ESSENTIAL OIL COMPOUNDS IN COMBINATION WITH CONVENTIONAL ANTIBIOTICS FOR DERMATOLOGY

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DECLARATION

I, Shivar Simbu (Student number 725952), declare that this dissertation is my own work and I have contributed in full towards the research findings published in the articles stated below.

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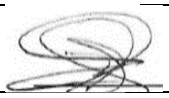
Author 3	Ané Orchard		27/03/2024
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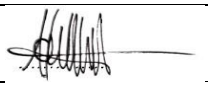
Author 5	Anna C. Hattingh		27 March 2024
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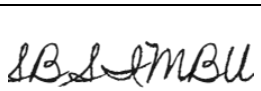
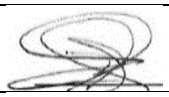
Author 1	Shivar Simbu		20 March 2024
Author 2	Ané Orchard		27/03/2024
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DEDICATION

To my endless pursuit of knowledge and understanding of the world.

LIST OF PRESENTATIONS ARISING FROM THIS STUDY

Presentation 1 – Abstract (Appendix A)

Simbu S., Orchard A., van de Venter M., van Vuuren S.F. Essential oil compounds in combination with conventional antibiotics for dermatology. School of Therapeutic Sciences Biennial Research Day, University of the Witwatersrand (Faculty of Health Sciences), Johannesburg, South Africa, September 5, 2023 (Oral presentation). **[Awarded best oral presentation]**

Presentation 2 – Abstract (Appendix B)

Simbu S., Orchard A., van de Venter M., van Vuuren S.F. Synergistic solutions: The potential of multitarget ibuprofen combinations for cutaneous infections. Molecular Biosciences Research Thrust (MBRT) Research Symposium, University of the Witwatersrand (Faculty of Science and Health Sciences), Johannesburg, South Africa, December 7, 2023 (Oral presentation).

ABSTRACT

Skin and soft tissue infections represent a heterogeneous array of clinical entities with varying severity, causative pathogens, and rates of progression. The slow development and overuse of antimicrobial agents have perpetuated the spread and severity of antimicrobial resistance. Natural products such as essential oils and their compounds are often investigated for their pharmacological properties, with particular interest in their antimicrobial properties. This study aimed to investigate the effects of combining six essential oil compounds (α-pinene, γ-terpinene, ±linalool, eugenol, carvacrol, and cinnamaldehyde) with eight conventional antimicrobials (amoxicillin, ciprofloxacin, erythromycin, gentamicin, meropenem, tetracycline, miconazole, and nystatin) against six commonly encountered skin pathogens (*Staphylococcus aureus* ATCC 25923, *Staphylococcus epidermidis* ATCC 12228, *Pseudomonas aeruginosa* ATCC 27853, *Acinetobacter baumannii* ATCC 19606, *Cutibacterium acnes* ATCC 11827, and *Candida albicans* ATCC 10231) to elucidate the interactive profiles, toxicity, and anti-inflammatory properties.

The antimicrobial analysis involved determining the minimum inhibitory concentrations (MIC) of the conventional antimicrobials and essential oil compounds, singularly and in combination, using the broth microdilution assay. The sum of the fractional inhibitory concentrations (ΣFICs) was calculated to investigate the interactive profile of the combinations. Synergistic interactions were further analysed at varying ratios and depicted on isobolograms. Eight synergistic interactions were identified, with seven against Gram-positive bacteria (ΣFIC 0.07 – 0.42) and one against *P. aeruginosa* (ΣFIC 0.32). In addition, it was demonstrated that when in combination, the selected combinations resulted in reduced toxicity (Brine-shrimp lethality assay). The combination of amoxicillin and eugenol demonstrated the lowest toxicity (LC_{50} = 1081 µg/mL) and the highest selectivity index (14.41) when in a (70:30) ratio with the antibiotic in the higher ratio.

Based on the synergistic results from the antimicrobial analysis, a selection of essential oil compounds with conventional antimicrobials were assessed for cytotoxicity and anti-inflammatory properties. The cytotoxicity properties were determined using the MTT assay on HaCAT keratinocytes. The anti-inflammatory properties were determined using lipopolysaccharide (LPS) activated RAW 264.7 macrophages, and the reduction in nitrate (NO)

production was measured. Cinnamaldehyde demonstrated the highest cytotoxicity (IC_{50} = 28.63 µg/mL, $p < 0.05$) and the greatest reduction (77.44%) in nitrite production, which was also concentration dependent. The combination of ciprofloxacin and cinnamaldehyde demonstrated the lowest cytotoxicity (88.42% ± 3.72 cell viability; combination index of 0.12) and the highest reduction in nitrite production (77.42%; ΣFa = 0.44).

Further investigations on the interactive properties of ibuprofen were undertaken. The antimicrobial, cytotoxicity, and anti-inflammatory properties of ibuprofen were analysed singularly and in combination with all essential oil compounds and conventional antimicrobials against reference and clinical skin pathogens. For the MIC results, four synergistic interactions were identified between ibuprofen and conventional antimicrobials (ΣFIC 0.33 - 0.50). For the cytotoxicity (MTT assay), none of the combinations demonstrated a cytotoxic effect (cell viability of 93.6-100%) and significant reduction on nitric oxide production. Additionally, higher order combinations involving the synergistic combinations were investigated with the inclusion of the essential oil compounds. Three synergistic interactions were identified (One against *C. acnes* and two against *A. baumannii*). The triple combinations were slightly cytotoxic (cell viability of 77.59 - 90.44%; combination index of 0.95 -1.10) on the HaCAT cell line and did not reduce nitric oxide production.

Based on the overall results from this study, combinations of essential oil compounds and some conventional antimicrobials demonstrate promising therapeutic approaches to attenuate antimicrobial resistance. These results demonstrated that combinations that comprise cinnamaldehyde have noteworthy antimicrobial and anti-inflammatory properties which may warrant further investigation. Combining ibuprofen with conventional antimicrobials and essential oil compounds may also offer potential advantages in managing resistant infections through direct and indirect antimicrobial mechanisms.

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Equation 1: Fractional inhibitory concentration (FIC)

$$\text{FIC (I)} = \frac{(A^*) \text{ combined with (B)}}{\text{(A) independently}}$$

$$\text{FIC (II)} = \frac{(B^*) \text{ combined with (A)}}{\text{(B) independently}}$$

From these values, the sum of the FIC (ΣFIC) was calculated:

$$\Sigma\text{FIC} = \text{FIC (I)} + \text{FIC (II)}$$

Equation 2: Percentage mortality (PM)

The percentage mortality was calculated using the following equation:

$$\text{PM} = \frac{\text{Number of dead shrimps at 24 hrs (Or 48 hrs) - Number of dead shrimps at } T_0 \text{ (Before acetic acid)}}{\text{Total number of dead shrimp (After acetic acid)}} \times 100$$

Equation 3: Selectivity index (SI)

The selectivity index was calculated using the following equation:

$$\text{SI} = \frac{LC_{50}^*}{\text{MIC}}$$

*Where the LC_{50} is the lethal concentration required to cause 50% percentage mortality at 24 hrs and 48 hrs and MIC is the minimum inhibitory concentration of the antimicrobial synergistic combination

Equation 4: Combination index (CI)

The combination index was calculated using the following equation:

$$CI = [(D)_1/(D_x)_1 + (D)_2/(D_x)_2]$$

*Where $(D)_1$ and $(D)_2$ represent the dose of each drug in combination necessary to produce the same effect, and $(D_x)_1$ and $(D_x)_2$ represent the individual dose of each drug required to inhibit a given level of cell growth.

$$D_x = D_m [F_a/(1/F_a)]1/m$$

*Where Dm refers to the median effect dose (Potency of drug expressed as IC_{50}), m is the slope of the graph, and Fa denotes the fraction effect (Expressed as % cell viability) in dose-effect curves.

Equation 5: Fractional effect analysis (Fa)

The fractional-effect analysis (Fa) was used to calculate the expected dose-response relationship for anti-inflammatory combinations.

$$\text{FIC (I)} = \frac{(A^*) \text{ combined with (B)}}{(A) \text{independently}}$$

$$\text{FIC (II)} = \frac{(B^*) \text{ combined with (A)}}{(B) \text{ independently}}$$

*****Where (A) is the NO produced by ibuprofen alone and (B) is the NO produced by the conventional antimicrobial/EOC alone.

GLOSSARY

Barrier to resistance – Describes how likely or easily a micro-organism can develop resistance to an antimicrobial agent (Chopra *et al.*, 2003). An antimicrobial agent that loses its efficacy if a micro-organism undergoes a single mutation, has a low barrier to resistance (ciprofloxacin and rifampicin) whilst multiple mutations define an antimicrobial agent with a high barrier to resistance (vancomycin)(Andersson, 2003).

Extensively-drug resistant – Describes a micro-organism that is non-susceptible to at least one agent in two or fewer antimicrobial categories or isolates remain susceptible to only one or two categories of antimicrobial agents (Magiorakos *et al.*, 2012).

Multi-drug resistant – Describes a micro-organism that is non-susceptible to at least one agent in three or more antimicrobial categories (Magiorakos *et al.*, 2012).

Pathobiont – Opportunistic micro-organisms that emerge when specific genetic, environmental, or host-related disturbances cause a change in the microbiome that leads to their selection and expansion (Jochum and Stecher, 2020). Pathobionts differ from contaminant micro-organisms as they colonize other anatomical locations and can even be beneficial (Chow *et al.*, 2011).

Polypharmacology – Refers to the ability of a drug to interact simultaneously and specifically with multiple targets to produce multiple biological responses on a single disease (Kabir and Muth, 2022; Wang and Yang, 2022a). It also includes drug effects that differ from its primary target and produce additional therapeutic effects (Ryszkiewicz *et al.*, 2023).

LIST OF ABBREVIATIONS

α	Alpha
β	Beta
γ	Gamma
˚C	Degrees Celsius
%	Percentage
<	Less than
=	Equal to
>	Greater than
≥	Greater than or equal to
≤	Less than or equal to
±	Plus-minus
AG	Amino-guanidine
AMR	Antimicrobial resistance
ARG	Antimicrobial resistance genes
ATCC	American type culture collection
ATP	Adenosine triphosphate
BSLA	Brine-shrimp lethality assay
CC	Culture control
CFU	Colony forming unit
CI	Combination index
CoNS	Coagulase-negative *Staphylococcus*
CRAB	Carbapenem-resistant *Acinetobacter baumannii*
CLSI	Clinical and Laboratory Standards Institute
COX	Cyclooxygenase
DMEM	Dulbecco's Modified Eagle's medium
DMSO	Dimethyl sulfoxide
EO	Essential oil
EOC	Essential oil compound
eNOS	Endothelial nitric oxide synthase
ESBL	Extended Spectrum Beta-Lactamase
EUCAST	European Committee on Antimicrobial Susceptibility Testing

Fa	Fractional effect
FBS	Foetal bovine serum
FIC	Fractional inhibitory concentration
ΣFIC	Sum of the fractional inhibitory concentration
g	Gram
HaCAT	Cultured human keratinocytes
HIV	Human immunodeficiency virus
hrs	Hours
IC_{50}	Half-maximal inhibitory concentration
IDSA	Infectious Diseases Society of America
IL	Interleukin
iNOS	Inducible-nitric oxide synthase
INT	*p*-iodonitrotetrazolium violet
LC_{50}	Lethal dose to kill 50.00%
LDH	Lactate dehydrogenase
LPS	Lipopolysaccharide
LTA	Lipoteichoic acid
MAPK	Mitogen-activated protein kinase
mg	Milligram
mg/L	Milligram per litre
mg/mL	Milligram per millilitre
MDR	Multi-drug resistant
MHB	Muller Hinton broth
MBC	Minimum bactericidal concentration
MIC	Minimum inhibitory concentration
min	Minutes
mL	Millilitre
mM	Millimolar
MMPs	Matrix metalloproteinases
MRSA	Methicillin-resistant *Staphylococcus aureus*
MSSA	Methicillin-susceptible *Staphylococcus aureus*
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NCTC	National Collection of Type Cultures
NF-κB	Nuclear factor kappa B

nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
NOS	Nitric oxide synthase
NSAIDs	Non-steroidal anti-inflammatory drugs
PGE_2	Prostaglandin E_2
PM	Percentage mortality
RPMI	Roswell Park Memorial Institute
SD	Standard deviation
SI	Selectivity index
SIRS	Systemic inflammatory response syndrome
SSTIs	Soft skin and tissue infections
TNF-α	Tumour necrosis factor - alpha
TSA	Tryptone Soya agar
TSB	Tryptone Soya broth
v/v	Volume/volume percentage
VRSA	Vancomycin-resistant *Staphylococcus aureus*
WHO	World Health Organisation
XDR	Extensively-drug resistant
XTT	2,3-Bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide
µg	Microgram
µg/mL	Microgram per millilitre
µM	Micromolar

DISSERTATION STRUCTURE

This dissertation was undertaken by publication and comprises the following chapters:

- Chapter One introduces the study and explains the design
- Chapter Two provides a comprehensive literature review
- Chapter Three – Publication 1 (*Essential oil compounds in combination with conventional antibiotics for dermatology*).
- Chapter Four – Publication 2 (*Cytotoxicity and anti-inflammatory properties of essential oil compounds in combination with conventional antimicrobials in dermatology*).
- Chapter Five – Publication 3 (*Ibuprofen as an adjuvant to conventional antimicrobials and essential oil compounds against skin pathogens*)
- Chapter Six – Recommendations, limitations, and conclusion

CHAPTER 1: INTRODUCTION

1.1 Skin infections

Skin and soft tissue infections (SSTIs) are multifactorial disease states that are a frequent cause of morbidity and mortality (Kaye *et al.*, 2019). A skin infection can be described as a microbial invasion of the skin or underlying tissue that results in an immune response and causes systemic symptoms such as pain, inflammation, redness, and swelling (Bouza *et al.*, 2022). Most skin infections are caused by Gram-positive bacteria such as *Staphylococcus aureus* and *Streptococcus pyogenes* (Esposito *et al.*, 2017). Gram-negative and fungal skin infections are less common and usually occur in hospital settings or have specific risk factors (Russo *et al.*, 2022a). According to several reviews, all skin infections can be classified into two broad categories: complicated and uncomplicated (DiNubile and Lipsky, 2004; Bassetti *et al.*, 2023). Uncomplicated infections are superficial, purulent wounds and can be managed effectively with common outpatient treatments (Esposito *et al.*, 2017). Some common examples include impetigo, cellulitis, and abscesses (Buckley, 2021). In contrast, complicated infections are severe, non-purulent, deep-tissue infections that generally present with systemic inflammatory response syndrome (SIRS) or sepsis (Dryden, 2010). These infections require extensive antibiotic therapy and surgical interventions (Bouza *et al.*, 2022). The most common examples are necrotizing fasciitis and surgical site infections (Poulakou *et al.*, 2019).

1.2 Inflammation

Cutaneous inflammation can be defined as a defensive mechanism in response to microbial signals (Ahmed, 2011). The main aim of inflammation is to reduce or remove foreign pathogens and restore cellular homeostasis (Medzhitov, 2010). Inflammation is predominantly mediated by the innate immune system, which stimulates a sequence of biochemical interactions between immune cells, plasma proteins, and infected tissue (Galvão *et al.*, 2018; Mathias, 2020; Bombassaro *et al.*, 2024). The extent of these interactions and duration can be used to classify the type of inflammation as acute or chronic (Medzhitov, 2008). Clinically, acute inflammation is characterized by the cardinal signs of inflammation *rubor* (redness), *calor* (increased heat), *tumor* (swelling), *dolor* (pain), and *functio laesa* (loss of function) (Galvão *et al.*, 2018). Each symptom is centred on migrating leukocytes out of the blood vessels

and into the surrounding tissue (Rodrigues *et al.*, 2019). For instance, vascular changes increase blood volume (redness) and permeability, which contributes to the leakage of cells and other molecules into the surrounding tissue (swelling) (Galvão *et al.*, 2018; Rodrigues *et al.*, 2019). This sensitizes afferent fibres to stimuli (pain) and cumulatively increases the temperature (warmth). Although most skin infections are superficial (Buckley, 2021), virulent pathogens may overstimulate immune responses, resulting in prolonged inflammation (chronic) and can even lead to sepsis (Jarczak *et al.*, 2021).

1.3 Antibiotics

The discovery of antibiotics revolutionized medicine and heralded a new era that greatly improved the quality of life and increased the lifespans of humans (Haney and Hancock, 2022). Antibiotics represent the cornerstone treatment for all skin infections and can be used topically, systemically, or both (Ki and Rotstein, 2008). Antibiotics inhibit the growth and proliferation of the micro-organism (-static) or induce cell death (-cidal) through interactions with essential cellular processes such as cell wall synthesis, protein synthesis, or DNA replication (Bhattacharjee, 2016). The inhibition of these micro-organisms reduces the expression of virulence factors and aids the immune system in clearing the infection (Hotinger *et al.*, 2021). The fundamental characteristic of an antibiotic is the selectivity for prokaryotes, which leaves eukaryotic cells unaffected (Yadav *et al.*, 2023). Currently, approximately 15 classes of conventional antimicrobials (including antifungals) and are classified according to their parent chemical structure (Bhardwaj *et al.*, 2022). Additional chemical modifications allow for the synthesis of analogues which demonstrate varying spectrums of pharmacokinetic/pharmacodynamic activity (Fischbach and Walsh, 2009). This means that a single conventional antimicrobial class can have many variants that can be used for different infections (Bhattacharjee, 2016). Antibiotics represent some of the most critical drugs in modern medicine as they have equipped humanity with a silver bullet to combat infectious diseases (Rolain et al., 2016). However, these victories are becoming short-lived due to the emergence and dissemination of antimicrobial resistance.

1.4 Antimicrobial resistance

Antimicrobial resistance (AMR) represents one of the principle public health problems of the 21st century and threatens the effective treatment and prevention of infectious diseases (Prestinaci *et al.*, 2015). The World Health Organization (WHO) has predicted that by the year 2050, approximately 10 million people may succumb to antimicrobial-resistant infections or associated conditions (O'Neill, 2016). In 2019, it was estimated that 4.95 million deaths occurred due to AMR with the majority of these deaths being attributed to the ESKAPE (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa,* and *Enterobacter*) pathogens (Oliveira *et al.*, 2020; Murray *et al.*, 2022). Another study by Smith *et al.* (2023) reported that COVID-19 contributed to a 14% increase in the acquisition of antibiotic-resistant bacteria and a 10% increase in the overall occurrence of AMR. Both these studies highlight the ongoing rise in AMR which correlates with an increase in morbidity and mortality.

Antimicrobial resistance can be described as a multifaceted arms race between modern medicine and micro-organisms (Baran *et al.*, 2023). As older antimicrobials lose their efficacy, newer antimicrobial drugs are needed to replace redundant ones (Rolain *et al.*, 2016). Thus, new antimicrobial drugs must be developed faster to effectively treat diseases than the emergence and spread of antimicrobial resistance genes (ARGs)(Cheesman *et al.*, 2017). Many reviews suggest that the injudicious use of antimicrobial agents is the primary culprit for antimicrobial resistance (Aljeldah, 2022; Haney and Hancock, 2022); however, there are other factors, such as climate change and the increased presence of non-antimicrobial pharmaceutical drugs in the environment (Hornischer and Häußler, 2016; Dutta and Ramamurthy, 2020; Paul and Varghese, 2020). In contrast to the increase in antimicrobial resistance, the stagnation in antibiotic development represents a serious humanity crisis that extends beyond the healthcare sector (Tarín-Pelló *et al.*, 2022; Muteeb *et al.*, 2023). The slow development of antibiotics has prompted the investigation into alternative strategies involving natural chemical reservoirs such as essential oils and their compounds (Trifan *et al.*, 2020; Yu *et al.*, 2020).

1.5 Essential oils and essential oil compounds

Essential oils (EOs) are complex natural mixtures of volatile compounds that plants produce as secondary metabolites (Bakkali *et al.*, 2008). Essential oils can comprise between 20 to ≥ 60 chemical compounds at varying concentrations, with two to three in high concentrations (20-70%) (Baptista-Silva *et al.*, 2020). However, due to several intrinsic (seasonal changes, geographical location, and subspecies) and extrinsic (method of extraction) factors systematic evaluation of EOs pharmacological properties is complex, which has shifted focus to the individual compounds (Dorman and Deans, 2000; Ju *et al.*, 2022).

Essential oil compounds (EOCs) are the individual chemical compounds that make up an essential oil and can be classified into three broad categories: terpenes, terpenoids, and phenylpropanoids (Baptista-Silva *et al.*, 2020; Angane *et al.*, 2022). Terpenes are the major compounds of EOs and form several structurally diverse classes based on the number of isoprene units (Masyita *et al.*, 2022). Terpenoids are oxygenated terpenes which are classified according to their functional groups such as alcohols, aldehydes, ketones, and phenols (Dorman and Deans, 2000; Carson and Hammer, 2011). Phenylpropanoids are non-terpene-derived compounds with a three-carbon chain attached to a six-carbon aromatic ring (Dhifi *et al.*, 2016). Most plant-derived phenylpropanoids are formed from cinnamic or *p*-coumaric acids (Vergis *et al.*, 2015). Essential oil compounds' antimicrobial and anti-inflammatory properties have been Investigated as natural alternatives to conventional therapies (Costa *et al.*, 2018).

1.6 Ibuprofen

Ibuprofen (2-(3-Isobutylphenyl)propionic acid) belongs to the non-steroidal anti-inflammatory (NSAID) class of drugs and is used for the treatment of pain, fever, and inflammation (Upadhyay *et al.*, 2021). Ibuprofen inhibits prostaglandin synthesis through the non-selective inhibition of cyclooxygenase (COX) enzymes (Varrassi *et al.*, 2020). Ibuprofen is commonly administered as adjunctive therapy to manage systemic symptoms induced by skin infections (Stevens *et al.*, 2014). Some studies have reported that ibuprofen may elicit other pharmacological properties such as anti-cancer properties (Shen *et al.*, 2020), reducing proteotoxicity in neurological disorders (Upadhyay *et al.*, 2016), and potentiating antimicrobial therapy (Pina-Vaz *et al.*, 2000; Ricardo *et al.*, 2009; Chan *et al.*, 2017; Rastgar *et al.*, 2022). Ibuprofen's antimicrobial properties have garnered interest and have been investigated in

respiratory and urinary tract infections (Byrne *et al.*, 2006; Vilaplana *et al.*, 2013; Ildikó *et al.*, 2015; Shah *et al.*, 2018; Aloush *et al.*, 2019). Although these studies are promising, there are still uncertainties regarding skin infections, citing idiopathic interactions between host defences and some causative pathogens, which warrants further investigations

1.7 Combination therapy

Combination therapy represents a type of polypharmacological strategy whereby two or more drugs are combined for co-administration or sequential administration to increase the overall therapeutic benefit (Wang and Yang, 2022a). Antimicrobial combination therapy offers several benefits such as increasing the spectrum of activity, reducing the emergence of resistance, lowering the dose of toxic drugs, and modulating the drug's pharmacological effects (Pillai *et al.*, 2005; Coates *et al.*, 2020). The combinational interactions between antimicrobial drugs are classified according to the degree of the combination's effectiveness (Keith *et al.*, 2005). Synergistic interactions occur when the overall effect of the combination is greater than the sum of the individual drugs (Bush, 2018). This means there is a greater reduction in microbial growth or killing than the sum of the individual drugs. The term "additive" or "additivity" is used to describe interactions where the combination exhibits an effect that is equal to the sum of the individual drugs (Dawis *et al.*, 2003; Sun *et al.*, 2016). Antagonism occurs when the combination demonstrates a decrease in the effectiveness that is less than the sum of the individual drugs (Bush, 2018; Zhu *et al.*, 2021). Lastly, indifference or non-interactive occurs when the combination effect is equal to the individual drugs, i.e., one of the drugs was inactive or had no effect (Tyers and Wright, 2019).

The combinational effects of conventional antimicrobials and EOCs have also been investigated and reviewed by several authors (Langeveld *et al.*, 2014; Owen and Laird, 2018; Bhattacharya *et al.*, 2021). In combination, EOCs augment an antibiotic's efficacy by modulating the micro-organism's physiology or reducing virulence factors (Lahmar *et al.*, 2017). However, most studies have only focused on investigating the effect of antimicrobial combinations with limited consideration of host-directed therapy, such as modulating the inflammatory pathway. Ibuprofen has also demonstrated positive therapeutic benefits in combination with conventional antimicrobials against bacteria and fungi (Zimmermann and Curtis, 2017; Yimer *et al.*, 2019).

1.8 Problem statement and rationale

Skin and soft tissue infections are diverse pathological conditions that burden patients and healthcare establishments. These infections are complex and increasingly challenging to treat due to antimicrobial resistance. There has been growing interest in essential oil compounds to address antimicrobial resistance. These aromatic compounds have demonstrated broad-spectrum antimicrobial activity and may also elicit anti-inflammatory properties. In addition, ibuprofen is a well-known anti-inflammatory drug that can elicit secondary pharmacological effects, including antimicrobial effects. Considering the multifaceted pathophysiology of skin infections, which involves numerous interactions between the host defences and causative pathogens, there is very little research investigating a multidrug combination of EOCs, antibiotics, and ibuprofen. This study investigated the *in vitro* antimicrobial, cytotoxicity, and anti-inflammatory effects of combining EOCs, conventional antimicrobials, and ibuprofen against common skin pathogens.

Although antimicrobial combinations are effective, they only target the causative pathogen and require additional adjunctive therapy to manage systemic symptoms. Investigating combinations comprising EOCs, conventional antimicrobials, and ibuprofen represents a comprehensive and holistic therapeutic approach. Essential oil compounds are naturally derived bioactive compounds that can potentiate conventional antimicrobials and elicit anti-inflammatory effects. Ibuprofen can alleviate inflammation and given its emerging status as a "non-antibiotic", could synergize with conventional antimicrobials. Therefore, these combinations offer a multitargeted strategy that could potentially improve the efficacy of conventional antimicrobials, reduce inflammation, and enhance the overall therapeutic effect.

1.9 Essential oil compound selection

The selection of the EOCs (Table 1.1) was based on compounds that have demonstrated both antimicrobial and anti-inflammatory properties (De Cássia da Silveira Sá *et al.*, 2014; Nazzaro *et al.*, 2017). In addition, compounds were selected from different chemical classes with limited or no previous studies investigating the combinational effects against the selected micro-organisms.

1.10 Conventional antimicrobials selection

The conventional antimicrobials selected for this study (Table 1.2) were based on several factors: a.) Conventional antimicrobials that were poorly studied in combination with essential oil compounds, b.) frequency of clinical use for both complicated and uncomplicated skin infections (Stevens *et al.*, 2014; Tamma *et al.*, 2023), c.) "Drug-bug" matching whereby specific antimicrobial agents have a counterpart pathogen (De Waele *et al.*, 2020), d.) antimicrobials from different classes and mechanisms of action and e.) the availability of the conventional antimicrobials.

1.11 Ibuprofens selection

The inclusion of ibuprofen was based on the established status as an anti-inflammatory drug (Rainsford, 2012b). In addition, the emerging evidence of ibuprofen as a polypharmacological drug was also considered, particularly for its antimicrobial properties (Paes Leme and da Silva, 2021; Babaei *et al.*, 2023).

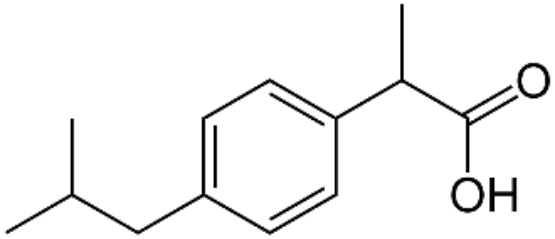


Figure 1.1: Chemical structure of structure of (±) – Ibuprofen

1.12 Micro-organisms selection

The micro-organisms selected for this study (Table 1.3) were based on their frequency in causing both complicated and uncomplicated skin infections (Dryden, 2010; Tognetti *et al.*, 2012; Olaniyi *et al.*, 2017; Severn and Horswill, 2023). In addition, micro-organisms were also selected based on their increasing prevalence of antimicrobial resistance (Aslan Kayiran *et al.*, 2020; Vale de Macedo *et al.*, 2021).

Table 1.1: Essential oil compounds selected for this study

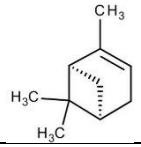
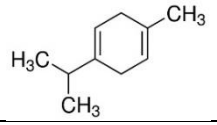
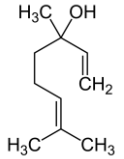
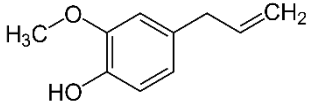
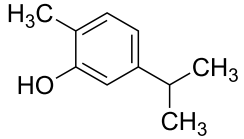
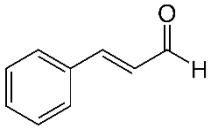
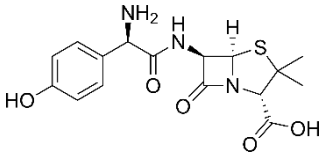
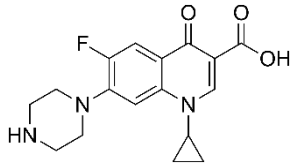
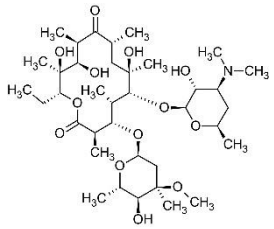
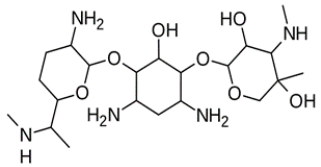
Essential oil compound	Classification	Chemical structure	Antimicrobial spectrum	Effect on inflammation	References
(-) α-Pinene	Monoterpene hydrocarbon		Weak broad-spectrum activity against bacteria and fungi.	Elicits multiple anti-inflammatory properties through modulation of NF-kB signalling and suppression of the MAPK pathway.	(De Cássia da Silveira Sá *et al.*, 2013; Kim *et al.*, 2015; Suganya *et al.*, 2022)
γ-Terpinene	Monoterpene hydrocarbon		Weak broad-spectrum activity against bacteria and fungi.	Reduces prostaglandin synthesis and inhibits pro-inflammatory cytokines.	(Ramalho *et al.*, 2016)
(±)-Linalool	Alcohol terpenoid		Moderate broad-spectrum activity against bacteria with limited evidence against fungi.	Elicits anti-inflammatory effects through indirect inhibition of interleukins and reduces NO production.	(An *et al.*, 2021; Mączka *et al.*, 2022)
Eugenol	Phenylpropanoid		Strong broad-spectrum activity with higher efficacy against Gram-negative bacteria and fungi.	Elicits anti-inflammatory effects through inhibition of prostaglandin and leukotriene production. Can also suppress arachidonic acid metabolism and thromboxane A_2.	(De Cássia da Silveira Sá *et al.*, 2014; Nazzaro *et al.*, 2017; Suganya *et al.*, 2022)
Carvacrol	Phenolic		Strong broad-spectrum activity with higher efficacy against Gram-positive bacteria and some fungi. Has stronger antimicrobial properties than eugenol.	Modulates prostaglandin synthesis and other pro-inflammatory interleukins.	(De Cássia da Silveira Sá *et al.*, 2013)
Trans-Cinnamaldehyde	Phenylpropanoid		Although weaker than eugenol and carvacrol, has strong activity against Gram-negative bacteria with limited evidence against fungi.	Numerous anti-inflammatory mechanisms including inhibition of NO production and PGE_2 synthesis. Can also reduce ROS release from cells.	(De Cássia da Silveira Sá *et al.*, 2014)

Table 1.2: Conventional antimicrobial selected for this study

Conventional antimicrobial (Classification)	Chemical structure	Mechanism of action	Antimicrobial spectrum	Clinical use in dermatology	Effect on inflammation	References
Amoxicillin (Beta-lactam – penicillin)		Inhibits the transpeptidation reaction of cell wall synthesis. (Bactericidal – time dependent)	Mainly Gram-positive bacteria with limited Gram-negative coverage.	Normally used as first-line therapy for uncomplicated infections. Pivotal treatment for cutaneous infections.	Can increase inflammation through its antibacterial mechanism.	(Melhus, 2001; Kapoor *et al.*, 2017)
Ciprofloxacin (Fluroquinolone)		Inhibits the unwinding of DNA by binding to DNA gyrase and topoisomerase IV which prevents cell division. (Bactericidal – concentration dependent)	Broad spectrum activity against both Gram-positive and Gram-negative bacteria.	Predominantly used in complicated infections to increase the spectrum of cover. Commonly used before gentamicin due to lower toxicity.	Demonstrates immunomodulatory effects and can reduce the production of pro-inflammatory interleukins and NO.	(Kapoor *et al.*, 2017)
Erythromycin (Macrolide)		Binds to the 50S subunit of the bacterial rRNA complex and inhibits protein synthesis. (Bacteriostatic – time dependent)	Mainly Gram-positive bacteria with limited Gram-negative coverage	Can be used interchangeably with amoxicillin. Widely used for uncomplicated infections including acne.	Has multiple anti-inflammatory properties; accumulates inside macrophages and can reduce pro-inflammatory interleukins and NO production.	(Labro, 1998; Tamaoki *et al.*, 2004; Pradhan *et al.*, 2016)
Gentamicin (Aminoglycoside)		Binds to the 30S subunit of the bacterial ribosome and inhibits protein synthesis. (Bactericidal – concentration dependent)	Broad-spectrum activity against both Gram-positive and Gram-negative bacteria.	Used as empirical therapy for life-threatening infections. Normally added to treatment regiments to increase the spectrum of cover.	Elicits anti-inflammatory properties through inhibition of neutrophil activation and impairing angiogenesis.	(Umeki, 1995; Olekson *et al.*, 2017; Dallo *et al.*, 2023)

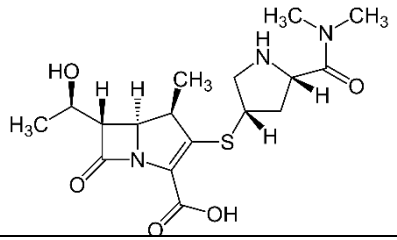
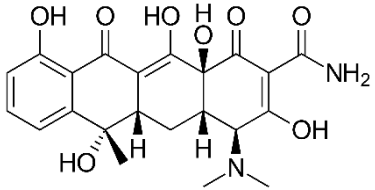
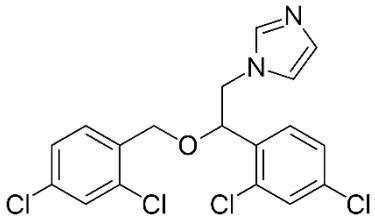
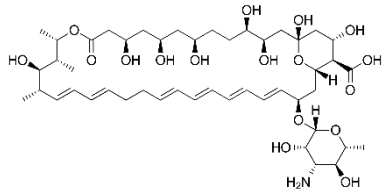
Conventional antimicrobial (Classification)	Chemical structure	Mechanism of action	Antimicrobial spectrum	Clinical use in dermatology	Effect on inflammation	References
Meropenem (Beta-lactam-carbapenem)		Inhibits the transpeptidation reaction of cell wall synthesis. (Bactericidal – time dependent)	Broad spectrum activity against both Gram-positive (Not MRSA) and Gram-negative bacteria. Demonstrates higher resistance to beta-lactamase enzymes. Used as an agent of "last resort".	Used for complicated infections caused by multi-drug resistant Gram-negative bacteria such as *P. aeruginosa* and *A. baumannii*.	Noted to increase inflammation in some *in vitro* studies due to its antibacterial mechanism of action.	(Papp-Wallace *et al.*, 2011; Ye *et al.*, 2021)
Tetracycline (Tetracycline)		Binds reversibly to the bacterial 30S ribosomal subunit and inhibits protein synthesis by blocking the addition of new amino acids. (Bacteriostatic – time and concentration-dependent)	Broad spectrum activity against Gram-positive and some Gram-negative bacteria.	Predominantly used to treat mild, superficial infections. Demonstrates excellent lipid penetration which increases its efficacy in acne vulgaris.	Inhibits pro-inflammatory enzymes involved in dermal inflammation; can reduce interleukin and NO production.	(Pradhan *et al.*, 2016; Dallo *et al.*, 2023)
Miconazole (Azole-imidazole)		Inhibits the fungal enzyme 14-α demethylase which reduces the synthesis of ergosterol. Can also induce oxidative stress intracellularly. (Fungicidal – concentration dependent)	Demonstrates intermediate spectrum activity with reported Gram-positive activity.	Used as a topical ointment for superficial fungal infections.	Has numerous anti-inflammatory properties and can promote wound healing.	(Quatresooz *et al.*, 2008; Nenoff *et al.*, 2017)
Nystatin (Polyene)		Ionophore. Induces pore formation in the fungal cell membrane resulting in leakage of ions and eventual cell death. (Fungicidal – concentration dependent)	Elicits the broad spectrum of activity against *Candida* species, dermatophytes, and *Cryptococcus* species.	Used as a topical ointment for superficial candidiasis infections.	Elicits pro-inflammatory properties via induction of interleukin secretion.	(Darisipudi *et al.*, 2011; Bongomin *et al.*, 2017)

Table 1.3: Micro-organisms selected for this study

Micro-organism	Classification	Description	Role in skin infections	Reference
Cutibacterium acnes	Gram-positive	Forms part of the natural skin microbiota. Regulates skin immune responses.	Widely recognized as the microbial cause of acne vulgaris. Can also increase the severity of mild infections.	(Fournière *et al.*, 2020; Mayslich *et al.*, 2021)
Staphylococcus aureus	Gram-positive	Pathobiont. Colonizes the upper respiratory tract as a commensal. Highly pathogenic.	The most frequently isolated pathogen in acute and chronic skin infections. Demonstrates high resistance to antibiotics and virulence factors.	(Nandhini *et al.*, 2022)
Staphylococcus epidermidis	Gram-positive	The most common Coagulase-negative *Staphylococcus* species (CoNS) is found on the skin and plays an integral role in microbiota-mediated immunity.	Increasing occurrence of skin infections. Can also worsen superficial skin infections through the expression of virulence factors.	(Fournière *et al.*, 2020; Severn and Horswill, 2023)
Pseudomonas aeruginosa	Gram-negative	Pathobiont can also be acquired from the environment. Has high levels of antibiotic resistance.	Can cause skin infections in healthy and immunosuppressed individuals. Increases the severity of acute skin infections.	(Kerr and Snelling, 2009; Oliveira *et al.*, 2020)
Acinetobacter baumannii	Gram-negative	Multi-drug resistant nosocomial pathogen that is acquired from the environment. Elicits high levels of antibiotic resistance with limited therapeutic options.	Implicated in nosocomial skin infections with exceedingly high mortality rates. Also contributes to chronic skin infections.	(Peleg *et al.*, 2008; Castanheira *et al.*, 2023)
Candida albicans	Yeast	Commensal yeast that colonizes various parts of the skin and other mucosal areas.	Has the highest incidence rate in fungal infections. Elicits high virulence factors that can induce recurrent infections.	(Fisher *et al.*, 2022)

1.13 Aim and objectives

This study investigated the *in vitro* antimicrobial, cytotoxicity, and anti-inflammatory effects of combining six essential oil compounds with eight conventional antimicrobials and ibuprofen against common skin pathogens (Figure 1.2).

The objectives of this study:

Objective 1: To investigate the antimicrobial activity of the selected essential oil compounds, conventional antimicrobials, and ibuprofen singularly and in combination.

Objective 2: To determine the antimicrobial interactive profiles of all combinations involving essential oil compounds, conventional antimicrobials, and ibuprofen.

Objective 3: To determine the optimal 1:1 ratios of combinations that have demonstrated synergy using isobolograms as a graphical tool to demonstrate interactions.

Objective 4: To investigate the toxicity of the essential oil compounds and conventional antimicrobials singularly and for the synergistic antimicrobial combination using the brine-shrimp lethality and MTT cytotoxicity assays. Dose-response curves were used for the brine shrimp lethality assay to determine the LC_{50} for essential oil compounds and the synergistic combinations' selectivity index (SI). For the MTT assay, the cell viability, $IC_{50,}$ and combination index (CI) were determined for the conventional antimicrobials, essential oil compounds, and synergistic combinations.

Objective 5: To determine the anti-inflammatory activity of the essential oil compounds, conventional antimicrobials, and ibuprofen singularly and for the synergistic combinations using the LPS - NO-induced anti-inflammatory assay.

Objective 6: To determine the cytotoxicity and anti-inflammatory properties of ibuprofen singularly and for the antimicrobially synergistic combinations (Ibuprofen with conventional antimicrobial, and ibuprofen with conventional antimicrobials and essential oil compound) using the MTT and LPS - NO-induced assays.

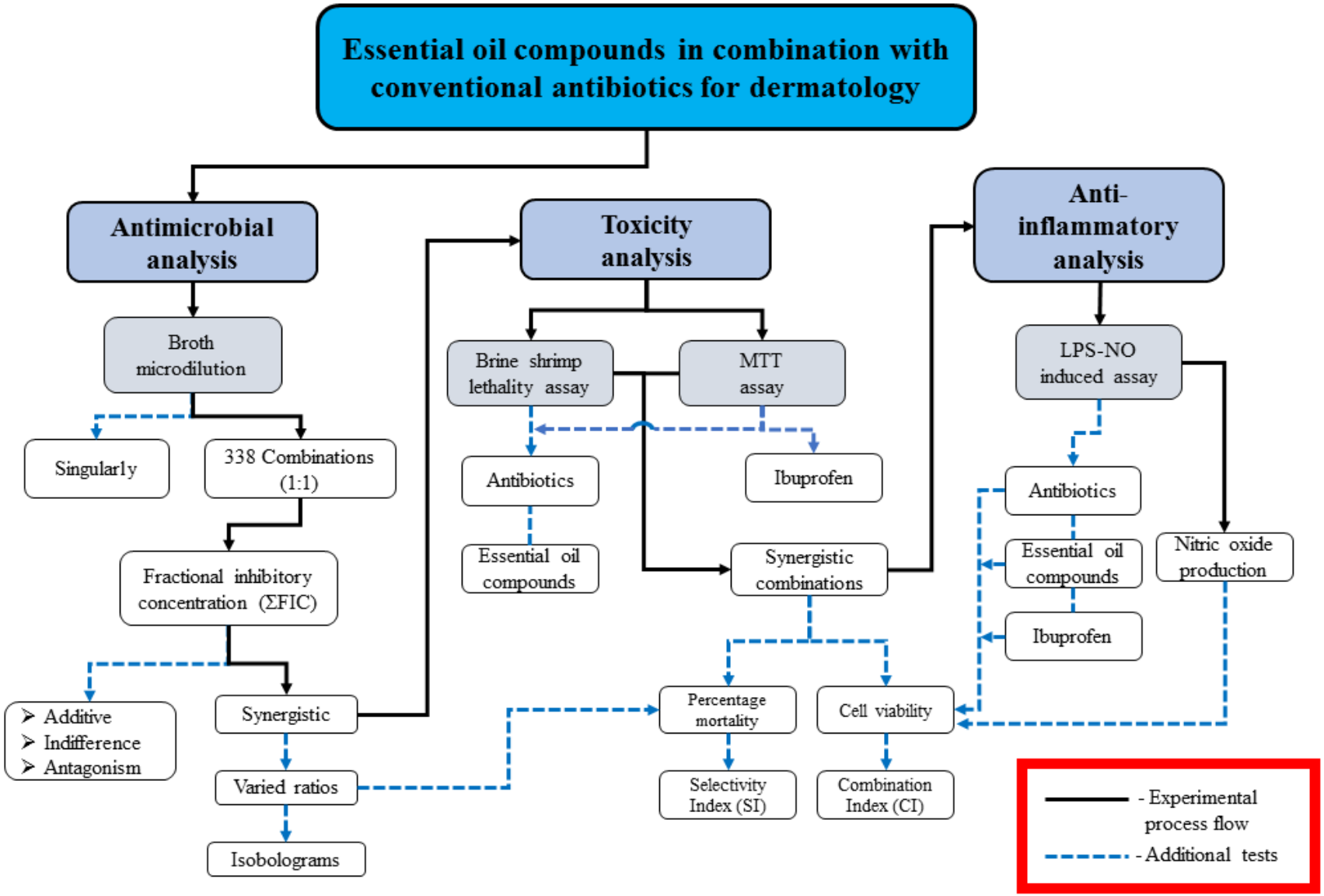


Figure 1.2: Experimental schematic of study objectives

CHAPTER 2: LITERATURE REVIEW

2.1 Skin physiology and immunology

The skin is the largest organ in the human body and comprises multiple layers of continuous tissue that act as the primary barrier against physical, chemical, and biological assailants (Ojeh *et al.*, 2015). The three main layers of the skin are the epidermis, the dermis, and the subcutaneous tissue (Powell, 2006). These layers have various physiological functions, including producing collagen, maintaining homeostasis, providing nutritional support, thermoregulation, and insulation (Rodrigues *et al.*, 2019). The proper functioning of the skin requires close communication and collaboration between various cell types, including the stromal cells (keratinocytes, fibroblasts, endothelial cells, and adipocytes) as well as those derived from bone marrow (dendritic cells, macrophages, natural killer cells, mast cells, and T cells)(Vale de Macedo *et al.*, 2021). Additionally, plasma mediators contribute to innate immune defence by bridging the transition from innate to adaptive immune responses (Turner, 2017).

2.2 Skin microbiota

The skin microbiota is a consortium of bacteria, viruses, and fungi that colonize the skin and exhibit complex relationships with each other and the host immune system (Smythe and Wilkinson, 2023). The most common Phyla are Actinobacteria (52%), Firmicutes (24%), Proteobacteria (17%), and Bacteroidetes (7%), whilst *Corynebacteria*, *Cutibacterium*, and *Staphylococci* are the most prevalent genera (Zhu *et al.*, 2023). The composition of the skin microbiome is influenced by several host-related factors such as age, anatomical site, and associated co-morbidities (Canchy *et al.*, 2023). Each genus tends to colonize specific areas of the skin, which is determined by the chemistry of the skin's ecotone and moisture content (Ersanli *et al.*, 2023). For instance, seborrheic areas are inhabited by lipophilic species such as *C. acnes* and CoNS, whilst high moisture sites are dominated by *Corynebacterium* species (Grice and Segre, 2011). The function of the skin microbiome can be broadly classified into three groups: directly inhibit the growth of pathogenic bacteria, enhance host innate immunity, and educate the adaptive immune responses (Egert and Simmering, 2016). For example, *S. epidermidis* produces antimicrobial peptides that inhibit the growth of *S. aureus* whilst *C. acnes* metabolizes free fatty acids from sebum and decreases the surface pH, contributing to barrier

homeostasis (Fournière *et al.*, 2020). These interactions shape the resident microbial community and play an integral role in regulating cutaneous inflammatory responses and cell differentiation (Sachdeva *et al.*, 2022). However, changes in the microbial communities have been linked to several skin disorders such as atopic dermatitis, psoriasis, and acne vulgaris (Dessinioti and Dreno, 2023). Skin microbiome dysbiosis can occur when there is a loss of commensal microbial diversity due to host genetics or environmental factors (Nørreslet *et al.*, 2020). This dysbiosis changes the immune system's reactivity and subsequently leads to the development of inflammatory diseases or allows colonization of transient micro-organisms (Zeeuwen *et al.*, 2013).

2.3 Pathogenesis of skin infections

The development of a skin infection is a multi-step process that involves detecting a pathogen, stimulating acute inflammation, antigen presentation, activation of adaptive immunity, and pathogen destruction (Mathias, 2020). Skin infections usually result from an imbalance between the pathogenicity of micro-organisms and the host's immunological defences (Buckley, 2021). All pathogens that cause cutaneous infections are associated with the infection's anatomical location, such as being commensals or are acquired from the environment (Chiller *et al.*, 2001; Miller, 2017). Various physical, chemical, or immunological alterations can predispose the skin to pathogen penetration, facilitating the entry of these micro-organisms into the underlying tissue (Buckley, 2021). Upon entry into the tissue, the pathogens must evade the host defences and multiply to a point whereby the infection is self-sustaining (Coates *et al.*, 2018). Most skin pathogens elicit virulence factors, which enhance their intracellular survival and induce damage to host cells (Miller, 2017). Some common examples include exotoxins and adhesin proteins (Gatica *et al.*, 2023). The increased presence of these pathogens stimulates other cascades of the adaptive immune system and subsequently triggers systemic symptoms such as pain and inflammation (Balato and Megna, 2017).

2.4 Inflammatory mediators for skin infections

Inflammatory mediators are regulatory molecules that control inflammation's generation, maintenance, and resolution. (Galvão *et al.*, 2018). Cell mediators include immune cells (keratinocytes, macrophages, dendritic cells, and neutrophils), vasoactive amines (histamine and serotonin), lipid mediators (prostaglandins and leukotrienes), and cytokines (interleukins and chemokines) (Balato and Megna, 2017). Plasma mediators include the complement system

and associated proteins, kinins, and platelets (Jain *et al.*, 2015). Together, these mediators control all inflammatory processes, clearing the invading pathogens and restoring cellular homeostasis (Stingl *et al.*, 2017).

2.4.1 Pathogen-associated molecular patterns

Pathogen-associated molecular patterns (PAMPs) are conserved microbial structures that bind to pathogen-recognition receptors (PRR), which are present in keratinocytes and stimulate inflammation (Silva-Gomes *et al.*, 2015). Pathogen-associated molecular patterns are not inflammatory mediators. These molecules act as signalling alarms to activate the immune system (Galvão *et al.*, 2018). There are many types of PAMPs, the most studied being lipopolysaccharide (LPS), a glycolipid that makes up the outer layer of the cell membrane in Gram-negative bacteria (Coates *et al.*, 2018). Other relevant PAMPs include lipoteichoic acid (LTA) peptidoglycan (Gram-positive bacteria) and mannan derivatives or β-glucans (*C. albicans*) (Silva-Gomes *et al.*, 2015). Both bacteria and fungi can have many different PAMPs, which stimulate varying degrees of inflammation (Kumar *et al.*, 2011). Additionally, the increased presence or impaired recognition of PAMPs is directly related to chronic inflammatory disorders and septic shock (Coates *et al.*, 2018).

2.4.2 Cytokines

Cytokines are a diverse group of polypeptides that are produced by various cells in response to different stimuli (PAMPs) and function as mediators of inflammation (Table 2.1) (Balato and Megna, 2017). Interleukins are a type of cytokine primarily produced by immune cells and exhibit many functions, including activating the immune system, modulating inflammation, and controlling the growth of granulocytes (Balato and Megna, 2017). The function of a specific interleukin depends on its binding to specific receptors that mediate paracrine and autocrine signalling. This activates other immune system branches or can also reduce inflammation (Jain *et al.*, 2015). Interleukins also demonstrate overlapping actions which means that some interleukins elicit similar responses (Galvão *et al.*, 2018). Chemokines are chemotactic cytokines that control the migration and residence of immune cells into the infected tissue (Turner, 2017). Tumor Necrosis Factor (TNF) is a transmembrane cytokine that is one of the most important activators of inflammation and predominantly activates the immune system (Wallach and Kovalenko, 2016). Interleukins, chemokines, and TNF function

synergistically or independently, whereby each cytokine may modulate the production or inhibition of each other to regulate inflammation (Chousterman *et al.*, 2017).

2.4.3 Nitric oxide

Nitric oxide (NO) is a type of reactive oxygen species (ROS) that plays a vital role in physiological processes, host defence, and inflammation (Boscá *et al.*, 2005). Nitric oxide is produced through the oxidative deamination of L-arginine to L-citrulline by nitric oxide synthetase (NOS) (Aktan, 2004). There are three isoforms of NOS which include neuronal nitric oxide synthase (nNOS), endothelial nitric oxide synthase (eNOS), and inducible nitric oxide synthase (iNOS). Inducible nitric oxide synthase is the main isoform associated with inflammation and can be activated by macrophages (Jain *et al.*, 2015). The upregulation of iNOS stimulates the production of NO, which attracts immune cells, induces the release of inflammatory cytokines (IL-1β and TNF-α), and directly inhibits the growth of the invading pathogens (Sharma *et al.*, 2007). Nitric oxide also exhibits detrimental effects, whereby overstimulation induces cytotoxicity, premature apoptosis of immune cells, and autoimmune disorders such as atopic dermatitis (Aktan, 2004).

2.4.4 Prostaglandin

Prostanoids are a group of eicosanoids consisting of 20 carbon fatty acids that are produced through the cyclooxygenase pathway from arachidonic acid in response to inflammatory stimuli (Nüsing, 2016). Prostaglandin E_2 (PGE_2) is the most abundant prostanoid and is an important mediator of biological functions such as regulating blood pressure, kidney function and immune responses (Balato and Megna, 2017). Under inflammatory conditions PGE_2 regulates the cardinal signs of acute inflammation, promotes the proliferation or apoptosis of immune cells, and can also control the production of cytokines and chemokines which stimulates the remodelling phase in wound healing (Nicolaou, 2013).

Table 2.1: Summary of some important inflammatory mediators involved in skin infections

Inflammatory mediators	Examples	Inflammatory function	Role in skin infections	References
Interleukins	IL-1β	Pro-inflammatory. Stimulates proliferation of keratinocytes and activates leukocytes. Also induces the release of histamine from mast cells which increases localized inflammation.	Increased levels of IL-1β activate other pro-inflammatory cytokines which induces tissue damage and eventual sepsis.	(Turner, 2017)
	IL-6	Pro-inflammatory. Stimulates the healing process, activates immune cells (B and T cell proliferation), and functions as an endogenous pyrogen. Also induces the release of acute-phase proteins.	Overproduction of IL-6 impairs wound healing (proliferation and remodelling phases) resulting in scar formation. High concentrations of IL-6 can also cause sepsis, especially in deep-tissue infections.	(Turabelidze and Dipietro, 2011)
	IL-8 (CXCL1)	Pro-inflammatory. Primarily functions as a signalling molecule for neutrophils.	Overproduction of IL-8 can induce cell necrosis and chronic inflammation.	(Balato and Megna, 2017)
	IL-10	Anti-inflammatory. Inhibits cytokines and chemokine production and regulates allergen tolerance.	Reduced levels of IL-10 causes chronic inflammation due to uncontrolled activation of macrophages. Overproduction of IL-10 increases the severity of infections.	
	IL-17 (Including sub-groups)	Pro-inflammatory. Promotes neutrophil trafficking and stimulates the production of antimicrobial peptides, chemokines, and metalloproteinases.	Reduced levels of IL-17 increase the ability of transient and contaminant pathogens to colonize the skin, especially *C. albicans*. This can lead to recurrent skin infections and even sepsis. In addition, low levels of IL-17 impair wound-healing.	(Turner, 2017)
Tumour necrosis factor (TNF)	TNF-α	Pro-inflammatory. One of the main pro-inflammatory mediators. Elicits multifunctional effects on immune cells including cell signalling (Enhances the release of other cytokines), growth, and motility. Also activates the adaptive immune system.	Increased levels of TNF-α with IL-1β contributes to poor wound healing, tissue damage, and autoimmune skin disorders.	(Wallach and Kovalenko, 2016)
Chemokines	CC, CXC, CX3C and C	Pro-inflammatory. Attracts leukocytes to the wound site and induces the release of histamine.	Overproduction of chemokines continuously stimulates resident immune cells which results in prolonged inflammation and impair wound healing.	(Sugaya, 2015)
Reactive oxygen species (ROS)	Nitric oxide (NO)	Concentration-dependent (Pro- and anti-inflammatory). Functions as a signalling molecule for macrophages and neutrophils and inhibits the growth of pathogens.	Increased levels of NO impair wound healing and can induce sepsis. Prolonged upregulation of iNOS has been linked with autoimmune disorders and toxic epidermal necrolysis.	(Sharma *et al.*, 2007)
Arachidonic acid metabolites	Prostaglandin (PGE_2)	Receptor specific (Pro- and anti-inflammatory). Promotes vasodilation, and other signs of inflammation. Can also function as a pyrogen.	Dysfunction in PGE_2 can induce prolonged inflammatory responses resulting in necrosis and facilitate an increase in the colonization of pathogenic bacteria. High levels of PGE_2 can also impair host defences against certain pathogens.	(Balato and Megna, 2017; Cai *et al.*, 2018)

2.5 Types of skin infections

The classification of a skin infection is complex and varies according to different treatment guidelines. All skin infections can be broadly classified as complicated or uncomplicated infections (Esposito *et al.*, 2017; Duane *et al.*, 2021). This classification considers a wide range of risk factors such as anatomical location, depth of infection, causative pathogen, rate of progression, and co-morbidities (Poulakou *et al.*, 2019). The Infectious Disease Society of America (IDSA) proposed grouping skin infections based on the wound being purulent or non-purulent (Stevens *et al.*, 2014). Non-purulent wounds are further divided into necrotizing or non-necrotizing. This classification simplifies clinical diagnosis and facilitates an empirical treatment approach (Kaye *et al.*, 2019). However, both classifications can be summarized as uncomplicated infections being purulent, and complicated infections being non-purulent.

2.5.1 Impetigo

Impetigo is a highly contagious, superficial bacterial skin infection commonly occurring in children with a global disease burden of approximately 140 million (Johnson, 2020). It presents as non-bullous (70%) or bullous (30%) with yellow, erythematous plaques and may be itchy or painful. Impetigo is generally transmitted through direct contact with risk factors, including poor hygiene, malnutrition, and environmental factors (humidity and high temperatures) (Veraldi and Benzecry, 2023). Impetigo can be classified as either primary, which involves direct bacterial invasion into the skin, or secondary which involves infection at a pre-existing skin condition such as eczema (Johnson, 2020). Non-bullous impetigo is caused by *S. aureus* alone or in combination with Group A *Streptococcus*, while bullous impetigo is almost exclusively *S. aureus* (Olaniyi *et al.*, 2017). Impetigo is mostly self-limiting and resolves within three weeks with minimal scarring or associated complications (Koning *et al.*, 2012).

2.5.2 Folliculitis

Folliculitis is a superficial inflammatory reaction of the hair follicles in the epidermis (Luelmo-Aguilar and Santandreu, 2004). Folliculitis is characterized by scattered or extensive follicular pustules that can be surrounded by a ring of erythema located within follicular orifices (Durdu and Ilkit, 2013). The pustules may be present from the onset of the infection and usually heal without scarring. Deep tissue folliculitis can also occur, involving plaques or nodules, and can

enlarge to form an abscess (Ioannides and Lazaridou, 2023). Risk factors for folliculitis include poor hygiene, diabetes, and immunodeficiencies (Esposito *et al.*, 2017). Bacteria and fungi can cause folliculitis, with the most common being Gram-positive bacteria such as *S. aureus* (David and Daum, 2017). Gram-negative folliculitis (Hot-tub folliculitis) involves *P. aeruginosa* and arises from exposure to contaminated water or improperly treated swimming pools or hot tubs (Wu *et al.*, 2011).

2.5.3 Cellulitis

Cellulitis is an infection involving the deep dermis and subcutaneous tissue with a global incidence rate of approximately 600000 hospitalizations per year (Ki and Rotstein, 2008). The clinical presentation of cellulitis varies according to the anatomical location and causative pathogen and usually involves a rapid onset of a fever and chills, followed by a painful lesion with a red oedematous appearance (Balabanova, 2023). Cellulitis usually arises from trauma to the skin, such as scratching or abrasions and can progress rapidly to necrosis (Dryden, 2010). Other risk factors include invasive surgeries, pre-existing interdigital infections, and chronic swelling (Chlebicki and Oh, 2014). The most common causative pathogens are *S. pyogenes* and *S. aureus* (Ki and Rotstein, 2008).

2.5.4 Necrotizing infection

Necrotizing soft-tissue infections are serious, life-threatening wounds involving any or all soft-tissue compartment layers (dermis, subcutaneous tissue, fascia, and muscle) (Hua *et al.*, 2023). These infections have an average mortality of 30%, which can increase depending on host-related factors such as diabetes, chronic liver or kidney disease, systemic lupus erythematosus, and cirrhosis (Misiakos *et al.*, 2014). Necrotizing infections occur through previous trauma or abrasions to the skin and can also occur from surgical site incisions (Misiakos *et al.*, 2014). These infections initially present with swelling, tenderness, and pain and can rapidly progress to septic shock and multiorgan failure (Peetermans *et al.*, 2020). One of the major contributing factors in the development of necrotizing infections is pathogen virulence factors and patient co-morbidities (Stevens and Bryant, 2017). The microbial aetiology of necrotizing infections is complex with approximately 50-85% being polymicrobial infections (Dhanasekara *et al.*, 2021; Hua *et al.*, 2023). According to several reviews and standard therapeutic guidelines, a classification system has been used to describe the causative pathogens, rate of progression,

and recommended treatment (Stevens *et al.*, 2014; Stevens and Bryant, 2017; Peetermans *et al.*, 2020). Type I is the most common and accounts for the majority (70-80%) of all necrotizing infections (Paramythiotis *et al.*, 2007). This type is polymicrobial and involves a mixture of Gram-positive (*S. aureus* and *S. pyogenes*), Gram-negative (*Enterobacteriaceae* species, *P. aeruginosa*), and anaerobic (*Clostridium perfringens*) bacteria (Davoudian and Flint, 2012). A major contributing factor is that the virulence factors exhibited by the bacteria work together synergistically, increasing the severity (Hua *et al.*, 2023). Type II is less common (20-30%) and can also develop from a varicella infection (Secondary infection) (Raulin *et al.*, 2010). Type II is monomicrobial involving *S. pyogenes* or *S. aureus* and can progress to toxic shock syndrome (TSS) (Davoudian and Flint, 2012). In recent years, type III infections have become increasingly common (3-5%) due to the increased prevalence of *Vibrio vulnificus* and *Aeromonas* species in seafood (Huang *et al.*, 2016). Type III almost exclusively involves marine-related Gram-negative bacteria and can also include some *Clostridium* species (Paramythiotis *et al.*, 2007). Type IV infections are rare (< 1%) and are predominantly fungal infections involving *Candida* species (Immunocompetent) and Zygomycetes (Immunocompromised) (Dhanasekara *et al.*, 2021). Type II and Type IV infections can overlap due to synergistic interactions between *Candida* and *Staphylococcus* species (Förster *et al.*, 2016).

2.5.5 Acne vulgaris

Acne vulgaris is an inflammatory dermatosis of the pilosebaceous unit and is among the most common dermatological condition worldwide, with an estimated 650 million people affected (Coenye *et al.*, 2022). The pathogenesis of acne is multifactorial and involves four main factors: increased sebum production (commonly androgen-induced), follicular hyperkeratinisation, increased presence of skin bacteria (*C. acnes*), and inflammation (Moradi Tuchayi *et al.*, 2015; Kumtornrut and Noppakun, 2021). Acne vulgaris occurs through host-related factors such as hormone changes, which stimulate sebum production (Walsh *et al.*, 2016). The accumulation of sebum and dead skin cells block or alter follicular growth and increase the growth of *C. acnes*, which colonizes the pilosebaceous unit (Dessinioti and Dreno, 2023). The increased presence of *C. acnes* stimulates immune cells and the production of pro-inflammatory cytokines, which induces inflammation and the formation of pustules or nodules (Dagnelie *et al.*, 2021). Although acne is described as an inflammatory skin condition, skin lesions and prolonged inflammation may contribute to other cutaneous infections, such as folliculitis

(O'Neill and Gallo, 2018). Other factors that may increase the severity of acne include nutrition and some medications (Dessinioti and Dreno, 2023).

2.5.6 Cutaneous candidiasis

Candidiasis refers to infections caused by *Candida* species (Sharma *et al.*, 2024). Cutaneous candidiasis is classified according to the site of the primary infection as superficial (limited to the top layer of the epidermis with no inflammation), cutaneous (involves the upper layers of the epidermis with inflammation), subcutaneous (extends through all layers of the epidermis and can reach the dermis) and systemic (involves previous trauma to the skin) infections (Guidry *et al.*, 2017). Most cutaneous candidiasis is acute and presents as lesions with intense erythema and creamy exudate, with the formation of satellite pustules. Chronic infections are described as being thick with a white layer and mainly occur in skin folds (intertrigo) or are interdigital (Toe-web) infections (Ostrosky-Zeichner and Sobel, 2023). Deep tissue (systemic) infections predominantly develop from existing wounds and can progress rapidly to candidemia (Guidry *et al.*, 2017). Other cutaneous infections include penile, vaginal, and mucocutaneous candidiasis (Hay, 2016). Cutaneous candidiasis is generally associated with predisposing factors such as prolonged use of antibiotics, co-morbidities (diabetes, cancer) and other infections (Human Immunodeficiency Virus (HIV)) (Talapko *et al.*, 2021).

2.6 Epidemiology and microbiology of skin infections

The causative pathogens for all skin infections can be classified into three groups: resident (commensal), transient or pathobionts, and contaminant (Tognetti *et al.*, 2012; Jochum and Stecher, 2020). Resident pathogens are bacteria or fungi that colonize the skin and can enter the tissue through breakage of the epidermis (Kong and Segre, 2012). These micro-organisms form part of the skin microbiota and can display pathogenic characteristics due to changes in the host defences or external factors such as prolonged antibiotic therapy (Chow *et al.*, 2011). Examples include *C. acnes* and *S. epidermidis* (Otto, 2009). Transient pathogens are bacteria that colonize other body areas and can only colonize the skin if there is cutaneous dysbiosis or the host is immunocompromised (Kong and Segre, 2012). The most common example is *S. aureus*. Contaminant pathogens are acquired from the environment and are not present in the body (Marshall *et al.*, 2009).

These micro-organisms are primarily implicated in nosocomial infections or have specific risk factors that contribute to the pathogen's ability to colonize the skin and cause an infection (Ki and Rotstein, 2008).

2.6.1 Staphylococcus aureus

Staphylococcus aureus is a Gram-positive, aerobic coccus commonly identified through its characteristic golden colour (Monaco *et al.*, 2017). Humans are natural carriers of *S. aureus,* with approximately 50% of the population being colonized as intermittent or permanent carriers (van Belkum *et al.*, 2009). *Staphylococcus aureus* colonizes the upper respiratory tract and can also colonize the skin where it forms part of the skin microbiota (Zhu *et al.*, 2023). *Staphylococcus aureus* is a major human pathogen that can cause infections in any organ system which has been attributed to its co-evolution with animals and humans (van Belkum *et al.*, 2009). *Staphylococcus aureus* is classified as one of the ESKAPE pathogens and a priority pathogen that requires new antibiotics (Tacconelli *et al.*, 2018). A systematic analysis of 204 countries by the GBD 2019 Antimicrobial Resistance Collaborators (2022), reported that in 2019, *S. aureus* was the leading cause of mortality in 135 countries and contributed to approximately one million deaths. The success of *S. aureus* as a human pathogen is due to its ability to adapt to new environments, upregulate a plethora of virulence factors, and acquire antibiotic resistance genes (ARGs) (Monaco *et al.*, 2017; Cruz *et al.*, 2021). *Staphylococcus aureus* is the most frequently isolated pathogen in skin infections and accounts for 35-80% of all infections (Linz *et al.*, 2023). In addition, *S. aureus* can cause a diverse array of skin disorders, including superficial infections such as abscesses to life-threatening necrotizing wounds (Thammavongsa *et al.*, 2015). The pathogenesis of *S. aureus* involves an imbalance between the skin microbiome and host defences, allowing *S. aureus* to colonize the skin (Balasubramanian *et al.*, 2017). Under normal circumstances, *S. aureus* is outcompeted or inhibited through skin microbiota-mediated immunity (Chiller *et al.*, 2001). A traumatic skin breakage allows *S. aureus* entry into the underlying tissue, where it upregulates its virulence factors (Cheung *et al.*, 2021). These virulence factors enable *S. aureus* to evade the immune system, acquire nutrients, persist at the site of the infection, and spread to neighbouring tissue (Liu, 2009; Cruz *et al.*, 2021). The production of these virulence factors coupled with additional host co-morbidities is directly correlated with the severity of *S. aureus* infections (Olaniyi *et al.*, 2017). The increased use of antibiotics has also contributed to the emergence and dissemination of antibiotic-resistant phenotypes (Gopikrishnan and Haryini, 2024).

Methicillin-resistant *S. aureus* (MRSA) is the most recognized of these phenotypes that have acquired resistance to all beta-lactam antibiotics (Appelbaum, 2006). Unlike other clinically relevant pathogens, MRSA can colonize the skin, disrupt the skin microbiome, and ultimately become a permanent colonizer (Ong, 2014; Shi *et al.*, 2018).

The treatment of S. *aureus* skin infections is based on the suspected phenotype (*S. aureus*, MSSA, and MRSA) and clinical presentation of the infection (Nandhini *et al.*, 2022). First-line agents for monotherapy include amoxicillin/amoxicillin-clavulanic acid, cephalosporins (cephalexin or cefazolin), and clarithromycin/erythromycin (David and Daum, 2017). Methicillin-sensitive *Staphylococcus aureus* displays dose-dependent resistance to beta-lactams and may require broader spectrum agents (ceftriaxone/ceftazidime) or non-beta-lactam agents such as doxycycline (Gould, 2013). Although the treatment of MRSA relies heavily on glycopeptides, the emergence of resistance has prompted the investigation of combinations of other antibiotic classes (Deresinski, 2009). Some reviews have discussed that other agents such as gentamicin, ciprofloxacin, co-trimoxazole, or rifampicin should be used in combination with vancomycin to lower the risk of resistance (Bal *et al.*, 2017; Rose *et al.*, 2021). However, the clinical efficacy has been met with mixed results, suggesting that the severity of the infection and host-related factors contribute to the combination efficacy (Jorgensen *et al.*, 2019). Nevertheless, an overview of the treatment of MRSA involves monotherapy vancomycin/teicoplanin as first-line with linezolid, ceftaroline, tigecycline, daptomycin, or combinations of linezolid and daptomycin/ertapenem as second line (Gould, 2013; Stevens *et al.*, 2014; Linz *et al.*, 2023).

2.6.2 Staphylococcus epidermidis

Staphylococcus epidermidis is a coagulase-negative Gram-positive coccus related to *S. aureus* and colonizes the skin and upper respiratory tract (Fournière *et al.*, 2020). *Staphylococcus epidermidis* plays an integral role in maintaining skin homeostasis and inhibits the growth of pathogenic micro-organisms such as *S. aureus* and *C. albicans* (Carolus *et al.*, 2019; Brown and Horswill, 2020). *Staphylococcus epidermidis* can also display pathogenic behaviour and has been increasingly implicated in implant-associated infections and hospital-acquired skin infections (Severn and Horswill, 2023). Previous reviews have described *S. epidermidis* as an ‘accidental pathogen’, given that many *S. epidermidis* infections are derived from the same strains that inhabit the skin (Otto, 2009). However, shifts in *S. epidermidis* abundance have

been associated with skin diseases, including atopic dermatitis, dandruff, seborrheic dermatitis, and acne vulgaris (Brown and Horswill, 2020). Unlike *S. aureus*, *S. epidermidis* displays a low virulence, which has been hypothesized to be a possible mechanism for its ability to colonize the skin (Massey *et al.*, 2006). Many virulent factors that *S. epidermidis* displays in infections are also used to colonize the skin (Otto, 2009). For instance, the production of biofilm proteins aids in *S. epidermidis* ability to adhere to epithelial cells; however, the same proteins can also shelter other pathogenic bacteria from both antimicrobial therapy and the immune system (McCann *et al.*, 2008).

The treatment of *S. epidermidis*-associated infections is similar to *S. aureus* with narrow-spectrum beta-lactams, macrolides, and doxycycline, demonstrating high efficacy (Ziebuhr *et al.*, 2006). However, it must also be noted that *S. epidermidis* is a colonizer of the skin, and whilst antibiotic regiments may aid in reducing the bacterial load, it can also contribute to antimicrobial resistance (Marshall *et al.*, 2009). In these cases, *S. epidermidis* acts as a reservoir for ARGs, which can easily be passed to *S. aureus*, resulting in recurrent abscesses or chronic inflammatory conditions (Otto, 2013). Hospital-acquired *S. epidermidis* demonstrate high antibiotic resistance, with approximately 70% of isolates exhibiting multi-drug resistance (Siciliano *et al.*, 2023). Hospital isolates of *S. epidermidis* can colonize patients and persist on abiotic surfaces due to its ability to form robust biofilms (Ziebuhr *et al.*, 2006). Some treatment guidelines recommend empirical monotherapy of vancomycin or daptomycin/linezolid with the incorporation of other agents based on the severity and site of the infection (Siciliano *et al.*, 2023).

2.6.3 Cutibacterium acnes

Cutibacterium acnes (formerly *Propionibacterium acnes*) is an anaerobic Gram-positive bacilli and forms part of the skin microbiota (Dagnelie *et al.*, 2021). *Cutibacterium acnes* play an integral role in modulating skin immunity, maintaining the skin barrier, and preventing the colonization of pathogenic micro-organisms (Fournière *et al.*, 2020). Similarly to *S. epidermidis*, *C. acnes* can also exhibit pathogenic characteristics, the most popular being its role in acne vulgaris (Dréno *et al.*, 2018). In addition, *C. acnes* has become the second most common pathogen in prosthetic-associated infections (Boisrenoult, 2018). *Cutibacterium acnes* colonizes the deeper layers of the epidermis, such as pilosebaceous units where it uses free fatty acids (from sebum) as a nutrient source (Dagnelie *et al.*, 2021). In acne vulgaris,

changes in sebum production within these pilosebaceous units increases the growth of *C. acnes*, contributing to inflammation and skin lesions (Sá *et al.*, 2023). The pathogenesis of *C. acnes* in skin infections occurs through indirect mechanisms, whereby virulence factors expressed by *C. acne*, contribute to the colonization and growth of other pathogenic bacteria (Monteiro and Fernandes, 2024). Other factors include skin dysbiosis and changes in the phenotypic population of *C. acnes* (Mayslich *et al.*, 2021). The growth of *C. acnes* is also modulated by other members of the skin microbiota such as *S. epidermidis* (Aslan Kayiran *et al.*, 2020). Dysregulation or imbalances within the skin microbiota applies a selective pressure which allows the growth of these virulent phenotypes (Lomholt and Kilian, 2014).

The treatment of cutaneous infections caused by *C. acnes* involves macrolides and tetracyclines (Layton, 2016; Sá *et al.*, 2023). These antibiotics reduce the presence of *C. acnes*, decreasing the expression of virulence factors and exhibit anti-inflammatory effects (Monteiro and Fernandes, 2024). As a commensal, *C. acnes* is a permanent colonizer of the skin, and whilst antimicrobial therapy reduces the symptoms associated with these infections, it also contributes to antibiotic resistance (Aslan Kayiran *et al.*, 2020). Some studies have reported that oral and topical use of erythromycin contributes to macrolide cross-resistance (Lomholt and Kilian, 2014; Paugam *et al.*, 2017). A few reviews have discussed using benzoyl peroxide with oral macrolides/tetracyclines to reduce resistance (Taylor and Shalita, 2004; Sagransky *et al.*, 2009). Additionally, other non-antibiotic drugs that impair or inhibit the virulence mechanisms of *C. acnes* are also effective, such as retinoids and anti-androgen medications (Habeshian and Cohen, 2020; Shah *et al.*, 2022). Retinoids inhibit sebum production by inducing apoptosis in sebaceous gland cells and reduces nutrient availability for *C. acnes* (Walsh *et al.*, 2016). Anti-androgen medicines modulate sebum production, reducing the proliferation of *C. acnes* (Kumtornrut and Noppakun, 2021).

2.6.4 Acinetobacter baumannii

Acinetobacter baumannii is a Gram-negative, obligate aerobe, coccobacillus that occurs naturally in soil and water (Harding *et al.*, 2018). Most *Acinetobacter* species are non-pathogenic and colonize healthy individuals' skin, nose, and digestive tracts (Maure *et al.*, 2023). These initial observations led to the classification of most *Acinetobacter* species as low-grade pathogens (Joly-Guillou, 2005). *Acinetobacter baumannii* currently ranks as the main

critical threat to global public health and the priority pathogen amongst the ESKAPE group of bacteria (Tacconelli *et al.*, 2018; Ogyu *et al.*, 2020).

The emergence of *A. baumannii* as a nosocomial pathogen is primarily based on its environmental persistence and antibiotic resistance (Bartal *et al.*, 2022). This pathogen typically colonizes moist surfaces, including door handles, medical syringes, catheters, and other hospital equipment (Peleg *et al.*, 2008). The colonization of abiotic surfaces facilitates the spread of *A. baumannii* through contamination (typically outbreaks) or cross-infection between patients (Lucidi *et al.*, 2024) . Similarly to antibiotic resistance, *A. baumannii* can tolerate a variety of xeric stresses (desiccation and disinfectants) and alter its metabolism whereby more than 20 different metabolic pathways have been identified in *A. baumannii* (Nwugo *et al.*, 2012; Zeidler and Müller, 2019; Djahanschiri *et al.*, 2022). *Acinetobacter baumannii* also displays several virulence factors including secretion systems, biofilm formation, siderophores, and expression of outer membrane protein A (OmpA), which are relevant to skin infections (Lucidi *et al.*, 2024). *Acinetobacter baumannii* can cause a broad spectrum of infections and are usually associated with high mortality rates (45-84%)(Appaneal *et al.*, 2022; Yu *et al.*, 2024). These infections include skin infections, urinary tract infections (UTIs), meningitis, bacteraemia, and ventilator-associated pneumonia (Peleg *et al.*, 2008). Several risk factors such as prolonged hospital stay, underlying health conditions, prior antibiotic exposure, and immunosuppressive medicine increase the likelihood of infection (Reina *et al.*, 2022; Tamma *et al.*, 2023). The pathogenesis of *A. baumannii* in cutaneous infections is poorly studied with most reviews referencing case reports from wounded soldiers and hospital outbreaks (Sebeny *et al.*, 2008; Eveillard *et al.*, 2013). The initial seed of the infection is believed to occur through colonization of the skin from hospital equipment or other infected patients (Wong *et al.*, 2017). *Acinetobacter baumannii* infections present with a "*peau d'orange*" (orange-like) appearance (Erysipelas) followed by "sandpaper-like" translucent vesicles on the skin (Sebeny *et al.*, 2008). At the site of the infection, haemorrhagic bullae are formed with visible necrotizing tissue damage commonly associated with bacteraemia (Cisneros and Rodríguez-Baño, 2002). Although *A. baumannii* is likely responsible for this clinical presentation, other pathogens, such as *K. pneumoniae*, *C. albicans*, and *E. faecalis* can also influence the severity of the infection (Howard *et al.*, 2012). These co-pathogens contribute to the initial necrosis of the skin, which later facilitates the entry of *A. baumannii* into the bloodstream (Sebeny *et al.*, 2008).

Acinetobacter baumannii is notoriously difficult to treat due to its genetic plasticity, which accelerates genetic mutations and recombination, resulting in the phenotypic expression of intrinsic and acquired resistance mechanisms (Castanheira *et al.*, 2023). A review of 204 countries conducted by Murray *et al.* (2022) reported that 60-80% of *A. baumannii* isolates were resistant to carbapenems. According to several treatment guidelines (Abdul-Mutakabbir *et al.*, 2021; EUCAST, 2022; Tamma *et al.*, 2023; Onorato *et al.*, 2024), combination therapy involving polymyxin B (Colistin) with other antibiotics such as minocycline/tigecycline or carbapenems (Imipenem or meropenem), ampicillin-sulbactam and new cephalosporins (Cefiderocol) are recommended. Given the increasing rate of resistance, coupled with the poor accessibility of new antibiotic agents as well as the multifaceted aetiology of *A baumannii* infections, it is likely that this pathogen could soon become untreatable (Karakonstantis *et al.*, 2021).

2.6.5 Pseudomonas aeruginosa

Pseudomonas aeruginosa is a rod-shaped, flagellated, aerobic Gram-negative bacterium that is commonly recognized by its production of a blue-green pigment and "grape-like" smell (Horcajada *et al.*, 2019). *Pseudomonas aeruginosa* has long been recognized as a priority pathogen that requires new antibiotics and alternative treatments (Oliveira *et al.*, 2020). A systematic review of 204 countries by the GBD 2019 Antimicrobial Resistance Collaborators (2022) reported that in 2019, *P. aeruginosa* caused approximately 559000 deaths. Another review by Lansbury *et al.* (2020) identified *P. aeruginosa* as the second most common pathogen that co-infected COVID-19 patients and also contributed to an increase in mortality.

The classification of *P. aeruginosa* as a ubiquitous pathogen is based on its metabolic versatility, antibiotic resistance, and virulence factors (Kerr and Snelling, 2009). As a nosocomial pathogen, *P. aeruginosa* colonizes medical equipment such as respirators, ventilators, and other moist surfaces (Horcajada *et al.*, 2019). *Pseudomonas aeruginosa* can cause infections in every organ system and infect healthy and immunocompromised individuals (Poole, 2011). In addition, *P. aeruginosa* exhibits many virulence factors that allow it to proliferate at the wound site and intercellularly (Kerr and Snelling, 2009). These virulence factors induce damage to the host, facilitate evasion from the immune system, increase nutrient acquisition, and aid in biofilm formation (Anantharajah *et al.*, 2016; Liao *et al.*, 2022). Some risk factors for *P. aeruginosa* infections include diabetes, long-term antibiotic therapy, use of

corticosteroids, prolonged hospital stays and skin dysbiosis (Wood *et al.*, 2023). Cutaneous infections caused by *P. aeruginosa* are diverse and range from superficial infections such as hot tub folliculitis, green nail syndrome, and interdigital (toe-web) infections to gangrenous cellulitis and necrotizing infections (Ecthyma gangrenosum) (Wu *et al.*, 2011). Other skin-related infections include otitis externa (perichondritis) and infections arising from burn wounds (Spernovasilis *et al.*, 2021).

The treatment of superficial *P. aeruginosa* infections is usually self-limiting and can be managed with topical antibiotics such as gentamicin or tobramycin (Spernovasilis *et al.*, 2021). In contrast, severe infections (deep tissue) are difficult to treat and require combinations of antibiotics (Tamma *et al.*, 2023). *Pseudomonas aeruginosa* can rapidly develop resistance to monotherapy antibiotics such as ciprofloxacin, whilst other agents can upregulate pleiotropic resistance genes and contributes to treatment failure (Tam *et al.*, 2010; Morita *et al.*, 2014; Khalili *et al.*, 2019). A common example is in diabetic foot ulcers where amoxicillin-clavulanic acid induces the expression of AmpC which is a chromosomal beta-lactamase gene that provides resistance to most cephalosporins (Kumari *et al.*, 2014; Tooke *et al.*, 2019). Several treatment guidelines recommend monotherapy of piperacillin-tazobactam as first-line or ciprofloxacin with additional antibiotics such as anti-pseudomonal aminoglycosides (gentamicin, tobramycin, and amikacin) (Stevens *et al.*, 2014; EUCAST, 2022). For hospital-acquired infections, it is not uncommon for these antibiotics to fail as most clinical isolates are highly resistant (Wood *et al.*, 2023). In such cases, combinations of carbapenems (meropenem or imipenem) with tigecycline/aztreonam or colistin are commonly used as well as newer beta-lactam with beta-lactamase inhibitor combinations (ceftazidime-avibactam and ceftolozane-tazobactam) (Karaiskos *et al.*, 2019; Tamma *et al.*, 2023).

2.6.6 Candida albicans

Candida albicans is a dimorphic yeast that is the most prevalent fungal species of the human microbiota and asymptomatically colonizes many areas of the body, including the skin, gastrointestinal and genitourinary tracts (Ostrosky-Zeichner and Sobel, 2023). *Candida albicans* exhibit a dual lifestyle that can be beneficial and pathogenic (Bongomin *et al.*, 2017). As a commensal, *C. albicans* suppresses the growth of pathogenic micro-organisms such as *P. aeruginosa* and modulates inflammatory mechanisms (Ader *et al.*, 2011). *Candida albicans* is also an opportunistic pathogen and can cause various infections in both immunocompetent and

immunocompromised individuals (Mayer *et al.*, 2013; Hay, 2016). *Candida albicans* cause most skin-related infections and makeup 80-90% of all cutaneous fungal infections (Talapko *et al.*, 2021). *Candida albicans* upregulates its pathogenic mechanisms in response to host immunity alterations or microbiota changes due to antibiotic therapy (Fisher *et al.*, 2022). Post-antibiotic bacterial depletion decreases stimulation and, subsequently, less effective innate immunity, which benefits the commensal-pathogen transition of *C. albicans* (Peghin and Ruiz-Camps, 2022). As a polymorphic fungus, *C. albicans* can grow as a budding yeast or elongated ellipsoid cells with hyphae (Mayer *et al.*, 2013). Under environmental stresses, *C. albicans* can transition between these two growth states, triggering its pathogenicity mechanisms (Sudbery, 2011). Several virulence factors also contribute to its ability to cause infection, which includes the ability to withstand environmental stresses, production of adhesion proteins, acid proteases, phospholipase, and mycotoxins (Candidalysin) (Ostrosky-Zeichner and Sobel, 2023). *Candida albicans* can also exhibit synergistic relationships with Gram-positive bacteria and other *Candida* species (Förster *et al.*, 2016; Hu *et al.*, 2021). These mixed-species infections are among the best examples of "lethal synergism", whereby the co-infection increases the mortality of an otherwise survivable monomicrobial infections (Carolus *et al.*, 2019).

Candidiasis (*C. albicans*) treatment involves topical or systemic antifungal agents (Rigopoulos, 2023). Topical agents such as imidazoles (clotrimazole or miconazole) are used for superficial infections, whilst systemic antifungals (mainly fluconazole or echinocandins/amphotericin B) are used for severe, invasive infections (Guidry *et al.*, 2017).

2.7 Antimicrobial therapy

The selection of specific antimicrobial agents (Table 2.2) depends on many factors, such as the causative pathogen (virulence factors), aetiology of the infection (Community-acquired or hospital-acquired), the spectrum of activity, anatomical location, and patient co-morbidities (Golan, 2019). Skin infections are generally first treated empirically (Russo *et al.*, 2022b). Empirical therapy uses antibiotics based on specific pathogens' clinical presentations and prevalence (Fung *et al.*, 2003). Empirical therapy is the starting point of treatment and usually comprises a beta-lactam or another agent (companion antibiotic) that aims to cover the "most probable pathogens" (Golan, 2019; Singhal, 2020). Companion antibiotics may be added or removed depending on the clinical improvement/deterioration of the patient, results from microbial culturing, availability of antibiotic agents, and local treatment guidelines (De Waele

et al., 2020). The prevalence of MDR-pathogens also means that first-line agents may not work for the infection, requiring broader spectrum antibiotics or combinations (Karaiskos *et al.*, 2019).

Table 2.2: An overview of antimicrobial agents used for skin infections

Empirical therapy	**Companion antibiotic reasoning**	**Antibiotic escalation ranking**			**References**
		1st line	2nd line	3rd line	
Amoxicillin-clavulanic acid/ clarithromycin/ cephalosporin + topical agents (mupirocin, neomycin)	+ Gram-negative	Piperacillin-tazobactam	Ciprofloxacin, gentamicin, tobramycin	Amikacin, carbapenems, tigecycline or combinations	(Gould, 2013; Stevens *et al.*, 2014; De Waele *et al.*, 2020; EUCAST, 2022; Fioriti *et al.*, 2022)
	+ MRSA	Vancomycin, co-trimoxazole, doxycycline	Linezolid, daptomycin, fifth generation cephalosporins, or combinations	Salvage therapy or empirical combinations	
	+ Antifungal	Fluconazole	Voriconazole/ echinocandins	Amphotericin B or combinations	
	+ Anaerobic	Piperacillin-tazobactam /Azithromycin/ metronidazole	Fluroquinolones/ clindamycin	Carbapenems	
	+ Anti-toxin	Clindamycin		Linezolid	

Topical antimicrobial agents can also treat certain skin infections and are commonly paired with oral antibiotics (Singal and Thami, 2003). Topical agents have the advantage of being applied directly to the site of infection, which allows high concentrations of the antimicrobial agent to be localized at the site of the infection (Dallo *et al.*, 2023). Some common examples include mupirocin or gentamicin.

2.8 Toxicity of antibiotics and antifungals

Antibiotics are among the safest therapeutic drugs due to their selectivity for prokaryote molecular targets (Yadav *et al.*, 2023). However, antibiotics can also elicit unpleasant, adverse effects and organ-associated drug toxicity (Table 2.3). Adverse effects (also called adverse drug reactions) are reversible side effects resulting from an intervention related to the use of the antibiotic (Guengerich, 2011). These effects are reversible and resolve as soon as the antibiotic is stopped. An example is gastrointestinal disturbances from beta-lactams or macrolides. Most cutaneous adverse drug reactions are unpredictable, dose-dependent, and idiosyncratic since symptoms differ according to the individual (Wang and Hung, 2019). Antibiotic toxicity refers to irreversible anatomical changes occurring in specific organ systems

and is usually encountered with prolonged use of the antimicrobial agent (Bhattacharjee, 2016). The most common examples are hepatoxicity, ototoxicity and nephrotoxicity (Bhattacharjee, 2016). Antibiotic toxicity can be categorized according to the mechanism, which includes on-target toxicity (based on the antibiotic's mechanism), hypersensitivity or immunological responses, off-target toxicity, and bioactivation (Guengerich, 2011). These mechanisms are predominantly associated with pharmacokinetics/pharmacodynamics, chemical structure, and host-related factors (Williams, 2001).

Table 2.3: Associated toxicity and adverse effects of studied antibiotics

Antibiotic	Associated toxicity	Adverse effects	References
Amoxicillin	Hepatoxicity, nephrotoxicity and encephalopathy (rare).	Hypersensitivity (rashes) gastrointestinal disturbances (vomiting, diarrhoea), and neutropenia (prolonged use).	(Geddes *et al.*, 2007; Garnier *et al.*, 2023)
Ciprofloxacin	Hepatotoxicity, hematologic toxicity, and ototoxicity.	Hypersensitivity, arthropathy tendonitis, and impaired renal function.	(Badawy *et al.*, 2021)
Erythromycin	Hepatoxicity, ototoxicity, and cardiotoxic.	Gastrointestinal disturbances (diarrhoea).	(Williams, 2001)
Gentamicin	Ototoxicity, nephrotoxic neurotoxic	Skin rashes and sensitization, gastrointestinal disturbances (diarrhoea).	(Avent *et al.*, 2011)
Meropenem	Hepatotoxicity	Hypersensitivity (rashes) gastrointestinal disturbances (vomiting, diarrhoea), and central nervous system disturbances (seizures, headaches).	(Papp-Wallace *et al.*, 2011)
Tetracycline	Hepatoxicity, neurotoxic and nephrotoxicity.	Gastrointestinal disturbances (vomiting, nausea, heartburn, diarrhoea), teeth discolouration and hypersensitivity (rashes and photosensitization).	(Bradford and Jones, 2012)
Miconazole	Hepatoxicity	Contact dermatitis (burning, itching).	(Tverdek *et al.*, 2016)
Nystatin	Hepatoxicity	Contact dermatitis	(Tverdek *et al.*, 2016)

2.9 Anti-inflammatory properties of antibiotics and antifungals

Antibiotics can also elicit other therapeutic effects, such as immunomodulation, which refers to the activation or suppression of the immune system (Yadav *et al.*, 2023). One of the primary mechanisms by which antibiotics affect the immune system is the modulation of inflammatory processes (Hamilton-Miller, 2001). Most antibiotics elicit anti-inflammatory properties (Table 2.4) by directly inhibiting cytokines and chemokines (Tamaoki *et al.*, 2004). The extent of these anti-inflammatory effects is related to the antibiotic's molecular structure and antimicrobial mechanism (Labro, 1998). A review by Nau and Eiffert (2005) discussed that bactericidal agents increase inflammation whilst bacteriostatic agents reduce and can even

inhibit pro-inflammatory processes. For skin infections, macrolides and tetracyclines are widely recognized for their anti-inflammatory properties, favouring their use for most cutaneous infections (Giamarellos-Bourboulis, 2008).

2.9.1 Beta-lactams

Beta-lactams are regarded as pro-inflammatory antibiotics because they increase inflammatory cytokines and chemokines (Mondemé *et al.*, 2023). Beta-lactams inhibit cell wall synthesis, which causes bacterial cells to burst due to changes in osmotic pressure (Wong *et al.*, 2021). This beta-lactam-induced apoptosis releases the cell contents (PAMPs) into the surrounding tissue, stimulating inflammation (Holzheimer, 2001). The ability of beta-lactams to affect inflammatory processes has been studied using endotoxemia models (Tsuji *et al.*, 2003; Vianna *et al.*, 2004). Some reviews have discussed that the extent of endotoxin release from beta-lactams is associated with the chemical structure, binding affinity for specific penicillin-binding-proteins (PBPs), type of bacteria, and the concentration of the beta-lactam (Periti and Mazzei, 1998; Holzheimer, 2001). For example, carbapenems (PBP-2 specific) release the lowest concentrations of endotoxins (Produce a reduced inflammatory response), whilst cephalosporins (PBP-3 specific) are associated with higher concentrations (Produce a higher inflammatory response) (Nau and Eiffert, 2002). A recent review by Mondemé *et al.* (2023) discussed that beta-lactams could synergize with the innate immune system through the activation of granulocytes (macrophages, basophil, and eosinophil) and increase the effectiveness of beta-lactam therapy. The authors argued that triggering pro-inflammatory responses by releasing PAMPs from beta-lactams may enhance the clearing of the infection and resolve inflammation. A few studies have demonstrated that some penicillins may increase the proliferation of neutrophils and stimulate the release of pro-inflammatory cytokines (IL-β and IL-6) in the absence of endotoxins (Bode *et al.*, 2014; Bystrzycka *et al.*, 2016), however whether these effects reduce inflammation and hasten the healing process still requires investigation.

Table 2.4: Inflammatory effects of studied antibiotics in response to PAMP stimulation using cell and animal models

Antibiotic	Experimental design	Cell line/animal model	Dose	Inflammatory effect	References
Ciprofloxacin	LPS-activated rodents	C57/BL6 mice	1, 50 and 100 mg/kg	Inhibition of pro-inflammatory cytokines (IL-1β, IL-6 and TNF-α)	(Ogino *et al.*, 2009)
	LPS-activated microglia	Rodent microglia	50 – 200 µg/mL	Inhibition of pro-inflammatory cytokines (IL-1β and TNF-α).	(Zusso *et al.*, 2019)
	LPS-activated macrophages	RAW 264.7 macrophages	10 – 80 µg/mL	Inhibition of pro-inflammatory cytokines (IL-1β, IL-6, IL-10 and TNF-α)	(Liu *et al.*, 2022)
Erythromycin	LPS-activated macrophages	THP-1 macrophages	2 µg/mL	Reduced the production of IL-1β and IL-6, downregulated TLRs associated with inflammation	(Bode *et al.*, 2014)
	LPS-activated macrophages	RAW 264.7 macrophages	1 and 10 µM	Dose-dependent reduction in IL-6 and PGE_2 production	(Sato *et al.*, 2007)
	LPS-activated macrophages	J774A.1 murine macrophages	12.5, 25 and 50 µM	Inhibited the production of IL-6 and PGE_2	(Munić *et al.*, 2011)
Gentamicin	LPS-activated liver cells	HK-2 cells (proximal tubule cells)	10 – 2000 µg/mL	Dose-dependent reduction in TNF-α and CCL2 (Pro-inflammatory chemokine) production.	(Zager *et al.*, 2007)
	Sepsis model using rodents (injected with LPS)	C57/BL6 mice and isolated spleen cells	7.5 mg/kg per day	Decreased production of IL-1β, TNF-α and IL-2. Increased the production of IL-10. Enhanced the survivability of endotoxemic mice and reduced organ damage.	(Li *et al.*, 2022a)
Tetracycline	LPS-activated macrophages	Human whole blood samples	1 and 10 µM	Reduced the production of IL-1β, IL-6 and IL-8.	(Cazalis *et al.*, 2009)
	LPS-activated macrophages	J774A.1 murine macrophages	62.5, 250 and 500 µM	Dose-dependent reduction in iNOS expression.	(D'Agostino *et al.*, 1998)
Miconazole	LPS-activated macrophages	RAW 264.7 macrophages	1, 5, 10, 50 and 100 µM	Dose-dependent reduction in NO production	(Vermuyten *et al.*, 1997)
	Fungal-activated monocytes	THP-1 monocytes	0.5 – 512 µg/mL	Increased the production of IL-1β, TNF-α IL-6 and IL-8.	(Cantürk, 2016)
	LPS-activated astrocytes and microglia cells	Astrocytes and microglial BV2 cells	1.25, 2.5, 5, 10 µM	Dose-dependent reduction in NO production and TNF-α, reduction in iNOS and NF-κB expression	(Yeo *et al.*, 2020)
Nystatin	LPS-activated macrophages	RAW 264.7 macrophages	1 – 10 µg/mL	A dose-dependent increase in NO production.	(Koide *et al.*, 2009)
	LPS-stimulated macrophages and dendritic cells	Macrophages and dendritic cells isolated from C57BL/6 mice	50 µg/mL	Increased the production of IL-1β in dendritic cells and macrophages by activating the NLRP3 inflammasome.	(Darisipudi *et al.*, 2011)

2.9.2 Macrolides

Macrolides exhibit multiple anti-inflammatory effects by inhibiting inflammatory cytokines (IL-1, IL-6, and TNF-α), reducing ROS production and modulating neutrophilic-associated inflammation (Labro, 1998; Rubin *et al.*, 2005). These anti-inflammatory effects have also been linked to the ability of macrolides to accumulate in polymorphonuclear leukocytes and be transported to the site of the infection (Zalewska-Kaszubska and Górska, 2001). Several studies have demonstrated erythromycin's ability to reduce the production of pro-inflammatory cytokines (IL-1β, IL-6) and PGE_2 using LPS-activated macrophages (Sato *et al.*, 2007; Munić *et al.*, 2011).

2.9.3 Tetracyclines

Tetracycline exhibits potent anti-inflammatory effects, which has increased its use in treating inflammatory dermatoses such as acne vulgaris and rosacea (Sadarangani *et al.*, 2015). A review by Monk *et al.* (2011) discussed several mechanisms in which tetracycline may modulate inflammatory processes, the most widely reported being the inhibition of metalloproteinases (MMPs). Metalloproteinases are proteolytic enzymes that are activated under inflammatory conditions and recruit inflammatory cells to the site of the infection (Caley *et al.*, 2015). Tetracycline inhibits these MMPs, which regulate the activation of interleukins and chemokines (Tauber and Nau, 2008). Other relevant anti-inflammatory mechanisms include the inhibition of NO production and neutrophil trafficking, as well as the inhibition of the cyclooxygenase pathway (Sapadin and Fleischmajer, 2006). Several studies have also reported tetracycline's ability to reduce inflammatory cytokines (IL-1β, IL-6 and IL-8) in a dose-dependent manner using LPS-activated macrophages (D'Agostino *et al.*, 1998; Cazalis *et al.*, 2009).

2.9.4 Fluroquinolones

Fluroquinolones can function as pro- and anti-inflammatory agents, depending on the upregulated cytokine and inflammatory (NF-κB signalling) pathway (Assar *et al.*, 2021). Ciprofloxacin exhibits multiple anti-inflammatory effects by suppressing pro-inflammatory interleukins (IL-1β, IL-2, IL-6 and TNF-α) and modulating the proliferation and activity of T and B lymphocytes (Dalhoff and Shalit, 2003). Several studies have reported that

concentrations of 10 – 200 µg/mL modulate the production of inflammatory cytokines using LPS-activated macrophages and rodent models (Ogino *et al.*, 2009; Zusso *et al.*, 2019; Liu *et al.*, 2022).

2.9.5 Aminoglycosides

Aminoglycosides can exhibit both pro- and anti-inflammatory effects depending on the site of infection (Rubin *et al.*, 2005). A study by Bode *et al.* (2014) demonstrated that gentamicin reduced the mRNA expression of IL-1β and TNF-α in LPS-activated THP-1 monocytes; however, the reduction was significantly less than minocycline and erythromycin. Another study by Li *et al.* (2022a) reported that gentamicin improved survivability and reduced inflammatory responses in a rodent sepsis model. The same study also demonstrated that gentamicin modulated the activation of regulatory T cells (Tregs) through the STAT5 signalling pathway. These studies suggest that gentamicin can modulate cell signalling and inhibit the gene expression of pro-inflammatory cytokines and chemokines. Gentamicin can also increase inflammation through mechanisms similar to beta-lactams, whereby destabilization of the cell membrane induces cell lysis and increases the presence of endotoxins (Kadurugamuwa and Beveridge, 1997).

2.9.6 Azoles

Azole antifungals exhibit pro- and anti-inflammatory properties (Ami *et al.*, 2008). Triazoles (fluconazole, itraconazole, and sertraconazole) and ketoconazole exhibit the highest anti-inflammatory effects, with clotrimazole and miconazole exhibiting weaker effects (Simitsopoulou *et al.*, 2011; Gupta *et al.*, 2021). Some studies have demonstrated that miconazole decreases the production of NO by reducing the expression of iNOS in a dose-dependent manner (Vermuyten *et al.*, 1997; Yeo *et al.*, 2020). Liebel *et al.* (2006) reported that miconazole reduces cytokine (IL-2, TNF-α) production and macrophage growth factors associated with skin irritation using phytohemagglutinin stimulated T-cells and rodent models. In contrast, Cantürk (2016) demonstrated that miconazole increased the production of IL-1β, TNF-α, IL-6, and IL-8 using THP-1 cells that were activated using *C. albicans* supernatants.

2.9.7 Polyenes

Nystatin exhibits pro-inflammatory responses through the activation of TLRs (Sau *et al.*, 2003; Razonable *et al.*, 2005a). A study by Razonable *et al.* (2005b) demonstrated that THP-1 monocytes exposed to nystatin increased the production of pro-inflammatory cytokines (IL-1β, IL-8, and TNF-α) without prior activation from a known fungal PAMP such as mannoproteins and glucans. A similar study by Darisipudi *et al.* (2011) also reported an increased production of IL-1β in LPS-activated macrophages and dendritic cells. These studies suggest that nystatin may act as a PAMP for TLRs and stimulate inflammation. However, the mechanism of action has not been fully elucidated (Sau *et al.*, 2003). This draws attention to the possibility that some naturally derived antibiotics may act as pro-inflammatory stimulants even in the absence of conventional PAMPs. Other studies have also shown that nystatin increases both NO and macrophage inflammatory proteins (MIPs) production (Koide *et al.*, 2009; Baek *et al.*, 2013). In contrast, other studies have reported that nystatin can modulate the signalling of macrophages through the production of cytokines (IL-17) and chemokines (CXCL8), which may promote the immune system's ability to clear fungal infections (Kim *et al.*, 2013; Zhang *et al.*, 2018).

2.10 Antimicrobial properties of ibuprofen

Ibuprofen's antimicrobial properties were first described by Hersh *et al.* (1991), who investigated the effects of ibuprofen against periodontal pathogens. This was followed by Sanyal *et al.* (1993) and Elvers and Wright (1995), who demonstrated ibuprofens' antimicrobial activity against several *Staphylococcus* species, Gram-negative bacteria, and dermatophytes. A study by Konstan *et al.* (1990) demonstrated that high doses of ibuprofen reduced inflammation and slowed the degradation of lung tissue in cystic fibrosis, which serendipitously modulated the virulence properties of *P. aeruginosa*. Konstan *et al.* (1990) were the first to identify ibuprofen's ability to modulate virulence factors and prompted investigation against other micro-organisms. Initial studies reported that ibuprofens MIC values exceeded therapeutic dosages (> 5000 µg/mL) (Konstan *et al.*, 1990; Hersh *et al.*, 1991; AL-Janabi, 2010), suggesting that it could only be used as an antimicrobial agent in instances where the therapeutic dosage matched the MIC value of the suspected pathogen.

This led to the investigation of ibuprofen to treat urinary tract infections (UTIs), as ibuprofen is concentrated in urine and can reach inhibitory (≥ 2500 µg/mL) concentrations (Arai *et al.*, 2005; Carey *et al.*, 2020).

2.10.1 Antimicrobial activity of ibuprofen against studied bacteria

The antibacterial activity of ibuprofen against the studied bacteria has been presented in Table 2.5. In general, ibuprofen demonstrates broad-spectrum activity, with Gram-positive bacteria being more susceptible than Gram-negative bacteria (Yimer *et al.*, 2019). Most studies have investigated ibuprofen's antibacterial activity against *S. aureus* and *P. aeruginosa* and have reported that at high concentrations (≥ 2500 µg/mL), ibuprofen exhibits bacteriostatic effects, whilst at sub-inhibitory concentrations (0.25 - 0.50 x MIC), ibuprofen impairs metabolic processes and virulence factors (Abd El-Baky, 2015; Oliveira *et al.*, 2019; Babaei *et al.*, 2022; Rastgar *et al.*, 2022). A study by Obad *et al.* (2015) reported that commercial formulations of ibuprofen inhibited the growth of *S. aureus* (ATCC 6538) and *S. epidermidis* (ATCC 14990) at concentrations of 100 - 800 mg/mL using the disk diffusion assay. Laudy *et al.* (2016) investigated ibuprofen's antimicrobial activity against *A. baumannii*, with a reported MIC of 3200 µg/mL against an ATCC 19606 strain. Compared to other studied pathogens, the investigation of ibuprofen against *S. epidermidis* and *C. acnes* is very limited. A few studies have reported ibuprofen MICs against *S. epidermidis* (Elvers and Wright, 1995; Tabatabaeifar *et al.*, 2022), with no studies investigating the direct antimicrobial activity of ibuprofen against *C. acnes*.

Table 2.5: Antibacterial activity of ibuprofen against studied bacteria

Micro-organism	Strain/isolate	MIC (µg/mL)	Method	Reference
S. aureus	ATCC (35556, 13565), SA1199B (fluroquinolone resistant), XU212 (MDR strain)	500 – 2000 µg/mL	Checkerboard assay	(Oliveira *et al.*, 2019)
	ATCC 10537	> 600 µg/mL	Broth microdilution	(Elvers and Wright, 1995)
	ATCC 6538	952 µg/mL	Broth microdilution	(Abd El-Baky, 2015)
	ATCC 25923	2500 µg/mL	Checkerboard assay	(Chan *et al.*, 2017)
	ATCC 33591 (MRSA) and clinical isolates (*n* = 2)	2500 µg/mL	Checkerboard assay	
	Clinical isolates (*n* = 10) (MRSA and VRSA)	1024 µg/mL	Broth microdilution	(Tabatabaeifar *et al.*, 2022)

Micro-organism	Strain/isolate	MIC (µg/mL)	Method	Reference
S. epidermidis	Not stated	350 µg/mL	Broth microdilution	(Elvers and Wright, 1995)
	ATCC 35984	1024 µg/mL	Broth microdilution	(Tabatabaeifar *et al.*, 2022)
P. aeruginosa	Clinical isolates (*n* = 3)	1024 – 2048 µg/mL	Checkerboard assay	(Rastgar *et al.*, 2022)
	ATCC 9027 and clinical isolates (*n* = 2)	1024 – 2048 µg/mL	Broth microdilution	(Babaei *et al.*, 2022)
	PA01 (BAA-07)	2048 µg/mL	Broth microdilution	(Tabatabaeifar *et al.*, 2022)
	Clinical isolates (*n* = 10) (carbapenem-resistant)	1024 – 2048 µg/mL		
	ATCC 10145	1292 µg/mL	Broth microdilution	(Abd El-Baky, 2015)
	Clinical isolate (cystic fibrosis isolate)	512 µg/mL	Broth microdilution	(Chen *et al.*, 2023)
	ATCC 85327 and clinical isolates (*n* = 2)	2048 µg/mL	Broth microdilution	(Khodaparast *et al.*, 2022)
	ATCC (27853, 25616, NCTC 6749, and PA01)	> 3200 µg/mL	Broth microdilution	(Laudy *et al.*, 2016)
	ATCC 27853	> 5000 µg/mL	Checkerboard assay	(Chan *et al.*, 2017)
A. baumannii	ATCC 19606	3200 µg/mL	Broth microdilution	(Laudy *et al.*, 2016)

2.10.2 Antifungal activity of ibuprofen against Candida albicans

The antifungal properties of ibuprofen have been known for decades and have been reviewed by Babaei *et al.* (2023). A study by Pina-Vaz *et al.* (2000) demonstrated that concentrations of 5.00 mg/mL exhibited fungistatic effects while 10 - 20 mg/mL were fungicidal against an ATCC 10231 strain of *C. albicans*. The study also showed that high doses of ibuprofen changed the permeability of the fungal cell membrane through the induction of potassium efflux and has also been reported by other studies (Costa-de-Oliveira *et al.*, 2015; Oliveira *et al.*, 2019). Some studies have reported varying MIC values (500 – 2000 µg/mL) against ATCC and clinical isolates of *C. albicans*, which may be attributed to the strains and methodology (de Quadros *et al.*, 2011; Rosato *et al.*, 2016; Król *et al.*, 2018). Ibuprofens antifungal activity is also related to its ability to inhibit fungal-prostaglandin, which is a multifunctional molecule that aids in the upregulation of virulence mechanisms and the dimorphic transition of *C. albicans* (Noverr *et al.*, 2001; Erb-Downward and Noverr, 2007).

2.11 Toxicity of ibuprofen

The most frequently encountered adverse effect of ibuprofen is gastrointestinal ulceration and

bleeding, which is related to the inhibition of cyclooxygenase enzymes and ibuprofens chemical structure (Rainsford, 2012c). However, ibuprofen has a lower risk of inducing gastrointestinal adverse effects compared to other NSAIDs (Varrassi *et al.*, 2020). Most of the associated adverse reactions or toxicities (renal, cardiovascular or hepatic) involve several risk factors, such as prolonged use, poor lifestyle choices and concomitant use of other medicines (Varrassi *et al.*, 2020). Other associated cutaneous drug reactions include photosensitivity, Steven-Johnson's syndrome and idiopathic dermatitis or rashes (Sánchez-Borges *et al.*, 2005).

2.12 Anti-inflammatory properties of ibuprofen

Ibuprofen exhibits multiple modes of action, which underlies its efficacy in treating acute and chronic inflammation (Rainsford, 2012b). Similarly to other NSAIDs, ibuprofen inhibits both the constitutive cyclooxygenase 1 (COX-1), which is responsible for the production of prostanoids and the inducible cyclooxygenase 2 (COX-2), whose production increases during an inflammatory response (Breyer *et al.*, 2008). The disruption of prostaglandin synthesis affects other inflammatory pathways and immune cells, further reducing inflammation (Nüsing, 2016; Balato and Megna, 2017). Ibuprofen can also affect the accumulation of activated leukocytes (neutrophils, macrophages) by reducing the expression of adhesion molecules in inflamed tissue (Rainsford, 2012d). Other anti-inflammatory effects include the reduction of pro-inflammatory cytokines (TNF-α, IL-1), modulation of the NF-κB pathway and inhibition of NO production (isoform dependent)(Rainsford, 2012d).

2.13 Antimicrobial characteristics of studied essential oil compounds

All EOCs demonstrate some degree of antimicrobial activity, which is dependent on the chemical structure, chirality, and hydrophobic/hydrophilic nature of the compound (Griffin *et al.*, 1999; Kalemba and Kunicka, 2003; Van de Vel *et al.*, 2019). Phenolic monoterpenes exhibit the highest antimicrobial activity and are frequently described in the literature (Nazzaro *et al.*, 2017; Suganya *et al.*, 2022). Previous studies have demonstrated that the high antimicrobial activity of these compounds is related to a structure-activity relationship based on the hydrophobic nature of the aromatic ring coupled with the presence of a hydroxyl group (Kachur and Suntres, 2020; Jeyakumar and Lawrence, 2021). Aldehydes and alcohol-containing EOCs also show strong antimicrobial effects, which vary according to additional R-groups, structural changes, polarity, and electronegativity of the compound (Griffin *et al.*,

1999; Dorman and Deans, 2000). Other EOCs, such as ketones, esters, and hydrocarbons, show the least antimicrobial activity and have little significance in antimicrobial testing (Aelenei *et al.*, 2016; Angane *et al.*, 2022). A proposed antimicrobial ranking of different EOCs can be described as follows (Kalemba and Kunicka, 2003):

Phenols > Alcohols > Aldehydes = Ketones > Esters > Hydrocarbons

2.13.1 Antibacterial activity of studied essential oil compounds

Essential oil compounds exhibit broad-spectrum activity, with Gram-positive bacteria demonstrating greater susceptibility than Gram-negative bacteria (Guimarães *et al.*, 2019). Gram-negative bacteria have more lipopolysaccharides in their cell well which reduces the ability of the lipophilic EOCs to enter the cell (Dhifi *et al.*, 2016). In addition, hydrolases in the periplasmic space of Gram-negative bacteria help degrade certain EOCs (Aelenei *et al.*, 2016). Gram-positive bacteria lack these natural barriers, thus allowing the lipophilic active EOCs to directly contact the cell membrane's phospholipid bilayer, resulting in cell membrane rupture and, ultimately, cell lysis (Ju *et al.*, 2022).

The investigation of the antibacterial properties of different EOCs predominantly involves terpenoids and phenylpropanoids, as these compounds demonstrate the most potent antibacterial activity (Table 2.6). Carvacrol and cinnamaldehyde have been investigated by numerous studies which have demonstrated their antibacterial properties against *S. aureus, P. aeruginosa*, and *A. baumannii* (Iscan, 2017; Owen *et al.*, 2019; Tapia-Rodriguez *et al.*, 2023; Ferrando *et al.*, 2024). Both compounds exhibit comparable MIC values against Gram-positive bacteria, with carvacrol exhibiting better activity (MIC values 32 – 400 µg/mL) compared to cinnamaldehyde (MIC values of 300 - 500 µg/mL). A similar trend is also noted for both compounds efficacy against Gram-negative bacteria. Carvacrol's antibacterial properties is mainly attributed to its hydrophobicity and chemical structure, which disrupts the cytoplasmic membrane, causing intracellular content leakage, and eventual cell lysis (Kachur and Suntres, 2020). Cinnamaldehyde elicits concentration-dependent mechanisms against bacteria whereby at low concentrations it inhibits metabolic enzymes and at high concentrations it induces destabilization of the cell membrane (Doyle and Stephens, 2019).

Table 2.6: Antibacterial activity of selected essential oil compounds against studied bacteria using the broth microdilution assay

Essential oil compound	Micro-organisms	Strains/isolates	Minimum inhibitory concentration (µg/mL)	References
Carvacrol	*S. aureus; S. epidermidis*	*S. aureus* - clinical strains (*n* = 6); *S. epidermidis* (ATCC 35984 and clinical strains (*n* = 5))	150 – 310 µg/mL	(Nostro *et al.*, 2007)
	A. baumannii	ESBL positive	160 µg/mL	(Montagu *et al.*, 2016)
	S. aureus, *S. epidermidis*, *P. aeruginosa*	*S. aureus* (ATCC 43300), *S. epidermidis* (ATCC 14990) and *P. aeruginosa* (ATCC 27853)	120 µg/mL (*S. aureus* and *S. epidermidis*); 250 µg/mL (*P. aeruginosa*)	(Iscan, 2017)
	S. aureus	ATCC 25923 and clinical isolate (*n* = 7)	32 – 256 µg/mL	(Miladi *et al.*, 2017)
	S. aureus, *P. aeruginosa*	ATCC 12598 (MSSA), NCTC 12497 (MRSA), *P. aeruginosa* (NCTC 6749 and clinical isolate)	13.18 µg/mL – MSSA 6.59 µg/mL – MRSA 105 – 210.50 µg/mL – *P. aeruginosa*	(Owen *et al.*, 2019)
	S. aureus (Including MRSA and enterotoxin-producing isolates)	ATCC (6538, 25923, 9144, 13565, 19095, 23235, and 27664); Agricultural isolates from milk (*n* = 6) and meat (*n* = 4).	400 µg/mL	(Rúa *et al.*, 2019)
	MRSA	ATCC 33591 and clinical isolates (*n* = 2)	150 µg/mL	(Selvaraj *et al.*, 2020)
	S. aureus	IS-58 (Tetracycline resistant)	256 µg/mL	(Sousa Silveira *et al.*, 2020)
	A. baumannii	ATCC 19606 and clinical isolates (*n* = 3)	7 – 28 µg/mL	(Aleksic Sabo *et al.*, 2021)
	S. aureus	SA-1199 and SA-1199B (Fluroquinolone resistant)	256 µg/mL	(dos Santos Barbosa *et al.*, 2021)
	P. aeruginosa	ATCC 15442	2000 µg/mL	(Gobin *et al.*, 2022)
	S. aureus	ATCC 6538	128 µg/mL	
	S. aureus	Not stated	125 µg/mL	(Li *et al.*, 2022b)
	A. baumannii	ATCC 19606	300 µg/mL (600 µg/mL – MBC)	(Tapia-Rodriguez *et al.*, 2023)
Trans-cinnamaldehyde	*S. aureus* (MRSA and enterotoxigenic strains)	ATCC (25923, 33591, 13565, 14558, 19095 and 23235)	200 – 400 µg/mL	(Albano *et al.*, 2016)
	P. aeruginosa	ATCC 27853	150 µg/mL	
	A. baumannii	ESBL positive	310 µg/mL	(Montagu *et al.*, 2016)
	S. aureus, *S. epidermidis*, *P. aeruginosa*	*S. aureus* (ATCC 43300), *S. epidermidis* (ATCC 14990) and *P. aeruginosa* (ATCC 27853)	2000 µg/mL (*S. epidermidis*); 4000 µg/mL (*P. aeruginosa* and *S. aureus*)	(Iscan, 2017)
	S. epidermidis	ATCC 155	125 µg/mL	(Sharma *et al.*, 2017)

Essential oil compound	Micro-organisms	Strains/isolates	Minimum inhibitory concentration (µg/mL)	References
Trans-cinnamaldehyde	*S. epidermidis*	ATCC (12228, 35983, 35984) and clinical isolates (*n* = 8)	300 µg/mL (ATCC), 400 – 500 µg/mL (Clinical)	(Albano *et al.*, 2019)
	P. aeruginosa	ATCC 27853 and clinical isolates (*n* = 2)	250 – 1000 µg/mL	(Ferro *et al.*, 2019)
	A. baumannii	ATCC 19606	500 µg/mL	(Ferrando *et al.*, 2024)
	S. aureus	ATCC 9144	500 µg/mL	
	P. aeruginosa	ATCC 27853	1000 µg/mL	
Eugenol	*S. aureus*, *P. aeruginosa*	*S. aureus* (ATCC 6538), *P. aeruginosa* (ATCC 10145)	38.06 µg/mL – *S. aureus* 152 µg/mL – *P. aeruginosa*	(Abd El-Baky and Shawky, 2016)
	S. aureus (MRSA and enterotoxigenic strains)	ATCC (25923, 33591, 13565, 14558, 19095 and 23235)	1300 – 1600 µg/mL	(Albano *et al.*, 2016)
	P. aeruginosa	ATCC 27853	150 µg/mL	
	S. aureus, *S. epidermidis*, *P. aeruginosa*	*S. aureus* (ATCC 43300), *S. epidermidis* (ATCC 14990) and *P. aeruginosa* (ATCC 27853)	4000 µg/mL (All bacteria)	(Iscan, 2017)
	S. aureus	ATCC 25923 and clinical isolates (*n* = 7)	32 – 256 µg/mL	(Miladi *et al.*, 2017)
	S. epidermidis	ATCC 155	250 µg/mL	(Sharma *et al.*, 2017)
	A. baumannii	ATCC 19606 and clinical isolates (*n* = 3)	91 – 304 µg/mL	(Aleksic Sabo *et al.*, 2021)
± Linalool	*C. acnes*, *S. epidermidis*	*C. acnes* (ATCC 6919), *S. epidermidis* (ATCC 14990)	536 µg/mL	(Kim *et al.*, 2008)
	S. aureus, *P. aeruginosa*	*S. aureus* (ATCC 6538), *P. aeruginosa* (ATCC 10145)	648 µg/mL – *S. aureus* 324 µg/mL – *P. aeruginosa*	(Abd El-Baky and Shawky, 2016)
	S. aureus, *S. epidermidis*, *P. aeruginosa*	*S. aureus* (ATCC 43300), *S. epidermidis* (ATCC 14990) and *P. aeruginosa* (ATCC 27853)	8000 µg/mL (All bacteria)	(Iscan, 2017)
	S. aureus, *P. aeruginosa*	ATCC 12598 (MSSA), NCTC 12497 (MRSA), *P. aeruginosa* (NCTC 6749 and clinical isolate)	740 µg/mL – MSSA 370 µg/mL – MRSA 1480 – 5917 µg/mL - *P. aeruginosa*	(Owen *et al.*, 2019)
	A. baumannii	MDR and XDR clinical isolates (*n* = 51)	2 - 32 µg/mL	(Kim *et al.*, 2020)
	P. aeruginosa	ATCC 9027	431 µg/mL	(Liu *et al.*, 2020)
	S. aureus, *P. aeruginosa*	*S. aureus* (ATCC 25923), *P. aeruginosa* (ATCC 27853)	1250 µg/mL – *S. aureus* 625 µg/mL – *P. aeruginosa*	(Varia *et al.*, 2020)

Essential oil compound	Micro-organisms	Strains/isolates	Minimum inhibitory concentration (µg/mL)	References
(-) - α-Pinene	*C. acnes*	ATCC 6919	> 2500 µg/mL	(Raman *et al.*, 1995)
	S. epidermidis	ATCC (12228, 35984) and clinical isolates (*n* =1)	625 µg/mL	(Sieniawska *et al.*, 2013)
	S. aureus, *S. epidermidis*, *P. aeruginosa*	*S. aureus* (ATCC 43300), *S. epidermidis* (ATCC 14990) and *P. aeruginosa* (ATCC 27853)	4000 µg/mL (*S. epidermidis*); 8000 µg/mL (*P. aeruginosa* and *S. aureus*)	(Iscan, 2017)
	S. aureus	IS-58 and RN-4220 (Tetracycline and macrolide resistant)	> 1024 µg/mL	(Freitas *et al.*, 2021)
γ-Terpinene	*C. acnes*, *S. epidermidis*	*C. acnes* (ATCC 6919), *S. epidermidis* (ATCC 14990)	No activity – *C. acnes* > 8000 µg/mL – *S. epidermidis*	(Kim *et al.*, 2008)
	S. aureus, *S. epidermidis*, *P. aeruginosa*	*S. aureus* (ATCC 43300), *S. epidermidis* (ATCC 14990) and *P. aeruginosa* (ATCC 27853)	8000 µg/mL (*P. aeruginosa* and *S. aureus*); 16000 µg/mL (*S. epidermidis*)	(Iscan, 2017)
	S. aureus	ATCC 25923 and clinical isolates (*n* = 7)	256 – 1024 µg/mL	(Miladi *et al.*, 2017)
	S. aureus, *P. aeruginosa*	ATCC 12598 (MSSA), NCTC 12497 (MRSA), *P. aeruginosa* (NCTC 6749 and clinical isolate)	456 µg/mL – MSSA 919 µg/mL – MRSA > 3676 µg/mL - *P. aeruginosa*	(Owen *et al.*, 2019)
	A. baumannii	ATCC 19606 and clinical isolates (*n* = 3)	> 32000 µg/mL	(Aleksic Sabo *et al.*, 2021)

Other studies have also reported that both carvacrol and cinnamaldehyde are able to reduce toxin production and induce metabolic stress against *S. aureus* and *A. baumannii* (Albano *et al.*, 2016; Montagu *et al.*, 2016; Mohammadzamani *et al.*, 2020).

The antibacterial properties of eugenol have predominantly been investigated against Gram-positive bacteria with MIC values of 32 – 4000 µg/mL. A few studies have demonstrated that eugenol exhibits stronger antibacterial activity compared to linalool, cinnamaldehyde and carvacrol against *S. aureus*, *S. epidermidis* and *P. aeruginosa* (Iscan, 2017; Abd El-Baky and Shawky, 2016).

A handful of reviews have discussed the antibacterial properties of linalool and have also proposed that could be a lead compound for antibiotic development (An *et al.*, 2021; Mączka *et al.*, 2022). Linalool demonstrates better activity against Gram-positive bacteria (MIC values 350 – 8000 µg/mL), whilst reported MIC values vary against *A. baumannii* (2 - 32 µg/mL) and *P. aeruginosa* (Owen *et al.*, 2019; Liu *et al.*, 2020; Kim *et al.*, 2020).

The antibacterial properties of α-pinene have been reviewed by Salehi *et al.* (2019). In comparison to the other studied EOCs, both α-pinene and γ-terpinene exhibit weaker antibacterial properties, with α-pinene exhibiting stronger antibacterial activity (Hammer *et al.*, 2003). γ-Terpinene has very weak antimicrobial activity with MIC values of > 8000 µg/mL against *P. aeruginosa* and *A. baumannii* (Owen *et al.*, 2019; Aleksic Sabo *et al.*, 2021).

2.13.2 Antifungal activity of studied essential oil compounds

Essential oil compounds elicit multiple antimicrobial mechanisms against *C. albicans*, including cell membrane destabilisation, disruption of metabolic processes, inhibition/modulation of virulence factors, and denaturation of fungal enzymes (Mani-López *et al.*, 2021). These mechanisms can be fungistatic or fungicidal effects which is based on the EOCs chemical structure and concentration (Zhao *et al.*, 2023a). Several reviews have discussed that phenolic and phenylpropanoid compounds exhibit strong antifungal activity, whilst most hydrocarbon terpenes demonstrate weak activity or are unable to inhibit the growth of *C. albicans* (Nazzaro *et al.*, 2017; D'agostino *et al.*, 2019).

A common investigative approach shared by most studies is to evaluate structurally similar EOCs that can be found in the same EO or include two different EOCs with noteworthy anti-*Candida* activity.

Ahmad *et al.* (2011) reported that carvacrol demonstrated MICs of 75 - 100 µg/mL against ATCC and resistant (polyene and azole) isolates of *C. albicans*. The study also demonstrated that carvacrol inhibits ergosterol synthesis and that the hydroxyl group's position changes phenolic compounds' antifungal activity. This observation was also reported by several other studies which demonstrated similar MIC values against ATCC and clinical isolates of *C. albicans* (Lima *et al.*, 2013a; Niu *et al.*, 2020). Ergosterol is the most common sterol in the fungal cell membrane and controls membrane permeability and fluidity (Douglas and Konopka, 2014). Carvacrol is relatively hydrophilic, increasing its ability to penetrate the membrane and interact with fungal enzymes (Sikkema *et al.*, 1995).

Cinnamaldehyde also exhibits potent activity against *C. albicans*, mainly attributed to its aldehyde group (Doyle and Stephens, 2019). A study by Shreaz *et al.* (2011) demonstrated that MIC values for cinnamaldehyde against fluconazole-resistant *C. albicans* ranged from 100 – 500 µg/mL whilst Chen *et al.* (2019) reported similar MIC values (130 - 260 µg/mL) against an ATCC (64547) of *C. albicans*. A study by Miranda-Cadena *et al.* (2021) investigated the antifungal activity of carvacrol and cinnamaldehyde against azole-resistant clinical strains (*n* = 10) of *C. albicans* that were isolated from patients infected with oral candidiasis. Cinnamaldehyde demonstrated higher efficacy (32-64 µg/mL (MIC); 64 - 128 µg/mL (MFC)) as compared to carvacrol (128 µg/mL (MIC); 256 µg/mL (MFC)) against all isolates. Saracino *et al.* (2022) evaluated the α-pinene, eugenol, and cinnamaldehyde against clinical isolates (*n* = 15) of *C. albicans*. Cinnamaldehyde was the most effective (mean MIC 50.05 µg/mL; mean MFC 109.25 µg/mL), followed by α-pinene (mean MIC 195.41 µg/mL; mean MFC 251.27 µg/mL) with eugenol (mean MIC 455.42 µg/mL; mean MFC 690.09 µg/mL) being the least effective. The study also evaluated the fungicidal effects of each compound using the time-to-kill assay, and it was reported that eugenol killed all fungal cells within one hour, whilst results for cinnamaldehyde (four hrs) and α-pinene (24 hrs) varied respectively. A few reviews have discussed eugenols potent anti-*Candida* activity, due to eugenols exhibiting multiple antifungal mechanisms simultaneously (Didehdar *et al.*, 2022; Shariati *et al.*, 2022). Some studies have demonstrated eugenols strong antifungal activity (MIC values of 32- 4000 µg/mL) against ATCC strains of *C. albicans* (Buket, 2017; Iscan, 2017).

Table 2.7: Antifungal activity of selected essential oil compounds against *C. albicans* using the broth microdilution or time-kill assays

Essential oil compound	Strain/isolate	Minimum inhibitory concentration (µg/mL)	References
Carvacrol	ATCC 10261,44829 90028, clinical isolates (fluconazole sensitive (*n* = 32), amphotericin B resistant (*n* = 8))	75 – 100 µg/mL	(Ahmad *et al.*, 2011)
	ATCC 40042, 13803, 76485 and clinical isolates (*n* = 14)	128 – 256 µg/mL	(Lima *et al.*, 2013a)
	ATCC (10231 and 24433)	120 – 250 µg/mL	(Iscan, 2017)
	ATCC (18804, 07318 and SC5314)	247 – 497 µg/mL	(Niu *et al.*, 2020)
	Clinical isolates (*n* = 10)	128 µg/mL	(Miranda-Cadena *et al.*, 2021)
Trans-Cinnamaldehyde	Clinical isolates (Fluconazole resistant (*n* = 18))	100 – 500 µg/mL	(Shreaz *et al.*, 2011)
	Amphotericin and fluconazole resistant clinical isolates (*n* =3)	200 – 400 µg/mL	(Khan *et al.*, 2012)
	ATCC 60193	25 µg/mL	(Buket, 2017)
	ATCC (10231 and 24433)	1000 - 4000 µg/mL	(Iscan, 2017)
	ATCC 10231 and one clinical strain (135BM2/94)	2600 µg/mL (MFC)	(Serra *et al.*, 2018)
	ATCC 64547	130 - 260 µg/mL	(Chen *et al.*, 2019)
	ATCC (90028 and 60193) and clinical isolates (*n* = 4)	40 µg/mL – ATCC 40 – 78 µg/mL – Clinical	(Martorano-Fernandes *et al.*, 2021)
	Clinical isolates (*n* = 10)	32 - 64 µg/mL	(Miranda-Cadena *et al.*, 2021)
	Clinical isolates (*n* = 15)	21 – 164 µg/mL (50.05 µg/mL (MIC); 109.25 µg/mL (MFC) –average across all isolates)	(Saracino *et al.*, 2022)
Eugenol	ATCC 60193	32 µg/mL	(Buket, 2017)
	ATCC (10231 and 24433)	2000 – 4000 µg/mL	(Iscan, 2017)
	Clinical isolates (*n* = 15)	82 – 1325 µg/mL (455.42 µg/mL (MIC); 690.09 µg/mL (MFC) –Average)	(Saracino *et al.*, 2022)
(±) Linalool	ATCC 14053 and clinical isolates (*n* = 13)	52 – 200 µg/mL	(Hsu *et al.*, 2013)
	ATCC 60193	482 µg/mL	(Buket, 2017)
	ATCC (10231 and 24433)	500 – 1000 µg/mL	(Iscan, 2017)
	ATCC 10231 and one clinical strain (135BM2/94)	2600 µg/mL (MFC)	(Serra *et al.*, 2018)
	ATCC (2091, 10231, 64124) and clinical isolates (*n* = 20)	2.0 – 8.0 µg/mL (ATCC), 0.50 – 8 µg/mL (Clinical)	(Biernasiuk and Malm, 2023)
(-) - α-Pinene	ATCC (10231 and 24433)	500 – 1000 µg/mL	(Iscan, 2017)
	ATCC (14065, 32354) and clinical isolates (*n* = 2)	500 – 4000 µg/mL	(Rivera-Yañez *et al.*, 2017)
	ATCC 10261 and clinical isolates (*n* = 10)	46 – 2960 µg/mL	(Iraji *et al.*, 2020)

Essential oil compound	Strain/isolate	Minimum inhibitory concentration (µg/mL)	References
(-) - α-Pinene	ATCC 60193	64 – 128 µg/mL	(Nóbrega *et al.*, 2021)
	Clinical isolates (*n* = 15)	17 – 536 µg/mL (195.41 µg/mL (MIC); 251.27 µg/mL (MFC) –Average)	(Saracino *et al.*, 2022)
	ATCC 76485	128 µg/mL	(Bomfim de Barros *et al.*, 2023)
γ-Terpinene	ATCC (10231 and 24433)	4000 µg/mL	(Iscan, 2017)
	ATCC (14065, 32354) and clinical isolates (*n* = 2)	2000 – 16000 µg/mL	(Rivera-Yañez *et al.*, 2017)

A similar trend to the antibacterial activity of linalool and γ-terpinene's is also observed for their antifungal activity with linalool (MIC values 0.50 – 2600 µg/mL) being more effective than γ-terpinene's (MIC values of 2000 – 16000 µg/mL) against ATCC and clinical isolates of *C. albicans* (Rivera-Yañez *et al.*, 2017; Serra *et al.*, 2018; Biernasiuk and Malm, 2023).

2.13 Toxicity of studied essential oil compounds

Essential oil compounds have been noted to exhibit inherent toxicities and has been evaluated using cell cultures (Table 2.8) and animal models (Table 2.9). The toxic properties demonstrated by an EOC is related to their lipophilic nature, low molecular weight, and non-specific affinity for a molecular target (Bakkali *et al.*, 2008; Dhifi *et al.*, 2016). The chemical characteristics which gives an EOC its antimicrobial mechanisms can also affect eukaryotes, resulting in cell damage or even cause cell death (Fuentes *et al.*, 2021). In general, terpenoids (alcohols, aldehydes, and phenols) are the most toxic, with other EOCs demonstrating varying results according to the toxicity model studied (Dhifi *et al.*, 2016; Stepić *et al.*, 2020). Essential oil compounds exhibit many toxic mechanisms whereby these compounds can cross cell membranes and induce leakage of cytoplasmic content (Wojtunik-Kulesza, 2022). Other mechanisms include reducing ATP production, changing the zeta-potential of the cell's surface, and increasing ROS production (Tisserand and Young, 2014). Despite these noted toxic mechanisms, EOCs are classified as generally recognized as safe (GRAS) compounds by the Food and Drug Administration (FDA) (Adams *et al.*, 2011; Jackson-Davis *et al.*, 2023). Essential oil compounds can also cause adverse reactions which includes contact dermatitis, photosensitivity, and skin corrosion (Tisserand and Young, 2014).

The cytotoxic properties of EOCs have also been investigated against various cell lines using the MTT assay. The MTT assay is a colorimetric bioassay used to measure the metabolic activity of cells in the presence of a cytotoxic compound (Ghasemi *et al.*, 2021). Similarly, to the antimicrobial properties, phenolic and phenylpropanoid compounds exhibit the highest cytotoxicity. A review by Tavvabi-Kashani *et al.* (2024) discussed that the cytotoxic properties of eugenol are attributed to the compound's ability to inhibit metabolic processes, denature enzymes and induce oxidative stress. A study by de Araújo Lopes *et al.* (2018) demonstrated that eugenol at 50 µg/mL (11.93% cell viability) and 100 µg/mL (26.69% cell viability) did not affect the proliferation of HaCAT keratinocytes.

Table 2.8: Cytotoxicity of studied essential oil compounds using cell culture assays

Essential oil compound	Experimental design	Cell line	Toxicity result	References
Carvacrol	MTT assay	Caco-2 cells (Human colon cells)	LC_{50} = 460 µM (24 hrs) LC_{50} = 343 µM (48 hrs)	(Llana-Ruiz-Cabello *et al.*, 2014)
	MTT assay	HaCAT keratinocytes	> 0.028 M (considered cytotoxic)	(Rojo-Ruvalcaba *et al.*, 2020)
	LDH and MTT assay	Healthy skin cells (CCD-1123Sk - fibroblasts) and HeLa cells	100 µg/mL (induced cell membrane damage and impaired metabolic activity)	(Ranjitkar *et al.*, 2021)
	MTT assay	HaCAT keratinocytes and THP1 human monocytes	0.030 mg/mL (70% cell viability) for both cell lines	(García-Salinas *et al.*, 2018)
Trans - Cinnamaldehyde	MTT assay	Human cancer cells (HT1080 (fibrosarcoma), A431(skin carcinoma), SK-N-MC (neuroblastoma), MG63(osteosarcoma), SiHa (cervix carcinoma)) Murine cells (Neuro2a (neuroblastoma), L929 (connective tissue), RAW264.7 (murine macrophages), fibroblast and hepatocytes	All cell lines were classified based on the cell viability at different concentration ranges: 0.80 – 10 µM (≥ 100% cell viability) 20 – 40 µM (58.66 - ≤ 100% cell viability) 80 – 320 µM (1.65 – 93.70% cell viability)	(Singh *et al.*, 2009)
	LDH and MTT assay	Healthy skin cells (CCD-1123Sk - fibroblasts) and HeLa cells	200 µg/mL (impaired metabolic activity)	(Ranjitkar *et al.*, 2021)
	MTT assay	A549 (lung cancer) cells	~ 200 µg/mL (induced cytotoxicity)	(Park and Baek, 2020)
	XTT assay	J774A.1 murine macrophages	IC_{50} = 199.80 µM (24 hrs), 207.74 µM (48 hrs)	(Ávila Brustolin *et al.*, 2022)
		HaCAT keratinocytes	IC_{50} = > 1000 µM	
	MTT assay	Human cervical cancer cells (CaSki, SiHa, C33); HeLa cells; human breast cancer cells (MCF-7, MDA-MB-231)	IC_{50} = > 100 µM (For all cell lines)	(Anselmo *et al.*, 2021)
	MTT assay	HaCAT keratinocytes and human erythrocytes	HaCAT = 227 µM (58% cell viability) Erythrocytes (≥ 1210.6 µM Cell lysis)	(da Nóbrega Alves *et al.*, 2020)
	MTT assay	HaCAT keratinocytes and THP1 human monocytes	0.090 mg/mL (70% cell viability) for both cell lines	(García-Salinas *et al.*, 2018)
	MTT assay	Mouse fibroblasts (NIH 3T3)	IC_{50} = 30 µg/mL (1 hrs), 20 µg/mL (24 hrs)	(Serra *et al.*, 2018)
Eugenol	MTT assay	HaCAT keratinocytes	50 µg/mL (11.93% cell viability) 100 µg/mL (26.69% cell viability)	(de Araújo Lopes *et al.*, 2018)
	MTT assay	Healthy skin cells (CCD-1123Sk - fibroblasts) and HeLa cells	100 – 400 µg/mL (induced cell membrane damage and at high doses blocked metabolic activity)	(Ranjitkar *et al.*, 2021)

Essential oil compound	Experimental design	Cell line	Toxicity result	References
Eugenol	LDH and MTT assay	HaCaT keratinocytes and A375 (human melanoma)	IC_{50} = 79.08 µg/mL (HaCAT); IC_{50} = 55.65 µg/mL (A375) - MTT > 100 µg/mL did not impair cell membrane integrity – LDH	(da Silva Bruckmann *et al.*, 2022)
(±) - Linalool	MTT assay	Human cancer cells (SW 620 (colon), T-47D (Breast), A549 and Hep G2 (hepatoblastoma)	SW 620 (IC_{50} = 222 µM), T-47D (IC_{50} = 224 µM), A549 (IC_{50} = 438 µM), Hep G2 (IC_{50} = 290 µM)	(Chang and Shen, 2014)
	MTT and Neutral red assay	HepG2 and A549 cells	HepG2 - IC_{50} = 1550 µM (MTT), IC_{50} = 1600 µM (NR) A549 - IC_{50} = 1093 µM (MTT), IC_{50} = 1750 µM (NR)	(Rodenak Kladniew *et al.*, 2014)
(-) - α-Pinene	MTT assay	HaCaT keratinocytes	No cytotoxic effect on cells (≥ 217.5 µg/mL)	(Xanthis *et al.*, 2021)
	MTT assay	HaCaT keratinocytes	IC_{50} = 1024 µg/mL	(Bomfim de Barros *et al.*, 2023)

Table 2.9: Toxicity of studied essential oil compounds using animal models

Essential oil compound	Experimental design	Animal model	Toxicity result	References
Carvacrol	Fumigation assay	Fruit fly (*Drosophila melanogaster*)	LC_{50} = 31 µg/mL	(Sousa Silveira *et al.*, 2020)
	Fumigation assay	Fruit fly (*D. melanogaster*)	LC_{50} = 0.04 µg/mL LC_{90} = 0.11 µg/mL	(Zhang *et al.*, 2016)
	Larval mortality assay	Asian tiger mosquito (*Aedes albopictus*)	LC_{50} = 13 µg/mL LC_{90} = 27 µg/mL	(Giatropoulos *et al.*, 2018)
	Lethality assay	*Leishmania infantum* (Promastigotes)	IC_{50} = 9.80 µg/mL	(Youssefi *et al.*, 2019)
Trans - Cinnamaldehyde	Lethality assay	Brine shrimp (*Artemia franciscana*)	LC_{50} = 25.60 µg/mL	(Buket, 2017)
	Exposure assay	Nematode (*Caenorhabditis elegans*)	LC_{50} = 800 µg/mL	(Lu *et al.*, 2020)
	Lethality assay	*Leishmania amazonensis* (Promastigotes)	IC_{50} = 28 µg/mL (24 hrs) IC_{50} = 28 µg/mL (48 and 72 hrs)	(Ávila Brustolin *et al.*, 2022)
Eugenol	Fumigation assay	Fruit fly *(D. melanogaster*)	LC_{50} = 0.03 µg/mL LC_{90} = 0.32 µg/mL	(Zhang *et al.*, 2016)
	Lethality assay	Brine shrimp (*Artemia franciscana*)	LC_{50} = 16.90 µg/mL	(Buket, 2017)
(±) - Linalool	Fumigation assay	Fruit fly (*D. melanogaster*)	LC_{50} = 0.35 µg/mL LC_{90} = 0.96 µg/mL	(Zhang *et al.*, 2016)
	Lethality assay	Brine shrimp (*Artemia franciscana*)	LC_{50} = 70.30 µg/mL	(Buket, 2017)
	Lethality assay	Zebrafish (*Danio rerio*)	LC_{50} = 193.3 µg/mL	(Singulani *et al.*, 2018)
	Lethality assay	*L. infantum* (Promastigotes)	No effect	(Youssefi *et al.*, 2019)
	Fumigation assay	Rice weevil (*Sitophilus oryzae*)	LC_{50} = 75.20 µg/mL LC_{90} = 112.91 µg/mL	(Fouad *et al.*, 2021)
	Lethality assay		LC_{50} = 36.90 µg/mL LC_{90} = 174.17 µg/mL	
(-) - α-Pinene	Fumigation assay	House mosquito (*Culex pipiens*)	LC_{50} = 94.88 µg/mL LC_{90} = 153.99 µg/mL	(Vourlioti-Arapi *et al.*, 2011)
	Fumigation assay	Fruit fly (*D. melanogaster*)	LC_{50} = 4.12 µg/mL LC_{90} = 9.84 µg/mL	(Zhang *et al.*, 2016)
	Fumigation assay	Rice weevil (*S. oryzae*)	LC_{50} = 68.47 µg/mL LC_{90} = 92.21 µg/mL	(Fouad *et al.*, 2021)
	Lethality assay		LC_{50} = 91.66 µg/mL LC_{90} = 116.23 µg/mL	
γ-Terpinene	Fumigation assay	Fruit fly (*D. melanogaster*)	LC_{50} = 1.68 µg/mL LC_{90} = 3.57 µg/mL	(Zhang *et al.*, 2016)
	Larval mortality assay	Asian tiger mosquito (*A. albopictus*)	LC_{50} = 20.20 µg/mL LC_{90} = 32.30 µg/mL	(Giatropoulos *et al.*, 2018)

Another study by da Silva Bruckmann *et al.* (2022) reported an IC_{50} = 79.08 µg/mL against HaCAT keratinocytes. Different cell lines demonstrate varying inhibitory concentrations, which is noted for cinnamaldehyde and carvacrol (Singh *et al.*, 2009; Ranjitkar *et al.*, 2021). A recent review by Bansal and Sharma (2024) discussed carvacrol's emerging classification as a lead compound for anti-cancer therapy. Carvacrol exhibits a high cytotoxic effect and has been demonstrated by some studies against various cell lines (García-Salinas *et al.*, 2018; Rojo-Ruvalcaba *et al.*, 2020). A few studies have investigated the cytotoxicity of linalool and α-pinene, with no studies investigating γ-terpinene (Rodenak Kladniew *et al.*, 2014; Xanthis *et al.*, 2021).

The toxicity of EOCs has been studied extensively using insect models since some EOCs have been used as "green insecticides". Several reviews have discussed the insecticidal properties of various EOCs using the fumigant or contact assay (Senthil-Nathan, 2020; Chaudhari *et al.*, 2021). A similar trend is noted for the animal models, whereby carvacrol and eugenol exhibit high toxicity against different species of insects (Zhang *et al.*, 2016; Sousa Silveira *et al.*, 2020). Interestingly, γ-terpinene exhibits toxicity against insects with LC_{50} values comparable to linalool (Zhang *et al.*, 2016; Giatropoulos *et al.*, 2018).

2.14 Anti-inflammatory properties of studied essential oil compounds

Essential oil compounds have also been investigated for their ability to modulate inflammatory pathways (Table 2.10). The anti-inflammatory properties demonstrated by individual EOCs are related to their chemical structure, whereby terpenoids and phenylpropanoids demonstrate the highest anti-inflammatory properties (De Cássia da Silveira Sá *et al.*, 2014; Zhao *et al.*, 2023b). Hydrocarbon terpenes exhibit lesser anti-inflammatory properties and can also depend on the compound's chirality and studied model (Dhifi *et al.*, 2016). Essential oil compounds demonstrate multiple anti-inflammatory mechanisms, including modulation of the arachidonic acid metabolic pathway, inhibiting the production of pro-inflammatory mediators (cytokines, chemokines, and ROS) and inflammatory pathways (Miguel, 2010). Essential oil compounds also exhibit strong antioxidant properties that influence an EOC's ability to reduce inflammation, as ROS production is integral to the inflammatory process. (Amorati *et al.*, 2013; Masyita *et al.*, 2022).

Table 2.10: Anti-inflammatory properties of studied essential oil compounds in response to lipopolysaccharide (LPS) activation using cell and rodent models

Essential oil compound	Experimental protocol	Cell line/model	Dosage (mg/kg)/Concentration (µM; µg/mL)	Effect on inflammation	References
Carvacrol	LPS-activated macrophages	U937 cells (Human myeloid leukaemia)	400 – 1000 µM	Suppressed COX-2 expression and reduced PGE_2 production.	(Hotta *et al.*, 2010)
	Rodent model assessing paw oedema (mixed in food)	Peritoneal macrophages	1, 10, and 100 µg/mL	Dose-dependent reduction in TNF-α and NO levels.	(Guimarães *et al.*, 2012)
	Rodent model assessing paw oedema (mixed in food)	Swiss mice (Paw tissue)	50 mg/kg, 100 mg/kg	Reduced the production of IL-1β and PGE_2 and not TNF-α. Increased IL-10 production	(Lima *et al.*, 2013b)
	Rodent model assessing endotoxemia (Injected with LPS)	Sprague–Dawley rats	20, 40, and 80 mg/kg	Dose-dependent reduction in TNF-α and IL-6. Also inhibited arginase activity which blocked NO production.	(Kara *et al.*, 2015)
	LPS-activated chondrocytes	Human chondrocytes	0 - 10 µg/mL	Inhibited NO and PGE_2 production and decreased the expression of iNOS and COX-2. Also suppressed the protein expression of MMP-3 and MMP-13.	(Xiao *et al.*, 2018)
	LPS-activated macrophages	RAW 264.7 macrophage	11 – 45 µg/mL	Inhibited production of TNF-α, IL-1β and NO. Also blocked phagocytosis.	(Somensi *et al.*, 2019)
	LPS-activated macrophages	RAW 264.7 macrophage	0.16 - 2.50 µg/ml	Dose dependent reduction in TNF-α, IL-1β, and IL-6 levels.	(Niu *et al.*, 2020)
	Rodent model (Injected with LPS)	Wistar rats	25–100 mg/kg	Decreased the production of NO and other ROS.	(Hosseini *et al.*, 2023)
Trans - Cinnamaldehyde	LPS-activated macrophages	Murine J774A-1 macrophages	24- 80 µM (3.18 – 10.57 µg/mL)	Inhibited IL-1β and TNF-α and reduced the release of ROS.	(Chao *et al.*, 2008)
	LPS-activated macrophage	RAW 264.7 macrophage	10 µg/mL	Inhibition of NO and PGE_2 production	(Tung *et al.*, 2008)
	LPS-activated macrophages	Murine J774A-1 macrophages	100 µM (13.22 µg/mL)	Reduced the production of NO and suppressed the expression of COX-2.	(Ballabeni *et al.*, 2010)
	LPS-activated microglia	BV2 microglia	25 – 100 µM (3.30 – 13.22 µg/mL)	Reduced the production IL-1β, IL-6, and TNF-α. Also suppressed the expression of iNOS.	(Ho *et al.*, 2013)

Essential oil compound	Experimental protocol	Cell line/model	Dosage (mg/kg)/Concentration (µM; µg/mL)	Effect on inflammation	References
Trans - Cinnamaldehyde	LPS-activated macrophages	Murine J774A.1 macrophages	1.25 – 20 µM (0.17 – 2.64 µg/mL)	Inhibited the production of IL-1β, IL-6, and TNF-α and reduced the expression of iNOS	(Pannee *et al.*, 2014)
	Rodent model assessing endotoxemia (Injected with LPS)	C57BL/6J mice	0.45 and 0.9 mg/kg	Reduced the levels of IL-1β, IL-18, TNF-α and IFN-γ. Downregulated TLR4 activates pro-inflammatory responses.	(Lee *et al.*, 2015)
	Rodent model assessing systemic inflammatory response syndrome (SIRS) (Injected with LPS)	Swiss mice	250 mg/kg	Reduced the production of NO, IL-10, and TNF-α. Also modulated activation of transient receptor potential channel ankyrin 1 (TRPA1) which reduces organ damage during sepsis.	(Mendes *et al.*, 2016)
	LPS-activated macrophages	RAW 264.7 macrophage	0 – 100 µM (0 – 13.22 µg/mL)	Dose-dependent reduction of iNOS expression and IL-1β, IL-6, and TNF-α levels.	(Kim *et al.*, 2018)
	LPS-activated macrophages	RAW 264.7 macrophage	15, 25, and 50 µM (1.98, 3.30 and 6.61 µg/mL)	Reduced the production of TNF-α, IL-1β, IL-6, and NO.	(Gulec Peker and Kaltalioglu, 2021)
Eugenol	LPS-activated macrophages	RAW 264.7 macrophage	40, 80, and 120 µg/mL	Dose-dependent inhibition of IL-6 and TNF-α.	(Huo *et al.*, 2013)
	Rodent model assessing lung inflammation (Injected with LPS)	BALB/c mice	15, 50, 100, 150, 500, 1000, and 1500 mg/kg	Inhibited TNF-α, IL-1β, and IL-6 and impaired NADPH oxidase activity.	(Magalhães *et al.*, 2019)
	LPS-activated epithelial cells	IPEC-J2 porcine cells	100 µM	Reduced the production of TNF-α and IL-8.	(Hui *et al.*, 2020)
	LPS-activated macrophages	RAW 264.7 macrophage	15, 25, and 50 µM	Reduced the production of TNF-α, IL-1β, IL-6, and NO.	(Gulec Peker and Kaltalioglu, 2021)
	LPS-activated macrophages	THP-1 macrophages	5, 10 and 15 µM	Reduced IL-1β production, however, increased TNF-α levels.	(Gojani *et al.*, 2023)
	LPS-activated macrophages	RAW 264.7 macrophage	50 µM	Modulated expression of NLRP3 inflammasome and NF-κB, which inhibited the production of IL-1β and NO.	(Bakshi *et al.*, 2024)
(±)-Linalool	LPS-activated microglia	BV2 microglia	162, 324 and 648 µM (25, 50 and 100 µg/mL)	Dose-dependent inhibition of TNF-α, IL-1ß, IL-6, and reduced production of PGE_2.	(Li *et al.*, 2015)

Essential oil compound	Experimental protocol	Cell line/model	Dosage (mg/kg)/ Concentration (µM; µg/mL)	Effect on inflammation	References
(±)-Linalool	Rodent model assessing endotoxemia (Injected with LPS)	C57BL/6J mice	2.6 and 5.2 mg/kg	Reduced the production of IL-1β, IL-18, TNF-α and IFN-γ. Also downregulated TLR4 which activates pro-inflammatory responses.	(Lee *et al.*, 2018)
	LPS-activated macrophages	RAW 264.7 macrophages	10, 50 and 100 µM (1.50, 7 and 15 µg/mL)	Dose-dependent inhibition of NO with no effect on PGE_2	(Varia *et al.*, 2020)
(-) - α- Pinene	LPS-activated macrophages	RAW 264.7 macrophages	25 µM (6.81 µg/mL)	Reduced the production of NO	(Kazemi, 2014)
	LPS-activated chondrocytes	Human chondrocytes	10 – 200 µg/mL	Reduced the production of NO.	(Rufino *et al.*, 2014)
	LPS-activated macrophages	Macrophages isolated from rodents	20 – 200 µg/mL	Reduced the expression of iNOS and the production of IL-6 and TNF-α.	(Kim *et al.*, 2015)
γ-Terpinene	LPS-activated macrophages	RAW 264.7 macrophages	25 µM (6.81 µg/mL)	Reduced the production of NO	(Kazemi, 2014)
	Rodent model assessing acute lung inflammation (Injected with LPS)	Inflamed tissue and isolated macrophages	25 mg/kg and 50 mg/kg	Reduced the levels of TNF-α and IL-1β, with no effect on IL-6. Promoted neutrophil migration.	(Ramalho *et al.*, 2016)
	LPS-activated macrophages	Isolated from BALB/c mice	10 µg/mL	Reduced the expression of iNOS.	(Ranjbaran *et al.*, 2019)
	LPS-activated microglia	BV2 microglial cells	125 and 250 µg/mL	Dose-dependent reduction in NO and IL-1β production	(Asikin *et al.*, 2022)

Most EOCs elicit overlapping anti-inflammatory effects, whereby different compounds at the same concentrations may inhibit or reduce the same inflammatory mediator (de Lavor *et al.*, 2018). Cinnamaldehyde exhibits multiple anti-inflammatory effects through the modulation of signal transduction pathways and the molecular regulation of the NF-kB and MAPK pathways (De Cássia da Silveira Sá *et al.*, 2014). Several studies have demonstrated that at concentrations of 24 – 100 µM, cinnamaldehyde inhibited the production of pro-inflammatory interleukins (IL-1β, IL-6 and TNF-α) and reduced the expression of iNOS and COX-2 using LPS-activated macrophages (Ballabeni *et al.*, 2010; Ho *et al.*, 2013; Pannee *et al.*, 2014; Kim *et al.*, 2018). Eugenol exhibits comparable anti-inflammatory properties to cinnamaldehyde with similar modulatory mechanisms (das Chagas Pereira de Andrade and Mendes, 2020). A noted difference is that eugenol exhibits more potent inhibitory effects on arachidonic acid metabolism (Okada *et al.*, 2005) than cinnamaldehyde (Guo *et al.*, 2006). Gulec Peker and Kaltalioglu (2021) reported that concentrations of 15 – 50 µM reduced the production of several pro-inflammatory cytokines (TNF-α, IL-1β and IL-6) using LPS-activated macrophages. Other studies have also demonstrated similar results at the same concentrations using LPS activated epithelial and monocyte cell lines (Hui *et al.*, 2020; Gojani *et al.*, 2023).

Carvacrol's anti-inflammatory properties have been demonstrated *in vitro* and *in vivo* models and reviewed by Costa *et al.* (2018). Carvacrol has been identified as a lead natural compound in skin infections since it exhibits multiple therapeutic properties such as modulating cytokine production, reducing oxidative stress and stimulating the wound healing process (Imran *et al.*, 2022; Romo-Rico *et al.*, 2022). Several studies have demonstrated carvacrol's ability to inhibit the production of pro-inflammatory cytokines and NO production using LPS-activated cell lines (Lima *et al.*, 2013b; Xiao *et al.*, 2018; Somensi *et al.*, 2019).

The anti-inflammatory properties of linalool have been predominantly demonstrated using oxidative stress or inflammatory models related to the central nervous system (CNS) and have been reviewed elsewhere (An *et al.*, 2021; Vassiliou *et al.*, 2023). A few studies have reported linalool's ability to reduce pro-inflammatory cytokines and NO production, with higher concentrations (> 100 µM) required for inhibiting PGE_2 production using LPS *in vivo* and *in vitro* models (Lee *et al.*, 2015; Li *et al.*, 2015; Varia *et al.*, 2020).

A few reviews have discussed α-pinene's ability to reduce inflammation by reducing the production of cytokines and ROS and suppressing the MAPK pathway (Salehi *et al.*, 2019;

Allenspach and Steuer, 2021). Some studies have demonstrated α-pinene's ability to reduce pro-inflammatory cytokines (IL-6 and TNF-α) NO and PGE_2 production and reduce the expression of iNOS and MMP genes using LPS-activated macrophages and chondrocytes (Rufino *et al.*, 2014; Kim *et al.*, 2015). Kazemi (2014) evaluated EOCs isolated from *Nigella sativa* L. (black seed), including α-pinene and γ-terpinene, using LPS-activated murine macrophages. The study reported that γ-terpinene (55% NO reduction) and α-pinene (53% NO reduction) exhibit similar effects on NO production compared to the LPS control. Ranjbaran *et al.* (2019) demonstrated that γ-terpinene reduced the production of NO and NADH oxidase using LPS-activated murine macrophages. Another study by Asikin *et al.* (2022) reported that γ-terpinene elicits a dose-dependent reduction in IL-1β and NO production using LPS-activated microglial cells. Comparatively, Asikin *et al.* (2022) study reported similar anti-inflammatory effects at higher concentrations (125 – 250 µg/mL) as compared to Kazemi (2014) (6.81 µg/mL) and Ranjbaran *et al.* (2019) (10 µg/mL), which may be attributed to the different cell lines.

2.15 Antimicrobial combination therapy

Combination therapy is one of the most effective strategies to improve the efficacy of individual antimicrobial agents (Brooks and Brooks, 2014). Combinations of antibiotics were first introduced in the 1940s to increase the spectrum of activity of penicillin and were later implemented for treating *Mycobacterium tuberculosis* (Tyers and Wright, 2019). Currently, antimicrobial combinations have become the baseline therapy for many infections, with the primary rationale being the reduction of resistance and to increase the clinical efficacy (Wang and Yang, 2022b). Antimicrobial combination therapy is also used as empirical therapy to treat heterogeneous populations of micro-organisms that grow in different anatomical locations (Roemhild *et al.*, 2022). The main aim of any drug combination is to achieve a synergistic effect whereby the combination elicits a greater efficacy than the individual drugs (Bush, 2018).

Antimicrobial combinations are commonly evaluated using the sum of the fractional inhibitory concentration (ΣFIC)(Bush, 2018; Tyers and Wright, 2019). The ΣFIC is based on the Loewe additivity zero-interaction theory, which hypothesizes that any drug cannot interact with itself and that the effect of a self-drug combination will always be additive (ΣFIC 1.00)(Duarte and Vale, 2022). Initially, this definition included two interactions: synergy (ΣFIC < 1.00) and

antagonism (ΣFIC > 1.00); however, several authors suggested the inclusion of other interactions to account for doubling dilution methodology (Greco *et al.*, 1995; Odds, 2003). For example, MIC values are determined using the broth microdilution assay using drug concentrations in two-fold dilutions. Based on the old definition, a MIC value of 2 µg/mL could also be 1.00 or 4.00 µg/mL. This motivated a change in the FIC interpretation cut-offs and included ranges for defining additivity, indifference, and antagonism (Table 2.11)(Bush, 2018).

Table 2.11: Summary of combinational interactions

Interaction	Description	ΣFIC	References
Synergy	The combined effect of two drugs is greater than the sum of the effects when given separately (four-fold or greater decrease in MIC of both drugs in combination compared with the drugs tested individually).	ΣFIC < 0.50	(van Vuuren and Viljoen, 2011; Bush, 2018)
Additive	The effect of two drugs in combination equals the summation of their effects when given alone (a two-fold decrease in the MIC of both agents).	>0.50 - ≤ 1.00	(Dawis *et al.*, 2003; van Vuuren and Viljoen, 2011)
Non-interactive/indifference	There is no effect between two drugs in combination (no change in the MIC value of the agents alone or combined).	>1.00 – ≤ 4.00	(van Vuuren and Viljoen, 2011)
Antagonism	The combined effects of the two drugs are less than the sum of the effects when administered separately (a four-fold increase in the MIC values of both agents).	> 4.00	(Botelho, 2000; Odds, 2003)

All combinations of antimicrobial agents can be classified into one of these interactive profiles (Coates *et al.*, 2020). However, these definitions only provide an overview of the drug interaction and do not consider the mechanism of action or molecular target. A review by Jia *et al.* (2009) discussed that synergistic combinations can be achieved through various mechanisms, such as different targets of unrelated pathways (Beta-lactams and aminoglycosides), mutual suppression of the same pathway (sulphamethoxazole-trimethoprim) and modification of one binding site to increase the action of another drug (rifampicin with fusidic acid). Another consideration is the degree of antimicrobial effect that a single compound may exhibit. For instance, co-trimoxazole comprises sulphamethoxazole and trimethoprim, which are both bacteriostatic, whilst clavulanic acid is approximately 300 times weaker than amoxicillin (Finlay *et al.*, 2003). Based on these considerations and the need for a more precise definition of "synergy", several authors proposed a sub-grouping of synergistic and antagonistic combinations (Keith *et al.*, 2005). Congruous synergy refers to a combination of two conventional antimicrobials. Both antibiotics have comparable antimicrobial activity (Interpreted by MIC values) and generally act on different or sequential

molecular targets (Gray and Wenzel, 2020; Dhanda *et al.*, 2023). Syncretic synergy involves combinations of a conventional antibiotic and a compound with little or no antimicrobial properties (Keith *et al.*, 2005). Syncretic combinations have the most potential from any synergistic combination, as these combinations exhibit a high barrier to resistance (Wright, 2016). Currently, syncretic combinations are the most studied, with many antibiotics in clinical trials being combinations of beta-lactams and beta-lactamase inhibitors (Dubey *et al.*, 2020). Coalistic synergy occurs when two compounds with limited or no antimicrobial properties are combined to produce an enhanced antimicrobial effect. Coalistic interactions are understudied (Gill *et al.*, 2015).

Hyperantagonism occurs when the combination produces an effect that is less than one compound's effect (Beppler *et al.*, 2016). Hyperantagonistic interactions have been hypothesized as a possible method to induce hysteresis in which a resistant population of micro-organisms reverts to their sensitive state (Lozano-Huntelman *et al.*, 2021; Roemhild *et al.*, 2022). However, some *in vitro* studies have demonstrated that for complete sensitivity to be restored, it could take up to 20 generations, with increased persistence of heteroresistant mutants (Das *et al.*, 2020; Lázár *et al.*, 2022).

2.15.1 Antibiotic adjuvants

An adjuvant can be defined as a natural or synthetic compound with little or no antimicrobial properties and can potentiate an antibiotic when combined (Tyers and Wright, 2019). Adjuvants are classified according to their antimicrobial properties, which can be direct or indirect (Table 2.12). Class I adjuvants act on bacterial metabolism or physiology, while Class II adjuvants increase the efficacy of antibiotics by modulating host defences (Xiao *et al.*, 2023). Class I adjuvants can further be broken down into compounds that directly block acquired resistance mechanisms (Class Ia) and compounds which inhibit virulence factors or modulate the micro-organisms physiology (Class Ib) (Tyers and Wright, 2019; Kumar *et al.*, 2023).

Table 2.12: Classification of antibiotic adjuvants

Adjuvant class	Sub-class	Mechanism of action	Molecular targets	Examples	References
Class I	Class Ia	Inhibit or block active resistance mechanism.	Beta-lactamase enzymes	Clavulanic acid, tazobactam	(Dhanda *et al.*, 2023)
	Class Ib	Inhibit or block passive resistance through alteration of cell physiology or signalling.	Membrane permeabilizes, inhibition of efflux pumps, inhibition of quorum sensing, and biofilm formation	Loperamide, N-acetyl cysteine (NAC), Tween 80	(Gray and Wenzel, 2020; Kumar *et al.*, 2023)
Class II		Modulate host defences to increase the clearing of pathogens.	Increasing macrophage requirement, reducing inflammation	Antimicrobial peptides, Vitamin D	(Ahmed *et al.*, 2020; Xiao *et al.*, 2023)

Adjuvants offer a direct method to extend the lifespan of antibiotics and reduce the emergence of resistance mutations (González-Bello, 2017; Dubey *et al.*, 2020). Adjuvants are described as having weak or limited antimicrobial activity, which has not been well defined in the literature. A review by Kalan and Wright (2011) proposed that any compound that enhances the efficacy of an antibiotic can be considered an adjuvant. This definition was ambiguous as it included antibiotics such as polymyxin E and complicated the classification of antibiotic combinations. Later reviews suggested that adjuvants should be defined as compounds that demonstrate limited antimicrobial properties and modify the micro-organism's physiology or virulence properties (Tyers and Wright, 2019; Gray and Wenzel, 2020; Zhu *et al.*, 2021). Although this definition has been mentioned in several reviews (Bernal *et al.*, 2013; Gill *et al.*, 2015; González-Bello, 2017; Kumar *et al.*, 2023), there is still no definitive MIC range that classifies a compound as an adjuvant. A review by Dhanda *et al.* (2023) proposed that a compound is considered "inactive" or "has no antimicrobial properties" if the MIC values are $\geq$ 512 µg/mL, whilst minimal antimicrobial activity was defined as $\leq$ 64 µg/mL. These limits align with some reviews that have discussed possible adjuvants (Martins *et al.*, 2008; Lagadinou *et al.*, 2020) and more appropriately apply to clinically approved adjuvants such as clavulanic acid (MIC of 350 – 700 µg/ml)(Finlay *et al.*, 2003). Although exhibiting a low antimicrobial effect seems paradoxical for an antimicrobial agent, it increases the barrier to resistance. This predominantly applies to Class 1b adjuvants, whose compounds do not elicit enough stress to induce mutations (Tyers and Wright, 2019).

Adjuvants also display multifunctional properties, which are defined as compounds that exhibit direct and indirect antimicrobial effects simultaneously (Gray and Wenzel, 2020; Wang and

Yang, 2022b). For example, loperamide can directly inhibit microbial growth and modulate inflammation (Ejim *et al.*, 2011). This "drug promiscuity" or low affinity for a specific molecular target reduces the likelihood of resistance and can affect multiple cellular processes simultaneously (Kabir and Muth, 2022). For example, compounds that target the cell membrane can simultaneously induce pore formation, disrupt metabolic processes, and impair the function of efflux pumps (Gray and Wenzel, 2020). In an antimicrobial context, this is beneficial; however, a low specificity for a molecular target can also increase off-target effects resulting in toxicity (Wang and Yang, 2022b). This is particularly noted for natural products that may function as an adjuvant (Fuentes *et al.*, 2021).

2.15.2 Higher order combinations

Higher-order combinations are defined as combinations of three or more drugs. These combinations are employed to broaden the spectrum of activity or overcome multiple resistance mechanisms in a single micro-organism (Beppler *et al.*, 2016). The clinical success of higher-order antimicrobial combinations has already been proven for *M. tuberculosis*, and HIV treatment. Higher-order combinations demonstrate high barriers to resistance as multiple drugs interact with different molecular targets synergistically and independently (Zhu *et al.*, 2021). Natural products such as essential oils are the best example of a higher-order combination. These natural mixtures can comprise more than 60 individual compounds and have evolved to be impervious to resistance by multitargeted effects (Rather *et al.*, 2013).

Higher-order combinations interact differently than conventional pairwise (binary) combinations (Beppler *et al.*, 2016). For example, in a three-drug combination, seven different interactions contribute to the effect of the combination. The first three are the effects of the three single drugs singularly, followed by the three pairwise interactions, and lastly, the effect of all three drugs interacting together. Therefore, within a three-drug combination, there is a total of four interactions co-occurring. These interactions can be grouped as a net interaction (all possible combinations) or as an emergent/suppressive interaction (an interaction that only occurs when all drugs are in combination)(Lozano-Huntelman *et al.*, 2021). An emergent interaction can be described as a synergistic interaction whereby all the compounds in combination exhibit an enhanced effect greater than any pairwise combination (Figure 2.1) (Lozano-Huntelman *et al.*, 2021).

In contrast, a suppressive interaction is antagonistic, whereby the compounds counteract each other to produce a lower effect that may be less than the individual compounds and pairwise combinations (Lozano-Huntelman *et al.*, 2021). The rationale behind higher-order combinations is to achieve a higher degree of microbial killing, and whilst antagonistic interactions are avoided, some reviews have argued that suppressive interactions (Hyperantagonism) might also slow or reverse antimicrobial resistance (Yeh *et al.*, 2009; Singh and Yeh, 2017). An essential consideration for higher-order combinations is to assess off-target effects. Drug combinations can also demonstrate higher toxicities even though they may be effective for an infection (Wang and Yang, 2022a). Therefore, higher-order combinations that demonstrate emergent interactions for antimicrobial efficacy may exhibit suppressive interactions for toxicity.

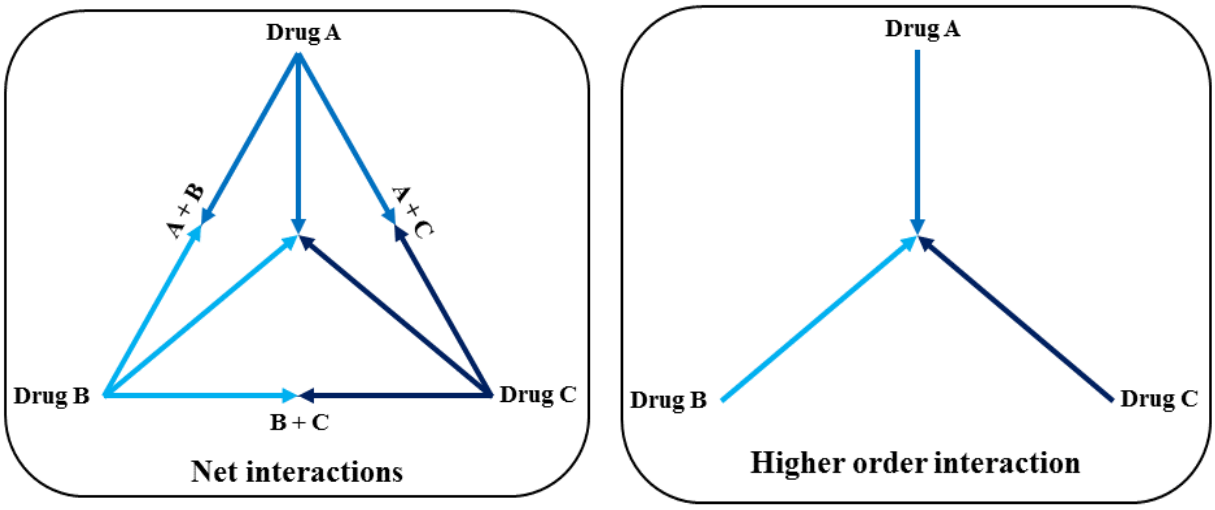


Figure 2.1: Net and higher-order (emergent/suppressive) interactions

2.16 Antimicrobial combinations of essential oil compounds and antibiotics

The ability of essential oil compounds to elicit broad-spectrum antimicrobial activity has facilitated their use in combinations with antibiotics (Trifan *et al.*, 2020). Review articles by Langeveld *et al.* (2014) and Owen and Laird (2018) have discussed combinations of various EOCs and antibiotics. However, there has been a growing trend amongst studies whereby the combinational interactions between antibiotics/antifungals and EOCs have been over-emphasized or portrayed in ways that suggest positive outcomes. For instance, some studies describe ΣFIC values of $0.50 \leq \Sigma FIC \leq 0.75$ as partial synergy, despite several reviews

discouraging it (Rand *et al.*, 1993; Greco *et al.*, 1995). Odds (2003) reviewed the inconsistencies of binary drug combinations and suggested that "partial synergy" is a non-interactive (Indifference) interaction that has been modified to illustrate a positive result. Another observation is reporting that EOCs lower the MIC values of antibiotics without including FIC calculations, which alludes to the conclusion that the combination exhibits synergy. Roell *et al.* (2017) discussed the principles of drug interactions and mentioned that the words "potentiation", "augmentation", and "supra-additivity" have all been used to describe synergy (Table 2.13), which further highlights the ambiguity of a synergistic interaction. These definitions have also been changed to describe the type of interaction according to the antimicrobial properties of the EOC. For instance: "potentiation" is commonly used to describe interactions whereby the EOC has reduced the MIC of the antibiotic. Generally, a drug interaction can be classified as potentiation when two drugs of different pharmacological mechanisms are combined, resulting in an increased effect of one of the drugs (Jia *et al.*, 2009). For this definition to be applied to EOC-antibiotic combinations, the EOC must demonstrate no antimicrobial effect, or a MIC limit should be established to classify a compound as having no antimicrobial properties.

Table 2.13: Summary of alternative interpretations used to describe interactions for essential oil compound and antibiotic combinations

Interpretation	Description	Definition	Reference
Augmentation	It is defined as the pharmacokinetic modification of an antimicrobial agent due to a drug interaction with another non-antibiotic drug. The "non-antibiotic" can either increase or decrease the antimicrobial effect of the antibiotic or function as an allosteric modulator.	No formal definition, however, is referred to as a "drug interaction". Examples include probenecid and meropenem, erythromycin and penicillin.	(Jia *et al.*, 2009)
Partial synergy	Defined as a four-fold or greater decrease in MIC for one agent with a two-fold decrease in the other.	$0.50 \leq \Sigma FIC \leq 0.75$	(Dawis *et al.*, 2003)
Potentiation	It occurs when one antimicrobial agent enhances the activity of another; however, the potentiator has no definable antimicrobial activity.	One compound exhibits "no antimicrobial activity".	(Roell *et al.*, 2017; Duarte and Vale, 2022)
Supra-additivity (Synergism)	A combined effect of two or more antimicrobial agents results in greater efficacy than the sum of their individual effects.	$\Sigma FIC \leq 0.50$ (Synonym for synergy)	(Roell *et al.*, 2017)

A review by Dias *et al.* (2022) reported that some studies investigating an EOCs' ability to block efflux pumps implement an MIC cut-off value of ≥ 1024 µg/mL. This means EOCs with higher or equivalent MIC values are antimicrobially inactive.

Another review by Ahirrao *et al.* (2022) discussed combinations of EOCs with antibiotics that inhibit efflux pumps against MRSA and suggested that EOCs that demonstrate MIC values of > 125 µg/mL should be considered "inactive antimicrobials". Both these MIC cut-offs differ significantly, which complicates comparisons between similar studies. Different methodologies have also made it challenging to compare EOC-antibiotic combinations. The broth microdilution method is the gold standard for determining the MIC of antimicrobial agents and has also been applied to EOC-antibiotic combinations (Tyers and Wright, 2019). Additional methods, such as the time-to-kill assay, are also used to describe micro-organisms' growth kinetics in the presence of the combination (Bush, 2018). Some studies use the disk diffusion method to categorize the combinational effects of EOCs and antibiotics (Albano *et al.*, 2016; Al-Tawalbeh *et al.*, 2024). Although the disk diffusion (Kirby-Bauer) method is established for antibiotic testing, it cannot be used as a quantitative method to evaluate the antimicrobial interactions of EOC combinations (Pillai *et al.*, 2005).

2.16.1 Essential oil compounds and antibiotic combinations against bacteria

The investigation of EOCs with antibiotics against bacteria is frequently described in the literature, with the main target pathogens being *S. aureus* and *P. aeruginosa* (Table 2.14). Most studies have investigated phenolic and phenylpropanoid compounds, whilst hydrocarbon terpenes have been reported to modulate resistance mechanisms such as efflux pumps (Dias *et al.*, 2022).

For Gram-positive bacteria, many studies have demonstrated interactions involving carvacrol and cinnamaldehyde with various classes of antibiotics against ATCC and clinical isolates of *S. aureus* and MRSA (Kwiatkowski *et al.*, 2020; Wang *et al.*, 2021; Al-Tawalbeh *et al.*, 2024; Ferrando *et al.*, 2024). Considering the results from these studies, combinations of EOCs with gentamicin has been demonstrated by some studies and may represent a species-specific combination against *S. aureus* (Hriouech *et al.*, 2020; Gan *et al.*, 2023). Species-specific combinations are based on the principle that a combination's efficacy is related to the unique characteristics of the micro-organism (Brochado *et al.*, 2018). This means that the combination could exhibit a narrow spectrum of activity against *Staphylococcus* species. The investigation of EOC-antibiotic combinations against *S. epidermidis* and *C. acnes* is limited.

Table 2.14: Combinational interactions of essential oil compounds and antibiotics against studied bacteria

Essential oil compound	Antibiotic	Micro-organism	Method	Antimicrobial Interaction (ΣFIC)	Reference
1.8 Cineole	Amoxicillin	Clinical isolate of MRSA	Checkerboard and time-kill assays	Synergism (0.30)	(Hriouech *et al.*, 2020)
	Amoxicillin – Clavulanic acid	Clinical isolate of MRSA	Checkerboard and time-kill assays	Synergism (0.375)	(Hriouech *et al.*, 2020)
		MSSA (*n* = 2)		Synergism (0.312 – 0.375)	
	Gentamicin	Clinical isolate of MRSA	Checkerboard and time-kill assays	Synergism (0.5)	
		MSSA (*n* = 2)		Additivity (0.75)	
	Methicillin	MRSA (ATCC 43300)	Checkerboard assay	Indifference (2.0)	(Kwiatkowski *et al.*, 2020)
	Penicillin G			Synergism (0.10)	
Carnosic acid	Ciprofloxacin	Clinical isolates of GMRSA (*n* = 3)	Checkerboard and time-kill assays	Additivity (0.87 – 0.92)	(Vázquez *et al.*, 2016)
	Gentamicin			Synergism (0.50); Additivity (0.53 – 0.75)	
Carvacrol	Ciprofloxacin	*A. baumannii* (ATCC 19606) and clinical isolates (*n* = 3)	Checkerboard and time-kill assays	Synergism (0.41) for ATCC strain whilst the other two additivity (ΣFIC not reported)	(Aleksic Sabo *et al.*, 2021)
	Gentamicin			Synergism (0.31) for ATCC strain whilst the other two additivity (ΣFIC not reported)	
	Linezolid	MRSA (ATCC 33591)	Broth microdilution	Additivity (1.00)	(Al-Tawalbeh *et al.*, 2024)
	Minocycline			Synergism (0.05)	
	Sulphamethoxazole			Indifference (1.13)	
	Trimethoprim			Synergistic (0.04)	
Trans - Cinnamaldehyde	Amoxicillin	*A. baumannii* (ATCC 19606)	Broth microdilution	Indifference (2.00)	(Ferrando *et al.*, 2024)
	Amikacin	MRSA (ATCC 33591) and clinical isolates of MRSA (*n* = 2)	Broth microdilution and time-kill assays	Synergism (0.19 - 0.38)	(Wang *et al.*, 2021)
	Ampicillin	MRSA (ATCC 33591) and clinical isolates of MRSA (*n* = 2)	Broth microdilution and time-kill assays	Additivity (0.75-1.00)	(Wang *et al.*, 2021)
		A. baumannii (ATCC 19606)	Broth microdilution	Additivity (0.75)	(Ferrando *et al.*, 2024)
		P. aeruginosa (ATCC 27853)		Additivity (0.62)	

Essential oil compound	Antibiotic	Micro-organism	Method	Antimicrobial Interaction (ΣFIC)	Reference
Trans - Cinnamaldehyde	Carbenicillin	*P. aeruginosa* (PA01)	Broth microdilution	Additivity (0.75)	(Topa *et al.*, 2020)
	Chloramphenicol	*A. baumannii* (ATCC 19606)	Broth microdilution	Additivity (1.00)	(Ferrando *et al.*, 2024)
		P. aeruginosa (ATCC 27853)		Antagonism (4.50)	
		S. aureus (ATCC 9144)		Indifference (>3.00)	
	Ceftazidime	MRSA (ATCC 33591)	Broth microdilution and time-kill assays	Additivity (0.75)	(Wang *et al.*, 2021)
		Clinical isolates of MRSA (*n* = 2)		Additivity (0.75 – 1.00)	
		A. baumannii (MDR - clinical isolates (*n* = 25))	Checkerboard and time-kill assays	Indifference (1.5 - 2.0)	(Thirapanmethee *et al.*, 2021)
	Colistin	*P. aeruginosa* (PA01)	Broth microdilution	Synergism (0.50)	(Topa *et al.*, 2020)
		A. baumannii (MDR - clinical isolates (*n* = 25))	Checkerboard and time-kill assays	84% (21/25) Indifference (1.5-2.0) with four isolates demonstrating synergism (0.50)	(Thirapanmethee *et al.*, 2021)
	Erythromycin	*A. baumannii* (ATCC 19606)	Broth microdilution	Indifference (1.50)	(Ferrando *et al.*, 2024)
		P. aeruginosa (ATCC 27853)		Indifference (1.50)	
		P. aeruginosa (PA01)	Broth microdilution	Additivity (0.75)	(Topa *et al.*, 2020)
	Gentamicin	*A. baumannii* (ATCC 19606)	Broth microdilution	Indifference (2.25)	(Ferrando *et al.*, 2024)
		P. aeruginosa (ATCC 27853)		Indifference (1.50)	
		P. aeruginosa (PA01)	Broth microdilution	Synergism (0.37)	(Chadha *et al.*, 2022)
		S. aureus (ATCC 9144)	Broth microdilution	Synergism (0.19)	(Ferrando *et al.*, 2024)
	Imipenem	*A. baumannii* (MDR - clinical isolates (*n* = 25))	Checkerboard and time-kill assays	96% (24/25) Additivity (> 0.50 – 1.00) with one isolate demonstrating synergism (0.50)	(Thirapanmethee *et al.*, 2021)
	Linezolid	ATCC (12228, 35983, 35984) and clinical isolates (*n* = 8)	Time-kill assay	Four to eight-fold decrease in CFU/mL for all isolates (Interpreted as synergism ≤ 0.50).	(Albano *et al.*, 2019)
	Oxacillin	MRSA (ATCC 33591)	Checkerboard and time-kill assays	Additivity (0.75)	(Wang *et al.*, 2021)
		Clinical isolates of MRSA (*n* = 2)			
	Streptomycin	*A. baumannii* (ATCC 19606)	Broth microdilution	Antagonism (4.50)	(Ferrando *et al.*, 2024)
		S. aureus (ATCC 9144)		Synergism (0.31)	
		P. aeruginosa (ATCC 27853)		Additivity (0.75)	

Essential oil compound	Antibiotic	Micro-organism	Method	Antimicrobial Interaction (ΣFIC)	Reference
Trans - Cinnamaldehyde	Tetracycline	*P. aeruginosa* (ATCC 27853)	Broth microdilution	Indifference (1.50)	(Ferrando *et al.*, 2024)
		S. aureus (ATCC 9144)		Indifference (1.50)	
	Tobramycin	*P. aeruginosa* (PA01)	Broth microdilution	Additivity (0.75)	(Topa *et al.*, 2020)
	Vancomycin	MRSA (ATCC 33591)	Checkerboard and time to kill assays	Synergism (0.25)	(Wang *et al.*, 2021)
		Clinical isolates of MRSA (*n* = 2)		Additivity (1.00)	
Eugenol	Ciprofloxacin	*A. baumannii* (ATCC 19606) and clinical isolates (n = 3)	Checkerboard assay	Synergism (0.50) for ATCC strain. Clinical isolates not reported.	(Aleksic Sabo *et al.*, 2021)
	Methicillin	MRSA (ATCC 43300)	Checkerboard and time to kill assays	Indifference (2.50)	(Kwiatkowski *et al.*, 2020)
	Penicillin G			Indifference (1.20)	
	Tetracycline	*S. aureus* (IS-58)	Checkerboard assays	Additivity (0.56)	(Bezerra *et al.*, 2022)
	Vancomycin	*S. aureus* (MTCC 3160)	Checkerboard assay	Synergism (0.25)	(Jafri and Ahmad, 2020)
(-) - α- Pinene	Norfloxacin	*S. aureus* (IS-58)	Checkerboard assay	Additivity (1.00)	(de Araújo *et al.*, 2021)
Thymol	Amikacin	*P. aeruginosa* (ATCC 9024) and one clinical strain	Checkerboard assay	Additivity (0.63-0.75)	(Jayakumar *et al.*, 2023)
	Amoxicillin	*A. baumannii (ATCC 19606)*	Broth microdilution	Additivity (1.00)	(Gan *et al.*, 2023)
	Ampicillin			Additivity (0.75)	
	Chloramphenicol	*A. baumannii* (ATCC 19606)		Synergism (0.37)	
		S. aureus (ATCC 9144)		Indifference (2.00)	
	Ciprofloxacin	*P. aeruginosa* (ATCC 9024) and one clinical strain	Checkerboard assay	Synergism (0.16 – 0.38)	(Jayakumar *et al.*, 2023)
	Colistin			Additivity (0.53 - 0.56)	
	Erythromycin	*A. baumannii* (ATCC 19606)	Broth microdilution	Indifference (2.00)	(Gan *et al.*, 2023)
	Gentamicin	*A. baumannii* (ATCC 19606)		Additivity (0.56)	
		S. aureus (ATCC 9144)		Synergism (0.37)	
	Penicillin G	*A. baumannii* (ATCC 19606)		Indifference (2.00)	
	Streptomycin	*A. baumannii* (ATCC 19606)		Additivity (1.00)	
	Vancomycin	*S. aureus* (MTCC 3160)	Checkerboard assay	Synergism (0.19)	(Jafri and Ahmad, 2020)

A study by Albano *et al.* (2019) reported a four- to eight-fold decrease in the MIC value for linezolid against ATCC and clinical isolates of *S. epidermidis*. In contrast, there are few studies investigating combinations of EOCs with antibiotics against *C. acnes*. Many EOCs have been tested with antibiotics against *P. aeruginosa,* with thymol and cinnamaldehyde being the most studied (Topa *et al.*, 2020; Gan *et al.*, 2023; Ferrando *et al.*, 2024). However, it should be mentioned that *P. aeruginosa* is not the "typical" Gram-negative bacterium as it displays numerous virulence characteristics and versatility, which differs from other Gram-negative bacteria (Carson and Hammer, 2011; Poole, 2011). Therefore, positive results regarding EOC-antibiotic combinations against *P. aeruginosa* should not be considered effective against other Gram-negative bacteria that may cause similar infections (Carson and Hammer, 2011). The investigation of EOC-antibiotic combinations against *A. baumannii* is very limited with only a few studies reporting varying results against ATCC and clinical isolates (Aleksic Sabo *et al.*, 2021; Gan *et al.*, 2023).

2.16.2 Essential oil compounds and antifungal combinations against Candida albicans

Many studies have attempted to identify EOC-antifungal combinations that may increase the efficacy of existing antifungals and reduce the virulence factors of *C. albicans* (Table 2.15). The investigation of EOC-antifungal combinations predominantly involves phenolic (carvacrol and thymol) and phenylpropanoids (eugenol) with azole antifungals (Didehdar *et al.*, 2022). Both phenolic compounds and phenylpropanoids are known for their strong antifungal activity, whilst azole drugs are the largest and most widely used antifungal class (Kathiravan *et al.*, 2012; Didehdar *et al.*, 2022). Another observation is that most studies investigate EOC-antifungal combinations against *C. albicans*' clinical isolates, demonstrating varying levels of resistance to fluconazole. Similarly to other azole antifungals, fluconazole elicits fungistatic effects against *C. albicans*, contributing to widespread resistance and cross-resistance to other azoles (Pfaller *et al.*, 2019). Considering the versatility of fluconazole for Candidiasis and cross-resistance between azole antifungals, the investigation of EOC-fluconazole combinations against *C. albicans* has become a popular target for many studies (Lu *et al.*, 2021).

Although cinnamaldehydes antimicrobial activity against *C. albicans* is well described (Shreaz *et al.*, 2016), very limited studies have investigated cinnamaldehyde-antifungal combinations(Khan *et al.*, 2013).

Table 2.15: Combinational interactions of essential oil compounds and antifungals against *C. albicans*

Essential oil compound	Antifungal	Strain/isolate	Method	Antimicrobial interaction (ΣFIC)	References
trans-Anethole	Clotrimazole	ATCC 10231 and clinical isolates (*n* = 4)	Checkerboard assay	Antagonism (6.00 – 8.50)	(Dąbrowska *et al.*, 2021)
	Econazole			Antagonism (6.20 – 8.50)	
	Miconazole			Indifference (1.50 -3.00)	
	Nystatin			Antagonism (4.80 – 7.80)	
Carvacrol	Fluconazole	ATCC (90028 and 10261) and clinical isolates (*n* = 15)	Checkerboard assay	Synergism (0.25 - 0.50) for 15/17 isolates, including ATCC isolates. Other isolates 2/17 Indifference (>1.00)	(Ahmad *et al.*, 2013)
	Voriconazole	ATCC 22972 and clinical isolates (*n* = 10)	Checkerboard assay	Synergism (0.27 - 0.47)	(Sharifzadeh *et al.*, 2019)
Trans-cinnamaldehyde	Amphotericin B	ATCC 18804 and clinical isolates (*n* = 3)	Checkerboard and time-kill assays	Synergism (0.093 - 0.25) for three isolates (including ATCC); Indifference (> 1.00) for one isolate	(Khan *et al.*, 2012)
	Fluconazole			Synergism (0.38) – ATCC Indifference (1.50) – Clinical	
± Linalool	Fluconazole	ATCC 10231	Checkerboard and time-kill assays	Synergism (0.14)	(Zore *et al.*, 2011)
	Nystatin	ATCC 10231	Checkerboard assay	Indifference (1.06)	(Biernasiuk and Malm, 2023)
Eugenol	Amphotericin B	ATCC 18804 and clinical isolates (*n* = 3)	Checkerboard and time-kill assays	Synergism (0.19 – 0.31)	(Khan *et al.*, 2012)
	Clotrimazole	ATCC 10231 and clinical isolates (*n* = 4)	Checkerboard assay	Indifference (1.10 – 3.00)	(Dąbrowska *et al.*, 2021)
	Econazole			Synergism (0.40) – One clinical isolate; Additivity (0.60 – 1.00) – all other isolates.	
	Fluconazole	ATCC (90028, 10261, 44829) and clinical isolates (*n* = 28)	Checkerboard assay	Synergism (0.31-0.50) for 25/28 isolates, including ATCC strains. Other strains 3/28 Indifference (> 1.00)	(Ahmad *et al.*, 2010)
		ATCC 10231	Checkerboard and time-kill assays	Synergism (0.27)	(Zore *et al.*, 2011)
		ATCC 18804 and clinical isolates (*n* = 3)	Checkerboard and time-kill assays	Synergism (0.093 – 0.38)	(Khan *et al.*, 2012)
		Clinical isolates (*n* = 2)	Checkerboard assay	Synergism (0.16-0.25)	(Jafri and Ahmad, 2020)

Essential oil compound	Antifungal	Strain	Method	Antimicrobial interaction (ΣFIC)	References
Eugenol	Miconazole	ATCC 10231 and clinical isolates (*n* = 4)	Checkerboard assay	Indifference (1.16) – ATCC Synergism (0.20 -0.40) – Clinical	(Dąbrowska *et al.*, 2021)
Geraniol	Amphotericin B	ATCC 18804 and clinical isolates (*n* = 3)	Checkerboard and time-kill assay	Additivity (0.75) – ATCC Synergism (0.25 - 0.38) – Clinical	(Khan *et al.*, 2012)
	Fluconazole			Additivity (1.00) – ATCC Synergism (0.19 -0.31) - Clinical	
Methyleugenol	Fluconazole	ATCC (90028, 10261, 44829) and clinical isolates (*n* = 28)	Checkerboard assay	Synergism (0.27 – 0.35) for 25/28 isolates, including both ATCC strains. Other strains 3/28 Indifference (>1.00)	(Ahmad *et al.*, 2010)
α-Pinene	Amphotericin B	ATCC 10231	Checkerboard assay	Additivity (0.62)	(Silva *et al.*, 2012)
Thymol	Fluconazole	Clinical isolates (*n* = 2)	Checkerboard assay	Synergism (0.19 - 0.25)	(Jafri and Ahmad, 2020)
	Nystatin	ATCC 18804	Checkerboard assay	Synergism (0.25)	(de Castro *et al.*, 2015)

Most studies have investigated cinnamaldehyde in combination with other EOCs (Khan and Ahmad, 2011; Martorano-Fernandes *et al.*, 2021), antiseptics (Priya and Pandian, 2022), or against other fungal species (Schlemmer *et al.*, 2019; Venturini *et al.*, 2021). The lack of studies investigating combinations of cinnamaldehyde with conventional antifungals is unclear, considering that several reviews have mentioned cinnamaldehyde as a lead compound for development as a novel antifungal drug (Shreaz *et al.*, 2016; Lu *et al.*, 2017; Shariati *et al.*, 2022). One reason may be attributed to cinnamaldehydes inhibitory effects on transmembrane ATPase activity (Shreaz *et al.*, 2013). This impairment of the ATPase pathway may reduce the uptake of azole antifungals, as these drugs require ATP (energy-dependent processes) to enter the cell (Mansfield *et al.*, 2010). Zore *et al*. (2011) showed that linalool was more effective than eugenol in combination with fluconazole an ATCC 10231 strain of *C. albicans*. The same study also reported that linalool was more effective (Higher degrees of synergy across multiple ratios) than eugenol using an isobologram Other studies reported indifference for linalool (ΣFIC 1.06) and eugenol (ΣFIC 1.13) with nystatin against an ATCC 10231 strain of *C. albicans* (Biernasiuk and Malm, 2023; Biernasiuk *et al.*, 2023). The investigation of terpenes (α-pinene and γ-terpinene) in combination with any antifungal is very limited with only one study by Silva *et al.* (2012). No studies have investigated combinations with γ-terpinene, as it has been documented to elicit weak antifungal properties (Hammer *et al.*, 2003; Nazzaro *et al.*, 2017).

2.17 Ibuprofen and antibiotic combinations against studied bacteria

The investigation of ibuprofen in combination with antibiotics stems from their therapeutic association with treating diseases (Table 2.16). Many studies have investigated combinations against *S. aureus* and *P. aeruginosa*, whilst studies against *C. acnes*, *S. epidermidis* and *A. baumannii* are lacking.

Several studies have demonstrated interactions between ibuprofen and various antibiotic classes against ATCC and clinical isolates of *S. aureus* and MRSA (Chan *et al.*, 2017; Aqib *et al.*, 2021; Maarouf *et al.*, 2021). A comparative analysis of Chan *et al.* (2017) and Maarouf *et al.* (2021) study suggest that the efficacy of ibuprofen-antibiotic combinations against MRSA isolates may be related to the expression of the *cfr* gene. The *cfr* gene is a natural ARG that resists several classes of antibiotics (phenicol's, lincosamides and streptogramins)(Li *et al.*, 2018). Both studies demonstrated synergistic interactions for clinical isolates of MRSA that

were resistant to chloramphenicol and linezolid. However, susceptible or ATCC strains demonstrated additivity or indifference, suggesting that the expression of specific ARGs may increase the efficacy of ibuprofen-antibiotic combinations.

Table 2.16: Combinational interactions of ibuprofen and antibiotic against studied bacteria

Antibiotic	Micro-organism	Strain	Method	Antimicrobial interaction (ΣFIC)	Reference
Amoxicillin	*S. aureus*	Livestock isolates (mecA positive)	Broth microdilution	Additivity (0.51)	(Aqib *et al.*, 2021)
Aztreonam	*P. aeruginosa*	Clinical isolates (cystic fibrosis)	Checkerboard and time- kill assay	Additivity (0.60)	(Chen *et al.*, 2023)
Ceftazidime				Synergism (0.35)	
Cefuroxime	*S. aureus*	ATCC 25923	Broth microdilution	Synergism (0.50)	(Chan *et al.*, 2017)
		ATCC 33591 (MRSA)		Synergism (0.08)	
		Clinical isolates (*n* = 2)		Additivity (0.51)	
Chloramphenicol		ATCC 25923	Broth microdilution	Additivity (0.52)	(Chan *et al.*, 2017)
		ATCC 33591 (MRSA)		Synergism (0.50)	
		Clinical isolates (*n* = 2)		Synergism (0.28 - 0.50)	
Ciprofloxacin	*P. aeruginosa*	ATCC 85327	Checkerboard assay	Synergism (0.40)	(Khodaparast *et al.*, 2022)
Gentamicin	*S. aureus*	Livestock isolates (mecA positive)	Broth microdilution	Additivity (0.63)	(Aqib *et al.*, 2021)
Linezolid	*S. aureus*	Clinical isolates (Linezolid resistant. (*n* = 6))	Broth microdilution	2/6 Synergism (0.5) 1/6 Additivity (1.00) 3/6 Indifference (1.25-1.50)	(Maarouf *et al.*, 2021)

Tabatabaeifar *et al.* (2022) investigated the effects of several antibiotics (ciprofloxacin, gentamicin, cefepime, imipenem and meropenem) in combination with sub-inhibitory concentrations of ibuprofen (200 µg/mL) against clinical isolates of MRSA and Carbapenem-resistant *P. aeruginosa*. Synergistic (> four-fold decrease in the antibiotics MIC) interactions were reported for combinations of ibuprofen with ciprofloxacin (four-eight-fold), gentamicin (two-16 fold) and cefepime, imipenem and meropenem (eight to 32-fold) against MRSA. The combinations of ibuprofen with ciprofloxacin, cefepime, meropenem and imipenem demonstrated synergy (two-eight-fold) whilst gentamicin (two-fold) was less effective against *P. aeruginosa*. Despite not using the FIC method to classify the interactions, these results demonstrate that ibuprofen may potentiate several antibiotics against MRSA and *P.*

aeruginosa. Furthermore, Tabatabaeifar *et al.* (2022) study contrasts the accepted investigative approach whereby high concentrations of ibuprofen (> 2000 µg/mL) are usually used in combination assays since these concentrations are usually bactericidal.

2.18 Ibuprofen and antifungal combinations against *Candida albicans*

The investigation of ibuprofen with antifungals is presented in Table 2.17. Most studies have demonstrated interactions between ibuprofen and azole antifungals. Arai *et al.* (2005) evaluated combinations of ibuprofen with fluconazole against fluconazole-sensitive and resistant isolates of *C. albicans*. The study reported that combinations of ibuprofen with fluconazole demonstrated additivity (ΣFIC 0.51 – 1.01) against the fluconazole-sensitive isolates. In contrast, synergism (ΣFIC 0.01 - 0.03) was demonstrated for the same combination against the fluconazole-resistant isolates.

Table 2.17: Combinational interactions of ibuprofen and antifungal against *C. albicans*

Antifungal	Strain	Method	Antimicrobial interaction (ΣFIC)	Reference
Anidulafungin	ATCC (10231, 90028, 24433 17A18)	Broth microdilution	Synergism (0.25 - 0.30)	(Rosato *et al.*, 2016)
Fluconazole	Clinical isolates (*n* = 4)	Broth microdilution	One additivity (0.63) two synergism (0.27 -0.28) and one indifference (2.00)	(Król *et al.*, 2018)
	ATCC 10231 and one clinical isolate (fluconazole resistant)	Broth microdilution	Indifference (1.03) – ATCC Synergism (0.18) - Clinical	(Pina-Vaz *et al.*, 2000)
	Fluconazole resistant (*n* = 3) and sensitive (*n* = 6)	Broth microdilution	Fluconazole resistant – Synergism (0.01-0.03) Fluconazole sensitive – Additivity (0.51-1.00)	(Arai *et al.*, 2005)
	NCYC 610	Broth microdilution	Synergism (0.16)	(Scott *et al.*, 1995)
Itraconazole	Clinical isolates (*n* = 2)	Broth microdilution	Additivity (0.73) and Synergism (0.08)	(Król *et al.*, 2018)
Isavuconazole			Indifference (2.0) and Synergism (0.38)	
Voriconazole			Additivity (0.51) and Synergism (0.41)	
Posaconazole			Synergism (0.37 – 0.38)	
Miconazole	NCYC 610	Broth microdilution	Synergism (0.38)	(Scott *et al.*, 1995)

Interestingly, the study also reported the "Eagle effect" for combinations of ibuprofen with fluconazole at concentrations higher than the MIC. The Eagle effect is defined as the paradoxical growth of micro-organisms in the presence of antimicrobial agents that exceed the

bactericidal concentrations (Prasetyoputri *et al.*, 2019). The identification of the eagle effect suggests that ibuprofen may induce persistence when combined with fluconazole or that the combination may demonstrate a synergy ceiling. A similar study by Ricardo *et al.* (2009) investigated combinations of ibuprofen with fluconazole and voriconazole against clinical isolates (n = 62) of azole-resistant *C. albicans*. The study demonstrated that combinations of ibuprofen (100 µg/mL) with triazoles (fluconazole or voriconazole) restored these antifungal efficacies against resistant *C. albicans* isolates. Interestingly, ibuprofen only restored the efficacy of resistant isolates (MIC value > 64µg/mL), with no effect on susceptible isolates. However, these observations were based on the presence of growth using the disk diffusion assay. A comparative analysis of these studies suggests that ibuprofen with azole antifungals demonstrates potential, particularly against azole-resistant *C. albicans*. In terms of other antifungals, only one study (Rosato *et al.*, 2016) investigated combinations of ibuprofen with an echinocandin with no studies for polyenes or other antifungals.

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Essential Oil Compounds in Combination with Conventional Antibiotics for Dermatology

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Abstract: Antimicrobial resistance has emerged as a significant threat to public health, prompting novel combinations comprising of natural sources such as essential oil compounds with conventional antibiotics. This study aimed to determine the possible interactions between six essential oil compounds with eight antibiotics/antifungals against six pathogens (*Staphylococcus aureus*, *Staphylococcus epidermidis*, *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Cutibacterium acnes*, and *Candida albicans*) commonly implicated in skin infections. The minimum inhibitory concentrations (MICs) for the antibiotics and essential oil compounds were evaluated singularly and in combination using the broth microdilution assay. The fractional inhibitory concentrations (FIC) were calculated to determine the interactive profile of the combinations. The synergistic interactions (FIC $\leq$ 0.5) were further analysed at varying ratios and depicted on isobolograms. The toxicity of the synergistic combinations was determined using the brine shrimp lethality assay. Eight synergistic interactions were identified against the selected Gram-positive and *P. aeruginosa* pathogens, and the combinations also demonstrated a reduced toxicity. The combination of amoxicillin and eugenol demonstrated the lowest toxicity (LC_{50} = 1081 µg/mL) and the highest selectivity index (14.41) when in a 70:30 ratio. This study provides insight into the in vitro antimicrobial interactions of essential oil compounds and conventional antibiotics that can form a basis for newer therapies.

Keywords: skin; toxicity; antimicrobial; minimum inhibitory concentration; selectivity index; synergy

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1. Introduction

Skin and soft tissue infections (SSTIs) are one of the most common types of infections in humans and occur in approximately 7–10% of all hospital patients [1,2]. These occur when there is a breakage in the epidermis layer that results in the microbial invasion of the skin or soft tissue, causing a cascade of biochemical reactions due to interactions between the host defences and pathogens [3,4]. The two main categories are uncomplicated and complicated skin infections [5]. Uncomplicated infections include superficial infections such as cellulitis or abscesses, which rarely require antibiotics and are often self-limiting [6]. In contrast, complicated infections are deep-tissue infections such as necrotizing fasciitis, which require broad-spectrum empiric antibiotic therapy or surgical intervention [5]. Although complicated infections can be treated with antibiotics, most treatment regimens are prolonged, contributing to increased antimicrobial resistance [7].

The injudicious use of antibiotics has allowed the dissemination of antibiotic resistance throughout the community and hospital settings, placing an ever-increasing burden on the healthcare system [8,9]. According to a recent review by Murray et al. [10], approximately 4.95 million deaths occurred in 2019 due to antimicrobial resistance. The six leading pathogens were the ESKAPE (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter)* species [11]. Moreover, these pathogens are frequently linked to skin infections, with *S. aureus* being responsible for 35–50% of all skin infections [12]. Antibiotic resistance amongst these pathogens has increased

substantially, with approximately 20–100% of methicillin-resistant *S. aureus* (MRSA) clinical isolates showing high resistance to several antibiotic classes, including glycopeptides [10,13]. Gram-negative pathogens such as *A. baumannii* have also exhibited widespread resistance, with approximately 90% of clinical isolates being resistant to meropenem [14–16], whilst *P. aeruginosa's* resistance rates have increased by between 15 to 30%, with some isolates showing extensively drug-resistant (XDR) profiles [17]. Antifungal resistance has also emerged as another public health crisis [18,19]. Recent epidemiological data suggest that fungal infections contribute to approximately 13 million infections annually with roughly 1.5 million deaths worldwide [20,21]. Candidiasis is the most common fungal infection, with *C. albicans* accounting for 70–90% of all infections [18]. The continuous increase in antimicrobial resistance in contrast to the slow development of new antimicrobial agents has expedited the need for alternative approaches, such as the investigation of natural antimicrobial agents from essential oils and related compounds.

Essential oils (EOs) are complex natural mixtures of bio-active compounds produced by plants as secondary metabolites and have been used for centuries to treat various diseases and ailments [22,23]. An EO is composed of between 20 and >200 different chemical compounds with varying molecular structures and can be broadly classified into three groups: terpenes, terpenoids, and non-terpene-derived compounds called phenylpropanoids [24,25]. These essential oil compounds (EOCs) are responsible for the pharmacological properties of an EO and, more specifically, their antimicrobial properties [24]. EOCs have been studied independently and are actively being sought as novel chemical entities for antimicrobial development [25,26].

In the past decade, antimicrobial combination therapy has become the mainstay for many clinically problematic infections requiring higher-order combinations, especially for nosocomial infections [11,17]. Many authors have argued and even alluded to the hypothesis that EOCs could be combined with conventional antibiotics as a type of syncretic combination [27–29]. Numerous studies have documented in vitro interactions of combinations between EOCs and conventional antibiotics with varying outcomes, which have been reviewed extensively [22,30–32]. However, there are limited studies investigating combinations against skin pathogens that have elucidated the type of interactions that can occur between the EOCs and any conventional antibiotic at varying ratios [33]. Since EOCs elicit broad-spectrum antimicrobial activity via multitarget mechanisms, combinations with conventional antibiotics could be a novel approach to reduce or attenuate resistance in pathogens, allowing ineffective antibiotics to be reclaimed [23].

Despite the overwhelming evidence which supports the antimicrobial properties of EOCs, the toxicity of these compounds remains understudied and has been recommended for further investigation [34]. Many EOCs elicit a high inherent toxicity due to their lipophilic nature, which causes damage to cell membranes and induces oxidative stress [35]. Several reviews have discussed various methods, such as formulating EOCs into nano-emulsions or adding adjunctive agents which can reduce the toxicity of individual EOCs [36,37], with only a handful of studies investigating the overall toxicity of these combinations [38,39].

This study evaluated the effects of combining essential oil compounds with conventional antibiotics against common skin pathogens to elucidate the effectiveness of these combinations with regard to their overall antimicrobial effect and toxicity.

2. Results and Discussion

2.1. Antimicrobial Analysis

The MIC results for conventional antimicrobials and essential oil compounds are presented in Table 1. For the conventional antibiotics, Gram-positive bacteria showed a greater susceptibility than the Gram-negative bacteria, which is a common observation due to physiological differences between the two types of bacteria [11]. *Cutibacterium acnes* showed the greatest susceptibility to conventional antibiotics, particularly erythromycin (0.12 μg/mL) and meropenem (0.20 μg/mL). All the Gram-positive bacteria demonstrated

Table 2. *Cont.*

Conventional Antibiotic	Essential Oil Compounds	*S. aureus* (ATCC 25923)			*S. epidermidis* (ATCC 12228)			*C. acnes* (ATCC 11827)		
		FIC(A) [1]	FIC(B) [2]	ΣFIC [3]	FIC(A)	FIC(B)	ΣFIC	FIC(A)	FIC(B)	ΣFIC
Miconazole	α-pinene	0.67	0.67	1.34	0.36	0.50	*0.86*	1.00	1.33	2.33
	γ-terpinene	0.33	0.50	*0.83*	0.36	0.67	1.03	1.00	1.33	2.33
	±Linalool	0.33	1.46	1.79	0.36	0.50	*0.86*	0.50	0.67	1.17
	Eugenol	0.33	1.20	1.53	0.36	1.00	1.36	1.25	0.50	1.75
	Carvacrol	0.08	0.25	**0.33**	0.14	1.00	1.14	0.12	0.50	*0.62*
	Cinnamaldehyde	0.02	0.30	**0.32**	0.01	0.06	**0.07**	0.06	0.60	*0.66*
Tetracycline	α-pinene	0.50	0.33	*0.83*	0.50	0.50	*1.00*	0.89	1.33	2.20
	γ-terpinene	0.50	0.50	*1.00*	1.00	1.33	2.33	0.89	1.33	2.22
	±Linalool	0.50	0.73	1.23	0.50	0.50	*1.00*	0.44	0.67	1.11
	Eugenol	0.25	0.60	*0.82*	0.25	0.50	*0.75*	0.22	0.67	*0.89*
	Carvacrol	0.25	0.50	*0.75*	0.25	0.67	*0.92*	0.11	0.50	*0.61*
	Cinnamaldehyde	0.13	1.20	1.33	0.13	0.50	*0.63*	0.05	0.60	*0.65*

[1] FIC(A)—fractional inhibitory concentration of antibiotic; [2] FIC(B)—fractional inhibitory concentration of EOC; [3] ΣFIC—sum of FIC values; **bold** values indicate synergy, while italicized values indicate additivity.

The synergistic interactions were further analysed in varied ratio concentrations and presented on isobolograms (Figure 1A–G). For *S. aureus*, three synergistic interactions were observed involving carvacrol with three antibiotics (Figure 1A–C). Comparatively, these combinations showed a similar trend in the ratio of antibiotic to EOC, with ratios of 60:40, 50:50, and 40:60 showing the highest degree of synergy. Figure 1D shows the isobologram of miconazole and cinnamaldehyde against *S. aureus*, which demonstrated higher levels of synergy (lower ΣFIC values) compared to the isobologram depicting the combination of miconazole and carvacrol (Figure 1C) against *S. aureus*. Figure 1E and F represent the isobolograms of synergistic combinations against *S. epidermidis*. The combination of erythromycin with cinnamaldehyde demonstrated the highest synergy compared to the combination of miconazole with cinnamaldehyde. In addition, six out of the nine ratios for the combination of erythromycin with cinnamaldehyde were synergistic. The combination of miconazole with cinnamaldehyde was synergistic against both *S. aureus* (Figure 1D) and *S. epidermidis* (Figure 1F). Very similar results in terms of synergistic ratios were noted, which highlights the potential of this combination. The isobologram for *C. acnes* is presented in Figure 1G. The general trend for *C. acnes* was that greater degrees of synergy were achieved when the ratio of antibiotic to EOC approached equal parts (1:1). In comparison to the combination of amoxicillin with carvacrol against *S. aureus* (Figure 1A), a similar trend was observed particularly at ratios where the EOC made up the majority of the concentration (10:90).

Several reviews have discussed carvacrol and cinnamaldehyde against food-borne pathogens [55,56], with the general consensus being that carvacrol has more potent antibacterial effects on Gram-positive bacteria whilst cinnamaldehyde has a more broad-spectrum effect against bacteria and fungi [57]. In the context of combinations, cell membrane damage brought about by carvacrol or cinnamaldehyde may increase the intracellular concentration of an antibiotic due to allosteric modulatory mechanisms that results in reduced MICs [46,48]. However, further studies are needed to investigate these mechanisms. The synergistic interactions elucidated in this study between miconazole, carvacrol, and cinnamaldehyde against *S. aureus* and *S. epidermidis* highlight its potential as a novel candidate broad-spectrum topical agent. Considering that most skin lesions are perpetuated by the presence of bacteria which may also increase the virulence of some fungal species [58], miconazole's combined antifungal and antibacterial properties may be an effective treatment. This has already been demonstrated in mixed fungal–bacterial infections such as dermatomycoses, with some studies suggesting miconazole's direct involvement in reducing the severity of this type of infection [59].

The results for the 1:1 combinations against Gram-negative bacteria are presented in Table 3. A total of 48 combinations, which only included the conventional antibiotics that had activity against Gram-negative bacteria. From the results, 40% (19/48) of the combinations were additive, while the remaining 58% (28/48) were non-interactive.

One synergistic interaction (ΣFIC 0.32) was observed against *P. aeruginosa*. Most combinations (16/24) against *P. aeruginosa* were additive, with combinations involving ciprofloxacin showing more favourable results than the rest. Figure 2 represents varied ratios for the synergistic interaction of ciprofloxacin with cinnamaldehyde against *P. aeruginosa*. In addition to the ratios in which the EOC and antibiotic components were in equal parts, the ratios (antibiotic/EOC) of 60:40, 40:60, and 30:70 also demonstrated synergy.

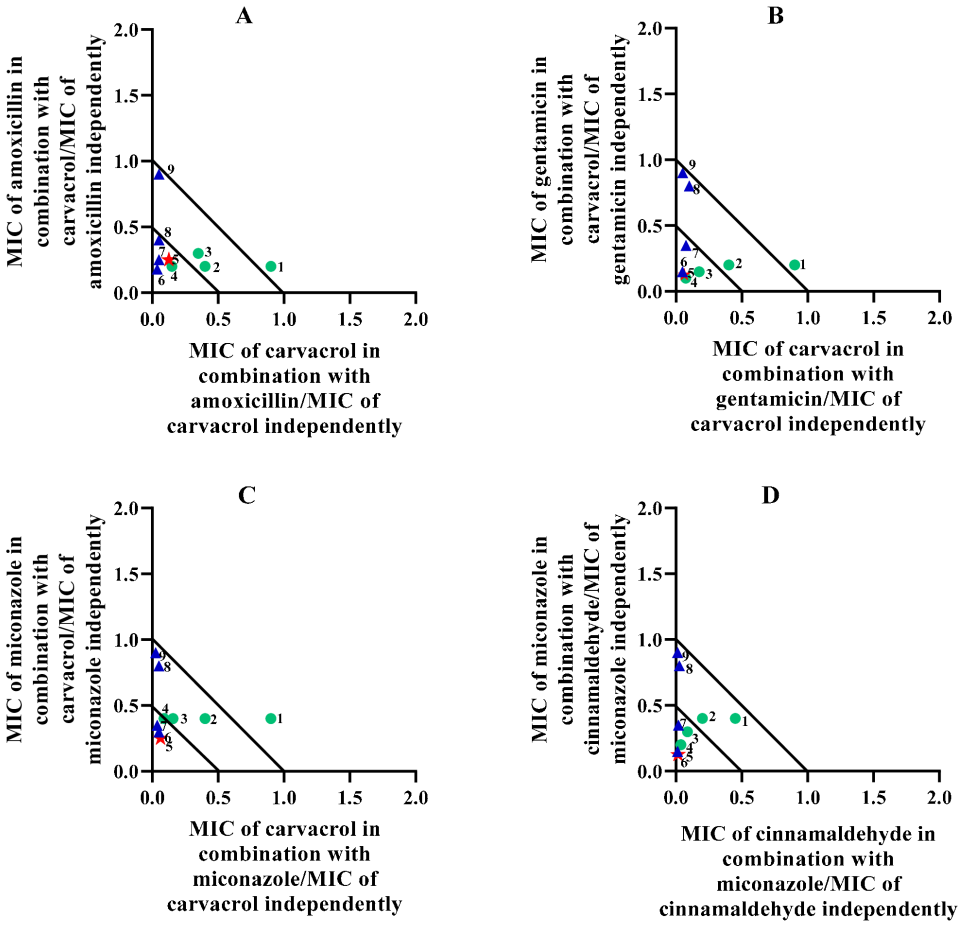

Figure 1. *Cont.*

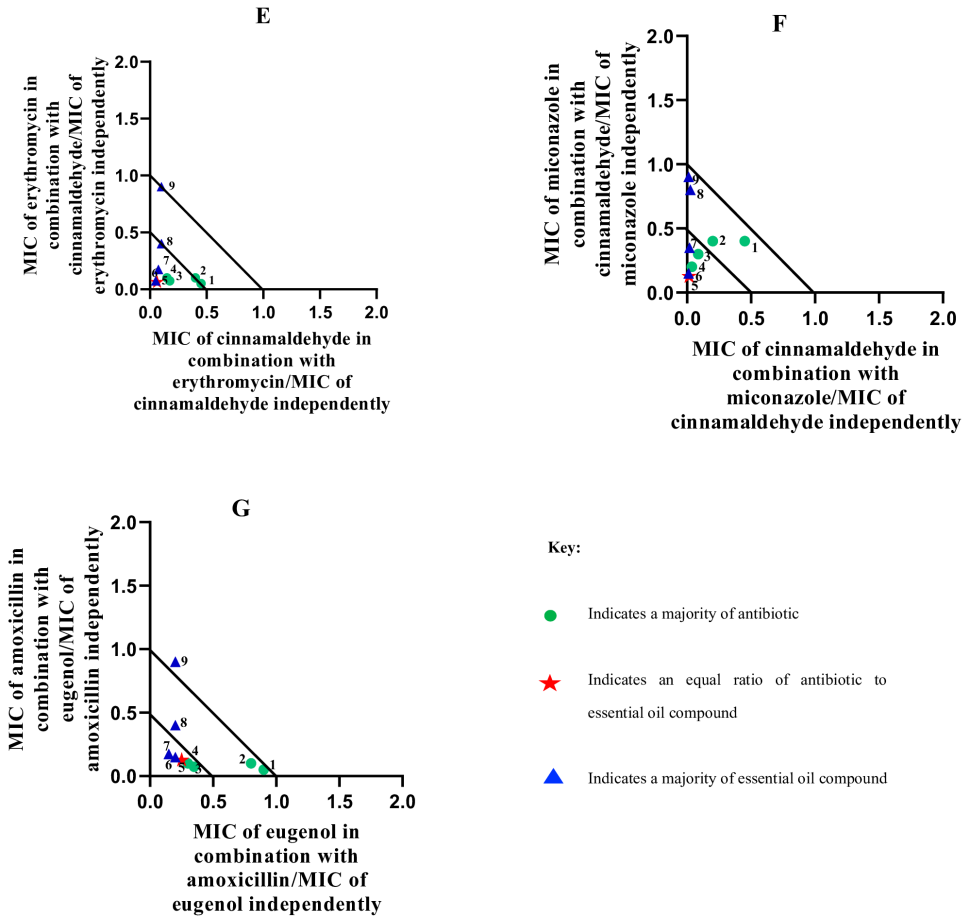

Figure 1. (**A**–**G**): Isobologram representation of synergistic interactions between different antibiotics and EOCs against Gram-positive bacteria. (**A**–**D**) was against *S. aureus* (ATCC 25923); (**E**,**F**) was against *S. epidermidis* (ATCC 12228); and (**G**) was against *C. acnes* (ATCC 11827).

Due to its high virulence potential and intrinsic resistance to several antibiotic classes [11,60], many studies have attempted to find combinations of EOCs and antibiotics against *P. aeruginosa*. However, only some have elucidated the precise synergistic combinations, with the majority being non-interactive [46]. A study by Miladinović et al. [61] demonstrated that geraniol and thymol were synergistic with tetracycline and chloramphenicol against *P. aeruginosa.* Recent studies [62,63] demonstrated synergistic interactions (ΣFIC 0.37–0.50) involving gentamicin and colistin in combination with cinnamaldehyde against the PA01 strain of *P. aeruginosa*. These antibiotics exhibit poor permeability through the pseudomonal outer membrane as well as being extruded out of the cell by efflux pumps [60]. From the results, the majority (17/24) of the combinations against *A. baumannii* were non-interactive (ΣFIC 1.07–2.20). However, gentamicin with eugenol (ΣFIC 0.62) and meropenem with eugenol (ΣFIC 0.64) demonstrated additivity.

Table 3. The FIC values of combinations of conventional antibiotics (A) and EOCs (B) against Gram-negative bacteria (n = 4).

Conventional Antibiotic	Essential Oil Compounds	*P. aeruginosa* (ATCC 27853)			*A. baumannii* (ATCC 19606)		
		FIC(A) [1]	FIC(B) [2]	ΣFIC [3]	FIC(A)	FIC(B)	ΣFIC
Ciprofloxacin	α-pinene	0.54	0.27	*0.81*	1.20	0.50	1.70
	γ-terpinene	0.54	0.29	*0.83*	1.20	0.50	1.70
	±Linalool	0.54	0.33	*0.87*	1.20	1.00	2.20
	Eugenol	0.27	0.33	*0.60*	0.60	0.67	1.27
	Carvacrol	0.27	0.67	*0.94*	0.60	1.00	1.60
	Cinnamaldehyde	0.07	0.25	**0.32**	0.30	1.00	1.30
Gentamicin	α-pinene	0.75	0.10	*0.85*	0.57	0.25	*0.82*
	γ-terpinene	1.51	0.21	1.72	1.14	0.50	1.64
	±Linalool	2.00	0.17	2.17	0.57	0.50	1.07
	Eugenol	1.00	0.33	1.33	0.29	0.33	*0.62*
	Carvacrol	0.50	0.33	*0.83*	0.57	1.00	1.57
	Cinnamaldehyde	1.00	1.00	2.00	0.29	1.00	1.29
Meropenem	α-pinene	2.00	0.53	2.53	1.04	0.25	1.29
	γ-terpinene	1.00	0.13	1.13	1.04	0.25	1.29
	±Linalool	1.00	0.17	1.17	1.04	0.50	1.54
	Eugenol	0.75	0.25	*1.00*	0.39	0.25	*0.64*
	Carvacrol	0.75	0.50	1.25	0.39	0.38	*0.77*
	Cinnamaldehyde	0.50	0.50	*1.00*	0.26	0.50	*0.76*
Tetracycline	α-pinene	0.33	0.53	*0.86*	0.40	0.25	*0.65*
	γ-terpinene	0.33	0.57	*0.90*	0.60	0.38	*0.98*
	±Linalool	0.33	0.67	*1.00*	0.80	1.00	1.80
	Eugenol	0.33	1.33	1.66	0.40	0.67	1.07
	Carvacrol	0.17	1.33	1.50	0.40	1.00	1.40
	Cinnamaldehyde	0.08	1.00	1.08	0.20	1.00	1.20

[1] FIC(A)—fractional inhibitory concentration of antibiotic; [2] FIC(B)—fractional inhibitory concentration of EOC; [3] ΣFIC—sum of FIC values; **bold** values indicate synergy, while italicized values indicate additivity.

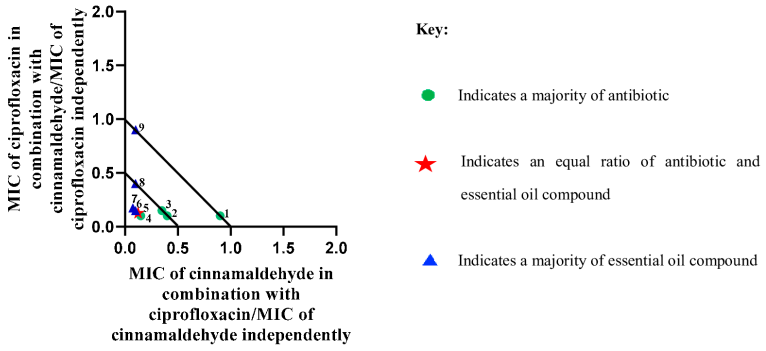

Figure 2. Isobologram represents synergistic interactions between ciprofloxacin and cinnamaldehyde against *P. aeruginosa* (ATCC 27853).

Previous studies investigating *A. baumannii* have used essential oils such as *Coriandrum sativum* L. (coriander) and *Origanum vulgare* L. (oregano) with conventional antibiotics, with some studies reporting that certain essential oils can increase the sensitivity of *A. baumannii*, resulting in synergy [64,65]. However, there are limited studies investigating EOCs against *A. baumannii* [66]. Karumathil et al. [67] demonstrated that triple combinations of cinnamaldehyde and eugenol with some beta-lactams enhanced the sensitivity of *A. baumannii* to these antibiotics, resulting in lower MIC values and reduced bacterial

cell counts. Aleksic Sabo et al. [68] reported that combinations of carvacrol, thymol, and eugenol with ciprofloxacin were synergistic (ΣFIC 0.11–0.50) against both reference and multi-drug resistant strains of *A. baumannii*, which differs from the results in this study, in which non-interactive interactions were observed (ΣFIC 1.27–1.60). A possible reason for this difference may be due to the solvent used in the MIC assays or the overall resistance profiles of the *A. baumannii* strains. There are limited studies investigating interactions between EOCs and conventional antibiotics against Gram-negative bacteria and fungi. Much of the focus has shifted to investigating how EOCs can attenuate virulence factors and resistance mechanisms [23,25,26]. Based on current therapies, many existing antibiotic strategies rely on combinations of an antibiotic with an adjuvant to target a resistance mechanism and "resensitize" the micro-organism to the antibiotic [16].

A well-known example of this is coupling beta-lactams with beta-lactamase inhibitors [69]. This strategy is often very effective and EOCs in combination with conventional antibiotics may be the best way to approach this type of combination therapy. Many studies published in the last five years have investigated the ability of certain EOCs to attenuate resistance mechanisms such as efflux pumps and beta-lactamase enzymes [67,70–73]. These studies have shown that EOCs can downregulate and even inactivate specific resistance mechanisms, re-establishing the antibiotic's effectiveness against the pathogen. Therefore, the results from this study support the concept that EOCs can be used as adjunctive agents alongside conventional antibiotics.

For *C. albicans*, 75% of the combinations (Table 4) were non-interactive (ΣFIC 1.02–2.13), whilst 25% were additive (ΣFIC 0.77–0.90). Eugenol showed the lowest overall ΣFIC values when combined with miconazole (0.87) and nystatin (0.77), whilst α-pinene had the highest ΣFIC values of 2.13 (miconazole) and 1.73 (nystatin). Eugenol exhibited noteworthy antifungal activity against various fungal species due to its ability to damage the fungal cell envelope and attenuate virulence factors [74]. Several studies have demonstrated synergistic interactions between eugenol and various antifungal agents, including fluconazole, micafungin, and amphotericin B, against *C. albicans* and related species [75–79]. These studies highlight the effectiveness of eugenol in combination with conventional antifungals against fungi. In this study, most of the interactions against *C. albicans* were non-interactive, which could be based on the fact that both the antifungal agent and the EOC may compete for the same molecular target [80]. Both nystatin and miconazole interact differently with the fungal cell membrane, which is also the primary molecular target for most EOCs [20,74]. Considering that EOCs can have multiple mechanisms of action which act sequentially and are not selective for a specific target site [48,80,81], the EOC may compete with the antifungal agent for the binding site and reduce the overall effectiveness of the combination.

Table 4. The FIC values of combinations of antifungals (A) and EOCs (B) against *C. albicans* (n = 4).

Antifungals	Essential Oil Compounds	*C. albicans* (ATCC 10231)		
		FIC(A) [1]	FIC(B) [2]	ΣFIC [3]
Miconazole	α-pinene	0.80	1.33	2.13
	γ-terpinene	0.80	0.80	1.60
	±Linalool	0.40	0.80	1.20
	Eugenol	0.20	0.67	*0.87*
	Carvacrol	0.15	0.75	*0.90*
	Cinnamaldehyde	0.05	1.00	1.05
Nystatin	α-pinene	0.40	1.33	1.73
	γ-terpinene	0.40	0.80	1.20
	±Linalool	0.20	1.33	1.53
	Eugenol	0.10	0.67	*0.77*
	Carvacrol	0.10	1.00	1.10
	Cinnamaldehyde	0.03	1.00	1.03

[1] FIC(A)—fractional inhibitory concentration of antibiotic; [2] FIC(B)—fractional inhibitory concentration of EOC; [3] ΣFIC—sum of FIC values; italicized values indicate additivity.

2.3. Toxicity Analysis

The toxicity of the conventional antimicrobials is presented in Table 5. Based on the results, none of the conventional antimicrobials were considered toxic (LC_{50} > 1000 µg/mL), which can be attributed to the selectivity of the antibiotics for prokaryotes as opposed to eukaryotes [82]. In addition, the negative controls did not affect the outcome of the assay.

The toxicity values of the EOCs expressed as LC_{50} after 24 h and 48 h of exposure are presented in Table 6. From the results for the 24 h of exposure, both α-pinene and γ-terpinene were considered non-toxic (LC_{50} > 1000 µg/mL), with the remaining four compounds (linalool, eugenol, carvacrol, and cinnamaldehyde) showing high toxicity (LC_{50} = 64.43–77.62 µg/mL). All EOCs showed a decrease in the LC_{50} after 48 h, except for linalool, which showed an increase (LC_{50} = 84.12 µg/mL) in toxicity, which may have been due to the pharmacokinetics and chemical stability of the compound [83].

Table 5. The mean percentage mortality and standard deviation (±SD) for the conventional antibiotics and controls (n = 3).

Conventional Antibiotics	Concentrations			
	0.01 mg/mL		0.05 mg/mL	
	24 h	48 h	24 h	48 h
Amoxicillin	0.00 ± 0.00	2.67 ± 3.06	0.00 ± 0.00	0.67 ± 1.15
Ciprofloxacin	0.00 ± 0.00	0.00 ± 0.00	1.67 ± 2.89	3.67 ± 6.35
Erythromycin	1.00 ± 2.31	5.67 ± 1.15	11.00 ± 7.94	15.67 ± 7.77
Gentamicin	0.00 ± 0.00	0.00 ± 0.00	2.67 ± 2.31	7.00 ± 2.64
Meropenem	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Tetracycline	1.00 ± 1.15	6.00 ± 5.29	5.67 ± 4.73	12.00 ± 3.61
Miconazole	0.00 ± 0.00	0.00 ± 0.00	7.67 ± 3.06	17.00 ± 1.73
Nystatin	3.00 ± 2.31	7.67 ± 3.79	5.33 ± 7.57	12.00 ± 7.94
Controls		**24 h**		**48 h**
Potassium dichromate (positive control)		100.00 ± 0.00		100.00 ± 0.00
2.00% DMSO (negative control)		3.00 ± 1.41		4.50 ± 0.71
Distilled water (negative control)		0.00 ± 0.00		0.00 ± 0.00
Saltwater (negative control)		1.93 ± 0.03		2.10 ± 0.01

Table 6. Lethal concentration (LC_{50}) with a 95% confidence interval (CI) of EOCs after 24 h and 48 h.

Essential Oil Compounds	LC_{50} (µg/mL) at 24 h	LC_{50} (µg/mL) at 48 h
α-pinene	>1000 [1] (>1000)	>1000 (>1000)
γ-terpinene	>1000 (>1000)	>1000 (>1000)
±Linalool	73.04 (71.24–74.85)	84.12 (82.23–86.01)
Eugenol	77.62 (75.41–79.83)	56.47 (54.80–58.14)
Carvacrol	64.43 (62.46–66.40)	37.14 (35.67–38.61)
Cinnamaldehyde	74.01 (71.91–76.11)	64.05 (62.26–65.84)

[1] The LC_{50} value represents the concentration of a test substance necessary to have a lethal effect on 50% of a brine shrimp sample. LC_{50} values < 249 µg/mL are considered highly toxic; 250–499 µg/mL are considered moderately toxic; 500–999 µg/mL are considered weak or low in toxicity; and ≥1000 µg/mL are considered non-toxic [84].

Carvacrol showed the highest increase in toxicity (LC_{50} = 37.14 µg/mL), with both α-pinene and γ-terpinene remaining non-toxic after 48 h. Previous studies have investigated the toxic effects of EOCs, with varying results based on the studied model [85,86]. Pattanasiri et al. [87] reported eugenol to cause 100.00% mortality in Siamese fighting fish at a concentration of 0.04 mg/mL, which supports the findings of this study, as 100.00% mortality of brine shrimp was observed at doses of 0.50 and 1.00 mg/mL. Youssefi et al. [88] demonstrated that carvacrol elicited a high toxicity in mosquito larvae with an LC_{50} = 15 µg/mL. Terpenoids such as eugenol and linalool are known for their high toxicity, whilst lower LC_{50} values are typically recorded for terpenes such as α-pinene and γ-terpinene [89].

The toxicity of the antimicrobial synergistic combinations is presented in Table 7. All the combinations showed weak/low toxicity (LC_{50} = 500–999 µg/mL) with the combination of ciprofloxacin and cinnamaldehyde being the least toxic (LC_{50} = 827.92 µg/mL) after 24 h, and the combination of gentamicin and carvacrol being the least toxic (LC_{50} = 696.07 µg/mL) after 48 h. A comparison of the toxicity of individual EOCs alone and when combined with conventional antibiotics shows a notable decrease in toxicity. This suggests that the conventional antibiotics used attenuate the toxicity of the EOCs. To the best of our knowledge, there have been minimal studies investigating the toxicity of combinations comprising conventional antibiotics and EOCs, supporting the novelty of this study.

Table 7. Lethal concentration (LC_{50}) with a 95% confidence interval (CI) of synergistic combinations after 24 h and 48 h.

Antimicrobial Synergistic Combination	LC_{50} (µg/mL) at 24 h	LC_{50} (µg/mL) at 48 h
Amoxicillin + Carvacrol	522.95 (520.99–524.91)	513.89 (511.77–516.01)
Gentamicin + Carvacrol	764.77 (762.61–766.94)	696.07 (693.85–698.29)
Miconazole + Carvacrol	707.03 (704.83–709.24)	657.45 (655.12–659.78)
Ciprofloxacin + Cinnamaldehyde	827.92 (825.85–829.99)	640.04 (638.25–641.83)
Erythromycin + Cinnamaldehyde	736.85 (734.84–738.86)	573.63 (571.86–575.40)
Miconazole + Cinnamaldehyde	704.23 (702.34–706.12)	636.05 (633.99–638.11)
Amoxicillin + Eugenol	806.43 (804.42–808.44)	628.81 (626.77–630.85)

2.4. Selectivity Index

The LC_{50} and SI for each synergistic ratio are presented in Table 8. Most of the ratios demonstrated a low toxicity (LC_{50} = 500–999 µg/mL), with the general trend being that increasing the amount of the EOC increased the toxicity. The combination of gentamicin and carvacrol at a ratio of 70:30, with the antibiotic making up the majority of the compound, was non-toxic, with an LC_{50} = 1025.32 µg/mL after 24 h. It showed a weak/low toxicity (LC_{50} = 931.44 µg/mL) after 48 h. In addition, the combination of amoxicillin and eugenol at a ratio of 70:30, with the antibiotic making up the majority of the compound, was also non-toxic, with an LC_{50} = 1081.17 µg/mL after 24 h and an LC_{50} = 843.04 µg/mL after 48 h.

For the SI values, the combination of amoxicillin and eugenol at a ratio of 70:30 demonstrated the highest SI of 14.41 at 24 h and 11.23 at 48 h. The lowest SI was noted for combinations comprising amoxicillin and carvacrol ranging from 0.31 to 1.05. Based on the results from Table 8, several combinations warrant further investigation, particularly against cell lines or a comparative model.

Table 8. Lethal concentration (LC_{50}) and selectivity index (SI) of synergistic ratios after 24 h and 48 h.

Synergistic Combinations	Pathogen	Synergistic Ratios (AB:EOC)	LC_{50} (µg/mL) of Combinations at Synergistic Ratios		SI of Combinations at Synergistic Ratios	
			24 h	48 h	24 h	48 h
Amoxicillin + Carvacrol	*S. aureus*	60:40	*612.03* [1]	*601.54*	1.53	1.50
		50:50	*522.95*	*513.89*	1.05	1.03
		40:60	433.87	426.24	0.87	0.85
		30:70	344.79	338.60	0.98	0.97
		20:80	255.71	250.95	0.32	0.31

Table 8. *Cont.*

Synergistic Combinations	Pathogen	Synergistic Ratios (AB:EOC)	LC_{50} (µg/mL) of Combinations at Synergistic Ratios		SI of Combinations at Synergistic Ratios	
			24 h	48 h	24 h	48 h
Gentamicin + Carvacrol	*S. aureus*	70:30	**1025.32** [2]	*931.44*	3.42	3.10
		60:40	*895.04*	*813.75*	***4.47*** [3]	***4.07***
		50:50	*764.77*	*696.07*	3.06	2.78
		40:60	*634.49*	*578.38*	2.11	1.93
		30:70	*504.21*	460.69	0.72	0.66
Miconazole + Carvacrol	*S. aureus*	60:40	*826.80*	*769.44*	2.07	1.92
		50:50	*707.03*	*657.45*	2.83	2.63
		40:60	*587.25*	*545.45*	1.96	1.82
		30:70	467.48	433.46	1.34	1.24
Miconazole + Cinnamaldehyde	*S. aureus*, *S. epidermidis*	70:30	*944.15*	*852.75*	3.14	2.84
		60:40	*824.19*	*744.40*	***4.12***	3.72
		50:50	*704.23*	*636.05*	***5.63***	***5.09***
		40:60	*584.27*	*527.70*	3.89	3.52
		30:70	464.31	419.35	1.32	1.20
Ciprofloxacin + Cinnamaldehyde	*P. aeruginosa*	60:40	*968.95*	*749.06*	***9.68***	***7.49***
		50:50	*827.92*	*640.04*	***6.62***	***5.12***
		40:60	*686.88*	*531.01*	***4.58***	3.54
		30:70	*545.85*	421.98	3.12	2.41
		20:80	404.81	312.95	1.01	0.78
Erythromycin + Cinnamaldehyde	*S. epidermidis*	70:30	987.89	769.06	6.58	5.12
		60:40	*862.37*	*671.34*	***4.31***	3.36
		50:50	*736.85*	*573.63*	***5.89***	***4.59***
		40:60	*611.33*	475.92	***4.07***	3.17
		30:70	485.81	378.20	1.39	1.08
Amoxicillin + Eugenol	*C. acnes*	70:30	**1081.17**	843.04	***14.41***	***11.23***
		60:40	*943.80*	*735.93*	***9.43***	***7.36***
		50:50	*806.43*	*628.81*	***6.45***	***5.03***
		40:60	*669.05*	*521.69*	***4.46***	3.48
		30:70	*531.68*	414.58	3.04	2.37

[1] Italicized values indicate a weak or low toxicity; [2] bold values indicate non-toxic concentrations; and [3] bold and italicized values indicate SI ≥ 4.

3. Materials and Methods

The overall experimental design can be found in Figure S1 (Supplementary Materials).

3.1. Preparation of Cultures

Due to their prevalence in skin infections, the following six American Type Culture Collection (ATCC) reference strains were included in this study: *Staphylococcus aureus* (ATCC 25923), *Staphylococcus epidermidis* (ATCC 12228), *Acinetobacter baumannii* (ATCC 19606), *Pseudomonas aeruginosa* (ATCC 27853), *Cutibacterium acnes* (ATCC 11827), and *Candida albicans* (ATCC 10231). All the bacteria were cultured in Tryptone Soya broth (TSB) (Oxoid) and incubated at 37 °C for 24 h, except for *C. acnes*, which was inoculated into Thioglycolate broth (TGB) (Oxoid) and incubated for seven days under anaerobic conditions (5.00% CO_2) at 37 °C. *Candida albicans* was cultured in TSB and incubated at 37 °C for 48 h. In addition, streak plates were prepared to ensure purity.

3.2. Antimicrobial Agents and Essential Oil Compounds

Based on their use in skin infections, the following antibiotics/antifungals (Sigma-Aldrich, St. Louis, MO, USA) were included; amoxicillin (potency ≥ 90.0%), ciprofloxacin (≥98.0%), erythromycin (≥85.0%), tetracycline (≥98.0%), gentamicin (≤100%), meropenem (≥95.0%), miconazole (99.5%), and nystatin (≥95.0%). The antibiotics were only tested against the microorganisms in which direct antibacterial activity had been noted. Miconazole was also tested against Gram-positive bacteria due to its known antibacterial activity [41,90]. The antibiotic/antifungal stocks were prepared as outlined by the Clinical and Laboratory Standards

Institute (CLSI) [91]. Stock solutions (0.01 mg/mL and 0.05 mg/mL) were stored at −20 °C, and working stocks were stored at 4 °C.

Based on the previous literature which has documented EOCs' antimicrobial properties [28,33,51], the following EOCs (Sigma-Aldrich, St. Louis, MO, USA) were selected: α-pinene, γ-terpinene, ±Linalool, eugenol, carvacrol, and cinnamaldehyde. For the combinations, the compounds were only combined with antibiotics/antifungals that had demonstrated direct antimicrobial activity against the tested organism. All the compounds had a purity range of 95.00–99.00%. The compounds were stored at 4 °C and prepared to a starting concentration of 32.00 mg/mL.

3.3. Minimum Inhibitory Concentration (MIC)

The broth microdilution assay (MIC) was used to evaluate the antimicrobial activity of the conventional antimicrobials and the selected EOCs independently and in combination [92]. Briefly, each well of a 96-well microtiter plate was filled with 100 µL of their respective sterile broth, followed by adding 100 µL of the antibiotic/antifungal or EOC into the top row. For the combinations, 50 µL of each antibiotic/antifungal and 50 µL of each EOC were added so that the ratio of antibiotic/antifungal to the EOC was 1:1. Serial doubling dilutions were then performed. The prepared microtiter plates were then inoculated with 100 µL of the relevant pathogen at colony-forming units (CFU) of approximately 1×10^6 (CFU/mL). The plates were then sealed with sterile adhesive to ensure no sample loss since EOCs are volatile. The plates were then incubated at their respective temperature and times. After incubation, 40.00 µL of a 0.40% *p*-iodonitrotetrazolium (INT) violet indicator solution was added to all the inoculated wells. A change in the colour of the wells from clear to pink or red was used to indicate the presence of microbial growth [93]. The MIC values were interpreted as the lowest concentration at which growth was inhibited. Each combination was performed in quadruplicate. Three controls were included: a culture control for the broth corresponding to the sample, a solvent control, and a conventional antimicrobial control to ensure susceptibility. The mean and standard deviations (±SD) for the MICs were calculated in MS Office (2016).

3.4. Interactive Profiles

The interactions between the combinations of the antibiotics and compounds were classified according to their fractional inhibitory concentration (FIC) (Equation (1)).

$$\text{FIC (I)} = \frac{\text{(A) combined with (B)}}{\text{(A) independently}} \quad \text{FIC (II)} = \frac{\text{(B) combined with (A)}}{\text{(B) independently}} \tag{1}$$

where (A) is the MIC of the essential oil compound, and (B) is the MIC of the antibiotic.

From these values, the ΣFIC was calculated following Equation (2):

$$\Sigma\text{FIC} = \text{FIC (I)} + \text{FIC (II)} \tag{2}$$

The ΣFIC for each EO compound combined with an antibiotic was interpreted as follows; an ΣFIC value of $\leq$0.5 was indicative of synergy; an ΣFIC value of >0.5–1.0 indicated an additive interaction; an ΣFIC of >1.0–$\leq$ 4.0 indicated non-interactive; and an ΣFIC value of >4.0 indicated antagonism [94].

3.5. Varied Ratio Combinations

For notable combinations demonstrating synergistic interactions in the 1:1 ΣFIC analysis, a further study was undertaken during which combinations were investigated at varied ratios of 90:10, 80:20, 70:30, 60:40, 50:50, 40:60, 30:70, 20:80, and 10:90. The data points for each ratio were then plotted on an isobologram using the GraphPad Prism® software (Version 9). Synergy was displayed for the data points closest to the apex and falling beneath or on the 0.5:0.5 line. Additive interactions referred to the data points between the

0.5:0.5 and the 1:1 lines. Non-interactive effects were those that were between the 1:1 and the 4:4 lines and antagonism was displayed for the data points above or on the 4:4 line [94].

3.6. Toxicity Studies

The brine shrimp lethality assay was used to determine the toxicity of the EOCs, antibiotics, and combinations demonstrating synergy [95]. An amount of 0.50 g of dried brine shrimp (*Artemia franciscana*) eggs (Ocean Nutrition) was weighed out and added to artificial seawater (16.00 g of sea salt (TropicMarine) in 500.00 mL of distilled water). A rotary pump was used to aerate the sea water to increase the hatch rate. The eggs were left for 48 h under a light source to allow the brine shrimp to hatch. A 48-well microtiter plate was prepared for the assay by adding 400.00 µL of saltwater containing 40–60 live brine shrimp to each well. A volume of 400.00 µL of either the EOC or antibiotic was added to each well. For the synergistic combinations, 200.00 µL of each sample (antibiotic/EOC) was mixed prior to adding to the wells. Before adding the samples, each well was examined using a light microscope (Olympus, 40× magnification) to check the viability of the brine shrimp. Six concentrations (2.0, 1.0, 0.5, 0.25, 0.125, and 0.06125 mg/mL) of each EOC were prepared using 2.00% DMSO and diluted in the well to achieve a 1:2 final concentration. All the antibiotic samples were prepared to a 0.1 mg/mL concentration using sterile distilled water. At 24 h and 48 h, the dead shrimp were viewed and counted. A lethal dose of 50 µL acetic acid (Saarchem) was added to each well. Thereafter, the percentage of mortality was calculated, and a percentage of 50% mortality or greater was considered biologically toxic [84]. The assay included a negative, non-toxic control of 32.00 g/L of artificial sea water to ensure the promotion of growth and the survival of the brine shrimp. The positive (toxic) control in the assay consisted of a 1.60 mg/mL potassium dichromate solution. The brine shrimp mortality was plotted against the logarithms of the concentrations using the Probit analysis tool in the IBM SPSS Statistics software (Version 27). The median lethal concentration (LC_{50}) at 95% confidence intervals (CI) was calculated [96].

3.7. Selectivity Index (SI)

The SI can be defined as the ratio of the toxic concentration of a sample against its effective bio-active concentration [36]. The SI represents the pre-clinical screening calculation used to determine the feasibility of novel compounds for in vivo testing [82]. When evaluating the SI, a cut-off value of $\geq$4 was used [97,98]. The SI was calculated using Equation (3) for each of the synergistic combinations.

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

* where the LC_{50} is the lethal concentration required to cause 50% mortality after 24 h and 48 h, and the MIC is the minimum inhibitory concentration of the antimicrobial synergistic combination.

4. Conclusions

This study investigated the emerging strategy of combining essential oil compounds with conventional antibiotics against six reference skin pathogens. Based on the findings, 48.39% of combinations were additive, 47.31% were non-interactive, and 4.30% were synergistic, with no antagonism observed. In addition, eight synergistic interactions were identified, seven being effective against Gram-positive bacteria and one against *P. aeruginosa*. Two synergistic interactions involving miconazole with two different EOCs were identified against *S. aureus* and *S. epidermidis*, which was the first to be reported. Furthermore, this study showed that synergistic interactions can exist at varying ratios for combinations of EOCs and conventional antibiotics. For *C. albicans*, it may be worth investigating combinations of different antifungals and EOCs at varying ratios to elucidate the potency of eugenol at different concentrations. Although the individual EOCs have high inherent toxicities, the overall toxicity can be reduced when combined with conventional antimicrobials. In addition, the combination of amoxicillin

and eugenol demonstrated an SI of 14.41, which warrants further investigation. Therefore, it can be proposed that some essential oil compounds enhance the antimicrobial efficacy of some conventional antibiotics.

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Abbreviations

EO	Essential oil
EOC	Essential oil compound
FIC	Fractional inhibitory concentration
LC	Lethal concentration
SI	Selectivity index

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Supplementary Data

Table S1 Synergistic interactions against *S. aureus*

Isobologram plot number	Ratio volumes (µl) (Antibiotic: EOC)	Amoxicillin + Carvacrol		Gentamicin + Carvacrol		Miconazole + Carvacrol		Miconazole + Cinnamaldehyde	
		MIC of antibiotic in combination (µg/mL)	MIC of EOC in combination (µg/mL)	MIC of antibiotic in combination (µg/mL)	MIC of EOC in combination (µg/mL)	MIC of antibiotic in combination (µg/mL)	MIC of EOC in combination (µg/mL)	MIC of antibiotic in combination (µg/mL)	MIC of EOC in combination (µg/mL)
1	90:10	1.13	400	1.13	400	1.13	400	1.13	400
2	80:20	0.50	400	0.50	400	0.50	400	0.50	400
3	70:30	0.44	600	0.22	300	0.22	300	0.22	300
4	60:40	0.19	400	0.09	200	0.19	400	0.09	200
5	50:50	0.16	500	0.08	250	0.08	250	0.04	125
6	40:60	0.06	500	0.06	300	0.06	300	0.03	150
7	30:70	0.05	350	0.09	700	0.05	350	0.05	350
8	20:80	0.06	800	0.13	1600	0.06	800	0.03	400
9	10:90	0.06	1800	0.06	1800	0.03	900	0.02	450

Table S2 Synergistic interactions against *S. epidermidis*, *C. acnes* and *P. aeruginosa*

Isobologram plot number	Ratio volumes (µl) (Antibiotic: EOC)	*S. epidermidis* (ATCC 12228)				*C. acnes* (ATCC 11827)		*P. aeruginosa* (ATCC 27853)	
		Erythromycin + Cinnamaldehyde		Miconazole + Cinnamaldehyde		Amoxicillin + Eugenol		Ciprofloxacin + Cinnamaldehyde	
		MIC of antibiotic in combination (µg/mL)	MIC of EOC in combination (µg/mL)	MIC of antibiotic in combination (µg/mL)	MIC of EOC in combination (µg/mL)	MIC of antibiotic in combination (µg/mL)	MIC of EOC in combination (µg/mL)	MIC of antibiotic in combination (µg/mL)	MIC of EOC in combination (µg/mL)
1	90:10	0.28	100	1.13	400	0.14	50	0.29	100
2	80:20	0.25	200	0.50	400	0.13	100	0.13	100
3	70:30	0.11	150	0.22	300	0.05	75	0.11	150
4	60:40	0.09	200	0.09	200	0.05	100	0.05	100
5	50:50	0.04	125	0.04	125	0.04	125	0.04	125
6	40:60	0.03	150	0.03	150	0.03	150	0.03	150
7	30:70	0.05	350	0.05	350	0.02	175	0.02	175
8	20:80	0.06	800	0.06	800	0.03	400	0.03	400
9	10:90	0.06	1800	0.03	900	0.03	900	0.03	900

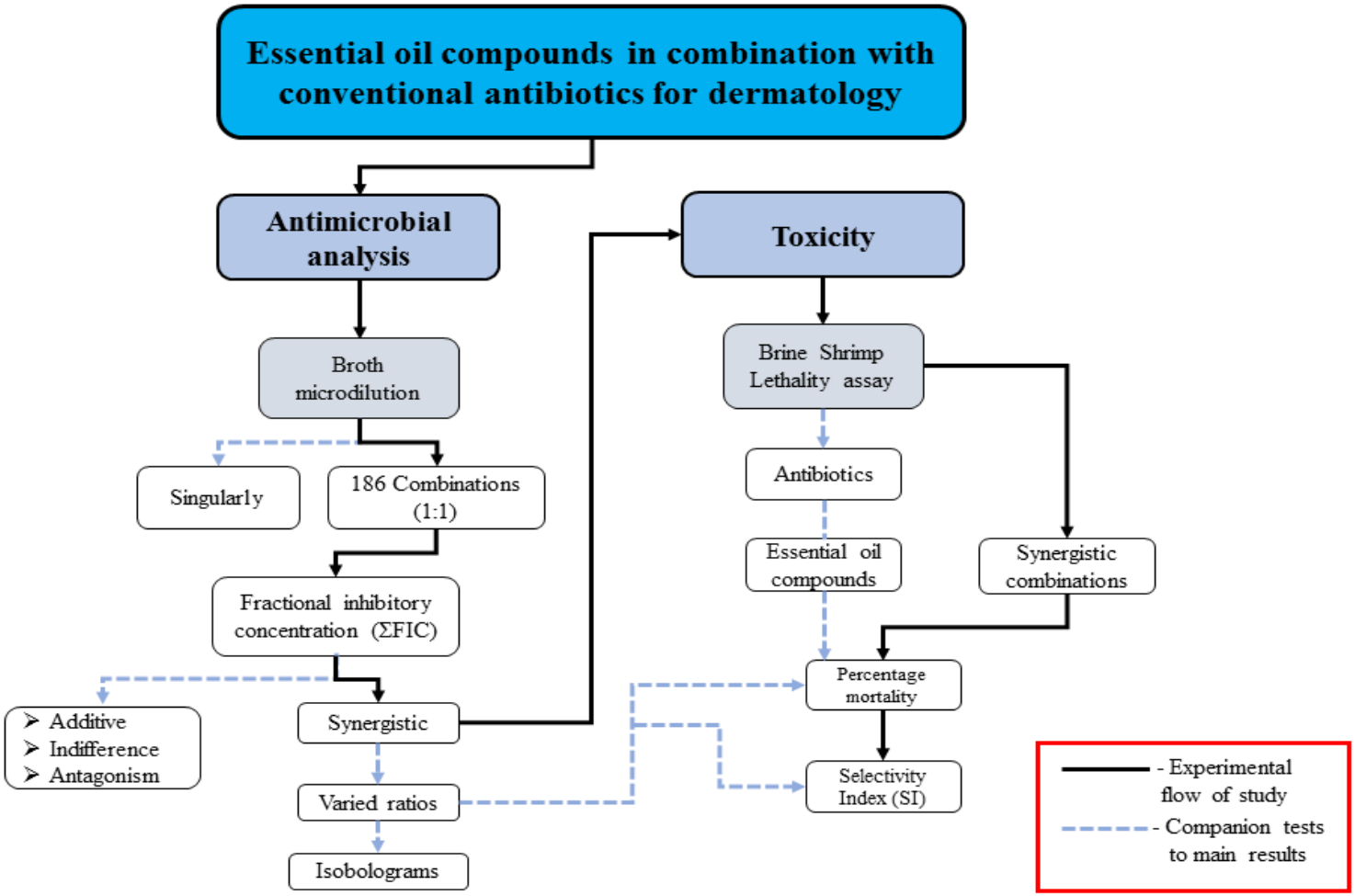
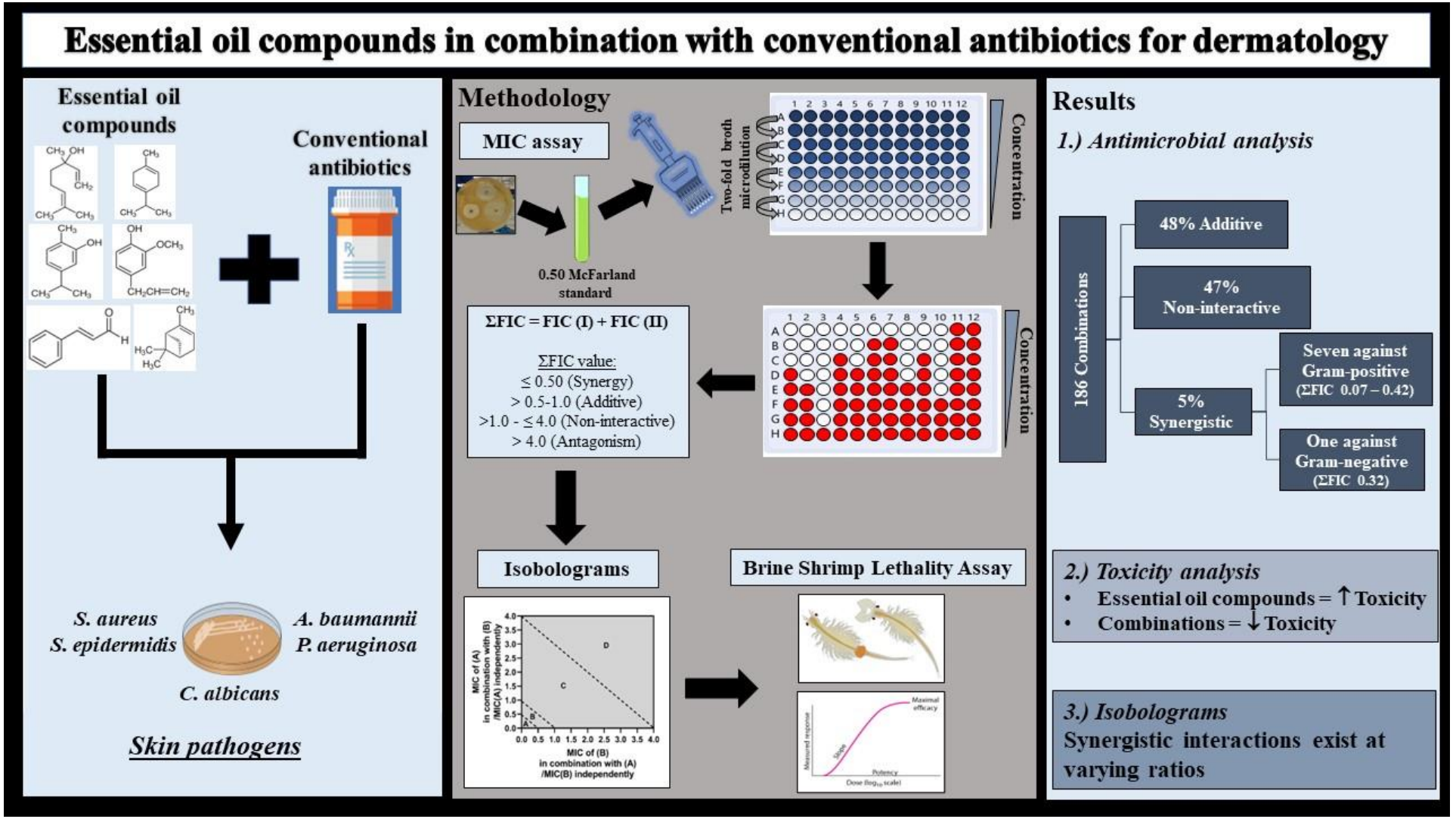


Figure S1: Study design

Graphical abstract: Publication one



CHAPTER 4: PUBLICATION TWO

Cytotoxicity and anti-inflammatory properties of essential oil compounds in combination with conventional antimicrobials for skin infections

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Abstract

Background: Skin inflammation is a common dermatological concern that arises from infections. Essential oil compounds have garnered interest due to their potent anti-inflammatory properties. Preliminary studies have shown antimicrobial synergistic interactions between essential oil compounds and conventional antimicrobials with limited evidence investigating the anti-inflammatory potential.

Objectives: We investigated the cytotoxicity and anti-inflammatory properties of six essential oil compounds in combination with eight conventional antimicrobials.

Methods: HaCAT keratinocytes were used to determine the cytotoxicity using the MTT assay whilst the anti-inflammatory properties were elucidated using Lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophages. The interactions of the combinations were analysed using combination index (toxicity) and fractional effect (anti-inflammatory).

Results: The results showed that the toxicity of the essential oil compounds was dose-dependent with low doses showing high cell viability and high doses showing low cell viability.

Cinnamaldehyde demonstrated the highest toxicity (IC_{50} = 28.63 µg/ml, $p < 0.05$) and the greatest reduction (77.44%) in nitrite production which was also concentration dependent. The combination of ciprofloxacin and cinnamaldehyde demonstrated the lowest toxicity (88.42% ± 3.72 cell viability; combination index of 0.12) and the highest reduction in NO production (78.42%; ΣFa = 0.44).

Conclusion: This study highlights the potential of essential oil compounds as possible adjunctive agents that can bestow anti-inflammatory effects when combined with conventional antimicrobials.

Keywords: antibiotics, essential oils, dermatology, nitric oxide, cinnamaldehyde, combination index

Abbreviations: EO, Essential oil; EOC, Essential oil compound; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

1. Introduction

The skin is the largest organ in the human body and functions as the primary barrier against biological invaders, which also makes it the main target for infections [1]. A skin infection can be characterized as a microbial invasion of the cutaneous layers and underlying tissue due to breakage or damage to the epidermis [2]. Upon entry into the wound, the microbial invaders are detected by resident skin cells such as keratinocytes and macrophages which initiate a cascade of biochemical reactions, that stimulate the immune system resulting in an inflammatory response [3]. The inflammatory process can be defined as a protective strategy in response to a foreign stimulus such as microbial infection or tissue damage [4]. The main aim of inflammation is to reinstate cellular homeostasis by removing or sequestering the source of the disturbance which can be considered an "adaptive response" [5]. The duration of inflammatory responses varies depending on the level of damage caused by the infection [6]. Most skin infections induce systemic effects through the excessive production of inflammatory cytokines, which subsequently mediate the secretion of acute phase proteins such as C-reactive protein [7]. This facilitates the production of prostaglandins resulting in the onset of common inflammatory symptoms such as pain, fever, redness, and swelling [8]. Although inflammation plays an integral part in wound healing, the sustained presence of pro-inflammatory mediators can be detrimental to the host [9]. This is particularly common in cutaneous infections caused

by multidrug-resistant (MDR) pathogens [10].

Antibiotic therapy remains the cornerstone treatment for all skin infections [11]. However, the dissemination of antimicrobial resistance amongst various microbial populations has escalated the occurrence of complicated skin infections [12]. A complex relationship exists between inflammation and skin infections caused by antibiotic-resistant pathogens. Micro-organisms that are resistant to antibiotics cannot be cleared from the site of the infection which induces a heightened and prolonged inflammatory response [13]. This results in an uncontrolled upregulation of both pro- and anti-inflammatory pathways, which causes a variety of detrimental effects such as progressive tissue damage, secondary infections, and even sepsis [14]. Most wound healing treatment guidelines have based the choice of antibiotic on the most probable pathogen, patient co-morbidities, and severity of the infection [15], with additional adjunctive therapy aimed at treating systemic effects such as pain and inflammation [16].

Several antibiotic classes such as macrolides and tetracyclines have been widely documented to affect inflammatory outputs that also contribute to their therapeutic benefits [17, 18]. However, the injudicious use of these antibiotic classes has contributed to widespread resistance thus prompting additional treatments such as corticosteroids to taper inflammatory responses [12]. Despite progress in the development of innovative therapeutic approaches, such as immunotherapy or drug repurposing, there are still no new adjunctive measures for which antimicrobial therapies may exhibit a polypharmacological effect [14, 19]. Therefore, the investigation of novel therapeutics that can elicit both antimicrobial and anti-inflammatory effects is warranted, thus prompting a renewed interest in phytomolecules.

Essential oils (EOs) are complex natural mixtures of volatile compounds that are produced by plants as secondary metabolites [20, 21]. Essential oils can be comprised of over 300 different chemical compounds that are mainly terpenes, terpenoids, and non-terpenic compounds called phenylpropanoids [22]. These essential oil compounds (EOCs) are responsible for different pharmacological profiles of essential oils [23]. In the past decades, the therapeutic potential of EOs and their compounds has been investigated extensively with particular interest in the antimicrobial, and anti-inflammatory properties [21, 24]. The anti-inflammatory properties of EOCs are well studied and have been demonstrated in both *in vitro* and *in vivo* models [25]. Several review articles have discussed the various direct and indirect anti-inflammatory mechanisms of some EOCs [7, 26] with no studies investigating the combinational anti-inflammatory effects of EOCs with conventional antimicrobials.

Many EOCs have been used as complementary medicines and natural antimicrobial agents, which are relatively safe when at low concentrations [27]. At high concentrations,

individual EOCs can elicit high inherent toxicity due to their lipophilic nature which can cause dermatitis thus limiting their use in topical therapies [28]. A few reviews have discussed various methods that can reduce the toxicity of individual EOCs [23, 29] with limited studies investigating the cytotoxic interactions of these combinations.

In our previous study [30], we investigated the effects of combining six essential oil compounds with eight conventional antimicrobials against skin pathogens. From this investigation, we identified seven combinations (EOC with conventional antimicrobials) that are antimicrobially synergistic against several reference strains of bacteria. Therefore, this study aimed to evaluate the pharmacological effects (cytotoxicity and anti-inflammatory properties) of these combinations.

2. Materials and methods

2.1. Preparation of samples

Based on their use in skin infections [31, 32], eight conventional antimicrobials (Sigma-Aldrich, St. Louis, MO, USA) were selected; amoxicillin (potency ≥ 90.0%), ciprofloxacin (≥ 98.0%), erythromycin (≥ 85.0%), tetracycline (≥ 98.0%), gentamicin (≤ 100%), meropenem (≥ 95.0%), miconazole (>99.5%) and nystatin (≥ 95.0%). Six EOCs (α-pinene, γ-terpinene, ± linalool, eugenol, carvacrol, and cinnamaldehyde (Sigma-Aldrich, St. Louis, MO, USA) were selected based on their anti-inflammatory properties [26, 33]. Selections were also based on the antimicrobial synergistic outcomes when tested in combination [30]. Both the conventional antimicrobials and EOCs were dissolved in DMSO (1.25%) and made to a stock concentration of 12.5 µg/mL and 32 mg/mL.

2.2. The MTT- cytotoxicity assay

The cytotoxicity of the EOCs, conventional antimicrobials, and combinations was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The procedure [34] was undertaken with HaCAT cells (Cellonex, South Africa) which were cultured in Dulbecco's Modified Eagle Medium (DMEM) (GE Healthcare Life Sciences, USA) and supplemented with 10.00% (v/v) FBS (GE Healthcare Life Sciences, USA) in a humidified, 5.00% CO_2 incubator at 37°C. The cells were then seeded into 96-well microtiter plates at a density of 4000 cells per well, using a volume of 100 µL in each well. The microtiter

plates were incubated for 24 h before the addition of the test samples, to allow for cell attachment. The cells were treated at three different concentrations (3.13, 6.25, and 12.5 µg/mL) for the conventional antimicrobials and five concentrations (15.6, 31.2, 62.5, 125.0, and 250.0 µg/mL) for the EOCs. These concentrations were selected as a preliminary screening to determine the concentrations that could be used for the anti-inflammatory assay. Melphalan (100 µM) (Sigma Aldrich, St. Louis, MO, USA) which is a known cytotoxic agent was used as a positive control. Each sample was tested in quadruplicate (n = 4). The cells were then incubated for 48 h. After incubation, the remaining media in each well was replaced with 0.5 mg/mL MTT and further incubated for 30 min at 37˚C. Thereafter, the MTT was removed and 100 µL of DMSO was added to each well to solubilize the formazan crystals. The absorbance was read at 540 nm using a BioTek® PowerWave XS spectrophotometer (Winooski, VT, USA). The cytotoxicity was interpreted as % cell viability.

2.3. Hoechst and phalloidin staining

The HaCAT cells were treated as described for the MTT assay and stained with phalloidin-TRITC as outlined by [35]. The cells were fixed in formaldehyde (37%, 20 µL/well) for 15 min at ambient temperature and washed three times with 200 µL Dulbecco's phosphate buffered saline (DPBS) per well. The cells' filamentous actin cytoskeletons were stained with phalloidin-TRITC (Sigma-Aldrich, St. Louis, MO, USA) (1 µM; 100 µL) in the dark for 15 min at 37 °C, followed by washing (two times) with DPBS. The nuclei were stained with Hoechst 33342 (Sigma-Aldrich, St. Louis, MO, USA) (5 µg/mL, 100 µL), and images were taken with a Molecular Devices ImageXpress® Micro XLS widefield microscope (CA, USA) using the TRITC and DAPI filters, and 10x objective.

2.4. The anti-inflammatory assay

The anti-inflammatory activity of the EOCs, conventional antimicrobials, and combinations were assessed by measuring the inhibition of nitric oxide (NO) using the lipopolysaccharide-nitric oxide (LPS-NO) induced assay. Lipopolysaccharide (LPS) induces the expression of inducible nitric oxide synthase (iNOS) in RAW264.7 macrophages resulting in the production of nitric oxide which stimulates an inflammatory response [34]. Briefly, RAW 264.7 cells (Cellonex, South Africa) were cultured in RPMI1640 medium (GE

Healthcare Life Sciences, USA) supplemented with 10.00% fetal calf serum in a humidified 5.00% CO_2 incubator at 37°C. Cells were seeded into 96-well plates at a density of 1.50 x 10^5 cells per well and allowed to attach overnight. The concentrations of the EOCs and combinations were based on the results from the preliminary MTT assay. Three different concentrations were used for both the EOCs (12.5, 25.0, and 50.0 µg/mL) and conventional antimicrobials (0.03, 0.31, and 3.13 µg/mL). The spent culture medium was replaced with 50 µL of the samples or complete medium only (Untreated cells and LPS control). Aminoguanidine (AG) (Sigma-Aldrich, St. Louis, MO, USA) was used as the positive control since it is a direct inhibitor of iNOS. To assess the anti-inflammatory activity, 50 µL of 1 µg/mL LPS (Sigma-Aldrich, St. Louis, MO, USA) in RPMI complete medium was added to all wells. The final concentration for the LPS control was 500 ng/mL and 100 µM for AG. The cells were incubated for a further 24 h. To quantify the NO production, 50 µL of the spent culture medium was transferred into a new 96-well plate and 50 µL Griess reagent (Sigma-Aldrich, St. Louis, MO, USA) was added. The samples were then incubated at ambient temperature for 10 min before absorbance was measured at 540 nm. A standard curve using sodium nitrite dissolved in culture medium was used to determine the concentration of NO in each sample. To confirm the absence of toxicity as a contributory factor, cell viability was assessed using the MTT assay against RAW 264.7 murine macrophages remaining in the 96 well culture plates (Section 2.2).

2.5. Combination index (CI)

The CI provides a quantitative measure (Equations 1 and 2) to determine the type of interactions between two drugs [36]. The method adopts the median-effect equation which was derived by merging the principle of the mass-action law with the mathematical principle of induction and deduction [37, 38].

$$CI = [(D)_1/(D_x)_1 + (D)_2/(D_x)_2] \quad - \quad \textbf{(Equation 1)}$$

*Where $(D)_1$ and $(D)_2$ denote the dose of each drug in combination necessary to produce the same effect, and $(D_x)_1$ and $(D_x)_2$ denote the individual dose of each drug required to inhibit a given level of cell growth.

This can be determined by:

$$D_x = D_m\,[F_a/(1/F_a)]1/m \quad \text{-} \quad \textbf{(Equation 2)}$$

*Where D_m refers to the median effect dose (Potency of EOC/conventional antimicrobial expressed as IC_{50}), m is the slope of the graph, and F_a denotes the fraction effect ($F_a = 0$ represents 100% cell viability and $F_a = 1.00$ represents 0% cell viability) in dose-effect curves. The CI values define the types of interactions and can be interpreted as $CI < 0.80$ indicates synergism, $CI = \leq 0.80 – 1.20$ indicates an additive effect, and $CI > 1.20$ indicates antagonism [39].

2.6. Interactive profiles

The fractional-effect (Fa) analysis refers to the index for calculating the expected dose-response relationship for drug combination therapy [38]. This type of analysis assumes that the two inhibitors of a given pharmacological target act via independent mechanisms [40]. In our previous study [30] we used this method to classify different antimicrobial interactions between combinations of conventional antimicrobials and EOCs. Here the method was used to interpret the anti-inflammatory interaction of the combinations.

$$\text{Fa (I)} = \frac{(A^*) \text{ combined with (B)}}{\text{(A) independently}} \qquad \text{Fa (II)} = \frac{(B^*) \text{ combined with (A)}}{\text{(B) independently}} \quad \text{-} \quad \textbf{(Equation 3)}$$

* Where (A) is the NO produced by the EOC alone and (B) is the NO produced by the conventional antimicrobial alone. From these values, the ΣFa index was calculated as (Equation 4):

$$\Sigma Fa = Fa\,(I) + Fa\,(II) \quad \text{-} \quad \textbf{(Equation 4)}$$

The ΣFa for each combination was interpreted as follows: $\Sigma Fa < 0.80$ indicates synergism, $\Sigma Fa \leq 0.80 – 1.20$ indicates an additive effect, and $\Sigma Fa > 1.20$ indicates antagonism [39].

2.7. Statistical analysis

The data analysis which included the calculation of the mean, standard deviation (±SD) and IC_{50} was done using GraphPad Prism® software version 9.0 (Graph Pad Prism Software, CA, USA). The Student t-test was used to determine whether data groups differed significantly from each other. Statistical significance was defined as having *P* values < 0.05. An ANOVA post hoc (Tukey) test was done to calculate the statistical significance of two or more groups.

3. Results

3.1. Cytotoxicity effects of the conventional antimicrobials

The results from the cytotoxicity assay for the conventional antimicrobials are presented in Fig. 1.

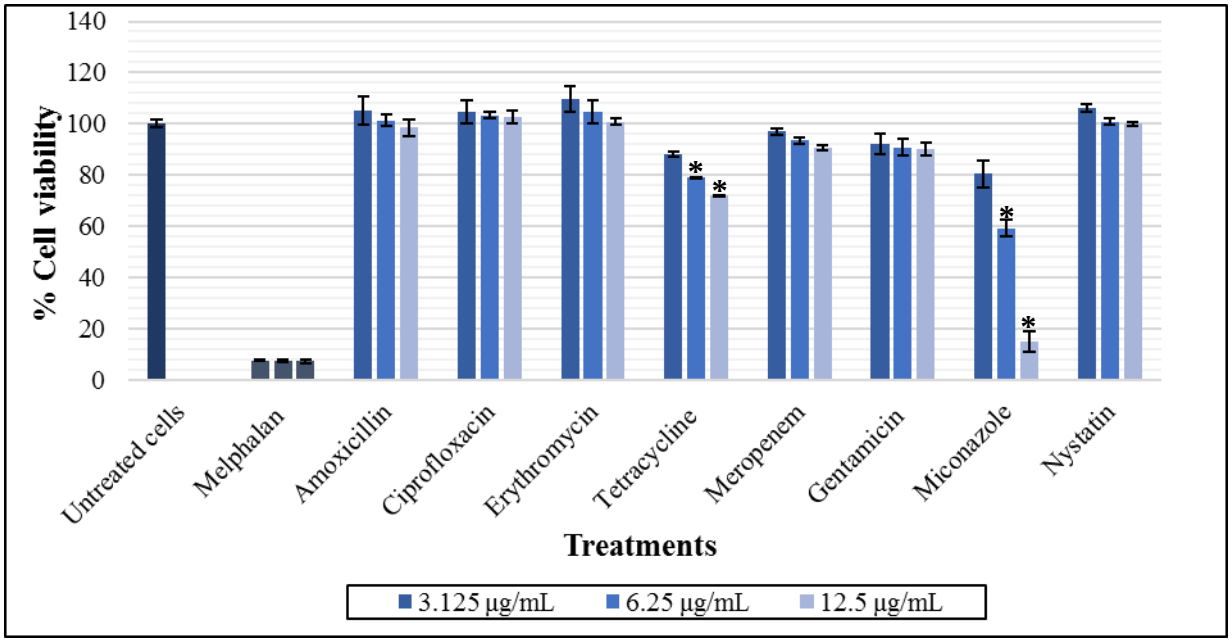


Fig. 1. Cell viability of the conventional antimicrobials and treatment controls on HaCAT keratinocytes. Melphalan was used as the positive control. Error bars represent mean ± SD. *****($p < 0.05$) indicates statistical significance when compared to the control (Untreated cells).

Most of the conventional antimicrobials demonstrated high cell viability apart from tetracycline and miconazole. For tetracycline, concentrations of 6.25 µg/mL ($p = 0.04$) and 12.5 µg/mL ($p = 0.004$) were statistically significant ($p < 0.05$). Miconazole demonstrated the

lowest overall cell viabilities, with concentrations of 6.25 µg/mL (p = 0.03) and 12.5 µg/mL (p = 0.001) being statistically significant. Melphalan demonstrated low cell viability which was expected considering it is an anticancer agent.

Fig. 2. (A-E) shows the effects of the different treatments on HaCAT cell density. The conventional antimicrobials demonstrated high cell density (Fig. 2C – ciprofloxacin) except for miconazole (Fig. 2E). A general trend that was noted was increasing the concentration of conventional antimicrobials resulted in decreased cell density. A study by Lam, et al. [41] demonstrated similar results for miconazole against HaCAT keratinocytes and showed that at concentrations of 12.5 µg/mL, miconazole induces an increased amount of reactive oxygen species (ROS) causing cell death.

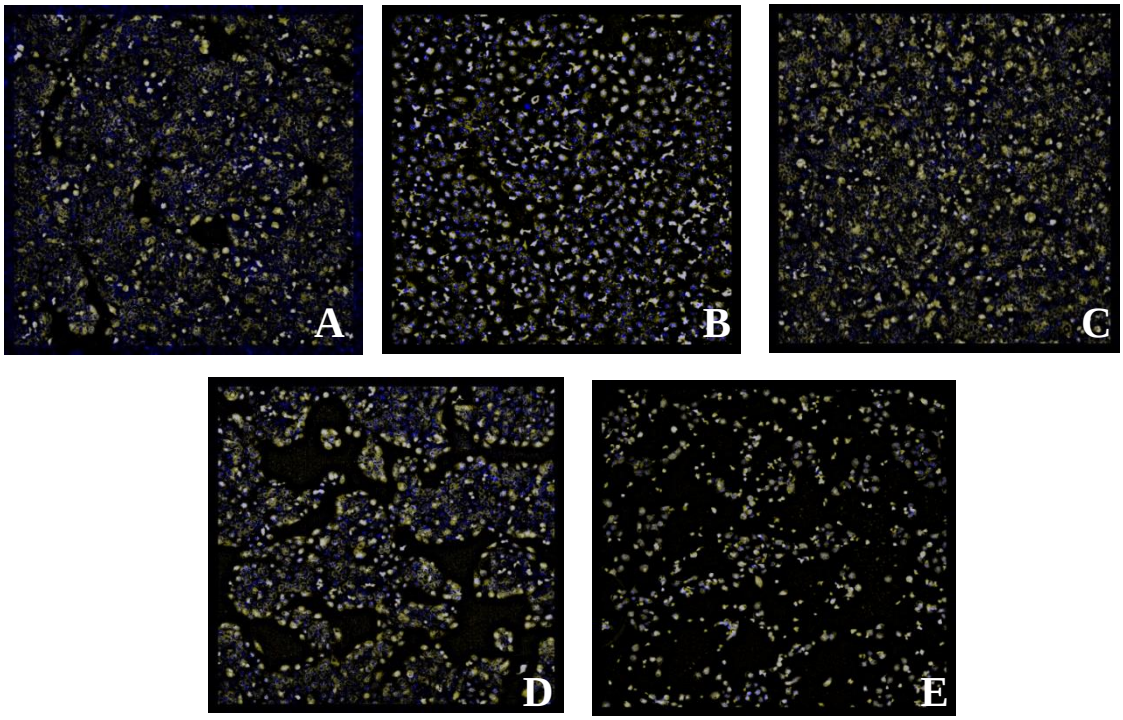
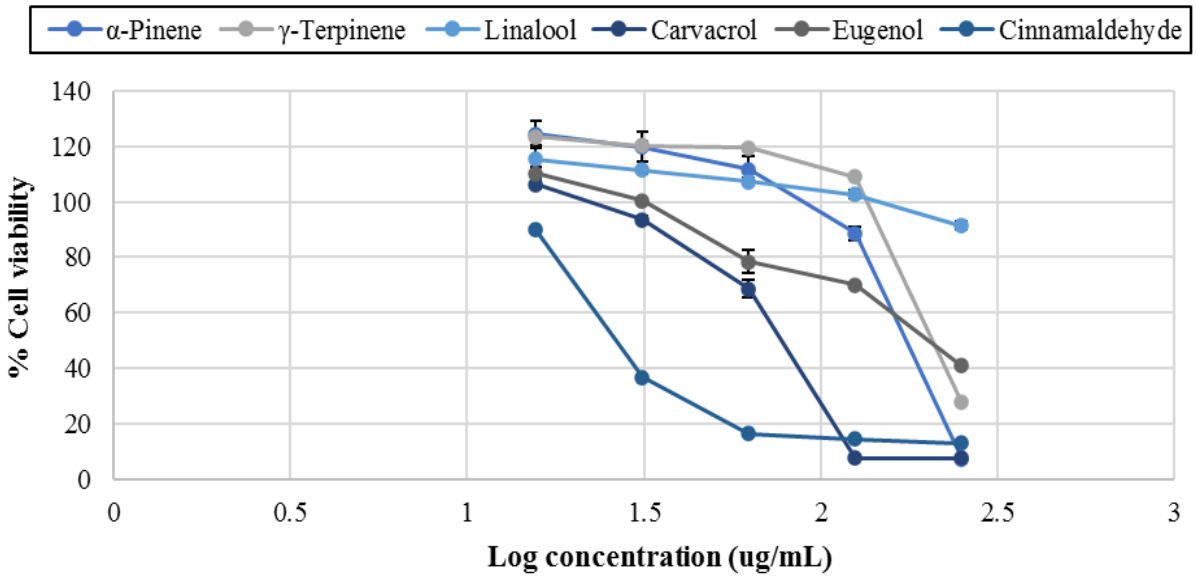


Fig. 2. HaCAT cells imaged using ImageXpress Micro XLS Widefield Microscope at 10X magnification. The cell nuclei (blue) were stained with Hoechst 33342 and the polymerized actin (yellow) with phalloidin-tetramethylrhodamine (TRITC). (A) Untreated cells (Control), (B) Melphalan (Positive control) (5 µM), (C) Ciprofloxacin (12.5 µg/mL), (D) Tetracycline (12.5 µg/mL), (E) Miconazole (12.5 µg/mL).

Some of the conventional antimicrobials such as amoxicillin, ciprofloxacin, and erythromycin demonstrated cell viabilities above 100% which was not statistically significant ($p > 0.05$). Although this suggests that the HaCAT cells were able to proliferate in the presence of these conventional antimicrobials, it may be due to these drugs affecting the metabolic activity of the HaCAT cells. The intracellular reduction of MTT relies on both oxidoreductase and dehydrogenase enzymes and electron donors such as nicotinamide adenine dinucleotide (phosphate) (NAD(P)H) occurring across glycolytic pathways to the mitochondrial electron transport chain [42]. High concentrations of bactericidal antibiotics can induce oxidative stress which increases the presence of superoxide [43, 44]. These superoxides can also contribute to intracellular reduction of the MTT dye [45]. However, the exact mechanism is still unclear as several other factors may also contribute to this observation [46].

3.2. Cytotoxicity effects of the essential oil compounds

The cytotoxicity results (Fig. 3.) demonstrates that at low concentrations (15.63 µg/mL) all EOCs elicited similar levels of toxicity, however, as the concentration increased, the cytotoxicity of each EOC varied. At higher concentrations (> 62.5 µg/mL), these EOCs demonstrated a noteworthy increase (up to 30% reduction in cell viability) in toxicity. Linalool demonstrated a negative linear relationship with a gradual decrease in cell viability. Both carvacrol and cinnamaldehyde showed low cell viability at concentrations greater than 15.63 µg/mL. For the IC_{50}, both cinnamaldehyde (IC_{50} = 28.6 µg/mL, $p = 0.007$) and carvacrol (IC_{50} 74.1 µg/mL, $p = 0.04$) demonstrated statistical significance when compared to the untreated control. Linalool had the lowest toxicity (IC_{50} = 274.0 µg/mL) with γ-terpinene (IC_{50} = 238.0 µg/mL) demonstrating a similar result. A two-way ANOVA was done between the EOCs at the same concentrations and linalool was statistically significant ($p = 0.01$) when compared to cinnamaldehyde at concentrations of 15.63, 31.3, and 62.5 µg/mL. The vehicle control (1.25% DMSO) showed no inhibition whilst melphalan demonstrated a low IC_{50} of 4.98 µg/mL.



IC_{50} (µg/mL)	α-pinene	γ-terpinene	± Linalool	Eugenol	Carvacrol	Cinnamaldehyde
	170	238	274	198	74.1*	28.6*

Fig. 3. Dose-response curve with calculated IC_{50} values for the EOCs on HaCAT keratinocytes. Error bars represent mean ± SD. *****($p < 0.05$) indicates statistical significance when compared to the control (Untreated cells). Melphalan (not shown) was a positive control.

Fig. 4 (A-G). shows the cell morphology of the HaCAT cells after exposure to the different EOCs at the same concentration. Both γ-Terpinene (Fig. 4C) and linalool (Fig. 4D) had a high cell density, however, carvacrol (Fig. 4E) and cinnamaldehyde (Fig. 4F) showed a low cell density further demonstrating their toxicity at high concentrations (250.0 µg/mL). Essential oil compounds exert their cytotoxic effects through the permeabilization of outer and inner mitochondrial membranes resulting in cell death by necrosis and apoptosis [47]. Generally, alcohol, aldehydes, and phenolic compounds elicit the highest toxicity with variations based on the chemical structure and lipophilic nature [20]. Unlike conventional antimicrobials, EOCs are non-selective and are toxic to both prokaryote and eukaryote cells [29]. Although this represents a caveat in the development of novel topical therapies there is a link between the type of organism (eukaryotes versus prokaryotes) and the lipophilic nature of the EOCs. In eukaryotes, the toxicity decreases with increasing lipophilicity whilst in prokaryotes, it increases with increasing lipophilicity [48]. This observation may explain the relatively low toxicity of linalool when compared to other compounds such as cinnamaldehyde and eugenol.

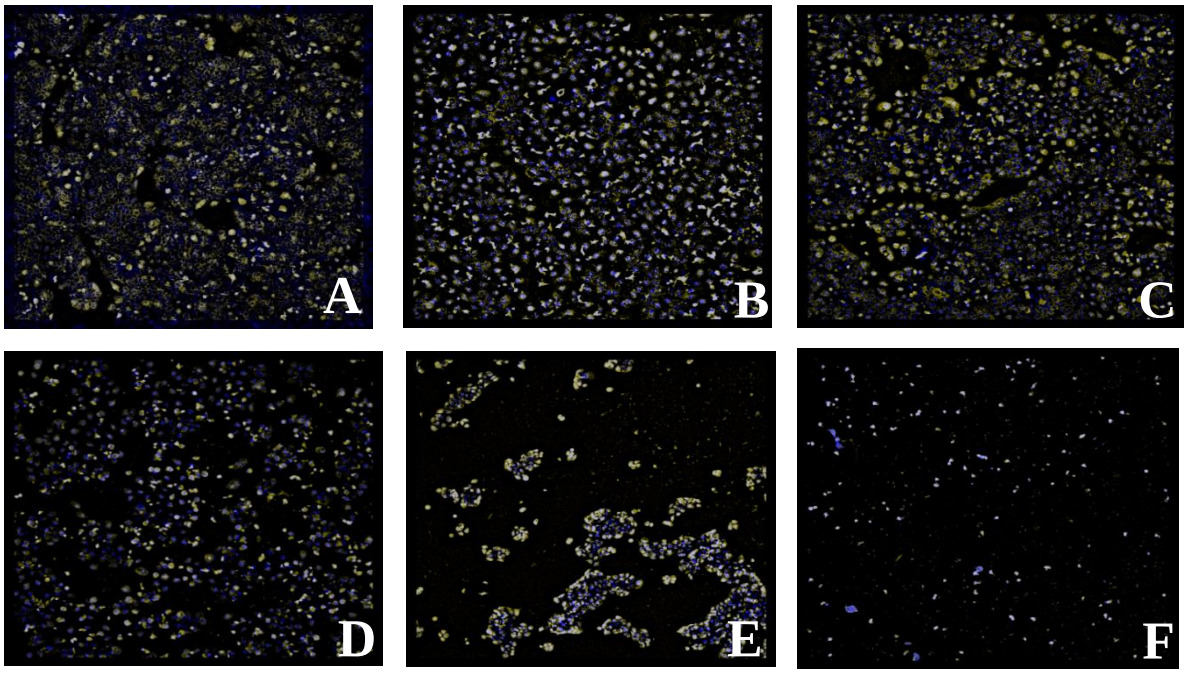


Fig. 4. HaCAT cells imaged using ImageXpress Micro XLS Widefield Microscope at 40X magnification. The cell nuclei (blue) were stained with Hoechst 33342 and the polymerized actin (yellow) with phalloidin-tetramethylrhodamine (TRITC). (A) Untreated cells (Control), (B) Melphalan (Positive control) (5 µM), (C) γ-Terpinene (250 µg/mL), (D) Linalool (250 µg/mL), (E) Carvacrol (250 µg/mL), (F) Cinnamaldehyde (250 µg/mL).

3.3. Cytotoxicity effects of the combinations

The cytotoxicity results for the EOC: antibiotic combinations are presented in Table 1. The concentrations of the conventional antimicrobial and EOC were determined based on a preliminary MTT assay. The combination of miconazole and cinnamaldehyde was the most toxic with a cell viability of 78.6%. The combination of amoxicillin and eugenol also demonstrated a comparable result with 79.4% cell viability. Although there is no definition of acceptable cell viability values, a study by Dahl, et al. [49] suggested that cell viabilities above 90% are considered non-toxic, and less than 30% are considered severely toxic. Based on this definition, two of the seven combinations were non-toxic whilst the remaining five were slightly cytotoxic. The CI was calculated for each of the combinations using the Chou-Talalay method.

Table 1
Cell viability (%; mean ± SD) and combination index (CI) for synergistic combinations (n = 4) on HaCAT keratinocytes

Combinations	Cell viability (% ± SD)	Interpretation of cytotoxicity	Combination index (CI)
Amoxicillin (0.31 µg/mL) + Eugenol (50 µg/mL)	79.42 ± 5.36	Slightly[1] cytotoxic	0.16[2]
Amoxicillin (0.31 µg/mL) + Carvacrol (50 µg/mL)	92.09 ± 4.62	Non-toxic	0.28
Erythromycin (0.16 µg/mL) + Cinnamaldehyde (25 µg/mL)	83.46 ± 1.49	Slightly cytotoxic	0.29
Ciprofloxacin (0.08 µg/mL) + Cinnamaldehyde (12.5 µg/mL)	88.42 ± 3.72	Slightly cytotoxic	0.12
Gentamicin (0.31 µg/mL) + Carvacrol (50 µg/mL)	84.41 ± 7.49	Slightly cytotoxic	0.34
Miconazole (0.31 µg/mL) + Carvacrol (50 µg/mL)	91.82 ± 6.03	Non-toxic	0.31
Miconazole (0.08 µg/mL) + Cinnamaldehyde (12.5 µg/mL)	78.57 ± 2.71	Slightly cytotoxic	0.17
Untreated cells	100 ± 0.00	N/A	N/A
Melphalan (Positive control)	6.55 ± 0.44	Severely cytotoxic	N/A

[1]Cytotoxicity interpreted based on cell viability: > 90% (Non-toxic), 60-90% (Slightly cytotoxic), 30–59% (Moderately toxic), and < 30% (Severely toxic) [49]. [2]CI < 0.80 (Synergism), CI = ≤ 0.80 – 1.20 (Additivity) and CI > 1.20 (Antagonism) [36].

All combinations were interpreted as synergistic (CI < 0.80) with the lowest CI being for the combination of ciprofloxacin and cinnamaldehyde (CI = 0.12). Based on these results, it can be suggested that when EOCs are combined with conventional antimicrobials, the combined toxicity is less than the individual components.

3.4. Anti-inflammatory effects of the conventional antimicrobials

The results for the anti-inflammatory assay for the conventional antimicrobials are presented in Fig. 5. and expressed as nitrite production. In comparison to the LPS control, none of the conventional antimicrobials demonstrated a significant reduction in nitrite production. Some of the conventional antimicrobials such as amoxicillin and tetracycline increased the nitrite production suggesting a pro-inflammatory effect, however this was not statistically significant (p > 0.05). Antibiotics can function as immuno-activators or immuno-suppressors depending on type of immuno-modulatory actions exhibited [19]. Macrolides such as erythromycin are known for their immunomodulatory properties, however, based on the results erythromycin did not elicit any noteworthy anti-inflammatory activity.

A possible reason for this is that erythromycin may act to inhibit other pro-inflammatory processes such as decreasing interleukins and cytokines [50].

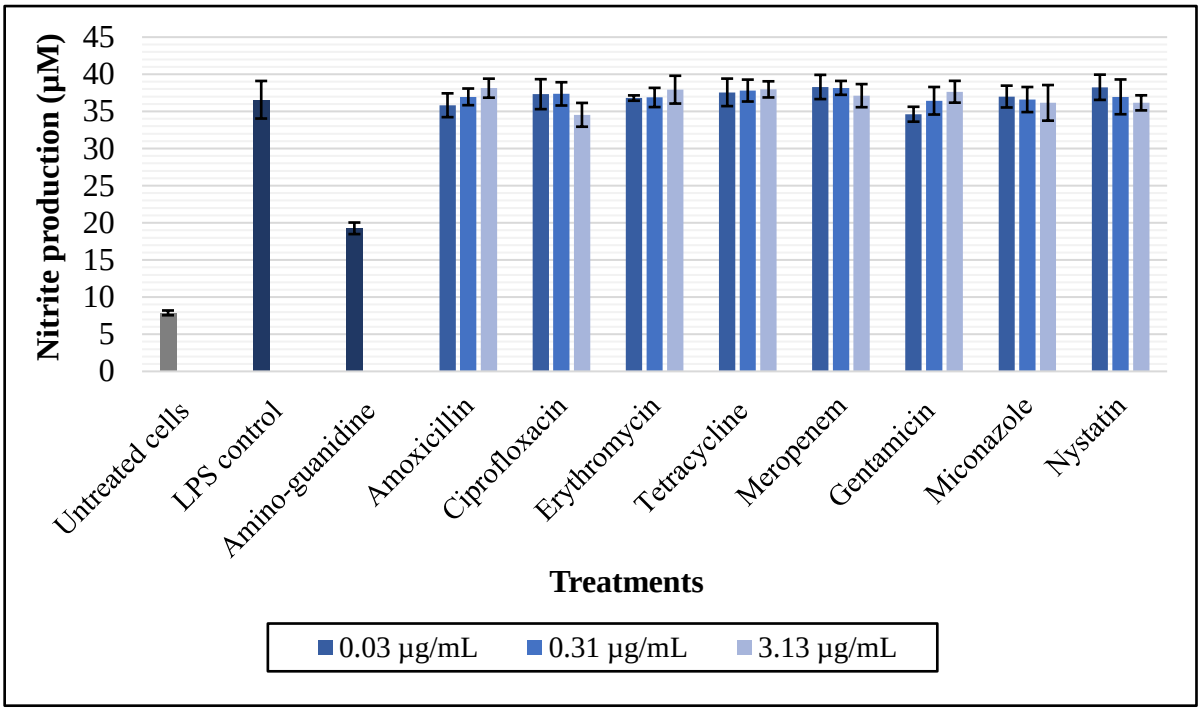


Fig. 5. Nitrite production (µM) of conventional antimicrobials at three different concentrations on RAW 264.7 macrophages. Error bars represent mean ± SD (n = 4). LPS was added to all treatments except for the untreated cells.

3.5. Anti-inflammatory effects of the essential oil compounds

The results from the LPS-NO-induced assay for the EOCs are presented in Figure 6. Linalool and γ-terpinene increased the nitrite production beyond the LPS control whilst α-pinene and eugenol showed a decrease in nitrite production in a concentration-dependent manner, which was not statistically significant. Cinnamaldehyde demonstrated the highest reduction in nitrite production (12.5 µg/mL – 67.9% reduction and at 25.0 µg/mL, 77.4% reduction). Both concentrations for cinnamaldehyde were statistically significant (12.5 µg/mL, p = 0.04, and 25.0 µg/mL, p = 0.05) when compared to the control. In addition, the reduction of nitrite production demonstrated by cinnamaldehyde was even higher than the control, amino-guanidine (47.0% reduction). The anti-inflammatory properties demonstrated by some EOCs are well documented, particularly for phenylpropanoid compounds such as eugenol and cinnamaldehyde [33].

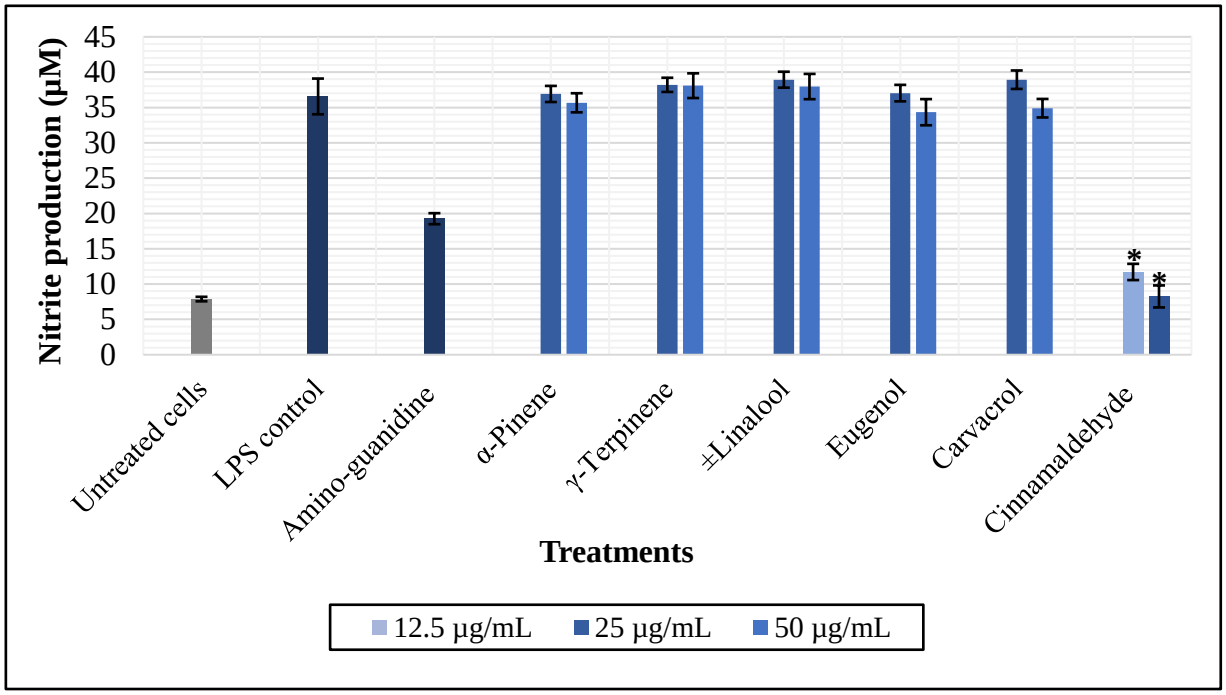


Fig. 6. Nitrite production (µM) of the essential oil compounds. Error bars indicate mean ±SD ($n = 4$). *****($p < 0.05$) indicating statistical significance when compared to the control. LPS was added to all treatments except for the untreated cells.

The results for cinnamaldehyde agree with numerous other studies which have demonstrated that cinnamaldehyde can elicit multiple anti-inflammatory mechanisms through the inhibition of iNOS, prostaglandin production, and suppression of various pro-inflammatory interleukins in both *in vivo* and *in vitro* models [51-53]. A study by Gulec Peker and Kaltalioglu [54] demonstrated that both eugenol and cinnamaldehyde were able to decrease the upregulation of various pro-inflammatory cytokines and decrease NO production in RAW264.7 cells. The compounds, α-pinene, γ-terpinene, and linalool did not reduce the production of nitric oxide which differs from other studies. A study by Kim, et al. [55] demonstrated that α-pinene inhibited the upregulation of iNOS, IL-6, and TNF-α in murine macrophages. Another study by Huo, et al. [56] reported that linalool reduced NO production and other pro-inflammatory cytokines (TNF-α, IL-1β) in both rodent and cell models. Although the results from this study differ from other previous studies, it is important to note that NO production is one of many pro-inflammatory processes [57, 58]. Different EOCs may act via alternative pathways to inhibit NO production [48].

3.6. Anti-inflammatory effects of the combinations

The anti-inflammatory results for the antimicrobial combinations are presented in Table 2. All combinations that involved cinnamaldehyde demonstrated a noteworthy reduction in NO production. In addition, all cinnamaldehyde combinations were statistically significant ($p < 0.05$) when compared to the LPS control. Other combinations with carvacrol also demonstrated a reduction in NO production, with amoxicillin and carvacrol (10.06 % reduction) and miconazole and carvacrol (9.95% reduction) demonstrating similar results, however these combinations were not statistically significant. Amino-guanidine demonstrated a high reduction of NO production which was expected as it is a noted inhibitor of iNOS [59]. Synergistic interactions (ΣFa < 0.80) were observed for combinations with cinnamaldehyde, whilst the remaining four combinations demonstrated additivity (ΣFa ≤ 0.80 – 1.20). These results suggest that cinnamaldehyde can potentiate the anti-inflammatory ability of some conventional antimicrobials.

Table 2
Nitrite production (μM; mean ± SD) of synergistic combinations ($n = 4$) with the fractional effect (ΣFa) index on RAW 264.7 macrophages

Combinations	**Nitrite production**	**% Reduction in NO**	**ΣFa[1]**
Amoxicillin (0.31 μg/mL) + Eugenol (50 μg/mL)	34.70 ± 1.10	5.11	0.98
Amoxicillin (0.31 μg/mL) + Carvacrol (50 μg/mL)	32.89 ± 1.48	10.06	0.95
Erythromycin (0.16 μg/mL) + Cinnamaldehyde (25 μg/mL)	7.89 ± 0.63*****	78.42	0.46
Ciprofloxacin (0.08 μg/mL) + Cinnamaldehyde (12.5 μg/mL)	8.17 ± 1.15*****	77.66	0.44
Gentamicin (0.31 μg/mL) + Carvacrol (50 μg/mL)	35.23 ± 1.31	3.66	0.99
Miconazole (0.31 μg/mL) + Carvacrol (50 μg/mL)	32.93 ± 1.01	9.95	0.92
Miconazole (0.08 μg/mL) + Cinnamaldehyde (12.5 μg/mL)	7.85 ± 0.24*****	78.53	0.44
Untreated cells	7.88 ± 0.32	N/A	N/A
LPS control	36.57 ± 2.53	N/A	N/A
Amino-guanidine (AG)	19.26 ± 0.26	47.33	N/A

*($p < 0.05$) indicates statistical significance compared to LPS control.[1]ΣFa ≤ 0.80 (Synergy); ΣFa = ≤ 0.80 – 1.20 (Additive); ΣFa > 1.20 (Antagonism). LPS was added to all combinations except for the untreated cells N/A = not applicable.

Although numerous studies have investigated the anti-inflammatory properties of EOCs against different cell lines and in animal models [55, 60, 61], there are no studies

involving combinations of EOCs and conventional antimicrobials. Most of the studies have focussed on combinations of EOs which have been reviewed by [7, 48].

The conventional antimicrobials showed no significant anti-inflammatory properties, which does not mean these drugs do not affect the immune system. Antibiotics are generally considered immuno-restorers as they kill or inhibit the growth of immuno-modulating pathogens and restore immune homeostasis [19]. Considering that EOCs have been actively sought as possible adjunctive agents that can potentiate conventional antimicrobials from an antimicrobial perspective, the possibility of these combinations eliciting anti-inflammatory effects is promising [62].

Based on the results from this study, combined with the results from our previous study [30], combinations of EOCs and some conventional antimicrobials are promising therapeutic approaches to attenuate antimicrobial resistance. The results have demonstrated that combinations that comprise of cinnamaldehyde have noteworthy antimicrobial and anti-inflammatory properties, which may warrant further investigation.

4. Conclusion

This study investigated the emerging strategy of combining essential oil compounds with conventional antimicrobials to investigate the toxicity and possible anti-inflammatory effects. Based on the findings, all essential oil compounds demonstrated concentration-dependent toxicity whereby low concentrations were non-toxic and higher concentrations were toxic. Cinnamaldehyde demonstrated the highest anti-inflammatory properties with the greatest reduction in nitrite production. For the conventional antimicrobials, only miconazole demonstrated noteworthy toxicity against the HaCAT cells. The combination of ciprofloxacin and cinnamaldehyde demonstrated the lowest toxicity and highest reduction in nitrite production highlighting its potential for further investigation. Furthermore, this study showed that although the individual EOCs have high inherent toxicities, the overall toxicity can be reduced when combined with conventional antimicrobials. The results from this study highlight the potential of EOCs as possible anti-inflammatory adjunctive agents with conventional antimicrobials. However, several challenges need to be addressed such as optimal dosages needed to maximize the targeted anti-inflammatory biochemical pathways whilst also minimizing potential adverse effects.

Declaration of Competing Interest

The authors have no conflicts of interest to declare.

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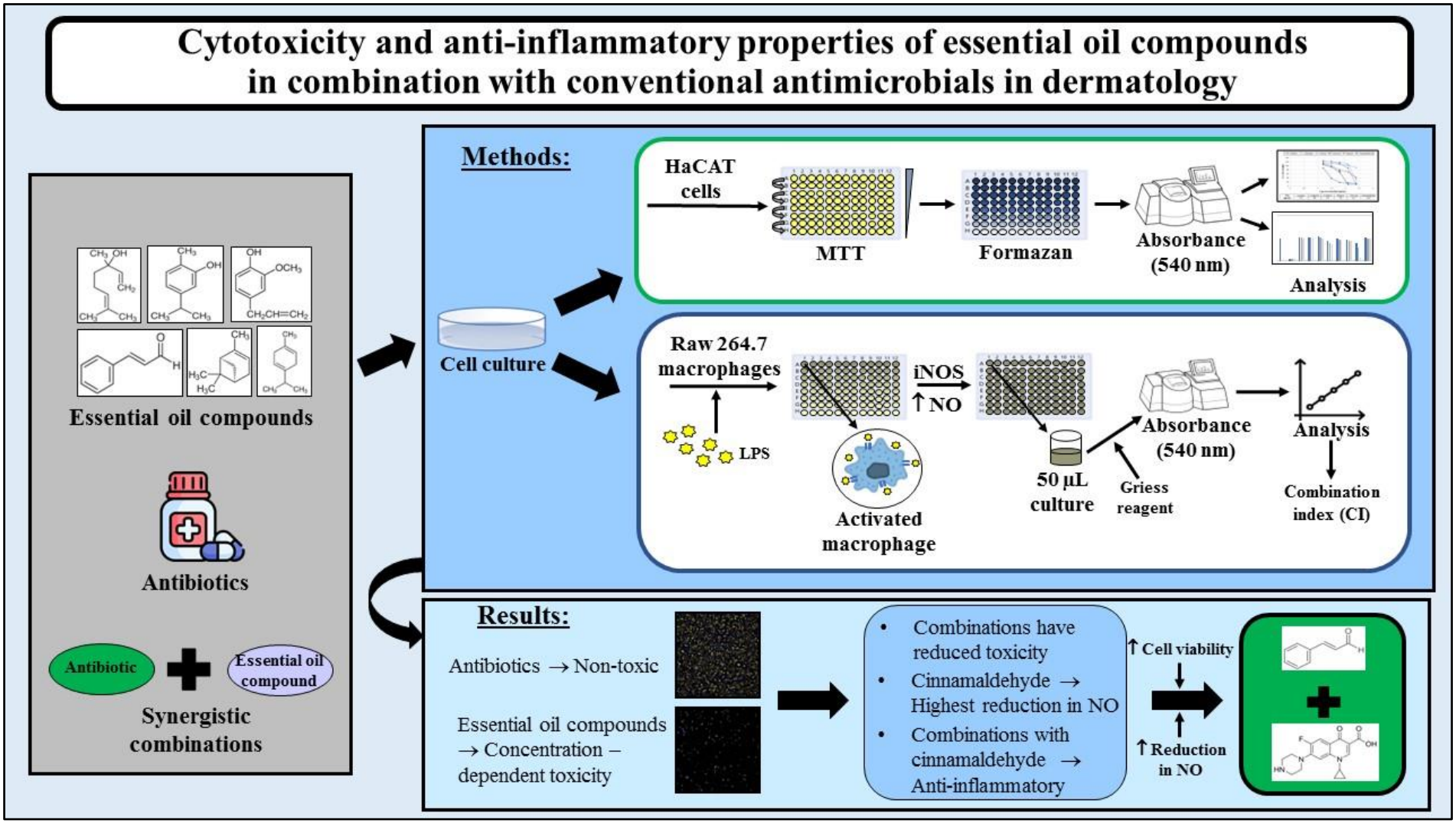
Graphical abstract: Publication two



CHAPTER 5: PUBLICATION THREE

Ibuprofen as an adjuvant to conventional antimicrobials and essential oil compounds against skin pathogens

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Abstract

Objectives: Antimicrobial resistance continues to be a growing concern to healthcare systems and has prompted the increased use of drug combinations to improve treatment efficacy. The use of antibiotic adjuvants is an emerging combinational strategy whereby natural or synthetic compounds may potentiate an antibiotics efficacy. This study aimed to investigate the potential of a novel multidrug combination involving essential oil compounds and conventional antimicrobials with ibuprofen against skin pathogens.

Methods: The minimum inhibitory concentrations of ibuprofen, conventional antimicrobials, and essential oil compounds was determined singularly and in combination against reference and clinical strains. The cytotoxicity was analysed using the MTT assay whilst the anti-inflammatory effects was evaluated using lipopolysaccharide activated murine macrophages.

Results: Four pairwise (Ibuprofen with antibiotic) (ΣFIC 0.33 - 0.50) and three triple

(Ibuprofen with antibiotic and essential oil compound) (ΣFIC 0.44 - 0.47) synergistic antimicrobial interactions were identified. These combinations demonstrated cell viabilities of 77.59 - 100%. For the anti-inflammatory assay, none of the combinations produced a significant anti-inflammatory effect.

Conclusion: The results from this study provide insight into the potential of a multidrug combination involving ibuprofen with conventional antimicrobial and essential oil compounds.

Keywords: synergy; cytotoxicity; Gram-negative; antimicrobial; combinations

Introduction

Infectious skin infections are one of the most common types of infections and are an important cause of morbidity and mortality [1]. A skin infection encompasses a broad range of pathological conditions that range from superficial to life-threatening necrotic infections [2]. The clinical manifestation of skin infections differs according to the anatomical location, causative pathogen, depth of the infected tissue, and underlying co-morbidities of the patient [3, 4]. Most skin infections are treated empirically with antibiotics [5], however, there has been an increase in chronic and recurrent skin infections due to antibiotic failure [6]. Antibiotic failure can be defined as any situation where the causative pathogen survives antibiotic treatment and the clinical symptoms of the infection persist or worsen [7]. The primary cause of antibiotic failure is antimicrobial resistance which has become a major threat to public health [8, 9]. One strategy to counteract antimicrobial resistance is combination therapy which is an established and clinically proven therapeutic approach [10]. The successes of drug combinations in infectious diseases have prompted investigation into other types of combinations such as compounds that may potentiate an existing antibiotic.

Antibiotic adjuvants (also called potentiators) are defined as synthetic or natural

molecules that potentiate the activity of existing antibiotics by either reversing resistance mechanisms or enhancing host defences [11-13]. Antibiotic adjuvants have been used as a type of combinational approach to recycle redundant antibiotics with the most documented examples being beta-lactamase inhibitors such as clavulanic acid (Amoxicillin-clavulanic acid) and tazobactam (Piperacillin-tazobactam) [14, 15]. The concept of antibiotic adjuvants is similar to the use of two or more antibiotic agents in congruous combinations [16]. However, antibiotic-adjuvant or adjuvant-adjuvant combinations differ as these compounds typically have little or no antimicrobial activity [13, 17]. Due to the unremitting increase in antimicrobial resistance, other therapeutic drug classes have also been investigated for their passive and active antimicrobial properties such as anti-inflammatory, cardiovascular, and psychotropic drugs [18, 19].

Ibuprofen is one of the most widely used non-steroidal anti-inflammatory drugs (NSAIDs) and is commonly used to relieve pain and inflammation [20]. Similarly, to other NSAIDs, ibuprofen is a non-selective inhibitor of cyclooxygenase (COX) enzymes which decreases the synthesis of prostaglandin and results in different pharmacological effects [21]. In recent years, ibuprofen has garnered interest due to increasing evidence that supports ibuprofen's identity as a polypharmacological drug that can elicit additional therapeutic benefits for other disease states [22-24]. These off-target effects or drug promiscuities have antimicrobial potential with some reviews proposing ibuprofen as a candidate for drug repurposing [25-27]. The antimicrobial properties of ibuprofen have been well-documented against bacteria and fungi [28-30]. In addition, the combinational effects of ibuprofen and some antibiotics have also been investigated for different diseases such as cystic fibrosis [31-33], tuberculosis [34, 35] urinary tract infections [36] and otitis media [37]. In contrast, ibuprofen use in cutaneous infections remains controversial as there is an increased risk of secondary bacterial infections [38]. Several clinical studies have demonstrated that patients who were given ibuprofen whilst being infected with Varicella-Zoster (Chickenpox) incurred a higher risk of developing

necrotizing Group A streptococcus (GAS) soft tissue infections [39-42]. However, in contrast animal studies demonstrated that non-selective COX-2 inhibitors such as ibuprofen limited the extent of necrotizing infections and lowered the bacterial numbers in infected tissue [43, 44]. This inconsistency between animal and human studies suggest that ibuprofen's ability to predispose individuals to necrotizing infections is multifactorial with the bulk of the literature focusing on only GAS-Varicella co-infections [45, 46]. Furthermore, the impact of ibuprofen as an antibiotic adjuvant against other skin micro-organisms such as *Pseudomonas aeruginosa*, *Acinetobacter baumannii* and *Candida* species is unknown and highlights the need for investigation.

Essential oils (EOs) are complex secondary metabolites comprised of anything between 20 and 200 different chemical compounds in varying concentrations [47]. These essential oil compounds (EOCs) give an EO its therapeutic benefits such as antimicrobial and anti-inflammatory properties [48]. The antimicrobial properties of EOCs are well-known and have been reviewed extensively [49, 50]. Recent reviews have discussed the emerging strategy of using EOCs as potential antibiotic-adjuvants [15, 51]. Combinations of EOCs and antifungals are also well documented with numerous studies demonstrating the EOCs ability to potentiate certain antifungal agents [52-54]. However, there is no evidence of the antifungal effects of ibuprofen-EOC combinations.

In our previous study [55], the antimicrobially synergistic combinations of EOCs and conventional antimicrobials against different skin bacteria were demonstrated. Most cutaneous infections have complex pathophysiology which induce systemic effects as a result of interactions between host defences and the causative pathogen [56, 57]. Considering the established status of ibuprofen as an anti-inflammatory drug and the increased recognition of EOCs as multifunctional compounds, it is justified to explore a novel combination that could produce a multitargeted therapeutic effect. Therefore, this study aimed to evaluate the *in vitro*

antimicrobial, cytotoxic, and anti-inflammatory effects of a multidrug combination involving EOCs, and conventional antimicrobial with ibuprofen against skin pathogens.

Materials and methods

Preparation of cultures

Due to their prevalence in skin infections [58, 59], the following American Type Culture Collection (ATCC) reference strains were included in the study; *Staphylococcus aureus* (ATCC 25923 and ATCC 6538), *Staphylococcus epidermidis* (ATCC 12228), *Acinetobacter baumannii* (ATCC 19606), *Pseudomonas aeruginosa* (ATCC 27853), *Cutibacterium acnes* (ATCC 11827) and the yeast *Candida albicans* (ATCC 10231). Most of the bacteria were cultured in Mueller Hinton broth (MHB) (Oxoid) and incubated at 37°C for 24 h except for *C. acnes* which was cultured in Thioglycolate broth (TGB) (Oxoid) and incubated for seven days under anaerobic conditions (5.00% CO_2) at 37°C. *Candida albicans* was cultured in Tryptone Soy Broth (TSB) and incubated at 37°C for 48 h. In addition, streak plates were prepared to ensure purity. Clinical isolates of *S. aureus* (Methicillin-resistant *S. aureus*), *S. epidermidis* (Erythromycin-resistant), *A. baumannii* (Carbapenem-resistant), two strains of *P. aeruginosa*, and two strains of *C. albicans* (Azole resistant) were also included in the study. These isolates were cultured in the same way as the reference strains. The antimicrobial resistance profiles of all studied micro-organisms were analysed using the disk diffusion assay as outlined by the Clinical Laboratory Standards Institute [60] (Supplementary data Table S1 and S2). A waiver for the use of these micro-organisms was obtained from the University of the Witwatersrand Human Research Ethics Committee (HREC) (Reference number: W-CBP-220411-02).

Preparation of antimicrobial agents, essential oil compounds, and ibuprofen

Ibuprofen (>99.0%) (Sigma-Aldrich, St. Louis, MO, USA) was initially dissolved in 15%

DMSO and then made up to a concentration of 10000 mg/L (1.25% DMSO). Based on the results from our previous study [55], the following antibiotics/antifungals (Sigma-Aldrich, St. Louis, MO, USA) were included; amoxicillin (potency ≥ 90%), ciprofloxacin (≥ 98%), erythromycin (≥ 85%), tetracycline (≥ 98%), gentamicin (≤ 100%), meropenem (≥ 95%), miconazole (99.5%) and nystatin (≥ 95%). The preparation of the antibiotic/antifungal stock solutions was performed as outlined by the CLSI [60]. Stock solutions (10 mg/L and 50 mg/L respectively) were stored at -20°C and working stocks were stored at 4°C.

The following six essential oil compounds (Sigma-Aldrich, St. Louis, MO, USA) were selected from our previous study [55]; α-pinene, γ-terpinene, ± linalool, eugenol, carvacrol, and cinnamaldehyde. All compounds had a purity range of 95 - 99%. The compounds were stored at 4°C and made up to a starting concentration of 32000 mg/L.

Minimum inhibitory concentration (MIC)

The broth microdilution assay was used to evaluate the minimum inhibitory concentration (MIC) of the conventional antimicrobials, EOCs, and ibuprofen independently and in combination. Briefly, each well of a 96-well microtiter plate was filled with 0.1 mL of sterile broth, followed by adding 0.1 mL of the antibiotic/EOC/Ibuprofen into the top row. For the combinations, 0.05 mL of the conventional antimicrobial/ EOC, as well as 0.05 mL of ibuprofen, was added so that the ratio of the antibiotic/EOC to ibuprofen was 1:1. Serial doubling dilutions were then performed. Based on the type of interaction (Section 2.4), triple combinations (higher order) comprising ibuprofen with the conventional antimicrobial and EOC were also included. Two methods were incorporated, firstly equivalent volumes (0.033 mL) of ibuprofen, conventional antimicrobial, and EOC were mixed and then added to the microdilution assay. The second method followed the procedure outlined by O'Shaughnessy, *et al.* [61] and involved adding fixed concentrations of the synergistic pairwise combination into

each well, which was followed by adding the third compound/drug. The prepared micro-titre plates were then inoculated with 0.1 mL of the relevant pathogen an approximate colony-forming unit of 1×10^6 (CFU/mL). The plates were then sealed with sterile adhesive to ensure no loss of sample since EOCs are volatile. The plates were then incubated at the respective temperature and times. After incubation, 0.04 mL of a 0.40% *p*-iodonitrotetrazolium (INT) violet indicator solution was added to all inoculated wells. A change in the colour of the wells from clear to pink or red was used as an indication of the presence of microbial growth. The MIC values were interpreted as the lowest concentration at which growth was inhibited. Each combination was performed in quadruplicate. Negative controls were included which consisted of a culture control for the respective broth and solvent controls for the tested samples. The MIC data for the conventional antibiotic and EOCs against both the reference and clinical isolates is presented under supplementary data Tables S3-S4.

Interactive profiles

The interactions between the combinations of the conventional antibiotic and EOCs and ibuprofen were classified according to the fractional inhibitory concentration (FIC) (Equation 1).

$$\text{FIC (I)} = \frac{(A^*) \text{ combined with (B)}}{\text{(A) independently}} \qquad \text{FIC (II)} = \frac{(B^*) \text{ combined with (A)}}{\text{(B) independently}} \quad \text{-} \quad \text{Equation 1}$$

*Where (A) is the MIC of the EO compound/conventional antibiotic and (B) is the MIC of ibuprofen.

From these values, the sum of the FIC was calculated (Equation 2):

$$\Sigma\text{FIC} = \text{FIC (I)} + \text{FIC (II)} \quad \text{-} \quad \text{Equation 2}$$

The ΣFIC for each combination was interpreted as follows: ΣFIC value of ≤ 0.5 is indicative of synergy; ΣFIC value of > 0.5 - ≤ 4.0 indicates non-interactive (Indifference) and ΣFIC value of > 4.0 indicates antagonism [62]. For the triple combinations, all pairwise and single interactions were evaluated for possible emergent or suppressive interactions [61, 63].

Varied ratio combinations

For combinations demonstrating synergistic interactions in the 1:1 ΣFIC analysis, further examination was undertaken where combinations were investigated at varied ratios of 90:10, 80:20, 70:30, 60:40, 50:50, 40:60, 30:70, 20:80, and 10:90. The data points for each ratio were then plotted on an isobologram using the GraphPad Prism® software (Version 9). Synergy is displayed for the data points closest to the apex and fall beneath or on the 0.5:0.5 line, additive interactions are for the data points between the 0.5:0.5 and on the 1:1 line, non-interactive effects are those that lay between the 1:1 or on the 4:4 line and antagonism is displayed for data points above or on the 4:4 line [64].

The MTT cytotoxicity assay

The cytotoxicity of ibuprofen, conventional antibiotic EOCs, and selected synergistic combinations was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay according to Leigh-de Rapper, *et al.* [65]. The HaCAT cells (Cellonex, South Africa) were cultured in Dulbecco's Modified Eagle Medium (DMEM) (GE Healthcare Life Sciences, USA supplemented with 10% (v/v) FBS (GE Healthcare Life Sciences, USA) in a humidified, 5% CO_2 incubator at 37°C. The cells were seeded into 96-well microtiter plates at a density of 4000 cells per well, using a volume of 0.1 mL in each well. The microtiter plates were incubated for 24 h before the addition of the test samples, to allow for cell attachment. The cells were treated at three different concentrations (3.13, 6.25, and 12.5 mg/L) for the

conventional antimicrobials, five concentrations (15.63, 31.3, 62.5, 125, and 250 mg/L) for the EOCs, and five concentrations (1.25, 2.50, 12.5, 125 and 1250 mg/L) for ibuprofen. Melphalan (100 µM) (Sigma Aldrich, St. Louis, MO, USA) which is a known cytotoxic agent was used as a positive control. Each sample was tested in quadruplicate (n = 4). The cells were then incubated for 48 h. After incubation, the remaining medium in each well was replaced with 0.5 mg/mL MTT in complete medium and further incubated for 30 min at 37˚C. Thereafter, the MTT was removed and 0.1 mL of dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO, USA) was added to each well to solubilize the formazan crystals. The absorbance was read at 540 nm using a BioTek® PowerWave XS spectrophotometer (Winooski, VT, USA). The cytotoxicity was interpreted as % cell viability when compared to the untreated control. The data analysis which included the calculation of the mean, standard deviation (±SD), and IC_{50} was done using GraphPad Prism® software (Version 9). The Student t-test was used to determine whether data groups differed significantly from each other. Statistical significance was defined as having P values < 0.05.

Combination index (CI)

The CI provides a quantitative measure to determine the type of interactions between two drugs and was used to assess the cytotoxicity [66-68]. The CI was calculated as per Equation 3.

$$CI = [(D)_1/(D_x)_1 + (D)_2/(D_x)_2] \quad - \quad \text{Equation 3}$$

*Where $(D)_1$ and $(D)_2$ denote the dose of each drug in combination necessary to produce the same effect, and $(D_x)_1$ and $(D_x)_2$ denote the individual dose of each drug required to inhibit a given level of cell growth and was calculated as per Equation 4.

$$D_x = D_m [F_a/(1/F_a)]1/m \quad - \quad \text{Equation 4}$$

*Where D_m refers to the median effect dose (Potency of drug expressed as IC_{50}), m is the slope of the graph, and F_a denotes the fraction effect expressed as % cell viability in dose-effect curves. The CI values were interpreted as CI < 0.80 indicates synergism, CI = ≤ 0.80 - ≤ 1.20 indicates an additive effect, and CI > 1.20 indicates antagonism [69].

The anti-inflammatory assay

The anti-inflammatory activity of the EOCs, conventional antimicrobials, and combinations were assessed by measuring the inhibition of nitric oxide (NO) using the lipopolysaccharide-nitric oxide (LPS-NO) induced assay. Lipopolysaccharide (LPS) induces the expression of inducible nitric oxide synthase (iNOS) in RAW264.7 macrophages (Cellonex, South Africa) resulting in the production of nitric oxide which stimulates an inflammatory response [65]. The RAW 264.7 cells were cultured in RPMI1640 medium supplemented with 10% fetal calf serum in a humidified 5% CO_2 incubator at 37°C. Cells were seeded into 96-well plates at a density of 1.50 x 10^5 cells per well and allowed to attach overnight. The concentrations of the ibuprofen, EOCs, and combinations were based on the results for the individual compounds from the preliminary MTT assay. Three different concentrations were used for ibuprofen (6.25, 12.50, and 25.00 mg/L), EOCs (12.50, 25.00, and 50.00 mg/L), conventional antimicrobial (0.03, 0.31, and 3.13 mg/L). The spent culture medium was replaced with 0.05 mL of the samples or complete medium only (Untreated cells and LPS control). Aminoguanidine (AG) (Sigma-Aldrich, St. Louis, MO, USA) was used as the positive control since it is a direct inhibitor of iNOS. To assess the anti-inflammatory activity, 0.05 mL of 1 mg/L LPS (Sigma-Aldrich, St. Louis, MO, USA) in RPMI complete medium was added to all wells. The final concentration for the LPS control was 0.5 mg/L and 100 µM for AG. The cells were incubated for a further 24 h. To quantify the NO production, 0.05 mL of the spent culture medium was transferred into a new 96-well plate and 0.05 mL Griess reagent (Sigma-Aldrich, St. Louis, MO, USA) was added. The samples were then

incubated at ambient temperature for 10 min before absorbance was measured at 540 nm. A standard curve using sodium nitrite dissolved in culture medium was used to determine the concentration of NO in each sample. The anti-inflammatory properties were interpreted as the amount of NO produced, followed by the calculation of the % reduction in NO. To confirm the absence of toxicity as a contributory factor, cell viability was assessed using the MTT assay against RAW 264.7 murine macrophages remaining in the 96 well culture plates (Section 2.6).

The fractional-effect analysis (Fa) was used to calculate the expected dose-response relationship for the anti-inflammatory combinations [68] (Equation 5):

$$Fa = \frac{(A^*) \text{ combined with (B)}}{(A)\text{independently}} \qquad Fa\ (II) = \frac{(B^*) \text{ combined with (A)}}{(B)\text{independently}} \quad - \quad \text{Equation 5}$$

*Where (A) is the NO produced by Ibuprofen alone and (B) is the NO produced by the conventional antibiotic/EOC alone.

From these values, the ΣF_a index was calculated in Equation 6.

$$\Sigma Fa = Fa\ (I) + Fa\ (II) \quad - \quad \text{Equation 6}$$

The ΣFa for each combination was interpreted as follows: $\Sigma Fa < 0.80$ indicates synergism, $\Sigma Fa = \leq 0.80 - \leq 1.20$ indicates an additive effect, and $\Sigma Fa > 1.20$ indicates antagonism [69].

Results and discussion

Ibuprofen exhibits broad spectrum activity

The results of the MICs for ibuprofen against the reference and clinical isolates are presented in Table 1. For the Gram-positive bacteria, all strains of *S. aureus* demonstrated similar susceptibility patterns with the clinical MRSA isolate being the least susceptible (2500 mg/L). A similar trend was also noticed for *S. epidermidis* whilst *C. acnes* was the most susceptible to ibuprofen with the lowest MIC value (313 mg/L). The Gram-negative bacteria

were less susceptible to ibuprofen which is reflected in overall higher MIC values. All strains of *P. aeruginosa* demonstrated the same MIC value (2500 mg/L) whilst the clinical strain of *A. baumannii* was the least susceptible overall, with the highest MIC (5000 mg/L). For *C. albicans*, the clinical isolates were less susceptible when compared to the reference strain (ATCC 10231). Neither the negative controls nor the culture controls affected the growth of the micro-organisms. The differences in the MIC against the Gram-positive and Gram-negative bacteria can be attributed to the limited penetration of ibuprofen through the outer cell membrane [70]. This observation is similar to the differences in susceptibility to conventional antibiotic, where Gram-negative bacteria, are more resilient and demonstrate higher resistance profiles to several antibiotic classes [71].

Table 1: The mean MIC values (mg/L) and standard deviation (± SD) for ibuprofen against reference and clinical isolates (n = 6)

Micro-organisms	Ibuprofen (mg/L)	Ciprofloxacin[1] (mg/L)	Nystatin[2] (mg/L)
Gram-positive bacteria			
S. aureus (ATCC 25923)	1250 ± 0.00	1.06 ± 0.24	R[3]
S. aureus (ATCC 6538)	1250 ± 0.00	1.25 ± 0.00	R
MRSA (Clinical)	2500 ± 0.00	2.81 ± 0.34	R
S. epidermidis (ATCC 12228)	625 ± 0.00	0.94 ± 0.36	R
S. epidermidis (Clinical)	1250 ± 0.00	1.56 ± 0.00	R
C. acnes (ATCC 11827)	313 ± 0.00	1.25 ± 0.00	R
Gram-negative bacteria			
P. aeruginosa (ATCC 27853)	2500 ± 0.00	0.57 ± 0.28	R
P. aeruginosa (Clinical isolate 1)	2500 ± 0.00	1.56 ± 0.00	R
P. aeruginosa (Clinical isolate 2)	2500 ± 0.00	1.46 ± 0.18	R
A. baumannii (ATCC 19606)	2500 ± 0.00	0.52 ± 0.16	R
A. baumannii (Clinical)	5000 ± 0.00	9.34 ± 3.60	R
Yeast			
C. albicans (ATCC 10231)	625 ± 0.00	R	1.56 ± 0.63
C. albicans (Clinical isolate 1)	1250 ± 0.00	R	6.25 ± 0.00
C. albicans (Clinical isolate 2)	2500 ± 0.00	R	4.69 ± 1.80

[1]Ciprofloxacin (Positive control for bacteria); [2]Nystatin (Positive control for *C. albicans*); [3]R – Not susceptible; Both culture control and solvent (5.00% DMSO) showed no inhibition of growth (MIC > 8000 mg/L).

A study by Oliveira, *et al.* [25] demonstrated that ibuprofen had MIC values of 500 – >2000 mg/L against both clinical and reference strains of *S. aureus*. Another study by Laudy, *et al.* [72] demonstrated that ibuprofen was effective against both clinical and reference strains of *P. aeruginosa* and a reference strain (ATCC 19606) of *A. baumannii* with MIC values of ≤ 3200 mg/L.

The antifungal activity of ibuprofen is well documented with several studies demonstrating MIC values of 500 – 4000 mg/L against clinical strains of various *Candida* species [30, 73, 74]. Most *Candida* species produce a fungal prostaglandin which aids in biofilm formation and facilitates yeast-to-hypha conversion which are all virulence factors [75-77]. Ibuprofen's ability to inhibit fungal prostaglandin offers a unique mechanism of action and may represent a potential strategy to modulate the virulence properties of *C. albicans*.

Ibuprofen combinations with conventional antibiotic and EOCs against Gram-positive bacteria

The results for the 1:1 combination against the Gram-positive bacteria is presented in Table 2. A total of 78 interactions were studied with three demonstrating synergy (4.00%), 33 (42%) additive, and 42 (54%) non-interactive. No antagonism was observed. For *S. aureus*, most of the interactions were additive (56%) with 41% being non-interactive. One synergistic interaction was observed for the combinations of ibuprofen with meropenem (ΣFIC 0.46) against the reference strain (ATCC 25923) which demonstrated additivity against the other two strains (ΣFIC 0.65 - 0.81). Combinations of ibuprofen and miconazole demonstrated additivity (ΣFIC 0.67 – 0.92) against all strains. Although this observation may not be synergistic, it highlights a positive combinational effect against *S. aureus*.

Table 2: The MIC values (mg/L) of ibuprofen (A) and conventional antimicrobials/EOCs (B) in combination against Gram-positive bacteria (n = 4)

Antibiotics	***Staphylococcus aureus***								
	ATCC 25923			ATCC 6538			MRSA (Clinical)		
	MIC (A)[1]	MIC (B)[2]	ΣFIC[3]	MIC (A)	MIC (B)	ΣFIC	MIC (A)	MIC (B)	ΣFIC
Amoxicillin	1875	0.47	*1.00*(*Add*)[4]	1250	1.56	*0.75*(*Add*)	2500	3.13	*0.83*(*Add*)
Ciprofloxacin	2500	0.63	1.29(Ind)	1250	1.56	1.12(Ind)	1250	1.56	*0.53*(*Add*)
Erythromycin	2500	0.63	1.50(Ind)	2500	3.13	1.70(Ind)	2500	3.13	1.30(Ind)
Gentamicin	3750	0.94	1.88(Ind)	2500	3.13	2.04(Ind)	5000	6.25	2.25(Ind)
Meropenem	625	0.60	**0.46(Syn)**	1250	1.56	*0.81*(*Add*)	2500	1.56	*0.65*(*Add*)
Tetracycline	625	0.78	*1.00*(*Add*)	625	0.78	*0.56*(*Add*)	2500	3.13	1.01(Ind)
Miconazole	1250	1.56	*0.92*(*Add*)	1250	1.56	*0.75*(*Add*)	2500	3.13	*0.67*(*Add*)
Essential oil compounds									
α-Pinene	2500	2000	1.17(Ind)	2500	2000	1.50(Ind)	5000	4000	2.00(Ind)
γ-Terpinene	2500	2000	1.25(Ind)	5000	4000	2.50(Ind)	2500	2000	*0.75*(*Add*)
± Linalool	2500	2000	1.36(Ind)	1250	1000	*1.00*(*Add*)	2500	2000	*1.00*(*Add*)
Eugenol	2500	2000	1.60(Ind)	1250	1000	*0.75*(*Add*)	2500	2000	*1.00*(*Add*)
Carvacrol	1875	1000	*1.00*(*Add*)	1250	1000	*0.83*(*Add*)	1250	1000	*0.92*(*Add*)
Cinnamaldehyde	625	500	*0.85*(*Add*)	625	500	*0.75*(*Add*)	625	500	*0.79*(*Add*)

Antibiotic	***Staphylococcus epidermidis***					
	ATCC 12228			Clinical isolate		
	MIC (A)	MIC (B)	ΣFIC	MIC (A)	MIC (B)	ΣFIC
Amoxicillin	1250	0.31	1.21(Ind)	1250	3.13	1.30(Ind)
Ciprofloxacin	1250	0.31	1.08(Ind)	1250	3.13	*1.00*(*Add*)
Erythromycin	625	0.15	*0.60*(*Add*)	625	1.56	*0.54*(*Add*)
Gentamicin	625	0.78	*0.62*(*Add*)	625	1.56	*0.58*(*Add*)
Meropenem	1250	0.50	1.48(Ind)	2500	6.25	2.33(Ind)
Tetracycline	1250	1.56	1.22(Ind)	313	1.56	*0.63*(*Add*)
Miconazole	1250	1.56	1.45(Ind)	5000	6.25	3.60(Ind)

Essential oil compounds	*Staphylococcus epidermidis*					
	ATCC 12228			Clinical isolate		
	MIC (A)	MIC (B)	ΣFIC	MIC (A)	MIC (B)	ΣFIC
α-Pinene	2500	2000	2.25(Ind)	2500	2000	2.00(Ind)
γ-Terpinene	2500	2000	2.50(Ind)	2500	2000	1.25(Ind)
± Linalool	2500	2000	2.25(Ind)	2500	2000	1.25(Ind)
Eugenol	1250	1000	1.25(Ind)	1250	1000	*0.75*(*Add*)
Carvacrol	1250	1000	1.67(Ind)	1250	1000	1.70(Ind)
Cinnamaldehyde	1250	1000	*0.75*(*Add*)	625	500	*0.55*(*Add*)

Antibiotics	*Cutibacterium acnes*		
	ATCC 11827		
	MIC (A)	MIC(B)	ΣFIC
Amoxicillin	156	0.04	**0.33(Syn)**
Ciprofloxacin	1250	0.31	2.13(Ind)
Erythromycin	156	0.04	**0.41(Syn)**
Gentamicin	1250	0.31	1.25(Ind)
Meropenem	625	0.78	*0.75*(*Add*)
Tetracycline	156	0.20	2.22(Ind)
Miconazole	1250	1.56	3.25(Ind)
Essential oils compounds			
α-Pinene	1250	1000	2.33(Ind)
γ-Terpinene	1250	1000	2.33(Ind)
± Linalool	1250	1000	2.33(Ind)
Eugenol	625	500	1.25(Ind)
Carvacrol	625	500	1.50(Ind)
Cinnamaldehyde	156	125	*0.55*(*Add*)

[1]MIC(A) – Minimum inhibitory concentration of ibuprofen in combination; [2]MIC(B) – Minimum inhibitory concentration of antibiotic/EOC in combination; [3]ΣFIC – Sum of FIC values; [4]*Add* – Additive; Ind – Indifference; **Syn** – Synergism.

For *S. epidermidis*, most of the interactions were non-interactive (65%) with combinations involving erythromycin (ΣFIC 0.60) and gentamicin (ΣFIC 0.62) being the most effective. For *C. acnes* two synergistic interactions were observed for the combination of ibuprofen with erythromycin (ΣFIC 0.41) and ibuprofen with amoxicillin (ΣFIC 0.33). For the combinations involving the EOCs, 50% (18/36) were additive with the other 50% being non-interactive. For *S. aureus*, combinations of ibuprofen with carvacrol (ΣFIC 0.83 – 1.00) and

cinnamaldehyde (ΣFIC 0.75 - 0.85) were the most effective as these combinations demonstrated the lowest overall ΣFIC values. Comparatively, combinations of ibuprofen and the EOCs demonstrated better efficacy against the MRSA isolate as compared to the ATCC strains. A similar trend was observed for both *S. epidermidis* isolates and *C. acnes* with cinnamaldehyde being the most effective EOC with ibuprofen (ΣFIC 0.55 – 0.75). Ibuprofens antimicrobial activity against Gram-positive bacteria such as *S. aureus* is well documented and has been reviewed elsewhere [78, 79] however, there are limited studies which have investigated combinational effects with conventional antibiotic. A study by Chan, *et al.* [80] reported synergistic interactions (ΣFIC 0.08 - 0.50) between chloramphenicol and ibuprofen against ATCC (25923 and 33591) and clinical strains of *S. aureus*.

Another study by Tabatabaeifar, *et al.* [81] demonstrated that combinations of ibuprofen with different beta-lactams could reduce the MIC and minimum-bactericidal concentration (MBC) by between four- to 16-fold against clinical isolates of MRSA and Vancomycin-resistant *S. aureus* (VRSA). Although ibuprofen is commonly prescribed to treat acne related inflammation [82], there are no studies investigating ibuprofens antimicrobial activity against *C. acnes*. Most antibiotics that are used for skin infections also exhibit an anti-inflammatory effect which may also explain their effectiveness [83, 84]. Given the rise in resistance to these antibiotics and the capacity of *C. acnes* to worsen skin inflammation[85], a potentially effective strategy appears to be a combinational approach that may include ibuprofen.

The three synergistic interactions (Ibuprofen with meropenem, amoxicillin, and erythromycin) against the Gram-positive bacteria were analysed at different ratios (Supplementary data Table S5).

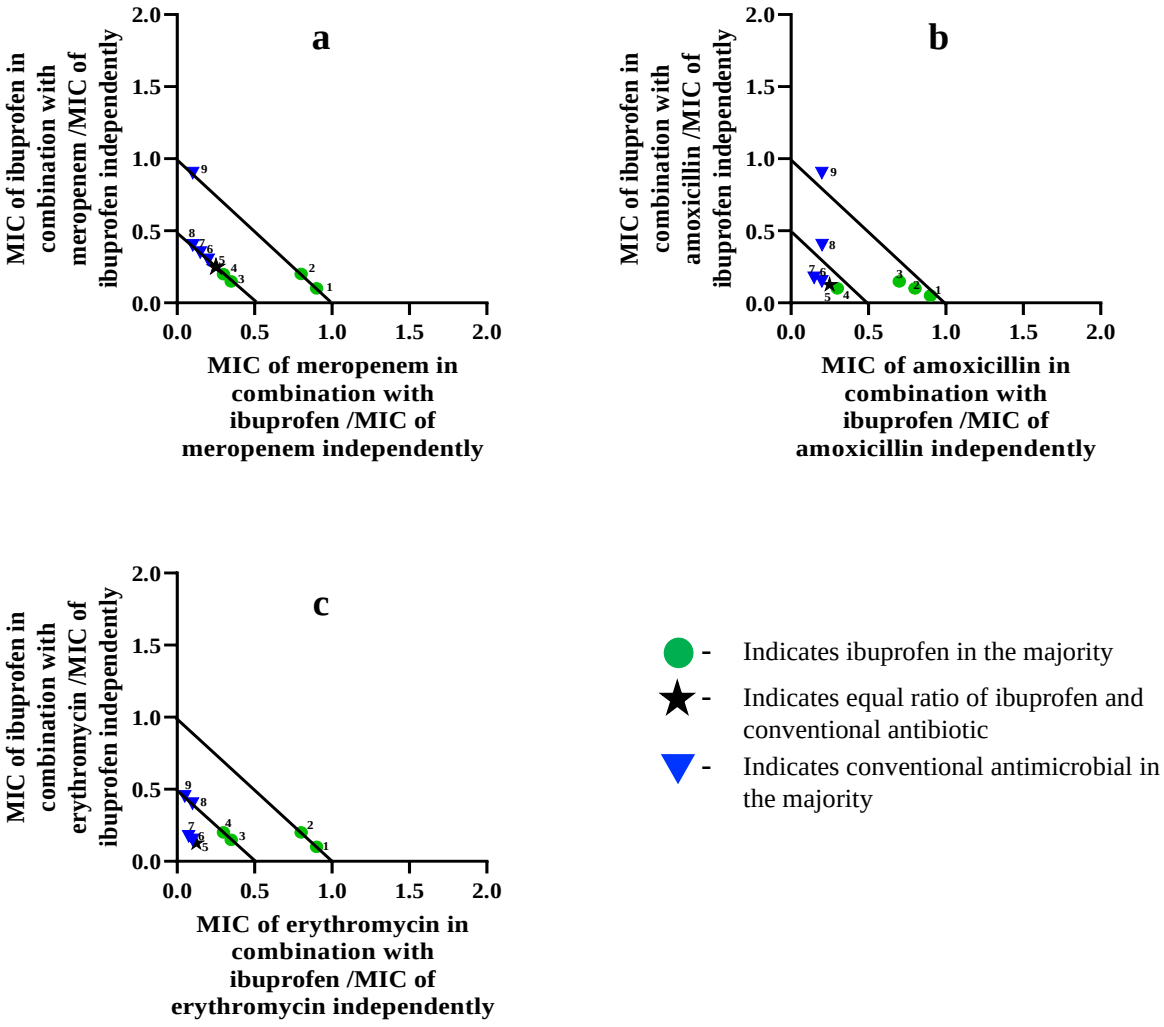

Figure 1 (a-c). Isobologram representation of the synergistic interactions between Ibuprofen and antibiotics against Gram-positive bacteria. *S. aureus* (ATCC 25923) (a); *C. acnes* (ATCC 11827) (b & c).

Figure 1a represents the combination of ibuprofen and meropenem against *S. aureus* (ATCC 25923) with ratios close to equal quantities (50:50) demonstrating the best synergy (ΣFIC 0.50). However, when meropenem was in the majority (10:90), additivity (ΣFIC 1.00) was observed. Figures 1b and 1c represent the synergistic combinations of ibuprofen with

amoxicillin and ibuprofen with erythromycin against *C. acnes* (ATCC 11827) respectively.

For the combination of ibuprofen with amoxicillin, synergy was observed when the quantities were close to equal parts (50:50). When the ratio of amoxicillin to ibuprofen exceeded (30:70) an additive interaction was observed which eventually became non-interactive (10:90). A similar trend was noted for the combination of ibuprofen with erythromycin where synergy was observed at ratios close to equal parts (50:50). Comparatively, the combination of ibuprofen with amoxicillin demonstrated synergy over more ratios as compared to ibuprofen with erythromycin.

Ibuprofen combinations with conventional antibiotic and EOCs against Gram-negative bacteria

The results for 1:1 combinations against the Gram-negative bacteria are presented in Table 3. A total of 50 combinations were studied with one interaction (2.00%) demonstrating synergy, 25 interactions (50%) additive, and 24 (48%) being non-interactive. No antagonism was observed. Combinations involving meropenem were the most effective, demonstrating additivity (ΣFIC 0.56 - 0.83) for all isolates of both bacteria.

Table 3: The MIC values (mg/L) of Ibuprofen (A) and antibiotics/EOCs (B) in combination against Gram-negative bacteria (n = 4)

Antibiotics	***Pseudomonas aeruginosa***								
	ATCC 27853			Clinical isolate 1			Clinical isolate 2		
	MIC (A)[1]	MIC (B)[2]	ΣFIC[3]	MIC (A)	MIC (B)	ΣFIC	MIC (A)	MIC (B)	ΣFIC
Ciprofloxacin	625	0.78	*0.89*(*Add*)[4]	1250	1.56	*0.87*(*Add*)	1250	1.56	*0.79*(*Add*)
Gentamicin	1250	1.56	1.25(Ind)[5]	1250	1.56	*0.87*(*Add*)	1250	1.56	*0.55*(*Add*)
Meropenem	313	0.39	*0.56*(*Add*)	1875	1.25	*0.58*(*Add*)	1250	1.56	*0.83*(*Add*)
Tetracycline	2500	6.25	*0.67*(*Add*)	5000	12.5	1.25(Ind)	5000	6.25	1.14(Ind)
Essential oil compounds									
α-Pinene	5000	4000	1.53(Ind)	5000	4000	2.00(Ind)	2500	2000	*0.75*(*Add*)
γ-Terpinene	5000	4000	1.57(Ind)	5000	4000	1.50(Ind)	5000	4000	1.50(Ind)
± Linalool	5000	4000	1.67(Ind)	2500	2000	1.50(Ind)	2500	2000	*0.75*(*Add*)
Eugenol	2500	2000	1.17(Ind)	2500	2000	1.50(Ind)	1250	1000	*0.75*(*Add*)
Carvacrol	1875	1500	1.38(Ind)	1250	1000	1.25(Ind)	1250	1000	*0.85*(*Add*)
Cinnamaldehyde	1250	1000	1.25(Ind)	625	500	*0.63*(*Add*)	1250	1000	1.25(Ind)

Antibiotics	***Acinetobacter baumannii***					
	ATCC 19606			Clinical isolate 1		
	MIC (A)	MIC (B)	ΣFIC	MIC (A)	MIC (B)	ΣFIC
Ciprofloxacin	2500	0.63	1.10(Ind)	2500	6.25	*0.58*(*Add*)
Gentamicin	625	0.78	**0.27**(**Syn**)[6]	5000	12.5	*0.75*(Add)
Meropenem	1250	1.56	0.76(*Add*)	1250	12.5	*0.63*(*Add*)
Tetracycline	1250	1.56	1.30(Ind)	2500	12.5	*0.58*(*Add*)
Essential oil compounds						
α-Pinene	2500	2000	*0.75*(*Add*)	2500	2000	*0.75*(*Add*)
γ-Terpinene	5000	4000	1.50(Ind)	5000	4000	*0.75*(Add)
± Linalool	2500	2000	1.00(Add)	3750	3000	0.75(Add)
Eugenol	2500	2000	1.17(Ind)	1250	1000	1.13(Ind)
Carvacrol	1250	1000	0.75(Add)	2500	2000	2.25(Ind)
Cinnamaldehyde	1875	1500	1.88(Ind)	1250	1000	2.13(Ind)

[1]MIC(A) – Minimum inhibitory concentration of Ibuprofen in combination; [2]MIC(B) – Minimum inhibitory concentration of antibiotic/EOC in combination; [3]ΣFIC – Sum of FIC values; [4]*Add* – Additive; [5]Ind – Indifference; [6]**Syn** – Synergism.

One synergistic interaction was observed for the reference strain of *A. baumannii* which

was ibuprofen with gentamicin (ΣFIC 0.27). However, when the same combination was tested against the clinical isolate, additivity (ΣFIC 0.75) was observed. For the EOCs, most of the interactions (15/30) were non-interactive. Combinations of ibuprofen and α-pinene displayed additivity (ΣFIC 0.75) which was especially noted for the clinical isolates of both bacteria. Both *P. aeruginosa* and *A. baumannii* are clinically problematic pathogens due to their genomic plasticity and intrinsic resistance to several antibiotic classes [86, 87]. This has made both pathogens a popular target for the investigation of novel antimicrobial agents [88, 89]. Combinations of ibuprofen and antibiotics against *P. aeruginosa* is an emerging therapeutic strategy due to ibuprofen's empiric use in cystic fibrosis [90]. Initially, it was believed that ibuprofens main function was to slow the progression of the disease and reduce inflammation, however, some studies have shown that ibuprofen may suppress virulence mechanisms and even potentiate some antibiotics [31, 91, 92]. A study by Chen, *et al.* [33] demonstrated that ibuprofen was synergistic with ceftazidime (ΣFIC = 0.35) and additive with aztreonam (ΣFIC = 0.60). The same study also demonstrated that ibuprofen-antibiotic combinations significantly improved the survivability of mice in an acute pneumonia model. Another study by Khodaparast, *et al.* [93] demonstrated a synergistic interaction (ΣFIC = 0.40) of ciprofloxacin and ibuprofen against clinical isolates of *P. aeruginosa*. Based on both these observations, combinations of ibuprofen with antibiotics may be a promising strategy to attenuate virulence factors, potentiate anti-pseudomonal antibiotics, and reduce inflammation. In contrast, there are no studies investigating the combinational effects of antibiotics and ibuprofen against *A. baumannii*. The results of this study demonstrated that ibuprofen in combination was able to reduce the MICs of the antibiotics against *A. baumannii*, which suggests that ibuprofen may represent a potential adjuvant which warrants further investigation.

The synergistic interaction of ibuprofen with gentamicin against *A. baumannii* (ATCC

19606) was analysed at different ratios (Figure 2). It was observed that ratios close to equal quantities (40:60, 50:50, and 60:40) demonstrated synergy whilst ratios with gentamicin in the majority (70:30, 80:20, and 90:10) and on the other scale (ibuprofen in the majority 10:90) were additive.

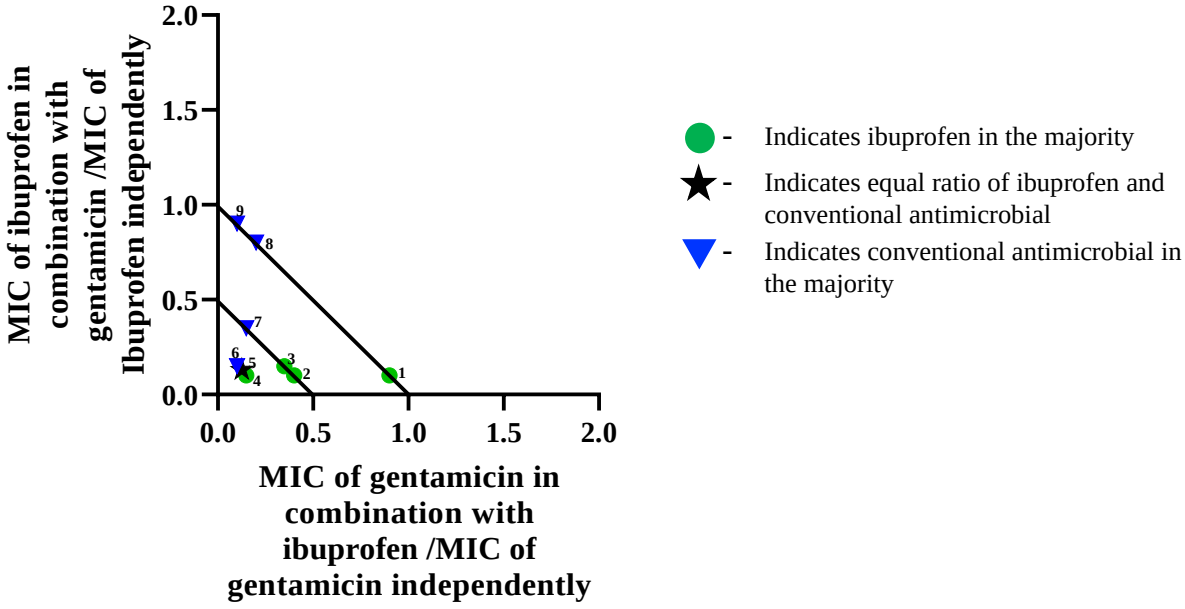

Figure 2. Isobologram representation of the synergistic interaction between ibuprofen and gentamicin against *A. baumannii* (ATCC 19606).

Ibuprofen combinations with antifungals and EOCs against C. albicans

The results for the 1:1 combinations against *C. albicans* are presented in Table 4. For both the conventional antifungals and EOCs, most of the interactions (58%) were non-interactive. However, several additive interactions (ΣFIC 0.63 - 0.93) were observed for combinations involving cinnamaldehyde. The antifungal properties of ibuprofen are well documented and have been reviewed extensively by Babaei, *et al.* [94]. Several studies have demonstrated synergistic interactions between ibuprofen and different azole antifungals against

clinical strains of *C. albicans* [30, 95, 96].

Table 4: The MIC values of ibuprofen (A) and antifungals/EOCs (B) in combination against *C. albicans* (*n* = 4)

Antifungals	*Candida albicans*								
	ATCC 10231			Clinical isolate 1			Clinical isolate 2		
	MIC (A)[1]	MIC (B)[2]	ΣFIC[3]	MIC (A)	MIC (B)	ΣFIC	MIC (A)	MIC (B)	ΣFIC
Miconazole	2500	0.63	2.20(Ind)[4]	2500	3.13	1.14(Ind)	2500	3.13	*0.90*(*Add*)[5]
Nystatin	1250	1.56	2.00(Ind)	2500	3.13	1.25(Ind)	2500	3.13	*0.83* (*Add*)
Essential oil compounds									
α-Pinene	1250	1000	1.33(Ind)	2500	2000	1.67(Ind)	2500	2000	1.50 (Ind)
γ-Terpinene	625	500	*0.60*(*Add*)	2500	2000	1.33(Ind)	2500	2000	*1.00*(*Add*)
± Linalool	1250	1000	1.40(Ind)	2500	2000	1.50(Ind)	2500	2000	*1.00*(*Add*)
Eugenol	625	500	*0.83*(*Add*)	1250	1000	1.50(Ind)	1250	1000	*0.75*(*Add*)
Carvacrol	1250	1500	2.50(Ind)	625	500	1.25(Ind)	625	500	1.13(Ind)
Cinnamaldehyde	156	125	*0.63*(*Add*)	313	250	*0.93*(*Add*)	625	500	*0.63*(*Add*)

[1]MIC(A) – Minimum inhibitory concentration of Ibuprofen in combination; [2]MIC(B) – Minimum inhibitory concentration of antibiotic/EOC in combination; [3]ΣFIC – Sum of FIC values; [4]Ind – Indifference; [5]*Add* – Additive.

Combination therapy for candidiasis is becoming more frequent due to the increase in antifungal resistance [97]. Unlike antibiotics, there are no clinically approved adjuvants for antifungal drugs. Several studies have highlighted the potential of ibuprofen as an adjuvant considering its ability to reverse azole resistance and modulate fungal virulence [77, 98, 99]. Combinations of ibuprofen with eugenol and cinnamaldehyde demonstrated additivity (ΣFIC 0.63 - 0.93) and may represent an adjuvant-adjuvant combination. Adjuvant-adjuvant combinations are poorly understood drug combinations and are based on the principle that two individual compounds with limited antimicrobial activity, enhance each other to produce a greater antimicrobial effect [16]. Most of the evidence describing these combinations is limited to synthetic compounds and serendipitous interactions between non-antibiotics or natural products [100, 101]. Additionally, adjuvant-adjuvant combinations have also been proposed as a potential method to induce collateral sensitivity in MDR pathogens [102, 103], however existing

studies have predominantly focussed on antibiotic combinations [104, 105], with investigation using non-antibiotic combinations still warranted.

Combinations – Triple (1:1:1)

Based on the synergistic interaction between either the EOC or conventional antimicrobial with ibuprofen, combinations, further 1:1:1 combinations (Ibuprofen, antibiotics, and EOCs) were investigated (Table 5). For the synergistic interactions against *S. aureus*, five out of the six were non-interactive. Two combinations of ibuprofen and antibiotics were synergistic against *C. acnes*. The combination of ibuprofen and amoxicillin demonstrated several additive interactions (ΣFIC 0.52 – 0.92) with one synergistic interaction for eugenol (ΣFIC 0.47). Most interactions for the combination of ibuprofen and erythromycin were non-interactive except for cinnamaldehyde which was additive (ΣFIC 0.90). For *A. baumannii*, two synergistic interactions were observed for combinations containing α-pinene (ΣFIC 0.44) and γ-terpinene (ΣFIC 0.44).

The investigation of triple combinations represents a type of higher-order combination that has the potential to simultaneously amplify individual drug effects and overcome multiple resistance mechanisms in a single organism [100]. The clinical use of higher order combinations against *A. baumannii* has become the accepted treatment due to its high antimicrobial resistance profile [70, 106]. In recent years, the clinical use of adjuvant agents against *A. baumannii* has become more frequent with the investigation and implementation of new beta-lactamase inhibitors [107]. Given the clinical success of antibiotic-adjuvant combinations, it may be worth investigating compounds that may target other mechanisms of resistance or stimulate host responses against *A. baumannii*.

Table 5: Triple combinations of ibuprofen (A), conventional antimicrobial (B), and EOC (C) against selected pathogens (*n* =4)

Micro-organism	Synergistic combination	Essential oil compounds	MIC (A)[1]	MIC (B)[2]	MIC (C)[3]	ΣFIC[4]
S. aureus (ATCC 25923)	Ibuprofen + Meropenem	α-Pinene	5000	1.25	4000	2.23 (Ind)[5]
		γ-Terpinene	5000	1.25	4000	2.34 (Ind)
		± Linalool	2500	1.56	1000	1.56 (Ind)
		Eugenol	625	0.78	500	*0.69* (*Add*)[6]
		Carvacrol	2500	1.56	2000	1.86 (Ind)
		Cinnamaldehyde	625	0.78	500	*1.00* (*Add*)
C. acnes (ATCC 11827)	Ibuprofen + Amoxicillin	α-Pinene	1250	1.56	1000	3.68 (Ind)
		γ-Terpinene	625	0.78	500	1.84 (Ind)
		± Linalool	313	0.39	250	*0.92* (Add)
		Eugenol	156	0.20	125	**0.47 (Syn)[7]**
		Carvacrol	156	0.20	125	*0.52* (*Add*)
		Cinnamaldehyde	156	0.20	125	*0.63* (*Add*)
C. acnes (ATCC 11827)	Ibuprofen + Erythromycin	α-Pinene	625	0.78	500	2.91 (Ind)
		γ-Terpinene	625	0.78	500	2.91 (Ind)
		± Linalool	625	0.78	500	2.91 (Ind)
		Eugenol	313	0.39	250	1.49 (Ind)
		Carvacrol	313	0.39	250	1.57 (Ind)
		Cinnamaldehyde	156	0.20	125	*0.90* (*Add*)
A. baumannii (ATCC 19606)	Ibuprofen + Gentamicin	α-Pinene	1250	1.56	1000	**0.44 (Syn)**
		γ-Terpinene	1250	1.56	1000	**0.44 (Syn)**
		± Linalool	2500	3.13	2000	1.04 (Ind)
		Eugenol	2500	3.13	2000	1.15 (Ind)
		Carvacrol	2500	3.13	2000	1.37 (Ind)
		Cinnamaldehyde	2500	3.13	2000	2.03 (Ind)

[1]MIC(A) – Minimum inhibitory concentration of ibuprofen in combination; [2]MIC(B) – Minimum inhibitory concentration of conventional antimicrobial in combination; [3]MIC (C) – Minimum inhibitory concentration of EOC in combination; [4]ΣFIC – Sum of FIC values. [5]Ind – Indifference; [6]*Add* – Additive; [7]**Syn** – Synergistic.

A study by Laudy, *et al.* [72] demonstrated that NSAIDs including ibuprofen may enhance or inhibit efflux pumps against several species of Gram-negative bacteria. Efflux pumps are a diverse group of inner membrane proteins which extrude antibiotics or other pollutants from the bacterial cell and thus contribute to multi-drug resistance [108]. The ability of ibuprofen to interact with the functioning of efflux pumps represents a promising observation and may represent a potential target for a novel adjuvant. Combination therapy against *C. acnes* is common and has been predominantly studied for acne vulgaris [109]. The pathogenesis of acne is

multifactorial and is generally treated with antimicrobial agents such as oral antibiotics and/or topical ointments and washes [110, 111]. However, the rates of antimicrobial resistance in *C. acnes* have increased [112] and have also become more frequent in non-cutaneous associated infections [113, 114]. The results of the triple combinations against *C. acnes* support the concept of using combinations involving both antibiotics and non-antibiotic agents which represents a multitargeted approach and may warrant further investigation.

Cytotoxicity of ibuprofen

The cytotoxicity of ibuprofen at both low and high concentrations and combinations on HaCAT keratinocytes is presented in Table 6. From the results, the cytotoxicity was concentration dependent with low doses (1.25, 2.5, and 12.5 mg/L) demonstrating high cell viability and high doses (125 and 1250 mg/L) demonstrating low cell viability. The concentration of 1250 mg/L was statistically significant (p = 0.04), when compared to the untreated control.

Table 6: Cell viability (%; mean ± SD) of ibuprofen (n = 4) on HaCAT keratinocytes

Concentrations of ibuprofen (mg/L)	**% Cell viability**	**Interpretation of cytotoxicity**
1.25	95.60 ± 0.09	Non-toxic[1]
2.5	94.00 ± 5.60	Non-toxic
12.5	92.87 ± 2.22	Non-toxic
125	81.41 ± 4.60	Slightly cytotoxic
1250	14.91 ± 8.50*****	Severely cytotoxic
Untreated cells	100 ± 2.45	-
Melphalan (100 µM)	7.29 ± 2.75	-
Vehicle control (1.25% DMSO)	91.74 ± 3.12	-

*****($p < 0.05$) indicates statistical significance when compared to the vehicle control. [1]Cytotoxicity interpreted based on cell viability: > 90% (Non-toxic), 60-90% (Slightly cytotoxic), 30–59% (Moderately toxic), and < 30% (Severely toxic) [116].

A study by Melendez-Ortiz, *et al.* [115] demonstrated that cell viability of RAW 264.7 murine macrophages remain above 90% provided that the concentration of ibuprofen does not

exceed 400 mg/L.

The cytotoxicity of ibuprofen is well-documented in clinical practice [117]. The most common adverse effect of ibuprofen is the development of gastrointestinal (GI) tract ulcers and associated haemorrhaging [118]. However, the risk of developing GI ulcers at over-the-counter dosages is low and is only achievable at very high concentrations (> 2400 mg/day)[119]. Cutaneous reactions from topical use of ibuprofen have also been reported with most cases being mild and superficial and have been reviewed by Rainsford [118].

Cytotoxicity of combinations

All combinations demonstrated a high cell viability indicating a non-toxic effect. Based on the criteria outlined by Dahl, *et al.* [116] compounds with cell viabilities above 90% are considered non-toxic. For the CI, all combinations were interpreted as additive (CI = ≤ 0.80 - ≤ 1.20). From these results, it can be proposed that when ibuprofen is combined in pairwise combinations with conventional antimicrobials, the combined cytotoxicity is the same as the sum of the individual drugs.

For the triple combinations, all combinations demonstrated slightly cytotoxic (60-90% Cell viability) effects on HaCAT keratinocytes. In comparison to the pairwise combinations (Supplementary data Figure S1-S3), the triple combinations demonstrated similar cytotoxic effects as pairwise combinations and individual compounds. All triple combinations demonstrated an additive effect which was based on the combination index. Comparatively, the triple combinations demonstrated higher cytotoxic effects on the RAW264.7 macrophages as compared to the HaCAT keratinocytes (Supplementary data figures S1-S3). The pairwise combinations demonstrated similar cytotoxic responses as the triple combinations, especially for combinations with the EOCs.

Table 7: Cell viability (%; mean ± SD) and combination index (CI) of synergistic combinations (n = 4) on HaCAT keratinocytes

Combinations	% Cell viability	Interpretation of cytotoxicity	Combination index (CI)
Ibuprofen (25 mg/L) + Amoxicillin (0.31 mg/L)	100.06 ± 5.31	Non-toxic[1]	0.94 (*Add*)[2]
Ibuprofen (50 mg/L) + Gentamicin (3.13 mg/L)	100.21 ± 5.66	Non-toxic	0.88 (*Add*)
Ibuprofen (6.25 mg/L) + Erythromycin (0.04 mg/L)	93.55 ± 8.14	Non-toxic	0.97 (*Add*)
Ibuprofen (25 mg/L) + Meropenem (0.78 mg/L)	100.15 ± 5.14	Non-toxic	1.01 (*Add*)
Ibuprofen (6.25 mg/L) + Amoxicillin (0.31 mg/L) + Eugenol (50 mg/L)	77.59 ± 10.59	Slightly cytotoxic	0.95 (*Add*)
Ibuprofen (25 mg/L) + Gentamicin (3.13 mg/L) + α-pinene (25 mg/L)	82.79 ± 8.52	Slightly cytotoxic	1.10 (*Add*)
Ibuprofen (25 mg/L) + Gentamicin (3.13 mg/L) + γ-terpinene (25 mg/L)	90.44 ± 5.70	Slightly cytotoxic	1.01 (*Add*)
Untreated cells	100 ± 5.15	N/A	N/A
Vehicle control (1.25% DMSO)[3]	93.45 ± 2.17	N/A	N/A

[1]Cytotoxicity interpreted from % cell viability: > 90% (Non-toxic), 60-90% (Slightly cytotoxic), 30–59% (Moderately toxic), and < 30% (Severely toxic) [116]. [2]**Syn** (Synergism, CI < 0.80); *Add* (Additivity, CI = ≤ 0.80 - ≤ 1.20); Ant (Antagonism, CI > 1.20).

Anti-inflammatory properties of combinations

The anti-inflammatory properties of the combinations that demonstrated synergy in the antimicrobial combinations were analysed (Table 8). For the 1:1 combination, none of the combinations significantly reduced the nitric oxide production with all interactions demonstrating additivity (ΣFa 0.98). In comparison to the pairwise combinations, the triple combinations demonstrated increased cytotoxicity which impaired the macrophages' ability to produce nitric oxide. None of the controls impaired the growth of the cells.

Table 8: Nitrite production (mean ± SD) and fractional effect (ΣFa) of synergistic combinations (n = 4) on RAW264.7 macrophages

Combinations	Nitrite production (µM)	% Reduction in nitrite production	Fractional effect (ΣFa)
Ibuprofen (25 mg/L) + Amoxicillin (0.31 mg/L)	35.47 ± 1.02	3.01	0.98 (*Add*)[2]
Ibuprofen (25 mg/L) + Gentamicin (3.13 mg/L)	35.27 ± 1.74	3.57	0.98 (*Add*)
Ibuprofen (6.25 mg/L) + Erythromycin (0.04 mg/L)	35.47 ± 1.09	3.01	0.98 (*Add*)
Ibuprofen (25 mg/L) + Meropenem (0.78 mg/L)	36.92 ± 1.59	N/A[3]	N/A
Ibuprofen (6.25 mg/L) + Amoxicillin (0.31 mg/L) + Eugenol (50 mg/L)	Cytotoxic[1]	N/A	N/A
Ibuprofen (25 mg/L) + Gentamicin (3.13 mg/L) + α-pinene (25 mg/L)	Cytotoxic	N/A	N/A
Ibuprofen (25 mg/L) + Gentamicin (3.13 mg/L) + γ-terpinene (25 mg/L)	Cytotoxic	N/A	N/A
Untreated cells	7.88 ± 0.32	N/A	N/A
LPS control	36.57 ± 2.53	N/A	N/A
Amino-guanidine (AG)	19.26 ± 0.26	47.33	N/A

[1]Combination demonstrated high cytotoxicity (< 10% Cell viability). [2]**Syn** (Synergism, CI < 0.80); *Add* (Additivity, CI = ≤ 0.80 - ≤ 1.20); Ant (Antagonism, CI > 1.20). [3]N/A – Not applicable. LPS was added to all combinations except for the untreated cells.

Ibuprofen is a well-established anti-inflammatory drug with its ability to modulate prostaglandin synthesis being well documented [20]. However, ibuprofen may also exhibit additional anti-inflammatory effects such as reducing the accumulation and activation of leukocytes and reducing the production of inflammogens such as nitric oxide. Nitric oxide is an important inflammatory mediator that has a dual function: at physiological concentrations it regulates house-keeping functions, whereas its overproduction causes a cytotoxic effect [120]. A study by De Palma, *et al.* [121] demonstrated that ibuprofen can reduce the production of nitric

oxide in HeLa cells and showed that ibuprofens ability to modulate the nitric oxide pathway may depend on arginine.

In an *in vivo* setting, L-arginine is reduced to L-citrulline which causes the release of free nitric oxide ions [122]. Reduced availability of L-arginine impairs ibuprofens ability to interact with the nitric oxide pathway [121]. This may support the results of low reduction in nitric oxide.

Conclusion

The combination of ibuprofen with conventional antimicrobials and EOCs may offer potential advantages in managing resistant infections. From the results, 152 ibuprofen combinational interactions were studied with four (2%) synergistic, 68 (45%) additive, and 80 (68%) non-interactive with no antagonism. Triple combinations comprising ibuprofen, gentamicin, and both α-pinene and γ-terpinene were synergistic against *A. baumannii*. For the anti-inflammatory assay, none of the combinations demonstrated antagonism which suggests that conventional antimicrobials do not impair the anti-inflammatory properties of ibuprofen. All triple combinations demonstrated an increased cytotoxic effect especially on the RAW264.7 cell line to an extent whereby reliable anti-inflammatory activity could not be evaluated. Combination therapies represents a proven strategy to combat multidrug resistance and restore the efficacy of redundant antimicrobials. Considering that both ibuprofen and essential oil compounds can elicit multifunctional pharmacological effects, further investigations is warranted to investigate the underlying mechanisms and optimized dosing.

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Supplementary data

Table S1: Antibiogram for reference and clinical strains

Antibiotic	Antibiotic abbreviation	Micro-organisms									
		S. aureus (ATCC 25923)	*S. aureus* (ATCC 6538)	*S. aureus* (Clinical isolate)	*S. epidermidis* (ATCC 12228)	*S. epidermidis* (Clinical isolate)	*P. aeruginosa* (ATCC 27853)	*P. aeruginosa* (Clinical isolate 1)	*P. aeruginosa* (Clinical isolate 2)	*A. baumannii* (ATCC 19606)	*A. baumannii* (Clinical isolate)
Amikacin	AK30	S	S	R	S	S	S	S	S	S	I
Amoxicillin	AML10	S	I	R	S	S	R	R	R	R	R
Amoxicillin – Clavulanic acid	AMC3	S	S	R	S	S	R	R	R	S	R
Ampicillin	AMP10	S	S	R	S	S	R	R	R	R	R
Ceftriaxone	CRO30	S	S	R	S	S	R	R	R	S	R
Cephalexin	CL30	S	I	R	S	R	R	R	R	R	R
Cephalothin	KF30	S	S	R	S	S	R	R	R	R	R
Chloramphenicol	C30	S	S	R	S	S	S	R	R	S	R
Ciprofloxacin	CIP5	S	S	R	S	S	S	S	S	S	R
Clindamycin	DA2	S	S	S	S	S		R	R	R	R
Erythromycin	E10	S	S	R	S	R	R	R	R	R	R
Fosfomycin	FOS5	S	S	I	S	R	R	R	R	R	R
Gentamicin	CN10	S	I	R	S	S	S	R	R	S	R
Meropenem	MEM10	S	S	I	S	S	S	S	S	S	R
Neomycin	N10	S	I	R	S	R	R	R	R	R	R
Penicillin	P10	S	S	R	S	S	R	R	R	R	R
Piperacillin-Tazobactam	TZP36	S	S	R	S	S	S	S	S	S	R
Sulfamethoxazole-Trimethoprim	SXT	S	S	R	S	S	R	R	R	R	R
Tetracycline	TE30	S	S	R	S	S	S	R	R	S	R
Vancomycin	VA5	S	S	S	S	S	R	R	R	R	R

***S** – Susceptible = Microbe is "susceptible" to the antibiotic
I – Intermediate = Microbe is somewhat "susceptible" to the antibiotic which can further be described as dose-dependent. MICs values may exceed breakpoints outlined in CLSI and EUCAST.
R – Resistant = Microbe is resistant to antibiotic

Table S2: Antibiogram for reference and clinical isolates of *C. albicans*

Antifungal	**Antifungal abbreviation**	**Yeasts**		
		C. albicans (ATCC 10231)	*C. albicans* (Clinical isolate 1)	*C. albicans* (Clinical isolate 2)
Amphotericin B	AMB	S	S	S
Nystatin	NY	S	S	I
Fluconazole	FLU	S	R	R
Miconazole	MCL	S	R	R
Caspofungin	CAS	S	S	S

Table S3: The mean MIC values (µg/mL) and standard deviation (±SD) of conventional antimicrobials and essential oil compounds against the reference strain pathogens (n = 6)

Conventional antimicrobials	**Micro-organisms**					
	S. aureus (ATCC 25923)	*S. epidermidis* (ATCC 12228)	*C. acnes* (ATCC 11827)	*P. aeruginosa* (ATCC 27853)	*A. baumannii* (ATCC 19606)	*C. albicans* (ATCC 10231)
Amoxicillin	0.90 ± 0.13	0.51 ± 0.16	0.24 ± 0.09	NS[a]	NS	NS
Ciprofloxacin	1.06 ± 0.24	0.94 ± 0.36	1.25 ± 0.00	0.57 ± 0.28	0.52 ± 0.16	NS
Erythromycin	0.63 ± 0.00	0.42 ± 0.22	0.12 ± 0.06	NS	NS	NS
Gentamicin	1.41 ± 0.18	3.13 ± 0.00	1.56 ± 0.00	0.78 ± 0.00	2.73 ± 0.78	NS
Meropenem	0.60 ± 0.00	0.52 ± 0.45	0.20 ± 0.00	0.78 ± 0.00	1.53 ± 0.31	NS
Tetracycline	1.25 ± 0.00	1.25 ± 0.00	0.70 ± 0.09	18.75 ± 7.21	3.91 ± 1.91	NS
Miconazole	1.88 ± 0.96	1.72 ± 0.64	0.63 ± 0.00	NS	NS	0.78 ± 0.29
Nystatin	NS	NS	NS	NS	NS	1.56 ± 0.63
Essential oil compounds						
α-Pinene	6000 ± 0.00	4000 ± 0.00	1500 ± 577	3750 ± 250	4000 ± 0.00	1500 ± 577
γ-Terpinene	4000 ± 0.00	3000 ± 1154	1500 ± 577	3500 ± 1788	4000 ± 0.00	2500 ± 1000
± Linalool	2750 ± 1035	4000 ± 0.00	1500 ± 547	3000 ± 1673	2000 ± 0.00	1250 ± 500
Eugenol	1667 ± 517	2000 ± 0.00	1000 ± 0.00	1500 ± 837	1500 ± 837	750 ± 288
Carvacrol	2000 ± 0.00	750 ± 274	500 ± 0.00	750 ± 478	1000 ± 0.00	500 ± 0.00
Cinnamaldehyde	417 ± 129	1000 ± 250	208 ± 65.0	500 ± 0.00	500 ± 0.00	125 ± 0.00
Negative control	>8000 ± 0.00	>8000 ± 0.00	>8000 ± 0.00	>8000 ± 0.00	>8000 ± 0.00	>8000 ± 0.00
Culture control	>8000 ± 0.00	>8000 ± 0.00	>8000 ± 0.00	>8000 ± 0.00	>8000 ± 0.00	>8000 ± 0.00

[a]Not susceptible

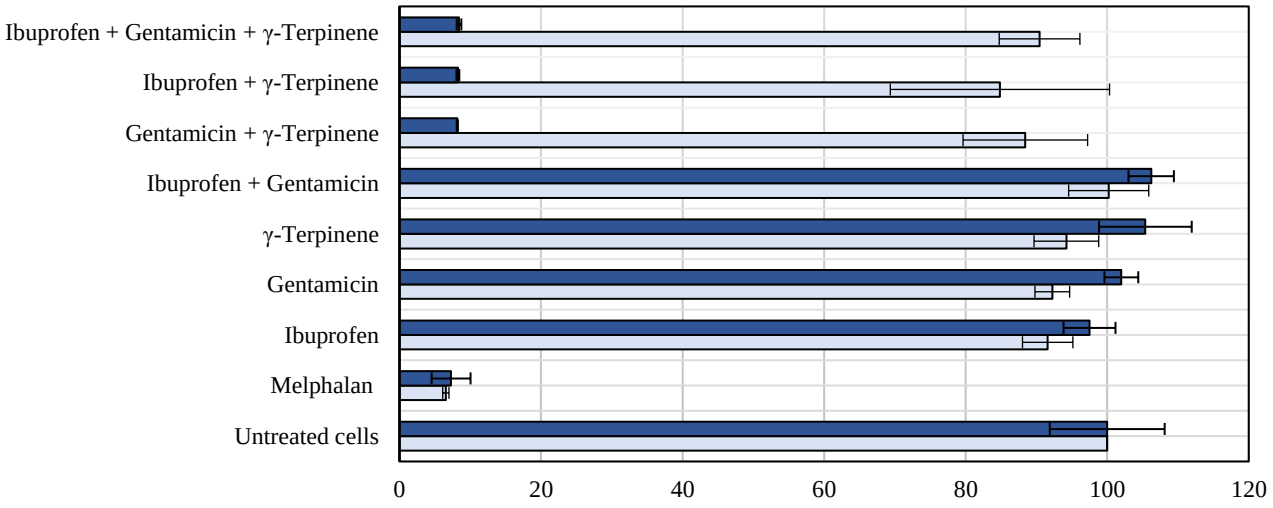
Table S4: The mean MIC values (µg/mL) and standard deviation (±SD) of conventional antimicrobials and essential oil compounds against the clinical strain pathogens (n = 6)

Conventional antimicrobials	**Micro-organisms**							
	S. aureus (ATCC 6538)	MRSA (Clinical)	*S. epidermidis* (Clinical)	*P. aeruginosa* (Clinical 1)	*P. aeruginosa* (Clinical 2)	*A. baumannii* (Clinical)	*C. albicans* (Clinical 1)	*C.albicans* (Clinical 2)
Amoxicillin	3.13 ± 0.00	4.69 ± 1.80	1.95 ± 0.78	NS[a]	NS[a]	NS	NS	NS
Ciprofloxacin	1.25 ± 0.00	2.81 ± 0.34	1.56 ± 0.00	1.56 ± 0.00	1.46 ± 0.18	9.34 ± 3.60	NS	NS
Erythromycin	2.25 ± 0.10	1.95 ± 0.78	2.73 ± 0.78	NS[a]	NS	NS	NS	NS
Gentamicin	2.50 ± 0.00	5.13 ± 1.30	2.34 ± 1.11	3.13 ± 0.00	2.60 ± 0.90	12.50 ± 3.13	NS	NS
Meropenem	1.25 ± 0.00	3.06 ± 0.07	1.56 ± 0.00	1.25 ± 0.00	2.50 ± 0.00	18.75 ± 4.84	NS	NS
Tetracycline	1.50 ± 0.50	2.49 ± 0.86	2.34 ± 0.90	14.05 ± 1.70	21.88 ± 7.21	31.25 ± 0.00	NS	NS
Miconazole	3.13 ± 0.00	4.69 ± 1.80	1.95 ± 0.78	NS[a]	NS	NS	10.94 ± 3.10	3.91 ± 1.56
Nystatin	NS	NS	NS	NS[a]	NS	NS	6.25 ± 0.00	4.69 ± 1.80
Essential oil compounds								
α-Pinene	2000 ± 0.00	2000 ± 0.00	1000 ± 0.00	4000 ± 0.00	4000 ± 0.00	4000 ± 0.00	1500 ± 577	2000 ± 0.00
γ-Terpinene	4000 ± 0.00	4000 ± 0.00	4000 ± 0.00	4000 ± 0.00	4000 ± 0.00	8000 ± 0.00	4000 ± 0.00	4000 ± 0.00
± Linalool	1000 ± 0.00	2000 ± 0.00	4000 ± 0.00	4000 ± 0.00	4000 ± 0.00	4000 ± 0.00	4000 ± 0.00	1000 ± 0.00
Eugenol	2000 ± 0.00	2000 ± 0.00	2000 ± 0.00	1000 ± 0.00	1000 ± 0.00	1500 ± 837	1000 ± 0.00	1000 ± 0.00
Carvacrol	1500 ± 500	750 ± 288	417 ± 144	500 ± 0.00	833 ± 288	1000 ± 0.00	1000 ± 0.00	500 ± 151
Cinnamaldehyde	500 ± 0.00	375 ± 144	833 ± 288	500 ± 0.00	500 ± 0.00	500 ± 0.00	2000 ± 0.00	2000 ± 0.00
Negative control	>8000 ± 0.00	>8000 ± 0.00	>8000 ± 0.00	>8000 ± 0.00	>8000 ± 0.00	>8000 ± 0.00	>8000 ± 0.00	>8000 ± 0.00
Culture control	>8000 ± 0.00	>8000 ± 0.00	>8000 ± 0.00	>8000 ± 0.00	>8000 ± 0.00	>8000 ± 0.00	>8000 ± 0.00	>8000 ± 0.00

[a]Not susceptible

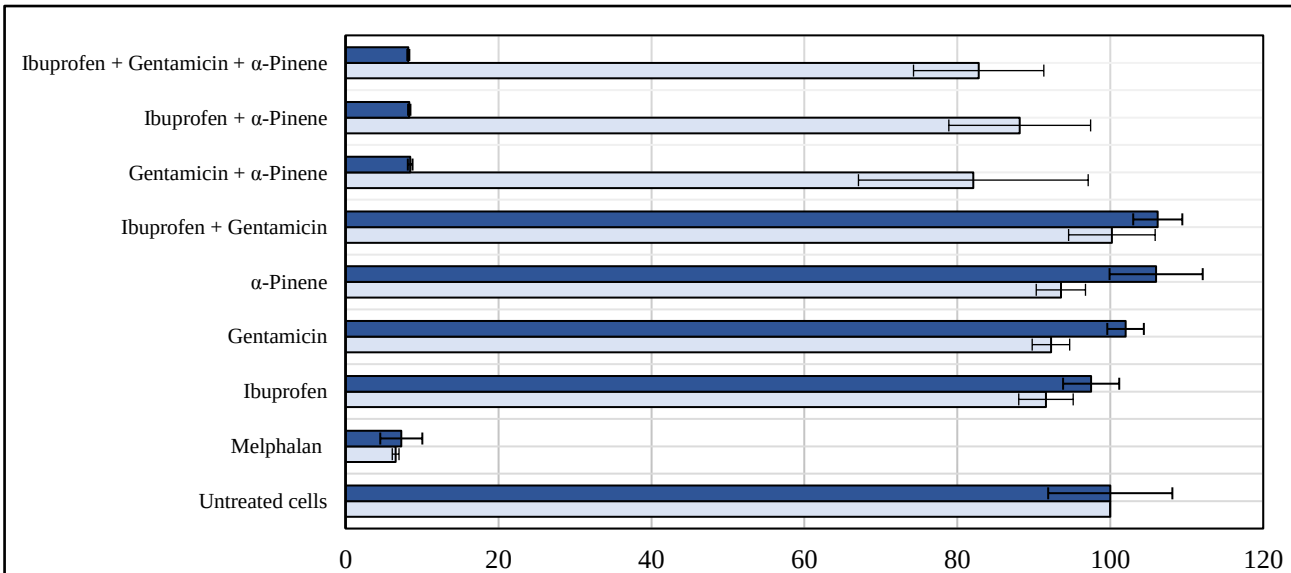
Table S5: Synergistic interactions (Isobologram ratios) against *S. aureus*, *C. acnes* and *A. baumannii*

Isobologram plot number	Ratio volumes (µl) (Ibuprofen: Antibiotic)	*S. aureus* (ATCC 25923)		*C. acnes* (ATCC 11827)		*C. acnes* (ATCC 11827)		*A. baumannii* (ATCC 19606)	
		Ibuprofen + Meropenem		Ibuprofen + Amoxicillin		Ibuprofen + Erythromycin		Ibuprofen + Gentamicin	
		MIC of ibuprofen in combination (µg/mL)	MIC of antibiotic in combination (µg/mL)	MIC of ibuprofen in combination (µg/mL)	MIC of antibiotic in combination (µg/mL)	MIC of ibuprofen in combination (µg/mL)	MIC of antibiotic in combination (µg/mL)	MIC of ibuprofen in combination (µg/mL)	MIC of antibiotic in combination (µg/mL)
1	90:10	0.90	0.10	0.90	0.05	0.90	0.10	0.90	0.10
2	80:20	0.80	0.20	0.80	0.10	0.80	0.20	0.40	0.10
3	70:30	0.35	0.15	0.70	0.15	0.35	0.15	0.35	0.15
4	60:40	0.30	0.20	0.30	0.10	0.30	0.20	0.15	0.10
5	50:50	0.25	0.25	0.25	0.13	0.12	0.13	0.13	0.13
6	40:60	0.20	0.30	0.20	0.15	0.10	0.15	0.10	0.15
7	30:70	0.15	0.35	0.15	0.18	0.07	0.18	0.15	0.35
8	20:80	0.10	0.40	0.20	0.40	0.10	0.40	0.20	0.80
9	10:90	0.10	0.90	0.20	0.90	0.05	0.45	0.10	0.90



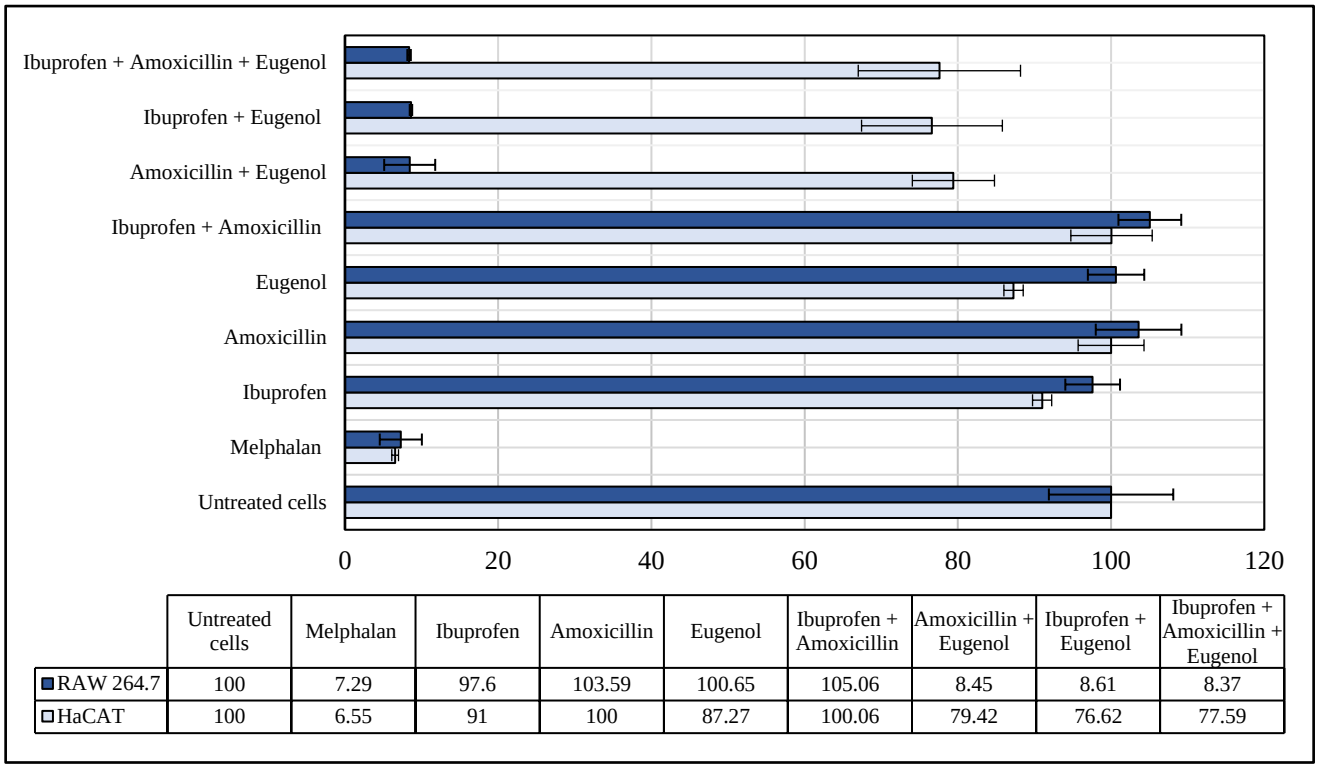
	Untreated cells	Melphalan	Ibuprofen	Gentamicin	γ-Terpinene	Ibuprofen + Gentamicin	Gentamicin + γ-Terpinene	Ibuprofen + γ-Terpinene	Ibuprofen + Gentamicin + γ-Terpinene
RAW 264.7	100	7.29	97.5	102	105.39	106.21	8.17	8.25	8.41
HaCAT	100	6.55	91.59	92.24	94.23	100.21	88.43	84.84	90.44

Figure S1: Cell viability of net interactions (Ibuprofen + amoxicillin + eugenol) against HaCAT and RAW 264.7 cell lines



	Untreated cells	Melphalan	Ibuprofen	Gentamicin	α-Pinene	Ibuprofen + Gentamicin	Gentamicin + α-Pinene	Ibuprofen + α-Pinene	Ibuprofen + Gentamicin + α-Pinene
RAW 264.7	100	7.29	97.5	102	106	106.21	8.45	8.33	8.21
HaCAT	100	6.55	91.59	92.24	93.54	100.21	82.09	88.16	82.79

Figure S2: Cell viability of net interactions (Ibuprofen + gentamicin + α-pinene) against HaCAT and RAW 264.7 cell lines



	Untreated cells	Melphalan	Ibuprofen	Amoxicillin	Eugenol	Ibuprofen + Amoxicillin	Amoxicillin + Eugenol	Ibuprofen + Eugenol	Ibuprofen + Amoxicillin + Eugenol
RAW 264.7	100	7.29	97.6	103.59	100.65	105.06	8.45	8.61	8.37
HaCAT	100	6.55	91	100	87.27	100.06	79.42	76.62	77.59

Figure S3: Cell viability of net interactions (Ibuprofen+ gentamicin + γ-terpinene) against HaCAT and RAW 264.7 cell lines

CHAPTER 6: RECOMMENDATIONS, LIMITATIONS AND CONCLUSION

6.1 Study highlights

Publication one: Essential oil compounds in combination with conventional antibiotics for dermatology

The antimicrobial effects of six essential oil compounds with eight conventional antimicrobials were investigated against skin pathogens. A total of 186 combinations (126 against Gram-positive, 48 against Gram-negative, and 12 against *C. albicans*) were studied. The following highlights were observed:

- Miconazole demonstrated antibacterial properties against *S. aureus*, *S. epidermidis*, and *C. acnes* and in combination with the EOCs.

- Cinnamaldehyde demonstrated the strongest antimicrobial properties, whilst γ-terpinene was the least effective against all studied micro-organisms.

- Seven synergistic interactions were identified against the Gram-positive bacteria (Four against *S. aureus*, two against *S. epidermidis*, and one against *C. acnes*).

- One synergistic interaction (ciprofloxacin with cinnamaldehyde) was identified against *P. aeruginosa.*

- Synergistic interactions were observed at varying ratios, with the highest degrees of synergy observed at ratios close or equal to equivalence (50:50)

- Carvacrol (LC_{50} = 64.43 µg/mL (24 hrs) and LC_{50} = 37.14 µg/mL (48 hrs)) demonstrated the highest toxicity, whilst α-pinene (LC_{50} = >1000 µg/mL (24 and 48 hrs) and γ-terpinene (LC_{50} = >1000 µg/mL (24 and 48 hrs)) were the least toxic.

- When the essential oil compounds were combined with the conventional antibiotics,

the combination demonstrated a reduced toxicity.

- The combination of amoxicillin and eugenol demonstrated the lowest toxicity (LC_{50} = 1081 µg/mL) and the highest selectivity index (14.41) when in a (70:30) ratio.

Publication two: Cytotoxicity and anti-inflammatory properties of essential oil compounds in combination with conventional antimicrobials in dermatology

The cytotoxicity and anti-inflammatory properties of conventional antimicrobials, essential oil compounds, and synergistic antimicrobial combinations were investigated. The following highlights were observed:

- At high concentrations (> 3.13 µg/mL), tetracycline and miconazole elicited toxic effects on HaCAT keratinocytes.

- Amoxicillin, ciprofloxacin, and erythromycin demonstrated high cell viability even at high concentrations (12.5 µg/mL).

- The cytotoxicity properties of the essential oil compounds were dose dependent.

- Cinnamaldehyde demonstrated the highest cytotoxicity (IC_{50} = 28.6 µg/mL), whilst linalool was the least toxic (IC_{50} = 274 µg/mL) on HaCAT keratinocytes.

- All combinations demonstrated reduced cytotoxicity.

- The combination of ciprofloxacin with cinnamaldehyde demonstrated the lowest cytotoxicity (88.42 % cell viability) and was synergistic according to the combination index (0.44).

- No conventional antimicrobial significantly reduced nitric oxide production.

- Cinnamaldehyde demonstrated the highest reduction in nitric oxide production (12.5 µg/mL – 67.9% reduction and at 25.0 µg/mL, 77.4% reduction), which was higher than the positive control (aminoguanidine).

- All combinations with cinnamaldehyde demonstrated a significant ($p < 0.05$) reduction in nitric oxide production.

- The combination with the highest reduction in nitric oxide and lowest toxicity was ciprofloxacin with cinnamaldehyde (77.66% reduction in nitric oxide production and 88.42% cell viability).

- Based on the fractional effect analysis, the interaction between ciprofloxacin and cinnamaldehyde was synergistic ($\Sigma Fa = 0.44$).

Publication 3: Ibuprofen as an adjuvant to conventional antimicrobials and essential oil compounds against skin pathogens

Ibuprofen was studied as a multifunctional adjuvant to conventional antimicrobials and essential oil compounds against skin pathogens. The study evaluated ibuprofen's antimicrobial, cytotoxic, and anti-inflammatory properties in combination with the conventional antimicrobials and essential oil compounds.

- Ibuprofen demonstrated broad-spectrum activity against all studied pathogens.

- A total of 152 combinations were analysed (78 against Gram-positive bacteria, 50 against Gram-negative, and 24 against *C. albicans*). For the Gram-positive bacteria, 4.00% were synergistic, 42% were additive, and 54% were non-interactive. For the Gram-negative bacteria, 2.00% were synergistic, 50% were additive, and 48% were non-interactive. For *C. albicans*, 42% was additive and 58% non-interactive. No antagonism was observed.

- This study was the first to evaluate combinations of antibiotics and ibuprofen against Carbapenem-resistant *A. baumannii* and multi-drug-resistant *S. aureus* strains.

- Three synergistic interactions were observed against the Gram-positive bacteria (One against *S. aureus* and two against *C. acnes*). One synergistic interaction was observed against the Gram-negative *A. baumannii*.

- From the ratio analysis, synergistic interactions were observed at varying ratios, with the highest degree of synergy at ratios close to equivalence (50:50).

- Triple combinations involving the synergistic pairwise combinations (Ibuprofen with antibiotic) with the addition of each essential oil compound identified three synergistic interactions (Two against *A. baumannii* and one against *C. acnes*).

- Ibuprofen demonstrated dose-dependent cytotoxicity on HaCAT keratinocytes.

- Antimicrobial synergistic combinations (Ibuprofen with antibiotic) demonstrated low toxicity, with all interactions demonstrating additivity according to the combination index (0.97 - 1.01).

- No ibuprofen-antibiotic combination significantly reduced nitric oxide production.

- Triple combinations (Ibuprofen with antibiotic and essential oil compound) demonstrated similar cytotoxic effects as the pairwise combinations. Based on the combination index, it was interpreted as additivity.

- This study was the first to investigate higher-order combinations using an antibiotic with two adjuvants.

Table 6.1 shows a selection of the most positive combinations based on the initial results from the antimicrobial analysis.

Table 6.1: Summary of most positive combinations identified from this study

Combinations	Pathogen	Antimicrobial (ΣFIC)	Brine-shrimp lethality assay	MTT assay	Anti-inflammatory assay
			Toxicity (LC_{50})	% Cell viability (HaCAT)	% Reduction in NO production
Amoxicillin + carvacrol	*S. aureus*	0.42	LC_{50} = 522.95 µg/mL (24 hrs); 513.89 µg/mL (48 hrs)	92.09	10.06
Amoxicillin + eugenol	*C. acnes*	0.30	LC_{50} = 806.43 µg/mL (24 hrs); 628.81 µg/mL (48 hrs)	79.42	5.11
Ciprofloxacin + cinnamaldehyde	*P. aeruginosa*	0.32	LC_{50} = 827.92 µg/mL (24hrs); 640.02 µg/mL (48 hrs)	88.42	77.66
Erythromycin + cinnamaldehyde	*S. epidermidis*	0.44	LC_{50} = 736.85 µg/mL (24 hrs); 573.63 µg/mL (48 hrs)	83.46	78.42
Gentamicin + carvacrol	*S. aureus*	0.36	LC_{50} = 707.03 µg/mL (24 hrs); 657.45 µg/mL (48 hrs)	84.41	3.66
Miconazole + carvacrol	*S. aureus*	0.33	LC_{50} = 707.03 µg/mL (24 hrs); 657.43 µg/mL (48 hrs)	91.82	9.95
Miconazole + cinnamaldehyde	*S. epidermidis*	0.07	LC_{50} = 704.23 µg/mL (24 hrs); 636.05 µg/mL (48 hrs)	78.57	78.53
Ibuprofen + amoxicillin	*C. acnes*	0.33	N/A[1]	100.06	3.01
Ibuprofen + erythromycin	*C. acnes*	0.41	N/A	100.21	3.57
Ibuprofen + gentamicin	*A. baumannii*	0.27	N/A	93.55	3.01
Ibuprofen + meropenem	*S. aureus*	0.46	N/A	100.15	<1.00
Ibuprofen + amoxicillin + eugenol	*C. acnes*	0.47	N/A	77.59	<1.00
Ibuprofen + gentamicin + α-pinene	*A. baumannii*	0.44	N/A	82.79	<1.00
Ibuprofen + gentamicin + γ-terpinene	*A. baumannii*	0.44	N/A	90.44	<1.00

[1]N/A – Ibuprofen and the conventional antimicrobials were not evaluated using the Brine-shrimp lethality assay.

6.2 Limitations

The study investigated a limited cohort of essential oil compounds, which represents a small portion of potential EOCs that may exhibit positive interactions with antibiotics. The brine shrimp lethality assay can only be used as a preliminary screen for natural products. In addition, toxicity models using aquatic animals differ in toxicity results compared to fumigant and cell culture models. For the anti-inflammatory assay, only nitric oxide production was analysed. Inflammation is a complex process and involves several pro-inflammatory mediators. The effect of nitric oxide production represents one part of this process, and other cytokines could also affect the anti-inflammatory properties of the essential oil compounds, conventional antimicrobials, and ibuprofen.

6.3 Future recommendations

The study focused on the antimicrobial efficacy of common skin pathogens. Considering the multifaceted nature of antimicrobial resistance extending beyond these pathogens, investigating other emerging pathogens should be considered. Emerging bacterial pathogens are problematic as they are poorly studied and typically exhibit high antibiotic resistance or are highly virulent (Tiwari and Deshwal, 2023). Some examples include non-tuberculosis mycobacteria (NTM), *Stenotrophomonas maltophila*, and *Candida auris* (Falkinham, 2016; Misch *et al.*, 2018; Brooke, 2021; Montoya, 2024).

Ibuprofen is a propionic derivative that contains a chiral carbon in the α-position of the moiety (Nüsing, 2016). This means ibuprofen has two enantiomers demonstrating different pharmacological properties (Evans, 2001). Most commercially available ibuprofen formulations have equal ratios of both enantiomers (Varrassi *et al.*, 2020). The S-(+)-ibuprofen enantiomer is more pharmacologically active and is responsible for the therapeutic properties of ibuprofen (Rainsford, 2012b). In contrast, the R-(-)-ibuprofen enantiomer undergoes metabolic inversion to yield the pharmacologically active S-(+)-ibuprofen enantiomer (Evans, 2001). Combining both enantiomers increases the safety of ibuprofen, reduces adverse effects, and can also exhibit enhanced pharmacological properties for certain diseases (Rainsford, 2012a). The stereochemistry properties of conventional antimicrobials have also been well established with different enantiomers exhibiting varying antimicrobial properties (Paris, 2012; Wright *et al.*, 2014).

For example: only the S-(+)-linezolid enantiomer exhibits antimicrobial properties (Barbachyn, 2018), whilst levofloxacin is twice as potent as ofloxacin (Hurst *et al.*, 2002). Considering the current evidence of ibuprofen as a non-antibiotic that can block resistance mechanisms and virulence factors (Discussed in Chapter 2), the antimicrobial properties of ibuprofen enantiomers should be investigated.

Ibuprofen's antimicrobial properties have been described in Chapter 2. A noted method to increase the antimicrobial activity of ibuprofen is incorporating it into a hypertonic solution. Hypertonic solutions such as saline (Sodium chloride) are commonly used to clean or irrigate wounds and function as a type of debridement to reduce debris and biological contaminants (Wilkins and Unverdorben, 2013). Unlike other wound washes, such as chlorhexidine and alcohol, saline solutions do not contribute to antibiotic resistance (Mahamadou Harouna *et al.*, 2019). Several studies have demonstrated that ibuprofen's antimicrobial properties are amplified when dissolved in an ionic solution (Sanyal *et al.*, 1993; Elvers and Wright, 1995; García *et al.*, 2020). A study by Muñoz *et al.* (2018) demonstrated that ibuprofen dissolved in a sodium chloride (1.0M) solution increased the bactericidal effects of ibuprofen and synergistically improved the efficacy of gentamicin, tobramycin and fosfomycin against ATCC strains of *S. aureus* (ATCC 6538) and *P. aeruginosa* (ATCC 27853). Ibuprofen can change the permeability of the cell membrane in bacteria and fungi through potassium efflux (Oliveira *et al.*, 2019). This mechanism also changes the zeta potential of the membrane surface, which is amplified in an ionic solution (Halder *et al.*, 2015). A hypertonic saline formulation of ibuprofen (Ibuprofenate) has been investigated for treating respiratory infections such as COVID-19 and pneumonia (Zurita-Lizza *et al.*, 2023). Considering the anti-inflammatory properties of ibuprofen, these hypertonic-ibuprofen solutions may provide dual benefits in the initial management of wounds.

The synergistic combinations involving cinnamaldehyde should be investigated for its ability to modulate other pro-inflammatory cytokines. Other microbial PAMPs should be considered to identify EOC-specific mechanisms for reducing inflammation. Lipopolysaccharide stimulates inflammatory cytokines such as NO and interleukins; however, bacteria and fungi have multiple PAMPs that induce inflammation (Silva-Gomes *et al.*, 2015). Most skin infections are caused by Gram-positive bacteria with lower levels of LPS than Gram-negative bacteria.

Other PAMPs, such as lipoteichoic acid (LTA), mannan-derivatives or species-specific such as phenol-soluble modulins (PSM), should be investigated (Peschel and Otto, 2013). This may identify EOCs for reducing inflammation induced by specific skin pathogens.

6.4 Conclusion

This study provided a comprehensive investigation into the antimicrobial, cytotoxic, and anti-inflammatory properties of essential oil compounds, conventional antimicrobials, and the adjuvant ibuprofen against skin pathogens and has yielded several insightful outcomes. These findings accentuate the potential of combination therapies involving essential oil compounds, conventional antimicrobials, and adjuvants such as ibuprofen in combating skin pathogens effectively whilst mitigating cytotoxicity and enhancing anti-inflammatory effects. Further investigation regarding optimal ratios and other combinations could lead to the development of novel therapeutic strategies in dermatology.

CHAPTER 7: REFERENCES

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APPENDEX A: ABSTRACT FOR PRESENTATION 1 (SCHOOL OF THERAPEUTIC SCIENCES BIENNIAL RESEARCH DAY 2023)

Essential oil compounds in combination with conventional antibiotics for dermatology

Shivar Simbu, Ané Orchard, Maryna van de Venter, Sandy van Vuuren

Abstract:

Antimicrobial resistance has emerged as a significant threat to global public health, prompting interest in new chemical entities derived from natural sources such as essential oil compounds. This study aimed to determine the possible interactions between six essential oil compounds (α-pinene, γ-terpinene, ±linalool, eugenol, carvacrol, and cinnamaldehyde) with eight antibiotics (amoxicillin, ciprofloxacin, erythromycin, gentamicin, meropenem, tetracycline, miconazole, and nystatin) against six reference strains implicated in skin infections (*Staphylococcus aureus* (ATCC 25923), *Staphylococcus epidermidis* (ATCC 12228), *Pseudomonas aeruginosa* (ATCC 27853), *Acinetobacter baumannii* (ATCC 19606), *Cutibacterium acnes* (ATCC 11827), and *Candida albicans* (ATCC 10231)). The minimum inhibitory concentrations (MIC) of the antibiotics and essential oil compounds, singularly and when combined, were determined using the broth microdilution assay. The sum of the fractional inhibitory concentrations (ΣFIC) was calculated to investigate the interactive profile of the combinations. Synergistic interactions were further analyzed at varying ratios and depicted on isobolograms. The results identified eight synergistic interactions, seven against Gram-positive bacteria (ΣFIC 0.07 – 0.42) and one against *Pseudomonas aeruginosa* (ΣFIC 0.32). The cytotoxicity of the synergistic combinations was screened using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay using HaCAT keratinocytes. It was demonstrated that when combined, the overall toxicity of the essential oil compounds decreased. The combination of amoxicillin and carvacrol demonstrated the lowest toxicity (93% cell viability). This study provides insight into the interactions between essential oil compounds and conventional antimicrobials, which can form a basis for future antimicrobial therapies.

APPENDEX B: ABSTRACT FOR PRESENTATION 2 (MOLECULAR BIOSCIENCES RESEARCH THRUST (MBRT) POSTGRADUATE RESEARCH SYMPOSIUM 2023)

Title: Synergistic solutions: The potential of multitarget ibuprofen combinations for cutaneous infections

Authors: Shivar Simbu, Ané Orchard, Maryna van de Venter, Sandy van Vuuren

Affiliations: Pharmaceutical Microbiology (Department of Pharmacy and Pharmacology)

Background: The increasing occurrence of multi-drug resistant (MDR) pathogens in skin infections has limited the available therapeutic options. Repurposing non-antibiotics represents an emerging strategy that can help fast-track the drug development process. Ibuprofen represents a class of repurposed drugs called non-antibiotics which demonstrate antimicrobial activity. This study aimed to investigate the interactive properties of combining eight conventional antimicrobials with ibuprofen against common skin pathogens.

Methods: Eight conventional antimicrobials (amoxicillin, ciprofloxacin, erythromycin, tetracycline, gentamicin, meropenem, miconazole and nystatin) and ibuprofen. Reference strain pathogens (*Staphylococcus aureus* (ATCC 25923 & ATCC 6538), *Staphylococcus epidermidis* (ATCC 12228), *Acinetobacter baumannii* (ATCC 19606), *Pseudomonas aeruginosa* (ATCC 27853), *Cutibacterium acnes* (ATCC 11827) and the yeast *Candida albicans* (ATCC 10231). The antimicrobial analysis was conducted using the broth microdilution method, to determine the minimum inhibitory concentrations (MIC) and fractional inhibitory concentrations (FIC). The cytotoxicity analysis was conducted on HaCAT keratinocytes using the MTT cytotoxicity assay. The anti-inflammatory analysis was conducted on lipopolysaccharide (LPS) activated RAW 264.7 macrophages using the LPS-NO induced assay.

Results & discussion: For the antimicrobial analysis, four synergistic interactions (ΣFIC 0.33 - 0.50) were identified between ibuprofen and conventional antimicrobials, with the combination of amoxicillin and ibuprofen demonstrating the highest degree of synergy (ΣFIC = 0.33) against *Cutibacterium acnes*. For the cytotoxicity, none of the combinations

demonstrated a cytotoxic effect (cell viability of 93.6-100%). For the anti-inflammatory results, none of the combinations reduced nitrite production however, no antagonistic interaction was observed.

Conclusions: The combination strategy of ibuprofen and conventional antimicrobials can pave the way for developing new combination therapies to treat MDR infections. However, further studies of the underlying mechanisms supported by animal efficacy experiments are required for the development of safe drug combination usage.

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APPENDEX C: ANTIBIOTIC/ANTIFUNGAL BREAKPOINTS

Antibiotics/antifungals	Micro-organisms					
	Staphylococcus aureus	*Staphylococcus epidermidis*	*Cutibacterium acnes*	*Pseudomonas aeruginosa*	*Acinetobacter baumannii*	*Candida albicans*
Amoxicillin	1.) ≤ 2 - ≥ 4 2.) ≥ 2	1.) ≤ 0.25 - ≥ 0.5 2.) > 0.25	1.) No data[1] 2.) ≤ 2 - ≥ 8	1.) No data 2.) -[2]	1.) No data 2.) -	Resistant
Ciprofloxacin	1.) ≤ 1 - ≥ 4 2.) ≤ 0.001 - ≥ 1	1.) ≤ 1 - ≥ 4 2.) ≤ 0.001 - ≥ 1	1.) No data 2.) ≤ 0.25 - ≥ 0.5	1.) ≤ 0.5 - ≥ 2 2.) ≤ 0.001 – ≥ 0.5	1.) ≤ 1 - ≥ 4 2.) ≤ 0.001 - ≥ 1	Resistant
Erythromycin	1.) ≤ 0.5 - ≥ 8 2.) ≤ 1	1.) ≤ 0.5 - ≥ 8 2.) ≤ 1	1.) No data 2.) Insufficient evidence[3]	1.) No data 2.) -	1.) No data 2.) -	Resistant
Gentamicin	1.) ≤ 4 - ≥ 16 2.) ≤ 2	1.) ≤ 4 - ≥ 16 2.) ≤ 2	1.) No data 2.) ≤ 0.5	1.) ≤ 2 - ≥ 16 2.) Insufficient evidence	1.) ≤ 2 - ≥ 16 2.) ≤ 4	Resistant
Meropenem	1.) ≤ 2 - ≥ 4 2.) ≥ 4	1.) ≤ 0.25 - ≥ 0.5 2.) ≥ 4	1.) No data 2.) ≤ 2 - ≥ 8	1.) ≤ 2 - ≥ 8 2.) ≤ 2 - ≥ 8	1.) ≤ 2 - ≥ 8 2.) ≤ 2 - ≥ 8	Resistant
Tetracycline	1.) ≤ 4 - ≥ 16 2.) ≤ 1	1.) ≤ 4 - ≥ 16 2.) ≤ 1	1.) No data 2.) Insufficient evidence	1.) No data 2.) -	1.) ≤ 4 - ≥ 16 2.) -	Resistant
Miconazole	1.) - 2.) -	1.) - 2.) -	1.) - 2.) -	1.) - 2.) -	1.) - 2.) -	1.) ≤ 0.5 – ≥ 8 2.) ≤ 1 – ≥ 8
Nystatin	Resistant	Resistant	Resistant	Resistant	Resistant	1.) No data 2.) ≤ 1

[1]No breakpoints have been defined for this antibiotic/antifungal; [2]Antibiotic is considered "ineffective" against target micro-organism; [3]Not enough clinical evidence to support the antibiotic/antifungals use.

1.) CLSI (2020)

2.) EUCAST (2022)

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Faculty of Health Sciences, Phillip Tobias Health Sciences Building, 3rd Floor, Office 301/2/4, 29 Princess of Wales Terrace, Parktown, 2193
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Ref: W-CBP-220411-02 11/04/2022

TO WHOM IT MAY CONCERN:

Waiver:	This certifies that the following research does not require clearance from the Human Research Ethics Committee (Medical)
Investigator:	Mr Shivar Simbu
Supervisors:	Prof Sandy van Vuuren and Dr Ané Orchard
Department:	Pharmacy and Pharmacology
Project title:	Essential oil compounds with conventional antibiotics for dermatology
Reason:	*In vitro* laboratory study. No human participants will be involved in the study.

Dr CB Penny

Chairperson: Human Research Ethics Committee (Medical)

Copy – HREC (Medical) Secretariat: Ms Zanele Ndlovu, Ms Mapula Ramaila and Mr Rhulani Mkansi

APPENDEX F: TURNITIN REPORT

Shivar Thesis full - final

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