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Enhanced Efficacy Of Zingerone niosomes In Attenuation Of Virulence And Biofilm Formation By *Pseudomonas aeruginosa*: An *In-Vitro* And *Ex-Vivo* Study

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Abstract: Zingerone, a bioactive of *Zingiber officinale*, has enormous pharmacological properties and thus can be used as a promising candidate against various diseases. However, sparingly solubility in aqueous solution limits its potential as a therapeutic agent. To overcome this problem, Zingerone niosomes (Z-Niosomes) were prepared by thin-film hydration method using non-ionic surfactant and cholesterol. Synthesized Z-Niosomes were further characterised. The preparation showed a uniform size of 564.8 nm, PDI of 0.377 with -4.68 mV zeta potential and entrapment efficiency of 52%. The preparation was also found to be stable at different temperatures for 30 days. Z-Niosomes showed biofilm inhibition as well as eradication of the established biofilm, decreased the production of alginate. This was confirmed by field emission scanning electron microscope (FESEM). Various quorum sensing mediated phenotypes like protease, elastase, pyocyanin and hemolysin production were also attenuated on exposure to Z-Niosomes. Besides this, phagocytic uptake and intracellular killing of Z-Niosomes treated *P. aeruginosa* by murine macrophages were found to be more efficient and susceptibility to serum bactericidal killing was also enhanced. The results indicate that Z-Niosomes has the ability to attenuate the virulence of *Pseudomonas aeruginosa* resulting in increased vulnerability of the organism to innate immune responses. Z-Niosomes therefore can be used as anti-virulence agent against *P. aeruginosa* which can further potentiate the activity of zingerone.

Keywords: Niosomes, *Pseudomonas aeruginosa*, Zingerone, Anti-virulence, Biofilm

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1. INTRODUCTION

Multi-drug resistant opportunistic pathogen, *P. aeruginosa* (MDRP) produces surface associated communities called biofilms, which protect the pathogen by forming a complex permeability barrier for antibiotics and immune cells.[1] Biofilms of *P. aeruginosa* account for 57% of all nosocomial infections, particularly in immunocompromised individuals suffering from cystic fibrosis and infected burn wounds.[2,3] Such infections result in persistence of *P. aeruginosa* and are associated with increased mortality and morbidity. Virulence of *P. aeruginosa* including biofilm formation is regulated by quorum sensing (QS), the cell density dependent communication network, comprising of chemical signals, acyl homoserine lactones (AHLs) and *Pseudomonas quinolone* signal PQS), which play an important role in the disease process.[4] The ability of *P. aeruginosa* to form biofilms and tolerate high concentrations of antibiotics has led to the failure of current treatment regimens along with the emergence of multidrug resistance. The side effects or toxicity of antibiotics limit their use as therapeutic agents. Hence, novel and safe agents are required to combat such infections. Therapeutic agents from dietary products are gaining attention as they are confirmed safe and inexpensive and have no issues of drug resistance. Ginger (*Zingiber officinale*) is one of the most commonly used spices worldwide, to treat a number of different infections. More than 60 active components are known to be present in ginger.[5]Among active components, Zingerone, also called Vanillyl acetone, is pharmacologically active component of ginger and is converted from gingerol to zingerone while drying and boiling.[5] It has been reported that zingerone has a wide range of properties like anticancer[6]antioxidant[7] anti-quorum[8] and anti-inflammatory property.[9] Positive effects of zingerone on human health have been confirmed in the literature on the basis of number of studies. Recently, zingerone has been reported to inhibit colonic motility via direct action on smooth muscles *in-vivo*.[10] Besides, zingerone has also been found to have anti-diarrheal activity.[11,12] It is declared as a remedy for gastrointestinal disorders and also helps in improving stress-induced irritable bowel syndrome.[13]All these studies indicate that this natural product can be used as a natural therapy. Despite of these advantages, the use of zingerone is limited due to its sparing solubility in aqueous solution. It is previously reported that high molecular weight proanthocyanidin polymers cause much lower aqueous solubility resulting in low absorption.[14] Zingerone, is a phenolic phytochemical and has high molecular weight. Its low absorption rate and limited bioavailability is linked with much lower aqueous solubility that also explains its low absorption. Potency of any bioactive molecule is dependent upon its solubility, absorption rate, bioavailability and sustained release. A promising approach to address these issues is the use of vesicular nanocarrier such as niosomes. Niosomes, unilamellar or multilamellar vesicles are formed by auto-assembly of non-ionic surfactants in aqueous media. Although niosomes are similar to liposomes in physical properties and biopharmaceutical functions, they possess many advantages including high stability, easy handling, storage, and low cost, which make them a promising alternative.[15,16] Niosomes are biocompatible, biodegradable and non-immunogenic that are capable of delivering both hydrophilic and hydrophobic drugs. Non-ionic surfactants have also been found to improve the solubility of some sparingly soluble bioactive components, enhancing their delivery to localized sites. In the present study, zingerone niosomal formulation was developed and was further evaluated for attenuation of virulence and biofilm inhibition followed by assessment of susceptibility of *P. aeruginosa* to innate immune responses.

2. MATERIALS AND METHODS

2.1 Bacterial Strains

Standard strain of *P. aeruginosa* PAO1(MTCC-3541), was obtained from Dr. Barbara H. Iglewski, Department of Microbiology and Immunology, University of Rochester, New York, USA and stored in 20% glycerol Luria broth at -80°C. *Agrobacterium tumefaciens* A136, was obtained from Dr. J. Handlesman, Wisconsin University USA. Both these strains were employed as biosensor strains.

2.2 Ethical Approval

All animal protocols were approved by the Institutional Animal Ethics Committee at Panjab University, Chandigarh (Animal Ethical approval No. PU/45/99/CPCSEA/IAEC).

2.3 Preparation of Zingerone niosomes

Zingerone niosomes were prepared using thin film-hydration method. Accurately weighed quantities of the surfactant (Span 60) and cholesterol in different molar ratios, viz. 1:0, 1:1 and 1:1.5, were dissolved in 10 mL of chloroform:methanol mixture (2:1, v/v) in a round-bottom flask as at this particular ratio, niosomes were found to be small, circular with well-defined unilamellar vesicles.[17] Different concentrations of Zingerone (5, 10, 20, 30, 40 and 50 mg/mL) were prepared in methanol and then added to the lipid solution (Table.1). The organic solvents was removed under vacuum in a rotary evaporator at 40°C for 30 min to form a thin film on the wall of the flask, and kept under vacuum for 2h to ensure total removal of trace solvents. Hydration of the surfactant film was carried out using 10 mL of PBS (7.2) at 60°C. Resulting niosomal suspensions were sonicated (Branson 5510R- DTH, USA) in 3 cycles of 1 min "on" and 1 min "off" leading to the formation of multi-lamellar niosomes. All the niosomal suspension were left to mature overnight at 4°C and stored at 4°C for further studies.

2.4 Characterization of Zingerone niosomes

2.4.1 Particle size

Mean particle size of each prepared Z-Niosomes was determined by Zeta analyser. Stability of the prepared Z-Niosomes was determined by measuring zeta potential measurements using zetasizer (Zetasizer 2000HS, Malvern Instruments Limited, UK) for 360 seconds at a temperature of 25°C. Selection of the best preparation was made on the basis of vesicle size, PDI and entrapment efficiency.

2.4.2 Field emission scanning electron microscopy (FESEM)

Field emission electron microscope (JSM 6360, UK) was used to study surface morphology of the selected niosomes at voltage of 17 kV. Sample to be examined was placed on the

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FESEM holder and coated with a layer of gold of 150 Å for 2 min under argon with a pressure of 0.3 atm.

2.4.3 Niosomes entrapment efficiency (% EE)

Percentage entrapment efficiency of Zingerone loaded niosomal suspension was determined in triplicate. The niosomal suspension was centrifuged (REMI C-30BL) at 10000 rpm at 4°C for 10 min. Supernatant as well as vesicular sediment was collected after lysing with n-propanol. Appropriate dilutions were made and analyzed in UV spectrophotometer at 254 nm. Percentage EE of drug was calculated using the following equation:

$$\%EE = (T-C) \div T \times 100$$

Where, T is the total amount of drug that is detected both in the supernatant and sediment, and C is the amount of drug detected only in the supernatant.[18]

2.4.4 In vitro drug release studies

The *in vitro* drug release profile of Zingerone loaded niosomes was carried out at two different pH (5 and 7.4) using PBS (10 mM). Known quantity of Zingerone loaded Niosomes (Z-niosomes) were taken in dialysis bags (cellulose membrane, MW cut-off 10,000 Da, sigma-Aldrich chemical Co. Ltd., St Louis, MO). Dialysis bag was immersed in a beaker containing 100 mL of PBS (10 mM) and then incubated in a water bath shaker at 37°C. At definite time intervals, 3mL of sample was withdrawn which was analyzed for the release of Zingerone on a spectrophotometer at a wavelength of 254 nm. Simultaneously the same amount of fresh medium was replaced back.

2.4.5 Stability of niosomes

Physical stability studies were carried out to investigate the leaching of drug from niosomes during storage. Selected zingerone niosomal formulation, was sealed in 20 mL glass vial and stored at different temperatures (-20°C, 0°C, 4°C, 37°C) for a period of 3 months. Samples from each batch were withdrawn at definite time intervals and checked using wavelength scan of Z-Niosomes on UV spectrophotometer (Champion scientific Inc.) to determine λ_{max} and observed the change in terms of aggregation.

2.4.6 Quorum sensing inhibition by Zingerone niosomes

Quorum sensing inhibition (QSI) was checked in terms of colorless zone of inhibition around each well containing Zingerone niosomes .The QSI activity of Zingerone vesicles was assessed by qualitatively using the biosensor strain *Agrobacterium tumefaciens* (A136) which can detect quorum sensing signal molecules. Briefly, 20 µL of Zingerone niosomes was added in the wells on Luria agar plates spread with 0.8% X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside). After 10 minutes, AHL of *P. aeruginosa* along with that of *A. tumefaciens* A136 was swabbed on these plates. Plates were then incubated for 48 h at 28°C.

2.4.7 Estimation of virulence factors in vitro

Cell free supernatant of treated and untreated samples of *P. aeruginosa* was obtained to estimate various virulence factors.

2.4.8 Alginate

Alginate was precipitated out using an equal volume of culture supernatant and 2% (w/v) cetylpyridinium chloride followed by centrifugation at 6,000g for 20 min.[19] Pellet was suspended and precipitated with 5 mL of iso-propanol followed by centrifugation at 6,000g for 10 min. Pellet was suspended in 1 mL of normal saline. Alginate was determined using D-mannourate lactone to calibrate a standard curve.

2.4.9 Pyochelin

Pyochelin was estimated in cell free supernatant by 0.5NHCl, nitrite molybdate and 1N Sodium Hydroxide[20] to 1 mL of treated and untreated culture supernatants. Volume was raised to 5 mL with distilled water followed by taking absorbance at 510 nm.

2.4.10 Cell bound hemolysin

Treated and untreated cells were mixed with 2% sheep RBC suspension followed by incubation at 37°C for 2 h. After incubation, the mixture was centrifuged for 15 min at 8,000 g. Concentration of hemolysin was estimated by taking absorbance of supernatant at 545 nm. Haemolysin concentration was expressed in mg/mL using haemoglobin standard as reference.[20]

2.4.11 Cell free haemolysin

Sheep RBC suspension (2%, 1.5 mL) was mixed with 1.5 mL of cell free supernatant and incubated at 37°C for 2 h. After incubation, the assay mixture was centrifuged at 5,000 g for 5 min and absorbance was taken at 545 nm.[21]

2.5 Biofilm susceptibility assay

2.5.1 Biofilm inhibition of Zingerone niosomes

Biofilms were generated in a 96 well microtiter plate in presence and absence of Z-niosomes for seven days. Briefly, 100 µL of Luria broth (LB) containing Z-niosomes (5 mg/mL) was added into the wells. 20 µL of inoculum (0.4 O.D.) was added to each well. Positive control included only *P. aeruginosa* in LB media and negative control included sterile LB media. The microtiter plate was then incubated at 37°C for 24 h. Planktonic cells were removed and adhered cells were stained with crystal violet followed by removal of excessive dye and washing with PBS. Stained cells were solubilized with ethanol (95%) and absorbance was taken at 595 nm.

2.5.2 Biofilm eradication of Zingerone niosomes

Preformed biofilms, 24 h (young), 4 days (Peak) and 7 days (mature) were allowed to be formed in a 96 well microtiter plate. The preformed biofilms were grown in the presence and absence of zingerone alone and Zingerone niosomes. The microtiter plate was then incubated at 37°C for 24 h. Wells were stained with 0.1% crystal violet and then washed

and solubilized with 95% ethanol and absorbance was taken at 595nm.

2.5.3 Field emission scanning electron microscopy (FE-SEM)

The effect of zingerone alone and Z-Niosomes on biofilm formation was studied, *P. aeruginosa* was grown in presence and absence of zingerone and Z-Niosomes. Bacteria were grown in petri-dish containing sterile glass cover slips and incubated at 37°C for 48h. After incubation, cover slips were washed gently with sterile PBS. Cover slips were gently allowed to fix in a solution of 2.5% glutaraldehyde in 0.1mM PBS overnight at 4°C. The samples were rinsed once in the same buffer and fixed in 1% osmium tetroxide for 1 h at room temperature. Samples were dehydrated using increasing concentrations of ethanol (30%, 50%, 70%, 90%, and 100%) and air dried in a desiccator. Dried samples were fixed on stubs with conductive self-sticking adhesive tapes, coated with gold film. The cells in biofilm attached on the glass surface were visualised under FESEM.

2.5.4 Serum bactericidal assay

For determining the serum sensitivity, 250 µL of *P. aeruginosa,* treated and untreated cells (10^6cells/mL), were incubated with pre warmed human serum (500ul) at 37°C. Samples were withdrawn at 0, 1, 2, 3 h post incubation. Each sample was serially diluted in normal saline and plated on MacConkey's agar plates which were incubated at 37°C and viable bacteria was calculated.

2.6 Phagocytic and Intracellular killing assay

2.6.1 Isolation and culturing of mouse peritoneal macrophages

Mouse peritoneal macrophages were used for the phagocytic assay. BALB/c mice, 6–8 weeks old (20–25 g) were procured from the central animal house of Panjab University, Chandigarh, India. The animal protocols were duly approved by the Institutional Animal Ethics Committee, Panjab University, Chandigarh, India. Five BALB/c mice were injected with 3% thioglycollate broth (HiMedia, Mumbai, India) and mice were euthanized by cervical dislocation after 4 days. After swabbing the abdomen, 5 mL of ice cold (RPMI) (Sigma, St. Louis, MO, USA) supplemented with 10% fetal calf serum (FCS) (Gibco Invitrogen, Paisley, United Kingdom) was injected into the peritoneal cavity. Gentle massaging of peritoneal cavity was carried out to dislodge any attached cells into RPMI. Fluid was aspirated from the cavity using the same syringe. The fluid collected from each individual mouse was pooled, centrifuged and washed thrice using ice cold RPMI. The viability of macrophages was determined by counting cells in a haemocytometer using trypan blue (Sigma, St. Louis, MO, USA). Live, unstained cells were counted in all 4 sets of 16 squares. Viability was determined as number of viable macrophages/mL and the count was adjusted so as to achieve concentration of $1x10^5$ macrophages/well.

2.6.2 Phagocytic uptake of P. aeruginosa

Peritoneal mouse macrophages ($1x10^5$/well) suspended in RPMI supplemented with FCS and streptomycin (25 µg/mL) were seeded in 12-well plate. For determining bacterial uptake, bacterial suspension (10^6bacteria/well), treated with Z-niosomes was mixed with macrophages (10^5cells/well). Plate was then incubated overnight at 37°C in 5% CO_2 following withdrawal of 250 µL sample at different time intervals and an equal volume of ice-cold RPMI was added. Samples were then centrifuged at 1500g (Sigma Centrifuge) for 5 min. The supernatant was collected and pellets washed twice with 2 mL of ice-cold RPMI. The pellet was resuspended in 20 µL of ice cold RPMI having 0.5% Triton X 100 and incubated at room temperature for 30 min. Number of viable intracellular bacteria and extracellular bacteria was determined in the treated pellet and supernatant respectively, by plating the dilution on MacConkey agar plates.

2.6.3 Intracellular killing activity of P. aeruginosa

Peritoneal mouse macrophages supplemented with 5% foetal calf serum (FCS) were distributed in 12-well plates (10^5cells/well). For determining the intracellular killing, bacterial suspension (10^5 bacteria/well) treated with Z-Niosomes was then incubated for 20 minutes. Free bacteria were removed by centrifugation at 1800g for 5 min. Pellet was resuspended in 2 mL of RPMI-1640 containing 5% fetal calf serum (FCS). This was followed by addition of gentamicin (25 µg/well) for 1 h to kill extracellular bacteria. Cells were lysed at 0, 30, 60 and 90 min post incubation by adding Tween 20 (final concentration, 0.03%) to recover intracellular bacteria and each lysate was serially diluted in saline and plated to find the number of viable bacteria. The cells were also stained with BacLight Live/Dead fluorescent stain (Molecular Probes, Eugene, OR, USA) and observed under fluorescent microscope.

3. STATISTICAL ANALYSIS

All the experiments were performed in triplicate and repeated on different days. The treated and untreated groups were compared by using student-t test. *p* values were calculated and $p<0.05$ was considered significant.

4. RESULTS AND DISCUSSION

4.1 Preparation and optimization of Zingerone niosomes (Z-Niosomes)

Zingerone niosomal vesicles were prepared and assessed by varying the non-ionic surfactant (span 60) and cholesterol molar ratio from 1:1 to 3:1 by keeping cholesterol as constant. The optimization of all the prepared formulations was done on the basis of variable particle size, PDI, zeta potential and entrapment efficiency. The particle size of the niosomes was found to increase with increase in the surfactant concentration (Table.1).Due to the excess of surfactant concentration in niosomes leakage of zingerone was observed from vesicles leading to lower entrapment efficiency. Also the hydrophilic-lipophilic balance (HLB) value of a surfactant plays an important role in the drug encapsulation and formation of vesicle previously. Span60, (non-ionic surfactant) with an HLB value within the range (4.7) has been found to be appropriate for creating niosomal vesicles.[22] The maximum entrapment efficiency (52%) of Z-Niosomes was observed with span 60/cholesterol molar ratios 1:1. Mechanism for enhanced drug loading in niosomal formulations may be linked to hydrogen bonding, as well as kind of surfactant with bigger hydrophilic head groups. The presence of cholesterol is one of the common additives in

the preparation of stable niosomes. It makes the membrane more arranged and has the capacity for effective prevention of drug leakage from niosomes.[23]

Table.1: Characterization of Zingerone niosomes in terms of Zeta potential, Particle diameter (nm), Encapsulation efficiency and Polydispersity index (PI).

Formulation	Surfactant/ Cholesterol Molar ratios	Average particle size (nm)	Polydispersity index (PDI)	Zeta potential (mV)	Encapsulation efficiency (%)
F1	1:1	564.8± 32.56	0.377± 0.021	-4.68 ± 0.024	52.56 ± 3.39
F2	1.5:1	815 ± 40.34	0.74 ± 0.05	-2.85 ± 0.034	22 ± 2.3
F3	2:1	1847 ± 67.45	0.48 ± 0.034	-1.89 ± 0.013	43.45 ± 3.1
F4	2.5:1	823 ± 34.98	0.142 ± 0.013	-2.46 ± 0.012	59.60 ± 4.3
F5	3:1	1050 ± 56.22	0.69 ± 0.078	-0.635 ± 0.045	46 ± 3.8

p< 0.001 *n=3*

The size of the selected niosomal formulation (F1) was 564.8 ±32.56 indicating favourable interaction between drug and niosomal matrix. This might be the result of increased cohesion among the polar portions of the bilayer, as suggested earlier.[24,25] The PDI value was found to be between 0.1-0.5, indicating the size heterogeneity. Polydispersity index of formulation F1 was 0.377 and zeta potential -4.68 mV (Table.1). Zeta-potential of all the formulations was found to be negative. This might be due to the presence of free carbonyl groups in the cholesterol and surfactant molecules. The Zeta potential value also suggested the stability of the niosomes. The results revealed that formulation F1 was the best optimized formulation containing Span 60/ Cholesterol (50:50) and zingerone 5 mg/mL. It was therefore chosen for further studies.

4.2 Shape and surface morphology of Z-Niosomes

FE-SEM is usually used to determine the shape and lamellarity of niosomes (Fig.1). These results are in agreement with an earlier reports that field emission scanning electron microscopy (FE-SEM) showed well defined boundaries of zingerone based niosomes with smooth surface and spherical morphology.[26]

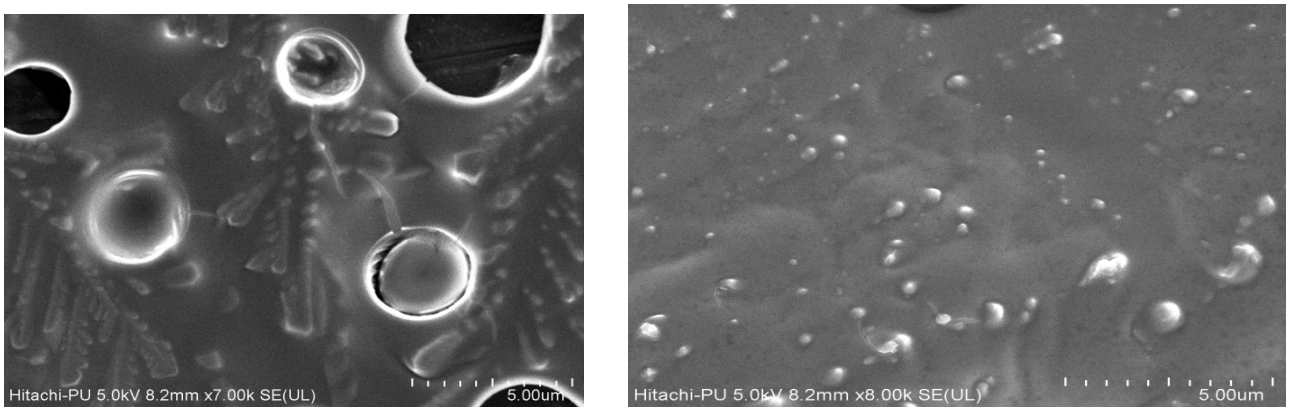

Fig 1. Field emission scanning electron microscopy of Zingerone niosomes formulation (F1) at 7000X

4.3 In vitro release and physical stability

Drug release rate is an important parameter in the development of drug delivery system. Biphasic release of zingerone, including primary rapid drug release followed by slow release was observed. *In vitro* release study of Z-Niosomes was performed at pH 5 (acetate buffer) and pH 7.4 (PBS) by the dialysis method. At pH 5 and pH 7.4, 70% and 60% release of zingerone was observed respectively. Rate of release of zingerone was slow at alkaline pH as compared to acidic pH. An initial burst release was observed up to 4 h and after 6 h, the release became slower. At the end of 9 h, 90% zingerone release was observed at pH 5 (acidic) as well as at pH 7.4 (neutral pH),confirming that niosomes having the ability for sustained release of zingerone(Fig.2).This unusual release behaviour may be related to the specific structure of zingerone and lipid bilayers interactions. The zingerone entrapment can help in sustained release to better absorption. This can prove beneficial during diseased condition where due to inflammation acidic conditions may prevail inside the tissue. Niosomes are found to be stable in solubilizing bile salt solutions and could effectively prolong the release of insulin in both simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) in a previous study.[27]The biphasic kinetic release of insulin showing fast release initially followed by an equilibrium state or a slower release by these workers is also in agreement with the result of this study. No change in absorbance and physical appearance of the formulation was observed at different temperatures until a period of one month (Data not shown). This may be attributed to the presence of cholesterol in the Zingerone niosomes which is known to increase the rigidity of niosomes making them less leaky.[28] The ability of niosomes to act as a local depot for the sustained release or permeation of compounds is well known.[29] These can interact with bioactive molecules through hydrogen bonds and hydrophobic interaction and can

encapsulate bioactives hence enhancing their aqueous solubility.[16]

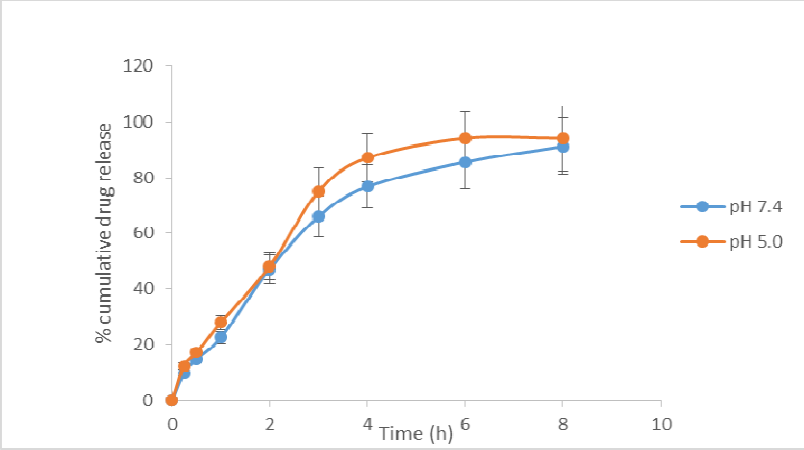

Fig 2. *In-vitro* release profile of Z-Niosomes at neutral pH (7.4) and acidic pH (5.0) conditions at 37°C.

4.4 *Z-Niosomes showed increased anti-quorum sensing and anti-virulent activity*

Bacteria use a cascade of quorum sensing to modulate virulence potential and biofilm formation, therefore anti-quorum sensing activity of Z-Niosomes was assessed by using the biosensor strain *Agrobacterium tumefaciens*A136, which is well known for the production of β-galactosidase in the presence of external AHL. (Fig.3). In the presence of exogenous AHLs, *lacI*:Z gene gets activated and transcripts the biosensor gene for the production of blue coloration by the activation of β- galactosidase[33]. Absence of colorless zone indicating inhibition was maximum in Z-Niosomes as compared to zingerone alone, remained almost same irrespective of temperature settings confirming no effect of temperature on QSI activity. Result of the present study clearly indicated the QSI potential of Z-Niosomes in comparison to zingerone alone. Since, QSSMs are involved in regulating virulence factors of *P. aeruginosa*, further studies were carried out to assess the anti-virulent activity of Z-Niosomes. The effect of Z-Niosomes on QS-controlled virulence factors of test pathogen *P. aeruginosa* was analysed. Reduced level of Pyocyanin, alginate, elastase, protease and hemolysin in presence of Z-Niosomes were indicative of attenuation of virulence. From these results it can be inferred that virulence factors required for the progression of *P. aeruginosa* mediated pathogenesis were reduced in presence of Z-Niosomes significantly (Table. 2).

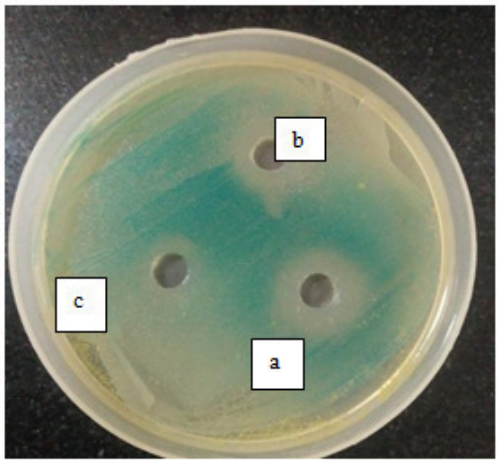

Fig.3 Quorum sensing inhibitorybioactivity of (a) Z-Niosomes, (b) zingerone alone and (c) blank niosomes as control using biosensor strain *Agrobacterium tumefaciens* A136.

Table 2. Estimation of virulence factors produced by *P. aeruginosa* grown in absence and presence of zingerone alone and Z-Niosomes.

Samples		Protease (UL^{-1})	Elastase (UL^{-1})	Pyocyanin (OD at 510nm)	Cell bound hemolysin (mg mL^{-1})	Cell free hemolysin (mg mL^{-1})	Alginate (ug mL^{-1})
Control	PAO1	329 ± 8.544	349 ± 4.00	5.35 ± 0.15	0.378 ± 0.002	0.431 ± 0.012	0.376 ± 0.012
Test	Zingerone	178 ± 2.00 **	175.6±3.512**	1.33±0.036**	0.330±0.002*	0.257±0.034*	0.189±0.006**
Test	Z-Nio	107.3 ± 2.517**	116±4.00**	0.84±0.037**	0.237±0.006**	0.185±0.004**	0.135±0.003**

Result expressed as mean of two independent experiments along with standard deviations. **and represents statistical significant values p<0.01 and p<0.001 respectively.***

4.5 Z-Niosomes showed increased anti-biofilm efficacy

4.5.1 Biofilm inhibition

Biofilm formation is most notable aspect of *P. Aeruginosa* mediated pathogenicity. These structures are able to resist high concentration of antibiotics and help evade the immune system.[30] Therefore, our next aim was to investigate inhibition of biofilms of *P. aeruginosa* by Z-Niosomes. Crystal violet staining method has been widely used because of its reproducibility. Blank Niosomes did not show any anti-biofilm effect whereas Z-Niosomes showed significant inhibition of biofilm formation by *P. aeruginosa* PAO1 as compared to control ($p<0.001$) (Fig.4). Reduced alginate production was also observed which was supportive of antibiofilm activity of Z-Niosomes. Further analysis of biofilm by FESEM also clearly revealed the similar effect. Control PAO1 formed thick biofilm where cells were seen entangled in dense exopolysaccharides (EPS) (Fig.5a).Intact cells were seen in biofilm treated with blank niosomes till 48 h (Fig.5b). Biofilm treated with Zingerone niosomes showed disintegration of EPS and cell membrane damage of bacterial cells as compared to untreated control (Fig.5c).

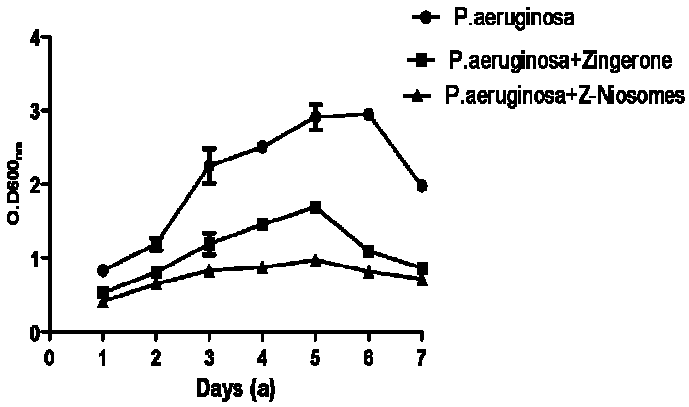

Fig 4 Inhibition of biofilm formation by *P. aeruginosa* in the presence of zingerone alone and Z-Niosomes by crystal violet assay.

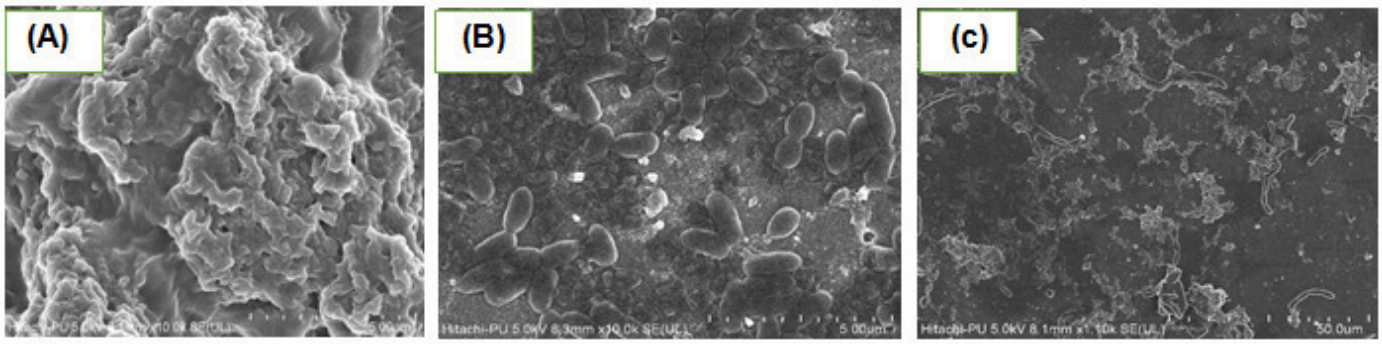

Fig 5. FESEM images showing (a) control untreated *P. aeruginosa* biofilm, (b) blank CSDS niosomes treated PAO1 biofilms (c) Zingerone niosomes (Z-Niosomes) treated 48 h *P. aeruginosa* biofilms.

4.5.2 Biofilm eradication

The age of biofilms is also an important factor in determining the outcome of a therapeutic regimen.[31]Older biofilms are known to resist higher concentrations of drugs than younger biofilms.[32] In the present study the preformed biofilms, young (1 day), old (4 days) and mature (7 days) biofilms were treated with Z-Niosomes and blank Niosomes (Fig.6). Z-Niosomes were able to reduce ($p<0.001$) 1 day old, 4 days old and 7 days old established biofilms significantly as compared to control group. On the other hand, blank Niosomes did not show any effect on pre-formed biofilms. Results of the present study indicate that niosomes can further complement zingerone in its action against biofilms. Preformed biofilms were targeted well due to specific attachment of Z-Niosomes which may allow the zingerone to be released in the vicinity of the microbe. Previously, biofilms of *P. aeruginosa* have been shown to be effectively inhibited by various other phytochemicals as well, like cranberry,[33] ajoene.[34]

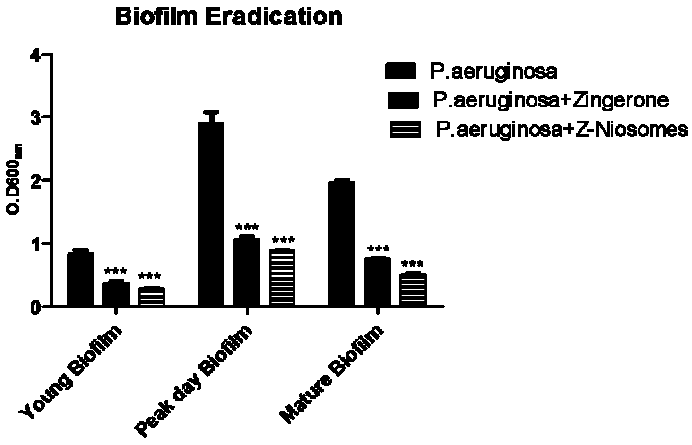

Fig 6. Eradication of preformed biofilm of *P. aeruginosa* after treatment with zingerone alone and Z-Niosomes.

4.5.3 Z-Niosomes showed increased serum sensitivity

Serum bactericidal activity has been considered as one of the most important host defence mechanisms against bacterial infections. Results of the present study indicate that the ability of phytochemicals to inhibit the production of virulence factors along with their anti-biofilm activity, can also sensitize the pathogen and can make them susceptible to the innate host immune responses.[35]The susceptibility of *P. aeruginosa* treated with Z-Niosomes was enhanced. It was observed that zingerone alone could also enhance the susceptibility of *P. aeruginosa* to serum by 85% in 3 h ($p<0.001$), as compared to the untreated control (30%) (Fig.6a). However, Z-Niosomes further increased the sensitivity of *P. aeruginosa* to serum to the maximum of (90%) in 3h ($p<0.001$). Changes in some cell surface alterations, surface hydrophobicity can lead to enhancement of serum sensitivity.[36]It is reported that some antibiotics like erythromycin at Sub-MIC concentrations can also enhance the serum sensitivity of *P. aeruginosa* strains.[37]

4.5.4 Z-Niosomes showed increased phagocytosis of P. aeruginosa

Macrophages as well as serum play a key role in the innate immune system by recognizing and removing bacteria. The process of phagocytosis takes place in two steps, uptake and killing. Increased susceptibility of *P. aeruginosa* to the action of phagocytic uptake and intracellular killing was observed when *P. aeruginosa* was grown in the absence and presence of zingerone alone and Z-Niosomes[34]. A significant increase in phagocytic uptake and intracellular killing was observed in presence of zingerone alone ($p < 0.05$) within half an hour. However, uptake and killing of *P. aeruginosa* in presence of Z-Niosomes was further enhanced significantly ($p < 0.01$) as compared to zingerone alone at 60 min and 90 min (Fig.7b,c). Alemi and workers[38] studied the cellular uptake behaviour of curcumin/paclitaxel cationic PEGylated niosomal formulations in cancer cells and normal human mammary epithelial cells monitored by fluorescence microscopy. It was observed that curcumin/paclitaxel loaded niosome formulations could enter healthy as well as cancerous cells. Fluorescence microscopy using Live/Dead staining kit also confirmed the difference in the killing activity of Z-Niosomes (Fig.8a,b) Both green (live cells) and red fluorescing (dead cells) intracellular bacteria were observed inside the macrophages post 90 minutes. Since, internalization of bacteria inside macrophages is preceded by interaction and attachment of bacteria to macrophages, Z-Niosomes treated *P. aeruginosa* must be attaching tightly to macrophages before being taken inside. Tight attachment of nano-niosomes to macrophage cell membrane was observed.[39]This attachment could be retained even after extensive washing with PBS. These workers also observed efficient phagocytosis of niosomes with a diameter of about 300-600nm. The uptake of niosomes was time dependent process. Uptake and phagocytosis of the liposomal preparation by phagocytic cells has also been observed by various other workers.[40]

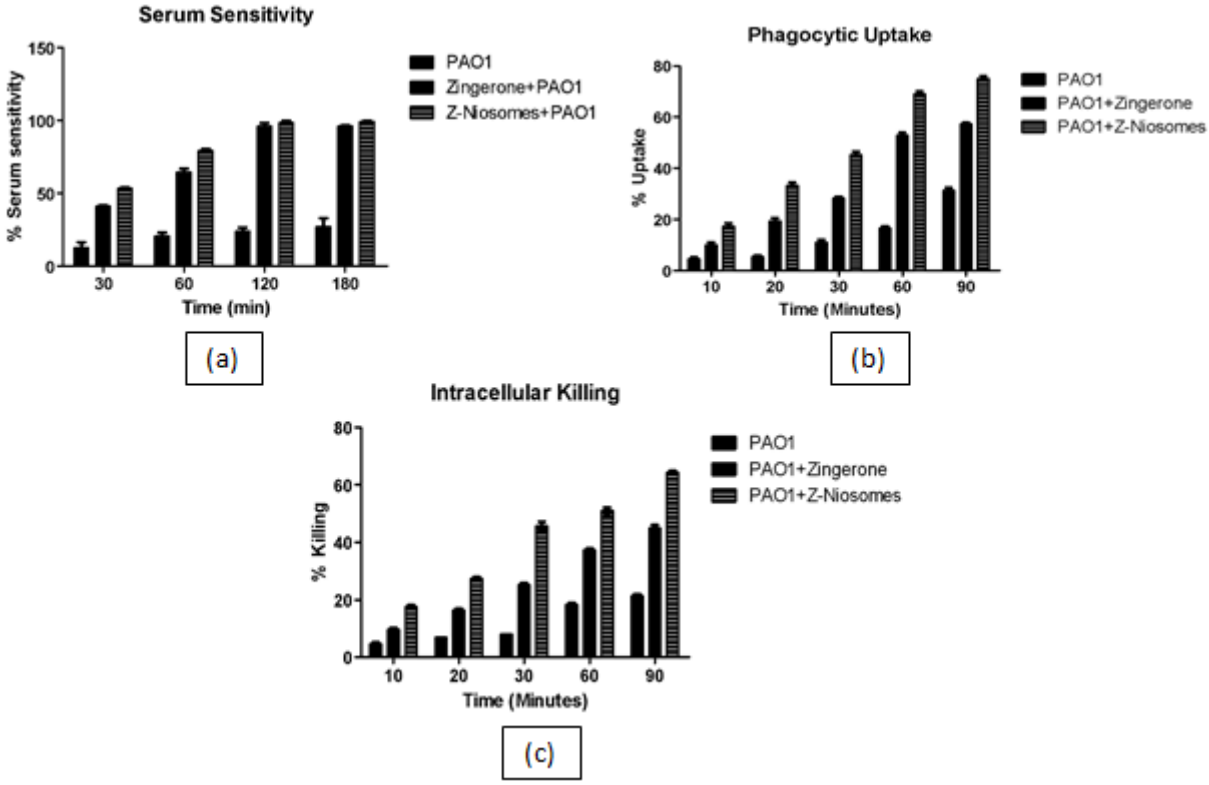

Fig 7. Influence of zingerone and Z-Niosomes on (a) serum sensitivity (b) phagocytic uptake (c) and intracellular killing against *P. aeruginosa* PAO1.

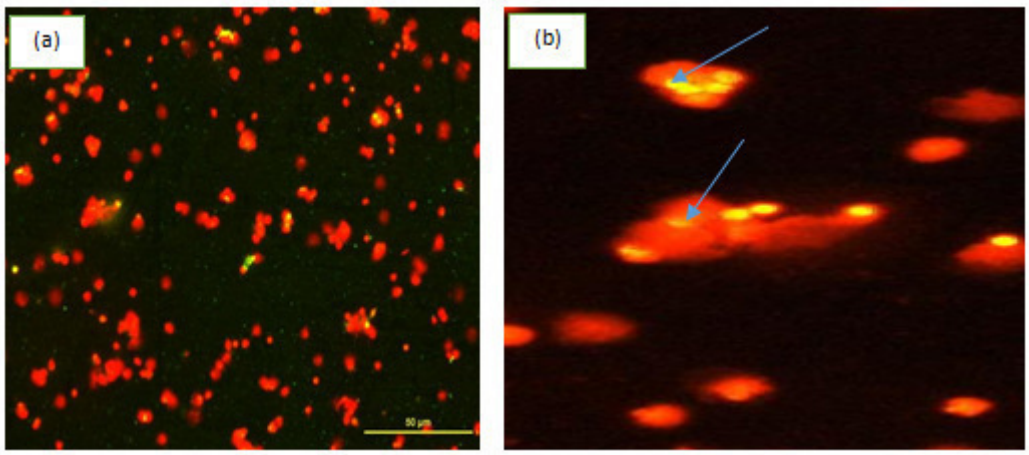

Fig 8. Samples were stained with syto9/PI dyes for visualization of live (green) and dead bacteria (red) present within the macrophages (seen as red stained) a) clumps of engulfed bacteria containing mixture of live and dead bacteria as seen in Zingerone niosomes treated sample b) *P. aeruginosa* after phagocytic uptake by macrophages post 90 minutes (Blue arrows indicate engulfed bacteria).

5. CONCLUSION

Zingerone loaded niosomes (Z-Niosomes) were synthesized by thin film hydration method and characterized for size, PDI, zeta potential and percentage entrapment efficiency. The prepared niosomal formulation exhibited spherical morphology, physical stability and sustained *in vitro* drug release profile. The present study reveals that encapsulation of the active constituent Zingerone inside the vesicles enhanced its anti-quorum sensing, anti-virulence and anti-biofilm activity. Also, Z-Niosomes accentuated intracellular uptake and killing by macrophages and increased serum sensitivity of *P. aeruginosa.* This study provides *in vitro* experimental evidence on the attenuation of virulence of *P. aeruginosa* including biofilm reduction and increased susceptibility to innate immune response.

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7. AUTHORS CONTRIBUTION STATEMENT

Ms. Karuna Sharma conceptualized and gathered the data with regard to this work. Prof. Kusum Harjai and Prof. Sanjay

Chhibber analysed the data and gave necessary inputs towards the designing of the study and manuscript. All authors discussed the methodology and results and contributed to the final manuscript.

8. CONFLICT OF INTEREST

Conflict of Interest declared none.

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