



Rapid-killing efficacy substantiates the antiseptic property of the synergistic combination of carvacrol and nerol against nosocomial pathogens

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Abstract

Globally, new classes of synthetic and natural antibiotics and antivirulents have continuously been validated for their potential broad-spectrum antagonistic activity with the aim of identifying an effective active molecule to prevent the spread of infectious agents in both food industry and medical field. In view of this, present study is aimed at evaluating the rapid killing efficacy of bioactive molecules Carvacrol (C) and Nerol (N) through British Standard European Norm 1276: phase2/step1 (EN1276) protocol. Active molecules C and N showed broad-spectrum antimicrobial activity against the test strains *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli* and *Enterococcus hirae* at concentration range of 78.125, 625, 156.25 and 312.5 µg/mL, respectively, for C, and 625 µg/mL for N. Whereas, combinatorial approach showed efficient activity with four times reduced concentration of C and N at 78.125 and 156.25 µg/mL, respectively, against test strains. Further, EN1276 results proved the rapid killing efficacy of test strains in 1 min of contact time with significant (> 5 log) growth reduction at 100X concentration of actives. SEM analysis and reduced concentration of protease, lipids and carbohydrate contents of treated group biofilm components ascertained preformed biofilm disruption potential of C + N on polystyrene and nail surfaces. C + N at synergistic concentration exhibited no adverse effect on HaCaT cells at 78.125 µg/mL (C) + 156.25 µg/mL (N). Taken together, based on the observed experimental results, present study evidence the antiseptic/disinfectant ability of C + N and suggest that the combination can preferentially be used in foam-based hand wash formulations.

Keywords Nosocomial infections · Antiseptics/disinfectants · Phytocompounds · Synergistic combinations · BS: EN1276 protocol · Hand hygiene

Abbreviations

ATCC	American Type Culture Collection Center
BIA	British Infection Association
BS EN 1276	British Standard European Norm 1276: 2009 phase 2/step 1 protocol
BSA	Bovine serum albumin
BTS	Bacterial test suspension
CDC	Centers for Disease Control and Prevention

CLSI	Clinical and Laboratory Standards Institute
COVID-19	Coronavirus disease 2019
C + N	Carvacrol and nerol combination
C + N*	Synergistic concentration of carvacrol and nerol combination which was used against all the test strains for BS EN 1276 validation and further assays
EC	<i>Escherichia coli</i>
EH	<i>Enterococcus hirae</i>
EOs	Essential oils
FDA	Food and Drug Administration
FIC	Fractional inhibitory concentration
FICI	Fractional inhibitory concentration indices
HAIs	Hospital-acquired infections
ICMR	Indian Council of Medical Research
IDSA	Infectious Diseases Society of America
MDR	Multidrug resistance

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MERS-CoV	Middle-east respiratory syndrome coronavirus
MIC	Minimum inhibitory concentration
MTP	Microtiter plate
NB	Nutrient broth
NI	Nosocomial infection
PA	<i>Pseudomonas aeruginosa</i>
PTS	Product test solution
QACs	Quaternary ammonium compounds
SA	<i>Staphylococcus aureus</i>
SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2
SDW	Sterile distilled water
TSA	Tryptone soya agar
TSB	Tryptone soya broth
Vc	Viable cells

Introduction

Controlling the spread of infectious diseases in healthcare settings has become a major global concern due to the substantial increase in nosocomial infections (NI) (Haque et al. 2018; Tomczyk et al. 2022). Though various approaches such as the identification of novel drugs and vaccination are aiming to treat and prevent the infection rate, hand hygiene is being considered one of the most critical measures in infection control activities (Jumaa 2005). In recent years, healthcare-associated infections caused by multidrug-resistant (MDR) pathogens are constantly increasing the severity of illness and complexity of treatments (Prestinaci et al. 2015; Frieri et al. 2017; Bassetti et al. 2019). Further, predominant bacterial strains of both resident and transient flora such as *Enterobacteriaceae*, *Pseudomonas* spp., and *Staphylococcus aureus* on the hands of healthcare workers have become a major source of cross contamination (Sax et al. 2007). Hand hygiene is considered to be an important criterion to prevent the spread of communicable diseases and nosocomial infections including highly contagious viral diseases such as Middle East respiratory syndrome coronavirus (MERS-CoV) and severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) (Akinyinka et al. 2019). Hand hygiene is the least expensive means of reducing the prevalence of hospital-acquired infections (HAIs) and the spread of antimicrobial resistance, especially in low- and middle-income countries (Loftus et al. 2019; Inauen et al. 2020; Clancy et al. 2021). Other than hospital environments, poor hand hygiene among individuals makes them prone to health impairments. Proper implementation of hand hygiene is evinced scientifically to reduce the peril of cross-transmission of infections. Globally, all the Government policies and clinical guidelines including Centers for Disease Control and Prevention (CDC), Food and Drug Administration (FDA),

Indian Council of Medical Research (ICMR) and Infectious Diseases Society of America (IDSA), British Infection Association (BIA), community health and primary healthcare practitioners are providing valuable suggestions and awareness to the public about the importance of hand hygiene (FDA 2017; Pires et al. 2017). In this situation, scientific studies are also aiming at selecting the efficient and dermal tolerant agents for hand hygiene formulations and developing the strategies to improve the hand hygiene practices through behavioral theories and methods. Hence, identification of the non-irritant and non-toxic active ingredients with rapid killing efficacy for hand wash formulations are still on the prior list in the prevention of disease transmission.

In this context, plant essential oils (EOs) have been validated for their potential broad-spectrum antimicrobial activity and most of them are being used in personal-care products, mouthwashes, and surface cleaners as microbicidal agents (Jirovetz et al. 2007; Guarda et al. 2011; Mulani et al. 2019; Qian et al. 2020; Khare et al. 2021; de Souza et al. 2021). To devise the phytochemical-based active ingredients for hand wash formulations, this investigation was intended to study the disinfectant potential of a synergistic combination of terpenes such as Carvacrol (C) (5-Isopropyl-2-methyl phenol; C₁₀H₁₄O) (NCBI, 2022a) and Nerol (N) (*cis*-3, 7-Dimethyl-2, 6-octadien-1-ol; C₁₀H₁₈O) (NCBI, 2022b). C is a monocyclic monoterpenoid with phenolic functional group otherwise called aromatic hydroxyl group (or) cyclic secondary alcohol (Patočka and Kuca 2013). The hydroxyl group and hydrophobicity provide enhanced antimicrobial efficacy to C than other phenolic monoterpenoids such as eugenol, menthol, carvacryl acetate and carvacrol methyl ether (Arfa et al. 2006; Kim et al. 2012). N, a *cis*-isomer of geraniol, is an acyclic monoterpenoid containing primary alcohol functional group with pleasant rose odor than geraniol (Elsharif and Buettner 2016). Antimicrobial activity/ bioactivity of alcohols is generally higher for primary alcohols followed by secondary and tertiary alcohols. The primary alcohol functional group makes N an efficient antibacterial compound than other alcoholic terpenoids such as menthol, verbenol and borneol (Ceylan and Fung 2004). In agreement, previous studies have demonstrated the efficient antibacterial activity of primary alcohol (N) and phenolic alcohol (C) against Gram-positive and Gram-negative organisms, respectively (Kubo et al. 1995; Arfa et al. 2006). C is found rich in *Origanum vulgare*, *Thymus vulgaris*, *Lepidium* spp. and *Monarda fistulosa*, and N is highly found in *Vitis vinifera*, *Juglans nigra*, *Aloysia citrodora*, and *Vaccinium membranaceum*. Remarkable studies are available on elaborating the antimicrobial activity and other biological benefits of extracts obtained from the aforementioned plants and by-products (Rota et al. 2008; Shen et al. 2014; Kashkooli and Saharkhiz 2014; Filocamo et al. 2015; Bi et al. 2016; Sharifi-Rad et al. 2018; Ghosh et al. 2020; Radulescu et al.

2020; Hasanvand et al. 2021; Jaradat et al. 2021; de Campos et al. 2022). Moreover, combinatorial therapy has become a choice of treatment for combating AMR pathogens (Tamma et al. 2012; Magi et al. 2015; Xu et al. 2018; Li et al. 2022; Köse 2022). Though previous reports have documented the bactericidal efficacy of C and N, no previous reports are available on bactericidal efficiency of the synergistic combination of C + N and its rapid killing efficacy. Hence in the present investigation, C and N was chosen for evaluating their bactericidal and rapid killing efficacy in combinatorial approach against both Gram-positive and Gram-negative organisms.

British Standard European Norm 1276: 2009 (BS EN 1276: 2009) phase 2 step 1 protocol (Referred to as EN1276 Protocol) is a quantitative method that evaluates the disinfectant/antiseptic properties of an active molecule and the method follows the test method of one minute of contact time as a major criterion to prove the rapid killing efficacy of bioactives ((BS EN 1276). In the present investigation, the efficacy of the C + N combination was validated using the EN1276 protocol. Some of the studies have evaluated the effect of antiseptics/ disinfectants using EN1500 phase 2/step 2 protocol as well wherein, the formulation containing the active molecules could be used in hand rub formulations (Babeluk et al. 2014; Pires et al. 2019). Generally, hand rub is a disinfectant formulation used to reduce the microbial viability without the need of an exogenous water and requiring no rinsing with water after topical application (WHO 2009). Further, scope of the EN1276 protocol also states that the compounds which clear test methods and procedures of EN1276 could be used in products diluted with hard water or in the case of ready-to-use products with water (BS EN 1276)). Explicitly, this study is also focused on the evaluation of active molecules that could preferentially be used as active ingredients in hand wash formulations which require rinsing with water. In this context, the study is aimed at evaluating the rapid killing efficacy of the synergistic combination of C + N using the test methods and procedures as given in EN1276 protocol. Furthermore, bacteria are well known for their tendency to form biofilm on various niches as a survival mechanism in resisting the hostile environments (Donlan 2001; Lindsay and von Holy 2006; Sanchez et al. 2013; Gupta et al. 2018; Muhammad et al. 2020). Disrupting the preformed biofilms is an effective strategy in eradicating the microbes residing inside the microbial biofilm (Meng et al. 2011; Musk et al. 2005; Marchese et al. 2003; Abenojar et al. 2018). In view of this, the present investigation is also addressed the preformed biofilm disruption potential of synergistic combination of C + N. Previously, Vila et al. 2015 have used the in vitro biofilm model on human nail surfaces for testing the laser and light therapies against onychomycosis. In the present study, mono-species and mixed-species in vitro bacterial

biofilms on nail surfaces are used to analyze the biofilm disruption efficiency of C + N. In this, SEM analysis is used to understand differences in bacterial cells and biofilm architecture between control and treated samples. In addition to the antibacterial efficacy, analyzing the safety profile of compounds is an important factor in the acceptance of compounds in personal-care products. The human keratinocyte cell line (HaCaT) is generally used for evaluating the toxicity profile of personal-care products (Kyadarkunte et al. 2014; Alnuqaydan et al. 2016; Alnuqaydan and Sanderson 2016; Colombo et al. 2017; Querido et al. 2022). Hence, the study also aimed at evaluating the safety profile of the C + N combination on HaCaT cells. Altogether, the current investigation evidences the rapid killing efficacy and preformed biofilm disruption potential of C + N combination through in vitro experiments and demonstrates the safety profile on HaCaT cells.

Methods

Phytocompounds and bacterial strains

Monoterpenoids Carvacrol (98%, Sigma Aldrich, USA; MW: 150.22; CAS: 499-75-2; LOT # STBH1940, Fig. 1a) and Nerol (97%, Alfa Aeser, UK; MW: 154.25; CAS: 106-25.2; LOT: 10,124,423, Fig. 1b) were prepared as 10 mg/mL stock in methanol and stored at 4 °C for microbroth dilution assays and 100 mg/mL stock in methanol for EN Protocol. Test strains such as *S. aureus* ATCC 6538, *Escherichia coli* ATCC 10,536, *P. aeruginosa* ATCC 15,442, and *Enterococcus hirae* ATCC 10,541 were selected as mentioned in the EN protocol and procured from American Type Culture Collection Center (ATCC), USA. Glycerol stocks of the cultures were prepared and stored at – 80°C. Nutrient broth (NB) for *S. aureus*, *P. aeruginosa* and *E. coli* and tryptone soya broth (TSB) for *E. hirae* were used as growth media for all the assays unless otherwise mentioned. Glycerol stocks were sub-cultured on tryptone soya agar (TSA) and stored at – 4°C for up to 2 months. A single colony of axenic culture

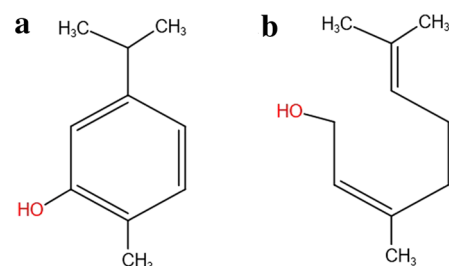


Fig. 1 Structure of phytocompounds **a** C (5-Isopropyl-2-methyl phenol), **b** N (3,7-dimethylocta-2,6-dien-1-ol) (Structures are drawn using KingDrawPc_V2.7.1.05)

from the stored plate was used to inoculate 2 mL of growth medium and incubated at 37 °C overnight at 160 rpm and used as primary inoculum for all the test strains.

Determination of minimum inhibitory concentration (MIC) of monoterpenoids

The MIC of monoterpenoids against four test strains was determined using microbroth dilution assay in 96 well microtiter plate (MTP) as provided in the Clinical and Laboratory Standards Institute (CLSI) manual (CLSI 2012). The brief protocol is given in the supplementary information.

Metabolic viability assay

The effect of C and N on cell viability was analyzed using a resazurin dye-based metabolic viability assay. The assay was carried out as described earlier for MIC determination and incubated at 37 °C for 18 h. After incubation, 160 µM resazurin dye was added to each well and incubated in dark at 37 °C for 1 h. Color change from blue to pink was recorded by reading the fluorescence intensity at 570 and 590 nm. Percentage reduction of cell viability in C- and N-treated samples was analyzed by comparing the fluorescence intensity (Swetha et al. 2020).

Determination of synergism between C and N using a two-dimensional checkerboard assay

A two-dimensional checkerboard assay was carried out against four test strains individually, to determine the combinatorial effect of C and N based on the fractional inhibitory concentration (FIC) (Swetha et al. 2020). The brief protocol is given in supplementary information.

Assessment of antimicrobial activity of synergistic combination using Live/Dead staining

Live/dead analysis of the control culture without phytochemicals and the culture treated with phytochemicals at synergistic concentration was done using acridine orange/ propidium iodide (AO/PI) staining as described in (Bogachev et al. 2018) Initially, 10 mL of growth media for each bacterial strain were added with the synergistic concentration of phytochemicals, and growth media without phytochemicals were maintained as control. Blank was maintained to ensure a sterile test environment by keeping the growth media alone in a test tube. Control and treated groups were added with 1% of overnight culture containing 1×10^8 CFU/mL. After 18 h incubation at 37 °C, 5 µL of culture from each sample was mixed with 5 µL of 1:1 ratio of AO/PI staining (Stock concentration of AO is 18 mM and PI is 7.5 mM) and kept at room temperature for 5 min in dark

condition. Then, the samples were examined by fluorescence microscope [Nikon Eclipse Ts2R, 400×] for identifying the presence of dead and live cells.

Evaluation of killing effect of synergistic combination using BS EN 1276: 2009 phase 2 step 1 protocol

The methods described in EN protocol were followed for the preparation of reagents and the procedure for evaluating the killing effect of bioactives (BS: EN1276). EN protocol is a quantitative suspension test for assessing the bactericidal activity of chemical disinfectants and antiseptics used in food, industrial, domestic and institutional areas. Among the obtained working synergistic concentrations of C + N against four test strains, the concentration which showed activity against all the test strains named C + N*. The synergistic combination of C + N* was further evaluated for its proficiency to outmaneuver the microbial bioburden using EN1276 protocol. Various ratios viz. 20X, 40X, 60X, 80X, and 100X (Table 3) of C + N* were tested using EN1276 protocol to identify the proficient ratio which can kill the microbes in one minute of contact time, and subsequently, viable cells (Vc) were determined by membrane filtration technique using 0.45 µ, 47 mm in diameter nitrocellulose membrane filter [HiMedia Laboratories Pvt. Ltd., Mumbai].

Preparation of product test solution (PTS)

Water comprising active ingredients, mineral salts, and the bovine serum albumin (BSA) was used as a product test solution. In brief, 209 mM MgCl₂, and 417 mM CaCl₂ were dissolved in 1 L of distilled water (DW), autoclaved, and stored at - 4 °C (Solution A). To 1 L sterile DW (SDW), 417 mM NaHCO₃ was dissolved and further sterilized using a 0.22 µ membrane filter and stored at - 4 °C (Solution B). The hard water was freshly prepared for each experiment by diluting solutions A and B at a ratio of 0.6: 0.8% in SDW. Active molecules were added to the hard water from the 100 mg/mL stock solution before each experiment and used as PTS. Further, the BSA stock solution at 3.0% was prepared in the autoclaved diluent containing 0.1% tryptone and 0.85% NaCl and further sterilized using a 0.22 µ membrane filter and stored at - 4 °C which was then used as an interfering substance.

Determination of the number of viable cells (Vc) present in the bacterial test suspension 'N'

Test strains were grown as mentioned earlier at 37 °C for 8 h and bacterial test suspension (BTS) was prepared by adjusting the OD₆₀₀ to 0.2 using diluent. The number of Vc (N) present in the BTS was calculated as follows:

Initially, 1 mL of 10^{-6} and 10^{-7} dilutions were spread on surface-dried TSA plates and incubated at 37 °C for 24 h and the N was calculated using the following formula, $N = (C / (n_1 + 0.1 n_2)) \times 10^{-6}$. Where, N = Number of Vc present in the bacterial suspensions, C = sum of Vc values taken into account, n_1 = Number of Vc values taken into account in lower dilution (10^{-6}), n_2 = Number of Vc values taken into account in higher dilution (10^{-7}), 10^{-6} is the dilution factor corresponding to the lower dilution.

Validation of membrane filtration method

The non-lethal effect of reagents and experimental procedure of EN1276 protocol was verified using validation controls using experimental conditions control (A), filtration control (B) and method validation (C). The brief protocol is given in supplementary information.

Assessment of bactericidal activity of the bioactive combination

Bactericidal activity of active molecules after one-minute contact time was assessed using the membrane filtration method under aseptic conditions at ambient room temperature (25 °C) as described in EN protocol (BS: EN1276). In brief, the filtration apparatus equipped with a membrane filter was preadjusted to filter 150 mL liquid in 30 s by leveraging the pressure gauge in the vacuum pump. To the 1 mL of BTS, 1 mL of interfering substance (final concentration: 3.0 mg/mL) was mixed and allowed to stand for 2 min at room temperature for homogenization. The homogenized mixture was added to 8 mL of hard water containing active molecules at C + N* concentration and vortexed. After one-minute contact time, 100 µL of test mixture was filtered through the filtration unit having 150 mL rinsing liquid followed by 50 mL of DW. The membranes were placed on the surface-dried TSA plates and incubated at 37 °C for 24 h and the colonies present on the membranes were counted and calculated for the 'Na' values using the formula, $Na = (C \times 10) / n$. Here, Na is the number of survivors in the test mixture at the end of contact time; C is the sum of Vc values taken into account; n is the number of Vc values taken into account. In the case of Vc values less than 14 and above 165, C was taken as < 14 and > 165 , respectively, regardless of the exact numbers obtained in the individual Vc values.

Microscopic analysis of preformed biofilms on nail surfaces using scanning electron microscope (SEM)

Initially, to verify the presence of microbial flora and biofilm on the free edge of nails, we analyzed the healthy volunteers' nail samples through SEM. Ethical clearance to collect and work on nail samples from healthy volunteers was

pre-approved by the Institutional Ethics Committee (Human Research), Alagappa University (AU) [IEC Ref. No. IEC/AU/2018/4]. For this study, we collected nail samples from four healthy volunteers from the Department of Biotechnology, AU. Volunteers were instructed to wash their hands with a commercial hand wash solution followed by air drying of hands. Then, using a nail clipper, nails were carefully collected and were excised with sterile scissors into 0.2 mm × 0.4 mm pieces and fixed with 2.5% glutaraldehyde for 1 h. Then, the samples were dehydrated with increasing concentrations of ethanol from 20 to 40%, 60%, 80%, and 100% at 2-min intervals in each concentration. After dehydration, nail clippings were air-dried for 40 min at room temperature and then, subjected to gold sputtering before SEM analysis with FEI QUANTA 250 FEG (Thermo Fisher Scientific, Waltham, MA, USA) (Swetha et al. 2021).

Subsequently, to evaluate the biofilm disruption potential of the C + N* combination, nail clippings were collected from the healthy volunteers as described earlier and further pre-processed for in vitro microbiological studies. In brief, nail clippings were excised into 0.2 mm × 0.4 mm pieces and washed with 75% ethanol followed by distilled water, and then sterilized by moist heat sterilization at 121 °C for 15 min at 15 lbs. Then, bacterial cells were allowed to form biofilm on the sterilized nails. As both monomicrobial (Archer et al. 2011) and polymicrobial species (Tomczak et al. 2017) were reported from the unhygienic clinical nail samples, *S. aureus* biofilm for mono-species and *S. aureus* and *P. aeruginosa* biofilms for mixed species were studied. The prepared nail samples were added to the growth media containing 1% of 1×10^8 cells of *S. aureus* for mono-species and 1% of mixed culture containing an equal volume of 1×10^8 cells of *S. aureus* and *P. aeruginosa* for mixed species. After 24 h incubation at 37 °C, planktonic cells on the nail surfaces were removed by gentle washing with SDW and then treated with a 100X ratio of C + N* combination as follows, nail samples having preformed biofilms were added with 1 mL of product test solution containing an interfering substance with and without actives for treated and control group, respectively, and immediately after one minute, the nail samples were gently washed with SDW and further processed for SEM analysis (VEGA 3 TESCAN, Czech Republic) as described earlier.

Analysis of preformed biofilm disruption potential of C + N* synergistic concentration

Analysis of preformed biofilm disruption potential of C + N* was done by following the method described in Kadam et al. (2020) with slight modification especially in treatment time of preformed biofilm with bioactive compounds. Initially, the cells were allowed to form biofilm on 6-well polystyrene surfaces in which the cultures were inoculated with TSB

and incubated for 48 h at 37 °C for *S. aureus*, *P. aeruginosa* and *E. coli*, and 72 h for *E. hirae*. Then, the supernatant was removed and washed with sterile PBS and then, 0.9 mL of hard water and 0.1 mL of BSA (30 mg/mL) were added to the each well. Finally, wells were added with 100X concentration of C + N* synergistic combination and immediately after 1 min contact time, the solutions were discarded by inverting the plates in a discarding jar and quickly washed by submerging the plates in SDW followed by rinsing liquid. Each well was then added with 1 mL of PBS and the sessile cells adhered on the walls of 24-well plates were gently scrapped off to made them utilize the resazurin present in the PBS. Subsequently, each well was added with 160 µM resazurin dye and incubated in dark at 37 °C for 24 h. Then, the percentage of reduction in cell viability was analyzed by measuring the fluorescence intensity at 560/590 nm as described earlier.

Estimation of components of treated and untreated biofilms

Initially, the cells were allowed to form biofilm on 6-well polystyrene surfaces as described earlier. Then, the supernatant was removed and washed with sterile PBS and then, subjected for treatment with 100X concentration of C + N* combination 1 min and washed with sterile PBS. Biofilm cells were then scraped off in 200 µL of PBS and used for further analysis. Estimation of carbohydrates, lipids and proteins contents were performed by following the methods described in Swetha et al. (2020) (The brief protocols are given in Supplementary information).

Analysis of cytotoxicity profile of C + N* combination on HaCaT (human skin keratinocyte cell line) cells

Immortalized human keratinocytes, HaCaT cells were used for the examination of the effect of C + N* combination on skin cells after one minute of contact time. The HaCaT cells were purchased from NCCS, Pune, India, and maintained in DMEM high glucose medium (Gibco) supplemented with 10% FBS along with the 1% antibiotic–antimycotic solution in the atmosphere of 5% CO₂, 18–20% O₂ at 37 °C in the CO₂ incubator and sub-cultured for every two days. For analysis of the cytotoxicity profile of the C + N* combination, the cells were cultured in a 12-well plate on the sterile coverslips coated with Poly-L-Ornithine solution at a density of 2 × 10⁵ cells/ 2 mL and incubated in a CO₂ incubator overnight at 37 °C for 24 h. Then, aspirated the spent medium and treated the cells with 78.125 µg/mL of C and 156.25 µg/mL of N, in 2 mL of culture medium. Immediately after one minute of contact time, the medium was removed from all the wells and washed with PBS. Then, the coverslips were removed from the 12-well plate and washed

with 1 mL of 1X D-PBS (HiMedia). The cells were stained with the staining solution containing 50 µg/mL of ethidium bromide (Thermo Fisher, USA) and 20 µg/mL of acridine orange (Thermo Fischer, USA) for 10 min. After incubation, the staining solution was removed and a drop of mounting medium was added to the coverslip before imaging. Images were acquired using a fluorescence microscope with ImageJ Software v1.48 (LSM 880 live cell imaging confocal system, Germany) (Colombo et al. 2017; Ding et al. 2022; Ortega-Llamas et al. 2022).

Statistical analysis

Microbroth dilution assays, metabolic viability assays, and preformed biofilm disruption assays were carried out in biological and experimental triplicates. Experiments on EN protocol and Live/Dead staining were carried out in biological triplicates and experimental duplicates. Data are represented in means ± standard deviations (SDs). One-way ANOVA with Dunnett's Post Hoc method was performed using the SPSS Software package (USA) to test the statistical significance between control and treated samples.

Results

Minimum inhibitory concentration and cell viability assay

The activity of C and N on *S. aureus*, *P. aeruginosa*, *E. coli*, and *E. hirae* was tested at various concentrations ranging from 5000 to 2.44 µg/mL using a microbroth dilution assay. C at the lowest concentrations of 78.125 µg/mL, 625 µg/mL, 156.25 µg/mL and 312.5 µg/mL exhibited ≥ 90% growth inhibition against *S. aureus*, *P. aeruginosa*, *E. coli*, and *E. hirae*, respectively. Similarly, N at the lowest concentration of 625 µg/mL showed ≥ 90% growth inhibition against all the test strains and hence the specified concentrations were determined as MICs for respective strains (Table 1). Significant reduction in pink fluorescence in resazurin-based metabolic viability assay also confirmed the absence of viable cells in MICs of C and N against test strains (Fig. 2a–d).

Synergistic interactions of antimicrobial agents

Checkerboard assay is used to assess the synergism between drug candidates when used concurrently. The C + N combination showed synergistic antimicrobial activity against test strains at various ratios from 1:1, 1:0.5, 0.5:1, 0.5:2 to 0.5:4 with the fractional inhibitory concentration indices (FICI) ranging from 0.5 to 0.25. Working synergistic concentrations of C + N and respective FICI were compared with MIC of individual actives (Table 1). Observed results confirmed that

Table 1 Comparison of minimum inhibitory concentrations of C and N with working synergistic concentrations of C+N combination and respective FICI values

S. no	Test strain	MIC of tested combination ($\mu\text{g}/\text{mL}$)	Working synergistic concentration(s) ($\mu\text{g}/\text{mL}$)	FICI index/ indices
1	<i>S. aureus</i> ATCC 6538	C: 78.125 N: 625	19.53 + 39.06	0.3125
			19.53 + 78.13	0.375
			19.53 + 156.25	0.5
2	<i>P. aeruginosa</i> ATCC 15,442	C: 625 N: 625	78.125 + 78.125	0.25
			78.125 + 156.25	0.375
			156.25 + 39.063	0.3125
			156.25 + 78.125	0.375
			156.25 + 156.25	0.5
3	<i>E. coli</i> ATCC 10,536	C: 156.25 N: 625	19.53 + 156.25	0.375
			39.0625 + 156.25	0.5
4	<i>E. hirae</i> ATCC 10,541	C: 312.5 N: 625	78.125 + 156.25	0.5

the working synergistic concentrations of C+N are lower than the cumulative concentration of individual actives with significantly higher antimicrobial activity. In the C+N combination, C at 78.125 $\mu\text{g}/\text{mL}$ and N at 156.25 $\mu\text{g}/\text{mL}$ (C+N*) showed significant killing effects on *S. aureus*, *P. aeruginosa*, *E. coli*, and *E. hirae*, which were taken for further studies.

Live/Dead analysis proves the efficient killing effect of C+N*

AO permeates viable cells and PI penetrates the cells with the compromised cell membrane and the binding of AO and PI to the dsDNA causes them to fluoresce green and red, respectively. The results of fluorescence analysis of control and treated cells (Fig. 3) confirmed the efficient bactericidal activity of C+N* at MIC. In the untreated control group, most of the cells are viable which fluoresce green whereas, in the treated group most of the cells fluoresce red; which indicates that C+N* has killed most of the viable cells of test strains.

The rapid killing effect of the synergistic combination of C+N*

The rapid killing effect of C+N* against both Gram-positive and Gram-negative bacteria was tested using the EN protocol and the reduction in the number of viable cells after one-minute contact time was expressed in terms of log reduction (R).

Validation of experimental conditions

To assess the killing effect of the C+N* synergistic combination using EN protocol, BTS was optimized, and 'N' of each strain was determined as 1.85×10^8 CFU/mL for

S. aureus, 4.0×10^8 CFU/mL for *P. aeruginosa*, 2.7×10^8 CFU/mL for *E. coli* and 1.5×10^8 CFU/mL for *E. hirae*. Similarly, the Vc value of validation suspension (Nv) for *S. aureus* was determined as 1.5×10^3 CFU/mL. Validation controls were used to verify the absence of lethal effects in experimental conditions and protocols (Control A) and also to ensure the proper filtration of liquids through the filtration unit (Control B) and proper removal of actives from membrane surfaces with rinsing liquid (Control C). Numbers of Vc were calculated as ≥ 165 , ≥ 165 , and 85 for control A, control B, and control C, respectively (Table 2 and Supplementary Fig. 1).

The bioactive combination kills the test strains in one-minute contact time

Synergistic concentrations of C+N* at 20X, 40X, 60X, 80X, and 100X were tested for their killing effect within a 1 min contact time. The C+N* concentrations at 80X and 100X ratios showed > 5 log reduction (R) in the viable count at the end of 1 min contact time. The R values are given in Table 3 (Detailed calculation can be seen in Supplementary Table 1) and the representative images of the membrane filters with viable cells that were used to calculate the R values are shown in Supplementary Fig. 2.

The C+N* combination impairs the exopolysaccharide matrix of matured biofilm of mono-species and mixed-species on nail surfaces

The observed SEM images of nail samples obtained from the healthy volunteers confirmed the presence of both mono-species (Fig. 4c) and polymicrobial species (Fig. 4a, b, d) with mucoid cell surface on the free edge of nails even after hand washing with a soap solution. Based on this background data, in vitro biofilm disruption assay

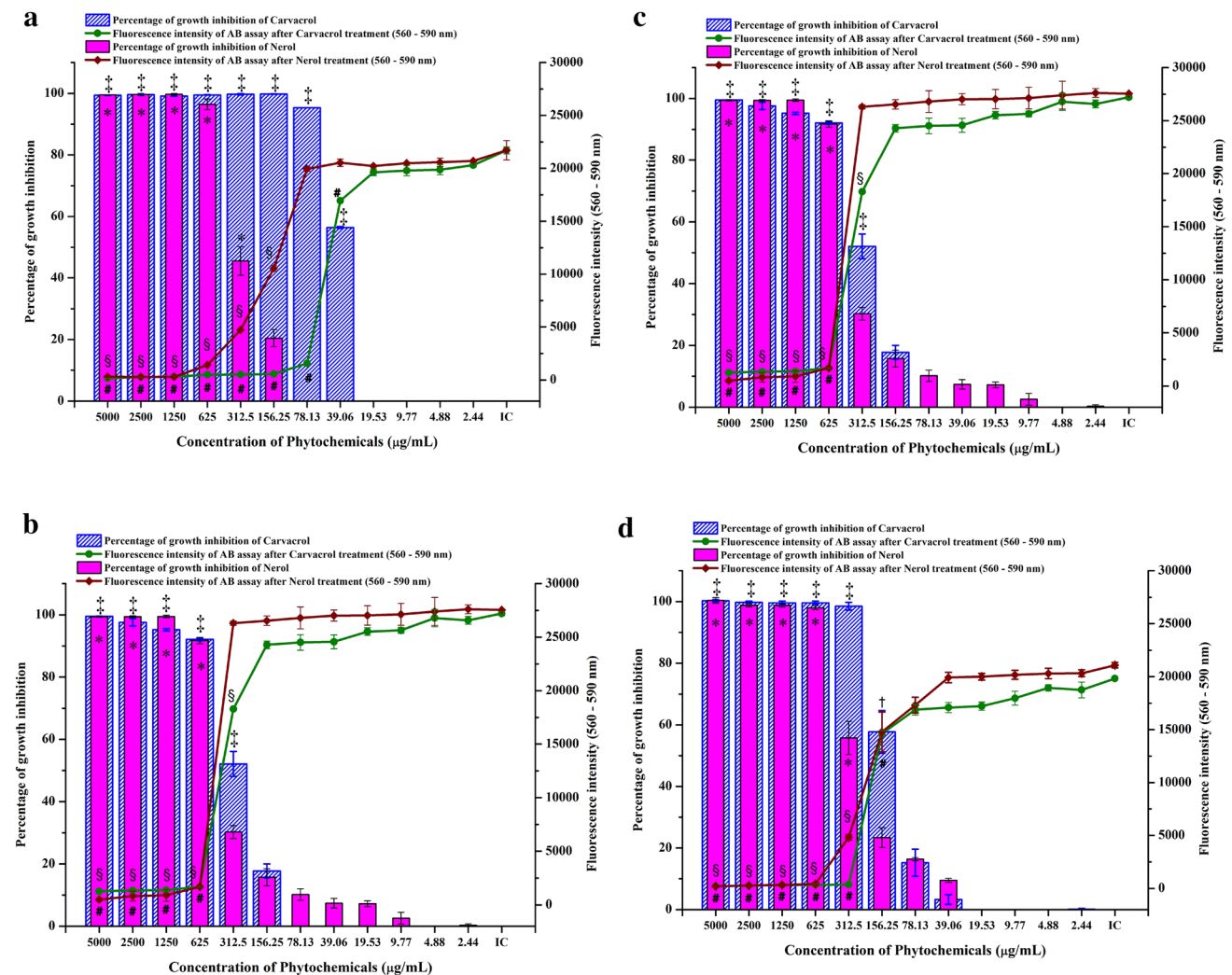


Fig. 2 Minimum inhibitory concentration of C and N against test strains. Bar charts represent the results of the microbroth dilution assay and linear graphs represent the resazurin-based metabolic viability assay results for **a** *S. aureus*; **b** *P. aeruginosa*; **c** *E. coli*; **d** *E. hirae*. Here, ‡ represents the significant growth inhibition of bacteria after C treatment ($P < 0.05$) in microbroth dilution assay. * represents

the significant growth inhibition of bacteria after N treatment ($P < 0.05$). § represents the significant reduction in fluorescence intensity of resazurin in metabolic viability assay for C treatment. # represents the significant reduction in fluorescence intensity of resazurin in metabolic viability assay for N treatment

was performed wherein, mono-species and mixed species of bacteria were allowed to form biofilm on nail samples. Then, the obtained results of in-depth microscopic analysis using SEM revealed that the 100X concentration of C + N* combination has efficiently disrupted the exopolysaccharide (EPS) of 24 h mature biofilm matrix. Substantial disruption of EPS matrix of both mono-species (*S. aureus*) (Fig. 5b) and mixed-species (*S. aureus* + *P. aeruginosa*) (Fig. 5d) biofilms was observed on C + N* treated nail samples when compared to the untreated biofilms. In the control group, a characteristic slimy exopolysaccharide matrix was observed as shown in Fig 5a (Mono-species) and Fig. 5c (Mixed species). Whereas,

the rough surfaces on the outer membrane as well as some damaged cells were observed in SEM images of treated groups. These results demonstrated that the C + N* concentration acted effectively on the bacterial membrane and removed the biofilm matrix from nail biofilms.

Synergistic concentration of C + N* combination efficiently disrupts the preformed biofilm on polystyrene surfaces

Preformed biofilm disruption potential of the synergistic combination of phytochemicals was confirmed using

Fig. 3 Representative images of the fluorescence analyses of AO/PI-stained control and C + N*-treated cells of bacterial strains. Green fluorescence represents the viable cells and red fluorescence represents the dead cells. **a** and **b**—*S. aureus* control and treated; **c** and **d**—*P. aeruginosa* control and treated; **e** and **f**—*E. coli* control and treated; **g** and **h**—*E. hirae* control and treated [Nikon Eclipse Ts2R, 200X, Scale Bar: 100 μ m]

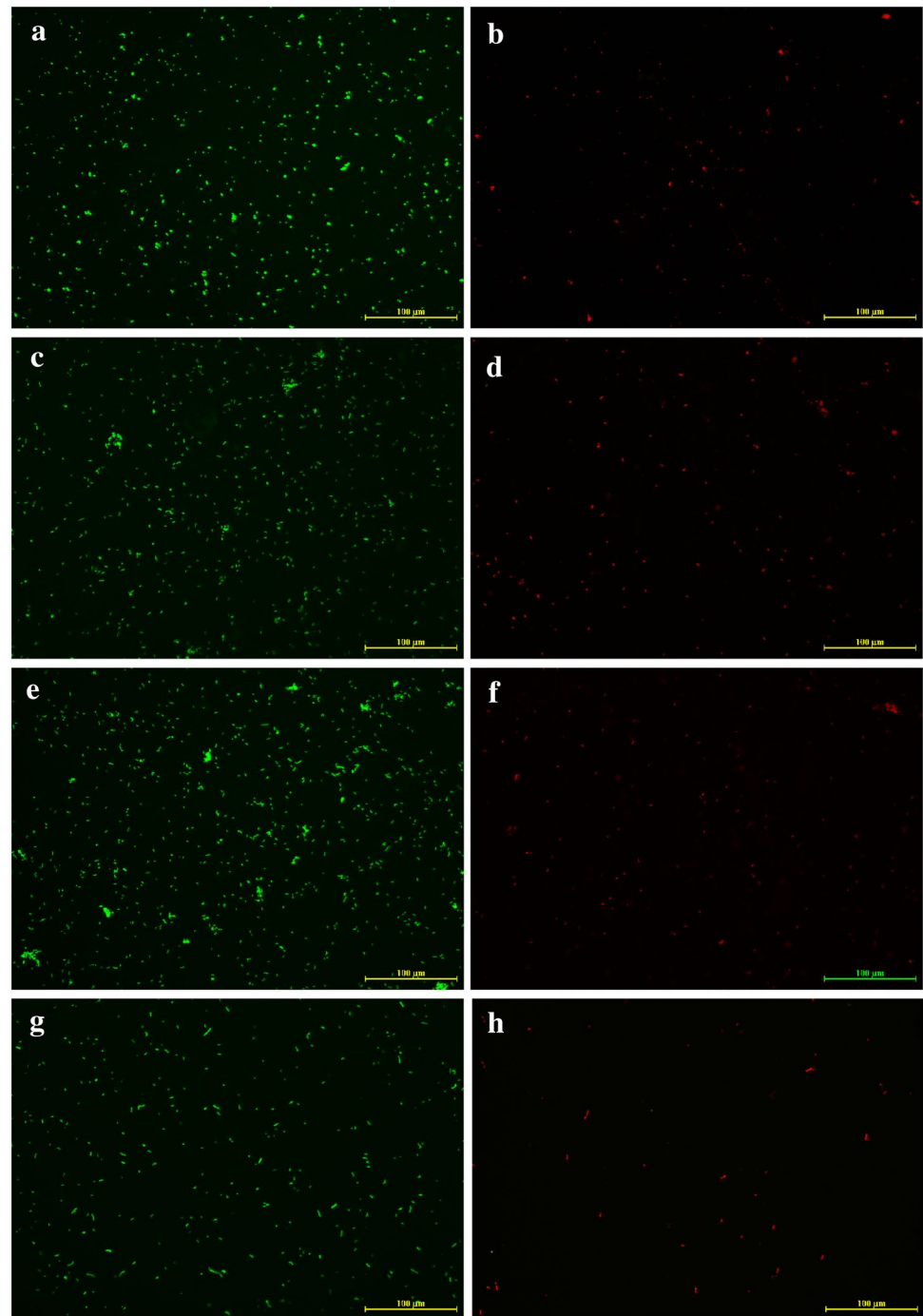


Table 2 Inference on controls and method validation of EN protocol

S no	Validation suspension N_v , N_{v_0} CFU/ mL	Test method	Number of colonies
1	$N_v = 1.5 \times 10^3$ $N_{v_0} = 150$	Experimental conditions control A (Control A)	≥ 165
2		Filtration control B (Control B)	≥ 165
3		Method validation C (Control C)	≥ 165

Here, N_v no. of cells/ mL in the validation suspension, N_{v_0} no. of cells/ mL in the validation mixture at the beginning of the contact time [$N_{v_0} = N_v / 10$]

Table 3 Effect of various ratios of synergistic concentration of C+N*against *S. aureus*, *P. aeruginosa*, *E. coli*, and *E. hirae*.

S no	The concentration of C and N combination (%)	Log reduction <i>R</i>			
		<i>S. aureus</i>	<i>P. aeruginosa</i>	<i>E. coli</i>	<i>E. hirae</i>
1	20X C: 0.156, N: 0.3125	4.0497	4.3889	5.8293	4.8239
2	40X C: 0.3125, N: 0.625	4.0497	6.2085	7.4314	5.7782
3	60X C: 0.4688, N: 0.9375	4.6044	7.6064	7.4314	7.2672
4	80X C: 0.625, N: 1.25	7.2672	7.6064	7.4314	7.2672
5	100X C: 0.781, N: 1.56	7.2672	7.6064	7.4314	7.2672

N number of viable cells/mL in test suspension, *N*₀ number of viable cells/mL in the test mixture at the beginning of the contact time, *N*_a number of viable cells in the test mixture at the end of the contact time. Concentrations that are exhibiting *R* values (Reduction in the number of viable cells on the log scale) of > 5 cleared EN 1276 criteria

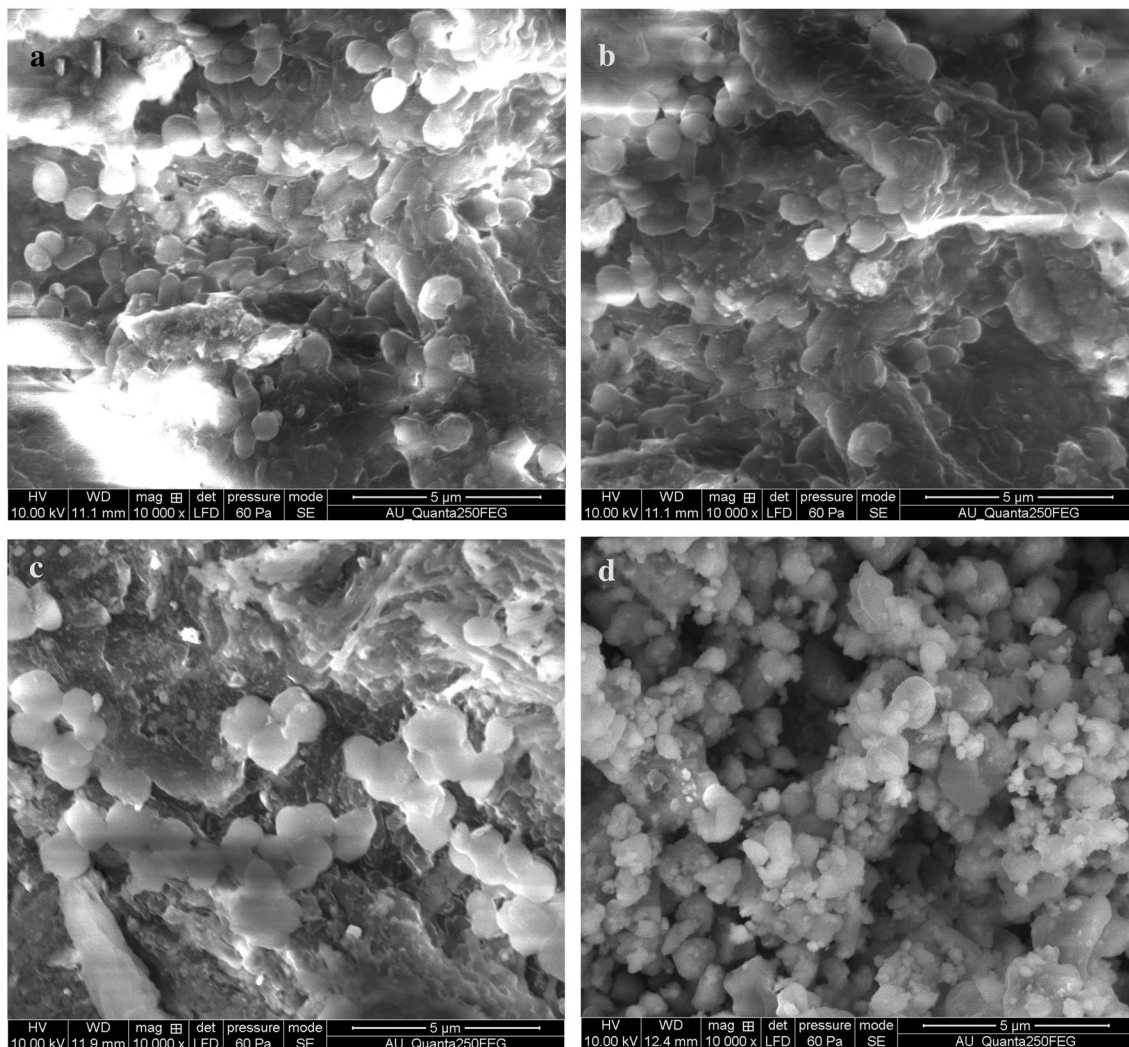


Fig. 4 Scanning Electron Microscopic images of mono-species **c** and mixed-species **a**, **b** and **d** bacterial cells on nail clippings of healthy human volunteers [FEI QUANTA 250 FEG, 10000X, Scale Bar: 5 μm]

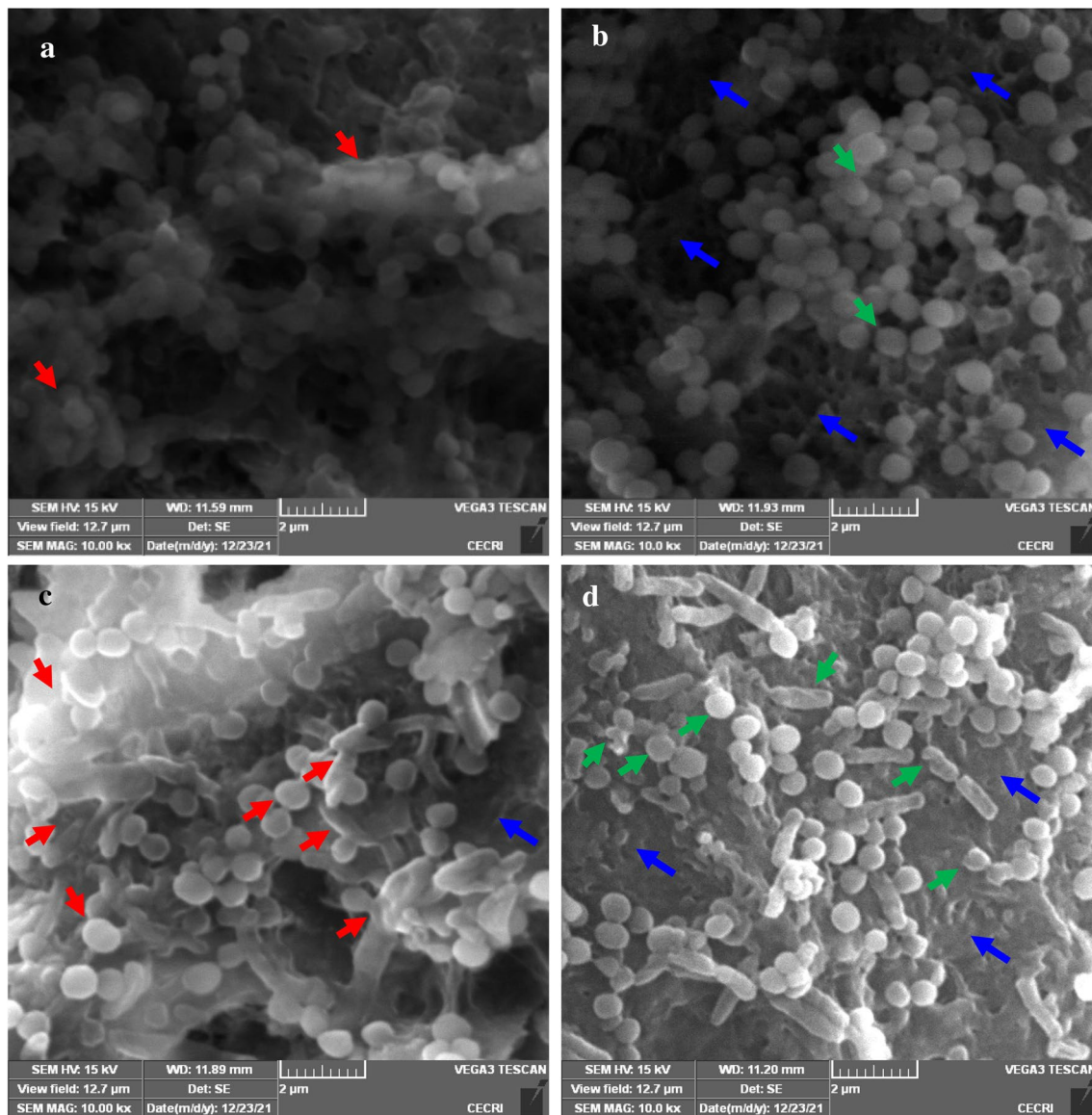


Fig. 5 Scanning Electron Microscopic images of mono-species [(a) Control, (b) Treated] and mixed-species [(c) Control, (d) Treated] bacterial cells [VEGA 3 TESCAN, 10000X, Scale Bar: 2 μ m]. Red arrows in control group represents the presence of mucoid biofilm

matrix on the bacterial cells. Green arrows in treated group represents the non-mucoid, less aggregated, rough cells and membrane damaged cells in the treated samples. Blue arrows represent the normal nail surfaces

resazurin-based metabolic viability assay. Significant loss in the viability of bacteria residing in the biofilm matrix was observed after treatment with the 100X concentrations of C + N* combination (Fig. 6).

The C + N* combination alters the components of biofilm matrices

The intact matured biofilm on polystyrene plates were observed after 48 h in all the test strains except *E. hirae* which was incubated for 72 h to obtain the mature biofilm. The 100X concentration of C + N-treated biofilm of *S. aureus*, *P.*

aeruginosa, *E. coli*, *E. hirae*, and *S. aureus* + *P. aeruginosa* showed altered biofilm components in the treated group. Carbohydrate content was found to be reduced by 50% in *S. aureus*, 60% in *P. aeruginosa*, 75% in *E. coli*, and 80% in *E. hirae* and *P. aeruginosa* + *S. aureus* biofilm matrices (Fig. 7a). Similarly, around 60–70% of protein contents were found to be reduced in biofilm matrices of all test strains (Fig. 7b). In the case of lipid contents analysis, the observed results showed significant reductions of about 25% and 50% in *S. aureus* and *E. coli* biofilm matrices. Whereas, characteristic reductions in lipid contents were observed in other species biofilms (Fig. 7c).

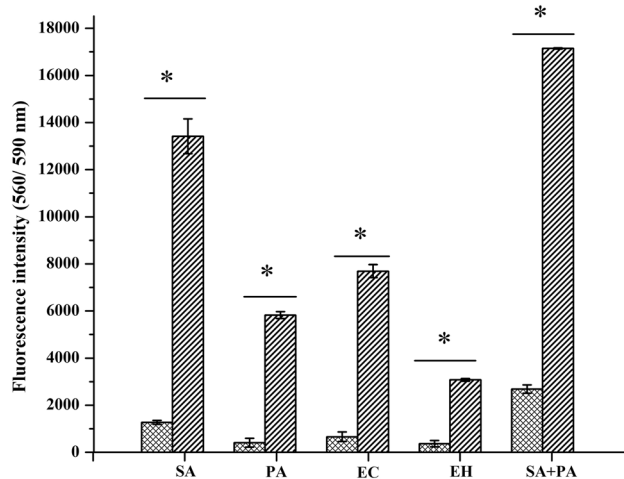


Fig. 6 Representative image illustrating the effect of C+N on the viability of cells residing in the biofilm matrix. Significantly reduced fluorescence intensities at treated groups reflect the absence of metabolically active cells and confirm the effective biofilm disruption potential of the C+N combination [* Significant reduction on the viability of treated cells, $p \leq 0.05$]. Here, SA—*S. aureus*, PA—*P. aeruginosa*, EC—*E. coli*, EH—*E. hirae* and PA+SA—Mixed species of *P. aeruginosa* and *S. aureus*

C + N* combination shows the biocompatibility on HaCaT cell lines

HaCaT cells have been widely employed to determine the epidermal skin toxicity of various agents. Nucleic acid-binding dyes acridine orange (AO) and Ethidium bromide (EtBr) based viability assay was performed to assess the cytotoxicity effect of C + N* on HaCaT cells. The microscopic images of live/ dead staining of treated cells (Fig. 8: d, e and f) were similar to that of untreated control (Fig. 8: a, b and c) without observable cell detachment and the results exemplified the non-deleterious effect of C + N* on keratinocytes after 1-min contact time.

Discussion

Controlling the spread of infections among both community-wide individuals and hospitalized patients, especially during therapeutics is the most challenging task worldwide. In addition, maintaining proper hand hygiene is considered one of the important critical measures in the process of mitigating the spread of communicable infections. The current Coronavirus disease 2019 (COVID-19) pandemic has also emphasized the importance of frequent hand washing in preventing the spread of infections (Gray et al. 2020; Rundle et al. 2020). In this scenario, the development of various antimicrobial formulations with fewer/ nil side effects is an essential need for the hospital environments as well as

community-wide hygienic practices. In general, antimicrobial agents of hygiene products should elicit rapid killing efficiency against test strains. The BS EN1276 protocol also specifies certain test methods and minimum requirements for an antimicrobial agent if it has to be established as a disinfectant and antiseptic agent. According to EN1276, the potential antiseptic agent should exhibit > 5 log reduction on the viability of the test strains within one minute of contact time (BS EN 1276). In agreement, observed results of the current research endorse the rapid killing efficacy of the synergistic combination of phytochemicals C and N against both Gram-positive and Gram-negative bacteria such as *S. aureus*, *P. aeruginosa*, and *E. coli*, and *E. hirae*. Based on the obtained results, the investigation supports the antiseptic/ disinfectant potential of the C + N combination and suggests that the C and N combination can be included as the active ingredients in hand wash formulation(s).

Initially, the bactericidal effect of monoterpenoids C and N was investigated against both Gram-positive and Gram-negative bacteria through microbroth dilution assay and results showed the absence of visible growth against test strains, which confirmed the broad-spectrum antimicrobial activity of C and N. Previous studies have also evidenced the antimicrobial activity of individual actives, C against *P. aeruginosa* (Lambert et al. 2001), *Clostridium perfringens* (Juneja and Friedman M 2007), *Mycobacterium avium* subsp. *Paratuberculosis* (Wong et al. 2008), *Listeria monocytogenes* (El Abed et al. 2014), *Streptococcus pyogenes* (Magi et al. 2015; Wijesundara et al. 2021), uropathogenic *E. coli* (Khan et al. 2017), *Klebsiella pneumoniae* (Köse, 2022) and N against *S. aureus*, *E. faecalis*, *E. coli*, *Proteus vulgaris*, *P. aeruginosa*, *Salmonella* sp., *K. pneumoniae* (Jirovetz et al. 2007; Wang et al. 2019). Moreover, a previous study has also investigated the effect of C against enteric viruses and suggested of using the C as a disinfectant (Sánchez et al. 2015). The remarkable chemical structure of C and N contributes to their broad-spectrum antimicrobial activity. C contains –OH functional group in benzene ring called a phenolic alcohol which is a secondary alcohol (Arfa et al. 2006; Kim et al. 2012). Acyclic monoterpenoid N contains the functional group –OH at acyclic ring structure called primary alcohol (Elsharif and Buettner 2016). Antibacterial activity of both primary alcohol (N) and phenolic alcohol/ secondary alcohol (C) are previously been reported against Gram-positive and Gram-negative organisms (Kubo et al. 1995; Arfa et al. 2006). Hence in the present investigation, combination of both C and N is used to obtain the efficient broad-spectrum antibacterial activity by combining the bactericidal effect of both monocyclic phenols and acyclic primary alcohols. The synergistic combination of C + N markedly reduced the net weight of active molecules than their individual counterpart with efficient bactericidal activity. In this study, the MIC of C was 2, 3, and 4 times

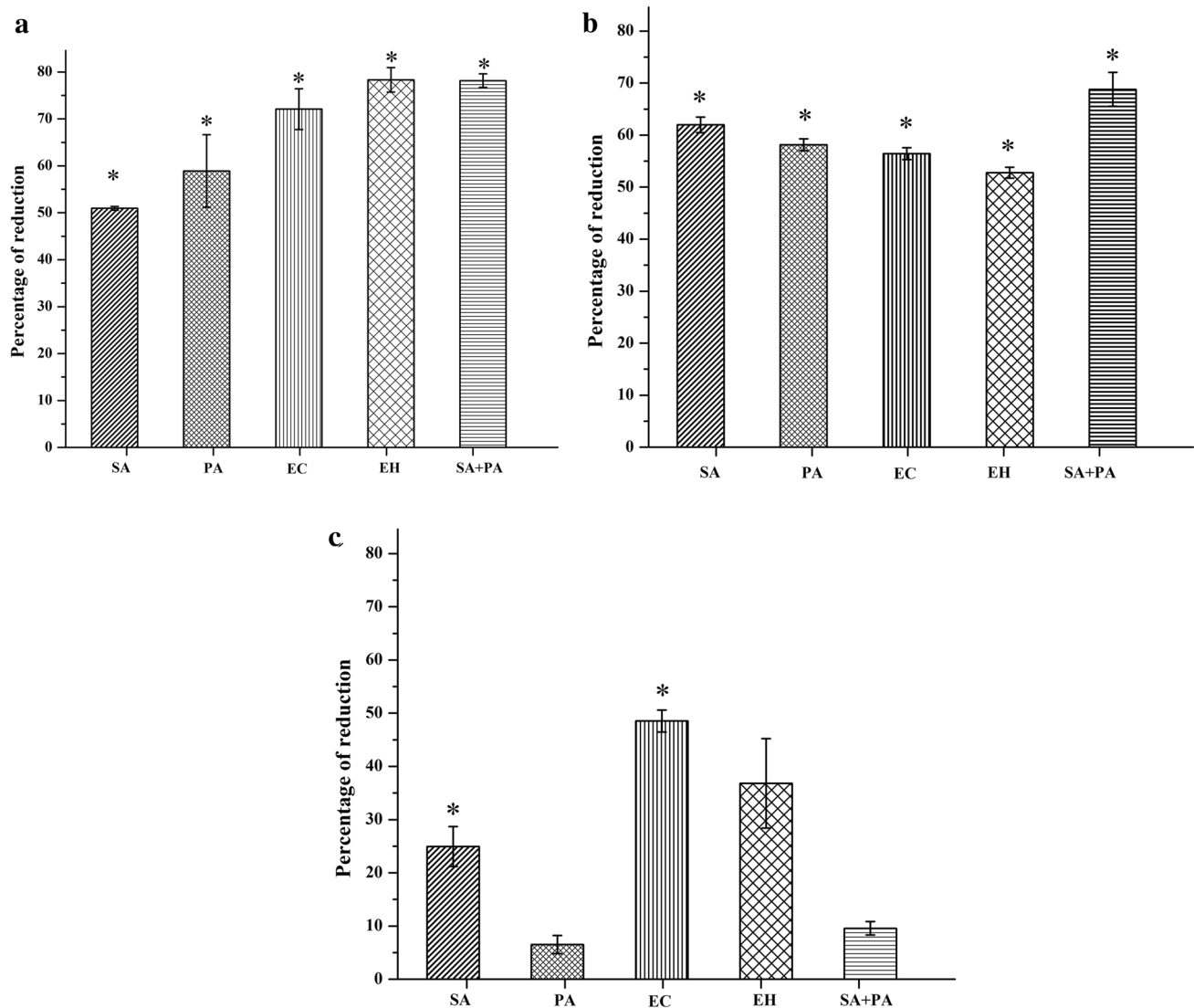


Fig. 7 Bar graphs representing the percentage reduction of biofilm components of treated groups. Assessment on the percentage reduction of carbohydrates **a**, proteins **b** and lipids **c**. [* Significant reduction

on the biofilm components of treated groups, $p \leq 0.05$]. Here, SA–*S. aureus*, PA–*P. aeruginosa*, EC–*E. coli*, EH–*E. hirae* and PA + SA–Mixed species of *P. aeruginosa* and *S. aureus*

higher for *E. coli*, *E. hirae*, and *P. aeruginosa*, respectively, when compared to *S. aureus* whereas, the MIC of N was similar for all the test strains. Resazurin-based metabolic viability assay also confirmed the significant reduction of metabolically active cells at MIC of C and N (Fig. 2a–d). Further, checkerboard assay results showed the potential synergistic activity of C + N against all test strains. Synergistic combination(s) significantly reduced the working concentration of monoterpenoids from the actual MICs with the FICI of ≤ 0.5 . In case of C, the concentration was reduced to 1/4 times for *S. aureus* and *E. hirae* and 1/4 and 1/8 times for *P. aeruginosa*. Similarly, the concentration of N was found to be reduced to 1/4, 1/8 and 1/16 times for *S. aureus* and *P. aeruginosa* and 1/4 times for *E. coli* and *E.*

hirae. The combined weight of C and N in working synergistic combination of C + N is 175.78 $\mu\text{g}/\text{mL}$, 97.66 $\mu\text{g}/\text{mL}$ and 58.59 $\mu\text{g}/\text{mL}$ for *S. aureus*, 312.5 $\mu\text{g}/\text{mL}$, 234.88 $\mu\text{g}/\text{mL}$, 195.31 $\mu\text{g}/\text{mL}$ and 156.26 $\mu\text{g}/\text{mL}$ for *P. aeruginosa*, 195.31 $\mu\text{g}/\text{mL}$, 175.78 $\mu\text{g}/\text{mL}$ for *E. coli* and 234.38 $\mu\text{g}/\text{mL}$ for *E. hirae*. These results reveal that the cumulative weight percentage of synergistic concentrations of C + N are lesser than their respective individual MICs. The reduced concentration of C + N combination is suggestive of compatibility between the two bioactive molecules C and N with enhanced concurrent effect at synergistic concentration than the sum of individual actives. Among various synergistic concentrations of C + N combination, C at 78.125 $\mu\text{g}/\text{mL}$ and N at 156.25 $\mu\text{g}/\text{mL}$ (C + N*) was further analyzed for

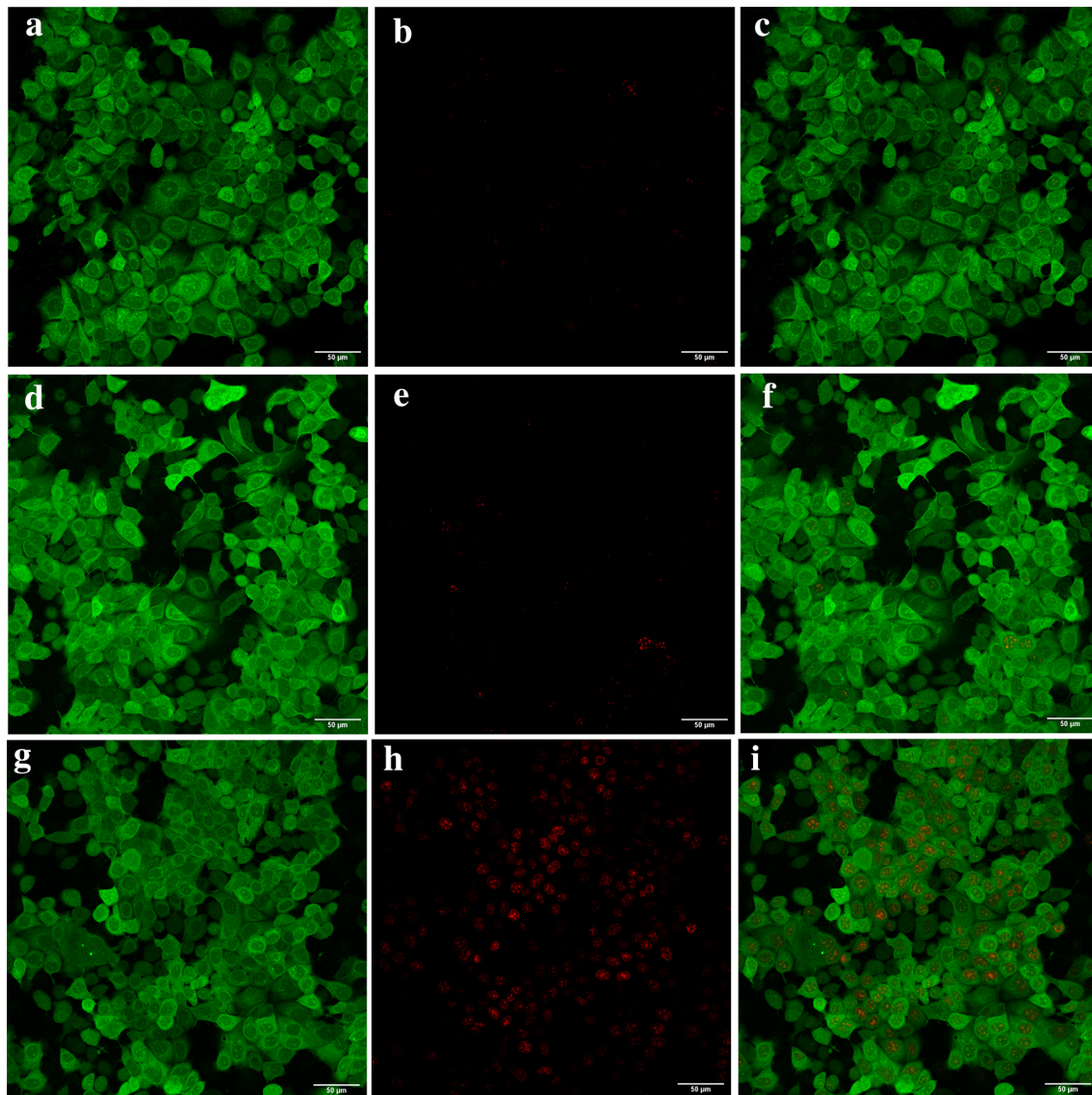


Fig. 8 Fluorescent Micrographs of acridine orange and ethidium bromide dual stained HaCaT cells after 1 min contact time with C+N* combination **a, b** and **c**—Untreated controls; **d, e** and **f**—Treated; **g, h** and **i**—Standards. Here, **a, d** and **g** are excitation and emission fluores-

cence of AO; **b, e** and **h** are excitation and emission fluorescence of EtBr; **c, f** and **i** are merged micrographs [LSM 880, 20X magnification, Scale Bar: 50 µm]

the antibacterial efficiency using live/ dead staining. Fluorescence images of control and treated cells confirmed the absence of viable cells in C+N* treated bacterial cultures even after 24 h incubation, which proved the complete killing effect of the monoterpenoids.

The hydrophobic molecules C (Log P: 3.20) and N (Log P: 2.89) (Log P values were determined using online software ALOGPS 2.1 (<http://www.vcclab.org/lab/alogps/>) (Tetko et al. 2005)), penetrate the lipid bi-layer of microbial cells eventually, the cells compromise the cell membrane leading to cell death (Arfa et al. 2016). As the phenolic terpenoid C has higher hydrophobicity, it easily partitions into phosphatidylethanolamine membrane (Rao et al.

2010). However, Ultee et al. 2002 compared the bactericidal efficacy of C with its precursor *p*-cymene, demonstrating that the hydrophobicity alone is not an important factor in exerting a potential killing effect on microbes. *p*-cymene has higher partition coefficient with lack of hydroxyl group (Nieto 2020). Further, the previous studies have also exemplified that the hydroxyl group is another important factor associated with the bactericidal efficacy of monoterpenoids. In agreement, it is speculated that the -OH groups of C and N in synergistic combination of C+N effectively depolarizing the cell membrane by dissipating pH and protons (H^+ and K^+) gradients across the cell membrane as explained by Ultee et al. (2002), Rao et al. 2010, Arfa et al. 2016.

Thus, the synergistic combination of C + N* could exhibit potential broad-spectrum killing activity. Apparently, the EN1276 protocol results of the product test solution with active molecules also confirmed the rapid killing efficacy of various ratios of C + N* against both Gram-positive and Gram-negative bacteria. Observed Vc values of validation controls confirmed the appropriate techniques used in the experiments. The presence of Vc on control A attested the non-lethal effect of reagents, control B results validated the proper filtration conditions and techniques followed in the protocol and control C results ensured thorough washing of membranes and complete removal of actives from filter membrane surface following the filtration of PTS containing active molecules. According to the EN1276 protocol, the active molecules can be considered effective disinfectants when they show > 5 log reduction in the viability of test pathogens. In the present study, various ratios of C + N* concentration showed an efficient killing effect within one-minute contact time against the test strains. The C + N* combination at the ratios of 80X, 20X, 40X, and 40X exhibited > 5 log reduction on viability of *S. aureus*, *P. aeruginosa*, *E. coli*, and *E. hirae*, respectively. The 80X and 100X ratios of C + N* concentration showed > 5 log reduction against all test strains; whereas, individual actives at 80X and 100X concentration did not exhibit a broad-spectrum killing effect. Thus, the study confirms that 80X and 100X concentrations of C + N* can be used to attain the broad-spectrum killing effect against both Gram-positive and Gram-negative bacteria.

Further, in many clinical incidences, microbial biofilms on nail surfaces are not removed completely and the viable cells encased in the biofilm matrix become the source of contamination. For instance, *P. aeruginosa* infection of the nail plate which is commonly known as chloronychia (or) green nail syndrome occurs due to the improper hand hygiene (Chiriac et al. 2015). Similarly, Tomczak et al. 2017 have reported the incidence of acute infection in nail fold of individuals with prolonged hand unhygienic condition wherein, authors observed the presence of mixed bacterial population. The isolated species from the nail fold of patients were *S. aureus*, *E. faecalis*, *E. coli*, *P. aeruginosa*, *Enterobacter* spp., and *Klebsiella* spp., and some fungi species. In these circumstances, CDC has recommended the guidelines for hand hygiene in healthcare settings. Further, CDC has stated that the alcohols possess broad-spectrum antimicrobial activity against various pathogens, including multidrug-resistant pathogens, *Mycobacterium tuberculosis*, various fungi, respiratory syncytial virus, vaccinia virus, etc. (<https://www.cdc.gov/mmwr/preview/mmwrhtml/rr5116a1.htm> accessed on 16th April 2022). Interestingly, one of the studies compared the efficiency of alcohol-based sanitizers with benzalkonium chloride (BKC) based hand sanitizers against the prototypical bacteria *S. aureus* and *E. coli*

(Chojnacki et al. 2021). The study demonstrated that the ethyl alcohol formulations had displayed improved activity with observed variation to different strains, in comparison to BKC containing sanitizers. This study further concluded that the efficacy of hand sanitizers has to be precisely evaluated, especially against bacterial pathogens (Chojnacki et al. 2021). Whereas another study reported that the BKC hand sanitizers reduced the transient *S. aureus* when compared with 70% ethanol-based hand sanitizers (Bondurant et al. 2020). Though these two results strongly evidence the potential antimicrobial effect of both alcohol and BKC based hand sanitizers, the variation may be due to the different test environments and conditions employed in the study methods. But these investigations support the potential antimicrobial activity of both alcohol and BKC based hand sanitizers. Most recently, Lehtinen et al. (2022) have also re-investigated the effect of non-alcohol-based hand disinfectants containing silver polymer, lactic acid, and benzalkonium chloride on inactivation of bacteria in hands of healthcare workers. The study demonstrated the low efficacy of non-alcohol-based hand sanitizers than the alcohol-based hand rubs in bacterial inactivation. Some of the studies have evaluated the effect of antiseptics/ disinfectants using EN1500 phase 2/step 2 protocol which has specified the test methods and protocols required for establishing a hand rub product with pathogen removal efficiency (Babeluk et al. 2014; Pires et al. 2019). Apart, explicitly, the current investigation focuses on the identification of bioactive(s) with rapid killing efficiency that could be used as active ingredients in hand wash products rather than hand rubs/sanitizers.

Initially, to know the architecture of bacterial cells on nail surfaces, we have imaged the nail surfaces of volunteers using SEM analysis. Interestingly, SEM images of four tested nail samples showed the presence of clumped, surface-bound, intact cells with uniform morphology (Monospecies) as well as cells with various morphology (Mixed species). Surface adhered clumped microbial cells embedded within the matrixome is called biofilm. The matrixome composed of extracellular polymeric substances (EPS) including exopolysaccharides, proteins, lipids, nucleic acids and extracellular vesicles (Karygianni et al. 2020; Wickramasinghe et al. 2020). The EPS of matrixome challenge the permeability of bioactive(s) into the matrix in the process of pathogen elimination (Zhang et al. 2020; Beloin and McDougald 2021). In general, both the transient flora and resident flora along with trapped dust particles may exist on the free edge of nails (Davis 1996). But, when the flora of the free edge of nails encounters the immunocompromised individuals (or) the individual having the flora become immunologically compromised under certain circumstances, either way, the incidences worsen the individuals' recovery (Taur and Pamer 2013; Mangutov et al. 2021; Talapko et al. 2021; Eltwisy et al. 2022). Hence, the ability of the

synergistic combination to disrupt the preformed biofilm on nail surfaces was analyzed. In-depth visualization of control and treated biofilm of nail samples through SEM analysis showed the significant variation in the biofilm matrix of the two groups. SEM images interpret that though all the adhered cells were not cleared off after the one minute of contact time, biofilm components have been removed completely. The cells of the treated group showed noticeable rough surfaces when relate to the cells encased in the pile of mucinous, slimy exopolysaccharide matrix of the control group. Furthermore, SEM images of C + N*-treated cells appeared to be loosely bound on the nail surface when compared to the cells present on the nail surfaces of healthy volunteers. It confirmed that the 100X concentration of C + N* had effectively disrupted the preformed biofilm.

After observing the SEM results, we were interested to assess the metabolic viability of the biofilm encased cells as well as the major components of the biofilm matrix of control and treated groups using in vitro quantification assays. Alamarblue assay results showed the significant reduction on the rate of metabolically active cells in treated groups when compared to the control groups. The reduction on viable cells opens up to the speculation that C + N* acted on biofilm encased cells by penetrating the biofilm matrix. This result highlights the biofilm disruption efficacy of 100X concentration of C + N* combination. Addition to this, quantitative comparison on the biofilm components of control and treated groups of mono-species biofilms of *S. aureus*, *P. aeruginosa*, *E. coli* and *E. hirae* and prototypical mixed species of *S. aureus* and *P. aeruginosa* biofilms is also confirmed the significant biofilm disruption ability of C + N*. The carbohydrate and protein contents of the treated groups were found to be significantly reduced in both mono-species and mixed-species biofilms when compared to the untreated control. After C + N* treatment, carbohydrates contents were reduced by about 50% in *S. aureus*, about 60% in *P. aeruginosa*, and > 70% in *E. coli*, *E. hirae*, and *S. aureus* + *P. aeruginosa* biofilms. Generally, carbohydrate content of the biofilm matrixome are cell bound or released polysaccharide moieties that are actively stabilizing the biofilm architecture (Angelin and Kavitha 2020). For instance, Nguyen et al. 2020 have discussed that the carbohydrate content of Gram-positive organisms including *S. aureus* and *S. epidermidis* contains the cationic, partially deacetylated homopolymer exopolysaccharide, polysaccharide intracellular adhesion (PIA) in their biofilm matrix. In *S. mutans*, the EPS, glucan interacts with the extracellular DNA (eDNA) of biofilm matrix and the resulting filamentous structure connects the bacterial cells of biofilms (Liao et al. 2014). Similarly, Psl and Pel exopolysaccharides strengthen the biofilm matrix of *P. aeruginosa* (Wang et al. 2015) and poly-N-acetylglucosamine fortifies the *A. baumannii* biofilms (Flannery et al. 2020). In order to slackening the biofilm matrix, some of the

previous studies have reported the ability of certain small molecules including curcumin against Psl and Pel exopolysaccharides of *P. aeruginosa* (Sethupathy et al. 2016), umbelliferone against *S. epidermidis* (Swetha et al. 2019), myrtenol against *A. baumannii* (Selvaraj et al. 2020), ursolic acid against *S. mutans* (Lyu et al. 2021), α -amylase against *S. aureus* and *P. aeruginosa* (Lakshmi et al. 2022) that are inhibiting the EPS production of microbes. Interestingly, the decreased carbohydrate contents in the present study indicates the eradication of exopolysaccharide substances of the mono-species and mixed-species biofilm matrix. Along with polysaccharides, proteins are also the prime factor in sustaining the biofilm architecture, in which proteins escalate the stability of biofilms in addition to favoring microbial surface attachment (Santschi et al. 2020). Lipids are another perceptible factor in nourishing the biofilm matrix. For instance, saturated fatty acids of biofilm cells of *S. aureus*, *Listeria monocytogenes*, *P. aeruginosa* and *S. Typhimurium* were found to be higher than their planktonic cells (Dubois-Brissonnet et al. 2016). The mycolic acid containing lipids on mycobacterial cell wall contribute to biofilm formation in *Mycobacterium smegmatis* (Pacheco et al. 2013). In another instance, increased lipid A of Gram-negative bacterial biofilms enhance the intracellular survival of bacteria in the host system (Chalabaev et al. 2014). Therefore, an efficient small molecule capable of eliminating biofilm matrix associated extracellular proteins and lipids could be used to disrupt bacterial biofilms in clinical settings. The C + N* combination of the present study reduced the protein contents of biofilm matrix by about > 50% in all the treated groups. Similarly, the lipid contents of *S. aureus* and *E. coli* biofilms were significantly reduced whereas, lipid contents of *P. aeruginosa*, *E. hirae*, and mixed-species biofilms were reduced by about 30% upon treatment with C + N* combinations. These results evidently proved that the C + N* combination can disrupt the preformed biofilm matrix and can strengthen the bacterial removal process in subsequent washing with water. Despite the current research has dealt with bacterial pathogens alone by following the test strains, test methods, and test environments given in the EN 1276 protocol, previous literature evinced the potential antifungal activity of C against *Candida albicans* (Rao et al. 2010), *Cryptococcus neoformans* (Nobrega et al. 2016), and N against *Aspergillus flavus* (Tian et al. 2013), *C. albicans* (Wang et al. 2020). Furthermore, previous studies on the anti-oxidant, anti-apoptotic, non-toxic, and anti-inflammatory effects of C and N (Cheng et al. 2021; Yildiz et al. 2021) also support the biocompatibility of C and N. However, cytotoxicity analysis of C + N* on keratinocytes was performed using HaCaT cells. The morphological features of C + N* treated HaCaT cells remained similar to that of untreated control cells. Hence the study concludes the non-deleterious effect

of C + N* combination on the human keratinocytes after one minute of contact time.

Altogether, the findings highlight the potential antimicrobial activity of the monoterpenoids C + N and the obtained results provide sufficient evidence for attesting the rapid killing efficacy of C + N. Although the rapid killing efficiency and preformed biofilm disruption potential of C + N are well documented, further studies are warranted before implementing the product.

Conclusion

In conclusion, the monoterpenoids C and N exhibited broad-spectrum antimicrobial activity with significant growth reduction against *S. aureus*, *P. aeruginosa*, *E. coli* and *E. hirae*. The binary complex checkerboard assay results showed significant reduction in monoterpenoids concentration with FICI of ≤ 0.5 against all the test strains. Though the concentrations of individual actives were lesser than their individual counterpart, the obtained synergistic concentration exhibited efficient growth reduction against the test strains. Live/dead analysis confirmed the efficient killing effect of the synergistic concentration of C + N combination. Further, EN1276 protocol results confirmed the efficiency of 80X and 100X concentrations of C + N combination with the weight percentage of C: 0.625% + N: 1.25% and C: 0.781% + N: 1.56% with > 5 log reduction in cell viability against all the test strains. Moreover, the C + N combination effectively killed the viable cells residing inside the preformed biofilm of test strains within one minute of contact time in the presence of the interfering substance. Non-deleterious effect of C + N on the human non-malignant keratinocyte (HaCaT) cells exemplified the biocompatibility of the synergistic combination. Hence, formulating a product with active ingredients such as C and N will not be potentially toxic to mankind. Thus, the current investigation concludes that the C and N combination can preferentially be used as active ingredients in the hand wash formulations along with other hand wash base ingredients for decontaminating the hand surfaces of individuals in hospital settings as well as domestic environments.

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Declarations

Conflict of interest The authors declare no Conflict of interests.

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