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Antibacterial Activity and Mechanism of Action of Lipid Nanoemulsions Against *Staphylococcus aureus*

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Pathogenic bacteria remain a major health problem as their treatments with antibiotics lead to bacterial resistance. Three nanoemulsions (NEa, NEb and NEc) were produced by mixing soya phosphatidylcholine (SPC), polyoxyethylenglycerol trihydroxystearate 40 (EU), sodium oleate (SO), cholesterol (CHO) and 0.1M of Tris-HCl (pH 7.22). The droplet sizes of all of NEs formulations were found in the range of 36-173.5 nm and their morphology were spherical and normally distributed, as determined by scanning electron microscope (SEM). Their antibacterial activities and mechanism of actions were examined against *Staphylococcus aureus (St. aureus)*. It has been found that the minimum inhibitory concentration (MIC) of NEa was 1.50ml/10ml of nutrient broth (NB), while it was 1.25 ml/10ml of NB for both of NEb and NEc. The NEs formulations have affected *St. aureus* through changing the cell morphology and cell wall composition, reducing the cell respiration and hydrophobicity, and enhancing the potassium leakage and cell permeability to the cytoplasmic constituents. This study suggests that the lipid-based NE formulations have great potential as antibacterial agents against *St. aureus.*

Key words: Minimum inhibitory concentration; Scanning electron microscope, Cell respiration; Cell permeability; Cell morphology; Pathogens.

Nowadays, many industries that develop antimicrobial agents are facing difficulties in curing infectious diseases since most of these agents are not completely penetrating the microbe cell membrane and even when they get transported into the cell, they get degraded and as a consequence they would have a reduced inhibition effect. In addition, several antibiotics are toxic to healthy tissues while they acquire resistance from the infectious microbes. Therefore, many research studies are concentrating on finding new therapeutic and diagnostic treatments for both of known and unknown pathogens. Nanoparticles, which can be metallic, semiconductor or polymeric, are one of the proposed materials that would be utilized as a nano-carrier for antibiotics or as an antimicrobial drug with increased efficiency and biocompatibility[1].

Emulsification systems are types of colloidal systems that produce nanoparticles. They consist of two or more immiscible liquids stabilized by surfactants which are chemical compounds with polar head and non-polar tail. These systems include emulsions, microemulsions and NEs. They differ in the composition, appearance, kinetic and thermodynamic stability. While emulsions appear opaque and microemulsions are transparent, NEs are in between slightly opaque and transparent[2]. NEs systems are like emulsions require less amount of surfactant fraction (around 2%) but more input of energy than the microemulsions which are easily

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prepared and include more than 5% surfactant. NEs can be identified as fine emulsions with dispersed phase aggregates within the submicron region and kinetically stable. The presence of oil-in-water (o/w) NEs droplets is likely to be produced where the volume fraction of oil is low. Conversely, water-in-oil (w/o) NEs droplets are likely to occur, when the volume fraction of water is low. In systems where the amounts of water and oil are similar, a bicontinuous NEs may results.

NEs have grabbed attention as antimicrobial drugs that would efficiently destroy HIV-1 and various tuberculosis pathogens without damaging the normal human cells or the cells of most other higher organisms. NEs containing soybean oil, with particles size range between 400 – 600 nm in diameter, have showed a mechanical force on the cell wall or membrane of the microbe to merge with the other lipids[3]. Soybean oil- based NEs have been reported to have antibactericidal properties against Gram- positive but not against enteric Gram-negative species[4]. They are sporicidal, in dilutions up to 1:1000[5, 6] and have antiviral properties against enveloped viruses[6, 7]. NEs have also fungistatic or fungicidal effect[6].

Bacterial susceptibility using NE was determined in vitro at concentrations of 1:100 to 1:10,000, which were effective against *Streptococcus mutans*[8]. Myc *et al*.[9] have reported that NEs have a spectrum of biocidal activity against a variety of microorganisms including Gram- positive and Gram-negative bacteria, spores and enveloped viruses. Moreover, 0.1% of NEs, designated with $X8W_{60}PC$, have fungicidal activity against yeast including *C. albicans* and *C. tropicalis* within 15 minutes.

The aim of this study was to prepare NEs formulas with different compositions of surfactant-to-oil (S/O) ratio and studying their effects against *S. aureus*. The mode of action of the nano-formulations on cellular respiration, permeability and morphological modification were also studied. In this work, the o/w NE formulations (NEa, NEb and NEc) were prepared by mixing different weight fractions for surfactant mixture of EU/ SPC/ SO and the oil phase of CHO. The Tris-HCl buffer (pH 7.22) and the cosurfactant 1-octanol were fixed. It is should be noted that the same NEs formulations were produced previously but with different weight fraction of the components[10, 11].

MATERIALS AND METHODS

Tris (hydroxymethyl) amino methane, SPC, SO, Eumulgin and HCl were purchased from Leo chem., S. Puran, Banglore-20, India. CHO was purchased from Jechno pharmacies Banadur Garh, Haryana, India. The 1-octanol was obtained from Alfa Aesar GmbH & Co KG Karlsruhe, Germany. Distilled water was purified using a water purification system from Bibby sterilin Itd, U.K. All types of media used in this work were sterilized by autoclaving at 121°C for 20 min. *S. aureus* was generously supplemented by King Abdulaziz Hospital, Jeddah, KSA. The nutrient agar (NA), used for cultivation, was obtained from Difco.

Preservation of *S. aureus*

It was maintained on slopes of NA at 4°C and was regenerated every six months, as described by Dadgar *et al*.[12].

Preparation of NEs formulations

Table 1. The NEs formulations prepared at different weight percentages of the mixture EU/ SPC/ SO, prepared at fixed weight ratio of 3.5:3.0:3.5, and CHO. It should be noted that the weight percentages of tris HCl-buffer (pH 7.22) and 1-octanol were fixed at 98.9 and 0.6, respectively in all of the three NE formulations.

NEa, NEb and NEc were prepared as described elsewhere with modifications on the weight fractions of surfactant mixture of EU/ SPC/ SO and the oil phase of CHO as shown in Table 1 [11]. The weight fraction of the aqueous phase, 0.1M of Tris-HCl buffer (pH 7.22) and the cosurfactant, 1- octanol, were fixed at 98.9 and 0.6 respectively.

Size and morphological characterization of NEs droplets

Table 1. The NEs formulations prepared at different weight percentages of the mixture EU/ SPC/ SO, prepared at fixed weight ratio of 3.5:3.0:3.5, and CHO. It should be noted that the weight percentages of tris HCl-buffer (pH 7.22) and 1-octanol were fixed at 98.9 and 0.6, respectively in all of the three NE formulations

Component	NEa	NEb	NEc
EU/SPC/SO	0.25	0.3	0.4
CHO	0.25	0.2	0.1

The particle size analysis and morphology was determined using SEM (Quanta FEG 450) at the unit of Electron microscope, Faculty of Science, King Abdulaziz University, Jeddah, KSA. To prepare specimens for the SEM, they were first fixed with Karnovsky's glutaraldehyde fixative (2g of Paraformaldehyde, 2 - 4 drops of 1M Sodium hydroxide, 5ml of 50% glutaraldehyde, and 20ml of 0.2M cacodylate buffer (pH7.4)) and then taken through a graded alcohol dehydration series. Once dehydrated, the specimens were placed in a critical point dryer, mounted and placed in a gold coater. Once gold coating was complete, specimens were ready to be viewed on the SEM. Images were scanned on a digital imaging system by computer enhancement.

Antibacterial activity of the prepared NE formulations

Pre-cultural preparation of *S. aureus*

Bacterial suspensions were prepared at a concentration visually equivalent to a 0.5 McFarland Standard as described elsewhere[13,14]. About 3-5 pure colonies of treated bacteria were taken by sterile cotton swab to glass tube containing 5ml of sterile distilled water. After shaking, sample suspension was visually compared to the standard. Accordingly, the concentration was adjusted with either more water or organisms until the suspension matches the Standard, which is equivalent to 1.5×10^8 CFU/ml.

Determination of the inhibition zone using agar well diffusion assay

Agar well diffusion procedure, as illustrated elsewhere, was used to determine the antimicrobial activity of the three NEs[15] Petri plates (85 mm×15 mm) were prepared by pouring 15 ml of sterile NA in each plate and the medium was allowed to solidify. About 0.1 ml of an inoculum suspension (tested bacterium, 1.5×10^8 cfu/ml) was poured and uniformly spread using sterile cotton swap. After inoculum absorption by agar, wells were made using sterile cork poorer (diameter 5 mm) and were filled with 100 µl of the NE. Plates were left for 45 min at room temperature to allow proper diffusion of the NE to occur in the medium. The plates were incubated at 37°C for 24 h. Inhibition of bacterial growth was measured as inhibition zone diameters (mm) and the average value was taken.

Effect of incubation period of time on antibacterial activity of NEs

In Erlenmeyer flask, 9 ml of sterile NB was mixed with 1ml of the tested NE. The flask was inoculated with 20 µl of the tested bacterium and incubated at 37°C for 15, 30, 45 and 60 min. After that, 0.1 ml of the culture was spread on petri dish containing 15 ml of NA and the plates were incubated at 37°C for 24 h. Finally, the number of bacterial colonies/ plate was counted using colony counters (SC6-R000103491) and compared with the counts of control (culture without NE).

Determination of MIC

The value of MIC is important in diagnostic laboratories to confirm the resistance of microorganisms to an antimicrobial agent and also to monitor the activity of new antimicrobial agents[16]. MIC is generally regarded as the most basic laboratory measurement of the activity of an antimicrobial[17]. The MIC of NEa, NEb and NEc was calculated using broth dilution method[18]. In 100 ml flasks, different concentration of NEa, NEb and NEc were prepared in 10 ml of NB. The prepared concentrations of 0.25, 0.5, 0.75, 1.0, 1.25, 1.5 and 1.75 ml of NE were added into 10 ml of NB medium. Each flask was inoculated with 10 µl of $1.5x10^8$ CFU/ml of the pre-culture. After 45 min of incubation at 37°C, the living bacterial cells were counted using NA plates. About 0.1 ml of each inoculated flasks were spread on NA plates and were incubated at 37°C for 24h to allow bacterial growth. The developed colonies were counted and mean numbers were calculated. The MIC was determined as the lowest concentration of NE which developed no growth or small colonies.

Mode of action of NEs against *S. aureus*

Cell morphology

Bacterial cells were cultivated on NB at 37°C for 24 h. Then, 9 ml of NB medium was inoculated with 20 µl of the pre-culture containing $1.5x10^8$ cfu/ml in a conical flask. The bacterial cells were treated with the MIC of the tested NE. An equal amount of distilled water was added as a control and all flasks were incubated for 45 min at 37°C. The treated and untreated cells were collected using centrifugation at 5000 rpm for 15 min. The collected cells were examined and photographed using SEM (Quanta FEG 450) at the unit of Electron microscope, Faculty of Science, King Abdulaziz University.

Cell permeability

The effect of NEs on the nucleic acids

and proteins components of *S. aureus* was determined as described by Hou *et al.*[19]. Approximately 1.5×10^8 cfu/ml of bacteria was incubated with 1.5 ml of the tested NE for 45 min. After that, cells were collected, washed three times with distilled water and suspended in sterile water for 8 h. The mixture was filtered and the absorbance (A1) of the filtrate was read at 260nm. The control sample was prepared by incubating the bacterial cells with Triton X-100 for 60 min (A0). Leakage of the nucleic acids materials were estimated as A1/A0×100.

Leakage of potassium

S. aureus cells were treated with the tested NEs in NB media, while the control cells were subjected into Amphotericin B. The cells were collected, washed and suspended in 10ml distilled water and left in the shaker for 30 min. After that, the cells were removed by centrifugation and the quantity of potassium in the solution was measured using a flame photometer (Kruss, model FP8800).

Cellular respiration

The quantity of oxygen consumed by the living cells in the presence or absence of the tested NEs was determined using an oxygraphe. After 5 min of incubation at room temperature, the quantity of oxygen was determined as µl/mg/h of oxygen and compared with the control.

Cell wall composition

The cell walls of *S. aureus* were obtained using a method described by Mahmoud and Aly[20]. A 5ml of the tested NE was mixed with 40 ml of NB medium followed by inoculation with 0.1 ml of bacterial suspension (1.5×10^8 cfu/ml) and incubation at 37°C for 24 h. After that, bacterial cells were collected by centrifugation at 5000 rpm for 15 min and then, washed several times and lyophilized. About 1 g of the lyophilized cells was suspended in 5 ml water and broken down using sonication for 3 min under cooling for three successive times. After centrifugation at 3000 rpm for 20 min, the cell walls were collected, dried and hydrolyzed by 6 N HCl at 105°C for 6 h. Control flask was prepared by adding 5 ml of sterile distilled water instead of active material. Then, the quantity of proteins, sugars and phosphors in the treated and normal cell walls were determined as described elsewhere[21-23].

Cell surface hydrophobicity

The effect of the NE on the hydrophobicity of the bacterial cells was examined using a method illustrated elsewhere[24].Cells were treated with ten-fold serial water dilutions of the tested NE at 37 °C for 60 min, while the control sample was incubated with an equal volume of sterile normal saline. After that, 0.2 ml of hexadecance was added to 1.2ml of cell suspension. Then, the mixture was vortexed for 2 min and left for 15 min to allow complete phase separation. The absorbance (Abs) of the aqueous phase was measured at 400 nm. The percentage of microbial adhesion was calculated as $(1-\frac{\text{Abs of treated sample}}{\text{Abs of control}})\times100$, and regarded as representative of cell surface hydrophobicity.

Statistical analysis

All experiments were carried out in triplicate and all values were expressed as mean ± standard deviation $(\overline{X}\pm SD)$. Statistical analysis was performed with one-way analysis of variance (ANOVA) using Megastat and $p \leq 0.05$ was considered significant.

RESULTS AND DISCUSSION

Characterization of NEs using SEM

SEM was employed to find out the droplet shape and distribution. As shown in Figure 1, the droplets morphologies for all of the NEs formulations were spherical and normally distributed. In order to assess the droplet sizes trend and their distribution, approximate measurements of their diameters and the percentages of the coefficient of variation (% CV), which is calculated through dividing the standard deviation by the mean of six replicates of the droplet sizes, were determined. The mean droplet sizes of all NEs formulations were found in the range of 36-173.5 nm. NEa, which has equivalent amount of surfactants and oil, was having the largest droplet size of 126.10 ± 28.48. NEb, which has surfactant-to-oil (S/O) ratio of 1.5, was having droplet size of 48.63 ± 8.04, whereas, NEc which has more amount of surfactant was having droplet size of 38.62 ± 4.97. The % CV's were 22.59, 16.53 and 12.86 for NEa, NEb and NEc, respectively, which mean that the droplets were normally distributed since they were lower than 25%. According to the statistical analyses, it has been found that there were significant differences

between all of the droplets sizes of the three NEs formulations ($p \leq 0.05$). These results were in agreement with a previous study as the droplet diameters of the NEs have decreased when the fractions of surfactants were increased[11]. It is worth noting that the NEs formulations in this study have increased fractions of surfactants and oil which resulted in the enlargement of the droplet sizes.

Antibacterial activity of NEs

According to agar well diffusion assay,

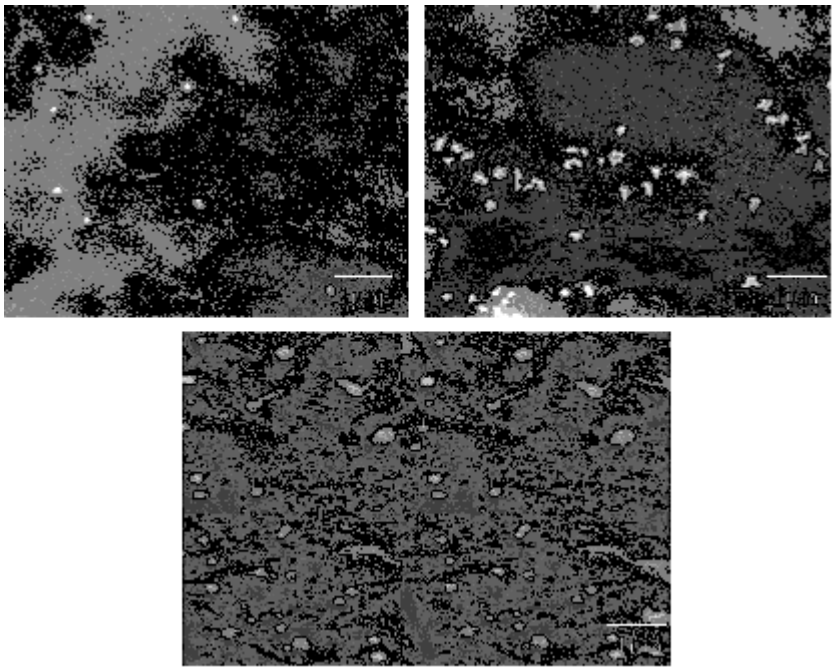

Fig. 1. Scanning electron micrographs of NEa, NEb and NEc

NEa, NEb and NEc gave inhibition zones of 11±0.58 mm, 17±1, 15±0.58, respectively. Further, MIC was determined as the lowest concentration which gave no growth or very small numbers of colonies of bacterial cells. The calculated MIC of NEb and NEc was 1.25 /10 ml of NB while it was 1.50 ml/10 ml of NB for NEa.

Myc *et al*.[9] found that the $X8W_{60}PC$ NE has great potential as a topical anti-fungal agent. Using MIC assay, 0.08% of the NE was inhibitory to *C. albicans* and *C. parapsilosis* in addition to filamentous fungi including *Microsporum gypseum*, *Trichophyton mentagrophytes*, *Trichophyton rubrum*, *Aspergillus fumigates* and *Fusarium oxysporum.* They found that the MIC of a different NE never exceeded 0.1%. In another study, it has been shown that one NE has antimicrobial activity against cariogenic planktonic and biofilms of S. *mutans*[8]. The results showed that NE inhibited *S. mutans* with MIC occurring at dilutions of 19,683.

The effect of the NEs formulations on the growth of *S. aureus* cells, determined by counting the number of cells/ml (cfu/ml) versus time (min), was the greatest for NEb as within 60 min, all of the cells were killed, while NEa and NEc killed 75% of cells at the same period of time (Figure 2). A previous research that studied the kinetics of killing demonstrated that the undiluted microemulsion caused a complete loss of viability of *E. coli or S. aureus* cells in 1 min[25]. It still had effective bactericidal effects even when diluted as more than 99% of viable *Escherichia coli* cells were killed within 15 min and a complete loss of viability was achieved at 45 min. While more than 99% of viable *S. aureus* cells were killed within 30 min, a complete loss of viability was achieved at 60 min in the presence of the 10-fold diluted microemulsion.

The fast killing kinetics of the ten-fold serial dilutions of microemulsions were in good

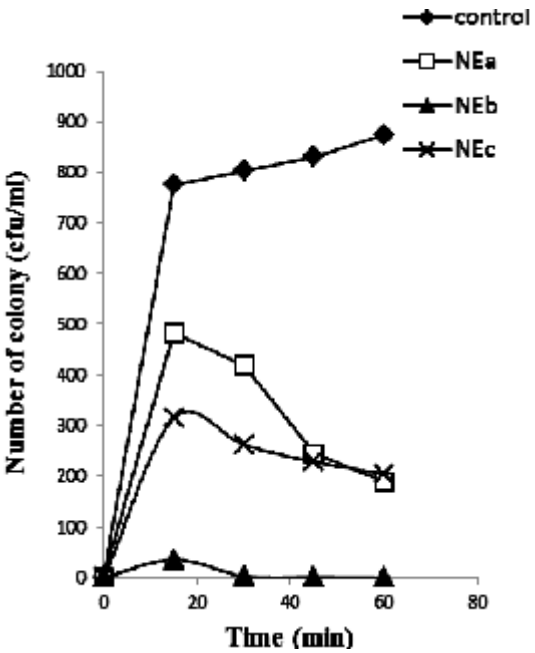

Fig. 2. The effect of NEa, NEb and NEc on growth of *St. aureus*

agreement with the mode of action studies, indicating that the interaction between the antimicrobial microemulsions and bacterial membranes significantly decreased the bacterial cell surface hydrophobicity and induced the quick release of 260 nm absorbing materials[15]. The kinetics of killing experiments demonstrated that the microemulsion caused a complete loss of viability of bacterial cells (*E. coli, S. aureus* and *B. subtilis*) in 1 min, killed over 99% *A. niger* and *P. expansum* spores and 99.9% *C. albicans* cells rapidly within 2 min and resulted in a complete loss of fungal viability in 5 min.

Mode of action of NEs against *S. aureus*

As displayed in Figure 3, all NEs formulations induced some morphological changes in *S. aureus* cells, which included the formation of membranous lesions through which cytoplasmic contents were loS. Cells treated with NEa and NEc were elongated, while the ones treated with NEb were accumulated, damaged and have lost viability. In order to detect the effect of NEs on the cell membrane, the release of the cytoplasmic constituents of the cells were monitored spectrophotometrically. As demonstrated in Table 2, a constant increase in the percentages leakages of nucleic acids and proteins was recorded, reaching 92.06, 84.12 and 77.50 when treated for 60 min with 10-fold dilutions of NEa, NEb and NEc, respectively. When the bacteria were incubated with the 100- fold dilution of NEa, NEb and NEc, leakages increased within 60 min to 98, 95 and 85, respectively.

In addition to the cell permeability of the cytoplasmic materials, the leakage of potassium

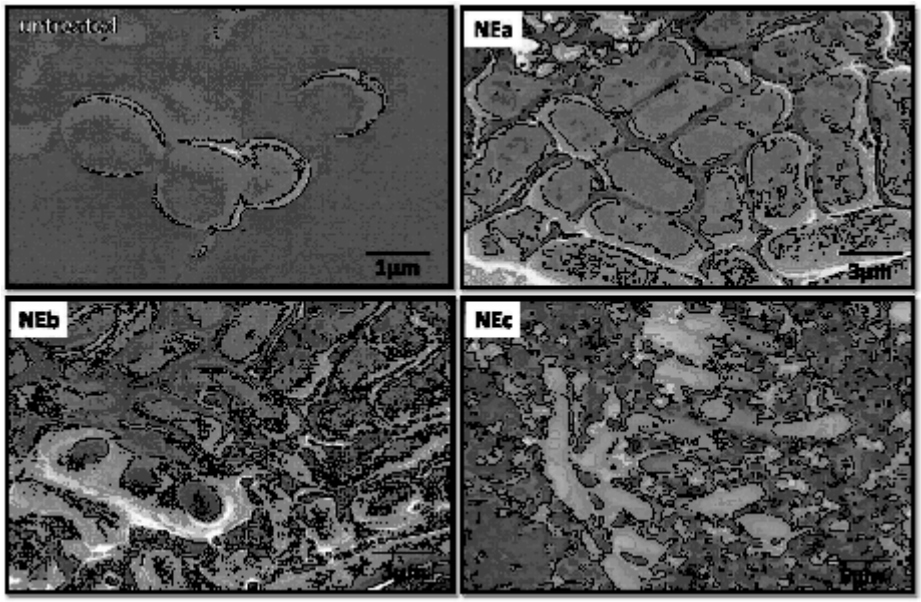

Fig. 3. Scanning electron micrographs of untreated cells of *Staphylococcus aureus* after incubations for 24 h at 37ºC and cells treated with NEa, NEb and NEc

Table 2. Leakage percentages of cytoplasmic constituents from *St. aureus* cells when incubated with 10 and 100 fold dilutions of NEs formulations

Time	10-Fold Dilutions of NEs			
	Control	NEa	NEb	NEc
15 min.	95	7.61	21.20	14.40
30 min.	96	18.48	25.60	20.96
45 min.	96	31.12	26.30	34.12
60 min.	99	92.06	84.12	77.50
	100-Fold Dilutions of NEs			
15 min.	95	34	26.11	33.92
30 min.	96	33.61	33.61	32.54
45 min.	96	95	90.92	60
60 min.	99	98	95	85

(Potassium flow $\times 10^{-6}$moles of $K^+/10^6$ cell/ ml) from the plasma membrane have significantly increased from 0.21 ± 0.51 into 0.33±0.16, 0.39±0.45 and 0.33±0.51 when treated with NEa, NEb and NEc, respectively. Moreover, the cellular respiration of *S. aureus*, determined by measuring the quantities of Oxygen consumed (µl of O_2/mg dry cells/ h) by *S. aureus*, have significantly decreased from 0.22 ± 0.05 into 0.15 ± 0.14, 0.17 ± 0.01 and 0.14 ± 0.16 when treated with NEa, NEb and NEc, respectively.

A previous research study reported that the amphotericin B and two synthetic antifungal polymers (polymer 1 and polymer 2) affects the fungal plasma membrane and impairs the barrier function of membranes enhancing the flow of K^+ from inside to outside the cells and decreasing the cells respiration[20]. The results obtained from polymer (2) indicates a slight effect on the plasma membrane and increasing cells respiration for exhausting the energy compound present inside the cell before its death. Another study indicated that primycin enhances the loss of K^+, Na^+ and perhaps Mn^{2+} at the high concentration from the mitochondria leading to cell death[26].

The effect of NEs formulations on the cell wall composition of *S. aureus* was determined by quantitatively measuring the fractions of sugars, proteins and phosphorus. According to Table 3, it is demonstrated that phosphorus have significantly changed and as consequence the sugars and proteins were affected as well. The disturbance in the cell wall composition of the treated bacteria generally lead to unnatural structure, weakness in the cell wall and finally to analysis and death of the bacterial cells.

Many authors found that antibiotics could kill bacteria by interfering with their normal

Table 3. Determination of cell wall fraction mg/g of *St. aureus* when treated with NE formulations

Cell wall composition	Untreated bacterial cell wall	Treated bacterial cell wall		
		NEa	NEb	NEc
Sugars	876 ±1. 8	800±0.019	876±0.018	852±0.014
Protein	266±0.031	279±0.060	279±0.025	269±0.175
Phosphors	24 ±0.003	35±0.009	39±0.006	42±0.008

intracellular functions (RNA, DNA and protein synthesis) or by weakening the bacteria's cellular structure or disrupting the cell wall, causing the cell to break or lyses. Penicillin functions by blocking a specific cross-linking step in cell wall production that is critical for the bacteria to form strong cell walls[27]. Vancomycin also acts to weaken newly made bacterial cell walls by interfering with the synthesis of the cell wall subunits (peptidoglycans)[28]. Bacitracin blocks another step in bacterial cell wall synthesis[29].

Another research study reported that the antibiotics (ampicillin, cefotaxime, methicillin, erythromycin, rifampicin and ciprofloxacin) inhibit cell-wall, protein and DNA synthesis of *Legionella pneumophila*[30]. The inhibitors of cell-wall synthesis ampicillin, cefotaxime and methicillin affected the greatest bactericidal activity and induced the most extensive morphological changes, which included the formation of membranous lesions through which cytoplasmic contents were loS. In terms of ultra-structural damage and loss of viability, the inhibition of protein and DNA synthesis were less effective than the antibiotics that acted on the microbial cell wall. Erythromycin- and rifampicin-treated cells

possessed irregular membranes and were partially or fully lysed, whereas ciprofloxacin induced abnormally elongated organisms with intermittently lysed and detached inner membranes.

Furthermore, the cell surface hydrophobicity, expressed as the percentages of cell adhesions, have been reduced significantly when treated with NEs formulations. When the *S. aureus* cells were incubated with sterile normal saline, 99.87 ± 0.15 of the cells were adhered to the apolar solvent demonstrating a hydrophobic surface. On the other hand, when they were treated with NEa, NEb and NEc, the percentages of cell adhesions have significantly decreased to 80.67 ± 0.33, 43.78 ± 0.19 and 33.89 ± 0.19, respectively.

It has been indicated that the microemulsions induced bacterial cells to be more hydrophilic[25]. The cell surface hydrophobicity is one of the most important factors to determine the interaction of cell and antimicrobials. The importance of cell surface hydrophobicity in microbial adhesion has been demonstrated by studying the kinetics of microbial adhesion to hexadecane as a function of pH of the aqueous phase[31]. The interaction between the antimicrobial microemulsions and bacterial membranes significantly decreased the bacterial cell hydrophobicity, leading to the rapid loss of bacterial viability. It is likely that the microemulsion resulted in the distortion of the lipid packing in the phospholipid bilayer, thereby affecting the fluidity of the membrane, and caused the plasma membrane to break apart leading to cell death[32].

CONCLUSIONS

The antibacterial activity and mode of actions of three NEs formulations against *S. aureus* were assessed. The MIC of NEb and NEc, which have decreased droplet sizes and more S/O ratios, have less MIC of 1.25 ml/10ml of NB. It has been demonstrated that all of NEs have altered the cell wall compositions and the cytoplasmic permeability besides affecting the proteins and nucleic acids materials. All of the metabolic functions of the bacteria were affected as the cellular respirations have diminished while the potassium leakages have risen. This study revealed that the small NEs droplets permeate the bacteria and hence, damage it. It is recommended to evaluate these NEs against various microbes and to employ it as a nanocarrier for many potent antibiotics that have issues with cell permeability.

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