

## LETTER

# Encapsulated virgin coconut oil as a nanoscale in vitro solution against multiple drug resistant *Staphylococcus aureus*

## 1 | INTRODUCTION

Rapid increase of severe systemic/non-systemic infections and the spread of resistant microorganisms are indisputable facts. The resistance among various microbial species (infectious agents) to different antimicrobial drugs has emerged as a cause of public health threat all over the world at a terrifying rate [1]. The currently available drugs result in failure of microbial response to the treatment, leading to prolonged illness, higher expenditures for health care and an immense risk of death. Some of the infecting microbes have employed high levels of multiple drug resistance (MDR) with enhanced morbidity and mortality [2]. Normally bacterial infections are treated by antibiotics and can be preventable but there are certain reasons behind the failure to eradicate them such as selection of wrong antibiotics, inappropriate use of antibiotics, poor patient compliance, inappropriate dose, inadequate duration of treatment, wrong prophylactics and empirical therapy, fewer resources for education and infection control; inability of some laboratories or laboratory procedures to detect microbial resistance; breakdown in aseptic techniques; community factors such as clustering or overcrowding and widespread use of broad-spectrum antibiotics as prophylaxis. All these factors contribute to the promotion of resistance in bacteria [3]. Sometimes outer membrane of bacteria acts as barrier and protect the entry of antibiotics inside the bacterial cell due to which it becomes difficult to treat that infection [4].

*Staphylococcus aureus* is a Gram positive bacterium. It is a part of human skin normal flora and can also act as an opportunistic pathogen. If the skin gets ruptured, it gets opportunity to infect by the production (and release) of toxins and/or by colonization which causes tissue invasion and destruction. It has many virulence factors due to which it develops resistance against many antimicrobial drugs [3]. Due to resistance towards antibiotics, superbugs are emerged such as methicillin-resistant *S. aureus* (MRSA), vancomycin-resistant *S. aureus* (VRSA), vancomycin-intermediate *S. aureus*—VISA, and borderline oxacillin-resistant *S. aureus*—BORSA. *S. aureus* is reported to develop resistance against all classes of antibiotics, for example by acquiring a plasmid (through horizontal gene transfer) that contains a gene (*blaZ*) which encodes a  $\beta$ -lactamase enzyme. All penicillin drugs (and related  $\beta$ -lactam drugs) have a  $\beta$ -lactam ring at the core of

their structure. The  $\beta$ -lactamase enzymes (also known as penicillinases) hydrolyse the peptide bond in the  $\beta$ -lactam ring, which opens up the ring and makes it impossible for the drug to bind to the penicillin binding proteins (PBP) rendering them incapable of killing the cell [5]. There are also reports which suggest that *S. aureus* has aminoglycoside-modifying enzyme (AME), it chemically modifies the drug (quite often by transfer of an acetyl group) and consequently decrease the drug's ability to bind to the 30S ribosomal subunit and leads to malfunctioning of the drug [6]. *S. aureus* has also developed resistance against fluoroquinolone, which inhibits gyrase or topoisomerase IV and stops DNA replication and transcription. This resistance is developed by causing mutations in either the GyrA subunit of gyrase (encoded by *gyrA* gene) or the GrlA subunit of topoisomerase IV (encoded by *grlA* gene), which reduces the ability of the drugs to bind to their targets. The aforementioned facts point to the fact why it is getting difficult to develop new drugs fast enough to keep up with developing resistance of these superbugs [7–9].

Niosomes are vesicles mainly consisting of non-ionic surfactants that encloses and encompasses the drug molecules. These nano-sized niosomes have composition similar to bacterial cell membrane so they can easily fuse with the bacterial cell membrane and carry drug inside cell. Thus the drugs loaded in niosomes vesicles exhibited improved bactericidal activity against a number of MDRs [3, 10].

Virgin coconut oil (VCO) is the finest grade of coconut oil obtained from fresh coconut meat/copra by cold or wet press processing method. It is rich in phenolic content, antioxidants and contains medium chain triglycerides (MCTs) such as lauric acid and mono lauric acid which have antibacterial activity. As VCO is rich in triglycerides, it would express antimicrobial activity when triglycerides break down into lauric acid or monolauric acid by lipase activity which is activated in an oil-water interface. Our (previous/unpublished) research showed that VCO has potent antimicrobial capacities against susceptible and MDR bacteria [11].

In this article a simple method is reported for the synthesis of a nanostructured lipid carrier (NLC) system, i.e. niosomes followed by encapsulation of VCO inside it. The VCO-encapsulated niosomes act as a potent nanoscale antibiotic against multidrug resistant strains of *S. aureus*. Strong

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antimicrobial properties of different types of nanosystems against a wide variety of both gram-positive and-negative bacterial pathogens had been observed so far [4]. But to the best of our knowledge, it is for the first time that VCO has been administered against MDR bacteria through a nanocarrier system. And such type of studies will promote the use of nanosystems in medical applications such as therapy, drug delivery and bio imaging in better ways [4].

## 2 | MATERIALS AND METHODS

### 2.1 | *S. aureus* strains

Three different clinical (isolates) strains of *S. aureus* were obtained from Microbiology & Public Health Lab, COMSATS Institute of Information Technology, Islamabad. All strains were already (biochemically) characterized and identified.

### 2.2 | Classification of *S. aureus* strains

The Kirby Bauer method [12] was used for antimicrobial susceptibility testing as recommended by the NCCLS M2-Performance Standards for Antimicrobial Disk Susceptibility Tests and M100-Performance Standards for Antimicrobial Susceptibility Testing to check if the strains were resistant or not (NCCLS: M100-S12; 2012). The 18 h incubated inocula ( $1.5-2 \times 10^8$  CFU mL<sup>-1</sup>) [13, 14] were swabbed on the surface of Müller Hinton agar plates. Commercially available antibiotic discs (Oxoid, UK) belongs to five different classes were placed on agar plates and incubated at 37 °C overnight.

### 2.3 | Synthesis of niosomes

The synthesis of empty niosomes was adopted from Ajera et.al [15] with some modifications. Briefly, 25 mg span-40 (Sigma-Aldrich) and 25 mg cholesterol (BioBasic) allowed to disperse in the aqueous phase (4 mL PBS) and sonicated firstly with probe sonicator (Cole Parmer CV 18) for 5 min at 60% ampule. The suspension (synthesized niosomes) were further ultrasonicated in a bath sonicator (E-30H, Elmasonic) for 5 min at 60 °C to achieve the formation of small unilamellar vesicles if there were any larger niosomes. The samples were then spun at  $20,500 \times g$  (Sigma) for 30 min. The supernatants were discarded, pellets were harvested and stored at 4 °C for until further investigations/testing. For VCO-encapsulated nanocarrier vehicles, a volume of 250  $\mu$ L VCO was mixed in 4 mL PBS and niosomes were synthesized exactly as the procedure given above. Due to rapid movement during sonication vesicles (niosomes) were formed and VCO would get encapsulated in niosomes.

### 2.4 | Electron microscopy imaging

The morphology of the niosomes encapsulated with VCO was determined through transmission electron microscope (TEM) (JEM 2100 TEM) imaging. Purified and concentrated niosome

VCO (7  $\mu$ L) dispersion was pipetted carefully onto a (carbon-coated) Cu grid (Ted Pella, USA) and the excess fluid was removed by touching a corner of a filter paper to one edge of the grid. The grids were allowed to air dry for 3–5 min and later 2% phosphotungstic acid was added. They were finally dried in a vacuum chamber for 1–2 h and imaged in the TEM.

### 2.5 | Particle sizing (dynamic light scattering) and zeta potential measurement

Particle sizing (through DLS) and zeta potential measurements were performed in a ZetaSizer (ZS Nano, Malvern, UK). A diluted sample of 50  $\mu$ L was poured in the special cuvette, and it was installed in the ZetaSizer for particle size (DLS) analysis. The zeta potential values were determined by adding 1 mL of samples in the specially designed (electrophoresis capable) tubes at 25 °C.

### 2.6 | Encapsulation efficiency

To measure the encapsulation efficiency of VCO-encapsulated niosomes, dilutions of VCO were prepared from 1000 to 100  $\mu$ g mL<sup>-1</sup>. The optical density of all dilutions was measured at 290 nm as proposed in Kumar et al [16].

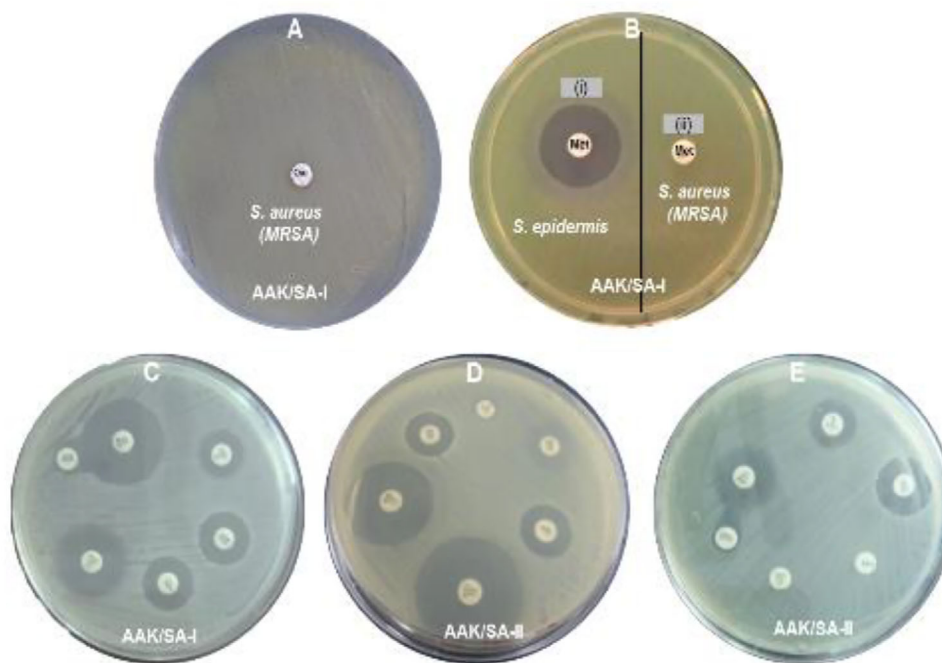
### 2.7 | Antibacterial assay and minimum inhibitory concentration (MIC) of encapsulated niosomes

Antibacterial activity of the pellets as well as supernatants of empty niosomes while only pellets of VCO-encapsulated niosomes were evaluated by disc diffusion assay. Inoculations were performed by swabbing on MH agar plates with cotton swabs dipped in bacterial strain inoculum ( $1.5 \times 10^8$  CFU mL<sup>-1</sup>). Discs impregnated with empty and (VCO) encapsulated niosomes were placed on the agar plates and incubated overnight at 37 °C. The zones of inhibition were measured the next day. The method of serial tube dilution [17, 18] was used for the calculation of MIC of VCO-encapsulated niosomes against the three strains of *S. aureus*.

## 3 | RESULTS AND DISCUSSION

### 3.1 | Antimicrobial susceptibility testing of *S. aureus* strains

The zones of inhibition obtained via disc diffusion assay were interpreted by referring to CLSI document M100-S23 (M02-A11): “Disc diffusion supplemental tables” performance standards for antimicrobial susceptibility testing to check either strain are susceptible, intermediate or resistant to antibiotics and whether they are multiple drug resistant or not [19]. Six antibiotics of different classes were selected; they were vancomycin



**FIGURE 1** Classification of the three clinical *S. aureus* strains. (a) AAK/SA-I was resistant to oxacillin. (b) AAK/SA-I was confirmed to be an MRSA when plated with a control of *S. epidermidis* with methicillin. (c) AAK/SA-I also showed resistance towards vancomycin and levofloxacin. (d) AAK/SA-II was found resistant towards vancomycin (VRSA). (e) AAK/SA-III was found to be resistant towards a number of antibiotics such as streptomycin, cefotaxime

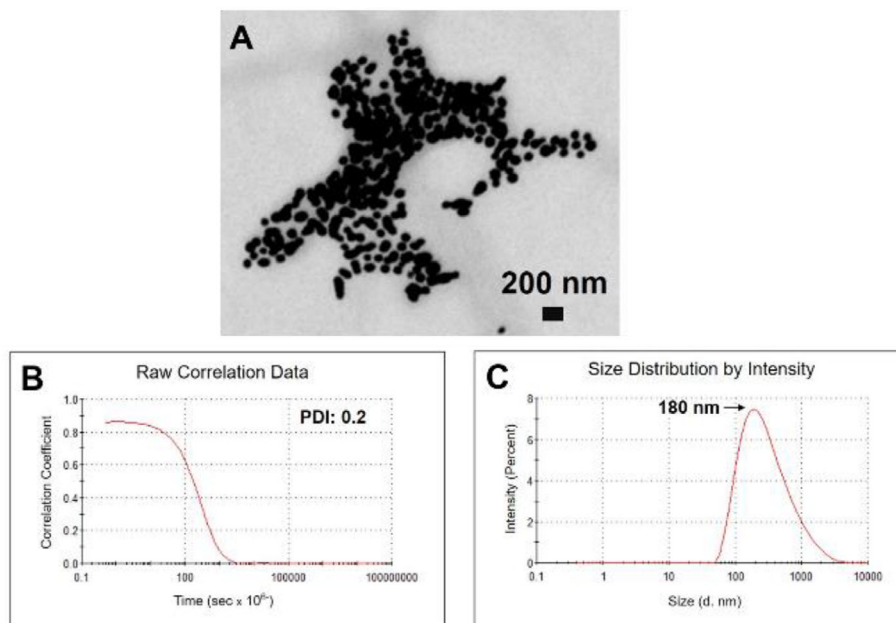
**TABLE 1** Antimicrobial susceptibility testing of strains by commercial disc diffusion method (diameter of the zone of inhibition is in mm). Vn: Vancomycin; St: Streptomycin, Im: Imipenem; Cf: Cefotaxime; Lv: Levofloxacin and Sm: Sulfamethoxazole

Strains	Zone of inhibition (in mm)					
	Vn.	St.	Im.	Cf.	Lv.	Sm.
<i>Staphylococcus aureus</i> (AAK/SA-I)	08	17	23	16	13	21
<i>Staphylococcus aureus</i> (AAK/SA-II)	00	10	30	17	06	28
<i>Staphylococcus aureus</i> (AAK/SA-III)	17	06	18	07	12	07
<i>Escherichia coli</i>	01	11	24	02	09	24
<i>Acinetobacter baumannii</i>	00	14	27	10	26	25
<i>Pseudomonas aeruginosa</i>	02	12	12	00	24	10

30 µg, imipenem 10 µg, cefotaxime 30 µg (cell wall synthesis inhibitors), streptomycin 10 µg (protein synthesis inhibitor), levofloxacin 5 µg (DNA synthesis inhibitors) and sulphamethoxazole 25 µg (folic acid synthesis inhibitor). The retrieved results were interpreted according to CLSI standards (as discussed above).

It was interpreted that the sample AAK/SA-I was resistant to oxacillin (Figure 1(a)); we found no zone of inhibition of this bacterium on the culture plate with antibiotic disc (10 µg). This indicated that it could be an MRSA and hence another confirmatory test was performed. An MH agar plate was split into two portions and one half was swabbed by the test (possibly MRSA) strain and the other half by *S. epidermidis* as a control. Both of the halves were tested with a disc each of methicillin. The *S. epidermidis* was found to be totally susceptible to it while the *S.*

*aureus* was resistant (Figure 1(b)). This proved that the sample AAK/SA-I was an MRSA strain. Moreover the same *S. aureus* strain was also resistant to vancomycin and levofloxacin; cefotaxime had intermediate activity on it while it was sensitive to streptomycin, imipenem and sulphamethoxazole (Figure 1(c)). Sample AAK/SA-II was vancomycin resistant *S. aureus* (VRSA) as it showed resistance to vancomycin, streptomycin, imipenem but has intermediate activity of cefotaxime. It was sensitive to sulphamethoxazole and levofloxacin (see Figure 1(d)). Sample AAK/SA-III was an MDR strain of *S. aureus* as it had been found to be resistant to streptomycin, cefotaxime, sulphamethoxazole and levofloxacin while sensitive to vancomycin and imipenem (see Figure 1(e)). Based on the above measurements and interpretation via CLSI standards, all these strains were found to be multiple drug resistant (Table 1).



**FIGURE 2** Physical characterization of VCO-encapsulated niosomes. (a) Electron microscopy image of niosomes. The VCO-encapsulated niosomes appeared as black spheres on the carbon coated Cu grid. Dynamic light scattering analysis. (b) The raw correlation data showed that it was a monodispersed sample from its signal decay. (c) Dynamic light scattering (DLS) analysis shows the hydrodynamic diameter was 180 nm

### 3.2 | Electron microscopy imaging

We performed transmission electron microscopy for the niosomes encapsulated with VCO. Microscopy analysis showed that the synthesized niosomes spherical shaped niosomes on a carbon coated Cu-grid. The size (diameter) range recorded via TEM fell between 170–180 nm in diameter. The niosomes appeared as dark (black) spherical balls (Figure 2(a)) because of the presence of (organic matter) VCO inside them and also due to the sticking of the (PTA) negative stain to them. This appearance of nanostructured lipid carriers as dark spheres was in coincidence with our published investigations where VCO was entrapped in solid lipid (nano/micro) particles when we exploited them as skin moisturizing agents [20].

### 3.3 | Particle sizing, zeta potential and encapsulation efficiency

The dynamic light scattering results through photon correlation spectroscopy (also known as DLS) proved that the population of synthesized niosomes was fairly monodispersed as seen in Figure 2(b). The raw correlation data showed a smooth signal decay which pointed to the fact that majority of the sample contained uniform distribution in terms of diameter of the nanoparticles. A low PDI (poly dispersity index) value (0.2) bore a testimony to aforementioned statement. The hydrodynamic diameter recorded for VCO filled niosomes was  $180 \pm 3.4$  nm (Figure 2(c)), which was clearly in agreement with electron microscopy data. A slight increase in the diameter was due to the fact that in this technique the machine determined hydrodynamic radius (or diameter) which would always be more than

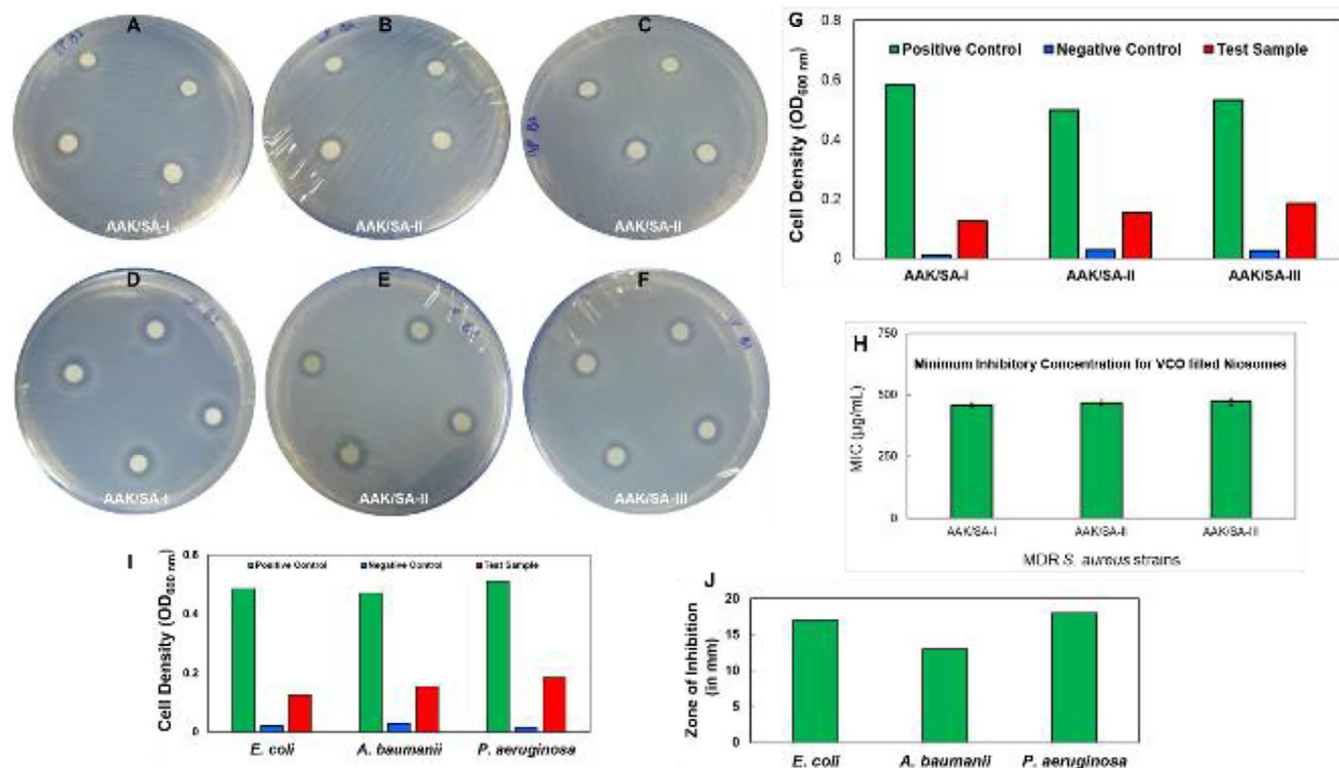
**TABLE 2** Summary of DLS and zeta potential analysis of VCO filled niosomes

Hydrodynamic diameter (via DLS) (nm)	Polydispersity index	Zeta potential (mV)
$80 \pm 3.4$	$0.2 \pm 0.08$	$-42 \pm 1.9$

diameter obtained through electron microscopy since a thin electric dipole layer of the solvent would adhere in the former case while the latter would give an estimation the projected area diameter [21]. Zeta potential values recorded for encapsulated niosomes was around  $-42 (\pm 0.35)$  mV, which indicated that the nanocarrier particles were having a good stability. The filled niosomes were negatively charged and were repelling each other due to electrostatics which made the suspension stable. A low polydispersity index and single (major) peak in the DLS analysis contemplated this too. This is also supported by the TEM images as no aggregation could be seen in these images. The results are summarized in the Table 2.

Since we had already reported entrapment of drugs in niosomes in our previous work [10], therefore we were interested to see the percent entrapment of VCO in our nanocontainers. The linear regression curve was plotted between concentrations on  $x$  axis and absorption on  $y$  axis. The value of free drug was measured as 904.58 mL. The encapsulation efficiency of VCO inside nanovesicles measured as 69% as shown in following calculation:

$$Y = 0.0025x - 0.1963$$



**FIGURE 3** (a–c) The antibacterial activities of a mixture of ZnO nanoparticles on the selected three *S. aureus* strains. The mixture of nanoparticles had zones of inhibition of 9, 7 and 8 mm respectively. (d–f) The antibacterial capacity of VCO filled niosomes that checked the growth of these superbugs with zones of inhibition of 15, 14 and 12 mm. (g) Broth culture inhibition assay showed the inhibition of resistant *S. aureus* cells. Positive control was culture broth, surfactants and bacterial cells while negative control was culture broth only. (h) MIC of VCO filled niosomes for the three *S. aureus* strains. (i,j) Lower cell viabilities and zones of inhibition of non-*Staphylococcal* bacteria

$$x = \frac{Y - 0.1963}{0.0025}$$

$$x = \frac{2.457 - 0.1963}{0.0025} = 904.58$$

$$EE\% = \frac{\text{total drug} - \text{free drug}}{\text{total drug}} \times 100$$

$$EE\% = \frac{3000 - 904.58}{3000} \times 100 = 69\%$$

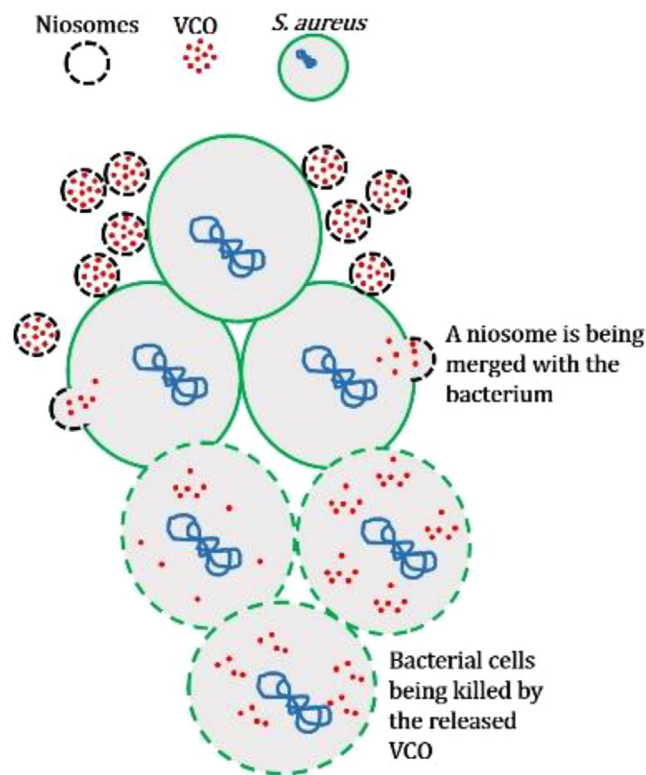
### 3.4 | Antibacterial activity

The antimicrobial potential of VCO-encapsulated niosomes was determined by disc diffusion method. It was rather essential to confirm that neither the synthesized nanostructured carriers nor the chemicals used for their synthesis were the reason of antibacterial activity. To test this we used two controls, i.e. the pellet that represented the empty/non-encapsulated niosomes and the supernatant which are the (unreacted) chemical ingredients used for the synthesis of niosomes. It was observed that neither the pellet nor the supernatant had resulted in retarda-

tion of the growth of the MDR *S. aureus* strains. No zones of inhibition were noticed around them proving their incapability to kill any of the test *S. aureus* cells cultured on the agar plates. It equipped us with the knowledge that neither the synthesized empty niosomes nor the chemicals used for the synthesis had any role in the antimicrobial activity and VCO would be the sole responsible factor for it.

VCO-entrapped niosomes prepared by sonication method [15] (with some modifications) were tested for antibacterial potential(s) in comparison with ZnO nanoparticles. Metallic (and/or metallic oxide) nanoparticles had emerged as the new (and novel) line of treatment (in vitro) against superbugs since so far there is no report of any resistance against them [8, 9, 22, 23]. As antimicrobial agents, nanoparticles present many distinctive advantages in reducing acute toxicity, overcoming resistance, and lowering costs, when compared to conventional antibiotics [24, 25]. The ZnO nanoparticles were previously reported (individually) for antibacterial activity [26–28].

We used a volume of 250  $\mu\text{L}$  ZnO (1 mM) nanoparticles in the disc diffusion assay. Susceptibility assays were run relative to each other on both, i.e. nanoparticles and niosomes encapsulated with VCO against MDR *S. aureus* strains. The disc diffusion antibacterial assay resulted in the fact that niosomes-entrapped VCO were relatively more effective in eradicating MDR bacteria than the nanoparticles. The diameter of zones of inhibition recorded for VCO-encapsulated niosomes for three *S. aureus*



**FIGURE 4** A model showing the mode of action of niosomes-encapsulated VCO to kill bacterial cells

strains were 15, 14 and 12 mm (Figure 3(d–f)) while for the ZnO nanoparticles 9, 7 and 8 mm (Figure 3(a–c)), respectively. Gram positive *S. aureus* strains were susceptible to encapsulated niosomes.

We also used the same nanosystem formulation with encapsulated VCO inside niosomes for broth culture assays. In these assays positive as well as negative controls were also run to confirm that the antibacterial effects are contributed by the VCO only and these results are summarised in Figure 3(g). The MIC calculated for VCO-encapsulated niosomes against the three *S. aureus* strains were  $459 \pm 10.5$ ,  $468 \pm 12.1$  and  $472 \pm 13.5 \mu\text{g mL}^{-1}$  (see Figure 3(h)). It was also important to determine the effect of niosomes filled with VCO could also prove antibacterial against pathogens other than *S. aureus*. We repeated the above mentioned experimental procedures for three more bacterial strains; *Escherichia coli*, *Acinetobacter baumannii* and *Pseudomonas aeruginosa*. The results are given as a bar chart diagram in Figure 3(i,j). It was noticed that VCO-entrapped niosomes could not only prove novel antibacterial agents against *S. aureus* but other gram positive and negative bacteria too as showed by significantly reduced cell viabilities and measured zones of inhibition obtained for non-*Staphylococcal* bacterial pathogens.

It has been postulated that the niosomes carrying VCO inside them were able to fuse with bacterial cell membrane and this fusion led to the release of drug either by micropinocytosis or (a specific type of) endocytosis [29–31]. One of the potential ways for the mode of action of VCO-filled niosomes is shown in Figure 4. The delivery of the (emulsified) VCO acted as a

potent antimicrobial agent. It is already established fact that VCO is rich in triglycerides; it would express antimicrobial activity when triglycerides are broken down into lauric acid and/or monolauric acid by lipase activity which is activated in an oil–water interface [32–34]. The presence of surfactants had aided in the development of the oil–water interface and led to (higher) lipase activity on the agar plate. Ample quantities of the lauric acid and/or monolauric acid were formed [35] and exert their antimicrobial activities and showed strong inhibition on all *S. aureus* MDR strains.

## 4 | CONCLUSION

Niosomes proved to be an excellent nanocarrier system that can encapsulate a number of bio-active compounds. VCO has an added capacity that it can act as a potent antibacterial agent against multiple drug resistant *S. aureus* infections. VCO-entrapped nanocarrier systems can eradicate superbugs synergistically if used in combination with conventional agents and pave the way towards green novel nano-antibiotics.

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