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Inhibition of Some Multidrug-Resistant Bacteria Using Prepared Essential Oil Nanoemulsion Formulas and Their Mode of Action

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Abstract

Objective: The use of essential oils for the preparation of different nanoemulsions (NEa, NEb and NEc) and detect their biological activities.

Methods: Nanoemulsiona were prepared by mixing the essential oil (10%) of *Coriandrum* seed of *Coriandrum sativum,* Ginger roots of *Zingiber officinale*, or *Achillea* leaves of *Achillea clavennae*), sterile water, and surfactants (Brij 30, Span 20, Triton X-100, Tween 60) in addition to the 0.1M Tris-HCl buffer (pH 7.22). The three prepared nanoemulsions were examined and characterized. Their antibacterial activities were examined against some multidrug-resistant Gram-positive and negative bacteria.

Results: The maximum activity was recorded by NEb against *Serratia marcescens, Staphylococcus aurous, Micrococcus luteus, Enterococcus faecalis, Streptococcus pyogenes,* and *Staphylococcus saprophyticus* while lower activity was obtained against *Klebsiella pneumonia* and *Escherichia coli.* The minimum inhibitory concentrations (MICs) of the NEb ranged from 40–60 µl/ml of the nutrient broth. NEb affects the cell counts and morphology of *S. marcescens* and destroys the bacterial cells by reducing cell respiration and enhancing the cell permeability and leakage of protein, DNA, and potassium of the bacterial cell membrane. NEb recorded no toxicity against *Artimia salina* and HaCaT cell line. It decreases biofilm formation by 51%, bacterial infection, and cell attachment.

Conclusion: This study suggests that NEs have great antibacterial activity against some human pathogens and process different modes of action, thus they can be used effectively to treat different bacterial infections.

Keywords: Antimicrobial activity, pathogenic, permeability, respiration, MIC, *Serratia marcescens,* biofilm

Introduction

Essential oils are complex volatile compounds synthesized naturally in different plant parts during secondary metabolism. They have great potential as they effectively destroy several pathogens and have great potential in the field of biomedicine as they effectively destroy several bacterial, fungal, and viral pathogens in addition they can be used to inhibit the growth of many foodborne pathogens.[1-4] Due to infection control problems and misuse of antibiotics, multidrug-resistant bacterial infections of soft tissue, skin, and bloodstream have increased in patients at many hospitals and they are now recognized as a major cause of nosocomial infections (Abou-assy et al., 2022, 2023),[5,6] and pose a significant threat to public health. Alternative therapies and new approaches to develop new antimicrobial therapeutic agents are needed. The use of essential oils to prepare nanoemulsions (NEs) was recommended by many authors.[7-9] Dispersing and stabilizing oil droplets in an aqueous solution using co-surfactants or surfactants form the nanoemulsions that have gained significant attention as potential antimicrobial agents due to their unique physicochemical properties and enhanced bioactivity.[3]

Nanoemulsions possess several advantages over conventional antimicrobial agents, including increased stability, improved solubility, and enhanced penetration into microbial cells. Numerous studies have demonstrated the efficacy of nanoemulsions in inhibiting pathogenic bacteria and have great potential in food preservation and safety. Nanoemulsions have broad antimicrobial activity against bacteria, fungi, and some enveloped viruses with low toxicity to animals.[10,11] Nanoemulsion systems are in between slightly opaque and transparent, differ in their composition, appearance, kinetic and thermodynamic stability, and their particle size ranges from 10 to 800 nm.[12] They may interact with lipid bilayers of bacterial cell membranes and the energy stored in the oil-and-detergent emulsion destroys the bacterial cell membrane of the pathogens.[13-15] Teixeira et al, (2007)[16] reported that NEs might have a mechanical force on the cell wall or membrane of the cell. In addition, the antimicrobial activity of nanoemulsions is due to their ability to fuse with the cell membranes and the electrostatic interaction between the cationic charge of the nanoparticles and the anionic charge on the microorganisms that destabilizes the lipid bilayers of the cell membrane. This increases membrane permeability and cell disruption.[15,17] Unlike some antibiotics, the nanoemulsion antimicrobial activity is nonspecific and has broad-spectrum activity, thus limiting the appearance of resistance.

Serratia marcescens is an opportunistic, gram-negative, nosocomial pathogen, that causes an outbreak in the ICU (Khanna et al., 2013),[18] which is often involved in epidemics of the colonization and infection with *the pathogens*.[19] The important reservoirs in epidemics are the digestive, respiratory, and urinary tracts in addition to medical equipment. The ability to form biofilms and the special cell surface properties made current medical approaches ineffective.[20,21] Recently, *S. marcescens* has gained attention as an emerging pathogen worldwide, provoking infections and outbreaks in debilitated individuals, particularly newborns, and patients in intensive care units.[19] NEs efficiently destroy bacterial pathogens, spores, enveloped viruses, HIV-1 and various tuberculosis pathogens without damaging the normal human cells or the cells of most other higher organisms.[2,13,22] This study aimed to determine the antimicrobial activity of some essential oil NEs formulas and study their effects on bacterial growth, and biofilm formation.

Materials and Methods

The Tested Bacteria, Cell Line, and Animal Larvae

The Gram-negative *Escherichia coli, Salmonella typhi, Shigella sonnei, Klebsiella pneumonia, Pseudomonas aeruginosa* and *Serratia marcescens* in addition to the Gram-positive *Staphylococcus aurous, S. saprophyticus, Streptococcus pyogenes, Micrococcus luteus, Bacillus cereus* and *Enterococcus faecalis* were obtained from King Abdulaziz University Hospital, Jeddah, KSA. Bacterial cultures were grown in Tryptic Soy agar supplemented with 1% w/v glucose (VWR Chemicals, Radnor, PA, USA). Stock culture of the previous isolates was routinely preserved on slants of Tryptic Soy agar at 4˚C until used and regenerated every six months.[23] For long-term preservation, they were maintained in 15% glycerol, frozen at −80˚C. Tryptic Soy broth was used for bacterial growth in a liquid medium; the isolate was grown in the broth medium at 37˚C on an orbital rotary shaker at 80 rpm.

The human epidermal keratinocyte cell line, HaCaT cells were obtained from the ATCC (American Type Culture Collection, Manassas, USA) and they are used for the investigation of toxicity and infection inhibition. They were grown in Dulbecco's Modified Eagle Medium (DMEM), containing 10% fetal calf serum, L-glutamine, and a mixture of penicillin, and streptomycin (200 units/ml) and incubated in a humidified incubator at 37˚C and 5% CO_2. The animal larvae of *Artemia salina* and *Galleria mellonella* were obtained from the Biology Department, Faculty of Science, Jeddah, Saudi Arabia.

Nanoemulsion Formation and Preparation

Plant materials of the original species were collected in June 2021 and were identified at the Biology Department, Faculty of Science, King Abdulaziz University. *Coriandrum seed oil of Coriandrum sativum and* Ginger root oil of *Zingiber officinale,* were extracted as described by Ashraf et al. (2011).[24] The fresh plant parts were subjected to steam distillation after being completely immersed in water and heated to boiling, after which the essential oil was evaporated together with water vapor and finally, the oil was collected and dried over sodium sulfate (water-free) and stowed at 4˚C until used. Pure *Achillea* oil of *Achillea clavennae* was acquired from Nature's Way Brands LLC Green Bay (USA). The obtained three oils were used to prepare the different nanoemulsions, NEa, NEb, and NEc. Preparation of NEs formulations was carried out from essential oil (10%), sterile water, and surfactants (Brij 30, Span 20, Triton X-100, Tween 60), obtained from (Saint Louis, MO, USA) as described by Tsai et al., (2010).[25] Using a magnetic stirrer, the components were dissolved and mixed under heating until became clear, transparent, and homogeneous. They were prepared and characterized as described by Pey et al. (2006).[26]

Characterization of Nanoemulsions

The morphology and particle size were studied using a Scanning electron microscope (SEM, Quanta FEG 450) at King Fahad Medical Research Center, Jeddah, KSA. A specimen of each solution was first fixed, treated with a graded alcohol dehydration series, dried, mounted, gold coated, and viewed on the SEM with the digital imaging system. The mean droplet diameter (nm) and Coefficient of variation (% CV) were calculated for each solution.[2]

Antibacterial Activity of the Prepared NE Formulations

All used media were prepared and sterilized using the autoclave at 121˚C for 20 min. before use. Bacterial suspensions were prepared at a concentration visually equivalent to a 0.5 McFarland Standard (2×10^6 CFU/ml). The agar well diffusion assay described by Zhang et al (2010)[27] was applied to determine the antimicrobial activities of the three prepared NEs by measuring the inhibition zone diameter (mm) after two days. The antibiotic, Ampicillin solution, and sterile phosphate buffer were used as positive and negative controls, respectively.

Determination of MIC

The MIC of NEb was calculated using the broth dilution method described by Turnidge et al (2007).[28] To 50 ml conical flasks containing 10 ml of Tryptic Soy Broth and inoculated with 100 µl of 2×10^6 CFU/ml of the pre-culture of the tested bacterial pathogens, different volumes of NEb were added and the counts of living bacterial cells were detected on Tryptic Soy agar plates after 2 days. About 0.1 ml of each inoculated flask was taken, spread on the agar surface and developed colonies were counted after incubation at 37˚C for 24 hrs. Ampicillin solution was used as a positive control. The MIC is the lowest concentration of NE that has no growth or very small numbers of colonies.

Effect of Incubation Period on Antibacterial Activity of Nes

In the 50 ml flask, 9 ml of sterile Tryptic Soy broth was mixed with the tested NE (20 µl/ml). The flask was inoculated with 20 µl of the tested bacterium (2×10^6 CFU/ml), and incubated at 37˚C for 0.0–48 hrs. The cell counts were determined in 0.1 ml of the culture using Petri dishes containing 15 ml of Tryptic Soy agar. The plates were incubated at 37˚C and the number of bacterial colonies/ ml was counted using colony counters (SC6-R000103491) and compared with the counts of control (culture without NE).

Effect of NEs on Cell Morphology and Counts

In 50 ml flask containing Tryptic Soy broth medium (9 ml) was inoculated with 20 µl of the pre-culture containing $2x10^6$ CFU/ml of the tested bacterium and the bacterial cells were treated with half the MIC of the tested NEb (20 µl/ml). All flasks were incubated at 37˚C for 12 hrs and distilled water was added to the control flasks instead of NEb. The cells were collected after centrifugation at 5000 rpm for 15 min, examined, and photographed after Gram staining at King Fahad Medical Research Centre.

Effect of Nes on Cell Permeability, Hydrophobicity, and Potassium Leakage

The effect of NEs on the nucleic acids, proteins, and potassium permeability from the bacterial cell was determined as described by Pelletier et al., (1997) and Hou et al (2007).[29,30] Sterile Tryptic Soy broth medium (18 ml) was inoculated with approximately 20 µl of the pre-culture (2×10^6 CFU/ml) and 20 µl/ml of the tested NEb for 12 hrs. After that, cells were collected, washed three times with distilled water, and suspended in sterile water for 8 hrs and the absorbance (A1) of the filtrate was read at 260 nm. The control sample was prepared by incubating the bacterial cells with Triton X-100 for 6 hrs (A0).

Leakage of the nucleic acid materials was estimated. Furthermore, the quantity of potassium in the solution was measured using a flame photometer (Kruss, model FP8800).

Effect of NEb on Cellular Respiration and Cell Wall Composition

Respiration of treated and untreated cells was determined using an oxygraphe, calculated the quantity of oxygen consumed (µl/mg/h) was recorded as described by Mahmoud and Aly (2004).[31] Total proteins and carbohydrate determinations of the bacterial cell walls were recorded in aliquots of the suspension of treated and untreated cells obtained as reported above. The cells were dried and lysed and the cell walls were collected, dried, weighed, and used to measure the protein, and carbohydrate using the Bio-Rad Protein assay Kit (Bio-Rad, CA, USA) and carbohydrate Assay Kit (MAK104, Sigma-Aldrich), respectively, following the manufacturer's instructions, and measuring sample absorbance with Microplate Reader (BioTek Instruments, Winooski, VT, USA).

Effect of NEb on Biofilm Assays

The biofilm formation by *S. marcescens* was quantified using protocols previously described (Stepanovic et al., 2002, 2007)[32,33] and modified by Jamal et al. (2022).[34] Briefly, bacteria cells (2×10^6 ml) were grown in TSB broth in 96-well microplates in the presence of NEb (20 µl/ml), and the plates were incubated at 37˚C for 12 hrs., then the free cells were removed from the plate, and the plates were washed two times with saline solution. The obtained biofilm was stained by 200 µl of 0.2% crystal violet and the plates were washed again with saline solution, and finally, the stained biofilm was re-suspended by adding 300 µl of 32% acetic acid (v/v) and the absorbance was recorded at 570 nm. The percentages of biofilm inhibition were calculated as:

$$\text{Biofilm reduction } (\%) = \frac{\text{OD at 570 nm of the control} - \text{OD at 570 of the treated}}{\text{OD 570 nm of the control}} \times 100$$

Toxicity of Nanoemulsions

In 96-well plates, HaCaT cells (2×10^5 cells/ml) were incubated in 5% CO_2 at 37˚C for 24 hrs. and the toxicity was evaluated by the 3-(4,5-Dimethylthiazol-2-yl)-2,5- Diphenyltetrazolium Bromide (MTT, Sigma-Aldrich, St. Louis, MO, USA) assay. After the incubation period, cells were treated with different concentrations of NEb (10 – 100 µl/ml and incubated for 8 hrs at 37˚C. After the removal of the medium, DMSO was used to dissolve the formazan crystals, and the absorbance was measured at 570 nm using a microplate reader (SYNER- GYH4, BioTek, Inc., Winooski, VT, USA). The viability of cells was evaluated compared to the control cells.

$$\text{Cell viability} = \frac{\text{Abs. of control} - \text{Abs. of sample}}{\text{Abs. of control}} \times 10$$

Toxicity against *Artemia salina* as a test organism was determined as described by Aly and Gumgumjee, (2011).[35] Toxic concentration is the concentration that kills 50% of the larvae or decreases cell viability to 50%.

$$\text{Cell viability} = \frac{\text{No. of living larvae in control} - \text{no. in treated sample}}{\text{No. of living larvae control}} \times 100$$

Adhesion Assay of Serratia marcescens to the Cell Line

The adhesion capacity of *Serratia marcescens* to HaCaT cells with and without NEb was evaluated. In brief, 2×10^5 cells ml of *Serratia marcescens* were seeded into 96-well polystyrene plates and incubated with pre-cultured HaCaT cells in DMEM for 2 hrs at 37˚C with or without NEb (10; 15; 20; 25 µl/ ml). Then, the cells were washed with PBS, and 100 µl of a solution of Trypsin/EDTA (Sigma Aldrich Co., St. Louis, MO, USA) was added to each well to separate cells. One ml of DMEM was added after 5 min at 37˚C, and the obtained cell suspensions in PBS were plated on TSA and incubated at 37˚C for 24 hrs to count *S. marcescens* cell forming units (CFU). The influence of NEs on the adhesion of the bacterial cells to HaCaT cells was calculated by the number of viable adherent bacteria and expressed by CFU per well.

Infection biocontrol activity by NEb

Galleria mellonella assay test was used to evaluate NEb capacity to protect larvae from *S. marcescens* infection. For the survival assay, five groups of 10 randomly chosen larvae with similar size were selected and were first injected with 10 µl of the NEb two successive times, and after 2 hrs of incubation at 37˚C, the larvae were infected with standardized suspension (2×10^6 cells/ml) of *S. marcescens* using a Hamilton syringe. Sterile PBS alone was used as a control and after 2 days of being kept at 37˚C, the number of dead larvae was monitored.

Statistical Analyses

The result was obtained from three independent experiments, which are shown as mean ± standard deviation ($\bar{X} \pm SD$). One or two-way ANOVA following Tukey's test was used to compare the results. Asterisks show significant differences, ($* = P < 0.05$).

Results and Discussion

Nanoemulsion systems are types of polymeric nanoparticles that consist of two or more immiscible liquids stabilized by surfactants (about 2%) which is a chemical compound with a polar head and nonpolar tail.[14] They differ in composition, appearance, kinetic, and thermal stability. Three oils of *Coriandrum seed,* Ginger root, *and Achillea* leaves, were used in this study to prepare three different Nanoemulsions. Table 1 shows the scientific names, families, and used parts. Nanoemulsion formulas were prepared from the three previous oils, the obtained Nanoemulsions were characterized, and their antimicrobial solutions were recorded. The nanoemulsion formulations (NEa, NEb and NEc) were prepared by mixing the surfactant mixture, and the oil phase as described by Formariz et al. (2006)[36] and Teixeira et al., (2007).[16] The solution appears opaque at the beginning but becomes transparent at the end and the droplet shape and distribution were determined using SEM (Figure 1). The droplet morphologies for all of the nanoemulsion formulations were spherical and normally distributed. The percentages of the coefficient of variation (% CV) for all of the NE formulations were assessed by dividing the standard deviation by the mean of six replicates of the droplet sizes and the obtained values were 9.82%, 8.41%, and 12.08% for NEa, NEb, and NEc which means that the droplets were well distributed. The mean droplet sizes of

Table 1. **The used medicinal plants, their families, the selected plant parts and the characters of their nanoemulsions**

Oil	Scientific name	Family	Used part	NE name	Nanoemulsions (NEs) characters	
					Mean droplet diameter (nm)	Coefficient of variation (% CV)
Coriandrum	*Coriandrum sativum*	Apiaceae	Seeds	NEa	131.19 ± 0.5	9.82
Achillea	*Achillea clavennae*	Asteraceae	Leaves	NEb	125.30 ± 1.74	8.41
Zingiber	*Zingiber officinale*	Zingiberaceae	Roots	NEc	137.69 ± 1.25	12.08

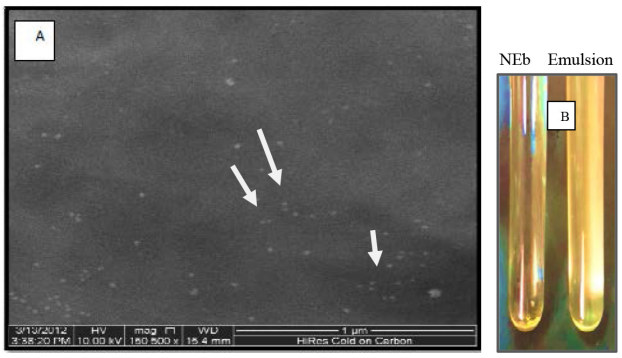

Fig. 1 **The droplet shape of the NEb under scanning electron microscope (A) and the clearance appearance of the prepared NEb (B).**

all NEs formulations were found in the range of 125.30–137.69 nm (Table 1). The droplet sizes for all NE formulations were found in the range 120.11–160.14 nm with mean values of 131.19 ± 0.5, 125.30 ± 1.74, and 137.69 ± 1.25 nm for NEa, NEb, and NEc. To assess the differences, NEb had the same amount of surfactant but contained *Achillea* leaves oil, which processes different chemical characteristics compared to *Coriandrum seed and* Ginger root oils which were used to prepare NEa and NEc. Similarly, the natural compounds carvacrol and thymol oils were used for nanoemulsions preparations to be used as inhibitory substances for food applications and they were more active compared with free natural compounds (da Silva et al., 2023).[37] Smaller spherical shape particles were recorded for NEb and the coefficient of variation percentage was little which means low variation in the droplet size. Thus, it was selected for more studies that are detailed. Similar solutions were prepared with small droplet sizes generally ranging between 20 and 200 nm, which creates a significant interfacial area for improving the solubility and bioavailability of weakly water-soluble drugs.[9]

The antibacterial activity of the three NEs was determined for some multidrug-resistant bacteria using an agar well diffusion assay (Table 2 and Figure 2). NEa, NEb, and NEc were active against all the tested bacteria and excellent activity was recorded for NEb (Figures 3 and 4). The inhibition zone diameter ranged between 10–14, 14–24, and 11–13 for NEa, NEb and NEc, respectively. Significant antibacterial activity was recorded using NEb for *S. marcescens, E. faecalis, S. saprophyticus,* and *S. pyogenes*. More inhibition of the bacterial growth was recorded by Ampicillin (positive control). Similar to these results, Abu Safe et al. (2023)[11] reported that the droplet size of *Achillea* (yarrow) NE was 151.21 ± 1.12 nm and it showed antibacterial and antioxidant properties that may be due to the presence of 51 different compounds detected using gas chromatography-mass spectrometry. Due to its excellent activity against microbes, NEs can be used as a strategy to preserve fresh fruits and vegetables.[38] The inhibitory effect of the NEs on the growth of *S. marcescens* (the number of cells/ml, CFU/ml) was affected by time. The greatest inhibitory effect was for NEb, followed by NEc and finally NEa, and all of the cells stopped growth within 18 hrs. (Figure 5). In the case of NEb, there is no growth up to 8 hrs, and then the growth slowly increases up to 18 hrs then becomes constant. In addition, NEc had nearly the same pattern but its activity was lower when detected using the agar well diffusion method (inhibition zone, mm). The control, which contains sterile phosphate buffer (without NEs), gives the highest growth. Zhang et al. (2009)[39] studied the kinetics of *E. coli* and *S. aureus* killing by microemulsion, which caused 99% of *E. coli* to kill within 15 min while 99% of viable *St. aureus* cells were killed within 30 min. The Nanoemulsions' antimicrobial activity is significant due to the denaturation of the bacterial membranes, and the decrease of the bacterial cell surface hydrophobicity, which induced the quick release of 260 nm absorbing materials.[27,39] The bactericidal properties of nanoemulsions were against Gram-positive but not against enteric Gram-negative species that cause many human health problems, and the evaluated MIC were against foodborne pathogens with reductions of up to four times in MIC compared with non-nanoemulsified versions.[13] They added that *E. coli* and *S. aureus* were the most sensitive bacteria to the carvacrol nanoemulsions, with MICs of 0.147 mg/ml. Khan and, Ramalingam (2019)[40] synthesized nanoemulsions and examined them against ESKAPE pathogens and recorded that statistically NE-12 showed maximum inhibitory activities of bacterial growth, cell counts, and biofilm formation compared with positive and negative control, therefore could be used for the improvement of promising antibiotics resistant pathogens.

The MIC value is important in diagnostic laboratories to confirm the resistance of microorganisms to an antimicrobial agent and to monitor the activity of new antimicrobial agents.[41] It is generally regarded as the most basic laboratory measurement of the activity of antimicrobial drugs.[42] The MIC of NEb (Figure 6), ranged from 30–50 µl/ ml while the MIC of the control was between 3.5–5.5 µg/ml (Table 2). Karthikeyan et al, (2011)[43] detected excellent antimicrobial activity against the biofilms of *S. mutans* and the recorded MIC was 0.08% for the tested microbes.[22] Cells of *S. marcescens* were grown and treated with NEb for 12 hrs. and the treated cells were collected and examined under light microspore. Treatment of cells with NEb induced some morphological changes and decreased cell size and numbers (Figure 7), which may be due to membranous lesions through which cytoplasmic contents

Table 2. **The effect of NEa, NEb, and NEc on growth inhibition (diameter of inhibition zone) of different bacteria and minimal inhibitory concentration (MIC) of NEb and control antibiotic**

Tested bacteria	Antimicrobial activity of nanoemulsions and control				MIC	
	NEa	NEb	NEc	Control##	NEb (µl/ml)	##Control µl/ml
Escherichia coli	11 ± 1.29	16 ± 0.90	12 ± 1.80	39 ± 1.12	40.0 ± 2.5	5.5 ± 1.5
Salmonella typhi	11 ± 0.09	17 ± 0.57	12 ± 0.46	27 ± 1.66	45.5 ± 2.5	5.5 ± 1.0
Shigella sonnei	14 ± 2.12	18 ± 0.37	12 ± 0.98	29 ± 0.60	50.0 ± 5.0	5.0 ± 0.1
Klebsiella pneumonia	11 ± 0.87	14 ± 0.07	11 ± 0.18	25 ± 1.57	50.5 ± 2.5	5.5 ± 1.0
Pseudomonas aeruginosa	11 ± 0.97	17 ± 0.50	11 ± 0.26	28 ± 2.67	50.0 ± 2.5	4.5 ± 1.0
Serratia marcescens	13 ± 0.64	24 ± 0.18	12 ± 0.33	38 ± 3.07	30.0 ± 5.0	5.5 ± 0.7
Staphylococcus aurous	10 ± 1.12	20 ± 1.45	13 ± 0.49	26 ± 0.57	45.0 ± 2.5	5.5 ± 1.5
Staphylococcus saprophyticus	12 ± 1.19	21 ± 1.23	13 ± 0.54	27 ± 0.56	45.0 ± 2.5	5.5 ± 1.7
Streptococcus pyogenes	13 ± 2.43	21 ± 0.29	13 ± 0.57	29 ± 0.09	45.0 ± 2.5	5.5 ± 1.7
Bacillus cereus	11 ± 0.0	17 ± 4.2	13 ± 0.0	24 ± 0.99	40.0 ± 2.5	5.5 ± 2.6
Enterococcus faecalis	10 ± 0.57	21 ± 0.96	12 ± 0.57	23 ± 0.87	42.5 ± 2.5	2.6 ± 1.0
Micrococcus luteus	14 ± 0.50	22 ± 0.07	12 ± 0.57	36 ± 0.54	50.0 ± 5.0	2.6 ± 0.1
*Bacterial index**	11.80**	21.91**#	12.16**	29.91	LSD$ = 122.65	

*Bacterial index: Total activities against bacteria divided by the number of the tested bacteria; **, significant results compared to the control at $P \leq 0.05$; #, significant results compared to the NEA or NEb at $P \leq 0.05$; LSD$: The least significant difference between MIC value of the effect of NEb on different bacteria; ##, Ampicillin: control antibiotic.

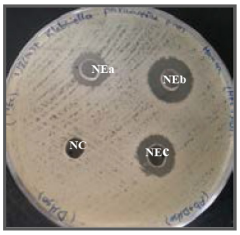

Fig. 2 **The effect of NEa, NEb and NEc on growth inhibition (Diameter of inhibition zone) of *Serratia marcescens* compared to sterile phosphate puffer (negative control, NC).**

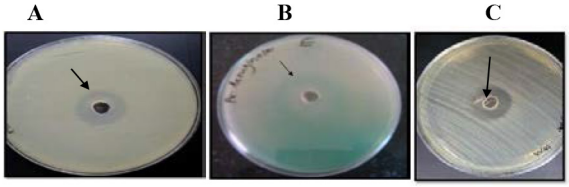

Fig. 3 **The antimicrobial activity of nanoemulsions NEb against *Serratia marcescens* (A), *Pseudomonas aeruginosa* (B) and *Streptococcus pyogenes* (C) using agar well diffusion assay.**

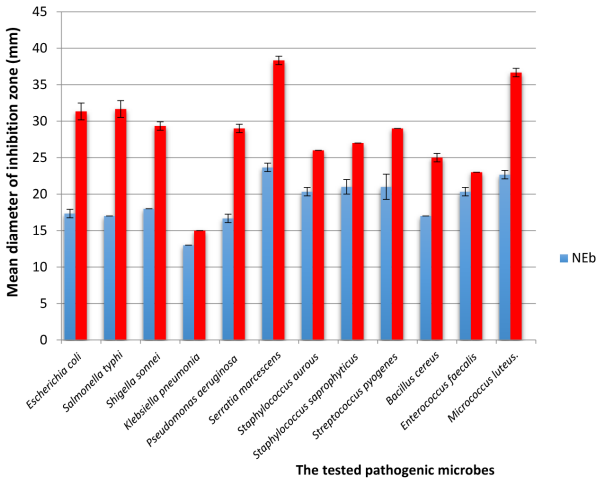

Fig. 4 **The antimicrobial activity of nanoemulsions NEb against some pathogenic bacteria using agar well diffusion assay and compared to control antibiotics. Error bars indicate the standard deviations of triplicate determinations (n = 3).**

were lost and disturbance in the cell wall composition lead to unnatural structure, weakness in the cell wall, and finally bacterial cell death.

The effect of NEb on the release of the cell cytoplasmic constituents was detected by measuring the absorbance at 260 nm after 12 hrs of growth in the presence of NEb. As demonstrated in Table 3, NEb at a concentration less than the MIC increased the leakage percentages of nucleic acids and proteins detected by the increase in the absorbance at 260 nm and K^+ leakage (10^{-6} moles of $K^+/10^6$ cell/ml) detected by atomic absorption where maximum leakage from the cell plasma membrane was found after 12 hrs. Moreover, the K^+ flow was

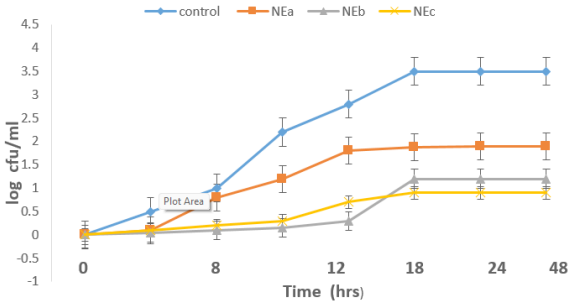

Fig. 5 **The effect of NEa, NEb, and NEc on growth (bacterial count) of *Serratia marcescens* after different periods.**

the lowest for untreated cells. A significant increase in the previous materials was found compared to the control. The cellular respiration of treated and normal cells was determined by measuring the quantities of Oxygen consumed (µl of O_2/ mg dry cells/hr). The cellular respiration was significantly decreased when cells were treated with NEb compared to the respiration of untreated cells (control) as shown in Table 3. Mahmoud and Aly (2004)[31] reported that synthetic antifungal polymers denatured the plasma membrane, enhanced the flow of K^+ from inside to outside the cells, and decreased the cell's respiration. High concentrations of the antibiotic primycin, increase the loss of K^+, Na^+, and perhaps Mn^{2+} from the cells leading to cell death.[44] Antimicrobial agents may damage the bacterial cell by interfering with RNA, DNA, and protein synthesis, weakening the bacteria's cellular structure, blocking a specific cross-linking step in cell wall production, interfering with the synthesis of the cell wall subunits, or damaging the plasma membrane.[45] Rodgers et al (1990)[46] reported that antibiotics inhibit the cell wall, protein, and DNA synthesis of *Legionella pneumophila*. Ampicillin, cefotaxime, and methicillin were cell wall synthesis inhibitors and induced many extensive morphological changes, which included the formation of membranous lesions through which cytoplasmic contents were lost. Some antibiotics act on the microbial cell wall while others like Erythromycin- and rifampicin affect cell membranes, which partially or fully leads to cell lysis, whereas ciprofloxacin induced abnormally elongated cells with intermittently lysed and detached inner membranes.[46] The fast killing of Nanoemulsions is due to bacterial membrane denaturation that significantly decreases the bacterial cell surface hydrophobicity and induces the quick release of 260 nm absorbing materials.[47]

The cell surface hydrophobicity is one of the most important factors that determine the interaction of cells and antibiotics. It was expressed as the percentages of cell adhesions and it has been reduced significantly when treated with NEs formulations. Zhang et al (2009)[38] indicated that the nanoemulsions induced bacterial cells to be more hydrophilic which affects microbial adhesion. The kinetics of microbial adhesion to hexadecane as a function of pH was studied.[48] The decreased bacterial cell hydrophobicity leads to the rapid loss of bacterial viability. The nanoemulsion likely distorts the lipid packing in the phospholipid bilayer, thereby affecting the fluidity of the membrane, and causing the plasma membrane to break apart leading to cell death.

Nanoemulsions were active against biofilm formation by *S. marcescens* (Table 3). Similarly, biofilms formation by *P. aeruginosa* was inhibited using NEs (Al-Adham et al., 2003)[49] while Teixeira et al., (2007)[16] found that Nanoemulsion were active against *Salmonella typhimurium, S. aureus, E. coli, P. aeruginosa,* and *Listeria monocytogenes.* The cell death may be due to a slight effect on the plasma membrane and increasing cell respiration that causes exhausting the energy compounds present inside the cell and its death. Meszaros *et al*., (1979)[43] indicated that prmycin enhances the loss of K^+, Na^+, and perhaps Mn^{2+} at the high concentration from the mitochondria leading to cell death. Concerning antibiofilm activity, nanoemulsions showed inhibition of bacterial adhesion >67.2% and removal of adhered cells >57.7%.[37] No toxicity was recorded for NEb against *Artemia salina* as a test organism or against HaCaT cells and the percentage of cell viabilities were 91 and 97% at 100 µl/ml, respectively (Table 3). Toxicity studies on NEs are scarce (Susana et al., 2023)[50] and among the few examples, oil of the *Nigella sativa* NE was not toxic to Sprague Dawley rats, administered orally at 20 ml/kg, considering the parameters of general behavior, body weight, food and water consumption, relative organ weight, hematology, histopathology, and clinical biochemistry).[51] After detecting the effect of NEb on *S. marcescens in vitro*, the capacity of NEb to protect *Galleria mellonella* larvae from bacterial infection was detected using different concentrations of NEb (10 –80 µl/ml) after 3, 6, 9, and 12 hrs and compared to control without nanoemulsion (Figure 8). The percentage of infection inhibition was increased by increasing NEb concentration and

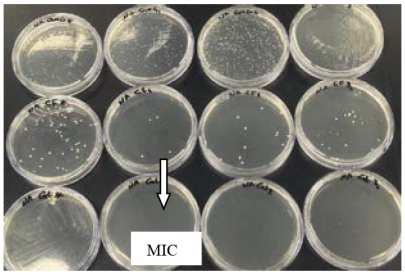

Fig. 6 **The effect of different concentration of NEb on the counts of *Serratia marcescens* after 24 hrs to detect the MIC (no growth or low bacterial counts).**

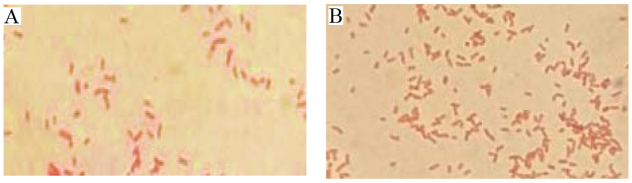

Fig. 7 **The cells of *Serratia marcescens* x1000, (A): treated with a sub-inhibitory dose of NEb (20 µl/ml) and (B): control (untreated cells) after growth at 37°C.**

Table 3. **Effect of NEb (20 µl/ml) on leakage of potassium and cytoplasmic constituents, Cellular respiration, inhibition of Biofilm inhibition (%), and cell toxicity. Data are the average of three experiments analyzed ± SD**

	K^+ leakage ($\times 10^{-6}$ moles of $K^+/10^6$ cell/ ml)	Cell surface hydrophobicity (A_{260nm})	Cellular respiration (µl of O_2/mg dry cells/ h)	Biofilm inhibition (%)	% Cell viability *Artemia* salina	HaCaT cells
Normal cells (control)	1.3 ± 0.881	0.94 ± 0.180	17.51 ± 2.105	0.0	100	100
Treated cells	2.12 ± 0.418*	1.97 ± 0.218*	29.11 ± 6.110*	51*	97#	90#

#, not toxic; *, significant result compared to control, Cell surface hydrophobicity is expressed as the $A_{260\ nm}$.

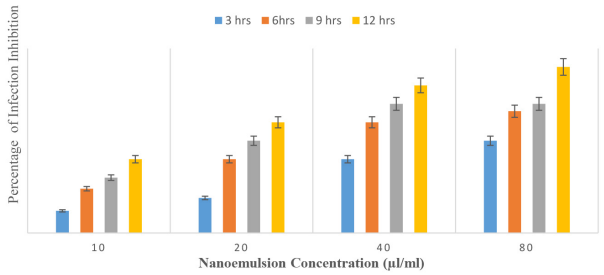

Fig. 8 **Percentage of infection inhibition of bacteria after 3, 6, 9, and 12 hrs using different concentrations of NEb (µl/ml) compared to control without nanoemulsion (100% infection).**

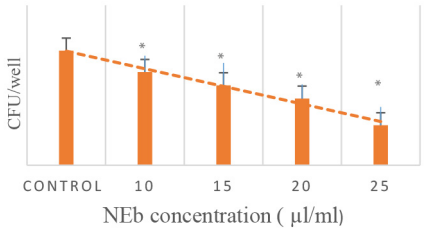

Fig. 9 **Assessment of anti-adhesion (CFU/well) after exposure of HaCaT cells to *Serratia marcescens* and different concentrations of NEb (10, 15, 20 and 25 µl/ml), treatment without nanoemulsion was used as a control, asterisks show significant differences at $P < 0.05$ compared to control.**

incubation time. Imparato et al. (2024)[52] used the same technique to test the activity of extracellular extract of *Candida albicans* on *Klebsiella pneumoniae* infection using *Galleria mellonella* as test larvae.

NEb prevents *Serratia marcescens* from adhering to HaCaT cells at concentrations of 10–25 µl/ml and the resulting numbers of the bacterial cells adhered to the HaCaT cells decreased at the highest NEb concentration, resulting in an attenuated adhesion, as shown in Figure 9. Imparato et al. (2024)[52] used the same technique to test the activity of the extracellular extract of *Candida albicans* on *Klebsiella pneumoniae* adherence and biofilm formation. The effect of NEb on the cell wall composition of *S. marcescens* was detected

Table 4. **The cell wall composition, total protein and carbohydrates of normal and treated *Serratia marcescens* cells**

Tested cell walls	Total protein mg/g	Total carbohydrates mg/g
Normal cells (control)	519	380
Treated cells	623*	297*

*, significant result compared to control.

(Table 4) and the result showed that the composition of the cell wall was changed in the tested bacterium compared to control. The sugar and protein quantity in the treated cell wall with all NEb were significantly different compared to the control (untreated cells). The disturbance in the cell wall composition of the treated bacteria generally leads to unnatural structure and weakness in the cell wall and finally to analysis and death of the bacterial cells.

Conclusions

NEs prepared from essential plant oils inhibit bacterial growth and development through increasing cytoplasmic membrane permeability for proteins, nucleic acids, and K^+, reduced cellular respirations, and finally induced cell death. Thus, NEs can be used against various microbes with high activity and low toxicity, thus, they can be used as potentially suitable alternatives to standard antibiotics for the treatment of antibiotic-resistant bacteria and those that form impenetrable biofilms.

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Conflicts of Interest

According to the authors, no conflicts of interest exist.

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