Synthesis and characterization of a nano-formulation for long lasting sterilization effect

Ria Ghosh [a,b,1], Soumendra Singh [a,1], Aniruddha Adhikari [c], Susmita Mondal [c], Dipanjan Mukherjee [c], Neha Bhattacharyya [a], Animesh Halder [a,d], Maitree Bhattacharyya [b,e], Samir Kumar Pal [a,c,*]

[a] Technical Research Centre, S. N. Bose National Centre for Basic Sciences, Block JD, Sector 3, Salt Lake, Kolkata 700106, India
[b] Department of Biochemistry, University of Calcutta, 35 Ballygunge Circular Road, Ballygunge, Kolkata 700019, India
[c] Department of Chemical, Biological and Macromolecular Sciences, S. N. Bose National Centre for Basic Sciences, Block JD, Sector 3, Salt Lake, Kolkata 700106, India
[d] Department of Applied Optics & Photonics, University of Calcutta, Block JD, Sector 3, Salt Lake, Kolkata 700106, India
[e] Jagadis Bose National Science Talent Search, 1300, Rajdanga Main Road, Kasba, Kolkata 700107, India

ARTICLE INFO

Article history:
Available online xxxx

Keywords:
Nano-sanitiser
Antimicrobial
COVID-19
Ozonated omega-9
Vesicle

ABSTRACT

The current COVID-19 pandemic has increased the use of alcohol based hand sanitisers globally. These available alcohol based sanitisers cannot provide an antibacterial effect for an extended period of time, after the evaporation of ethanol. Hence, the need for a sanitiser with an anti-microbial activity combined with a long lasting effect is the need of the hour. In this study, we report the synthesis of a long lasting sanitiser from ozonated omega 9 fatty acid esters in an ethanolic medium. The formed vesicles made of the fatty acids have been characterized by DLS, Zeta potential, and time resolved fluorescence anisotropy studies. Ethanol although, provides an antibacterial effect, the effect is more pronounced in our prepared formulation owing to its high peroxide value that generates additional oxidative stress. Finally, this additional antimicrobial effect will have relevance in the current COVID-19 scenario in providing a long lasting hand sanitiser.

© 2021 Elsevier Ltd. All rights reserved.
Selection and peer-review under responsibility of the scientific committee of the International Conference on Nanoelectronics, Nanophotonics, Nanomaterials, Nanobioscience & Nanotechnology.

1. Introduction

The current pandemic due to coronavirus (COVID-19) has caused a worldwide emergency by spreading rapidly and causing a high mortality rate [1]. Till date, the medication or vaccine to cope against the novel coronavirus is still under research. This disease is mainly characterized by fever and dry cough accompanied with respiratory and multi organ failure [2–4]. The mortality rate is particularly higher among the older group of people and those with underlying conditions owing to their immunosuppressive state. The rapid spread of SARS-CoV-2 virus among humans is through human to human transmission [5]. In the current scenario, the only preventive measures against COVID-19 is by maintaining a healthy lifestyle to have an efficient immune system [6]. In this regard,

adapting to have an effective hand hygiene is essential to decrease the rate of transmission of the virus [7]. Hence, WHO recommends to maintain hand hygiene by frequently washing and sanitizing your hands with soap or with an alcohol based hand sanitiser [8].

The alcohol based sanitisers, although effective in disinfection has widespread drawbacks. Direct contact of ethanol with the dermis can cause irritation and allergic reaction of the skin [9]. Along with this, exposure to ethanol for prolonged hours causes redness accompanied with itching and dryness or cracking of skin [9,10]. There has been several studies reporting that the regular use of ethanol has the potential to cause skin irritation or contact dermatitis, making it more prone to infections [10,11]. These secondary or hospital acquired infections are elevated among immune suppressive patients that predisposes them to infection more than the general population. Thus, a solution to these problems in such uncertain times is indispensable, which will provide an anti-microbial effect for an extended time and also prevent the cracking and peeling of skin.

In this present work, the main objective was to synthesise ethanol containing vesicular carriers composed of fatty acids for topical

---

R. Ghosh, S. Singh, A. Adhikari et al.

delivery of ethanol for the purpose of long lasting sanitation. The choice of the ethanol-containing vesicular carrier as a novel nano formulation lies in the fact that the ethanol content would evaporate leaving behind the oil emulsion which also imparts an additional antimicrobial effect. Ethanol containing vesicles composed of ozonized Omega 9 fatty acids has been formulated in unvaried (80%) ethanol concentration. Dynamic light scattering and spectroscopic studies using the auto fluorescence of Omega 9 has been used to characterize the vesicles formed from ozonized Omega 9 fatty acids, in the ethanol–water environment. Microbial studies has been used to determine the efficacy of the formulation in a long lasting sanitisation.

2. Materials and methods

2.1. Reagents

Potassium iodide, starch, sodium thiosulphate ($Na_2S_2O_3$), acetic acid (99.5%), chloroform were obtained from Sigma Aldrich (St Louis, MO, USA). DAPI, Propidium Iodide and Luria Bertini Agar was obtained from Himedia (Mumbai, India). Cold press olive oil was purchased from Solimo (Italy) as a source of Omega 9 and ethanol from Sigma Aldrich. Food grade H_2O_2 (3%) was obtained from Durox LR3. The chemicals were used without further purification. We used Millipore water whenever required.

2.2. Ozonation of omega 9

Air was passed at a constant rate through the generation of non-thermal atmospheric plasma (NTAP) for the production of ozone. The voltage was set to 15 kV using a 15 kV Neon Sign Transformer from Canon for all the experiments. The generated ozone/air mixture was bubbled in a jar containing Omega 9 fatty acids. The amount of ozonation of the Omega 9 fatty acids was determined by spectroscopic methods. The olive oil sample was ozonated in batches of 200 ml for 24 h, after which the respective batches were analysed and used for further study.

2.3. Preparation of sanitizer (formulation) (Patent Ref No. 202031026596)

Firstly, 1.45% of ozonated Omega 9 was dissolved in 80% ethanol–water mixture. The solution was stirred at 1000 rpm for 20 min till a turbid solution was formed. 3% food grade H_2O_2 was added to the solution in stirring condition. The entire solution was stirred at 1000 rpm for 10 min and allowed to stand overnight before use.

2.4. Determination of peroxide value

Peroxide value is the quantity (expressed in miliequivalents of active oxygen) of peroxide contained in 1000 g of the substance [12]. The peroxide value was determined according to previous literature [12]. The chemical reactions are as follows:

$$2KI + 2CH_3COOH \rightarrow 2HI + 2CH_3COO - K^+$$

$$R\bullet OO\bullet H + 2HI \rightarrow ROH + H_2O + I_2$$
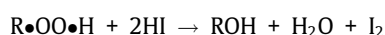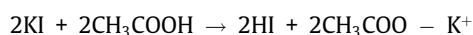
Peroxides (R•OO•H) present in the sample reacts with potassium iodide in the presence of acetic acid. The iodine liberated by the reaction is titrated with a known concentration of $Na_2S_2O_3$ solution. The peroxide value is calculated employing the formula:

$$PV \ (mEq/ml) = \frac{(V_1-V_2) * c * T * 1000}{m} \tag{1}$$

where PV is the peroxide value (mEq/ml of fat), V_1 and V_2 is the amount of $Na_2S_2O_3$ consumed in the test and in the blank sample, c is the molarity of the $Na_2S_2O_3$ solution, T is the titer of the $Na_2S_2O_3$ solution and m is the amount of substance taken in ml.

2.5. Bacterial cell culture

Stock *P. aeruginosa* strains were procured from Dey's Medical Stores Mfg. Ltd., Kolkata, India. All the glass wares and the culture media were sterilized in an autoclave at 0.1 MPa pressure at a temperature of 120 °C for 30 min before all the experiments. The bacteria was cultured in Luria Bertini (LB) media and incubated at 37 °C for 24 h. Cultures of *P. aeruginosa* were grown in duplicates on a non-selective medium of LB agar. The bacterial suspensions were prepared in LB and all the experiments were performed in freshly overnight grown cultures.

The freshly overnight grown bacterial culture was divided into three groups (2 ml in each group) One was incubated with our formulation for 30 min for the interaction with bacterial cells. Other groups included control (without any treatment), positive control (with 80% ethanol). All the samples were incubated at 37 °C with shaking and absorbance was recorded at an interval of 1 h to obtain the bacterial growth curve. For colony forming unit (CFU) assays, the bacterial culture was incubated with the prepared formulation for 30 min and subsequently diluted and spread over Luria-Bertani agar plates. All the plates were incubated for 24 h at 37℃ before the number of viable colonies were assessed. To assess the survivality ratio of *P. aeruginosa* the bacterial suspension was stained with DAPI and PI. DAPI stains all the bacterial cells while PI only
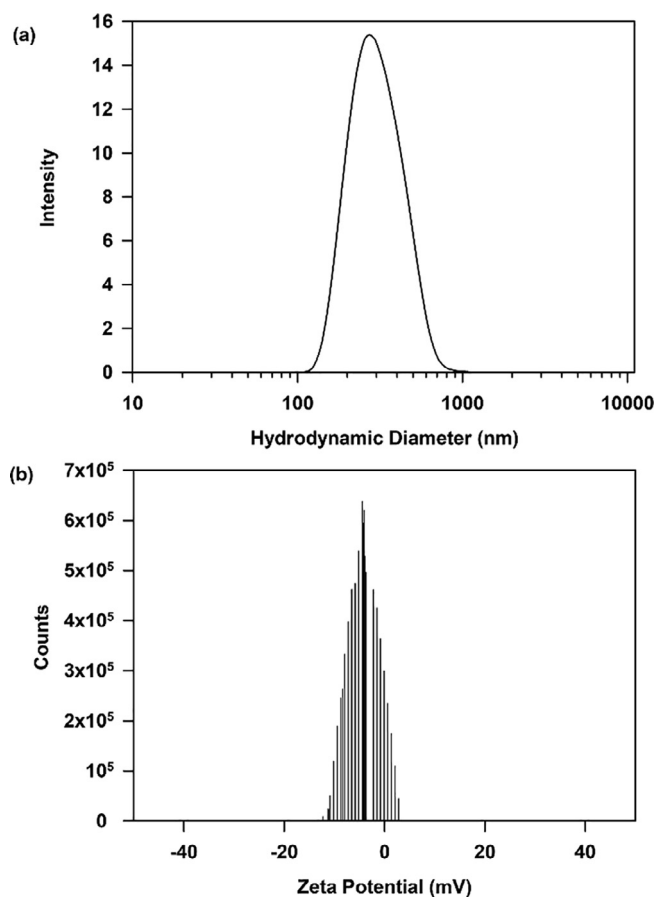

Fig. 1. Structural characterization of omega 9 vesicles in ethanol–water medium. (a) DLS of the prepared vesicles containing ethanol (b) Zeta potential of the vesicles in ethanol medium.

stains the dead cells. The Red/Blue ratio was obtained to assess the survivality of *P. aeruginosa*.

The well diffusion test was performed for screening of susceptibility of *P. aeruginosa* to the developed formulation. Bacterial suspensions adjusted to 1×10^9 CFU/ml were streaked on LB agar plates. Well (9 mm diameter) was impregnated on the inoculated agar surfaces. 100 µl of the stock solutions (Formulation and 80% ethanol) was used for the assay. Tetracyclin (40 µg/ml) was used as a positive control. The plates were incubated at 37 °C for 24 h before analysis. The anti-bacterial activity of the samples was indicated as the mean of the inhibition diameters (mm) produced by the samples.

2.6. Characterization and techniques

Dynamic light scattering (DLS) and Zeta Potential measurements were performed using a Nano S Malvern instrument equipped with a 4 mW He: Ne laser (l = 632.8 nm). The Fluorescence emission and excitation spectra were measured using spectrofluorometer, Model FP-8200 (Jasco, Tokyo, Japan). Picosecond-resolved fluorescence transients were measured by using a commercially available spectrophotometer (Life Spec-ps) from Edinburgh Instruments, UK as reported in our earlier publications [13–16]. For fluorescence anisotropy measurements the emission polarizer was adjusted in parallel (I_{VV}) and perpendicular position (I_{VH}) with respect to the excitation polarizer and the corresponding fluorescent transients were recorded Time-resolved fluorescence anisotropy was calculated as

$$r(t) = \frac{(I_{VV} - GI_{VH})}{(I_{VV} + 2GI_{VH})} \tag{2}$$

The magnitude of the grating factor (G) of the emission monochromator of the TCSPC system, was found using a long tail matching technique.

2.7. Statistical analysis

To show the quantitative data mean ± standard deviation was used, unless otherwise stated. The different parameters between the groups were compared using the computer program GraphPad Prism (version 5.00 for widows), GraphPad software, California, USA. The level of significance was considered using the p value with $p < 0.05$.

3. Results and discussion

Fig. 1 shows the hydrodynamic diameter of the prepared vesicles to be around ∼ 250 nm. The zeta potential (ξ) measurements revealed the surface charge of the vesicles to be anionic with
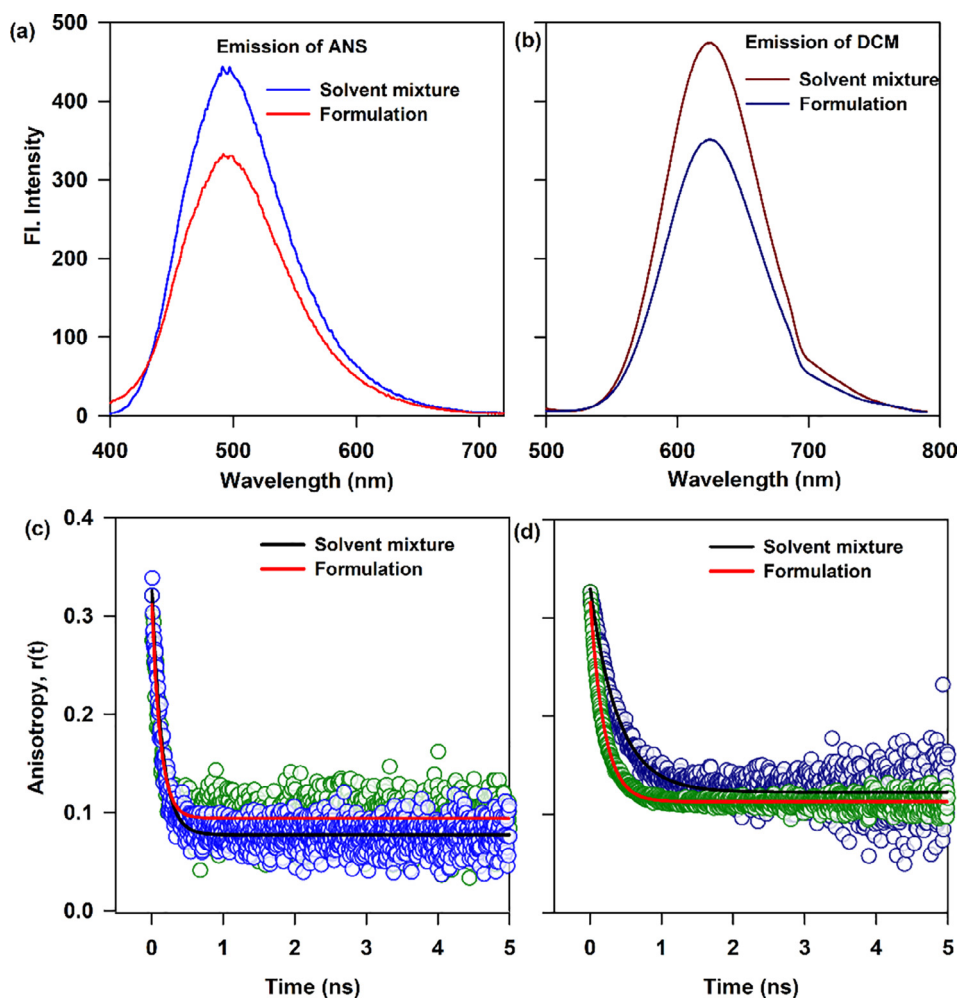
Fig. 2. Probing of omega 9 vesicles with ANS and DCM. (a) Steady-state fluorescence spectrum of ANS in presence (formulation) and absence of vesicles (solvent mixture). (b) Steady-state fluorescence spectrum of DCM in presence (formulation) and absence of vesicles (solvent mixture). (c) Fluorescence anisotropy of ANS in presence (formulation) and absence of vesicles (solvent mixture). (d) Fluorescence anisotropy of DCM in presence (formulation) and absence of vesicles (solvent mixture).

Table 1
Time-resolved decay parameters of ANS and DCM at emission peak maximum.

System	r_0	$\tau 1$ (%)	$\tau 2$ (%)
ANS in absence of vesicles	0.32	0.13 ns (89)	50 ns (11)
ANS in presence of vesicles	0.32	0.10 ns (86)	50 ns (14)
DCM in absence of vesicles	0.32	0.36 ns (71)	50 ns (29)
DCM in presence of vesicles	0.32	0.19 ns (90)	50 ns (10)

ξ = -4.5 ± 0.45 mV, and electrophoretic mobility of $-0.35 \pm 0.03 \ \mu cmV^{-1}s$.

In order to, have a more in depth insight into the dynamics of the structural relaxation of the prepared vesicles, we probed the vesicles using two fluorophores. For this purpose, the fluorophores used are 1-anilinonaphthalene-8-sulfonate) (ANS) and 4-(dicyano methylene)-2-methyl-6(p-dimethylaminostyryl)-4H-pyran (DCM) to probe the vesicles. Fig. 2 shows the steady state emission peak of ANS and DCM in ethanol–water mixture on excitation with 375 nm and 410 nm respectively. On excitation of ANS at 375 nm the emission maxima is at 496 nm which remains unchanged on addition of the nano vesicles. Similarly, the emission peak of DCM at 625 nm remains unchanged even after the addition of vesicles. As the solvent polarity and the structural flexibility of ANS determines its wavelength and yield of emission [17] Fig. 2a shows that bulk ANS was present in the ethanol and not bound to the surface of the fatty acid. Hence, to understand the residence of DCM and ANS in the vesicular structure we performed fluorescence anisotropy measurements. The time resolved anisotropy measurements were fitted bi-exponentially (Table 1). It was evident from the time resolved profiles that neither DCM nor ANS were able to probe the vesicles. Although DCM prefers to reside in the more hydrophobic region of the surface of SDS micelles [18] the emission of DCM at 625 nm in the presence of the fatty acids is due to the bulk DCM molecules in the ethanol and not bound to the hydrophobic interface of the vesicles. This is also in accordance to the comparable contribution of the faster component in presence of the vesicles (Table 1).

Since, both DCM and ANS were found to be delocalized from the interface of the vesicular structure, we used the auto fluorescence of omega 9 to investigate the relaxation dynamics of the vesicles. The presence of phenol derivatives (like vanillic and hydrocaffeic acid, etc) in ozonized omega 9 are excellent model for following their fluorescence behaviour in different solvents [19]. Fig. 3a represents the steady state emission of ozonated omega 9 on excitation at 375 nm in chloroform and in ethanol–water mixture. In comparison to chloroform, the fluorescence intensity of the molecules was increased in ethanol water mixture. This increase in fluorescence yield is attributed to the supress in the non-radiative process.

Along with the free rotation of molecules in homogeneous media the orientational motion of the molecules in organised assemblies (such as vesicles), is hindered due to the restrictions imposed by the surrounding architecture on the orientation of the probe [20]. Thus, fluorescence anisotropy decay measurements were performed to provide insights about the structural and dynamical changes in the molecule. In ethanol–water mixture, the fluorescence of the phenolic compounds did not decay to zero at long times, but remained at a constant value (Fig. 3b), indicative of hindered motion or restricted rotational diffusion of probe molecules in a restrictive environment [20–22]. The anisotropy decays patters have been fitted to a mono-exponential function which is in accordance in accordance with the wobbling-in-cone model [22]:

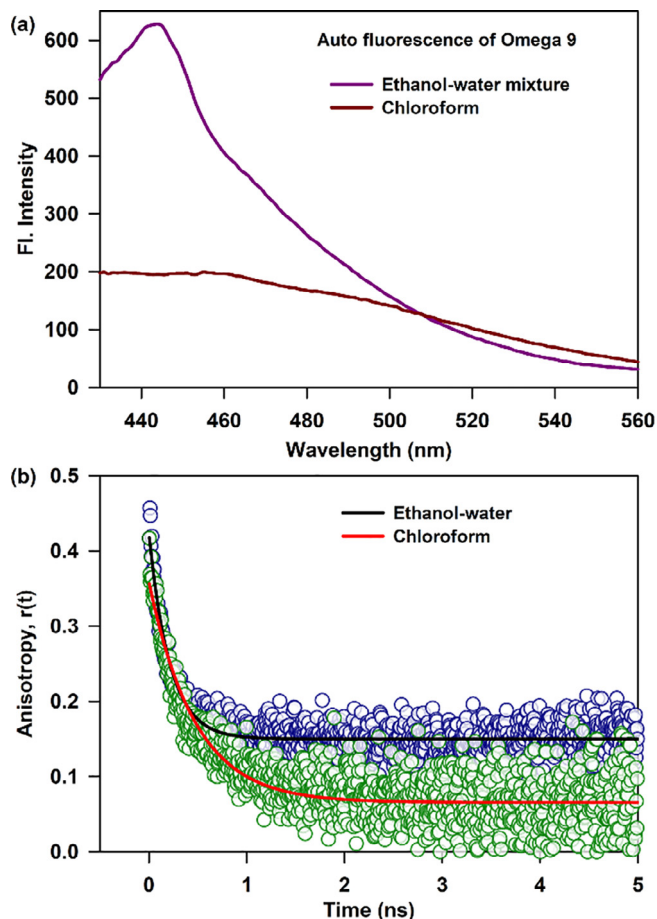$$r(t) = (r_0 - r_\infty)\exp(-t/\Phi) + r_\infty \tag{3}$$

Fig. 3. Probing the vesicles using the auto fluorescence of the polyphenolic compounds. (a) Steady state fluorescence of omega 9 in chloroform and ethanol medium. (b) Fluorescence anisotropy of omega 9 in chloroform and ethanol medium.

where the fundamental anisotropy is r_0, the residual emission anisotropy at significant long times is r_∞ and the time constant for orientational relaxation is Φ. An approximation of the degree of orientational constraint can also be measured from the cone angle (θ_c) [23]

$$\frac{r_0}{r_\infty} = [1/2\cos \theta_c (1 + \cos\theta_c)]^2 \tag{4}$$

The cone angle (θ_c) varies between $0°$ (when the probe molecule is in a restricted environment and its motion is completely hindered) to $90°$ (when the probe is completely free to rotate) [23]. In our study, the anisotropy decays of the polyphenols have a large residual anisotropy (0.14) which is indicative of hindered orientational motion of the probe molecules in a highly rigid environment [20,24]. Furthermore, the decrease in cone angle from 58.3° in chloroform to 45.2° in ethanol water mixture, is also consistent of a restrictive motion of the probe molecules. In ethanol water medium, the fatty acids form alcohol containing bilayer membrane vesicles due to interactions between the adjacent ionized carboxylates, thereby decreasing the electrostatic repulsion between the adjacent head groups [25]. The formation of vesicles in the ethanolic medium, increases the rigidity of the vesicles, which in turn restricts the orientational motion of the probe resulting in a decrease in the cone angle. (Table 2).

As the vesicles were prepared with ozonated omega 9 fatty acid esters, we evaluated the amount of peroxides generated by these fatty acids as a result of ozonation. Fig. 4 depicts the peroxide value

Table 2
Rotational relaxation parameters of Ozonated Omega-9 in different systems.

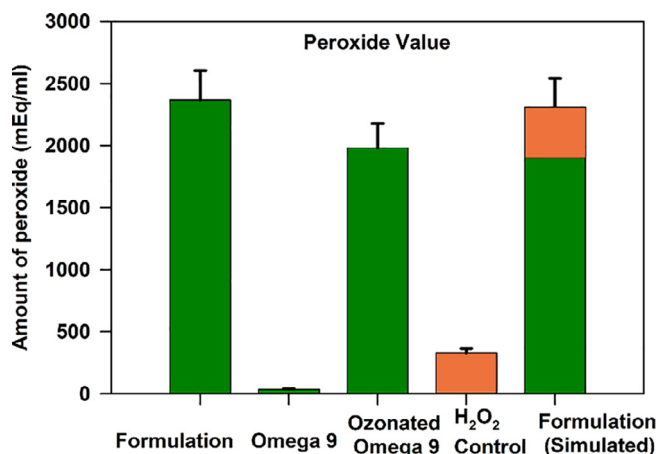| System	r_0	r_∞	Φ (ps)	r_0/r_∞	θ_c (°)
Ozonated omega 9 in chloroform	0.42	0.07	250	0.16	58.3
Ozonated omega 9 in 80% ethanol	0.38	0.14	300	0.36	45.2

Fig. 4. Peroxide value of the prepared sanitiser formulation.

of our formulated sanitiser, along with ozonized omega 9, omega 9 and H_2O_2 to be 2368 mEq/ml, 1980 mEq/ml, 36 mEq/ml and 331.2 mEq/ml respectively. It has been also found out that the cumulative peroxide value of our formulation is the total peroxide generated by the ozonized omega 9 along with H_2O_2. The amount of peroxide generated by the ozonized omega 9 is approximately ~ 55 times greater than the non-ozonized species and ~ 6 times more than the peroxides released by H_2O_2. This is mainly due to the formation of trioxolanes from omega 9 fatty acid esters which eventually decomposes to form lipid oxidation products (peroxides and hyperoxides), H_2O_2 along with reactive oxygen species (ROS) [26]. These compounds commonly termed as the "ozone messengers" mainly contribute to the anti-bacterial action of ozonized omega 9 by inducing oxidative stress.

The anti-microbial effect of our developed sanitiser (formulation) was assessed towards *P. aeruginosa*. Bacterial growth curve experiments were performed using the developed formulation and 80% ethanol control samples. For our sanitiser treated samples,
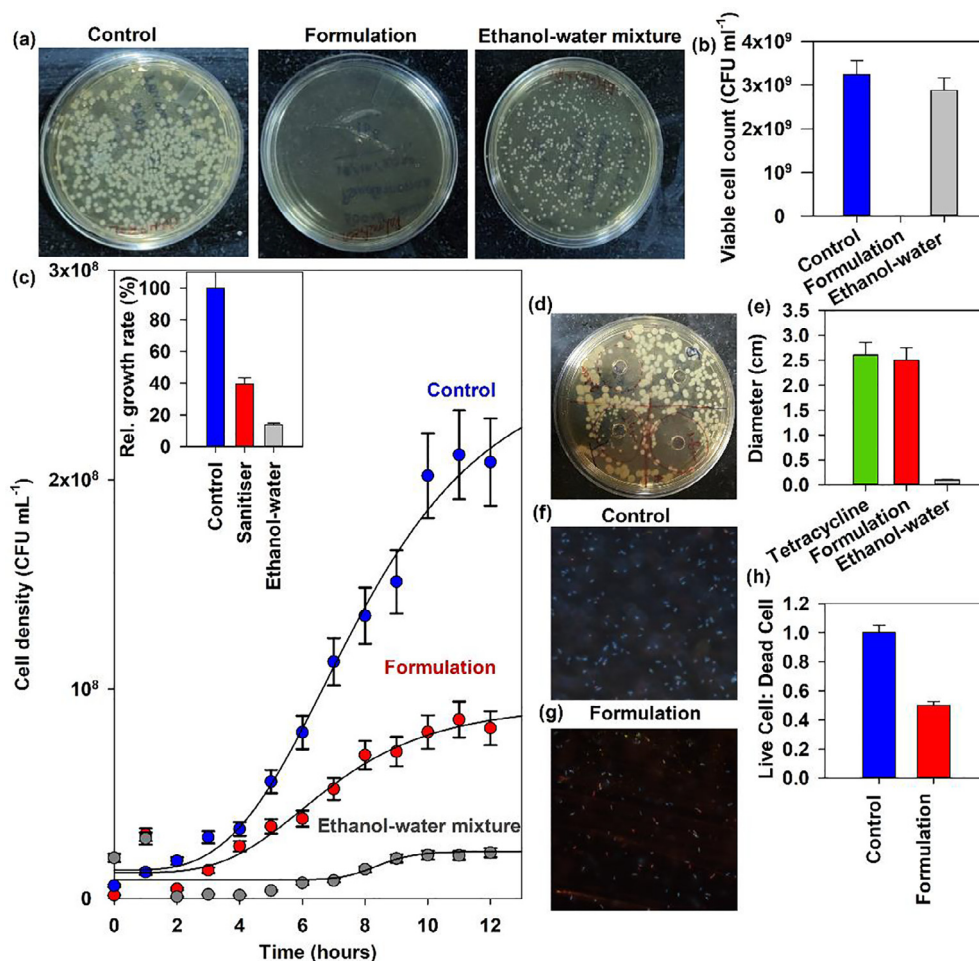

Fig. 5. Antibacterial effect of the prepared formulation. (a) Pictures of *P. aeruginosa* plates with treatment with the prepared formulation (b) Viable cell count from the plates (c) Growth curves of *P. aeruginosa* (d) and (e) Plate and graph showing the area of inhibition (f) and (g) fluorescence microscopy images stained with DAPI and PI (h) Count of live cell: dead cell from microscopy.

bacterial killing effect was observed as shown in Fig. 5. The bacterial growth was ~ 60% lower as compared to control. This trend is clearly indicative of the anti-bacterial effect of our developed formulation may be due to generation of oxidative stress owing to its high peroxide value. The growth rate of our formulation is also ~ 22% greater than the ethanol–water mixture, clearly indicating the difference in the mode of anti-bacterial action of both the samples. The visual difference in the number of *P. aeruginosa* colonies depicts antibacterial effect (~98% colony reduction) of our formulated sanitiser formulation even after the evaporation of ethanol from the plates during the time of incubation, thereby imparting long lasting effect to the prepared formulation. This additional therapeutic effect of the formulation may be attributed by the ozonized products (peroxides) generated which causes lethal structural integrity, disruption of the bacterial cell membrane by the induction of ROS and subsequently release of all the cellular constituents [27]. Although 80% ethanol has evident antibacterial activity with low growth rate (as shown in Fig. 5c subset), it doesn't have a long lasting effect. Hence, when the ethanol solution was evaporated, it resulted in more colony formation in comparison to the treatment plate (Fig. 5a). We further evaluated the number of dead cells and live cells in different matrix by staining with DAPI and PI. PI stains the dead cells whereas, DAPI stains all the nuclear material irrespective of their viability. The stained image of control shows more number of live cells than dead cells (ratio ~ 1.2) and the number of living cells were significantly reduced in case of the samples treated with sanitiser (~0.45),which further confirms the anti-bacterial efficacy of the formulation resulting due to oxidative stress generation.

4. Conclusion

In this study we report the synthesis of a long lasting sanitiser from ozonated omega 9 fatty acids which have an antibacterial effect against *P. aeruginosa*, owing to their high peroxide value. In 80% ethanolic medium ozonated omega 9 fatty acids forms ethanol containing bilayer membrane vesicles. Although 80% ethanolic solution has antimicrobial effect, the formation of vesicles from ozonated omega 9 fatty acids has an additional antibacterial effect, even after the evaporation of ethanol. This can give a long lasting

sanitising effect and have relevance regarding the current COVID-19 pandemic.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

References

[1] M.P. Ward et al., Transbound Emerg. Dis. 67 (2) (2020) 469.
[2] C. Huang et al., The Lancet 395 (10223) (2020) 497.
[3] N. Chen et al., The Lancet 395 (10223) (2020) 507.
[4] D. Wang et al., JAMA 323 (11) (2020) 1061.
[5] A. Kratzel et al., Emerg. Infect. Dis. 26 (7) (2020) 1592.
[6] L. Yang et al., Signal Transduct. Target Ther. 5 (1) (2020) 1.
[7] C.C. Lai et al., Int. J. Antimicrob. Agents 55 (3) (2020) 105924.
[8] World Health Organization and WHO Patient Safety, WHO guidelines on hand hygiene in health care, World Health Organization, Geneva, 2009, p. 30.
[9] S. Ophaswongse et al., Contact Derm. 30 (1) (1994) 1.
[10] W. Van Ketel et al., Contact Derm. 1 (1) (1975) 7.
[11] D.W. Lachenmeier, J. Occup. Med. Toxicol. 3 (1) (2008) 1.
[12] J. Sadowska et al., Chem. Phys. Lipids 151 (2) (2008) 85.
[13] A. Adhikari et al., ACS Omega 3 (11) (2018) 15975.
[14] A. Giri et al., J. Mater. Chem. C 1 (9) (2013) 1885.
[15] P. Majumder et al., J. Colloid Interface Sci. 290 (2) (2005) 462.
[16] A. Adhikari et al., ChemMedChem 15 (5) (2020) 420.
[17] P. Biswas et al., Soft Matter 16 (12) (2020) 3050.
[18] S.K. Pal et al., J. Phys. Chem. B 104 (18) (2000) 4529.
[19] M. Zandomeneghi et al., J. Agric. Food Chem. 53 (3) (2005) 759.
[20] K. Kinosita Jr et al., Biophys. J. 37 (2) (1982) 461.
[21] K. Kinosita Jr et al., Biophys. J. 20 (3) (1977) 289.
[22] G. Lipari et al., Biophys. J. 30 (3) (1980) 489.
[23] C.R. Guest et al., Biochemistry 30 (13) (1991) 3271.
[24] S.K. Pal et al., J. Phys. Chem. B 106 (48) (2002) 12376.
[25] I.A. Chen et al., Biophys. J. 87 (2) (2004) 988.
[26] Y. Günaydın et al., Ozone Sci. Eng. 40 (1) (2018) 37.
[27] S. Mondal et al., Mater. Today Proc. 43 (6) (2021) 3692, https://doi.org/10.1016/j.matpr.2020.10.981.