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

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Abstract

Aims: Antimicrobial resistance continues to be a growing concern, resulting in increased use of drug combinations. Antibiotic adjuvants are an emerging strategy that may potentiate an antibiotics efficacy. Ibuprofen's polypharmacological properties have been investigated for their antimicrobial and host-modulating potential. This study aimed to investigate the potential of a novel multidrug combination involving ibuprofen, essential oil compounds (EOCs), and conventional antimicrobials against skin pathogens.

Methods and Results: The minimum inhibitory concentrations of ibuprofen, conventional antimicrobials, and EOCs were determined and then combined and tested against 14 (reference and clinical) skin pathogens. The cytotoxicity was analysed using the MTT assay, whilst the anti-inflammatory effects were evaluated using lipopolysaccharide activated RAW264.7 murine macrophages. Four pairwise (Ibuprofen and antibiotic) (ΣFIC 0.33–0.50) and three triple (Ibuprofen and antibiotic with EOC) (ΣFIC 0.44–0.47) synergistic antimicrobial interactions were identified. These combinations demonstrated cell viability of 77.59%–100%. No combination significantly reduced nitric oxide production.

Conclusion: The results from this study provide insight into the potential of a multidrug combination involving ibuprofen with conventional antimicrobials and EOCs against common skin pathogens.

Impact Statement

The incorporation of ibuprofen as an adjuvant to some conventional antimicrobials for the treatment of skin infections offers an optimized treatment strategy that may broaden therapeutic options and reduce the risk of antimicrobial resistance. This study provides evidence of ibuprofens ability to potentate a conventional antimicrobials efficacy against common skin pathogens.

Keywords: synergy; essential oil compound; cytotoxicity; antibiotic; antimicrobial; combinations

Introduction

Skin infections represent some of the most common types of infections and account for approximately 7%–10% of all hospital patients (Ki and Rotstein 2008). Skin infections can be classified as complicated or uncomplicated infections (Lipsky et al. 2017, Bassetti et al. 2023). Uncomplicated infections are superficial or purulent wounds, whilst complicated infections are severe, non-purulent, and deep-tissue infections (Muhaj et al. 2022). Skin diseases are the 18th leading cause of global burden when measured in 'Disability-Adjusted Life Years'. Furthermore, skin diseases are the fourth leading cause of disability worldwide (Karimkhani et al. 2017). In recent times, there has been an increase in uncomplicated infections due to antibiotic resistance (Vale de Macedo et al. 2021, Xue et al. 2022). Antibiotic resistance is an ongoing public health crisis that threatens the effective treatment and prevention of infectious diseases (Aljeldah 2022). Combination therapy has been used for decades as a therapeutic strategy to counter antimicrobial resistance (Tyers and Wright 2019). 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 (Coates et al. 2020).

Antibiotic adjuvants are synthetic or natural molecules that are essentially nonantimicrobial in nature, but can potentiate the activity of an antimicrobial agent through the reversal of resistance mechanisms or by enhancing host defences (Bernal et al. 2013, Gill et al. 2015, Dhanda et al. 2023). Most antibiotic adjuvants have limited or no antimicrobial properties and have been investigated as a type of combination therapy to recycle redundant antibiotics (Tyers and Wright 2019).

Ibuprofen is one of the most widely used nonsteroidal anti-inflammatory drugs and functions as a non-selective inhibitor of cyclooxygenase enzymes, blocking prostaglandin synthesis (Rainsford 2009). In recent years, ibuprofen has garnered interest due to its potential for drug repurposing as an antimicrobial agent (Jampilek 2022, Babaei et al. 2023). However, there have been some varied reports on the antimicrobial properties of ibuprofen (bacteria with MIC values 300–5000 μg/ml and fungi with MIC values ranging between 600 and 5000 μg/ml) (Obad et al. 2015, Ogundeji et al. 2016, Król et al. 2018). Additionally, combinations of ibuprofen with some antibiotics have also been investigated for different diseases such as tuberculosis (Byrne et al. 2006, Vilaplana

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Table 1. Strains of micro-organisms investigated in this study.

Micro-organism	Strain	Reason for inclusion
Staphylococcus aureus (n =3)	ATCC 25923 ATCC 6538 Clinical (MRSA)	The most frequently isolated pathogen in acute and chronic skin infections (Nandhini et al. 2022)
Staphylococcus epidermidis (n = 2)	ATCC 12228 Clinical (Macrolide-resistant)	Increasing occurrence in superficial skin infections (Severn and Horswill 2023)
Cutibacterium acnes	ATCC 11827	Cause of acne vulgaris (Fournière et al. 2020)
Acinetobacter baumannii (n = 2)	ATCC 19606 Clinical (CRAB)	Implicated in nosocomial skin infections with exceedingly high mortality rates. Also contributes to chronic skin infections (Castanheira et al. 2023)
Pseudomonas aeruginosa (n = 3)	ATCC 27853 Clinical (n = 2)	Skin infections in healthy and immunosuppressed individuals. Increases the severity of acute skin infections (Oliveira et al. 2020)
Candida albicans (n = 3)	ATCC 10231 Clinical (Fluconazole-resistant; n = 2)	Colonizes various parts of the skin that can induce recurrent infections (Fisher et al. 2022)

et al. 2013) and urinary tract infections (Aloush et al. 2019, Whiteside et al. 2019). In contrast, ibuprofen use in cutaneous infections remains controversial as there is an increased risk of secondary bacterial infections (Bryant et al. 2015). Several reviews have discussed associations between ibuprofen use in Varicella-Zoster (chickenpox) infections and the development of necrotizing Group A *Streptococcus* infections (Souyri et al. 2008, Leroy et al. 2010, Das et al. 2012). However, other studies found no association or limited evidence relating ibuprofen and necrotizing infections (Lesko et al. 2001, Legras et al. 2009). These inconsistencies between studies and the overwhelming association with viral infections suggest that ibuprofen's role in necrotizing infections is multifactorial and may involve host-pathogen interactions (Zhao-Fleming et al. 2018, Quaglietta et al. 2021). Furthermore, investigating other skin pathogens, such as *Pseudomonas aeruginosa* and *Candida* species, is unknown and highlights the need for further investigation.

Essential oils (EOs) are complex secondary metabolites comprised of many chemical compounds in varying concentrations (Dhifi et al. 2016). These essential oil compounds (EOCs) give an EO its therapeutic benefits, such as antimicrobial and anti-inflammatory properties (Baptista-Silva et al. 2020). The antimicrobial properties of EOCs are well-known and have been reviewed extensively (Aelenei et al. 2016, Suganya et al. 2022). Recent reviews have discussed the emerging strategy of using EOCs as potential antibiotic adjuvants (Dias et al. 2022, Kumar et al. 2023).

Our previous studies have identified several antimicrobial synergistic combinations of EOCs and conventional antimicrobials against different skin bacteria and the anti-inflammatory effects of the same combinations (Simbu et al. 2024, Simbu 2024). 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 therapeutic effect. This study evaluated the *in vitro* antimicrobial, cytotoxic, and anti-inflammatory effects of a multidrug combination involving EOCs, and conventional antimicrobials with ibuprofen against skin pathogens.

Materials and methods

Preparation of cultures

Due to their prevalence in skin infections (Vale de Macedo et al. 2021, Russo et al. 2022, Rigopoulos 2023), American Type Culture Collection (ATCC) reference and clinical strains were included in the study (Table 1).

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% CO2) at 37°C. *Candida albicans* was cultured in Tryptone Soya broth and incubated at 37°C for 48 h.

The antimicrobial resistance profiles of all studied micro-organisms were analysed using the disk diffusion assay as outlined by the Clinical Laboratory Standards Institute (CLSI 2020) (Supplementary data Tables S1 and S2). A waiver for these micro-organisms was obtained from the University of the Witwatersrand Human Research Ethics Committee (Reference number: W-CBP-220411-02).

Preparation of antimicrobial agents, EOCs, and ibuprofen

Ibuprofen (>99.0%) (Sigma-Aldrich, St. Louis, MO, USA) was initially dissolved in 15% Dimethyl sulfoxide (DMSO) and then made up to a concentration of 10 mg/ml (starting concentration of 1.25% DMSO). Based on the synergistic interactions from our previous study (Simbu et al. 2024), 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 (2020).

The following six EOCs (Sigma–Aldrich, St. Louis, MO, USA) were selected for combination studies (Simbu et al. 2024); α-pinene, γ-terpinene, ± linalool, eugenol, carvacrol, and cinnamaldehyde. All compounds had a purity range of 95%–99% and were dissolved in 15% DMSO due to their

low solubility in water and made to an initial concentration of 32 mg/ml.

Minimum inhibitory concentrations

The broth microdilution assay was used to evaluate the minimum inhibitory concentration (MIC) of the conventional antimicrobials, EOCs, and ibuprofen independently and in combination (CLSI 2020). Briefly, each well of a 96-well microtiter plate was filled with 100 µl of sterile broth, followed by adding 100 µl of the antibiotic/EOC/ibuprofen into the top row. For the pairwise combinations, 50 µl of the conventional antimicrobial/EOC and 50 µl of ibuprofen were added as a 1:1 ratio followed by serial doubling dilutions. For favourable combinations (synergistic), triple combinations (higher order) comprising ibuprofen with the conventional antimicrobial or EOC were included. Equivalent volumes (33 µl) of ibuprofen, conventional antimicrobial, and EOC were mixed and assayed as outlined by O'Shaughnessy et al. (2006). The prepared micro-titre plates were then inoculated with 100 µl of the relevant pathogen with an approximate colony-forming unit of 2–8 $\times$ 10^5 (CFU/ml) (CLSI 2020). The MIC values were interpreted as the lowest concentration at which growth was inhibited. Each combination was performed in quadruplicate ($n = 4$). Negative controls (non-treated cultures and solvent controls) were included. The MIC data for the conventional antimicrobials and EOCs against reference and clinical isolates tested independently is shown in Supplementary data Tables S3 and S4.

Interactive profiles

The pairwise interactions of the conventional antimicrobials, EOCs, and ibuprofen were classified according to the fractional inhibitory concentration (FIC) (Equation 1)

$$\Sigma\text{FIC} = \text{FIC (A)} + \text{FIC (B)} = \frac{(\text{MIC}_\text{A})\ \text{in } \textit{combination}}{(\text{MIC}_\text{A})\ \textit{alone}} + \frac{(\text{MIC}_\text{B})\ \text{in } \textit{combination}}{(\text{MIC}_\text{B})\ \textit{alone}} \quad (1)$$

*where (A) is ibuprofen and (B) is EOC/conventional antimicrobial

The ΣFIC for each pairwise combination was interpreted as follows: ΣFIC value of ≤0.5 is indicative of synergy; an ΣFIC value of >0.5–≤4.0 indicates a non-interactive (indifferent) effect, and an ΣFIC value of >4.0 indicates antagonism (Odds 2003). For the triple combinations, all pairwise and single interactions were evaluated for possible emergent (favourable interactions that are not reliant on lower-order combinations) or suppressive interactions (unfavourable interactions that are less than the individual components) (Beppler et al. 2016).

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).

The MTT cytotoxicity assay

The cytotoxicity of ibuprofen, conventional antimicrobials, EOCs, and selected synergistic combinations was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay due to the cytotoxicity of the studied compounds. The assay was conducted as described by Leigh-de Rapper et al. (2021) using HaCAT cells (Cellonex, South Africa) cultured in Dulbecco's Modified Eagle Medium (GE Healthcare Life Sciences, USA) supplemented with 10% (v/v) Foetal bovine serum (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 100 µl in each well. The cells were treated at three different concentrations (3.13, 6.25, and 12.5 µg/ml) for the conventional antimicrobials, five concentrations (15.63, 31.3, 62.5, 125, and 250 µg/ml) for the EOCs, and five concentrations (1.25, 2.50, 12.5, 125, and 1250 µg/ml) for ibuprofen. Melphalan (100 µM) (Sigma Aldrich, St. Louis, MO, USA) was used as the positive control. The absorbance was read at 540 nm using a BioTek® PowerWave XS spectrophotometer (Winooski, VT, USA). The cytotoxicity was interpreted as % cell viability compared with the untreated control. The data analysis included the calculation of the mean and standard deviation (±SD) using GraphPad Prism® software (Version 9).

Combination index

The combination index (CI) provided a quantitative measure to determine the type of interactions between two drugs and was used to assess the cytotoxicity (Swanepoel et al. 2019, Duarte and Vale 2022). The CI was calculated as per Equation 2

$$\text{CI} = [(\text{D})_1/(\text{D}_\text{x})_1 + (\text{D})_2/(\text{D}_\text{x})_2], \quad (2)$$

*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 (Equation 3)

$$\text{D}_\text{x} = \text{D}_\text{m}\,[\text{F}_\text{a}/\,(1/\text{F}_\text{a})]\,1/\text{m}, \quad (3)$$

*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 (Chou 2018).

The anti-inflammatory assay

The anti-inflammatory activity of ibuprofen, EOCs, conventional antimicrobials, and synergistic combinations was assessed using the lipopolysaccharide-nitric oxide induced assay (Leigh-de Rapper et al. 2021). RAW 264.7 cells (Cellonex, South Africa) were cultured in RPMI1640 medium supplemented with 10% foetal calf serum in a humidified 5% CO_2 incubator at 37°C. A standard curve using sodium nitrite dissolved in culture medium was used to determine the NO concentration in each sample. The anti-inflammatory properties were interpreted as the amount of NO produced, followed by calculating 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. Aminoguanidine (AG) (Sigma–Aldrich, St. Louis, MO, USA) was used as the positive control since it is a direct inhibitor of iNOS, and negative controls were medium only and untreated cells with LPS. The fractional-effect analysis (Fa) was used to calculate the expected dose-response relationship for the anti-inflammatory

Table 2. The mean MIC values (μg/ml) and standard deviation (±SD) for ibuprofen against reference and clinical isolates micro-organisms ($n = 6$).

Micro-organisms	Ibuprofen (μg/ml)	Positive control (μg/ml)
S. aureus (ATCC 25923)	1250 ± 0.00	1.06 ± 0.24[1]
S. aureus (ATCC 6538)	1250 ± 0.00	1.25 ± 0.00[1]
MRSA (Clinical)	2500 ± 0.00	2.81 ± 0.34[1]
S. epidermidis (ATCC 12228)	625 ± 0.00	0.94 ± 0.36[1]
S. epidermidis (Macrolide-resistant)	1250 ± 0.00	1.56 ± 0.00[1]
C. acnes (ATCC 11827)	313 ± 0.00	1.25 ± 0.00[1]
P. aeruginosa (ATCC 27853)	2500 ± 0.00	0.57 ± 0.28[1]
P. aeruginosa (Clinical isolate 1)	2500 ± 0.00	1.56 ± 0.00[1]
P. aeruginosa (Clinical isolate 2)	2500 ± 0.00	1.46 ± 0.18[1]
A. baumannii (ATCC 19606)	2500 ± 0.00	0.52 ± 0.16[1]
A. baumannii (CRAB)	5000 ± 0.00	9.34 ± 3.60[1]
C. albicans (ATCC 10231)	625 ± 0.00	1.56 ± 0.63[2]
C. albicans (Clinical isolate 1)	1250 ± 0.00	6.25 ± 0.00[2]
C. albicans (Clinical isolate 2)	2500 ± 0.00	4.69 ± 1.80[2]

[1]Ciprofloxacin (positive control for bacteria).
[2]Nystatin (positive control for *C. albicans*); both the culture control and solvent (5.00% DMSO) showed no inhibition of growth (MIC > 8000 μg/ml).

combinations (Duarte and Vale 2022) (Equation 4) and interpreted as outlined by Chou (2018)

$$\Sigma Fa = Fa\ (A) + Fa\ (B) = \frac{(NO_A)\ in\ combination}{(NO_A)\ alone} + \frac{(NO_B)\ in\ combination}{(NO_B)\ alone} \quad (4)$$

*where (A) is the NO produced by Ibuprofen and (B) is the NO produced by the conventional antimicrobial/EOC.

Results

Antimicrobial activity of ibuprofen

The results of the MICs for ibuprofen against the reference and clinical isolates are shown in Table 2. For the Gram-positive bacteria, all strains of *S. aureus* had similar susceptibility patterns, with the clinical MRSA isolate being the least susceptible (2500 μg/ml). A similar trend was also noticed for *S. epidermidis*, whilst *C. acnes* was the most susceptible to ibuprofen with the lowest MIC value (313 μg/ml). The Gram-negative bacteria were less susceptible to ibuprofen, as reflected in the higher MIC values. All strains of *P. aeruginosa* had the same MIC value (2500 μg/ml), whilst the clinical isolate of *A. baumannii* was the least susceptible overall, with the highest MIC (5000 μg/ml) value. For *C. albicans*, the clinical isolates were less susceptible when compared with the reference strain (ATCC 10231).

Ibuprofen combinations with essential oil compounds

The results for the 1:1 ibuprofen: EOC combinations are shown in Table 3. A total of 66 interactions were studied, with all interactions demonstrating indifference (indifferent). No antagonism was observed. Comparatively, combinations of ibuprofen with carvacrol (ΣFIC 0.83–1.00) and cinnamaldehyde (ΣFIC 0.75–0.85) demonstrated better interactions. Combinations of ibuprofen and the EOCs demonstrated better efficacy against the MRSA isolate than the ATCC strains. A similar trend was also noted for combinations against *P. aeruginosa* and *A. baumannii*.

Ibuprofen combinations with conventional antimicrobials

Combinations of ibuprofen with conventional antimicrobials are shown in Table 4. A total of 62 interactions were investigated with the majority being non-interactive (94%). Four synergistic interactions were identified with three being against the Gram-positive bacteria [*S. aureus* ATCC 25923 with meropenem (ΣFIC 0.46), and *C. acnes* ATCC 11827 with erythromycin (ΣFIC 0.41) and amoxicillin (ΣFIC 0.33)] and one against the ATCC strain of *A. baumannii* (ΣFIC 0.27).

The four synergistic interactions (Ibuprofen with meropenem, amoxicillin, erythromycin, and gentamicin) were analysed at different ratios (Fig. 1). The combination of ibuprofen with meropenem against *S. aureus* (ATCC 25923) had six synergistic combinations when tested at varied concentrations (Fig 1a) with ratios close to equal quantities (50:50), demonstrating the best synergy (ΣFIC 0.50). For the combination of ibuprofen with amoxicillin against *C. acnes* (ATCC 11827) (Fig 1b), four synergistic combinations were observed, and synergy was also observed when the quantities were close to equal parts (50:50). When the ratio of amoxicillin to ibuprofen exceeded (30:70) non-interactive interactions was observed. A similar trend was noted for the combination of ibuprofen with erythromycin also against the same *C. acnes* strain (Fig 1c), with seven synergistic combinations. There were six synergistic interactions of ibuprofen with gentamicin against *A. baumannii* (Fig. 1d) at ratios close to equal quantities (40:60, 50:50, and 60:40), whilst ratios with gentamicin (70:30, 80:20, and 90:10) or ibuprofen in the majority (10:90) were indfferent.

Ibuprofen combinations with EOCs and conventional antifungals against *C. albicans*

The results for the 1:1 combinations against *C. albicans* are shown in Table 5. All interactions were indifferent for both the antifungals and EOCs. Comparatively, the clinical isolates were more susceptible to the ibuprofen combinations as opposed to the ATCC isolate.

Triple combinations (ibuprofen with conventional antimicrobial and EOC)

The triple combinations involving synergistic interactions with each EOC are shown in Table 6. The combination of ibuprofen with amoxicillin and eugenol was synergistic against *C. acnes* (ΣFIC 0.47). Two EOC combinations (α-pinene, ΣFIC 0.44 and γ-terpinene, ΣFIC 0.44) were synergistic with ibuprofen and gentamicin when tested against *A. baumannii* (ATCC 19606).

Cytotoxicity of combinations

All pairwise combinations demonstrated a high cell viability, indicating a non-toxic effect (Table 7). Based on the criteria outlined by Dahl et al. (2006), compounds with cell viability above 90% are considered non-toxic. For the CI, all combinations were interpreted as additive (CI = ≤0.80–≤1.20). These results demonstrate that when ibuprofen is combined in pairwise combinations with conventional antibiotics, the

Table 3. The MIC values (μg/ml) of ibuprofen (A) and EOCs (B) in combination ($n = 4$).

Essential oil compounds	*Staphylococcus aureus* ATCC 25923			ATCC 6538			MRSA (Clinical)		
	MIC(A)	MIC(B)	ΣFIC	MIC(A)	MIC(B)	ΣFIC	MIC(A)	MIC(B)	ΣFIC
α-Pinene	2500	2000	1.17(Ind)[1]	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(Ind)
± Linalool	2500	2000	1.36(Ind)	1250	1000	1.00(Ind)	2500	2000	1.00(Ind)
Eugenol	2500	2000	1.60(Ind)	1250	1000	0.75(Ind)	2500	2000	1.00(Ind)
Carvacrol	1875	1000	1.00(Ind)	1250	1000	0.83(Ind)	1250	1000	0.92(Ind)
Cinnamaldehyde	625	500	0.85(Ind)	625	500	0.75(Ind)	625	500	0.79(Ind)
	Staphylococcus epidermidis ATCC 12228			Macrolide-resistant (clinical)					
	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(Ind)			
Carvacrol	1250	1000	1.67(Ind)	1250	1000	1.70(Ind)			
Cinnamaldehyde	1250	1000	0.75(Ind)	625	500	0.55(Ind)			
	Cutibacterium acnes ATCC 11827								
	MIC(A)	MIC(B)	ΣFIC						
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(Ind)						
	Pseudomonas aeruginosa ATCC 27853			Clinical isolate 1			Clinical isolate 2		
	MIC(A)	MIC(B)	ΣFIC	MIC(A)	MIC(B)	ΣFIC	MIC(A)	MIC(B)	ΣFIC
α-Pinene	5000	4000	1.53(Ind)	5000	4000	2.00(Ind)	2500	2000	0.75(Ind)
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(Ind)
Eugenol	2500	2000	1.17(Ind)	2500	2000	1.50(Ind)	1250	1000	0.75(Ind)
Carvacrol	1875	1500	1.38(Ind)	1250	1000	1.25(Ind)	1250	1000	0.85(Ind)
Cinnamaldehyde	1250	1000	1.25(Ind)	625	500	0.63(Ind)	1250	1000	1.25(Ind)
	Acinetobacter baumannii ATCC 19606			Clinical (CRAB)					
	MIC(A)	MIC(B)	ΣFIC	MIC(A)	MIC(B)	ΣFIC			
α-Pinene	2500	2000	0.75(Ind)	2500	2000	0.75(Ind)			
γ-Terpinene	5000	4000	1.50(Ind)	5000	4000	0.75(Ind)			
± Linalool	2500	2000	1.00(Ind)	3750	3000	0.75(Ind)			
Eugenol	2500	2000	1.17(Ind)	1250	1000	1.13(Ind)			
Carvacrol	1250	1000	0.75(Ind)	2500	2000	2.25(Ind)			
Cinnamaldehyde	1875	1500	1.88(Ind)	1250	1000	2.13(Ind)			

combined cytotoxicity is the same as the individual drugs. All triple combinations demonstrated an additive effect on HaCAT keratinocytes based on the CI. The triple combinations demonstrated higher cytotoxic effects on the RAW264.7 macrophages than on the HaCAT keratinocytes.

Anti-inflammatory properties of combinations

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

Discussion

The antimicrobial properties of ibuprofen have been known for several decades, with few recent studies investigating its potential against clinically problematic pathogens (Rastgar et al. 2022, Tabatabaeifar et al. 2022). Gram-positive bacteria are generally more susceptible to ibuprofen than Gram-negative bacteria, which is related to their physiological differences such as cell wall composition and the presence of specific cellular structures (Stokes et al. 2019). Most studies have demonstrated ibuprofens' antibacterial activity against *S. aureus* and *P. aeruginosa* (Rastgar et al. 2022), with *C. acnes*, *S. epidermidis*, and *A. baumannii* being poorly studied.

Several studies have reported ibuprofens ability to potentiate and synergize with conventional antimicrobials against reference strains and resistant isolates of *P. aeruginosa* (Khodaparast et al. 2022, Chen et al. 2023). In contrast, no studies have investigated the combinational effects of ibuprofen with any conventional antimicrobials against *A. baumannii*.

Table 4. The MIC values (μg/ml) of ibuprofen (A) and conventional antimicrobials (B) in combination ($n = 4$).

Conventional antimicrobials	*Staphylococcus aureus* ATCC 25923			ATCC 6538			MRSA (Clinical)		
	MIC(A)	MIC(B)	ΣFIC	MIC(A)	MIC(B)	ΣFIC	MIC(A)	MIC(B)	ΣFIC
Amoxicillin	1875	0.47	1.00(Ind)[1]	1250	1.56	0.75(Ind)	2500	3.13	0.83(Ind)
Ciprofloxacin	2500	0.63	1.29(Ind)	1250	1.56	1.12(Ind)	1250	1.56	0.53(Ind)
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)**[2]	1250	1.56	0.81(Ind)	2500	1.56	0.65(Ind)
Tetracycline	625	0.78	1.00(Ind)	625	0.78	0.56(Ind)	2500	3.13	1.01(Ind)
Miconazole	1250	1.56	0.92(Ind)	1250	1.56	0.75(Ind)	2500	3.13	0.67(Ind)
	Staphylococcus epidermidis ATCC 12228			Macrolide-resistant (Clinical)					
	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(Ind)			
Erythromycin	625	0.15	0.60(Ind)	625	1.56	0.54(Ind)			
Gentamicin	625	0.78	0.62(Ind)	625	1.56	0.58(Ind)			
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(Ind)			
Miconazole	1250	1.56	1.45(Ind)	5000	6.25	3.60(Ind)			
	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(Ind)						
Tetracycline	156	0.20	2.22(Ind)						
Miconazole	1250	1.56	3.25(Ind)						
	Pseudomonas aeruginosa ATCC 27853			Clinical isolate 1			Clinical isolate 2		
	MIC(A)	MIC(B)	ΣFIC	MIC(A)	MIC(B)	ΣFIC	MIC(A)	MIC(B)	ΣFIC
Ciprofloxacin	625	0.78	0.89(Ind)	1250	1.56	0.87(Ind)	1250	1.56	0.79(Ind)
Gentamicin	1250	1.56	1.25(Ind)	1250	1.56	0.87(Ind)	1250	1.56	0.55(Ind)
Meropenem	313	0.39	0.56(Ind)	1875	1.25	0.58(Ind)	1250	1.56	0.83(Ind)
Tetracycline	2500	6.25	0.67(Ind)	5000	12.5	1.25(Ind)	5000	6.25	1.14(Ind)
	Acinetobacter baumannii ATCC 19606			Clinical isolate (CRAB)					
	MIC(A)	MIC(B)	ΣFIC	MIC(A)	MIC(B)	ΣFIC			
Ciprofloxacin	2500	0.63	1.10(Ind)	2500	6.25	0.58(Ind)			
Gentamicin	625	0.78	0.27(Syn)	5000	12.5	0.75(Ind)			
Meropenem	1250	1.56	0.76(Ind)	1250	12.5	0.63(Ind)			
Tetracycline	1250	1.56	1.30(Ind)	2500	12.5	0.58(Ind)			

[1]Ind—indifference; [2]Syn—synergism (bold).

Based on our results, ibuprofen reduced the MIC of some conventional antimicrobials by 1.5–2.0-fold, which suggests that ibuprofen could function as an antibiotic adjuvant against *A. baumannii*.

The investigation of triple combinations represents a higher-order combination that has the potential to simultaneously amplify individual drug effects and overcome multiple resistance mechanisms (Zhu et al. 2021). The clinical use of higher-order combinations against *A. baumannii* has become accepted due to its high antimicrobial resistance profile (Oliveira et al. 2020, Rodrigues et al. 2022). 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 (Watkins and Bonomo 2023). Given the clinical success of antibiotic-adjuvant combinations, it may be worth investigating compounds that may target other resistance mechanisms or stimulate host responses against *A. baumannii*.

Combination therapy against *C. acnes* is common and has been predominantly studied for acne vulgaris (Trivedi et al. 2018). The pathogenesis of acne is multifactorial and is treated with antimicrobial agents such as oral antibiotics and/or topical ointments and washes (Leyden 2003, Bienenfeld et al. 2017). The results of the triple combinations against *C. acnes* support the concept of using combinations involving antibiotics and non-antibiotic agents, representing a multitargeted approach and may warrant further investigation.

Ibuprofens activity against *C. albicans* and related yeasts is well documented, with several studies demonstrating MIC values of 500–4000 μg/ml against reference and clinical strains (Rosato et al. 2016, Król et al. 2018). Ibuprofen's antifungal properties are related to its ability to inhibit fungal prostaglandin which is a multifunctional molecule that aids in the upregulation of virulence factors and immune evasion. (Noverr et al. 2001, de Quadros et al. 2011, Ells et al. 2011). Combination therapy for candidiasis is becoming

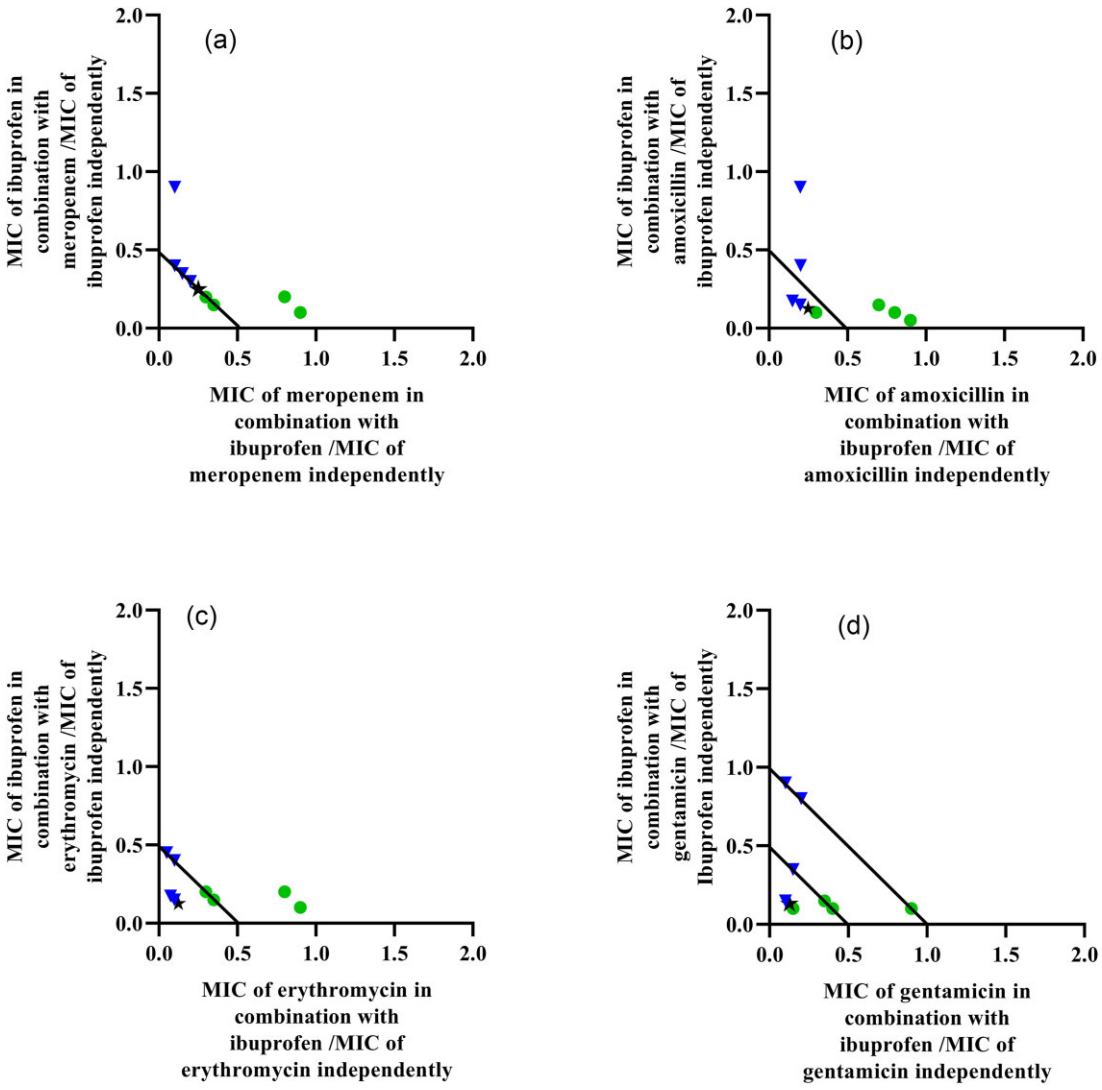

Figure 1. Isobologram representation of the synergistic interactions between Ibuprofen and conventional antimicrobials against bacteria. *S. aureus* (ATCC 25923) (a); *C. acnes* (ATCC 11827) (b and c); *A. baumannii* (ATCC 19606) (d). Synergy is displayed for the data points closest to the apex and fall beneath or on the 0.5:0.5-line, non-interactive interactions are for the data points between the 0.5:0.5 and 4:4 line with antagonism being greater than the 4:4 line. ●—Indicates ibuprofen in the majority; ★—indicates conventional antimicrobial in the majority; and ▼—indicates equal ratio of ibuprofen and conventional antimicrobial.

Table 5. 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)	MIC(B)	ΣFIC	MIC(A)	MIC(B)	ΣFIC	MIC(A)	MIC(B)	ΣFIC
Miconazole	2500	0.63	2.20(Ind)[1]	2500	3.13	1.14(Ind)	2500	3.13	0.90(Ind)
Nystatin	1250	1.56	2.00(Ind)	2500	3.13	1.25(Ind)	2500	3.13	0.83(Ind)
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(Ind)	2500	2000	1.33(Ind)	2500	2000	1.00(Ind)
± Linalool	1250	1000	1.40(Ind)	2500	2000	1.50(Ind)	2500	2000	1.00(Ind)
Eugenol	625	500	0.83(Ind)	1250	1000	1.50(Ind)	1250	1000	0.75(Ind)
Carvacrol	1250	1500	2.50(Ind)	625	500	1.25(Ind)	625	500	1.13(Ind)
Cinnamaldehyde	156	125	0.63(Ind)	313	250	0.93(Ind)	625	500	0.63(Ind)

[1]Ind—indifference.

Table 6. 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 (Ind)
		Carvacrol	2500	1.56	2000	1.86 (Ind)
		Cinnamaldehyde	625	0.78	500	1.00 (Ind)
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 (Ind)
		Eugenol	156	0.20	125	**0.47 (Syn)**[6]
		Carvacrol	156	0.20	125	0.52 (Ind)
		Cinnamaldehyde	156	0.20	125	0.63 (Ind)
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 (Ind)
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]**Syn**—synergistic.

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)
IBU (25 μg/ml) + AMX (0.31 μg/ml)	100.06 ± 5.31	Non-toxic[1]	0.94 (Add)[2]
IBU (50 μg/ml) + GEN (3.13 μg/ml)	100.21 ± 5.66	Non-toxic	0.88 (Add)
IBU (6.25 μg/ml) + ERY (0.04 μg/ml)	93.55 ± 8.14	Non-toxic	0.97 (Add)
IBU (25 μg/ml) + MERO (0.78 μg/ml)	100.15 ± 5.14	Non-toxic	1.01 (Add)
IBU (6.25 μg/ml) + AMX (0.31 μg/ml) + EUG (50 μg/ml)	77.59 ± 10.59	Slightly cytotoxic	0.95 (Add)
IBU (25 μg/ml) + GEN (3.13 μg/ml) + α-PIN (25 μg/ml)	82.79 ± 8.52	Slightly cytotoxic	1.10 (Add)
IBU (25 μg/ml) + GEN (3.13 μg/ml) + γ-TER (25 μg/ml)	90.44 ± 5.70	Slightly cytotoxic	1.01 (Add)
Untreated cells	100 ± 5.15	-	-
Vehicle control (1.25% DMSO)[3]	93.45 ± 2.17	-	-

[1]Cytotoxicity interpreted from % cell viability: >90% (nontoxic), 60%–90% (Slightly cytotoxic), 30%–59% (moderately toxic), and <30% (severely toxic) (Dahl *et al.* 2006).
[2]**Syn** (Synergism, CI < 0.80); Add (Additivity, CI = ≤0.80–≤1.20); Ant (Antagonism, CI > 1.20).
[3]IBU—Ibuprofen, AMX—Amoxicillin, GEN—Gentamicin, ERY—Erythromycin, MERO—Meropenem, EUG—Eugenol, α-PIN—α-Pinene, γ-TERP—γ-Terpinene.

more frequent due to increased antifungal resistance (Fioriti et al. 2022). Several studies have demonstrated synergistic interactions between ibuprofen and different azole antifungals against clinical strains of *C. albicans* (Arai et al. 2005, Król et al. 2018). Unlike antibiotics, there are no clinically approved adjuvants for antifungal drugs. A few studies have highlighted the potential of ibuprofen as an adjuvant, considering its ability to reverse azole resistance and modulate fungal virulence (Pina-Vaz et al. 2000, Ricardo et al. 2009, Ells et al. 2011).

The cytotoxicity and anti-inflammatory properties of the conventional antimicrobials and EOCs has been investigated and discussed in our previous study (Simbu 2024). Ibuprofen can modulate inflammation through multiple mechanisms related to its inhibitory effects on prostaglandin synthesis (Rainsford 2012). Some studies have reported ibuprofen's ability to reduce NO production using LPS-activated macrophages (Berg et al. 1999, De Palma et al. 2009). Ibuprofens inhibitory effects on NO production vary according to the NO source and isoform of nitric oxide synthase (Rainsford 2012). The evaluation of the anti-inflammatory properties of ibuprofen combinations with conventional antimicrobials and EOCs has been poorly studied with limited evidence. Pairwise combinations (ibuprofen and conventional antimicrobial) demonstrated lower cytotoxicity compared to combinations involving the EOCs which may be related to the non-selective toxicity of EOCs (Fuentes et al. 2021).

Table 8. Nitrite production (mean ± SD) and fractional effect (ΣFa) of synergistic combinations ($n = 4$) on RAW 264.7 macrophages.

Combinations	Nitrite production (μM)	% Reduction in nitrite production	Fractional effect (ΣFa)
IBU (25 μg/ml) + AMX (0.31 μg/ml)	35.47 ± 1.02	3.01	0.98 (Add)[2]
IBU (25 μg/ml) + GEN (3.13 μg/ml)	35.27 ± 1.74	3.57	0.98 (Add)
IBU (6.25 μg/ml) + ERY (0.04 μg/ml)	35.47 ± 1.09	3.01	0.98 (Add)
IBU (25 μg/ml) + MERO (0.78 μg/ml)	36.92 ± 1.59	N/A[3]	N/A
IBU (6.25 μg/ml) + AMX (0.31 μg/ml) + EUG (50 μg/ml)	Cytotoxic[1]	N/A	N/A
IBU (25 μg/ml) + GEN (3.13 μg/ml) + α-PIN (25 μg/ml)	Cytotoxic	N/A	N/A
IBU (25 μg/ml) + GEN (3.13 μg/ml) + γ-TERP (25 μg/ml)	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. IBU—Ibuprofen, AMX—Amoxicillin, GEN—Gentamicin, ERY—Erythromycin, MERO—Meropenem, EUG—Eugenol, α-PIN—α-Pinene, γ-TERP—γ-Terpinene.

Conclusion

The present study demonstrates the potential of combining ibuprofen with conventional antimicrobials and EOCs for the management of skin infections. From the results, four synergistic interactions were identified with no antagonism. Our study also was the first to identify higher order combinations involving EOCs and ibuprofen with antibiotics 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. Combination therapies represent 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 are warranted to identify the underlying mechanisms.

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Supplementary data

Supplementary data is available at *JAMBIO Journal* online.

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Author contributions

Shivar Simbu (Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Writing—original draft, Writing—review & editing), Ané Orchard (Conceptualization, Funding acquisition, Methodology, Writing—review & editing), Maryna van de Venter (Conceptualization, Data curation, Funding acquisition, Methodology, Resources, Supervision, Writing—review & editing), and Sandy van Vuuren (Conceptualization, Funding acquisition, Methodology, Project administration, Resources, Supervision, Writing—review & editing)

Data availability

The data underlying this article will be shared on request to the corresponding author.

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