U	U	Add.	**20.63**	**0.18**	**Syn.**	U	U	Ind.
Methylene blue + Steriscrub	U	U	Add.	**200.00**	1.00	Add.	U	U	Add.

Mean MBC: The mean MBC for the combination, Int: Interaction, Syn: Synergistic interaction, Add: Additive interaction, Ant: Antagonistic interaction, Ind: Indifferent interactions, Bold MBC: Reduced MBC values in comparison to independent values; Bold ΣFIC and Int: Synergy, U: The mean MBC values were not absolute values (either >8000.00 μg/ml or less than 0.01 μg/ml) and hence tentative FIC values were established

Synergistic interactions of the Methylene blue combinations were only observed with the Gram-negative clinical strains. Indifferent and additive interactions were mostly observed (70.83%) against the *E. coli* strains. Methylene blue combined with Neomycin demonstrated, the most synergistic interaction among the Gram-negative strains (and the *E. coli* strains) with a ΣFIC value of 0.06 against the *E. coli* clinical strain (Table 4.14). Only one antagonistic interaction was observed with Methylene blue combined with Betadine® against the *E. coli* resistant strain (ΣFIC value of 4.03).

Table 4.14: The mean MBC values (μg/ml) and ΣFIC values of Methylene blue and commercial antimicrobials in combination against *E. coli* strains

Combinations	*Escherichia coli*								
	Reference strain (ATCC 8379)			**Clinical strain (Clinical 66437873)**			**Resistant strain (DSM 22314)**		
	Mean MBC	**ΣFIC**	**Int.**	**Mean MBC**	**ΣFIC**	**Int.**	**Mean MBC**	**ΣFIC**	**Int.**
Methylene blue + Betadine®	**13.13**	2.25	Ind.	**26.25**	2.53	Ind.	**26.25**	4.03	Ant.
Methylene blue + Gentamycin	100.31	2.51	Ind.	**41.25**	**U**	**Syn.**	U	U	Add.
Methylene blue + Neomycin	**100.31**	2.50	Ind.	**41.25**	**0.06**	**Syn.**	U	U	Ind.
Methylene blue + Steriscrub	220.00	3.50	Ind.	**183.33**	**0.10**	**Syn.**	**55.00**	1.21	Ind.

Mean MBC: The mean MBC for the combination, Int: Interaction, Syn: Synergistic interaction, Add: Additive interaction, Ant: Antagonistic interaction, Ind: Indifferent interactions, Bold MBC: Reduced MBC values in comparison to independent values; Bold ΣFIC and Int: Synergy, U: The mean MBC values were not absolute values (either >8000.00 μg/ml or less than 0.01 μg/ml) and hence tentative FIC values were established

4.3.7. Combinations with Potassium permanganate

The MBC values of Potassium permanganate was >8000.00 μg/ml for all strains. All MBC values that were determined for the combinations demonstrated much lower MBC values, hence better activity. Combinations with Potassium permanganate did not demonstrate any synergistic interactions against the Gram-negative pathogens (Table 4.15 and 4.16). The mean MBC value of were not absolute values and were above >8000.00 μg/ml therefore the ΣFIC values could not be calculated hence, tentative interactions of additive, indifferent and antagonistic were observed based on the comparison of individual values. There are limited studies on Potassium permanganate used in combination, however, individually, it is known to have a strong microcidal effect on bacteria and fungi.

A 5.00% solution of Potassium permanganate in combination was able to hasten the healing process of diabetic foot ulcers (Delgado-Enciso *et al.*, 2018). While the exact combination is not known, this does show the potential of Potassium permanganate in combination for the treatment of skin infections.

Table 4.15: The mean MBC values (µg/ml) and ΣFIC values of Potassium permanganate and commercial antimicrobials in combination against *P. aeruginosa* strains

Combinations	*Pseudomonas aeruginosa*								
	Reference strain (ATCC 27853)			**Clinical strain (DSM 46316)**			**Resistant strain (ATCC 46316)**		
	Mean MBC	**ΣFIC**	**Int.**	**Mean MBC**	**ΣFIC**	**Int.**	**Mean MBC**	**ΣFIC**	**Int.**
Potassium permanganate + Betadine®	**501.56**	U	Add.	**167.19**	U	Add.	**417.97**	U	Add.
Potassium permanganate + Gentamycin	U	U	Ant.	U	U	Ind.	U	U	Ind.
Potassium permanganate + Neomycin	U	U	Ant.	U	U	Ind.	U	U	Ind.
Potassium Permanganate + Steriscrub	**1966.67**	U	Add.	**2200.00**	U	Add.	**1833.33**	U	Add.

Mean MBC: The mean MBC for the combination, Int: Interaction, Syn: Synergistic interaction, Add: Additive interaction, Ant: Antagonistic interaction, Ind: Indifferent interactions, Bold MBC: Reduced MBC values in comparison to independent values; Bold ΣFIC and Int: Synergy, U: The mean MBC values were not absolute values (either >8000.00 µg/ml or less than 0.01 µg/ml) and hence tentative FIC values were established

Table 4.16: The mean MBC values (µg/ml) and ΣFIC values of Potassium permanganate and commercial antimicrobials in combination against *E. coli* strains

Combinations	*Escherichia coli*								
	Reference strain (ATCC 8379)			**Clinical strain (Clinical 66437873)**			**Resistant strain (DSM 22314)**		
	Mean MBC	**ΣFIC**	**Int.**	**Mean MBC**	**ΣFIC**	**Int.**	**Mean MBC**	**ΣFIC**	**Int.**
Potassium permanganate + Betadine®	**250.78**	U	Add.	**250.78**	U	Add.	**250.78**	U	Add.
Potassium permanganate + Gentamycin	U	U	Ind.	**2000.63**	U	Add.	**2000.63**	U	Add.
Potassium permanganate + Neomycin	U	U	Ant.	**2000.63**	U	Add.	**2000.63**	U	Add.
Potassium Permanganate + Steriscrub	**2566.67**	U	Add.	**550.00**	U	Add.	**275.00**	U	Add.

Mean MBC: The mean MBC for the combination, Int: Interaction, Syn: Synergistic interaction, Add: Additive interaction, Ant: Antagonistic interaction, Ind: Indifferent interactions, Bold MBC: Reduced MBC values in comparison to independent values; Bold ΣFIC and Int: Synergy, U: The mean MBC values were not absolute values (either >8000.00 µg/ml or less than 0.01 µg/ml) and hence tentative FIC values were established

4.3.8. Overview of combinations against the Gram-negative pathogens

Figure 4.2 demonstrates the number of overall interactions for each Gram-negative pathogen. The highest number of synergistic interactions were observed with the *E. coli* clinical strain. In comparison to the other medicinal dyes tested, Iodine tincture in combination demonstrated a poorer interactive profile. The highest number of additive interactions were demonstrated against the *E. coli* reference strain. Methylene blue combinations demonstrated the most synergistic interactions against the *E. coli* clinical strain. The greatest number of antagonistic combinations were observed against the *P. aeruginosa* reference strain and the *E. coli* resistant strain. In comparison with the Gram-positive pathogens, the Gram-negative pathogens noted fewer synergistic interactions.

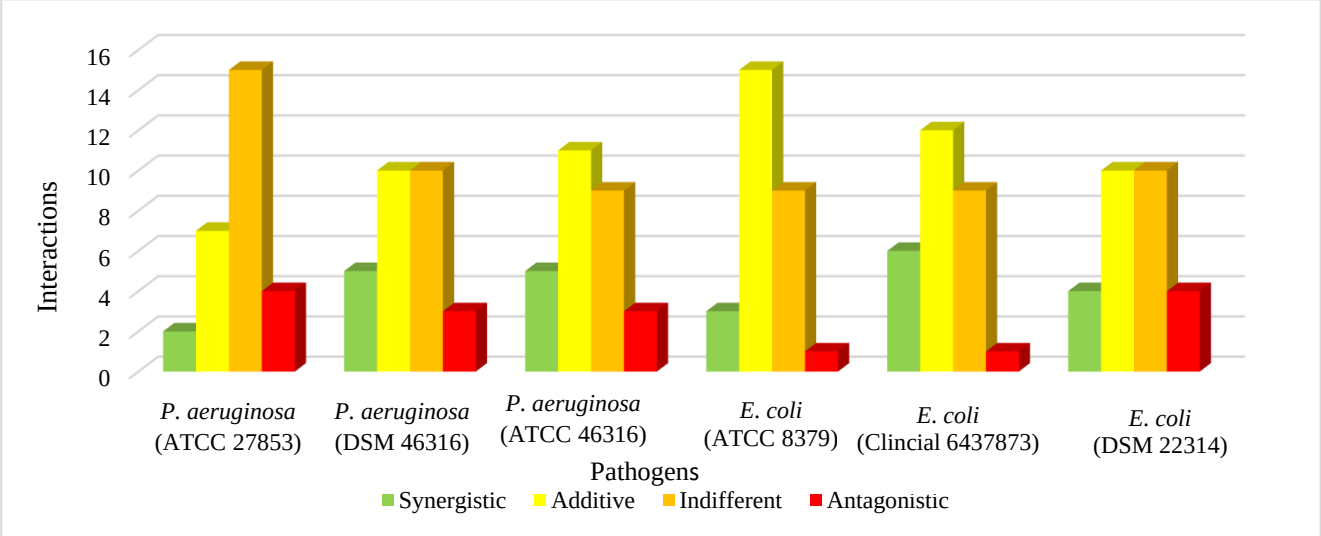


Figure 4.2: Summary of the interactions against the Gram-negative pathogens

4.4. Varied ratio combinations

As with the Gram-positive pathogens, combinations were selected based on their ΣFIC values and whether there a pattern demonstrated by that combination against the various pathogens. The combinations were also grouped into two groups: synergistic interactions and antagonistic interactions (Table 4.17).

Table 4.17: Combinations and their corresponding pathogens

Combination		Corresponding pathogen/s
Synergistic	Gentian violet + Gentamycin	*E. coli* resistant strain
	Fuchsine + Steriscrub	*P. aeruginosa* clinical strain and *E. coli* resistant strain
	Malachite green + Neomycin	*E. coli* resistant strain
	Fuchsine + Gentamycin	*P. aeruginosa* resistant strain
Antagonistic	Gentian violet + Neomycin	*P. aeruginosa* clinical strain
	Methylene blue + Gentamycin	*P. aeruginosa* resistant strain
	Mercurochrome + Steriscrub	*E. coli* resistant strain
	Methylene blue + Betadine®	*E. coli* resistant strain
	Gentian violet + Betadine®	*P. aeruginosa* reference strain

4.4.1. Synergistic varied ratio combinations of medicinal dyes with commercial antimicrobials against Gram-negative pathogens

Unlike the Gram-positive pathogens where a combination noted synergy against several of the same strains, for the Gram-negative pathogens the trend was more random and therefore combinations demonstrating the lowest ΣFIC values were expanded to determine if varied ratios would demonstrate similar synergistic interactions as observed with the 1:1 combination. Isobolograms were then constructed to visualize the varied ratio effects and to determine whether synergy was dose dependent.

Synergy was observed in the 1:1 ratio of Gentian violet and Gentamycin against the *E. coli* resistant strain (Figure 4.3). Ratios with Gentian violet in a higher concentration mainly demonstrated synergy with additive effects in selected ratios. Fuchsine and Steriscrub demonstrated synergy in a 1:1 ratio against two pathogens, *E. coli* and *P. aeruginosa* clinical strains (Figure 4.4.A and B).

Against the *E. coli* resistant strain, synergy was observed at the 1:1 ratio and mostly ratios with a higher Steriscrub concentrations whilst the other ratio in the combination noted additive effects. When tested against the *P. aeruginosa* clinical strain, all ratios demonstrated synergy. Malachite green with Neomycin tested against the *E. coli* resistant strain demonstrated a synergistic interaction only in a 1:1 ratio and additive interactions were predominantly noted for the other ratios (Figure 4.5). Fuchsine and Gentamycin predominantly demonstrated synergy against the resistant strain of *P. aeruginosa* with a slight variation (additive interaction) at a selected ratio (1:9), where Gentamycin was the dominant concentration (Figure 4.6).

The *E. coli* resistant strain demonstrated a trend of synergistic interaction, this is interesting due the attributes of a resistant strain. Gentamycin and Neomycin, both from the same family of aminoglycosides demonstrated synergistic activity (strong antimicrobial activity) (60% of the combinations) when in combination with the dyes. Combinations with Gentamycin and Neomycin did note synergistic interactions; however, they were randomly seen among the various strains and no pattern was determined.

Studies into varied ratio testing of medicinal dyes independently or in combination have been lacking with many studies focusing on varied ratio testing of antibiotics. When tested in varied ratios, the antibiotic combination of nalidixic acid with streptomycin demonstrated synergy against the *E. coli* strain. This is due to *E. coli* demonstrating susceptibility to streptomycin (Adamowicz and Harcombe, 2020). Ciprofloxacin and streptomycin (in varied ratios as well) have also demonstrated synergism against the *E. coli* strain as noted by Adamowicz and Harcombe (2020).

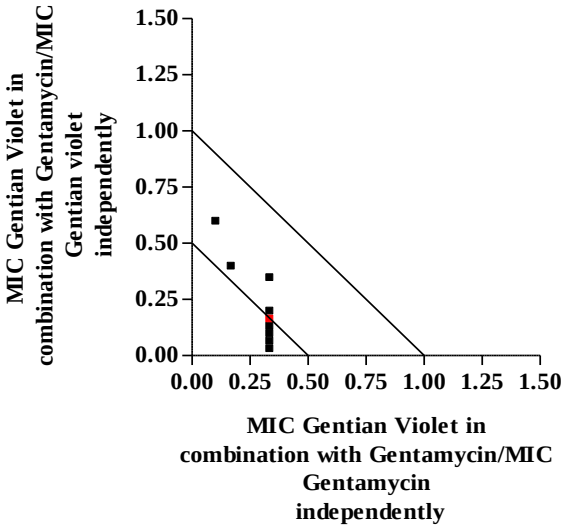


Figure 4.3: Isobologram of combinations of Gentian violet with Gentamycin against *E. coli* resistant strain.

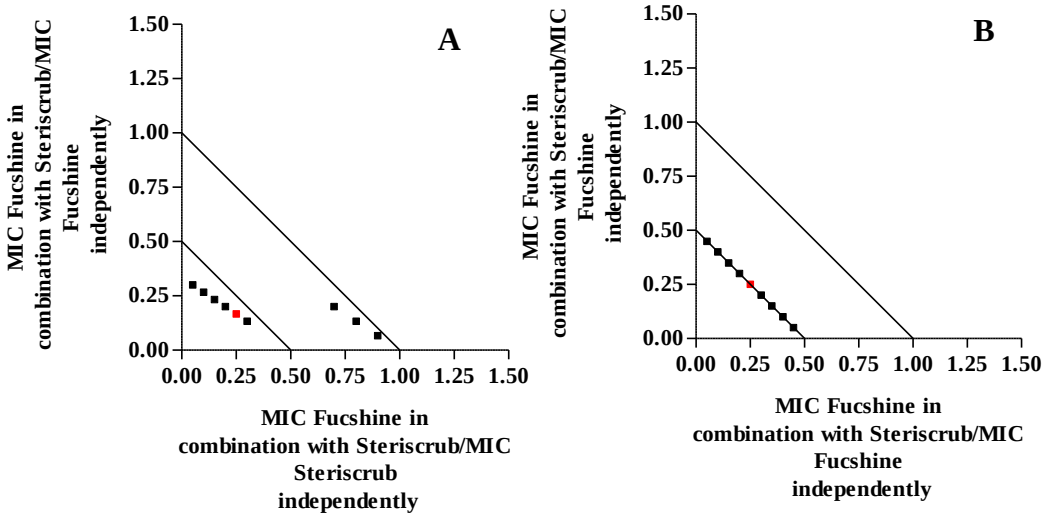


Figure 4.4: Isobologram of combinations A.) Fuchsine with Steriscrub against *E. coli* resistant strain, B.) Fuchsine with Steriscrub against *P. aeruginosa* clinical strain.

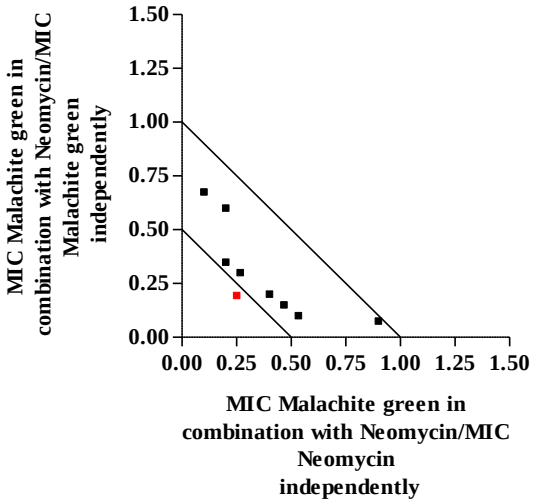


Figure 4.5: Isobologram of combinations of Malachite green with Neomycin against *E. coli* resistant strain

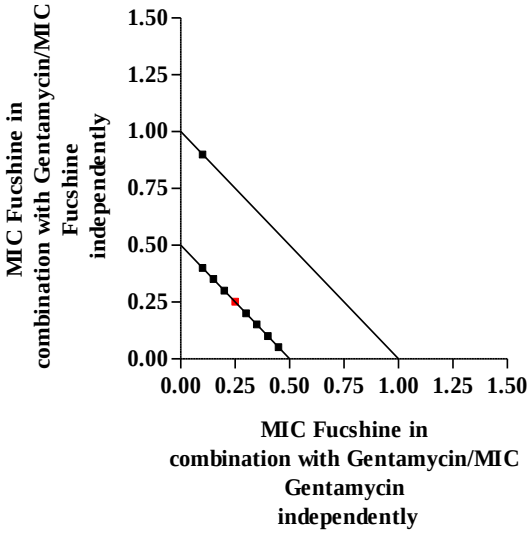


Figure 4.6: Isobologram of combinations of Fuchsine with Gentamycin against *P. aeruginosa* resistant strain.

4.4.2. Antagonistic varied ratio combinations of medicinal dyes with commercial antimicrobials against Gram-negative pathogens

As with the synergistic interactions, the antagonistic combinations selected for further assessment were based on the combinations with the highest ΣFIC values which were then expanded to determine if the varied ratios would demonstrate a similar interaction.

Gentian violet in combination with Neomycin against *P. aeruginosa* clinical strain noted an antagonistic 1:1 ratio. When tested in various ratios, indifferent interactions were observed for all other ratio's (Figure 4.7). An antagonistic interaction was observed with the combination of Methylene blue and Gentamycin in the 1:1 ratio tested, against the *P. aeruginosa* clinical strain, all other ratios in the combination have demonstrated an indifferent interaction (Figure 4.8).

Mercurochrome with Steriscrub mainly demonstrated antagonism in the varied ratios (when tested against the *E. coli* resistant strain) including the 1:1 ratio, however, ratios where Mercurochrome was dominant was observed to demonstrate an indifferent interaction (Figure 4.9). Methylene blue with Betadine® demonstrated an antagonistic interaction in the 1:1 ratio against the *E. coli* resistant strain, however, the other varied ratios predominantly demonstrated indifference (Figure 4.10). Lastly, the combination of Gentian violet with Betadine® tested against *P. aeruginosa* reference strain predominantly demonstrated antagonistic interactions including the 1:1 ratio, however, ratios 2:8, 8:2 and 9:1 (dye: commercial antimicrobial) have demonstrated indifference (Figure 4.11).

In a recent study by Dumisa (2020), varied ratio studies were conducted, however, none were conducted on antagonistic combinations. Varied ratio testing was conducted on *Moringa oleifera* extract with Gentamycin. The combination predominantly demonstrated antagonistic interactions against *Proteus mirabilis* (Ilanko *et al.*, 2019). While it has no relation to the current study, varied ratio testing has been used to determine interactions among antibiotic combinations and antibiotic: dye combinations remain a novel area of antimicrobial research.

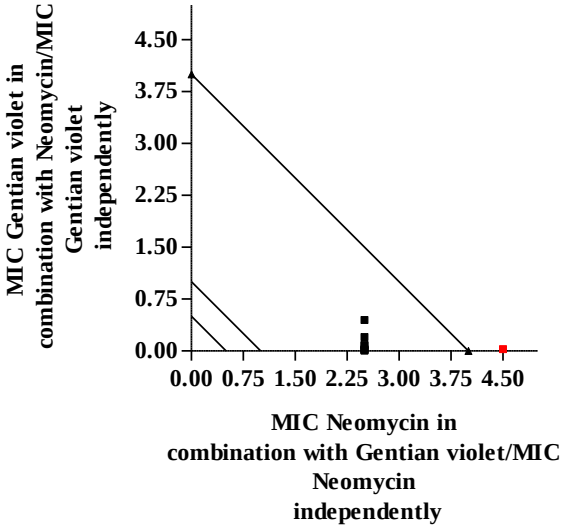


Figure 4.7: Isobologram of combinations of Gentian violet with Neomycin against *P. aeruginosa* clinical strain**.**

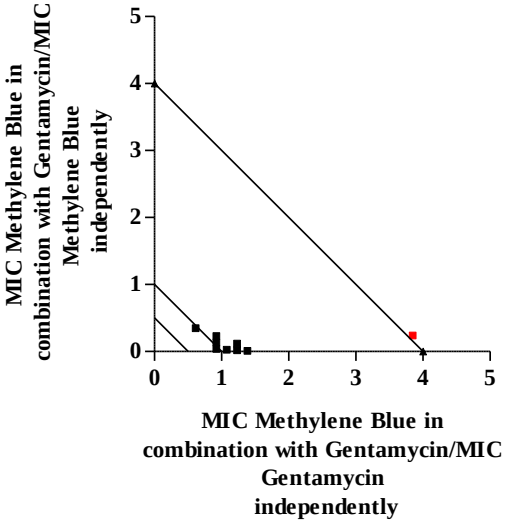


Figure 4.8: Isobologram of combinations of Methylene blue with Gentamycin against *P. aeruginosa* resistant strain.

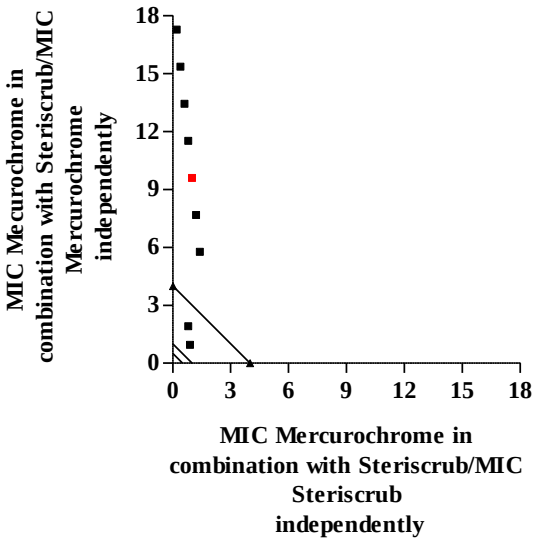


Figure 4.9: Isobologram of combinations of Mercurochrome with Steriscrub against *E. coli* resistant strain

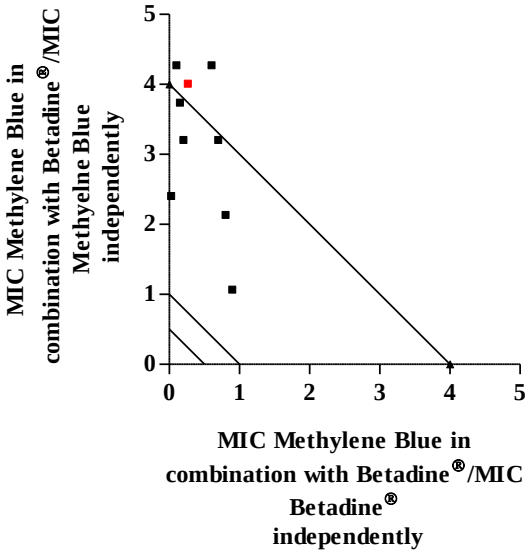


Figure 4.10: Isobologram of combinations of Methylene blue with Betadine® against *E. coli* resistant strain.

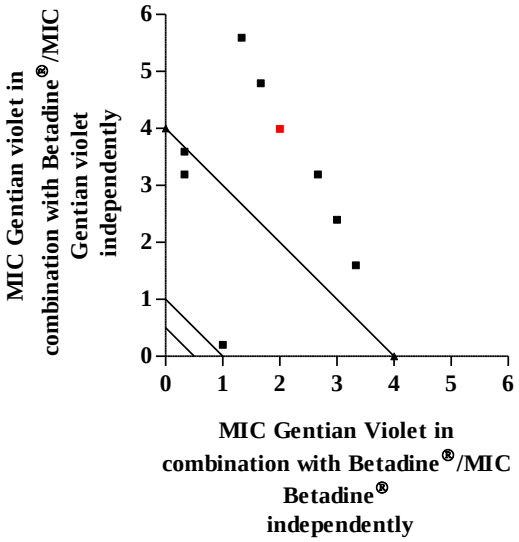


Figure 4.11: Isobologram of combinations of Gentian violet with Betadine® against *P. aeruginosa* reference strain

4.4.3. Overview of varied ratio combinations

Synergistic varied ratio interactions occurred mainly against the clinical and resistant strains rather than reference strains. Unlike the Gram-positive strains, where all ratios noted synergy, the 1:1 ratio was the only ratio that demonstrated synergy among all ratios tested. Additive interactions were mostly observed with other ratios. In the antagonistic combinations (as with the Gram-positive pathogens), not all ratios demonstrated antagonism (except for the 1:1 ratio tested) with most demonstrating indifference. No general pattern was noted among these combinations, however, combinations with Betadine® seem to more likely demonstrate an antagonistic interaction whereas, in the Gram-positive pathogens, it is most likely to demonstrate a synergistic interaction.

4.5. Summary

- Gentian violet demonstrated the lowest MBC values (0.01-0.52 μg/ml) against the Gram-negative strains.
- The *P. aeruginosa* and *E. coli* strains demonstrated the most resistance to Potassium permanganate with MBC values of >8000.00 μg/ml.
- Betadine® demonstrated the strongest efficacy against the Gram-negative strains with MIC/MBC values ranging from 1.56-2.60 μg/ml.
- Steriscrub noted the weakest activity against *E. coli* (clinical strain) with an MIC/MBC value of 2000.00 μg/ml.
- The highest number of synergistic interactions were observed against the *E. coli* clinical strain.
- When tested against *Pseudomonas aeruginosa* (reference strain) and *E. coli* (resistant train), the highest number of antagonistic interactions were noted.
- Fuchsine with Steriscrub was the only combination to demonstrate synergy among all varied ratios tested against the *P. aeruginosa* clinical strain.
- Among the antagonistic combinations used in varied ratio testing, the majority of the combinations noted antagonism only at the 1:1 ratio.

CHAPTER 5

Antimicrobial activity:

The yeasts

5.1. Introduction

Candida has increasingly been the cause of various serious infections partly due to the increased resistance to the few antifungal drugs available (Alves *et al*., 2021; de Lima *et al*., 2022). Research into new anti-candida/antifungal drugs have become rare therefore, it has become vital to determine new antifungal drugs of either synthetic or natural origin (Alves *et al*., 2021; de Lima *et al*., 2022). The objective of this chapter was to determine the antimicrobial activity of the dyes and the commercial antimicrobials as well as the combinations against *C. albicans*. Figure 5.1 demonstrates the flow in which the antimicrobial testing was carried out.

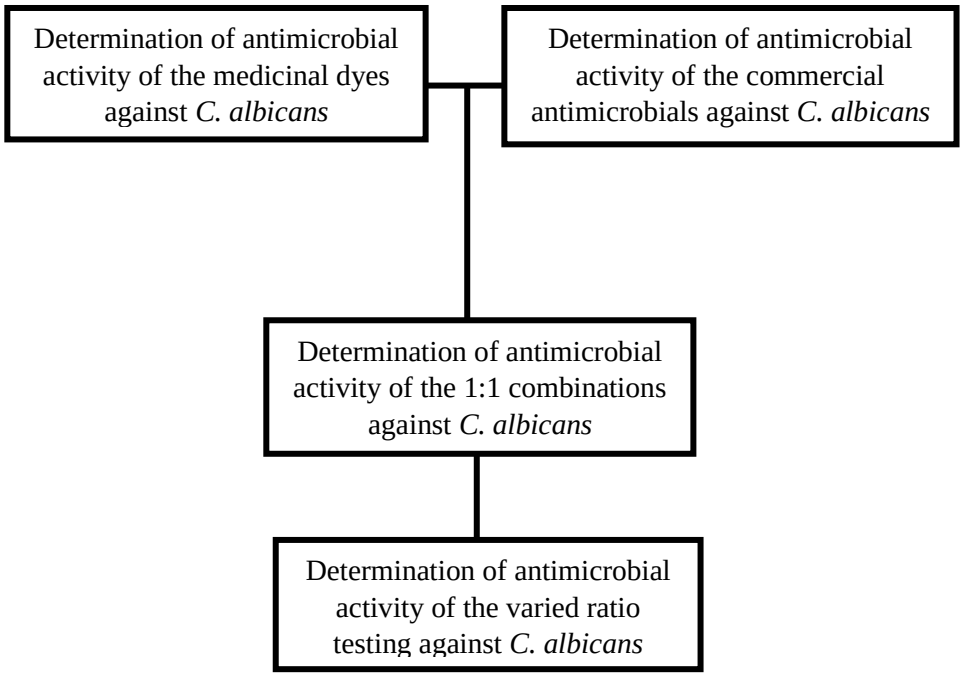


Figure 5.1: The flow of the study

5.2. Results and discussion

5.2.1. Antimicrobial activity of medicinal dyes against the yeasts

Fuchsine demonstrated the lowest MFC value of 200.00 µg/ml against the *C. albicans* clinical (A100) strain (Table 5.1). A cidal value of 208.33 µg/ml was noted for *C. albicans* resistant (ATCC 90028) and 250.00 µg/ml for *C. albicans* reference (ATCC 10231) strains.

Gentian violet proved to have the highest anti-candidal efficacy and demonstrated the lowest MFC value of 0.00025 µg/ml against the reference and clinical strains of *C. albicans*. A slightly lower MFC of 0.001 µg/ml was noted by the resistant strain. Gentian violet has been known to be used for the treatment of *Candida* infections (Kondo *et al*., 2012). A study testing the effectiveness of Gentian violet against various *Candida* strains, noted an MIC range of 0.25-1.00 µg/ml against *C. albicans* (Kondo *et al*., 2012). This proves interesting, as very little to no resistance has occurred over the years.

Iodine tincture also demonstrated a low MFC value against *C. albicans* reference and *C. albicans* resistant strains with a value of 0.78 µg/ml. Iodine tincture demonstrated an MFC value of 1.56 µg/ml against the *C. albicans* clinical strain. Otomycosis is a superficial infection of the external auditory canal caused by *Aspergillus* and *Candida* species (Mofatteh *et al*., 2021). A clinical study tested the antimicrobial activity of Iodine tincture as a treatment for otomycosis (Mofatteh *et al*., 2021). The study noted that after 20 days of treatment, 54% of test group had a good response to Iodine tincture with only 7% presenting with a reoccurring infection, which was higher than that observed with clotrimazole, the most frequently used antibiotic treatment (Mofatteh *et al*., 2021). In the study, it was concluded that Iodine tincture can be used to treat otomycosis (Mofatteh *et al*., 2021).

The reference strain of *C. albicans* noted the highest susceptibility to Malachite green with an MFC value of 0.32 µg/ml. The resistant strain of *C. albicans* noted the poorest susceptibility to Malachite green with a much higher MFC value of 5.00 µg/ml. This is to be expected as it is a resistant strain. A study demonstrated the *in vitro* effect of Malachite green against *C. albicans*

and non-*C. albicans* strains noted at concentrations above 0.20 µg/ml, Malachite green is fungicidal, and concentrations of 0.40 µg/ml observed little fungal growth (Dhamgaye *et al*., 2012). This study noted a MFC value above 0.20 µg/ml for all *C. albicans* strains, with values in accordance with Dhamgaye *et al*. (2012).

The reference strain of *C. albicans* demonstrated the highest susceptibility to Methylene blue with an MFC value of 12.50 µg/ml. Methylene blue demonstrate the same MFC of 26.67 µg/ml against the clinical and resistant strain of *C. albicans*. Ansari *et al*. (2016), demonstrated that Methylene blue had antifungal properties and *C. albicans* is susceptible to Methylene blue at a concentration of 100.00 µg/ml. The current study observed susceptibility at a much lower concentration, however, the methods chosen in the previous study were different (the broth spot assay and the filter disc assay) compared to the methods (MIC) used in the current study.

Potassium permanganate demonstrated the weakest efficacy against *C. albicans* when compared to the other dyes. It showed MFC values of 8000.00 µg/ml against *C. albicans* reference strain and 4000.00 µg/ml against *C. albicans* clinical strain. It is also noted that a higher value of >8000.00 µg/ml against *C. albicans* resistant strain was observed. A study of general antiseptics and their activity was conducted against *Aspergillus* spp. and *C. albicans* which noted *C. albicans* demonstrated susceptibility to potassium permanganate at 10 000.00 µg/ml (Abed and Hussein, 2016). This study used an initial concentration of 8000.00 µg/ml hence, no higher MFC value was noted, however, it can be noted that higher concentrations of potassium permanganate are needed to elicit an antimicrobial effect.

Table 5.1: The minimum fungicidal (MFC) value of the medicinal dyes against yeasts (µg/ml)

Dyes	***Candida albicans***		
	Reference strain (ATCC 10231)	**Clinical strain (A100)**	**Resistant strain (ATCC 90028)**
Fuchsine	250.00	200.00	208.33
Gentian violet	0.00025	0.00025	0.001
Iodine tincture	0.78	1.56	0.78
Malachite green	0.32	0.63	5.00

Dyes	*Candida albicans*		
	Reference strain (ATCC 10231)	**Clinical strain (A100)**	**Resistant strain (ATCC 90028)**
Mercurochrome	0.42	0.08	0.08
Methylene blue	12.50	26.67	26.67
Potassium permanganate	8000.00	4000.00	>8000.00
Controls			
Culture control	>8000.00	>8000.00	>8000.00
Negative control (water/acetone)	>8000.00	>8000.00	>8000.00
Positive control (Amphotericin B)	2.50	1.25	2.50

5.2.2. Overview of activity of the medicinal dyes against the yeasts

In summary, the yeasts noted slightly better susceptibility to Potassium permanganate compared the Gram-positive and Gram-negative pathogens, however, it still demonstrated poor activity overall. While potassium permanganate demonstrated high MBC values overall, the yeasts are the only group where some susceptibility was observed- one or two dilution difference. Fuchsine noted better activity against the yeasts compared to the Gram-negative strains, but not better than the Gram-positive strains, especially the *S. epidermidis* strains. Iodine tincture noted a similar efficacy to the Gram-negative pathogens and the *S. aureus* strains, but not higher than the *S. epidermidis* strains. Malachite green and Mercurochrome demonstrated low MBC values against the *C. albicans* strains, a similar pattern to the Gram-positive strains. Gentian violet demonstrated the best efficacy among all the strains tested.

5.2.3. Antimicrobial activity of commercial antimicrobials against yeasts

Antibiotics such as Gentamycin, Neomycin, Fusidic acid and Mupirocin are only effective against bacteria. Therefore, these were excluded for testing here. Three antifungals (Miconazole, Nystatin, and Ketoconazole) were selected for testing against the *C. albicans* strains. Table 5.2 shows the MIC/MFC values of antimicrobials tested.

Betadine® demonstrated MIC/MBC values of 1.30-4.16 µg/ml against all *C. albicans* strains. The strongest MIC/MFC value was 1.30 µg/ml against *C. albicans* reference strain. Betadine® noted values MIC/MFC values of 2.60 µg/ml against *C. albicans* clinical strain. A study tested the activity of antiseptics against *C. albicans*, whereby the MIC value for the ATCC strain ranged from 2.57-3.16 µg/ml and >4.46 µg/ml for the clinical isolate (Moore *et al*., 2017). The current study noted a slightly lower value (1.30 µg/ml) for the ATCC strain. The resistant strain demonstrated the poorest susceptibility to Betadine® with an MIC/MFC value of 4.16 µg/ml, as was expected from a resistant strain due the strains attributes.

Ketoconazole demonstrated the weakest activity among the antifungals tested against *C. albicans* with some strains exhibiting an MIC/MFC value of >25.00 µg/ml. The *C. albicans* clinical strain noted the highest susceptibility among the *C. albicans* strains with an MIC/MFC of 3.13 µg/ml. Behbehani *et al*. (2019), noted the MIC range of Ketoconazole between 4.00 and 32.00 µg/ml. While there is a slight difference in the MIC values against the *C. albicans* clinical strain, these values are in accordance with the current study.

Miconazole demonstrated the most susceptible efficacy against *C. albicans* clinical strain with an MIC/MFC value of 1.25 µg/ml. *C. albicans* demonstrated susceptibility to Miconazole with an MIC/MFC of 6.25 µg/ml noted for the antifungal while the resistant strain demonstrated the most resistance with an MIC/MFC of >25.00 µg/ml which is to be expected. A recent study noted the MIC value for *C. albicans* was 4.00 µg/ml (Arias *et al*., 2020). The MIC value in the current study (1.25 µg/ml) is only slightly lower than one reported in the study.

Nystatin noted an MIC/MFC value of 6.25 µg/ml against the *C. albicans* reference strain, a much higher MIC/MFC value than that for the *C. albicans* clinical strain (MIC/MFC of 0.20 µg/ml). This could be attributed to the resistance mechanisms developed by the reference strain, while the clinical strain has not developed the resistant mechanisms yet. As expected, the resistant strain of *C. albicans* demonstrated the least susceptibility to Nystatin with an MIC/MFC value of >25.00 µg/ml. A study conducted on *C. albicans* noted Nystatin had an MIC value of 4.00 µg/ml and an MFC value of 8.00 µg/ml, however, a different ATCC strain (ATCC 90028) of *C. albicans* was tested (Tonon *et al*., 2018). The results are congruent with the results observed with the reference strain for the current study.

Steriscrub demonstrated the same MIC/MFC values for all *C. albicans* strains with a value of 800.00 µg/ml. A study testing the antimicrobial activity of chlorhexidine (Steriscrub) against certain oral bacteria including *C. albicans*, noted an MIC value of 125.00 µg/ml against *C. albicans* (Dadpe *et al*., 2018). The current study demonstrated higher MIC/MFC values of Steriscrub in comparison to the previous study. The differences could be attributed to different strains of *C. albicans,* and different methods used to determine the antimicrobial activity.

Table 5.2: The minimum inhibitory and minimum fungicidal concentrations (MIC/MFC) of commercial antimicrobials against the yeasts (µg/ml)

Antimicrobials	***Candida albicans***					
	Reference strain (ATCC 10231)		**Clinical strain (A100)**		**Resistant strain (ATCC 90028)**	
	MIC	**MFC**	**MIC**	**MFC**	**MIC**	**MFC**
Betadine® (Povidone-Iodine)	1.30	1.30	2.60	2.60	4.16	4.16
Ketoconazole	18.75	18.75	3.13	3.13	>25.00	>25.00
Miconazole	6.25	6.25	1.25	1.25	>25.00	>25.00
Nystatin	6.25	6.25	0.20	0.20	>25.00	>25.00
Steriscrub (Chlorohexidine)	800.00	800.00	800.00	800.00	800.00	800.00
Controls						
Culture control	>8000.00	>8000.00	>8000.00	>8000.00	>8000.00	>8000.00
Negative control (water/acetone)	>8000.00	>8000.00	>8000.00	>8000.00	>8000.00	>8000.00
Positive control (Amphotericin B)	0.63	0.63	6.25	6.25	12.50	25.00

5.2.4. Overview activity of the commercial antimicrobials against the yeasts

Overall, the antifungals demonstrated the weakest efficacy again the *C. albicans* resistant strain. The Gram-positive pathogens demonstrated better susceptibility to Steriscrub in comparison to the yeasts. The majority of the Gram-negative pathogens noted better susceptibility to Steriscrub in comparison to the yeasts except for the *E. coli* resistant strain. The reference strain of *C. albicans* demonstrated lower susceptibility to the antifungals compared to the *C. albicans* clinical strain. However, Betadine® noted reasonable efficacy among all strains of *C. albicans*. Betadine® demonstrated the lowest MIC/MFC values against the *C. albicans* reference and resistant strains compared to the other commercial antimicrobials and while it did not demonstrate lower efficacy

compared to the antifungals, Betadine® has a better overall efficacy against the *C. albicans* strains compared to the commercial antimicrobials. Steriscrub demonstrated the weakest activity overall, against the *C. albicans* strains compared to the Gram-positive pathogens and Gram-negative pathogens.

5.3. 1:1 Combinations

The medicinal dye: commercial antimicrobial combinations were tested against the yeasts. The MIC and ΣFIC values were calculated and discussed under the appropriate sub sections. As with the independent values, all controls responded as expected. Amphotericin B was used as the positive control to ensure the yeasts were susceptible. The negative control (water/acetone) was used to ensure the sample and not the solvent demonstrated antifungal activity, and as expected, the negative control demonstrated microbial growth. A culture control was used to ensure the TSB supports the growth of the pathogen, and as expected, there was microbial growth. The ΣFIC value of medicinal dyes in combination with various commercial antimicrobials tested against the *C. albicans* strains could not be determined as no endpoint MBC value was observed and hence are depicted as "U" within Table 5.3 to 5.9, however, a tentative interpretation of synergy, additive, indifferent or antagonism was observed based on the comparison to individual MBC values of the individual medicinal dye and commercial antimicrobial respectively.

5.3.1. Combinations with Fuchsine

Fuchsine in various combinations tested against the *Candida* yeast strains noted only 40% of the combinations showing a reduced (lower) MBC value in comparison to the independent values. No synergy was noted when tested against the *C. albicans* reference strain. While some combinations did demonstrate lower MBC values, Fuchsine combined with Ketoconazole noted the lowest MBC value of 6.27 µg/ml against the *C. albicans* clinical strain (Table 5.3). This combination (Fuchsine with Ketoconazole) was the only combination to have demonstrated a synergistic interaction with an ΣFIC value of 0.04 (Table 5.3).

Combinations with Fuchsine demonstrated the most antagonistic interactions when compared to the other interactions among the yeasts. Antagonistic interactions were observed with Fuchsine in combination with three antifungals (Ketoconazole, Miconazole, and Nystatin) against the *C. albicans* resistant strain. The ΣFIC value of Fuchsine in combination with the antifungals tested against the *C. albicans* resistant strain could not be determined, however, a tentative interpretation of antagonism was observed based on the comparison to individual MBC values.

Castellini's paint which contains a combination of basic Fuchsine, boric acid, acetone, and alcohol has been used in previous years to treat various fungal infections in intertriginous areas of the body (Kyle and Dahl, 2004; Agnihotri *et al*., 2019). The previous studies have shown potential in the use of Fuchsine in combination against fungi, which is also observed in the current study.

Table 5.3: The mean MBC values (μg/ml) and ΣFIC values of Fuchsine and commercial antimicrobials in combination against *C. albicans* strains

Combinations	*Candida albicans*								
	Reference strain (ATCC 10231)			**Clinical strain (A100)**			**Resistant strain (ATCC 90028)**		
	Mean MBC	**ΣFIC**	**Int.**	**Mean MBC**	**ΣFIC**	**Int.**	**Mean MBC**	**ΣFIC**	**Int.**
Fuchsine + Betadine®	1671.88	10.67	Ant.	**125.39**	0.78	Add.	250.78	1.39	Ind.
Fuchsine + Ketoconazole	**250.78**	1.04	Ind.	**6.27**	**0.04**	**Syn.**	1003.13	U	Ant.
Fuchsine + Miconazole	**125.04**	0.51	Add.	250.08	1.31	Ind.	250.08	U	Ant.
Fuchsine + Nystatin	1338.54	4.08	Ant.	500.16	3.28	Ind.	501.56	U	Ant.
Fuchsine + Steriscrub	**229.17**	0.86	Add.	1100.00	5.13	Ant.	**275.00**	1.23	Ind.

Mean MBC: The mean MBC for the combination, Int: Interaction, Syn: Synergistic interaction, Add: Additive interaction, Ant: Antagonistic interaction, Ind: Indifferent interactions, Bold MBC: Reduced MBC values in comparison to independent values; Bold ΣFIC and Int: Synergy, U: The mean MBC values were not absolute values (either >8000.00 μg/ml or less than 0.01 μg/ml) and hence tentative FIC values were established

5.3.2. Combinations with Gentian violet

Gentian violet demonstrated much lower MBC values against all *C. albicans* strains in combination with the commercial antimicrobials (Table 5.4).

No synergistic interactions with the Gentian violet combinations were observed against the *C. albicans* strains (Table 5.4). Indifferent interactions were mainly observed with the Gentian violet combinations. The ΣFIC value of Gentian violet in combination with some of the antifungals tested against the *C. albicans* resistant strain could not be determined, however, a tentative interpretation of indifference was observed based on the comparison to individual MBC values.

A very old study tested the antimicrobial effects of Gentian violet in combination with sodium propionate against *Candida* infections in quails (Moorhead and Cross, 1972). The pathogen tested in this study was *C. albicans* (Moorhead and Cross, 1972). The study determined that the combination of both Gentian violet with sodium propionate reduced the incident rate of infection as well as the severity of the infection (Moorhead and Cross, 1972). While this study was not conducted on humans and is quite old, it does demonstrate the potential of Gentian violet combinations for the treatment of infections caused by *C. albicans.* Dumisa (2020), observed predominantly synergistic interactions (84%) among the Gentian violet: plant extract combinations when tested against *C. albicans* strains, while the current study, in comparison, demonstrated no synergy. This improved interaction noted in the previous study could possibly be explained by the mechanism in which the plant extracts interact with Gentian violet compared to the interaction with commercial antimicrobials as noted in this study, however, little research has been done in this area to further elaborate on this aspect.

Table 5.4: The mean MBC values (µg/ml) and ΣFIC values of Gentian violet and commercial antimicrobials in combination against *C. albicans* strains

Combinations	***Candida albicans***								
	Reference strain (ATCC 10231)			**Clinical strain (A100)**			**Resistant strain (ATCC 90028)**		
	Mean MBC	**ΣFIC**	**Int.**	**Mean MBC**	**ΣFIC**	**Int.**	**Mean MBC**	**ΣFIC**	**Int.**
Gentian violet + Betadine®	**0.10**	2.08	Ind.	**0.06**	1.19	Ind.	**0.10**	1.00	Add.
Gentian violet + Ketoconazole	**0.10**	2.01	Ind.	**0.01**	1.00	Add.	**0.02**	U	Ind.
Gentian violet + Miconazole	**0.10**	2.02	Ind.	**0.01**	1.00	Add.	**0.04**	U	Ind.

Combinations	*Candida albicans*								
	Reference strain (ATCC 10231)			**Clinical strain (A100)**			**Resistant strain (ATCC 90028)**		
	Mean MBC	**ΣFIC**	**Int.**	**Mean MBC**	**ΣFIC**	**Int.**	**Mean MBC**	**ΣFIC**	**Int.**
Gentian violet + Nystatin	**0.10**	2.02	Ind.	**0.01**	1.03	Ind.	**0.02**	U	Ind.
Gentian violet + Steriscrub	**62.50**	3.98	Ind.	**1.56**	1.00	Add.	**10.42**	1.64	Ind.

Mean MBC: The mean MBC for the combination, Int: Interaction, Syn: Synergistic interaction, Add: Additive interaction, Ant: Antagonistic interaction, Ind: Indifferent interactions, Bold MBC: Reduced MBC values in comparison to independent values; Bold ΣFIC and Int: Synergy, U: The mean MBC values were not absolute values (either >8000.00 µg/ml or less than 0.01 µg/ml) and hence tentative FIC values were established

5.3.3. Combinations with Iodine tincture

While all combinations with Iodine tincture demonstrated lower (enhanced) MBC values, not all demonstrated synergy and mostly additive and indifferent interactions were noted (Table 5.5). Overall, four synergistic interactions with Iodine tincture combinations were noted against the *C. albicans* strains. Fifty percent of the synergistic interactions observed, were against the *C. albicans* reference strain. Iodine tincture with Steriscrub noted the lowest ΣFIC of 0.14 against the *C. albicans* reference strain. Iodine tincture in combination with Ketoconazole demonstrated the lowest MBC value of 0.36 µg/ml against the *C. albicans* clinical strain, this combination also noted a synergistic interaction with ΣFIC value of 0.20 (Table 5.5).

The ΣFIC value of Iodine tincture in combination with Ketoconazole, Miconazole and Nystatin against the *C. albicans* resistant strain could not be determined, however, a tentative interaction of indifference was observed based on the comparison to individual MBC values.

There is various antimicrobial research that has been conducted into Iodine and Povidone-iodine (Felpel, 1997; Haapasalo *et al*., 2005; Davies and Patel, 2016; Cho *et al*., 2018; Mofatteh *et al*., 2021) Iodine tincture and Povidone-iodine has proven to be an effective fungicidal and have been used to irrigate wounds or treat fungal infections. But research is lacking on the combination therapy of Iodine tincture with antimicrobials and hence the studies as indicated herein prove a valuable tool in understanding antifungal combination therapy.

Table 5.5: The mean MBC values (μg/ml) and ΣFIC values of Iodine tincture and commercial antimicrobials in combination against *C. albicans* strains

Combinations	*Candida albicans*								
	Reference strain (ATCC 10231)			**Clinical strain (A100)**			**Resistant strain (ATCC 90028)**		
	Mean MBC	**ΣFIC**	**Int.**	**Mean MBC**	**ΣFIC**	**Int.**	**Mean MBC**	**ΣFIC**	**Int.**
Iodine tincture + Betadine®	**1.63**	1.42	Ind.	**1.63**	0.71	Add.	**3.12**	1.83	Ind.
Iodine tincture + Ketoconazole	**1.30**	**0.39**	**Syn.**	**0.36**	**0.20**	**Syn.**	**6.51**	U	Ind.
Iodine tincture + Miconazole	**1.09**	1.05	Ind.	**1.09**	0.75	Add.	**0.55**	U	Ind.
Iodine tincture + Nystatin	**2.60**	1.00	Add.	**0.55**	1.03	Ind.	**3.91**	U	Ind.
Iodine tincture + Steriscrub	**12.60**	**0.14**	**Syn.**	**33.59**	0.21	Ind.	**25.20**	**0.28**	**Syn.**

Mean MBC: The mean MBC for the combination, Int: Interaction, Syn: Synergistic interaction, Add: Additive interaction, Ant: Antagonistic interaction, Ind: Indifferent interactions, Bold MBC: Reduced MBC values in comparison to independent values; Bold ΣFIC and Int: Synergy, U: The mean MBC values were not absolute values (either >8000.00 μg/ml or less than 0.01 μg/ml) and hence tentative FIC values were established

5.3.4. Combinations with Malachite green

Malachite green combinations demonstrated lower (enhanced) MBC values; however, indifferent, and additive interactions were mostly observed against the *C. albicans* reference strain. Malachite green in combination with Ketoconazole demonstrating the lowest MBC value of 0.01 μg/ml and ΣFIC value of 0.02 against the *C. albicans* clinical strain (Table 5.6). As with the *C. albicans* reference strain, the Malachite green combinations mainly demonstrated indifferent interactions. While all combinations in the current study tested against *C. albicans* demonstrated lower MBC values than when studied independently, only two synergistic interactions were noted.

No antagonistic interactions were observed. The ΣFIC value of Malachite green in combination with Ketoconazole, Miconazole and Nystatin against the *C. albicans* resistant strain could not be determined, however, a tentative interaction of indifference was observed.

Dumisa (2020), observed varied interactions (synergistic, additive, and indifferent interactions) with synergistic interactions occurring most frequently when Malachite green was combined with medicinal plants.

Table 5.6: The mean MBC values and ΣFIC values of Malachite green and commercial antimicrobials in combination against *C. albicans* strains (μg/ml)

Combinations	*Candida albicans*								
	Reference strain (ATCC 10231)			**Clinical strain (A100)**			**Resistant strain (ATCC 90028)**		
	Mean MBC	**ΣFIC**	**Int.**	**Mean MBC**	**ΣFIC**	**Int.**	**Mean MBC**	**ΣFIC**	**Int.**
Malachite green + Betadine®	**0.82**	2.13	Ind.	**0.82**	1.08	Ind.	**3.28**	0.69	Add.
Malachite green + Ketoconazole	**0.22**	0.53	Add.	**0.01**	**0.02**	**Syn.**	**6.56**	U	Ind.
Malachite green + Miconazole	**0.33**	0.81	Add.	**0.33**	**0.46**	**Syn.**	**2.58**	U	Ind.
Malachite green + Nystatin	**0.33**	0.81	Add.	**0.64**	1.10	Ind.	**5.47**	U	Ind.
Malachite green + Steriscrub	**67.33**	2.20	Ind.	**1.29**	2.00	Ind.	**55.00**	1.06	Ind.

Mean MBC: The mean MBC for the combination, Int: Interaction, Syn: Synergistic interaction, Add: Additive interaction, Ant: Antagonistic interaction, Ind: Indifferent interactions, Bold MBC: Reduced MBC values in comparison to independent values; Bold ΣFIC and Int: Synergy, U: The mean MBC values were not absolute values (either >8000.00 μg/ml or less than 0.01 μg/ml) and hence tentative FIC values were established

5.3.5. Combinations with Mercurochrome

Mercurochrome in combination with Ketoconazole noted the best efficacy against the *C. albicans* reference strain, with a reduction in MBC value of 99.69% when compared to investigating the efficacy independently (Table 5.7). The combination noted the same MBC value of 0.06 μg/ml against both the *C. albicans* reference and clinical strain. A synergistic interaction was noted for the combination (Mercurochrome in combination with Ketoconazole) with an ΣFIC value of 0.09 against the *C. albicans* reference strain. The lowest ΣFIC value noted for the *C. albicans* strains was Mercurochrome in combination with Betadine® against the *C. albicans* resistant strain with an ΣFIC value of 0.07.

As with the Malachite green combinations, Mercurochrome combinations did not demonstrate any antagonistic interactions. The ΣFIC value of Mercurochrome in combination with Ketoconazole, Miconazole and Nystatin against the *C. albicans* resistant strain could not be determined hence, a tentative interaction of indifference was observed based on the comparison to individual MBC values.

Dumisa (2020), observed synergistic interactions for all combinations of Mercurochrome (in combination with medicinal plants) against a *C. albicans* clinical strain. Some synergy in other *C. albicans* strains was observed, however, Mercurochrome in combination with commercial antimicrobials have demonstrated more additive and indifferent interactions with only two synergistic interactions noted against the strains.

Table 5.7: The mean MBC values (μg/ml) and ΣFIC values of Mercurochrome and commercial antimicrobials in combination against *C. albicans* strains

Combinations	*Candida albicans*								
	Reference strain (ATCC 10231)			**Clinical strain (A100)**			**Resistant strain (ATCC 90028)**		
	Mean MBC	**ΣFIC**	**Int.**	**Mean MBC**	**ΣFIC**	**Int.**	**Mean MBC**	**ΣFIC**	**Int.**
Mercurochrome + Betadine®	**0.78**	0.81	Add.	**0.47**	1.15	Ind.	**0.03**	**0.07**	**Syn.**
Mercurochrome + Ketoconazole	**0.06**	**0.09**	**Syn.**	**0.06**	0.51	Add.	**2.34**	U	Ind.
Mercurochrome + Miconazole	**0.31**	0.52	Add.	**0.18**	1.71	Ind.	**0.40**	U	Ind.
Mercurochrome + Nystatin	**1.87**	1.00	Add.	**0.12**	1.20	Ind.	**0.23**	U	Ind.
Mercurochrome + Steriscrub	**50.31**	0.81	Add.	**16.77**	1.35	Ind.	**8.39**	0.68	Add.

Mean MBC: The mean MBC for the combination, Int: Interaction, Syn: Synergistic interaction, Add: Additive interaction, Ant: Antagonistic interaction, Ind: Indifferent interactions, Bold MBC: Reduced MBC values in comparison to independent values; Bold ΣFIC and Int: Synergy, U: The mean MBC values were not absolute values (either >8000.00 μg/ml or less than 0.01 μg/ml) and hence tentative FIC values were established

5.3.6. Combinations with Methylene blue

All Methylene blue combinations with conventional antifungals tested demonstrated lower MBC values (cidal activity) against the *C. albicans* clinical strain (Table 5.8). The most antagonistic

interaction was noted with the combination of Methylene blue and Betadine® with a ΣFIC value of 6.41 against the *C. albicans* reference strain. Methylene blue with Steriscrub demonstrated the best combined activity against all the *C. albicans* strains with lower (enhanced) MBC values. However, Methylene blue in combination with Ketoconazole demonstrated the lowest MBC value of 0.16 μg/ml and an ΣFIC value of 0.01 against the *C. albicans* clinical strain.

Another synergistic interaction with a low ΣFIC value of 0.06 was the combination of Methylene blue with Nystatin also against the *C. albicans* clinical strain. The ΣFIC value of Methylene blue in combination with Ketoconazole, Miconazole and Nystatin against the *C. albicans* resistant strain could not be determined, however, a tentative interaction of indifference and antagonism was observed.

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 then when tested individually (Lyon *et al*., 2012). This earlier study affirms 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.

Table 5.8: The mean MBC values and ΣFIC values of Methylene blue and commercial antimicrobials in combination against *C. albicans* strains (μg/ml)

Combinations	***Candida albicans***								
	Reference strain (ATCC 10231)			**Clinical strain (A100)**			**Resistant strain (ATCC 90028)**		
	Mean MBC	**ΣFIC**	**Int.**	**Mean MBC**	**ΣFIC**	**Int.**	**Mean MBC**	**ΣFIC**	**Int.**
Methylene blue + Betadine®	26.25	6.41	Ant.	**26.25**	3.07	Ind.	**26.25**	2.25	Ind.
Methylene blue + Ketoconazole	**10.94**	0.81	Add.	**0.16**	**0.01**	**Syn.**	52.50	U	Ind.
Methylene blue + Miconazole	41.25	3.40	Ind.	**20.63**	1.17	Ind.	**10.31**	U	Ant.
Methylene blue + Nystatin	26.25	2.60	Ind.	**0.32**	**0.06**	**Syn.**	**26.25**	U	Ind.

Combinations	*Candida albicans*								
	Reference strain (ATCC 10231)			**Clinical strain (A100)**			**Resistant strain (ATCC 90028)**		
	Mean MBC	**ΣFIC**	**Int.**	**Mean MBC**	**ΣFIC**	**Int.**	**Mean MBC**	**ΣFIC**	**Int.**
Methylene blue + Steriscrub	**220.00**	1.85	Ind.	**440.00**	1.83	Ind.	**220.00**	1.00	Add.

Mean MBC: The mean MBC for the combination, Int: Interaction, Syn: Synergistic interaction, Add: Additive interaction, Ant: Antagonistic interaction, Ind: Indifferent interactions, Bold MBC: Reduced MBC values in comparison to independent values; Bold ΣFIC and Int: Synergy, U: The mean MBC values were not absolute values (either >8000.00 µg/ml or less than 0.01 µg/ml) and hence tentative FIC values were established

5.3.7. Combinations with Potassium permanganate

All combinations with Potassium permanganate noted a reduced combined MBC value (better activity in combination) against the *C. albicans* reference strain and clinical strain. However, all reduced MBC values did not show synergistic interactions (Table 5.9). The most synergistic interaction noted was with the combination of Potassium permanganate with Miconazole with an ΣFIC value of 0.06 against the *C. albicans* reference strain. One antagonistic interaction was noted with Potassium permanganate in combination with Nystatin with an ΣFIC value of 7.25 against the *C. albicans* clinical strain (Table 5.9). Potassium permanganate combined with Betadine®, Miconazole and Steriscrub noted lower MBC values (better activity) against the resistant strain of *C. albicans.* The ΣFIC value of Potassium permanganate in combination with Ketoconazole, Miconazole and Nystatin against the *C. albicans* resistant strain could not be determined, however, a tentative interaction of indifference was observed based on the comparison to individual MBC values.

There are a number of studies observing the antimicrobial profile of Potassium permanganate for infections (Francis-Floyd and Klinger, 1997; Xiaoyan and Yuan, 2010; Korkut *et al*., 2013; Delgado-Enciso *et al.*, 2017; Street *et al*., 2018), These studies have been noted the antiseptic and antifungal activity of Potassium permanganate, however, research is lacking into combination therapy against *Candida* species.

Table 5.9: The mean MBC values (µg/ml) and ΣFIC values of Potassium permanganate and commercial antimicrobials in combination against *C. albicans* strains

Combinations	*Candida albicans*								
	Reference strain (ATCC 10231)			**Clinical strain (A100)**			**Resistant strain (ATCC 90028)**		
	Mean MBC	**ΣFIC**	**Int.**	**Mean MBC**	**ΣFIC**	**Int.**	**Mean MBC**	**ΣFIC**	**Int.**
Potassium permanganate + Betadine®	**250.78**	0.63	Add.	**125.39**	**0.18**	**Syn.**	**208.98**	U	Add.
Potassium permanganate + Ketoconazole	**250.78**	**0.07**	**Syn.**	**2000.63**	0.70	Add.	U	U	Ind.
Potassium permanganate + Miconazole	**100.31**	**0.06**	**Syn.**	**500.16**	**0.25**	**Syn.**	**4001.25**	U	Ind.
Potassium permanganate + Nystatin	**4006.25**	1.50	Ind.	**4001.25**	7.25	Ant.	U	U	Ind.
Potassium Permanganate + Steriscrub	**3666.67**	0.83	Add.	**2200**	0.75	Add.	**3666.67**	U	Add.

Mean MBC: The mean MBC for the combination, Int: Interaction, Syn: Synergistic interaction, Add: Additive interaction, Ant: Antagonistic interaction, Ind: Indifferent interactions, Bold MBC: Reduced MBC values in comparison to independent values; Bold ΣFIC and Int: Synergy, U: The mean MBC values were not absolute values (either >8000.00 µg/ml or less than 0.01 µg/ml) and hence tentative FIC values were established

5.3.8. Overview of combinations against the yeasts

Figure 5.2 shows the summary of all interactions among the *C. albicans* strains. The least number of synergistic combinations was observed when tested against the yeasts compared to the Gram-positive and Gram-negative pathogens. The *C. albicans* clinical strain demonstrated the highest number of synergistic interactions. The greatest number of indifferent and antagonistic interactions were noted when the combinations were tested against the *C. albicans* resistant strain. Unlike the Gram-positive and Gram-negative pathogens, Gentian violet combinations tested against the yeasts did not demonstrate any synergistic interactions. Potassium permanganate demonstrated better efficacy in combination against the yeasts when compared to the Gram-positive and Gram-negative strains.

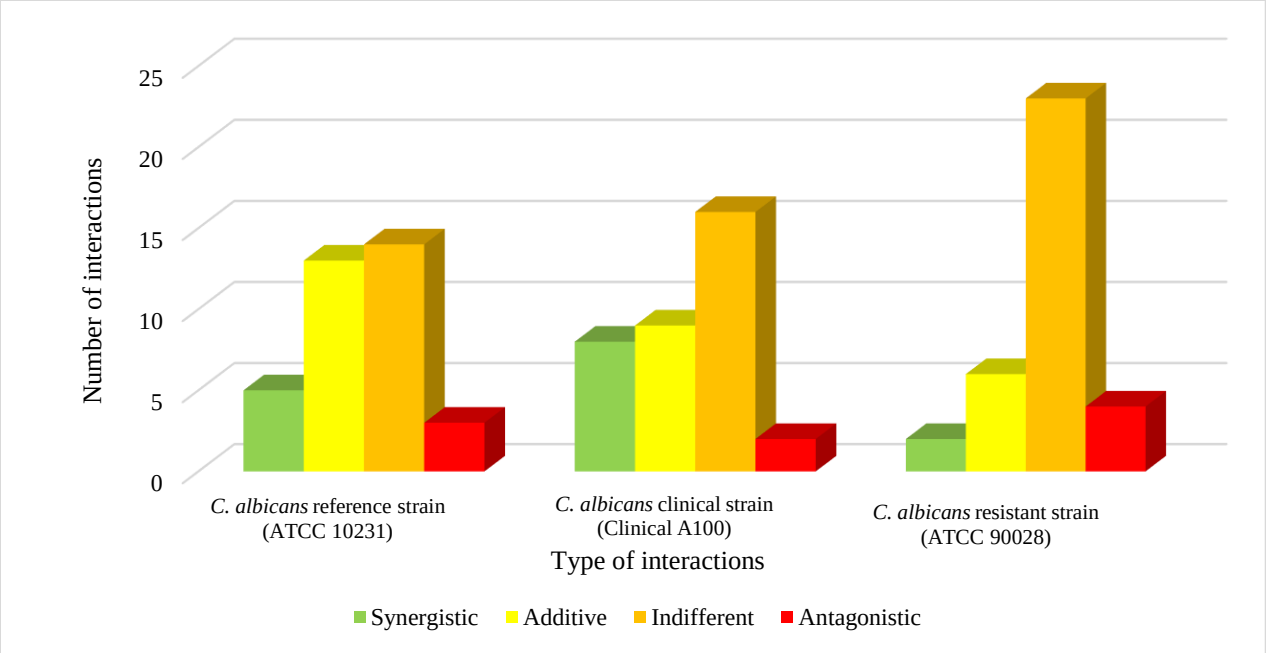


Figure 5.2: Summary of the interactions against the yeasts

5.4. Varied ratio combinations

There was no pattern demonstrated by the 1:1 combinations of medicinal dyes with the commercial antifungals therefore, the varied ratio combinations were determined by their respective ΣFIC values. Unlike the Gram-positives and Gram-negatives, where various options for selection was possible, here one synergistic and one antagonistic combination was chosen for further ratio assessment. These combinations were selected based on the ΣFIC values where the combination noted one or more synergistic or antagonistic interactions against the yeasts (Table 5.10). Isobolograms were then constructed to visualize the varied ratio effects and to determine whether synergy or antagonism was dose dependent.

Table 5.10: Combinations and their corresponding pathogens

Combination		Corresponding Pathogen/s
Synergistic	Malachite green + Miconazole	*C. albicans* clinical strain

Combination		Corresponding Pathogen/s
Antagonistic	Fuchsine + Nystatin	*C. albicans* reference strain

5.4.1. Synergistic varied ratio combinations of medicinal dyes with antimicrobials against yeasts

Malachite green and Miconazole in combination demonstrated synergy against *C. albicans* clinical strain at the 1:1 ratio. (Figure 5.3). All other varied ratios except 6:4 (where Malachite green is dominant) and 2:8 (where Miconazole is dominant) demonstrated a synergistic interaction and hence was included to represent the synergy ratio group. The synergistic interaction was observed to be independent of concentration when the dye and commercial antimicrobial was combined.

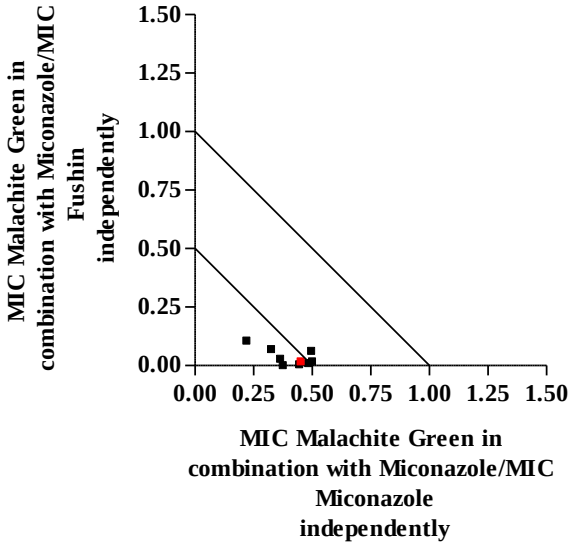


Figure 5.3: Isobologram of combination of Malachite green with Miconazole against *C. albicans* clinical strain

5.4.2. Antagonistic varied ratio combinations of medicinal dyes with antimicrobials against yeasts

Fuchsine and Nystatin demonstrated an antagonistic interaction against *C. albicans* reference strain at the 1:1 ratio when tested. The varied ratios in this combination predominantly demonstrated indifference and was thus included to represent the antagonistic ratio (Figure 5.4).

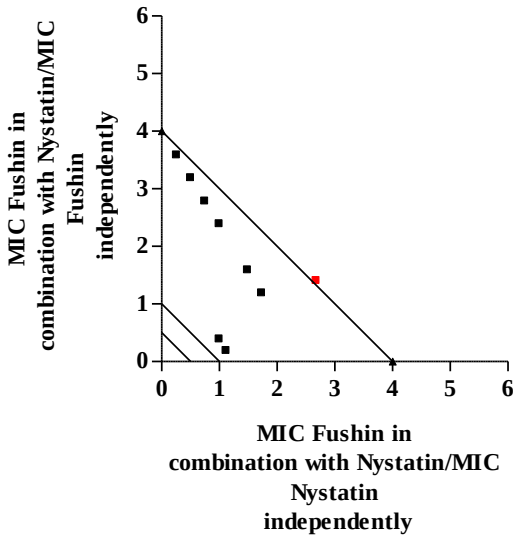


Figure 5.4: Isobologram of combination Fuchsine with Nystatin against *C. albicans* reference strain

Malachite green has previously demonstrated a synergistic interaction with Neomycin against *E. coli* resistant strain. In the case of the yeasts, a synergistic interaction was also observed. This could be attributed to the antifungal which possibly enhances the effects of the Malachite green. Fuchsine has demonstrated both antagonistic and synergistic interactions, however, the antagonism was observed only with the yeasts. This could possibly be due the antifungal when combined with Fuchsine, the dye's antimicrobial activity diminishes and hence an antagonistic interaction is observed.

5.4.3. Overview of varied ratios

A similar pattern was noticed among the yeasts as with the other pathogens tested.

In the combination of Malachite green and Miconazole the majority of the ratios noted synergy as with the other pathogens tested. Synergistic interactions were noted where higher ratios of Malachite green were observed. In the combination of Fuchsine and Nystatin, only the 1:1 ratio noted antagonism all other ratios were the medicinal dye or commercial antimicrobial were dominant noted indifference.

5.5. Summary

- Nystatin demonstrated the strongest activity against *C. albicans* (clinical strain) with an MIC value of 0.20 μg/ml.
- Gentian violet demonstrated the strongest antimicrobial activity against *C. albicans* (reference and clinical strain) with an MFC value of 0.00025 μg/ml.
- The highest number of synergistic interactions were noted against the *C. albicans* (clinical strain).
- The most antagonistic interactions were observed against the *C. albicans* (resistant strain).
- Fuchsine in combination with the antifungals (Ketoconazole, Miconazole, and Nystatin) demonstrated antagonism against the *C. albicans* resistant strain.
- When tested against the yeasts, the synergistic interaction (Malachite green with Miconazole) did not demonstrate synergy at all varied ratios.
- Fuchsine and Nystatin tested against the *C. albicans* reference strain demonstrated antagonism at the 1:1 ratio only and like the Gram-negatives, noted indifferent interactions for the other ratios.

CHAPTER 6

Toxicity studies

6.1. Introduction

The brine shrimp lethality assay (BLSA) was used to determine the toxic effects of the medicinal dyes and commercial antimicrobials. Medicinal dyes are known to have toxic effects (Luk *et al*., 1997; Clifton and Leiken, 2003; Owen, 2012; Maley and Arbiser, 2013; Gopinathan *et al*., 2015), however, Dumisa *et al*. (2020), suggested that combinations with medicinal dyes may demonstrate reduced toxicity. The objective of this chapter was to determine the toxicity of the dyes and their combinations with commercial antimicrobials, and to determine if the toxicity of the dyes in combination can be reduced to a safe level. Figure 6.1 demonstrates the order in which the toxicity studies were carried out.

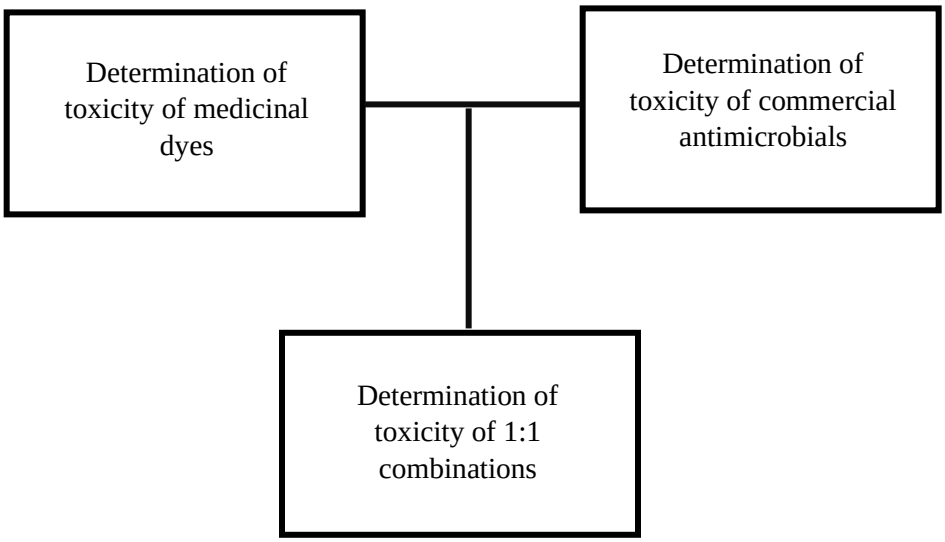


Figure 6.1: Order in which toxicity studies were carried out.

6.2. Results and discussion

6.2.1. Toxicity analysis of medicinal dyes

All seven dyes underwent toxicity studies using the BSLA (Table 6.1). At 24 and 48 hrs, and three of the seven dyes were highly toxic. The percentage mortality and LC_{50} values of each dye is shown in Table 6.1 and discussed under each dye individually.

Table 6.1: Percentage mortality (%) and LC_{50} values of medicinal dyes at 24 and 48 hrs

Medicinal dye	Percentage mortality (%)		LC_{50} (µg/ml)	
	24 hrs	48 hrs	24 hrs	48 hrs
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 32mg/ml)	1.86	6.38	N/A	N/A
Positive control (Potassium dichromate)	100.00	100.00	N/A	N/A

* Bold: Toxic values; N/A: No LC_{50} values are required as these are controls

6.2.1.1. Toxicity analysis of Fuchsine

At 24 hrs, Fuchsine demonstrated a percentage mortality of 39.38%, however, after prolonged exposure the toxicity increased. Fuchsine demonstrated percentage mortality of 90.36% at 48 hrs, while still a high value, in comparison to Gentian violet, Iodine tincture, Malachite green and Mercurochrome has one of the lowest percentage mortalities among the dyes confirming it as one of the least toxic dyes. Fuchsine demonstrated the second highest LC_{50} value (low toxicity) at 24

hrs with a LC_{50} value of 125.00 µg/ml and an LC_{50} value of 31.25 µg/ml at 48 hrs. The high LC_{50} value observed, confirms the low toxicity of the dye as well. To determine at which concentration, the dye is non-toxic, a dose-response was determined. Figure 6.2 displays the dose-dependent relationship of Fuchsine. Fuchsine was observed to be non-toxic at a concentration of 130.00 µg/ml. at 24 hrs and 47.00 µg/ml at 48 hrs (Figure 4.2).

Fuchsine is part of group of dyes known as the triphenyl-methanes which are considered carcinogens (Zhou *et al*., 2019). While Fuchsine is deemed a carcinogenic dye, Dumisa (2020), noted it to be one of the least toxic dyes which is affirmed in the current study. Dumisa observed a dose-dependent relationship with the percentage mortality dropping below 50% at a concentration of 125.00 µg/ml, slightly higher than the current study.

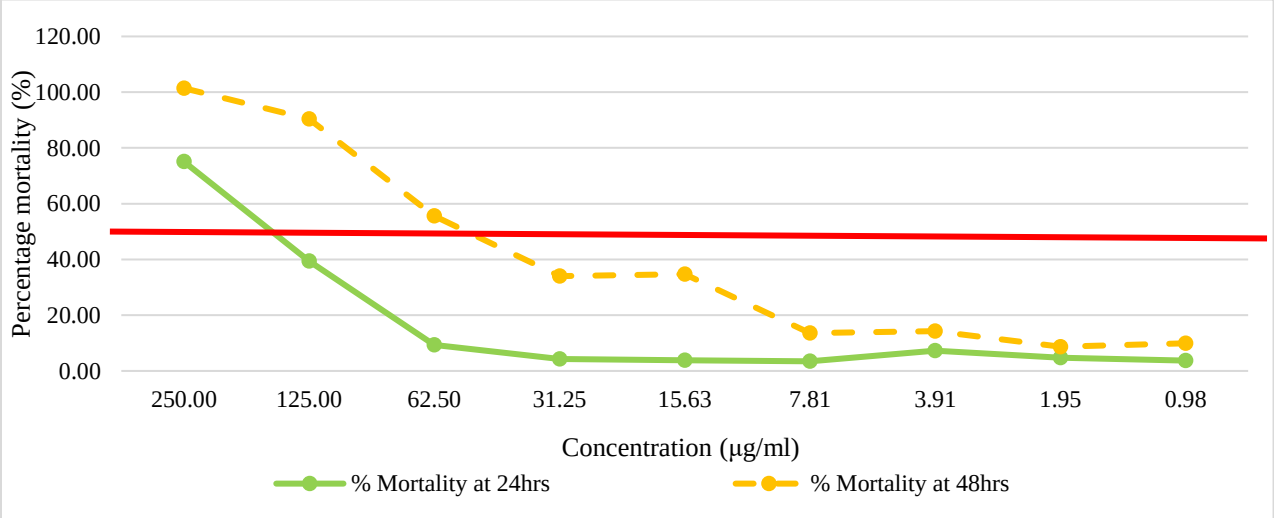


Figure 6.2: Dose dependent percentage mortality of Fuchsine over 24 and 48 hrs when exposed to brine shrimp. The red line delineates toxicity (>50%) from non-toxicity (≤50%)

6.2.1.2. Toxicity analysis of Gentian violet

Gentian violet proved to be highly toxic with a percentage mortality of 100% at 24 and 48 hrs (Table 6.1). A low LC_{50} value of 0.01 µg/ml at 24 hrs and 0.001 µg/ml at 48 hrs was noted for Gentian violet demonstrating the high toxicity. However, as the concentration of the dye decreased so did the percentage mortality, thereby, demonstrating a dose-dependent relationship (Figure 6.3), where Gentian violet was noted to be non-toxic at concentration of 0.016 µg/ml at 24 hrs and 0.001 µg/ml at 48 hrs.

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 when fed large quantities of gentian violet as well as an increase in thyroid cancers when fed to rats (Maley and Arbiser, 2013). Previous studies believed that Gentian violet is 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 being noted (Prabha *et al*., 2020), making the earlier findings by Maley and Arbiser (2013) substantiated. Gentian violet has been used topically to treat various conditions such as atopic eczema with minimal side effects (Prabha *et al*., 2020).

In a later study conducted by Dumisa *et al*. (2020), Gentian violet indicated a high percentage mortality of brine shrimp at 24 and 48 hrs with LC_{50} values of 46.00 µg/ml and 75.00 µg/ml respectively. A dose dependent relationship was noted by Dumisa (2020). While there is a difference in the values noted between Dumisa (2020) and the current study, Dumisa (2020), used a pure powder form after which a desired concentration was made up and in the current study, a pre-made solution was acquired from Dis-chem and the concentration that was already determined was tested. The current study affirms the high percentage mortality and dose dependent relationship as Dumisa (2020).

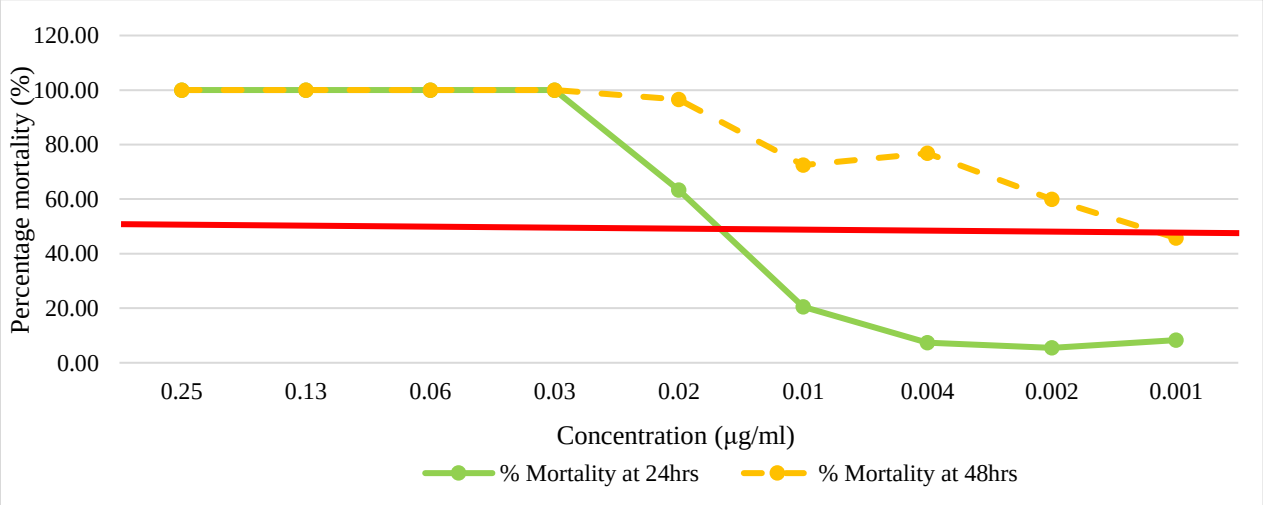


Figure 6.3: Dose dependent percentage mortality of Gentian violet over 24 and 48 hrs when exposed to brine shrimp. The red line delineates toxicity (>50%) from non-toxicity (≤50%)

6.2.1.3. Toxicity analysis of Iodine tincture

Iodine tincture demonstrated the greatest toxicity of all dyes tested with the percentage mortality rate of 100% (Table 6.1) at 24 and 48 hrs even when the dye concentrations were decreased (Figure 6.4). It also noted the lowest LC_{50} value of 0.0001 µg/ml at 24 and 48 hrs confirming the toxicity of the dye. From Figure 4.4, it can be observed that numerous dilutions had to be undertaken before Iodine tincture was deemed non-toxic for the brine shrimp. Iodine tincture observed to be non-toxic at a concentration of 0.004 µg/ml at 24 hrs and 0.00017 µg/ml at 48 hrs (Figure 6.4).

Iodine toxicity is dependent on use. It has been noted that when ingested, Iodine tincture can cause serious corrosive damage to the gastrointestinal tract and mucous membranes (Owen, 2012).

Exposure to the skin and eyes may can cause severe burns and lethal doses have varied in concentration from 200 mg to 20 g (Owen, 2012). Iodine tincture as with Gentian violet is sold in retail stores nationwide in South Africa such as Dis-Chem, Clicks and Med-rite pharmacies.

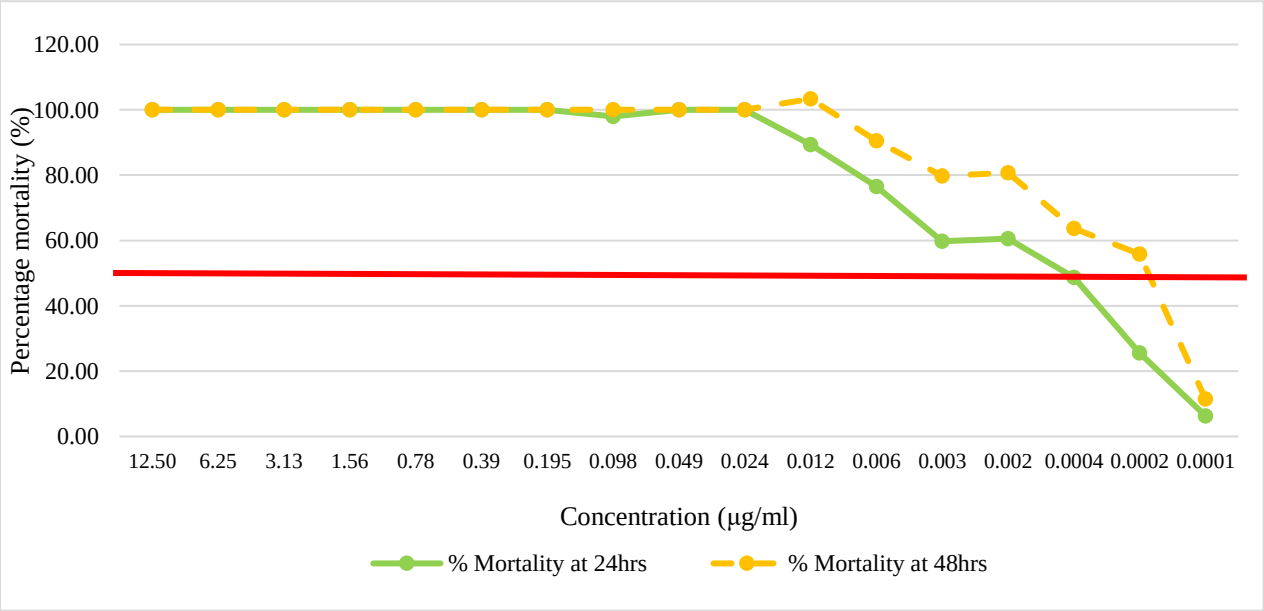


Figure 6.4: Dose dependent percentage mortality of Iodine tincture over 24 and 48 hrs when exposed to brine shrimp. The red line delineates toxicity (>50%) from non-toxicity (≤50%)

6.2.1.4. Toxicity analysis of Malachite green

Malachite green demonstrated a percentage mortality of 38.20% at 24 hrs and 100% at 48 hrs (Table 6.1). At 24 hrs, the low percentage mortality rate makes the dyes non-toxic, however, after prolonged exposure the percentage mortality increases considerably. The same low LC_{50} value of 3.91 µg/ml was noted at 24 and 48 hrs, proving it is a highly toxic dye. Malachite green also demonstrated a dose-dependent relationship as with the previous toxic dyes such as Gentian violet and Iodine tincture (Figure 6.5). At 24 hrs, a concentration of 1.47 µg/ml and at 48 hrs, a concentration of 1.80 µg/ml of Malachite green is deemed non-toxic (Figure 6.5). According to Dumisa (2020), Malachite green was also among the most toxic dyes which had a concentration dependent relationship. As the concentration reduced so did the toxicity.

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 United States, Canada, and Europe, however, 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).

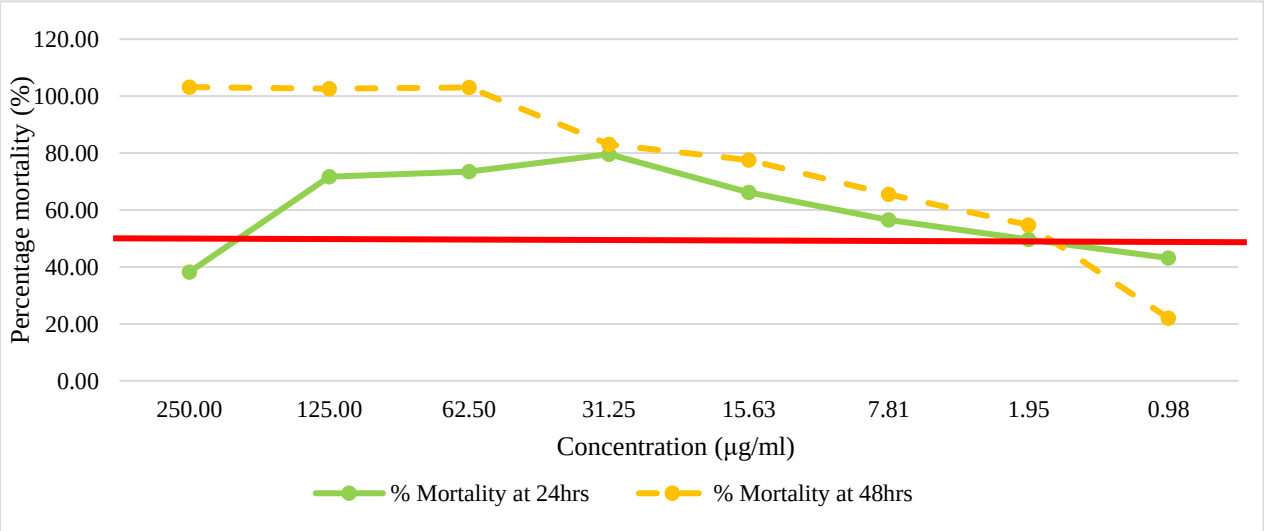


Figure 6.5: Dose dependent percentage mortality of Malachite green over 24 and 48 hrs when exposed to brine shrimp. The red line delineates toxicity (>50%) from non-toxicity (≤50%)

6.2.1.5. Toxicity analysis of Mercurochrome

Mercurochrome demonstrated high toxicity with a percentage mortality of 100% at both 24 and 48 hrs (Table 6.1). An LC_{50} value of 0.02 μg/ml was noted at 24 hrs and a value of 0.01 μg/ml at 48 hrs. It was observed that Mercurochrome was more toxic at 48 hrs in comparison to 24 hrs, however, as both values were very low, the dye is still highly toxic. Mercurochrome also displayed a dose-dependent relationship (Figure 6.6). Mercurochrome was noted to be non-toxic at a concentration of 0.025 μg/ml at 24 hrs and 0.004 μg/ml at 48 hrs (Figure 6.6).

Mercurochrome contains an inorganic compound called mercury which may be readily absorbed in the gastrointestinal tract and can lead to systemic toxicity (Luk *et al*., 1997; Borke and Zieve, 2019). Symptoms of poisoning may include bloody diarrhoea, severe stomach pain, vomiting, mouth sores and speech difficulties (Borke and Zieve, 2019). Most of these symptoms are when it is used intravenously or ingested. When use topically, dermatitis may occur and if in excess, (no quantitative value is specified) it may be absorbed into the blood stream (Cleveland, 1931). It needs to be noted that only if used in excess, toxicity will occur. Mercurochrome, just as Gentian violet, is sold in major retail grocery stores and pharmacies with no indications of its toxicity or side effects. Both Dumisa (2020) and the current study observed Mercurochrome to be one of the most toxic dyes, however, as with the other toxic dyes; a dose dependent relationship was observed. As the concentration was decreased so did Mercurochrome's toxicity.

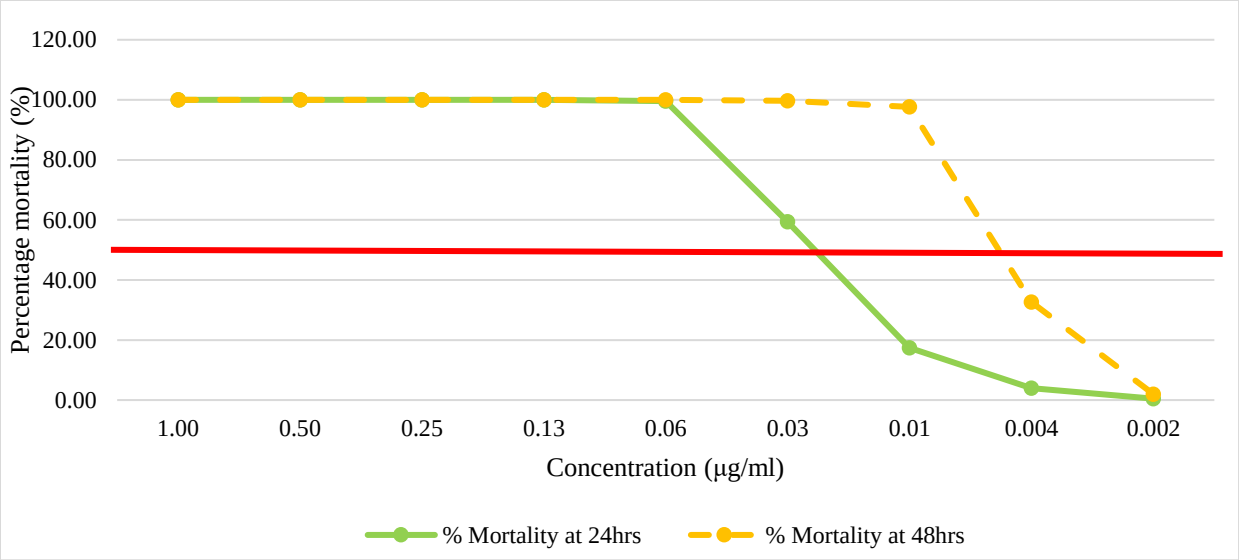


Figure 6.6: Dose dependent percentage mortality of Malachite green over 24 and 48 hrs when exposed to brine shrimp. The red line delineates toxicity (>50%) from non-toxicity (≤50%)

6.2.1.6. Toxicity analysis of Methylene blue

Methylene blue was observed to be the least toxic dye tested owing to the lower mortality rates and higher LC_{50} values (Table 6.1). The percentage mortality of the dye was at 33.33% at 24 hrs and 84.95% at 48 hrs, the lowest among all dyes. The dye demonstrated a LC_{50} value of 250.00 µg/ml at 24 hrs and 125.00 µg/ml at 48 hrs. Previous studies have noted a dose-dependent toxicity with Methylene blue (Clifton and Leiken, 2003). The current study notes a similar dose-dependent relationship (Figure 6.7). As the concentration decreases, so does the toxicity of Methylene blue. Methylene blue was observed to be non-toxic at a concentration of <250.00 µg/ml at 24 hrs and 180.00 µg/ml at 48 hrs (Figure 6.7).

Some toxic reactions to Methylene blue observed are fever, vomiting, nausea, abdominal pain, and a bluish discoloration of the skin (Clifton and Leiken, 2003). The discoloration was also observed in the current study and counting of the brine shrimp was difficult. A higher magnification was required to count the brine shrimp in the assay. A study noted methylene blue toxicity in a neonate causing acute renal failure, hemolytic anemia, and hyperbilirubinemia (Albert *et al*., 2003). Methylene blue has been known to prevent moss formation in fish tanks due to its antibacterial and antifungal activity at a diluted concentration of 0.0001% (Podrabsky, 1999; Ari *et al*., 2021).

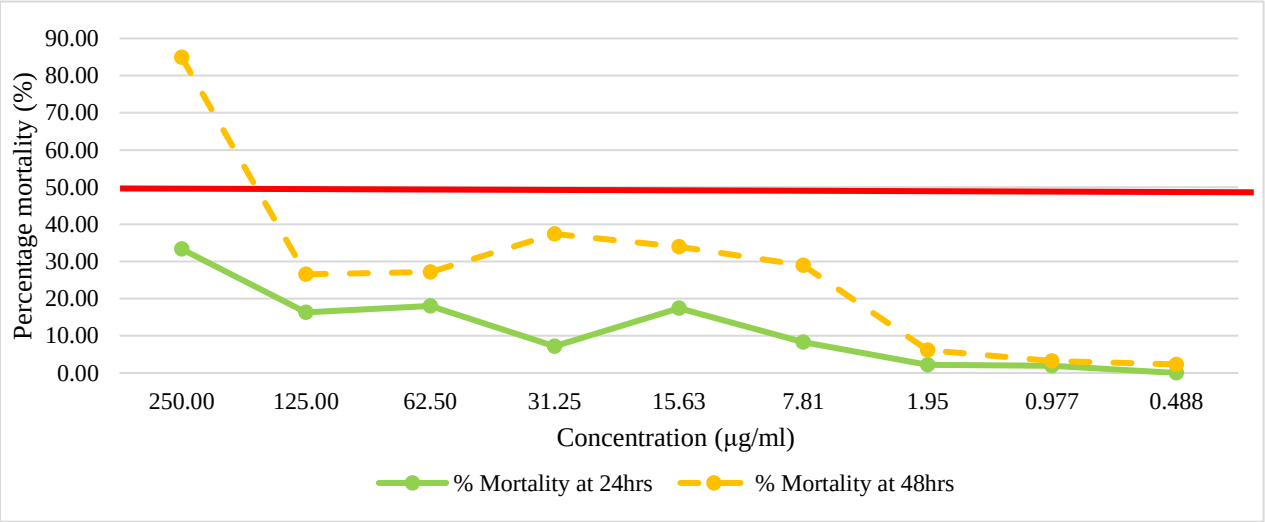


Figure 6.7: Dose dependent percentage mortality of Methylene blue over 24 and 48 hrs when exposed to brine shrimp. The red line delineates toxicity (>50%) from non-toxicity (≤50%)

6.2.1.7. Toxicity analysis of Potassium permanganate

A percentage mortality of 71.42% was observed at 24 hrs, the second highest among the medicinal dyes, and a percentage mortality of 94.30% was observed at 48 hrs. Relatively lower toxicity was observed in comparison to the other medicinal dyes. The LC_{50} values of 125 µg/ml at 24 hrs and 15.63 µg/ml at 48 hrs was observed for Potassium permanganate (Table 6.1). While non-toxic at 24 hrs, the LC_{50} value of the dye drastically decreased to 15.63 µg/ml at 48 hrs demonstrating its moderate toxicity. A concentration dependent relationship was observed with Potassium permanganate, as the concentration decreased so did the toxicity. Potassium permanganate demonstrated non-toxicity at a concentration of 185.00 µg/ml at 24 hrs and 15.63 µg/ml at 48 hrs, the same as the LC_{50} value (Figure 6.8).

The ingestion of Potassium permanganate crystals results in corrosive burns to the mouth, esophagus, and trachea (Southwood *et al*., 1987). The use of Potassium permanganate for skin infections such as perianal ulcers can result in necrotic areas and ulcerations (Riehemann *et al*., 2010). After investigating the cause of the ulcerations, Riehemann *et al*. (2010) determined that they were caused by caustic burns from the use of potassium permanganate. Poisoning with

Potassium permanganate is not common; however, it may lead to vomiting, nausea, gastrointestinal ulceration, and burn (Korkut *et al*., 2013).

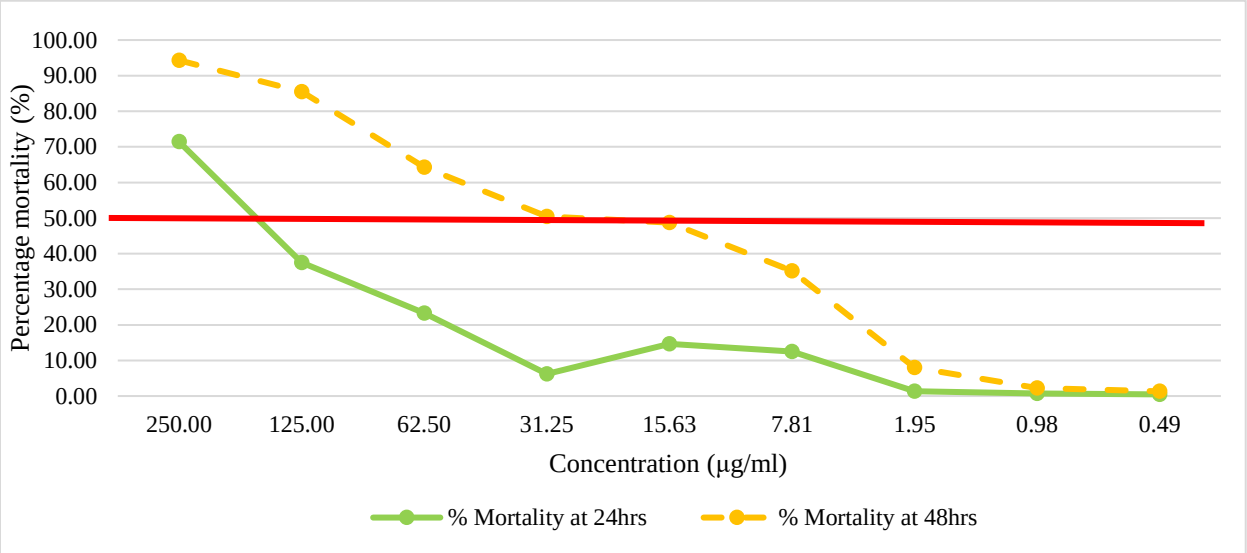


Figure 6.8: Dose dependent percentage mortality of Potassium permanganate over 24 and 48 hrs when exposed to brine shrimp. The red line delineates toxicity (>50%) from non-toxicity (≤50%)

6.3. Overview of the toxicity of the medicinal dyes

The BLSA has demonstrated that most of the medicinal dyes are toxic when tested at a starting concentration of 250 µg/ml. At 24 hrs, 57.14% of the dyes have a percentage mortality above 50% (Gentian violet, Iodine tincture, Mercurochrome and Potassium permanganate). At 48 hrs, all the dyes have percentage mortalities above 50%. According to the LC_{50} values, only four out of the seven dyes (57.14%) are toxic at both 24 and 48 hrs. Iodine tincture, however, is the most toxic of the dyes with a high mortality rate and the lowest concentration (0.0001 µg/ml) required to reach a percentage mortality of below 50%. A concentration dependent relationship was observed among the majority of the toxic dyes. As the concentration decreased so did the toxicity of the medicinal dye. According to the LC_{50} values, Methylene blue, Fuchsine and Potassium permanganate proved to be the least toxic. Table 6.2 shows the non-toxic (safe) concentrations (as determined in Figures 6.2 to 6.8) of the medicinal dyes when tested against the brine-shrimp. Methylene blue demonstrated the least toxicity with Iodine tincture proving to be the most toxic dye.

Table 6.2: Summary of non-toxic concentrations of the medicinal dyes

Medicinal dyes	Concentration at which dye is non-toxic at 24 hrs (µg/ml)	Concentration at which dye is non-toxic at 48 hrs (µg/ml)
Fuchsine	130.00	47.00
Gentian violet	0.02	0.001
Iodine tincture	0.004	0.00017
Malachite green	1.47	1.80
Mercurochrome	0.03	0.004
Methylene blue	250.00	180.00
Potassium permanganate	185.00	15.63

6.4. Toxicity analysis of commercial antimicrobials

The toxicity data of the commercial antimicrobials are shown together with the individual LC_{50} values and percentage mortalities in Table 6.3. The commercial antimicrobials demonstrated lower toxicity than the dyes. With the exception of Betadine®, Mupirocin and Steriscrub all commercial antimicrobials were non-toxic at the starting concentration, and hence no further concentration-dependent studies were undertaken.

Table 6.3: LC_{50} values and percentage mortality (%) of antimicrobials at 24 and 48 hrs

Conventional antimicrobial	Percentage mortality (%)		LC_{50} (µg/ml)	
	24 hrs	48 hrs	24 hrs	48 hrs
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				

Conventional antimicrobial	Percentage mortality (%)		LC_{50} (µg/ml)	
	24 hrs	48 hrs	24 hrs	48 hrs
Negative control (Salt water 32mg/ml)	1.86	6.38	N/A	N/A
Positive control (Potassium dichromate)	100.00	100.00	N/A	N/A

*Bold: Toxic values; N/A: No LC_{50} values are required as these are controls

6.4.1. Toxicity analysis of Betadine®

Betadine® demonstrated a high percentage mortality of 100% at 24 and 48 hrs and an LC_{50} value of 0.20 µg/ml at both 24 and 48 hrs (Table 6.3). Betadine® demonstrated the highest toxicity among all the antimicrobials. Betadine® (povidone-iodine) toxicity is dependent on concentration. Toxicity occurs at higher concentrations and lowering the concentration leads to a non-toxic (0.34 µg/ml at 24 hrs and 0.30 µg/ml at 48 hrs) effect (Figure 6.9).

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).

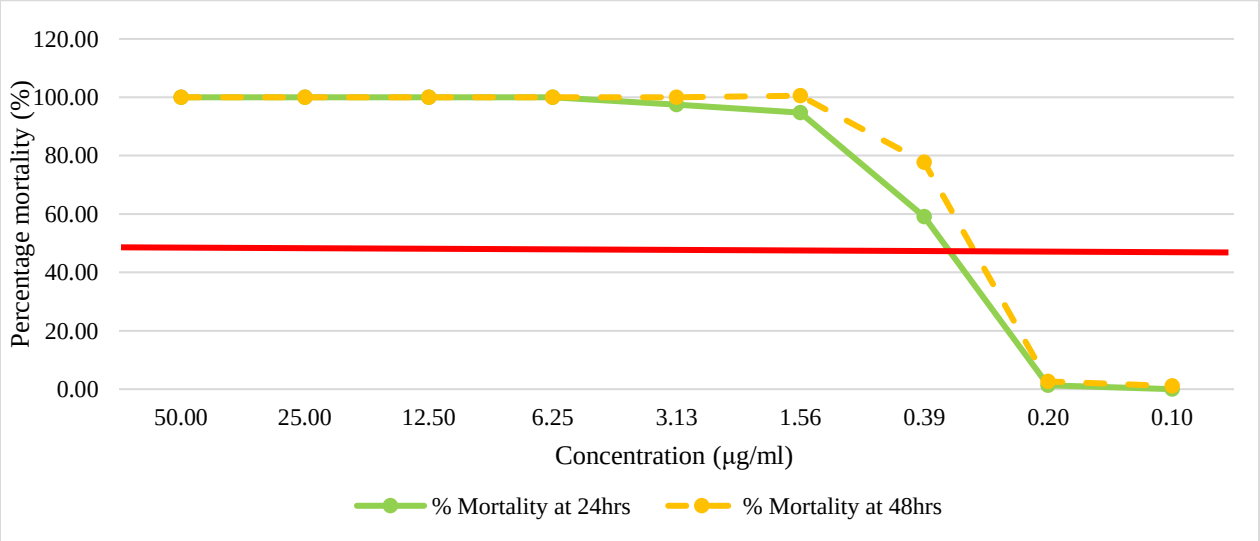


Figure 6.9: Dose dependent percentage mortality of Betadine® over 24 and 48 hrs when exposed to brine shrimp. The red line delineates toxicity (>50%) from non-toxicity (≤50%)

6.4.2. Toxicity analysis of Fusidic acid

A low percentage mortality of 9.78% was observed at 24 hrs and a percentage mortality of 46.79% was observed at 48 hrs (Table 6.3). While the percentage mortality at 48 hrs is higher than 24 hrs, the mortality is still below 50%. Fusidic acid demonstrated the same LC_{50} value of 500.00 µg/ml at 24 and 48 hrs.

Treatment with Fusidic acid is generally well tolerated and mild gastrointestinal side effects may occur such as diarrhoea and headaches (He *et al*., 2019). Very rarely, but more severe side effects may occur such as thrombocytopenia and Fusidic acid induced hepatotoxicity (liver damage) and hematologic toxicity (He *et al*., 2019). Fusidic acid topically, is well tolerated and is currently used in a cream and ointment called Fucidin (Rossiter *et al*., 2020).

6.4.3. Toxicity analysis of Gentamycin and Neomycin

Gentamycin demonstrated a percentage mortality of 0% at 24 hrs and a very low percentage mortality of 1.11% at 48 hrs. Neomycin noted a percentage mortality of 4.10% at 24 hrs and 5.84% at 48 hrs. Both antibiotics observed an LC_{50} value of 500 µg/ml at 24 and 48 hrs. Both antibiotics demonstrate low toxicity with concentrations of 500.00 µg/ml at 24 and 48 hrs (Table 6.3).

Gentamycin and Neomycin are both from the class of aminoglycosides and are common in treating Gram-negative infections, however, they have been associated with nephrotoxicity (Sandoval *et al*., 2006; Rossiter and Blackman, 2012). Current toxicity studies on Gentamycin and Neomycin have noted low toxicity of 500 µg/ml. While the brine shrimp assay does show low toxicity levels of Gentamycin, the antibiotic has been known to cause otoxicity (toxicity of the ear), hearing loss and vestibular disorders (Noack *et al*., 2017). Nephrotoxicity (kidney damage) 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). When the brine shrimp lethality assay was conducted in this study, only a single dose was used and effects were tested at 24 and 48 hrs, possibly leading to the low toxicity value found in the current study. When used topically, not many adverse effects

were noted apart from irritation such as erythema and pruritus and should these side effects occur, there is no need to discontinue treatment (Pasmooij, 2018). Neomycin is known to have the same side effect profile as gentamycin such as the ototoxicity and nephrotoxicity (Veirup and Kyriakopoulos, 2020).

6.4.4. Toxicity analysis of Ketoconazole

Ketoconazole was non-toxic at 24 and 48 hrs when tested in the brine shrimp lethality assay. A percentage mortality of 8.31% at 24 hrs and 29.58% at 48 hrs. An LC_{50} of 500 µg/ml was observed at 24 and 48 hrs. As with Fusidic acid, Gentamycin and Neomycin, Ketoconazole is noted to be a commercial antimicrobial with low toxicity at 24 and 48 hrs at a concentration of 500 µg/ml.

Ketoconazole is part of a family called imidazole's and they are known for their liver and hormonal toxicities when used systemically (Mourad and Perfect, 2018). Ketoconazole may also be known to cause abdominal pain, tongue discolouration, vomiting and constipation (Sinawe and Casadesus, 2020). When used topically, it may cause itchiness, dryness and stinging at the site of application. Hypersensitivity reactions such as anaphylaxis and urticaria may also occur (Sinawe and Casadesus, 2020). While the antifungal application has been noted to have some serious toxic effects (Mourad and Perfect, 2018; Sinawe and Casadesus, 2020), it demonstrated a non-toxic concentration of 500 µg/ml in the current study and displays lesser toxic effects when used topically.

6.4.5. Toxicity analysis of Miconazole

As with Ketoconazole, Miconazole was non-toxic when tested in the brine shrimp lethality assay. A low percentage mortality of 0.72% was observed at 24 hrs and 6.62% at 48 hrs (Table 6.3). An LC_{50} value of 500 µg/ml was observed for Miconazole at 24 and 48 hrs. Non-toxic concentrations of Miconazole was observed at <500.00 µg/ml at 24 and 48 hrs.

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).

6.4.6. Toxicity analysis of Mupirocin

Mupirocin noted a percentage mortality of 35.34% at 24 hrs and 72.22% at 48 hrs (Table 6.3), much higher percentage mortalities compared to some of the other commercial antimicrobials. Mupirocin, at 24 hrs demonstrated a LC_{50} value of 500 µg/ml, however, at 48 hrs, an LC_{50} value of 125 µg/ml was noted. In order to determine at which concentration Mupirocin is non-toxic, a dose response was determined. Mupirocin demonstrated non-toxic concentrations of 500.00 µg/ml at 24 hrs and 165.00 µg/ml at 48 hrs (Figure 6.10).

A study tested the cytotoxicity of Mupirocin against human fibroblast cells and noted that at all concentrations tested (1.95 - 1000 µg/ml), 80% or more of the cell population survived (Bakkiyaraj *et al.*, 2017). When used topically, Mupirocin is generally well tolerated, however, some side effects such as allergic dermatitis, burning, itching and redness have been noted (Punjataewakupt *et al.*, 2019).

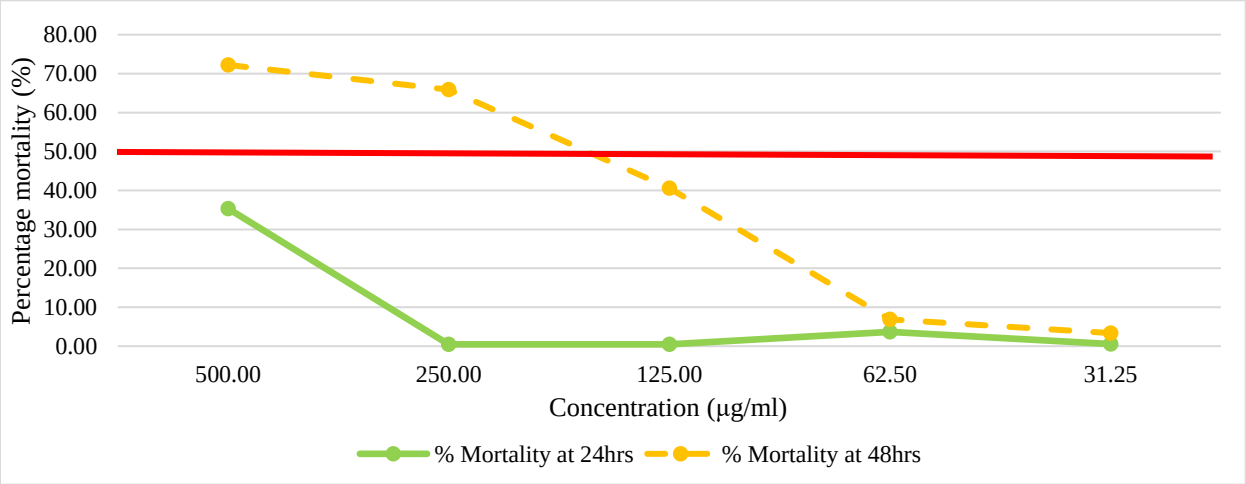


Figure 6.10: Dose dependent percentage mortality of Mupirocin over 24 and 48 hrs when exposed to brine shrimp. The red line delineates toxicity (>50%) from non-toxicity (≤50%)

6.4.7. Toxicity analysis of Nystatin

When testing Nystatin against brine shrimp, no mortality at 24 hrs was observed, but at 48 hrs the percentage jumps up to 40.17%. The percentage mortality is still below 50% therefore, Nystatin is

non-toxic at both 24 and 48 hr (Table 6.3). Compared to the other two antifungals (Ketoconazole and Miconazole), Nystatin noted a higher percentage mortality at 48 hrs. Nystatin demonstrated low toxicity at 24 and 48 hrs with a concentration of 500.00 µg/ml.

Hübsch (2014), tested the toxicity of Nystatin in the brine shrimp lethality assay and noted that Nystatin demonstrated no toxicity at 24 and 48 hrs. The current study supports these results. Intravenously, Nystatin is highly toxic, hence topically use is only recommended (Scheibler *et al*., 2017). Nystatin is deemed non-toxic when used topically as it is not absorbed through the skin or mucosa (Scheibler *et al*., 2017).

6.4.8. Toxicity analysis of Steriscrub

Steriscrub noted a percentage mortality value of 46.61% at 24 hrs and high percentage mortality of 92.84% at 48 hrs (Table 6.3). Steriscrub (chlorohexidine) was also the second commercial antimicrobial to demonstrate moderate toxicity with a LC_{50} value of 250.00 µg/ml at 48 hrs (Figure 6.11). An LC_{50} value of 500.00 µg/ml was observed at 24 hrs. Steriscrub demonstrated a concentration-dependent relationship (Figure 6.11). Non-toxic concentrations of Mupirocin were noted was <500.00 µg/ml at 24 hrs and 375.00 µg/ml at 48 hrs.

Previous literature noted that the toxicity of Steriscrub was dependent on the duration of exposure and the concentration used (Punjataewakupt *et al*., 2019). The current study affirms this statement as the toxicity of Steriscrub decreased as the concentration decreased when tested against the brine shrimp (Figure 6.11). Steriscrub toxicity has been noted in human osteoblast and cartilage and therefore, should be used with caution when used to irrigate wounds (Punjataewakupt *et al*., 2019). When compared to the other commercial antimicrobials, Steriscrub demonstrated higher toxicity. In a previous study (Punjataewakupt *et al*., 2019), Steriscrub displayed higher toxicity when used topically.

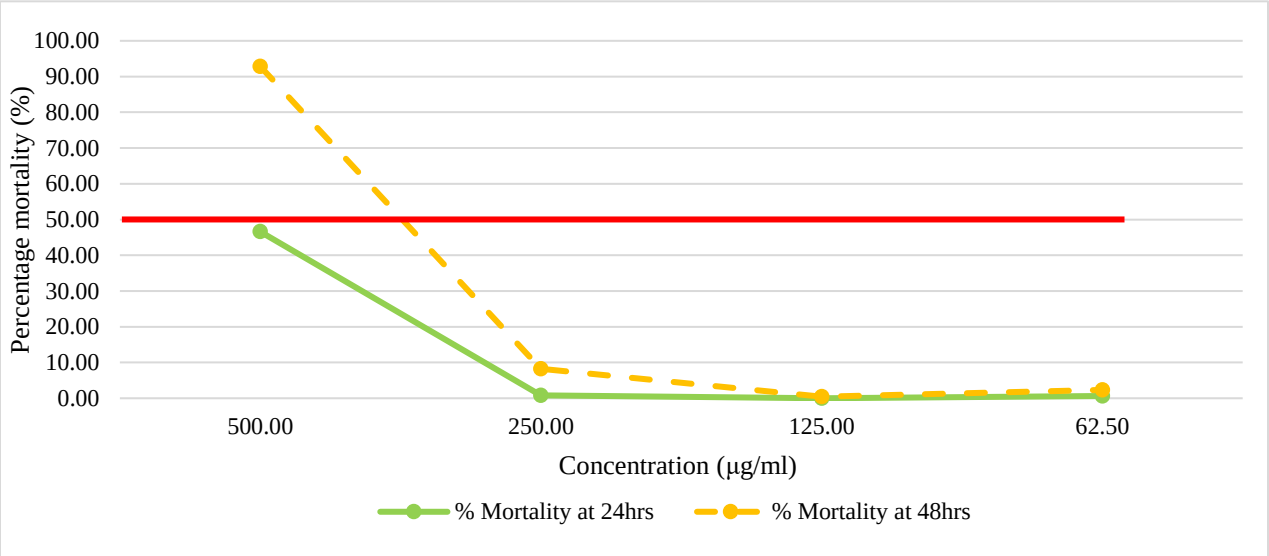


Figure 6.11: Dose dependent percentage mortality of Steriscrub over 24 and 48 hrs when exposed to brine shrimp. The red line delineates toxicity (>50%) from non-toxicity (≤50%)

6.5. Overview of the commercial antimicrobials

Unlike the medicinal dyes, 22.22% of commercial antimicrobials were toxic while the rest demonstrated minimal toxicity at the highest concentration tested. Betadine® and Steriscrub were the only two commercial antimicrobials to demonstrate toxicity at both 24 and 48 hrs. All antimicrobials demonstrated the same LC_{50} value of 500.00 µg/ml at 24 hrs, with only Betadine®, Mupirocin and Steriscrub) changing at 48 hrs. Betadine® is noted to be the most toxic dye with a percentage mortality of 100% at 24 and 48 hrs, requiring a concentration of 0.295 µg/ml as the least toxic value at 48 hrs. Table 6.4 demonstrated the concentrations of the commercial antimicrobials that are non-toxic (safe). Betadine® demonstrated the most toxicity in comparison to the other commercial antimicrobials tested.

Table 6.4: Summary of non-toxic concentrations of the commercial antimicrobials

Commercial antimicrobials	Concentration at which commercial antimicrobial is non-toxic at 24 hrs (µg/ml)	Concentration at which commercial antimicrobial is non-toxic at 48 hrs (µg/ml)
Betadine®	0.34	0.295

Commercial antimicrobials	Concentration at which commercial antimicrobial is non-toxic at 24 hrs (µg/ml)	Concentration at which commercial antimicrobial is non-toxic at 48 hrs (µg/ml)
Fusidic acid	≤500.00	≤500.00
Gentamycin	≤500.00	≤500.00
Ketoconazole	≤500.00	≤500.00
Miconazole	≤500.00	≤500.00
Mupirocin	≤500.00	165.00
Neomycin	≤500.00	≤500.00
Nystatin	≤500.00	≤500.00
Steriscrub	≤500.00	375.00

6.6. Toxicity analysis of 1:1 combinations

The medicinal dyes exhibited very high toxicity and therefore were combined with various commercial antimicrobials to determine if the toxicity could be reduced. As antimicrobial synergy was an important consideration the possible combinations were narrowed down to six synergistic combinations. Antimicrobial antagonism also may impact on the combined use and hence eight combinations were selected out of 11. The antagonistic combinations chosen were based on: if more than one strain (among the pathogens tested) demonstrated antagonism as well as if no synergy was observed. The antagonistic combinations demonstrated no pattern and therefore, were examined more closely. The selections and reason for selection is given in Table 6.5.

Table 6.5: Combinations chosen for Brine shrimp assay

Combinations of dye and commercial antimicrobial		Reasoning of chosen combination based on antimicrobial activity for toxicity studies
Synergistic	Fuchsine + Gentamycin	Synergy as well as mainly additive interactions among the Gram-negative pathogens (Chapter 4, Table 4.3 and 4.4)
	Fuchsine + Steriscrub	Synergy was noted against two Gram-negative pathogens, with additive interactions (Chapter 4, Table 4.3 and 4.4)

Combinations of dye and commercial antimicrobial		Reasoning of chosen combination based on antimicrobial activity for toxicity studies
	Gentian violet + Gentamycin	Synergy was noted with only one antagonistic interaction against the Gram-negative strains (Chapter 4, Table 4.5 and 4.6)
	Malachite green + Neomycin	Synergy was noted with only one antagonistic interaction against the Gram-negative strains (Chapter 4, Table 4.9 and 4.10)
	Malachite green + Miconazole	Synergy was noted against the yeast. No antagonistic interaction was noted with this combination. (Chapter 5, Table 5.6)
	Mercurochrome + Betadine®	Synergism was noted among majority of Gram-positive strains, no antagonism was noted with this combination (Chapter 3, Table 3.11 and 3.12)
Antagonistic	Fuchsine + Nystatin	Two out of three strains noted antagonism against this combination and no synergy. (Chapter 5, Table 5.3)
	Gentian violet + Betadine®	Antagonism was noted with these combinations, with mostly indifferent interactions when tested against the Gram-negative strains. (Chapter 4, Table 4.5 and 4.6)
	Gentian violet + Neomycin	Antagonism was noted with this combination (Chapter 4, Table 4.5 and 4.6)
	Gentian violet + Steriscrub	Antagonism was noted with this combination (Chapter 3, Table 3.5 and 3.6)
	Iodine tincture + Fusidic acid	Three antagonistic interactions noted against the combination when tested against the Gram-positive strains. No synergy was noted. (Chapter 3, Table 3.7 and 3.8)
	Malachite green + Betadine®	Three antagonistic interactions noted with no synergy among the Gram-positive strains tested. (Chapter 3, Table 3.9 and 3.10)
	Mercurochrome + Steriscrub	Antagonistic interactions noted when tested among the Gram-negative strains. No synergy noted. (Chapter 3, Table 3.11 and 3.12)

Combinations of dye and commercial antimicrobial		Reasoning of chosen combination based on antimicrobial activity for toxicity studies
	Methylene blue + Gentamycin	Antagonism was noted with this combination. (Chapter 4, Table 3.13 and 3.10)

All combinations tested against the brine shrimp are shown in Table 6.6. The table shows the toxicity of dyes independently as well as in combination with commercial antimicrobials when exposed to the brine shrimp.

The combination of Gentian violet with Gentamycin demonstrated the greatest decrease in dye toxicity. Hence, this combination was the most synergistic combination. An over fifteen-fold decrease is observed with Gentian violet when combined with the commercial antimicrobial. Three other combinations demonstrated a decrease in toxicity and were: Fuchsine with Gentamycin, Malachite green with Neomycin and Malachite green with Miconazole. Mercurochrome combined with Betadine® demonstrated a fivefold 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.

Figure 6.12 shows a visual representation of the individual toxicity and the toxicity when combined with the commercial antimicrobials of combinations which demonstrated antimicrobial synergy (as in Table 6.5). Out of the synergistic combinations tested, 66.67% of these combinations demonstrated a decrease in toxicity when tested against the brine shrimp.

As expected, a majority of the antagonistic combinations noted increased toxicity (i.e., antagonism), however, there were a few combinations that demonstrated decreased toxicity (synergism) while not having strong antimicrobial activity (Table 6.6). Figure 6.13 shows a visual representation of the combination's toxicity and the toxicities independently. 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 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.

Linezolid is used to treat tuberculosis, however, it is a toxic drug to the host; a combination of bedaquiline, pretomanid, and linezolid was 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 one in terms of medicinal dyes, it does demonstrate that combinations of antimicrobials are able to have possibly better efficacy as well as reduced toxicity to the host.

Dumisa (2020), noted a decrease in toxicity in the Gentian violet combinations tested. The current study also noted a decrease in the toxicity of Gentian violet tested, however, only one combination was tested. Malachite green when tested with plant extracts demonstrated reduced toxicity at 48 hrs (Dumisa, 2020). As with the current study, Dumisa (2020) noted concentration dependent toxicity. In the previous study, Methylene blue combinations noted toxicity, however, as the concentrations were decreased so did the toxicity. The same was noted for the current study and as with the previous dyes a concentration dependent relationship was noted.

Table 6.6: Combinations and their change in toxicity

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			
Syne... Fuchsine + Gentamycin	31.25	**62.50**	41.04	**125.00**	**Decrease**

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			
	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**

Bold: Decrease in toxicity

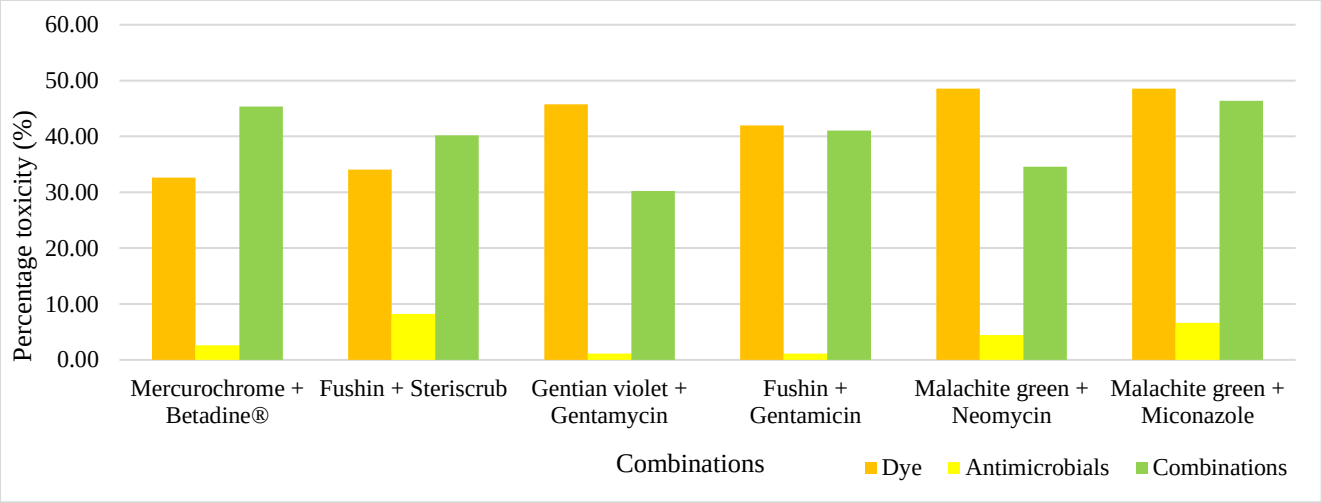


Figure 6.12: Toxicity of combinations which demonstrated antimicrobial synergy

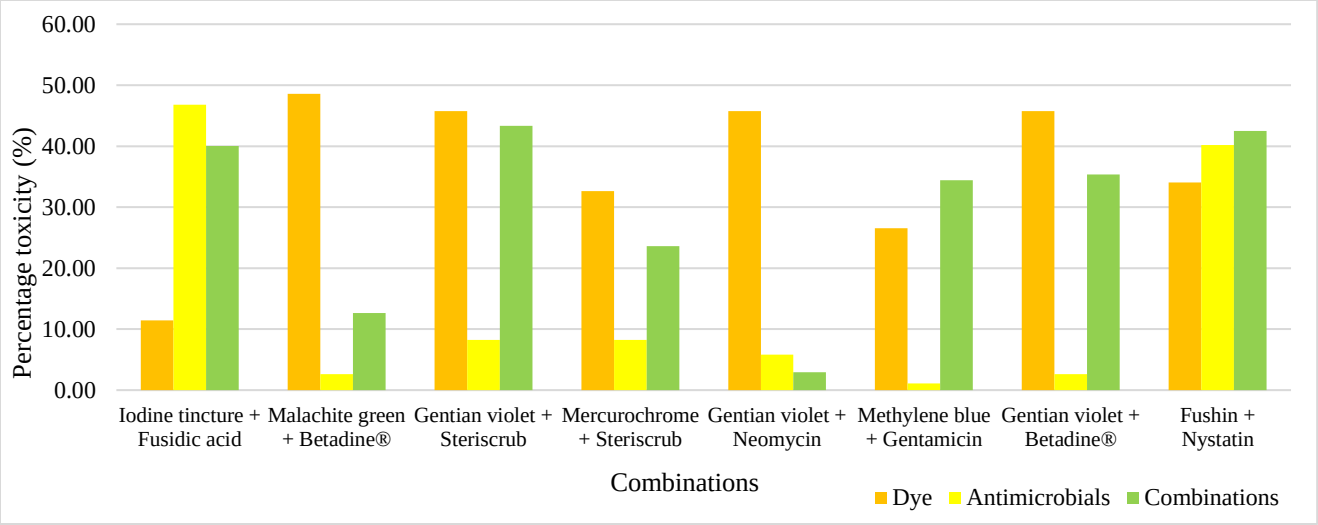


Figure 6.13: Toxicity of combinations which demonstrated antimicrobial antagonism

6.7. Overview of the combinations

The combinations had varying results overall i.e., not all the synergistic combinations demonstrated decreased toxicity and not all the antagonistic combinations demonstrated increased toxicity. All combinations demonstrated dose-dependent relationships. According to Table 6.5 and 6.6, four out of six synergistic combinations (66.67%) noted decreased toxicity when tested against the brine shrimp lethality assay. These four combinations: Fuchsine with Gentamycin, Gentian violet with Gentamycin, Malachite green with Neomycin and Malachite green with Miconazole, noted greatly reduced toxicity in the dyes (Figure 6.12). In comparison, the antagonistic

combinations noted lesser combinations with decreased toxicity. Among the combinations which demonstrated reduced toxicity, it was observed that mainly combinations with the commercial antibacterials (i.e., Gentamycin and Neomycin) and antifungals (i.e., Miconazole) noted a decrease in the toxicity of the dye. This change could be attributed to the antibiotics and antifungal with which the dye was combined with, as both (antibiotic and antifungal) note an LC_{50} values of 500.00 µg/ml which are non-toxic concentrations to begin with. This affirms the potential in combining medicinal dyes with commercial antimicrobials to reduce the toxicity. Table 6.7 notes the non-toxic (safe) concentrations of the combinations of interest.

Table 6.7: Summary of non-toxic combinations

Combinations	**LC_{50} value of combination (µg/ml)**	**Percentage mortality (%) at LC_{50} value (24 hrs)**	**Percentage mortality (%) at LC_{50} value (48 hrs)**
Fuchsine with Gentamycin	125.00	7.50	41.04
Gentian violet with Gentamycin	15.64	9.13	30.21
Malachite green with Neomycin	15.62	19.46	34.59
Malachite green with Miconazole	15.62	27.00	46.39

6.8. Summary

- All medicinal dyes tested, and a majority of the commercial antimicrobials demonstrated a concentration-dependent toxic relationship.
- Gentian violet, Iodine tincture and Mercurochrome demonstrate high toxicity at 24 and 48 hrs with a percentage mortality of 100%.
- Malachite green, while highly toxic at 48 hrs (percentage mortality of 100%), did demonstrate low toxicity at 24 hrs.
- Iodine tincture demonstrated the highest toxicity with an LC_{50} value of 0.0001 μg/ml at 24 and 48 hrs with a percentage mortality of 100.00% at both 24 and 48 hrs.
- Methylene blue (among the dyes) demonstrated the lowest toxicity with an LC_{50} value of 250.00 μg/ml at 24 hrs and 125.00 μg/ml at 48 hrs.
- Among the commercial antimicrobials, Betadine® demonstrated the highest toxicity with a percentage mortality of 100% and an LC_{50} value of 0.20 μg/ml at 24 and 48 hrs.
- In the synergistic combinations: Gentian violet and Gentamycin demonstrated the highest reduction in toxicity.
- Mercurochrome with Betadine® demonstrated the highest increase in toxicity among the synergistic combinations.
- Among the antagonistic combinations, Mercurochrome and Steriscrub demonstrated the highest reduction in toxicity.
- Four synergistic combinations noted reduced toxicity (Fuchsine with Gentamycin, Gentian violet with Gentamycin, Malachite green with Miconazole and Malachite green with Neomycin).

CHAPTER 7

Selectivity index of combinations and time-kill studies

7.1. Introduction

The selectivity index (SI) is used to determine the relationship of the safety of the host to toxicity of various compounds. Time-kill studies were then conducted on the selected combinations demonstrating least toxicity in relationship to highest antimicrobial activity over a period of time. Figure 7.1 demonstrates the order in which the assays were carried out.

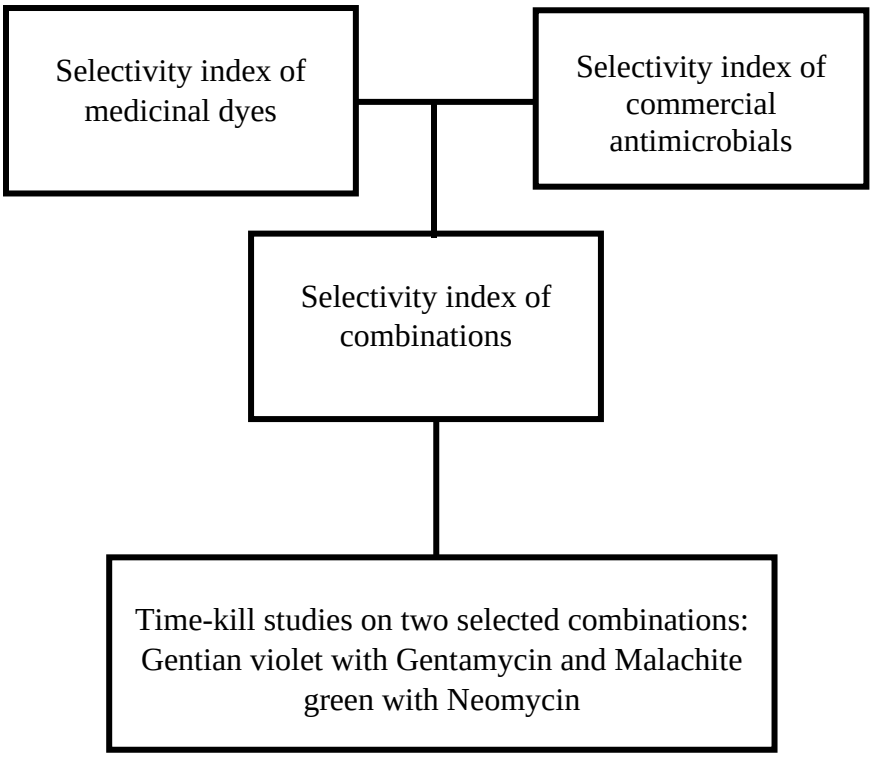


Figure 7.1: Order in which studies are carried out

7.2. Selectivity index of medicinal dyes against the Gram-positive strains

Table 7.1 demonstrates the SI of the dyes against the Gram-positive pathogens. The majority of the medicinal dyes demonstrated low SI values. The LC_{50} values (shaded area of the toxicity of each sample is provided here as well as previously in Chapter 5 for ease of reference). Fuchsine and Gentian violet noted a high SI value of 20.03 and 32.26 against the *S. epidermidis* clinical strain respectively. The high SI values (>10.00) mean that Fuchsine and Gentian violet are safe to use with low toxic effects.

Iodine tincture demonstrated the lowest SI value of 0.0003 against the *S. epidermidis* resistant strain. This means that while the dye has a strong antimicrobial activity, it is still highly toxic against the host as well. Iodine tincture also noted the lowest SI values of 0.0001-0.06 against the other Gram-positive strains.

Malachite green demonstrated the highest SI values of 130.33 against GMRSA and 4344.44 against the *S. epidermidis* reference strain. Methylene blue also notes an SI value of 10.00 against the *S. epidermidis* reference strain demonstrating that these dyes (Malachite green and Methylene blue) have strong antimicrobial activity against the strain while demonstrating low to no toxic effects against the host.

Potassium permanganate noted a low SI value of 0.002 against the *S. aureus* reference strain, the *S. epidermidis* clinical and the *S. epidermidis* resistant strain. The Gram-positive strains demonstrated low susceptibility to Potassium permanganate, however, it was one of the least toxic dyes. Certain SI values are denoted with a “U’ meaning the SI values could not be calculated as the MIC/MBC values were >8000.000 and an endpoint value was unable to be determined.

Dzoyem *et al*. (2016) suggested that a good selectivity index (high SI values) is due to the samples’ (the medicinal dye or commercial antimicrobial in this case) antimicrobial activity rather than a toxin associated with a metabolite of the sample. Therefore, this makes Malachite green an ideal dye to use against the respective pathogens. The opposite would then apply for Iodine tincture. The strong antimicrobial activity would possibly be due to a toxic metabolite rather than the dye itself.

7.3. Selectivity index of commercial antimicrobials against the Gram-positive strains

Unlike the medicinal dyes, the majority of the commercial antimicrobials demonstrated high SI values (a good selectivity index- which is a non-toxic relationship with the sample and the host while having strong antimicrobial activity) (Table 7.1). 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 the *S. epidermidis* reference strain. Gentamycin and Mupirocin demonstrated high SI values for all the *S. epidermidis* strains. This means while the dyes prove to have good antimicrobial activity against the strains, it is also safe to use (based on the BSLA studies).

Steriscrub was the second commercial antimicrobial to note low SI values ranging from 1.00-1.88 except against the *S. epidermidis* strains which noted a SI value of 10.00 making it ideal to use against the strain. While Steriscrub has a strong antimicrobial effect on the other strains tested, it has a toxic metabolite which may be harmful to the host.

Nizer *et al*. (2021) used the selectivity index to help determine the cytotoxicity of the hexane extract of roots of *Salacia crassifolia* (HER) and HER's isolate, pristimerin. Both samples were tested against a *S. aureu*s and *S. epidermidis* strain and had SI values of <1.00 which indicated that both samples are more toxic to the mammalian cells rather than the *S. aureus* and *S. epidermidis* strains (Mokoka *et al*., 2013). The SI index provides a relative toxicity of a sample, which enables changes to be made such as structural modifications of the molecule or change the mode of delivery i.e., to use topically (Nizer *et al*., 2021). Nizer *et al*. (2021) highlights the importance of a selectivity index which can be translated to this study.

Table 7.1: Selectivity index of medicinal dyes and antimicrobials against the Gram-positive pathogens

Sample	LC_{50}	Selectivity index						
		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**	**12500.00**	**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 less than 0.01 µg/ml) and hence the SI was unable to be calculated, Bold: Non-toxic values; LC_{50} values: Added as a reference

7.4. Selectivity index of medicinal dyes against the Gram-negative strains

All medicinal dyes noted low SI values ranging from 0.0001-6.21 (Table 7.2). While the medicinal dyes have proved to have strong efficacy against the Gram-negative pathogens, their efficacy could possibly be due to a toxic metabolite and therefore, is unsafe to use (Adamu *et al*., 2014). Iodine tincture demonstrated the lowest SI values of 0.0001 among all the *P. aeruginosa* and *E. coli* strains. The SI values of Potassium permanganate as well as Mercurochrome and Methylene blue (selected pathogens) have been denoted with a "U". These SI values could not be calculated as the MIC/MBC values were >8000.00 and the endpoint values were unable to be determined.

7.5. Selectivity index of commercial antimicrobials against the Gram-negative strains

Unlike the Gram-positive strains, many of the commercial antimicrobials noted low SI values (Table 7.2). Overall, Betadine® demonstrated the lowest SI values (as with the Gram-positive strains) with SI values ranging from 0.08-0.13. Steriscrub also noted low SI values against the *P. aeruginosa* and *E. coli* strains.

Gentamycin noted the highest SI value of 200.00 against the *P. aeruginosa* reference and resistant strain. This means that it does demonstrate strong antimicrobial activity and low toxic effects hence, it may be considered safe. Gentamycin also demonstrated high SI values against two *E. coli* strains: the reference strain (with an SI value of 20.00) and the resistant strain (with an SI value of 47.98). Neomycin, an antibiotic from the same group as Gentamycin demonstrated two high SI values as well: an SI value of 20.00 against the *P. aeruginosa* clinical strain and an SI of 125.00 against the *P. aeruginosa* resistant strain.

The cytotoxicity and antimicrobial activity of acetone leaf extracts were tested against various pathogens including *P. aeruginosa* and *E. coli* (Famuyide *et al*., 2019). While the study has no relation to the current study except for the two pathogens (*P. aeruginosa* and *E. coli*), it notes the importance of determining the safety of the sample for the specific pathogen.

Table 7.2: Selectivity index of medicinal dyes and antimicrobials against the Gram-negative pathogens

Sample	LC_{50}	Selectivity index					
		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 less than 0.01 µg/ml) and hence the SI was unable to be calculated, Bold: Non-toxic values; LC_{50} values: Added as a reference.

7.6. Selectivity index of medicinal dyes against the yeasts

The majority of the medicinal dyes demonstrated low SI values and hence, were toxic even though they demonstrated good antimicrobial activity. Iodine tincture noted the lowest SI value of 0.0001 (Table 7.3). While this dye did demonstrate strong antimicrobial activity against the yeasts, it also proved to be highly toxic hence, is not an ideal choice or safe to use. Only two medicinal dyes demonstrated high SI values-both against the *C. albicans* reference strain; Malachite green noted an SI value of 12.22 and Methylene blue demonstrated a SI value of 10.00. Both medicinal dyes noted an SI value >10.00 meaning they do have good antimicrobial efficacy while having minimal adverse effects to the host.

7.7. Selectivity index of commercial antimicrobials against the yeasts

Betadine® noted low SI values of 0.05-0.15 against the *C. albicans* strains (Table 7.3). Steriscrub also demonstrated the same low SI value of 0.31 against all the *C. albicans* strains. Both commercial antimicrobials Ketoconazole, Miconazole and Nystatin all demonstrated high SI values against the *C. albicans* reference and clinical strain. Nystatin noted the highest SI value of 2500.00 against the *C. albicans* clinical strain. The high SI value indicates that a good antimicrobial activity is shown, and the antimicrobial noted lesser toxic effects. The SI values of Ketoconazole, Miconazole and Nystatin have been denoted with a "U" against the *C. albicans* resistant strain (Table 7.3). These SI values could not be calculated as the MIC/MBC values were >125.00 μg/ml.

Dumisa (2020), predominantly observed lower SI values against Malachite green. A similar trend was noted by the current study. As with the previous study, Malachite green has shown good antimicrobial activity, but the activity is potentially due to a toxic metabolite hence the low SI. Interestingly, Fuchsine demonstrated moderate toxicity when tested in the brine shrimp assay, however, when calculating the SI values, highly toxic values were noted, indicating that the antimicrobial activity has selective toxicity against the host.

Table 7.3: Selectivity index of medicinal dyes and antimicrobials against the yeasts

Sample	LC_{50}	Selectivity Index		
		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

Sample	LC_{50}	Selectivity Index		
		Candida albicans		
		Reference strain (ATCC 10231)	**Clinical strain (A100)**	**Resistant strain (ATCC 90028)**
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 less than 0.01 µg/ml) and hence the SI was unable to be calculated, Bold: Non-toxic values; LC_{50} values: Added as a reference.

7.8. Selectivity index (SI) of medicinal dyes in combination with commercial antimicrobials (1:1)

The combinations chosen were synergistic and antagonistic combinations based on their MIC/MBC values 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 resistance and should not be used. The majority of the synergistic combinations (Table 7.4) have an SI of >1.00, however, not all show a significant difference in the change in SI values. Fuchsine in combination with Steriscrub demonstrated the lowest SI value of 0.17 against the *E. coli* strain. While this combination notes a strong antimicrobial activity, it may contain a toxic metabolite and is unsafe to use (Adamu *et al*., 2014). The combination also noted a low SI value against the *P. aeruginosa* clinical strain.

Mercurochrome in combination with Betadine® demonstrated strong antimicrobial activity against the Gram-positive strains. According to their SI values, the combination did note a higher SI value in comparison to the independent values however, the values are still below 10.00. Therefore, while a decrease in the combination's toxicity is observed, there is still a toxic relationship and even though the combination notes strong antimicrobial activity it cannot be used. Four combinations noted high SI values: 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). The four combinations noted strong antimicrobial activity against each of the pathogens which are possibly due to the samples itself and not a toxic metabolite as well as a non-toxic relationship with the host making them ideal combinations for use (Dzoyem *et al*., 2016).

As expected, 90% of the antagonistic combinations demonstrated a toxic relationship therefore, the combinations are not ideal due to their high toxicity (Table 7.4). Malachite green and Betadine® noted the lowest SI value of 0.12 against the *E. coli* resistant strain. Only one combination demonstrated a high SI value of 320.61 which was Methylene blue and Gentamycin against the *P. aeruginosa* resistant strain. While a non-toxic relationship was observed with this combination, it still is an antagonistic combination therefore, it does not demonstrate good antimicrobial activity. While it is important to note which are antagonistic combinations in order to know which combinations have the potential to increase resistance and therefore should not chose them; these combinations are not the focus of the study and will not be further investigated.

A previous study tested the antimicrobial activity and cytotoxicity of gold (III) and silver (I) complexes against various pathogens including *S. aureus*, *E. coli*, *P. aeruginosa* and *C. albicans*. The complexes were tested individually and in combination (Savić *et al*., 2016). The combinations of the gold (III) and silver (I) complexes demonstrated synergy, improved cytotoxicity, and had selectivity indices above 10.00 (Savić *et al*., 2016). This study has no relation to medicinal dyes but does demonstrate the importance of conducting a selectivity index on combinations and how combination therapy allows for strong antimicrobial activity while being safe to use on the host (non-toxic to the host).

Dumisa (2020), observed that combinations of Gentian violet and Malachite green demonstrated high SI values and hence those combinations were safe to use and had strong antimicrobial efficacy. In the current study, Gentian violet and Malachite green have noted high SI values in combination and depending on the pathogen, have demonstrated no toxicity.

Table 7.4: Selectivity index of synergistic and antagonistic combinations

Combinations		Corresponding pathogen	LC_{50}	Selectivity index
Synergistic combinatio	Fuchsine + Gentamycin	*P. aeruginosa* resistant strain	**125.00**	**100.03**
	Fuchsine + Steriscrub	*E. coli* resistant strain	7.81	0.17

Combinations		Corresponding pathogen	LC_{50}	Selectivity index
	Fuchsine + Steriscrub	*P. aeruginosa* clinical strain	7.81	0.52
	Gentian violet + Gentamycin	*E. coli* resistant strain	15.64	**977.50**
	Malachite green + Miconazole	*C. albicans* clinical strain	15.62	**128.87**
	Malachite green + Neomycin	*E. coli* resistant strain	15.62	**50.37**
	Mercurochrome + Betadine®	*S. aureus* clinical strain	0.20	5.11
	Mercurochrome + Betadine®	MRSA	0.20	2.25
	Mercurochrome + Betadine®	*S. epidermidis* reference strain	0.20	3.00
	Mercurochrome + Betadine®	*S. epidermidis* clinical strain	0.20	5.11
	Mercurochrome + Betadine®	*S. aureus* reference strain	0.20	1.55
Antagonistic combinations	Fuchsine + Nystatin	*C. albicans* reference strain	31.26	1.78
	Gentian violet + Betadine®	*P. aeruginosa* reference strain	0.20	1.68
	Gentian violet + Neomycin	*P. aeruginosa* clinical strain	0.24	4.35
	Iodine tincture + Fusidic acid	GMRSA	2.00	4.51
	Iodine tincture + Fusidic acid	*S. epidermidis* clinical strain	2.00	2.23
	Malachite green + Betadine®	*S. epidermidis* reference strain	2.15	0.90
	Malachite green + Betadine®	*S. epidermidis* clinical strain	2.15	0.23
	Malachite green + Betadine®	*E. coli* resistant strain	2.15	0.12
	Mercurochrome + Steriscrub	*E. coli* resistant strain	7.83	1.58
	Methylene blue + Gentamycin	*P. aeruginosa* resistant strain	**500.00**	**320.61**

LC_{50} values: Added as a reference; Bold: Non-toxic values

7.9. Overview of Selectivity index

The medicinal dyes were observed to have a lower antimicrobial efficacy to toxicity ratio when tested against the selected pathogens in comparison to the commercial antimicrobials. The antibiotics demonstrated the highest selectivity indices when calculated against the *S. epidermidis* strains. Iodine tincture was noted to have the lowest SI while Betadine® was noted to have the lowest SI among antimicrobials. Malachite green was the only dye that demonstrated a high SI value among three pathogens.

The majority of the combinations tested noted a high SI value, however, two synergistic combinations stood out in comparison to the other combinations tested. Gentian violet in combination with Gentamycin, and Malachite green in combination with Neomycin against the *E. coli* resistant strain demonstrated a high SI value meaning both have strong antimicrobial activity based on the combinations itself as well as low toxic effects to the host.

Fuchsine in combination with Steriscrub tested against the *E. coli* resistant strain and Malachite green in combination with Betadine® tested against the *E. coli* resistant strain demonstrated the lowest SI values, hence were the least favourable combinations. Amongst the antagonistic combinations, only one combination noted a high SI value; Methylene blue with Gentamycin while the others demonstrated SI values <10.00 meaning they were unsafe combinations.

7.10. Time-kill studies

Time-kill studies were conducted on the two combinations (Gentian violet in combination with Gentamycin, and Malachite green in combination with Neomycin) based on their selectivity index. Among the synergistic combinations that were investigated, the combination with the highest SI value and lowest SI value were chosen. Both combinations were tested against the *E. coli* resistant strain. As expected, the *E. coli* resistant strain did demonstrate susceptibility to the positive control (Ciprofloxacin). Microbial growth was also observed with the culture control.

7.10.1. Combination of Gentian violet with Gentamycin against the *E. coli* resistant strain

A slower killing action was observed with Gentian violet in comparison to the positive control, Ciprofloxacin (Figure 7.2). Gentian violet demonstrated a bacteriostatic effect on the *E. coli* resistant strain after 24 hrs. Gentamycin demonstrated a similar pattern to Gentian violet, however, at 6-24 hrs, Gentamycin demonstrates a more bactericidal effect which is indicated by the change in steepness of the curve. 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. (Figure 7.2).

Gentian violet has been observed to have a bacteriostatic effect on various bacteria (Ingraham, 1933; Rogosa, 1934). While Gentamycin is known for its bactericidal activity, a previous study conducting time-kill studies on Gentamycin observed bactericidal activity only after 6 hrs and 48 hrs (Bortolin *et al*., 2017). The current study noted the same pattern (interrupted black line). 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).

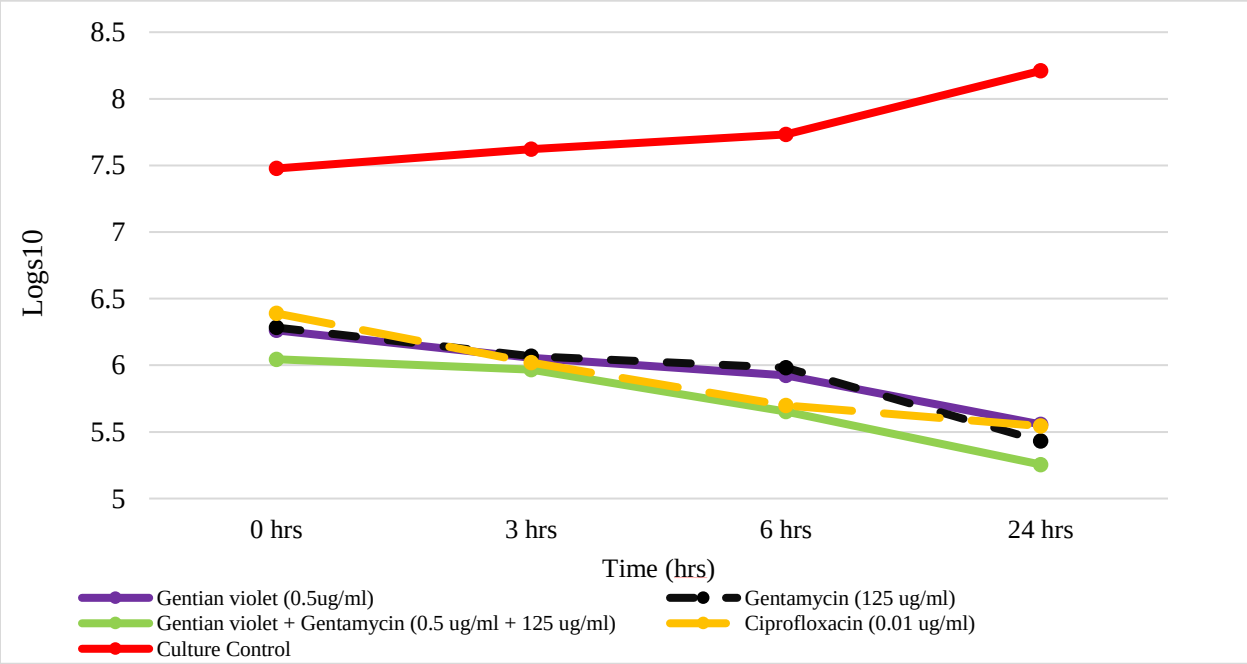


Figure 7.2: Time-kill studies of Gentian violet and Gentamycin

7.10.2. Combination of Malachite green with Neomycin against the *E. coli* resistant strain

As with the previous combination, Malachite green and Neomycin demonstrated a slow killing action when tested against the *E. coli* resistant strain (Figure 7.3). From 0-24 hrs, Malachite green demonstrated a bacteriostatic effect on the pathogen. Neomycin demonstrates bacteriostatic activity similar to Malachite green however, at 6-24 hrs, Neomycin noted slightly 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 0-6 hrs, the combination notes a bacteriostatic effect with a steady decline. Thereafter a steep decline of pathogen viability was noted. At 24 hrs, the pathogen is completely killed.

Malachite green is one of the dyes known to be bacteriostatic (Chaudhary *et al*., 2017). A study tested the antimicrobial effect of Malachite green against *Aeromonas hydrophila* and a bacteriostatic effect was noted (Gallani *et al*., 2020). *Aeromonas hydrophila* is also a Gram-negative pathogen like *E. coli* (Steinberg *et al*., 2020); and Malachite green also noted the same effect against the pathogen as was observed in the current study. 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).

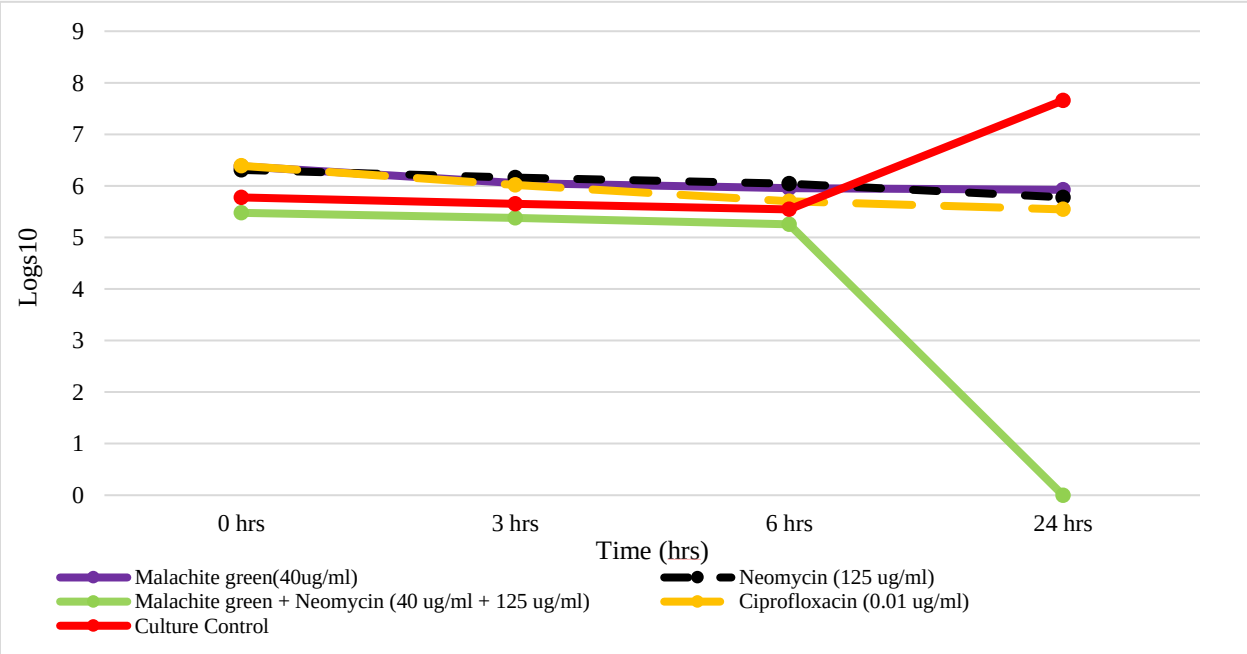


Figure 7.3: Time-kill studies of Malachite green and Neomycin

7.11. Overview of time-kill studies

Both combinations were tested against the *E. coli* resistant strain as both demonstrated a non-toxic relationship and strong antimicrobial activity based on their SI values. The medicinal dyes noted a bacteriostatic effect when tested alone as did the commercial antimicrobials. However, when tested in combination, both combinations demonstrated synergy (noting lower curves in each of their time-kill graphs). Both combinations demonstrated better activity in comparison to the controls as well as when tested independently. Malachite green in combination with Neomycin noted the better (cidal) activity against the *E. coli* resistant strain in comparison to Gentian violet and Gentamycin.

7.12. Summary

- Iodine tincture demonstrated the lowest SI value hence demonstrated the most toxicity irrespective of the pathogen tested against (SI=0.0001).
- Malachite green noted the highest SI values against GMRSA (SI value of 130.33) and *S. epidermidis* reference strain (SI value of 4344.44), hence, was non-toxic to the host.
- The commercial antimicrobials predominantly demonstrated high SI values hence were safe to use and demonstrated low toxicity.
- Higher SI values of the antibiotics were noted against the *S. epidermidis* strains.
- Betadine® demonstrated low SI values among all pathogens tested.
- Four synergistic combinations noted high SI values (Fuchsine with Gentamycin, Gentian violet with Gentamycin, Malachite green with Miconazole and Malachite green with Neomycin).
- Gentian violet with Gentamycin demonstrated the highest SI value of 977.50 against the *E. coli* resistant strain.
- In both time-kill studies conducted, both medicinal dyes demonstrated a bacteriostatic action.
- Gentamycin and Neomycin demonstrated similar activity in the time-kill studies due to them being from the same antibiotic group.
- Synergy was noted with both combinations however, Malachite green when combined with Neomycin completely killed the *E. coli* resistant strain at 24 hrs.

CHAPTER 8

Summary and conclusion

8.1. Summary

Antimicrobial resistance has been a growing problem especially when it comes to the treatment of skin and soft tissue infections. The increase in resistance has led to a change in treatment and an increase in combination therapy. The current study demonstrates the potential use of medicinal dyes in combination therapy to treat skin infections and possibly combat antimicrobial resistance. In this study, seven medicinal dyes: Fuchsine, Gentian violet, Iodine tincture, Malachite green, Mercurochrome, Methylene blue and Potassium permanganate were investigated. Medicinal dyes are a largely untouched source of antimicrobials for many years due to the promotion of antibiotics. The antimicrobial activity of the dyes was tested independently and in combination with eight commercial antimicrobials used to treat skin infections. The toxicity profiles of the medicinal dyes independently and in combination were determined. Lastly, the antimicrobial activity of selected combinations over a period of time (time-kill studies) were undertaken to determine a cidal/static response. An overview of the objectives of the study is given as follows.

Objective 1: To determine the antimicrobial inhibitory properties of selected commercial antibiotics and dyes against pathogens associated with skin infections using minimum inhibitory concentrations (MIC's) and determining the minimum bactericidal activity (MBC).

Figure 8.1 visually demonstrates the comparison of MBC values of the medicinal dyes and commercial antimicrobials. Good antimicrobial activity was noted with MIC/MBC values ≤1.00 μg/ml, moderate antimicrobial activity was noted between 1.01-1000.00 μg/ml and poor antimicrobial activity was noted with values above 1001.00 μg/ml (Figure 8.1)

Among all the pathogens tested, susceptibility to the medicinal dyes was observed except with Potassium permanganate (Figure 8.1). The commercial antimicrobials did note good antimicrobial activity, however, not better than with the medicinal dyes.

The Gram-positive strains proved to be the more susceptible than the Gram-negative strains when tested against the commercial antimicrobials. The yeasts were tested against antifungals rather than the antibiotics and therefore, no comparison could be made. The yeasts however, also noted more resilience to the antifungals as did the bacteria to the antibiotics.

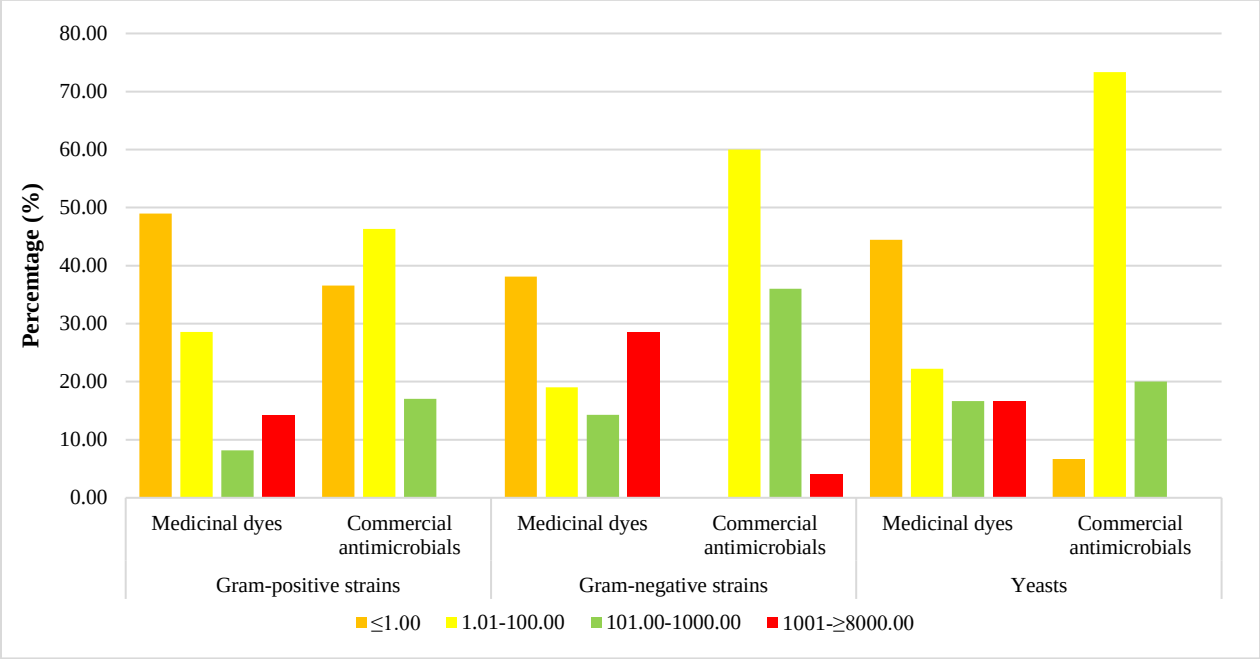


Figure 8.1: Overall the individual MBC values for all strains tested (µg/ml)

Objective 2: To assess the interactions of commercial antimicrobials and dyes by determining the sum of the fractional inhibitory concentration (ΣFIC) in 1:1 combinations and various ratios of medicinal dyes and commercial antimicrobial were combined and evaluated for interactive antimicrobial effects (isobologram analysis).

There was numerous medicinal dye: commercial antimicrobial combinations that demonstrated strong antimicrobial activity. When tested against the Gram-positive strains, Gram-negative strains, and yeasts, predominantly indifference (38.81%) was noted followed by additive interactions (27.87%), synergy (20.46%) and lastly antagonism with 12.87% (Figure 8.2). One

synergistic combination stood out among the Gram-positive pathogens: Mercurochrome with Betadine®. The most antagonistic combination (Malachite green and Betadine®) was observed against MRSA with an ΣFIC value of 1000.50. The lowest ΣFIC value 0.001 (Fuchsine with Gentamycin) was observed against the *P. aeruginosa* clinical strain. Methylene blue in combination with Ketoconazole demonstrated the lowest ΣFIC value of 0.01 against the *C. albicans* clinical strain. The most antagonistic interaction was noted with Methylene blue and Betadine® with an ΣFIC value of 10.67. Further varied ratio studies were conducted on notable synergistic and antagonistic combinations. The majority of the synergy in the combinations were attributed to the medicinal dyes possibly due to their strong antimicrobial activity while the antagonistic combinations only had the 1:1 ratio noting antagonism with majority of the other ratios noted indifference.

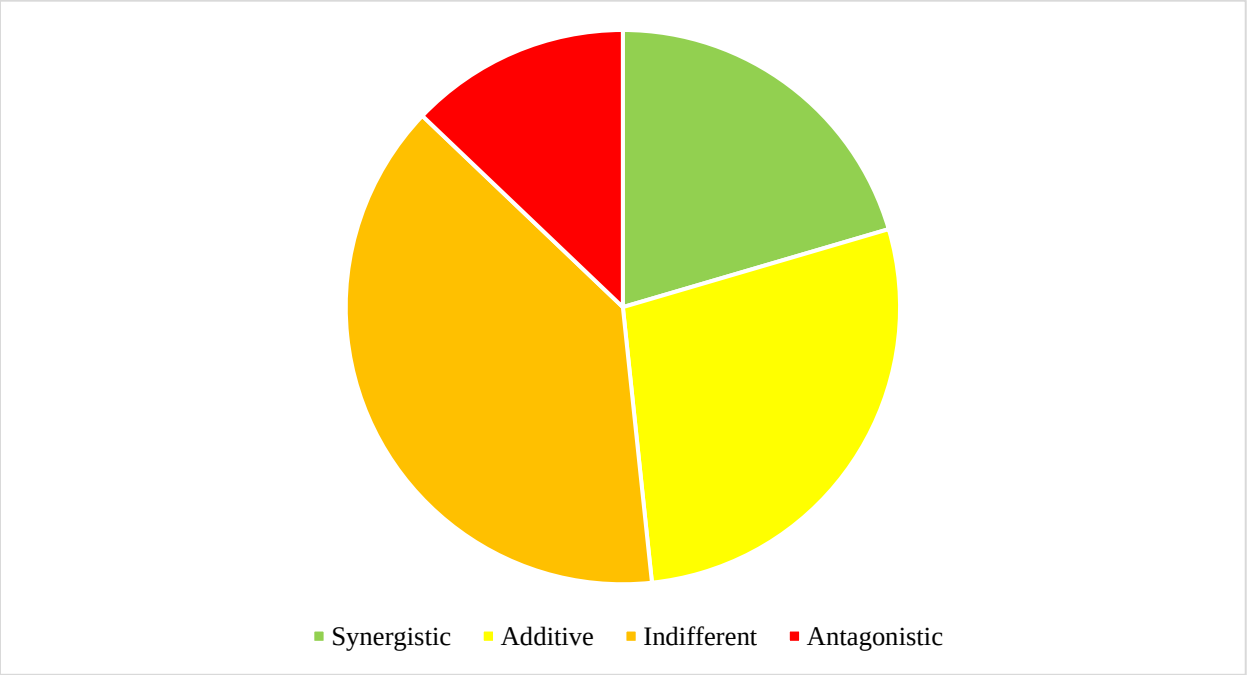


Figure 8.2: The distribution of interactions based on ΣFIC values

Objective 3: To perform toxicity studies on the medicinal dyes, antimicrobial agents and notable combinations using the brine-shrimp lethality assay.

The brine-shrimp lethality assay was conducted on the medicinal dyes, commercial antimicrobials, and selected combinations. According to the percentage mortalities, 43% of the dyes were toxic at 24 hrs which decreased to 29% at 48 hrs (Figure 8.3.A and 8.3.B).

At 24 hrs, Fuchsine, Malachite green and Methylene blue were observed to be non-toxic. At 48 hrs, majority of medicinal dyes demonstrated high percentage mortalities. Four medicinal dyes noted a percentage mortality of 100%: Gentian violet, Iodine tincture, Malachite green and Mercurochrome. The Iodine tincture was observed to have the most toxicity.

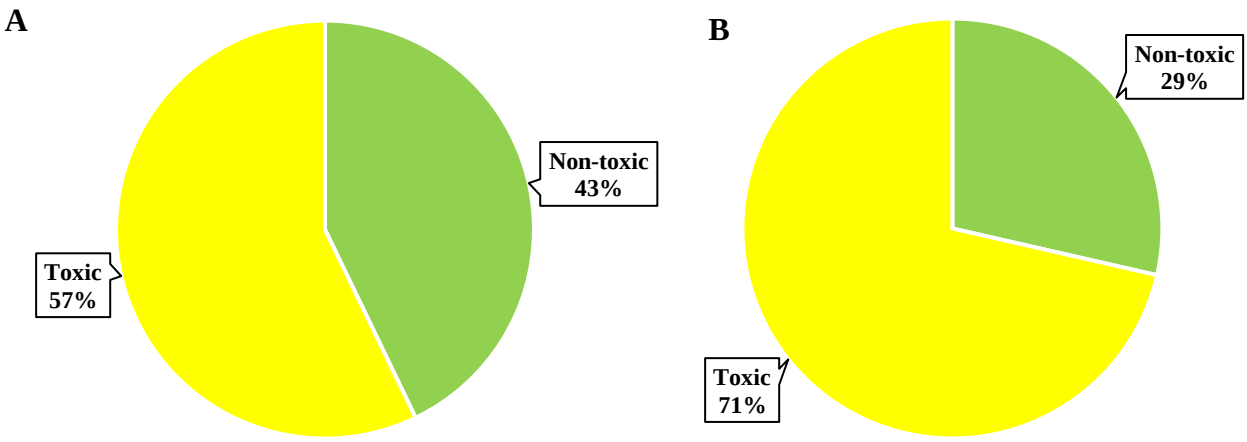


Figure 8.3: A.): Medicinal dye toxicity at 24 hrs, B.): Medicinal dye toxicity at 48 hrs

The commercial antimicrobials predominantly demonstrated low percentage mortalities (at 24 and 48 hrs) and were deemed non-toxic (Figure 8.4.A and 8.4.B). Gentamycin and Nystatin demonstrated the lowest percentage mortality of 0% at 24 hrs. At 48 hrs, Gentamycin and Neomycin noted the lowest percentage mortality of 1.11% and 5.84% respectively. Betadine® proved to be the most toxic dye at 24 and 48 hrs with a percentage mortality of 100%.

Among the synergistic combinations tested, 66.67% of the combinations noted lower toxicity in combination than when compared to the dyes and commercial antimicrobials tested independently. On the other hand, 50% of the antagonistic combinations noted decreased toxicity. Overall, Gentian violet in combination with Gentamycin noted the most synergy and the greatest decrease (fifteen-fold) in toxicity. Malachite green in combination with Neomycin also noted synergy (a

two-fold decrease in toxicity), however, not as pronounced as was noted with the Gentian violet: Gentamycin combination.

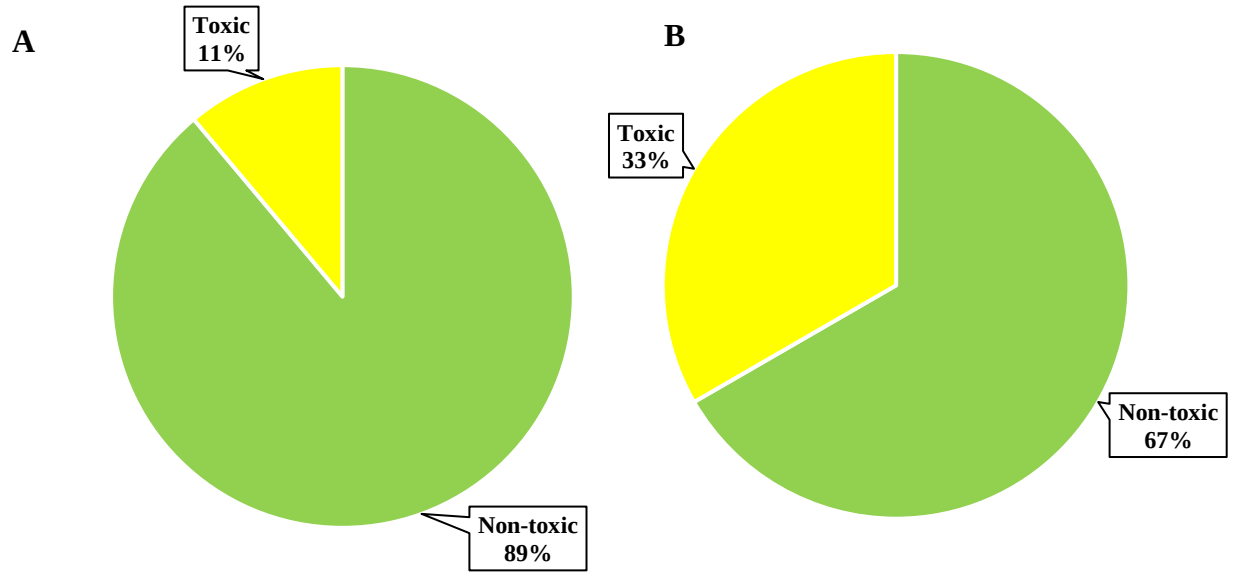


Figure 8.4: A.): Commercial antimicrobial toxicity at 24 hrs, B.): Commercial antimicrobial toxicity at 48 hrs

Objective 4: To determine the selectivity index of the medicinal dyes, commercial antimicrobials, and combinations.

When examined against the Gram-positive strains, very low SI values were observed. The majority of the medicinal dyes noted values <1.00 (Figure 8.5.A). The medicinal dyes were deemed toxic and unsafe for use independently. The SI's of certain dyes were unable to be calculated and are designated with a "U". In comparison, the majority of the commercial antimicrobials noted high SI values of >10.00 and were deemed safe for the host while still demonstrating an antimicrobial effect on the Gram-positive bacteria. (Figure 8.5.B). The exception was Betadine® which had an SI of <1.00 against all the Gram-positive pathogens.

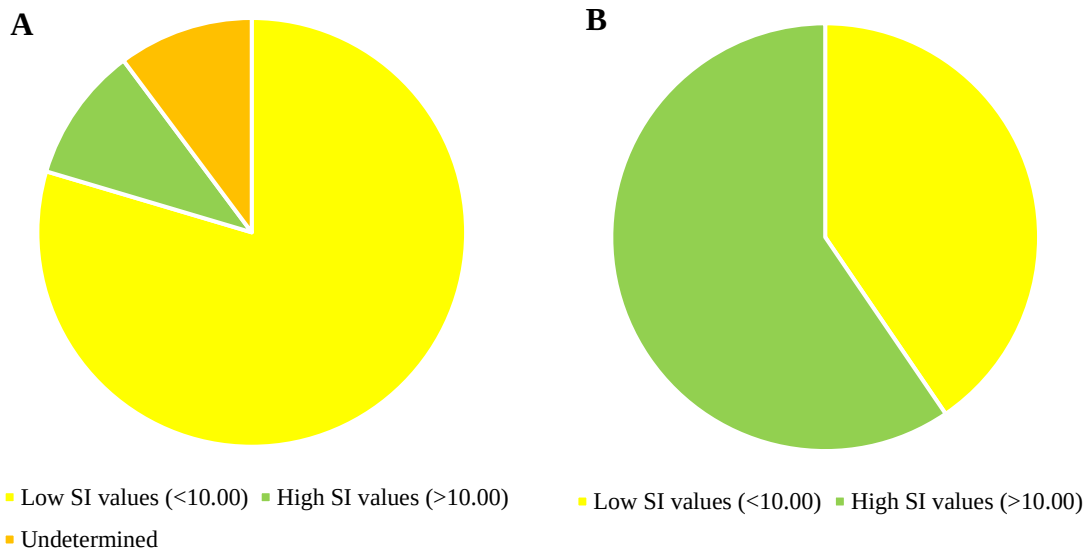


Figure 8.5: A.): The SI values against the medicinal dyes, B.): The SI values against the commercial antimicrobials when tested against the Gram-positive strains

For the Gram-negative strains, most of the medicinal dyes noted low SI values (76.19%) against the *P. aeruginosa* and *E. coli* strains (Figure 8.6.A). When examining the commercial antimicrobials, the majority of the SI values (66.67%) were less than 10.00 (Figure 8.6. B). Both the dyes and commercial antimicrobials when tested against the respective pathogens demonstrated low SI values making them unsafe for use on their own.

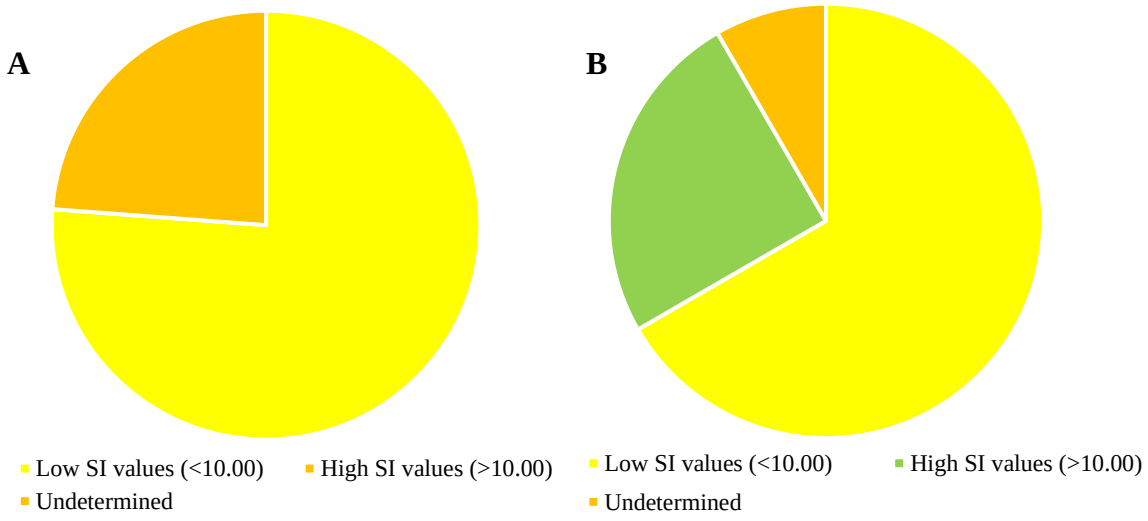


Figure 8.6: A.): The SI values against the medicinal dyes, B.): The SI values against the commercial antimicrobials when tested against the Gram-negative strains

A total of 85.71% of the dyes demonstrated low SI values (Figure 8.7.A). Only two dyes demonstrated high SI values – high enough to be used in a host with minimal toxic effects. Unlike the other pathogens, the SI values against the yeasts demonstrated an equal number of low and high SI values (Figure 8.7.B). All three antifungals (Ketoconazole, Miconazole and Nystatin) noted high SI values indicating minimal toxic effects in relation to antimicrobial activity. The commercial antimicrobials demonstrated higher SI values in comparison to the dyes, making them safer to use with minimal toxicity.

While the SI values of the medicinal dyes were higher when combined with the commercial antimicrobials, four combinations (synergistic combinations) demonstrated strong antimicrobial activity with high SI values in comparison to their independent values. These combinations have shown that the medicinal dyes while unsafe when used alone, may be safe to use when in combinations and still maintain a strong antimicrobial activity.

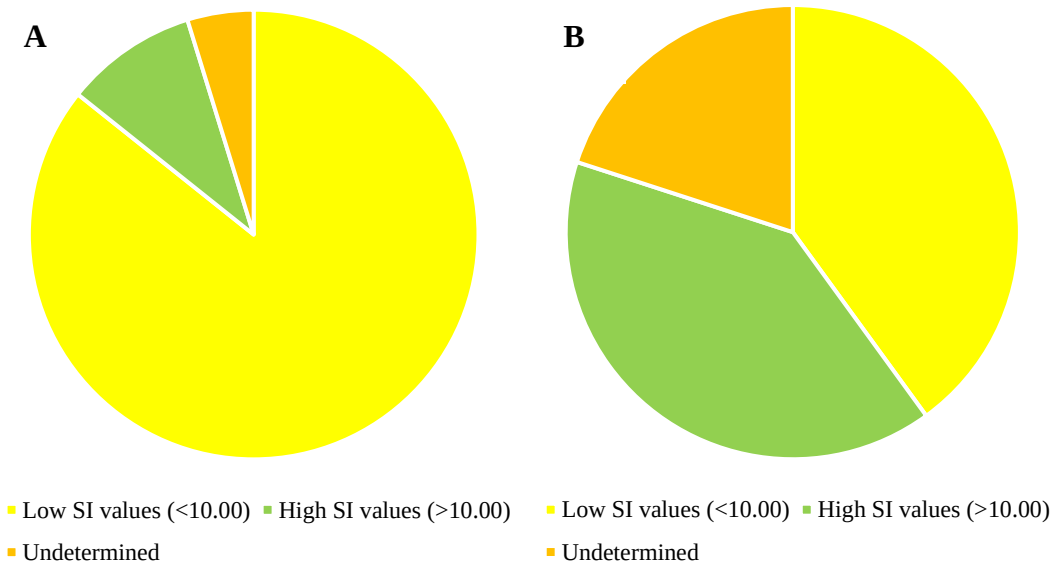


Figure 8.7: A.): The SI values against the medicinal dyes, B.): The SI values against the commercial antifungals when tested against the yeasts

Objective 4: To determine the bactericidal or static effect over time (time-kill assays) of the most promising combination s for synergy.

Two combinations (Gentian violet with Gentamycin and Malachite green with Neomycin) were selected for further investigation in the time-kill assay. These combinations were chosen based on their notable antimicrobial activity, reduced toxicity, and SI index. Both combinations were tested against the *E. coli* resistant strain. When combined, synergy was observed with the combination of Malachite green and Neomycin where a bactericidal effect was noted after 24 hr. Gentian violet and Gentamycin did note better activity in comparison to the individual samples, however, a bacteriostatic effect was noted. Overall, Malachite green in combination with Neomycin proved to have better activity against the *E. coli* resistant strain over a period of 24 hrs, which was surprising as it demonstrated the lower SI value when compared to the other pathogens tested. Both combinations demonstrated better activity than the controls proving the potential use of medicinal dye combinations.

8.2. Limitations of the study

The main limitation of this study was the COVID-19 pandemic which led to a National shutdown in March 2020. This led to time delays in the study. It also led to restricted laboratory access and delays in the procurement of samples and other materials required to conduct assays in this study.

Another limitation in the study was the colour and intensity of the medicinal dyes, as it led to difficulties in conducting assays especially the MIC assay which relies on a colour change to determine the inhibitory concentration. This was overcome by conducting the MBC assay. Another issue was conducting the brine-shrimp lethality assay. Due to the deep colour of the dyes especially Methylene blue, it was difficult to determine the number of shrimps under the microscope and dilutions were necessary to conduct the assay.

8.3. Future studies

This study has shown the possibility of the use of medicinal dyes for skin infections; however, certain assays were not within this scope of study, and it would be worthwhile to investigate these

further. Firstly, biofilm studies should be done to determine the medicinal dye's ability to eradicate a biofilm (Serralta *et al*., 2001; Nusbaum *et al*., 2012; Percival *et al*., 2015). This will help in chronic skin infections (Kwiecinski *et al*., 2015). One of the worries with medicinal dyes are that they have been shown to mutate *in vivo* (Wainwright, 2008). Therefore, further animal and human cell studies need to be conducted.

Further cytotoxicity studies should also be conducted on human skin cells to ensure the use of medicinal dyes are safe for use in humans. While this study has mainly focused on a few synergistic combinations, other options require more in-depth testing. Varied ratio testing was also conducted on a few ratios. Other combinations that noted additive or indifferent effects in the 1:1 ratio was not tested; therefore, other ratios may also note synergy with an increase in concentration in commercial antimicrobial or reducing the concentration of the dye.

8.4. Final conclusion

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. The medicinal dyes have demonstrated strong antimicrobial activity against the pathogens especially the resistant strains which are already difficult to effectively eradicate. This highlights the potential use of medicinal dyes in the current treatment of skin infections. Moreover, medicinal dye combinations have demonstrated synergy against all pathogen groups tested in various different mixes. Further varied ratio testing on combinations demonstrated the synergistic effect of combinations and highlighted the potential for synergy and for a potentially better therapeutic outcome. When used in combination with the commercial antimicrobials, the concentrations of the medicinal dyes are vastly lower which in turn lowers the staining ability of the dye. This provides an advantage, especially if used topically.

The toxicity of the medicinal dyes is well known and have in the past, overshadowed their strong efficacy. The dye's toxicity was largely dependent on the high concentrations used. Toxicity studies on combinations have demonstrated that this hurdle may be overcome with the combinations achieving decreased toxicity of the medicinal dyes with lower concentrations being utilized.

The high selectivity indices of combinations further demonstrated the toxicity may be reduced without affecting the antimicrobial activity of the dye, proving the medicinal dyes are able to be used safely. Time-kill studies conducted have demonstrated that the medicinal dyes when in combination with the commercial antimicrobials have improved bactericidal activity showing the importance of further combination studies of medicinal dyes.

Antimicrobial resistance has been a growing global threat and the formulation of topical combination therapy with medicinal dyes may reduce the burden caused by the resistance to commercial antimicrobials and minimize the cost in the treatment of skin infections. Medicinal dyes have been known to be cost effective; hence, the potential new treatment option of combination use of medicinal dyes may be especially helpful in South Africa due to the increased number of skin infections, the shortage of dermatologists and excessive costs for doctor consults (Asong *et al.,* 2019). Therefore, the current study provided some insight into alternate antimicrobial options against pathogens associated with the skin.

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APPENDIX A:

Abstract for presentation

The combination of medicinal dyes with conventional antimicrobials: Potential for synergy in topical skin infections

RAMFOL, R.[1], VAN VUUREN, S.F.[1]

[1]*Department of Pharmacy and Pharmacology, Faculty of Health Science, University of the Witwatersrand, Johannesburg, South Africa.*

Medicinal dyes have been used for centuries to treat ailments, however research into their use as an antiseptic predates 1945 and has been overshadowed by antibiotics. Many antibiotics have become ineffective in treating the simplest of infections thereby sparking new interest in combination antimicrobial therapy. This study aimed to explore potential synergistic effects of medicinal dyes and antimicrobials against five pathogens (reference, resistant and clinical strains) of skin infections. Studies undertaken were minimum inhibitory concentrations (MIC), minimum bactericidal concentration (MBC) assays and toxicity studies against Gram-positive, Gram-negative bacteria and yeasts. Individually, Gentian violet demonstrated the strongest antimicrobial activity against all pathogens with a notably strong MIC value of 0.000031 µg/ml against *Staphylococcus epidermidis* (clinical strain), 0.01µg/ml against *Pseudomonas aeruginosa* (reference and clinical strain) and *Escherichia coli* (reference strain). It also demonstrated the strongest activity against *Candida albicans* with an MIC value of 0.00025 µg/ml. Among the antibiotics, Mupirocin and Fusidic acid noted the strongest antimicrobial activity with an MIC of 0.04 µg/ml against *S. epidermidis* (clinical and reference strain), followed by Betadine® with an MIC of 1.56 µg/ml against *E. coli* (reference and resistant strains) and an MIC of 1.30µg/ml against *C. albicans* (reference strain). Individually, medicinal dyes noted stronger antimicrobial activity compared to conventional antimicrobials. Combination studies (1:1 ratio) noted 26% of dye-antibiotic combinations were synergistic against Gram-positive strains, 15% against Gram-negative and 14% against yeasts. Mercurochrome-Betadine® noted synergy among all the *Staphylococcus aureus* strains with fractional inhibitory concentration (FIC) values ranging from 0.05 to 0.48. Fuchsine-Gentamycin noted the most synergistic FIC value of 0.001 against *P. aeruginosa* (clinical) strain. Methylene blue-ketoconazole demonstrated the lowest synergistic FIC value of 0.01 among the yeasts. Toxicity studies performed on individual samples, five notable synergistic and antagonistic combinations for each group demonstrated the most toxic dye was Iodine tincture with a concentration of 0.0001µg/ml and least toxic Methylene blue with a concentration of 125 µg/ml. In combination, 62% of dyes noted decreased toxicity with a notable combination being Fuchsine-Gentamycin. This study has proven dye-antimicrobial have synergistic combinations, highlighting a gap (dyes) in treatment of skin infections.

APPENDIX B:

Abstract for publication

The combination of medicinal dyes with conventional antimicrobials: Potential for synergy in topical skin infections. (Ramfol, R. and van Vuuren, S.).

Infections caused by antibiotic-resistant bacteria has led to a change in the approach to empiric antibiotic use. Medicinal dyes have been used for centuries to treat infectious ailments; however, research has been lacking in recent years and most clinical work predates 1945 which has now been overshadowed by antibiotics. Many antibiotics have become ineffective in treating the simplest of infections thereby sparking new interest in combination antimicrobial therapy. This study aimed to explore potential synergistic effects of medicinal dyes and antimicrobials against pathogens responsible for skin infections. Antimicrobial testing was conducted by minimum inhibitory concentrations (MIC) and minimum bactericidal/fungicidal concentration (MBC/MFC) assays. The fractional inhibitory index (ΣFIC) of combinations were calculated and isobolograms were constructed for varied ratios on selected combinations. Toxicity studies were conducted by employing the brine-shrimp lethality assay. Gentian violet demonstrated the highest antimicrobial activity against all pathogens with a notably strong MBC value of 0.000031 µg/ml against *Staphylococcus epidermidis* (clinical strain). Mupirocin and Fusidic acid noted the highest antimicrobial activity with an MIC/MBC of 0.04 µg/ml against *S. epidermidis* (Clinical strain and ATCC 51625). Combination studies (1:1 ratio) noted that 26% of dye-antibiotic combinations were synergistic against the Gram-positive strains, 15% against the Gram-negative and 14% against the yeasts. The Mercurochrome: Betadine® combination noted synergy among all varied ratios against all the *Staphylococcus aureus* strains with ΣFIC values ranging from 0.05 to 0.48. Four dyes (Iodine tincture, Gentian violet, Malachite green and Mercurochrome) demonstrated a percentage mortality of 100% at 48 hrs. Most of the commercial antimicrobials were non-toxic at both 24 and 48 hrs. The combination of Gentian violet with Gentamycin noted a fifteen-fold decrease in toxicity. and a selectivity index of 977.50 against the *E. coli* (DSM 22314) strain. Time-kill studies were conducted on the combinations with the highest safe 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. This study highlights the potential impact medicinal dye combinations may have on skin infections.

APPENDIX C:

Ethics waiver

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

Office of the Deputy Vice-Chancellor (Research & Post Graduate Affairs)

TO: Ms R Ramfol
School: Therapeutic Sciences
Department: Pharmancy and Pharmacology
Medical School
University
E-mail: rhearamfol@yahoo.co.za

CC: Supervisor: Professor S van Vuuren <Sandy.vanVuuren@wits.ac.za>
and <HREC-Medical.ResearchOffice@wits.ac.za>

FROM: Iain Burns
Human Research Ethics Committee (Medical)
Tel: 011 717 1252

E-mail: Iain.Burns@wits.ac.za

DATE: 14/08/2020

REF: *R14/49*

PROTOCOL NO: W-CBP-200814-01 (This is your ethics application study reference number. Please quote this reference number in all correspondence relating to this study)

PROJECT TITLE: *The combination of medicinal dyes with conventional antimicrobials: potential for synergy in topical skin infections*

Please find attached the Ethics Waiver Certificate for the above project. I hope it goes well and that an article in a recognized publication comes out of it. This will reflect well on your professional standing and contribute to the Government funding of the University.

MSWorks2000/Iain0007/ClearScanWaiver.wps

APPENDIX D:

Turnitin Report

Rhea Masters Thesis

ORIGINALITY REPORT

9%	4%	7%	1%
SIMILARITY INDEX	INTERNET SOURCES	PUBLICATIONS	STUDENT PAPERS

PRIMARY SOURCES

1	Mthembeni Dumisa, Olayinka Ayobami Aiyegoro, Sandy Van Vuuren. "Medicinal plant: Dye combinations – Impact on antimicrobial potency and toxicity", South African Journal of Botany, 2020 Publication	1%
2	azdoc.site Internet Source	1%
3	www.ncbi.nlm.nih.gov Internet Source	1%
4	Submitted to University of Witwatersrand Student Paper	<1%
5	Karl T. Clebak, Michael A. Malone. "Skin Infections", Primary Care: Clinics in Office Practice, 2018 Publication	<1%
6	Claira Arul Aruldass, Santhana Raj Louis Masalamany, Chidambaram Kulandaisamy Venil, Wan Azlina Ahmad. "Antibacterial mode of action of violacein from Chromobacterium	<1%