

Fusidic Acid/Tea-Tree Oil Nanoemulsions: A Potentially Safe and Effective Anti MRSA/MSSA Topical Agent for Chronic Wound Healing

(Nanoemulsi Asid Fusidik/Minyak *Tea Tree*: Agen Topikal Anti MRSA/MSSA yang Berpotensi Selamat dan Berkesan untuk Penyembuhan Luka Kronik)

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Received: 11 October 2021/Accepted: 29 November 2021

ABSTRACT

Fusidic acid (FA) is clinically used as an antibacterial agent for the treatment of Gram-positive bacterial infections. It interferes with bacterial protein synthesis, specifically by preventing the translocation of the elongation factor G on the ribosome. In the present work, oil-in-water nanoemulsion (NE) was developed as a carrier for the transdermal delivery of FA. Different oils, surfactants and co-surfactants were screened. The solubility of FA, the emulsifying capacity of the surfactants and phase diagrams for each oil and surfactant mix were constructed. From the analysis, eight stable NE formulations were chosen, and their physicochemical properties were further evaluated. The antibacterial activity against methicillin-resistant *Staphylococcus aureus* (MRSA) and methicillin-sensitive *Staphylococcus aureus* (MSSA) were also evaluated, and cytotoxicity was conducted on HS-27 cell line to determine the safety of the formula. It was found that the NE produced from tea tree oil has the most optimal stability with promising antibacterial activity against MRSA as compared to a commercially available product. The safety profile of the NE was also comparable to the commercial product; thus, the formulated FA-NE is promising for clinical use.

Keywords: Fusidic acid; HS-27; MRSA; nanoemulsion; tea tree oil

ABSTRAK

Asid fusidik (AF) merupakan agen antibakteria yang digunakan untuk rawatan jangkitan bakteria Gram positif. Ia mengganggu proses sintesis protein dalam bakteria, secara khususnya melalui halangan translokasi faktor pemanjangan G pada ribosom. Dalam kajian ini, nanoemulsi minyak-dalam-air (NE) telah dibangunkan sebagai pembawa kepada penghantaran AF melalui laluan transdermal. Pelbagai jenis minyak, surfaktan and surfaktan bersama telah dinilai. Keterlarutan AF dalam eksipien, kapasiti pengemulsian bagi setiap surfaktan dan rajah fasa telah dibangunkan. Daripada analisis yang dijalankan, lapan formulasi NE yang stabil telah dipilih dan ciri farmakofizikal bagi setiap formulasi ini telah dinilai. Aktiviti antibakteria formulasi-formulasi NE terhadap bakteria *Staphylococcus aureus* rintang metisilin (MRSA) dan *Staphylococcus aureus* rentan metisilin (MSSA) juga telah dinilai dan kesan kesitotoksikan telah diuji pada sel HS-27 untuk menentukan keselamatan formula. NE yang dihasilkan daripada minyak *tea tree* ditemui mempunyai kestabilan yang paling optimum berbanding NE yang dihasilkan daripada minyak lain yang diuji, dengan aktiviti antibakteria yang baik terhadap bakteria MRSA berbanding produk komersial. Keselamatan formula NE juga adalah setara dengan produk komersial, jadi ia berpotensi untuk dibangunkan dengan lebih lanjut bagi kegunaan klinikal.

Kata kunci: Asid fusidik; HS-27; minyak *tea tree*; MRSA; nanoemulsi

INTRODUCTION

Chronic wound is the type of wound that do not progress through the normal healing process in a timely manner. The problem normally lies in the inflammation

phase of healing, which is due to excessive levels of proinflammatory cytokines, proteases, reactive oxygen species (ROS) and the presence of persistent infection further complicates the treatment process (Frykberg &

Banks 2015). The current treatment standard for chronic wound care includes systemic antibiotics and antiseptic solution, to overcome the deep-seated infections that is difficult to reach with simple topical application.

Nanoemulsion (NE) is a type of emulsion with droplet sizes between 20-200 nm with narrow distributions. They are transparent or translucent with a bluish colouration. NE is obtained by mixing two immiscible liquids with an emulsifier, followed by an introduction of high energy techniques such as ultrasonication, or homogenisation. The nanodroplets produced usually have excellent kinetic stability (Abolmaali et al. 2011). There are many advantages associated with NE over conventional emulsions. In terms of physical stability, the internal phase droplet size distribution would not be affected by the dilution of the external phase (Sugumar et al. 2015). In addition, with respect to the biological activity, NE allows adequate localisation and skin penetration of active ingredients, which render them effective locally as compared to conventional emulsion systems (Eleraky et al. 2020).

The oil phase may consist of natural or synthetic oils and lipids, such as medium or long-chain triglycerides or perfluorochemicals. Among the natural oils, plant-derived essential oils have garnered significant attention due to their insecticidal, anti-fungal and antibacterial properties with good safety profile (Sugumar et al. 2014). They could be a good option in the NE based antibacterial formulation as they may contribute synergistically to the effectiveness of the active ingredients incorporated in the formula (Panaitescu et al. 2018).

Fusidic acid (FA) is a tetracyclic triterpenoid that is structurally linked to cephalosporin P1. It originates from the fungus *Fusidium coccineum* and differs from cephalosporin by the presence of three acetyl groups, which contributes to its enhanced antibacterial activity (Fernandes 2016). The FA nucleus has properties common to other tetracyclic structures such as the adrenocorticoids and bile salts, particularly the cholate and taurocholate (Godtfredsen et al. 1962). It is correlated to other antibiotic groups, including the helvolic acids and the viridominic acids. Antibiotics parallel or identical to FA are produced by dermatophytes such as *Microsporum canis*, *Microsporum gypseum*, and *Epidermophyton floccosum* (Perry et al. 1983). Fusidic acid has bacteriostatic activity against staphylococci, including both methicillin-sensitive and methicillin-resistant strains, *Neisseria* spp., *Bordetella pertussis*, *Corynebacterium* spp., and Gram-positive anaerobes like *Clostridium difficile*, *Clostridium perfringens*, *Peptostreptococcus* spp. and *Propionibacterium acnes* (Frimodt-Møller 2010).

NE has the potential to increase the effectiveness of chronic wound treatment by improving the absorption of FA through the wound to eliminate possible infection. In this current study, FA was employed as the antibacterial agent and in combination with essential oils with antibacterial activity, a NE was formulated as a potentially effective topical antibacterial formulation. Eight different oils (palm oil, sesame seed oil, lavender oil, orange oil, lemon oil, tea tree oil, eucalyptus oil and peppermint oil) were formulated in combination with FA as the active ingredient. The obtained NE were evaluated in terms of physicochemical properties and antibacterial activity against MRSA and MSSA strains, and cytotoxicity against human skin fibroblast HS 27 cells to prove the safety of the NE formulations.

MATERIALS AND METHODS

MATERIALS

Pure FA powder was purchased from Sgonek Biological Technology Co. (China). Ethanol and methanol were purchased from QRec Asia (Malaysia). Tea tree oil, lavender oil, peppermint oil, lemon oil, orange oil, and eucalyptus oil were obtained from Soap Cart Co. (Malaysia) and palm oil was purchased from Sunlong Industrial and Trading Co. (China). Sesame seed oil and Span 20 were purchased from Moksha LifeStyle Products (India). Propylene glycol was purchased from Sigma-Aldrich (USA), polyethylene glycol 4000 was from Merck (Germany) while Tween 20, Tween 60, and Tween 80 were purchased from Euro-Chemo-Pharma (Malaysia). All reagents and chemicals used were of analytical grade.

Dulbecco's Modified Eagle Medium (DMEM) were obtained from Life Technologies (USA) and 1% Penicillin/Streptomycin were obtained from PAA Laboratories (Austria). Three-[4,5-dimethylthiazolyl]-2,5- diphenyltetrazolium bromide (MTT) was purchased from Biobasic Inc. (Canada), phosphate-buffered saline (PBS) and DMSO were obtained from Sigma Life Science (USA).

Methicillin-resistant *Staphylococcus aureus* (MRSA) ATCC 43300, methicillin-sensitive *Staphylococcus aureus* (MSSA) ATCC 12600 and human skin fibroblast HS 27 cells were purchased from American Type Culture Collection (ATCC) (USA).

CALIBRATION CURVE OF FA IN ETHANOL

Calibration curves of FA were prepared in ethanol using a UV/Vis spectrophotometer U-2800 (Hitachi, Japan).

Briefly, 10 mg of FA was accurately weighed using a calibrated digital weighing balance (Ohaus, USA) and dissolved in 10 mL ethanol, producing 1 mg/mL FA solution. This stock solution was further diluted to produce a range of working concentrations between 2 and 20 $\mu\text{g/mL}$. The UV/Vis absorbance of each working solution was measured at 235 nm in triplicates and a standard calibration curve was constructed accordingly.

DETERMINATION OF SATURATED SOLUBILITY OF FA

The saturated solubilities of FA in different solvents, oils and surfactants were determined by adding an excess amount of drug (100 mg) into 2 mL of each medium. With regards to Poloxamer 407 and polyethylene glycol 4000, 1% w/v solution of each surfactant in water was prepared to evaluate the solubility of the drug. The mixtures were then placed on a mechanical shaker (Thermo Shaker, Hangzhou Allsheng Instrument Co., China) at room temperature for 72 h. Samples were centrifuged (Hettich, Germany) at 5000 rpm for 15 min and the concentration of FA in the supernatant was assayed using UV Vis spectrophotometer at 235 nm. All tests were done in triplicates and the data is presented as mean (\pm s.d.).

SCREENING OF SURFACTANTS

Six surfactants (Tween 20, Span 20, Tween 80, Poloxamer 408, propylene glycol and polyethylene glycol (PEG) 4000) were screened for their ability to produce nanoemulsion, as previously described by Azeem et al. (2009). Briefly 2 mL of surfactant solution was prepared and 5 μL of oil was added with vigorous mixing by using a vortex for 30 s. The mixture was observed for presence of turbidity. If a clear mixture was obtained, the addition of oil was repeated until the mixture turned turbid following vortex mixing.

CONSTRUCTION OF TERNARY PHASE DIAGRAM

Pseudo-ternary phase diagrams were prepared for three oils: Palm oil (PO), sesame oil (SO), and tea tree oil (TTO). The pseudo-ternary phase diagrams consisting of oil, water and surfactant mixtures with different hydrophilic-lipophilic balance (HLB) values were constructed through the water titration method. The ratio of surfactant to co-surfactant was fixed at 1:1 and the ratio between oil and surfactant mix (Smix) was screened from 0.5:9.5 to 9.0:1.0 (oil:Smix). Distilled water was added to the oil and Smix mixture in increments of 100 μL by micropipette at room temperature. The samples

were mixed vigorously by a homogeniser for 2 min and kept at room temperature for 24 h to reach equilibrium before further addition of distilled water was made. The physical appearance of the mixture was observed after each addition of distilled water. The formation of nanoemulsion was identified as the transparent or translucent liquid. The results obtained were plotted on a ternary phase diagram using Mix-School 3.51 software (Gupta et al. 2011).

PREPARATION OF FA-LOADED NANOEMULSION

FA-loaded NE was prepared with the oil/Smix/water ratios that produced transparent NE as determined by the pseudoternary phase diagram. The aqueous phase was added to the oily phase (containing 2% w/v FA) under vortex for 30 s. The characteristics of the prepared NE were evaluated accordingly.

THERMODYNAMIC STABILITY

In brief, three types of mechanical and thermodynamic stresses were tested: Centrifugation, heating-cooling, and freeze-thaw cycle. Mechanical stress was applied through centrifugation at 3500 rpm for 30 min. Any change in physical appearances was recorded. The heating-cooling stress was done by storing the NE at 4 $^{\circ}\text{C}$ for 72 h, followed by storage at 40 $^{\circ}\text{C}$ for 72 h. This cycle was repeated three times and the homogeneity of the formulation was recorded. The freeze-thaw cycle was done by freezing the NE at -20 $^{\circ}\text{C}$ for 72 h, followed by storage at room temperature, which was also conducted for 72 h. This cycle was repeated three times and changes were noted.

CHARACTERISATION OF PHYSICOCHEMICAL PROPERTIES INVESTIGATION OF MICROMETRICS & ZETA POTENTIAL

The average droplet size (Z-average) and PDI of the formulated nanoemulsion system were analysed by dynamic light scattering method using LitesizerTM 100 (Anton Paar, Austria) with light at a scattering angle of 173 $^{\circ}$ at 25 $^{\circ}\text{C}$. The value of ζ -potential was determined at 25 $^{\circ}\text{C}$ with an electric field strength of 23.2 V/cm. All measurements were reported as an average of three replicates (\pm s.d) (Zhu et al. 2015).

DYE SOLUBILITY TEST

Two drops of 2% methylene blue were added to the nanoemulsion and visual observation was conducted after 5 min using a light microscope. Oil-in-water (o/w)

nanoemulsion will incorporate the dye rapidly whilst clumps could be observed under the microscope for the water-in-oil (w/o) nanoemulsion. Following this test, the nanoemulsion was diluted with distilled water to investigate the presence of any phase separation in the system.

OPTICAL CLARITY (PERCENTAGE TRANSMITTANCE)

The percentage of transmittance indicates the homogeneity and clarity of the nanoemulsion. Percentage of transmittance of the formulation was measured at 650 nm using UV-spectrophotometer against distilled water as blank.

REFRACTIVE INDEX

The refractive index of the nanoemulsion was determined using Abbes Brix Refractometer (Atago, Japan) and distilled water was employed as the standard. One drop of the sample was placed on the glass slide and the reading was taken accordingly.

pH MEASUREMENT

The pH value of the nanoemulsion was determined using a digital pH meter (Eutech Instruments, USA), calibrated by using a standard buffer solution at pH 4 and 7 before each use.

MORPHOLOGY ANALYSIS OF NANOEMULSION DROPLETS

The morphology of the NE was observed under a transmission electron microscope (TEM) (Hitachi High-Technologies Corp, Tokyo, Japan) at 100 \times magnification. The nanoemulsion was diluted 10 times and dropped on a copper grid coated with a carbon film, stained with 1% phosphotungstic acid (pH adjusted to 7.0) and air-dried before the analysis.

IN VITRO ANTIBACTERIAL STUDY

The antibacterial activity of the NE was evaluated in comparison to a commercially available 2% FA cream against MSSA and MRSA using the agar diffusion method. The agar was inoculated with log phase (McFarland 3) bacteria and 6 mm diameter holes were punched in the agar using a cork borer. The holes were filled with the NE formulations, formula without FA and the commercial FA cream. The plates were incubated at 37 °C for 24 h. The antibacterial activity was evaluated by measuring the diameter of the inhibition zone. All tests were done in triplicates.

IN VITRO CELL VIABILITY ASSAY

The cytotoxicity of the NE was assessed against human skin fibroblast HS-27 cells using MTT assay (Mosmann 1983). Cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 1% Penicillin/Streptomycin (PAA Laboratories, Austria) with 5% fetal bovine serum. Briefly, the cells were plated at a density of 5000 cells/well and was incubated for 24 h at 37 °C in 5% CO₂. After 24 h, cells were treated with NE formulations at different concentrations (0.025, 0.05, 0.1, and 0.2%) for 24 h. Following that, 3-[4,5-dimethylthiazol-yl]-2,5-diphenyltetrazolium bromide (MTT) was dissolved in PBS at 5 mg/mL, added to all wells, and the plates were incubated at 37 °C with 5% CO₂ for 4 h. The medium was discarded, replaced with 100 μ L DMSO and the absorbance was measured at 570 nm using a microplate reader (Fisher Scientific, USA) (Latif et al. 2019). The percentage of cell viability was calculated in comparison to the untreated control.

RESULTS AND DISCUSSION

Choosing a stable and safe combination of ingredients for a formulation requires careful investigation. In this study, the authors aimed to prepare a NE formulation with FA as the active ingredient. As FA is commonly available in semi-solid preparations such as creams and ointments, it would be a promising approach to explore the possibility of formulating FA in the form of liquid, suitable for spraying on the wound for the prevention of microbial infection. The preparation of stable NE will provide greater flexibility in choosing suitable dosage forms for the final product.

DETERMINATION OF SATURATED SOLUBILITY OF FA

The saturated solubility of FA in different solvents, oils and surfactants are presented in Table 1. Comparing between the solvents, the solubility of FA is higher in ethanol (251.73 \pm 0.01 μ g/mL) than methanol (187.19 \pm 0.55 μ g/mL) or distilled water (5.198 \pm 0.35 μ g/mL). Solubilisation power of a particular solvent gives the quantitative estimates on its ability to solubilise a drug. It was reported that solubilisation power is correlated with the solvent's polarity, besides the molecular structure of the solute (Desai & Park 2004). FA is a weakly acidic molecule, which exists in water in the protonated form. However, the presence of the huge hydrophobic moiety in the structure (Figure 1) prevents complete solubilisation of FA in water.

The solubility of FA in oil is an important factor in the selection of oil for a NE formulation. This is to

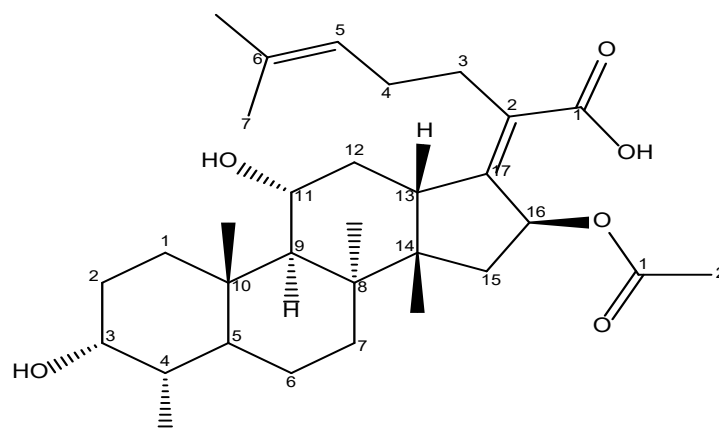
prevent the precipitation of the active ingredient during production and storage. It was found that the solubility of FA is highest in sesame oil ($89.08 \pm 5.2 \mu\text{g/mL}$), followed by palm oil ($72.62 \pm 5.2 \mu\text{g/mL}$), and tea tree oil ($57.71 \pm 1.2 \mu\text{g/mL}$). Sesame oil is known to have a high content of unsaturated fatty acids than many other vegetable oils, whilst palm oil has a similar portion of saturated and unsaturated fatty acids. This unsaturated fat helps in the solubilisation of drugs, and a high content of unsaturated fatty acids would help in solubilising the drug molecules. Most essential oils such as lavender and peppermint oil have a low proportion of unsaturated

fatty acids which lead to the low solubility of FA in these oils (Boateng et al. 2016).

The solubility profile of FA in surfactants is important, as it will be pre-solubilised in the oil and surfactant mix prior to the emulsification process. The FA will stay in the oil droplets as solubilised form and this will prevent precipitation in the system. The incomplete or low solubility of a surfactant will lead to the leaching of a drug from the system, which subsequently leads to inefficient drug loading. In regard to the ability of surfactants to solubilise FA, Tween 80 has the highest solubilising ability ($57.34 \pm 4.7 \mu\text{g/mL}$) as compared to other surfactants.

TABLE 1. Saturated solubility of FA in different solvents, oils, surfactants and co-surfactants

| Material | Solubility ($\mu\text{g/mL}$) |
|--------------------------------|---------------------------------|
| Solvent | |
| Distilled water | 5.1 ± 0.4 |
| Ethanol | 251.7 ± 0.1 |
| Methanol | 187.2 ± 0.6 |
| Oil | |
| Eucalyptus oil | 13.93 ± 1.3 |
| Lavender oil | 44.4 ± 3.9 |
| Lemon oil | 55.49 ± 3.5 |
| Orange oil | 55.19 ± 1.8 |
| Palm oil | 72.62 ± 5.2 |
| Peppermint oil | 42.89 ± 2.7 |
| Sesame oil | 89.1 ± 5.2 |
| Tea tree oil | 57.71 ± 1.2 |
| Surfactant | |
| 1% w/v Poloxamer 407 | 38.5 ± 3.5 |
| 1%w/v Polyethylene glycol 4000 | 40.78 ± 3.2 |
| Propylene glycol | 26.43 ± 2.3 |
| Span 20 | 34.45 ± 0.6 |
| Tween 20 | 43.90 ± 2.7 |
| Tween 80 | 57.3 ± 4.7 |



fusidic acid

FIGURE 1. Molecular structure of FA

DETERMINATION OF EMULSIFYING CAPACITY

The choice of surfactant plays a vital role in the formation of a stable NE system. As surfactants may produce toxicity, choosing a safe surfactant at a suitable concentration is important. Non-ionic surfactants such as Tween and Span have shown lower cytotoxicity as compared to cationic or anionic surfactants, which could cause skin irritation following topical applications.

Based on the solubility study of FA in the different oils, a pre-formulation study of the NE prepared from the three oils with the highest potential (sesame seed oil, palm oil, and tea tree oil) were conducted using six surfactants. The surfactants chosen were Tween 20, Span

20, Tween 80, Poloxamer 407, propylene glycol (PG) and polyethylene glycol (PEG) 4000. These are non-ionic surfactants from different groups and have different molecular structures and sizes. The solubility of oil in surfactant is important to determine the affinity of the surfactant to the oil used in the formulation. The greater amount of oil solubilised by the surfactant, the greater its nano emulsification capacity. This characteristic will also indicate the possibility of forming a NE by the system. Figure 2 shows the percentage of palm oil, sesame oil and tea tree oil solubilised by the six tested surfactants. Tween[®] 80 and PEG 4000 showed the highest oil solubilising capacity for all three oils.

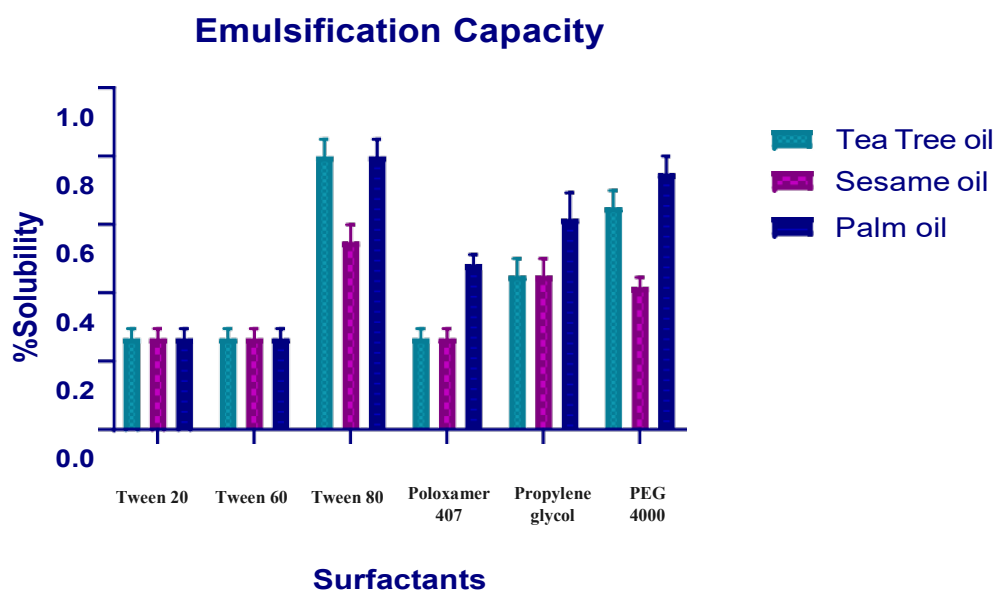


FIGURE 2. Emulsifying capacity of surfactants on palm oil, sesame oil and tea tree oil

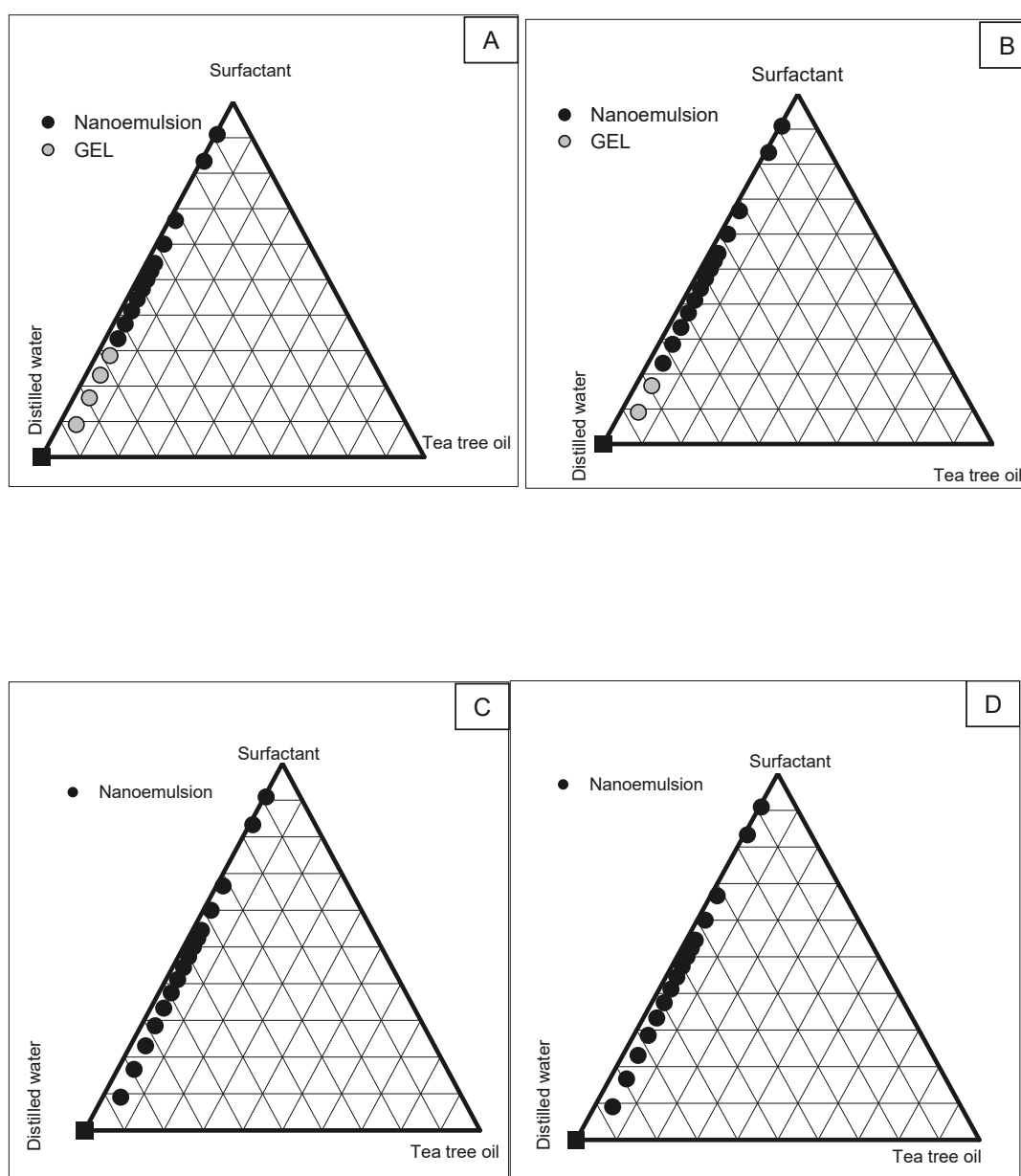
CONSTRUCTION OF TERNARY PHASE DIAGRAM

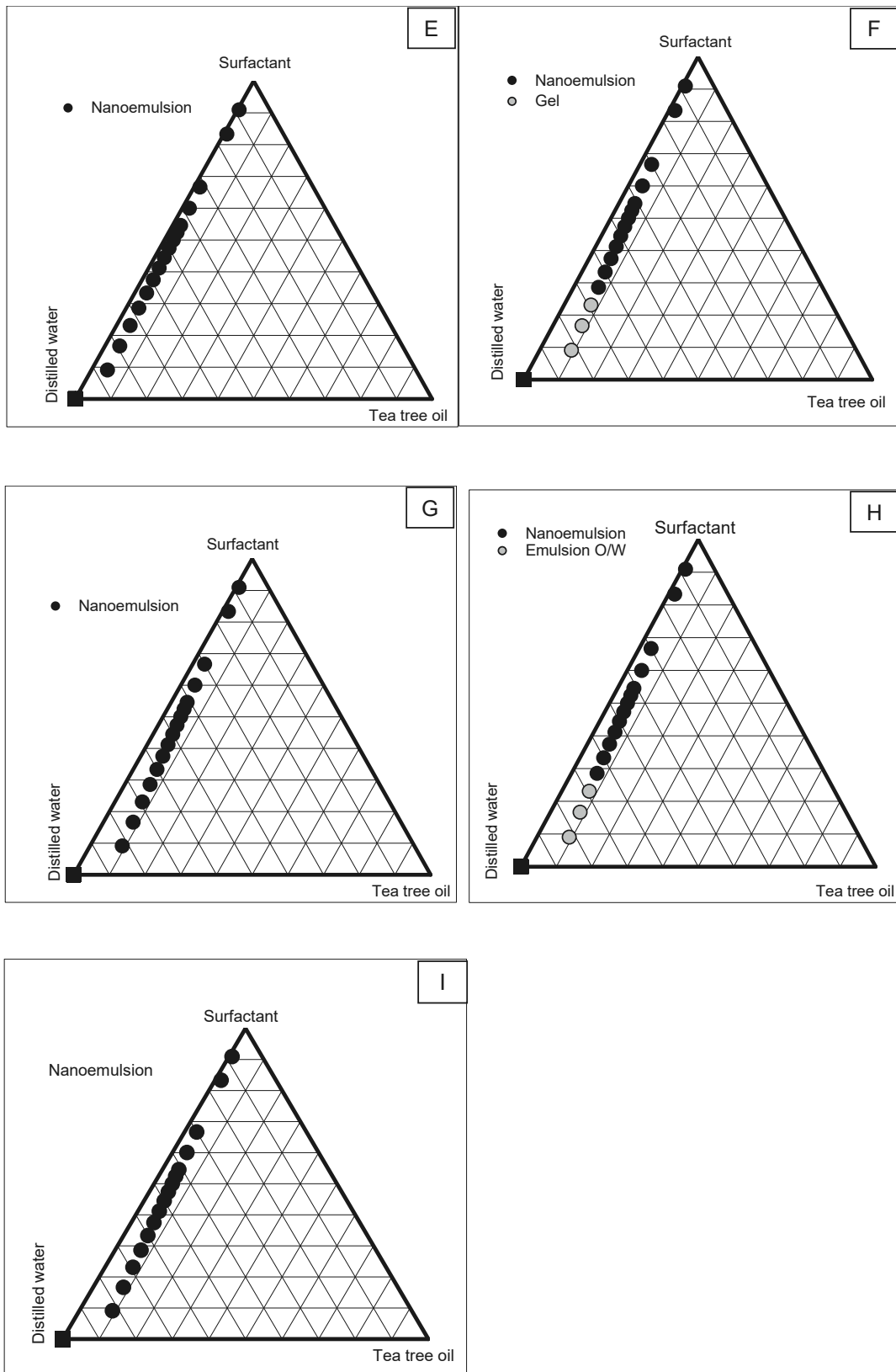
Pseudoternary phase diagrams were constructed to determine the quantity of each component needed to prepare a stable NE. Based on the experiment conducted on the solubility of FA, the following materials were used to construct phase diagrams; distilled water, oils (sesame oil, palm oil and tea tree oil), surfactants (Tween®80 and PEG 4000) and co-surfactants (PG, ethanol). Our attempts to produce NE from sesame oil and palm oil were not successful, in which all the different mixtures of oil, surfactants, co-surfactants and water produced gels or conventional emulsion. Only tea tree oil successfully produced NE. All the tea-tree based phase diagrams are included in Figure 3.

The presence of higher content of fats in the sesame and palm oil as compared to the tea tree oil have led

to difficulties in producing NE. Interestingly, El-Refai et al. (2019) reported the successful production of NE based on sesame oil, using Tween 80 as the surfactant. The authors used high energy techniques, which include high temperature and high amplitude sonication for 45 min. This may explain our results for not being able to produce sesame oil-based NE using the present method. The high-energy methods and long processing time may not be cost-efficient and may affect the stability of the drug incorporated in the NE (Tubtimsri et al. 2020). The percent of oil incorporated would also need to be reduced to enable dispersion of the oil droplets in the NE formulations.

The pseudo-ternary diagrams gave ideas on the efficiency of the surfactant system to produce NE. The size of the NE area in each diagram is different, with





A. Surfactant: 0.5:9.5 [Tea tree oil: Tween 80], B. Surfactant: 0.5:9.5 [Tea tree oil: (Tween 80/PG)], C. Surfactant: 0.5:9.5 [Tea tree oil: (Tween80/PEG4000)], D. Surfactant: 0.5:9.5 [Tea tree oil: (Tween 80/PG/Ethanol)], E. Surfactant: 0.5:9.5 [Tea tree oil: (Tween80/PEG4000/Ethanol)], F. Surfactant: 1:9 [Tea tree oil: (Tween80/PG)], G. Surfactant: 1:9 [Tea tree oil: (Tween80/PEG4000)], H. Surfactant: 1:9 [Tea tree oil: (Tween80/PEG4000/Ethanol)], I. Surfactant: 1:9 [Tea tree oil: (Tween80/PG/Ethanol)]

FIGURE 3. Pseudo-ternary diagrams of the tea tree oil-based NE, prepared by using different proportions of surfactant(s)

the biggest shown by diagram C, D, G, and I. These diagrams corresponded to the presence of PEG 4000 or PG/ethanol in the Smix, suggesting the importance of these ingredients in the production of NE. It has also been reported that a reduction in oil concentration will reduce the NE area (Azeem et al. 2009). However, this reduction was not observed in this study, perhaps due to the insufficient reduction of oil percentage between formulations.

From the pseudo ternary phase diagrams, 18 formulations were found to be stable, and they were

subsequently loaded with FA. The formulations were then characterised for mechanical and thermodynamic stability. As presented in Table 2, only eight formulations showed good mechanical and thermodynamic stability. As stability towards temperature changes is an important criterion that differentiates between NE and microemulsions (ME) (Aswathanarayan & Vittal 2019), this becomes the determining point in this study in choosing the formulas to be brought forward for further analysis. Table 3 summarises the eight formulas.

TABLE 2. Thermodynamic stability of the nanoemulsions (NE) produced

| Formula | Centrifugation test | Heating-cooling cycle | Freeze-thaw cycle |
|---------|---------------------|-----------------------|-------------------|
| FA-NE1 | / | / | / |
| FA-NE2 | X | / | / |
| FA-NE3 | / | / | / |
| FA-NE4 | / | X | / |
| FA-NE5 | / | / | / |
| FA-NE6 | / | / | / |
| FA-NE7 | / | / | X |
| FA-NE8 | X | / | / |
| FA-NE9 | / | / | / |
| FA-NE10 | / | / | / |
| FA-NE11 | X | / | / |
| FA-NE12 | / | / | X |
| FA-NE13 | / | / | / |
| FA-NE14 | / | X | / |
| FA-NE15 | / | X | / |
| FA-NE16 | / | / | X |
| FA-NE17 | / | / | / |
| FA-NE18 | X | / | / |

Note: The highlighted formulations were stable under mechanical and thermodynamic stresses

TABLE 3. The eight formulas based on tea tree oils

| Formula | FA (%) | TTO Oil (%) | Water (%) | Smix (%) | Surfactant | Co Surfactant | Smix ratio | Oil: Smix |
|---------|--------|-------------|-----------|----------|------------|---------------------|------------|-----------|
| FA-NE1 | 2 | 3.33 | 33.33 | 63.33 | Tween 80 | - | 1:0 | 0.5:9.5 |
| FA-NE3 | 2 | 3.33 | 33.33 | 63.33 | Tween 80 | PG | 1:1 | 0.5:9.5 |
| FA-NE5 | 2 | 3.33 | 33.33 | 63.33 | Tween 80 | PG/Ethanol | 1:1:1 | 0.5:9.5 |
| FA-NE6 | 2 | 2.5 | 50 | 47.5 | Tween 80 | PG/Ethanol | 1:1:1 | 0.5:9.5 |
| FA-NE9 | 2 | 3.33 | 33.33 | 63.33 | Tween 80 | PEG4000/ Ethanol | 1:1:1 | 0.5:9.5 |
| FA-NE10 | 2 | 2.5 | 50 | 47.5 | Tween 80 | PEG4000/ Ethanol | 1:1:1 | 0.5:9.5 |
| FA-NE13 | 2 | 7.14 | 28.57 | 64.29 | Tween 80 | PG/Ethanol | 1:1:1 | 1:9 |
| FA-NE17 | 2 | 7.14 | 28.57 | 64.29 | Tween 80 | PEG4000/ Ethanol | 1:1:1 | 1:9 |

CHARACTERISATION OF PHYSICO-CHEMICAL
PROPERTIES

The quantity of FA encapsulated within the NE was calculated by using Formula 1 below. It was found to be within the range of 92-99%. Both formula that contained PEG 4000 and ethanol (FA-NE9 and FA-NE17) showed the highest encapsulation as compared to the others, and this may be attributed to the entanglement of the PEG 4000 that helped in the encapsulation of drug molecules within the NE droplets. The difference in the oil content (3.33% in FA-NE9 as compared to 7.14% in FA-NE17) however, did not show any influence on the drug content percentage between the two formulations. Table 4 summarises the characteristics of the NE produced by each formulation.

$$\text{Encapsulation efficiency (\%)} = \frac{\text{Amount of drug incorporated (mg)} - \text{Amount of free drug (mg)}}{\text{Total amount of drug incorporated (mg)}} \times 100 \quad (1)$$

Droplet size, polydispersibility index (PDI), and zeta potential (ζ -potential) are the important characteristics of NE. The droplet size may be influenced by several factors that include the composition of materials and the preparation method, among others. As shown in Table 4, FA-NE1, FA-NE3, FA-NE5, FA-NE6, FA-NE9 and FA-NE10 gave oil droplet sizes of below 20 nm, which may be due to the low oil composition. FA-NE13 and FA-NE17 with the highest composition of oil showed significantly higher droplet sizes as compared to the others. This is attributed to the oil components concentration, as this was similarly obtained for both NE with PG (FA-NE13) and PEG (FA-NE17) as part of the Smix.

ζ -potential is a measure of the magnitude of electrostatic or charge repulsion or attraction between particles and may serve as a partial indicator for the physical stability of the NE. The ζ -potential values differ between formulas, ranging from -16.1 to -24.7 mV (Table 4). ζ -potential was also found to be highly influenced by the type of surfactants used in the formula. The presence of PG or PEG 4000 reduces the surface charges. FA-NE1 which contains only Tween 80 showed a higher surface charge (-24.7 mV) as compared to the formulas with PEG 4000 and PG. This is due to the shielding of the droplets by the surfactants, causing the reduction in the absolute charge value (Devalapally et al. 2013). A high surface charge ensures a stable NE, as it creates a high-energy barrier against coalescence of the dispersed droplets ((Kale & Deore 2016). In general, droplets with a surface charge of $>+25$ mV or <-25 mV will have a high stability, while those at lower dispersion values will be more susceptible to coalescence, which may lead to

emulsion cracking or creaming (Shnoudeh et al. 2019). The surface charges of FA-NE formulas prepared in this study gave an initial idea that the risk of coalescence may be higher, and this was subsequently evaluated in the stability study.

Optimised nanoemulsion showed PDI values of lower than 0.5. PDI of the NE droplets were aimed to be less than 0.5 to ensure that a monodisperse system would be obtained. Products with highly polydisperse droplets or internal phase present major hurdles in drug diffusion. In addition, polydispersity will also cause inconsistencies in drug absorbance and hence difficulties in predicting treatment response. The opacity of NE depends on their droplet size and would usually be transparent or slightly turbid. This optical property is usually determined by measuring light transmission. The FA-loaded NE obtained in this study showed light transmission between 81.37 ± 0.47 % and 96.47 ± 0.76 % which means that the preparations are clear and transparent (Rokad et al. 2014). The clarity of NE could be estimated by measuring the refractive index of the formulations. In the present study, the refractive index of distilled water was used as a comparison to the prepared formulations. As shown in Table 4, the refractive index of the NE was between 1.3 and 1.4 at 25 ± 0.5 °C. Hence, the formulations appeared nearly transparent in the visible spectrum and exhibited minimal light scattering effect (Rokad et al. 2014).

Formulation for topical application should match the pH of the skin, which in general ranges from 4 to 6. Products with alkaline pH would tend to cause skin irritation and subsequently bacterial infections upon continuous usage (Teo et al. 2015). Lucero et al. (1994) suggested that topical products should have a pH of between 4 and 6.5 to avoid any skin irritation. The pH values of all formulations were within this suggested limit, except for FA-NE1 (pH 7.47), which was prepared with Tween 80 as a single surfactant. This means that the quantity of Tween 80 should be well moderated to ensure that the pH of the product is acceptable for topical application. Elfiyani et al. (2017) suggested that the presence of ethanol as co-surfactant to Tween 80 may reduce the pH of a microemulsion due to the oxidation of the alcohol into carboxylic acid (Maddela et al. 2017). However, this could not be corroborated in the current study. The pH value of FA-NE3 and FA-NE5 were not significantly different ($p < 0.05$) although ethanol was present in FA-NE5. TEM analysis showed that the lipid emulsion droplets were almost spherical and homogenous (Figure 4). This finding confirms that the droplets dispersed in the NE system are in the nanometre range, between 30 and 100 nm.

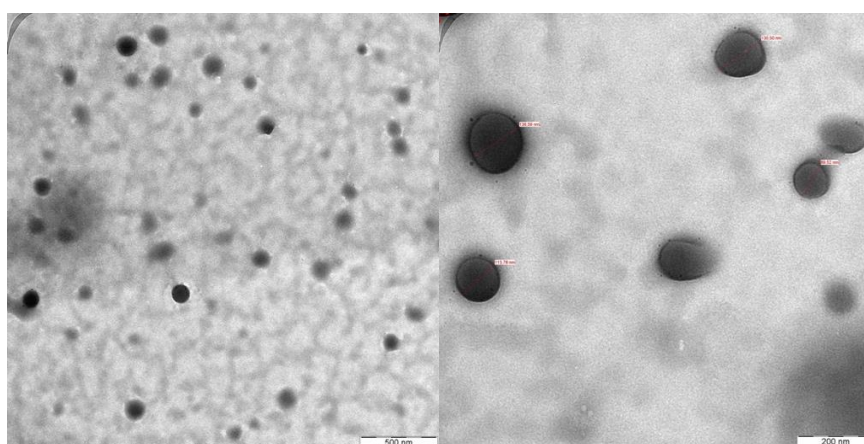


FIGURE 4. NE droplets as observed under TEM

TABLE 4. Physical characteristics of the produced NE

| Formula | Transmittance (%) | Particle size (nm) | PDI | ζ - potential (mV) | pH | Refractive index |
|---------|-------------------|--------------------|-------------------|--------------------------|-----------------|--------------------|
| FA-NE1 | 95.4 \pm 0.74 | 16.4 \pm 0.10 | 0.232 \pm 0.004 | -24.7 \pm 1.66 | 7.47 \pm 0.05 | 1.439 \pm 0.0016 |
| FA-NE3 | 89.9 \pm 0.86 | 17.5 \pm 2.15 | 0.213 \pm 0.022 | -21.4 \pm 9.54 | 6.39 \pm 0.03 | 1.413 \pm 0.0025 |
| FA-NE5 | 81.6 \pm 0.57 | 16.9 \pm 0.93 | 0.234 \pm 0.015 | -19.8 \pm 0.35 | 6.22 \pm 0.01 | 1.402 \pm 0.0011 |
| FA-NE6 | 82.8 \pm 0.55 | 11.4 \pm 1.08 | 0.214 \pm 0.011 | -16.1 \pm 0.29 | 6.07 \pm 0.02 | 1.390 \pm 0.0026 |
| FA-NE9 | 81.4 \pm 0.47 | 17.0 \pm 1.08 | 0.214 \pm 0.006 | -19.3 \pm 1.71 | 6.22 \pm 0.02 | 1.375 \pm 0.0013 |
| FA-NE10 | 84.4 \pm 1.29 | 13.2 \pm 0.48 | 0.235 \pm 0.006 | -16.7 \pm 0.80 | 6.02 \pm 0.02 | 1.366 \pm 0.0011 |
| FA-NE13 | 89.5 \pm 0.26 | 87.8 \pm 5.68 | 0.274 \pm 0.015 | -18.4 \pm 1.03 | 6.22 \pm 0.02 | 1.400 \pm 0.0018 |
| FA-NE17 | 96.5 \pm 0.76 | 97.0 \pm 1.92 | 0.262 \pm 0.015 | -17.5 \pm 0.90 | 6.27 \pm 0.02 | 1.379 \pm 0.0028 |

ANTIBACTERIAL STUDY

The antibacterial test was conducted against MSSA and MRSA and the results were compared to the commercially available fusidic acid cream. The effectiveness of all formulations in inhibiting the bacterial growth was determined through measuring the inhibition zone, as presented in Table 5.

When compared to the marketed medication, fusidic acid nanoemulsion demonstrated a significant improvement ($p < 0.05$) and a larger zone of inhibition in antibacterial activity. Several factors may have contributed to these results. First, due to the nano-size of the droplets, NE has a bigger surface area and could penetrate deeper, hence improving the activity (Zhang

et al. 2010). For instance, Marslin et al. (2015) tested the effect of *Withania somnifera* cream mixed with silver nanoparticles. They reported an increased in the penetration, which resulted in a greater suppression of bacterial growth.

The inclusion of tea tree oil, which has been widely reported to possess anti-microbial activity against Gram-negative and Gram-positive bacteria and fungi, is also responsible in the improvement of bacterial inhibition by the formulated NE. Tea tree oil has been used in numerous skincare products for its antibacterial effects, which are principally due to the presence of terpinen-4-ol and α -caryophyllene (Farag et al. 2004).

TABLE 5. The inhibition zone against MRSA and MSSA and half-maximal inhibitory concentration (IC₅₀) against human skin HS-27 cells of the NE formulations (n>3, ±SD)

| Formula | Zone of inhibition (cm) | | Half-maximal inhibitory concentration (IC ₅₀) (µg/mL) |
|--|-------------------------|----------|--|
| | MSSA | MRSA | |
| FA-NE1 | 4.8±0.23 | 4.4±0.17 | – |
| FA-NE3 | 4.8±0.29 | 4.6±0.17 | – |
| FA-NE5 | 5.1±0.36 | 4.6±0.28 | 313.6 |
| FA-NE6 | 5.0±0.50 | 4.5±0.00 | 132.2 |
| FA-NE9 | 4.3±0.82 | 4.5±0.30 | 66.6 |
| FA-NE10 | 4.8±0.58 | 4.6±0.17 | 99.9 |
| FA-NE13 | 5.5±0.26 | 5.3±0.35 | 211.5 |
| FA-NE17 | 5.1±0.79 | 4.5±0.00 | 44.6 |
| Tee Tree oil In Emulsion without FA | 2.7±0.41 | 2.5±0.00 | – |
| FA cream | 3.7±0.12 | 3.7±0.12 | 42.7 |

CELL VIABILITY ASSAY

The cytotoxicity of FA-E was expressed as the half maximal inhibitory of concentration (IC₅₀). The lower the IC₅₀ value, the higher the cytotoxic effect of the sample (Rivero-Cruz et al. 2020). The results of cell viability assay are also shown in Table 5. It was noted that the cells treated with formulations containing PEG 4000 as co-surfactant (FA-NE9, FA-NE10, FA-NE17) showed lower viability as compared to those with PG (FA-NE5, FA-NE6, FA-NE13). This may suggest that the formulations with PG could have a better safety profile and lower toxicity, which is preferred to be further evaluated as a potential antibacterial NE. The cell viability following the treatment with commercial FA cream showed lowest IC₅₀ value as compared to the FA-NEs which suggest better safety profiles of the FA-NEs.

CONCLUSIONS

In the present study, FA-loaded NE was prepared successfully. Tween 80, PG and PEG 4000 gave the NE with the best physical characteristics. The antimicrobial activity of the NE formulation against MRSA and MSSA and the cytotoxicity on the HS-27 cells suggests that the formula containing Tween80/PG as surfactants showed better profile with promising antibacterial and cytotoxicity properties. As the FA-NE prepared is

meant for antimicrobial activity on the chronic wound and not for deep skin penetration, the delivery of the formulation to the microorganisms will determine its effectiveness. At the same time, the safety of the surrounding healthy skin cells should be preserved, and this is more potentially suitable with PG as the surfactant in the FA-NE formulations, as suggested in the cell viability assay on HS-27 cells. Hence, FA/tea tree oil with Tween80/PG NE has the potential to be further developed for the treatment of chronic wounds.

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