

Water-in-oil microemulsions exhibit antimicrobial activity

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Abstract

Objectives: Previous research from this group has identified significant antimicrobial activity associated with oil-in-water (O/W) microemulsions. This activity has been exhibited against both bacteria and fungi (including yeasts) and bacterial biofilms and is dependent upon the position of the microemulsion within its stability zone. This novel work aims to exhibit antimicrobial activity of water-in-oil (W/O) microemulsions.

Materials & Methods: A simple, thermodynamically stable water-in-oil (W/O) microemulsion was tested for its time-related antimicrobial activity against a selected panel of four test microorganisms (i.e.: *Pseudomonas aeruginosa* ATCC 9027, *Escherichia coli* ATCC 8739, *Candida albicans* ATCC 10231 and *Staphylococcus aureus* ATCC 6538P) and its effectiveness as a self-preserving system against a similar panel (*Pseudomonas aeruginosa* ATCC 9027, *Candida albicans* ATCC 10231, *Staphylococcus aureus* ATCC 6538P and *Aspergillus niger* ATCC 16404).

Results: The microemulsion exhibited significant antimicrobial activity against all the selected microorganisms. Decreases in the viability of cultures (*P. aeruginosa*, *C. albicans*, *E. coli* and *S. aureus*) were observed over a short period of time after exposure to a known concentration of the first microemulsion. According to the European Pharmacopeia, the results of the preservative effectiveness test require a significant reduction in bacterial count, and this requirement was achieved against all test microorganisms.

Conclusions: Thermodynamically stable water-in-oil microemulsions are antimicrobially active self-preserving systems, as are their oil-in-water counterparts.

Keywords: *Pseudomonas aeruginosa*, microemulsion, self-preserving, antimicrobial, biocide

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Introduction

In recent years there has been a dramatic increase in the utilization of microemulsion systems in pharmaceutical systems, largely as carrier and delivery systems. However, there are few studies on their antimicrobial activity *per se*, but researchers have shown an increased interest in this field. The possibility of microemulsions having an antimicrobial action was suggested by Friberg [1], who also observed that bacteria cannot survive in pure fat or oil and that water is necessary for their growth and reproduction. In microemulsions the water present is efficiently bound by the structure of the microemulsion and, for that reason, access to the water by microorganisms is limited [1]. Friberg's comments suggested the hypothesis that the chemical structure of thermodynamically stable microemulsions is harmful to microbial cells. Our group eventually proved this hypothesis correct [2]. This work [2] concluded that microemulsions are stable, self-preserving antimicrobial agents with highly effective killing rates against *Staphylococcus aureus* and the particularly resistant bacterial species *Pseudomonas aeruginosa*, and that microemulsions have an effect on the structure and performance of the microbial cytoplasmic membrane.

Our group also undertook further studies on microemulsions as anti-biofilm agents by testing the viability of established biofilm cultures of *P. aeruginosa* for 4 h. The results exhibited a three log-cycle (1000-fold) reduction of viability in the microemulsion treated biofilms and a one log-cycle (10-fold) reduction in the viability of similar biofilms treated with planktonic MICs of sodium pyrithione and cetrime for the same period [3]. These results indicate that microemulsions have a potential role in treatment of industrial or environmental biofilms [3]. Some of our earlier observations about biofilms [3] were confirmed by Teixeira *et al.* [4], who extended the investigations to other pathogens, particularly the ones more commonly found in foods and on

food processing equipment. Recently Alkhatib *et al.* [5] have confirmed that microemulsions are both anti-fungal and anti-viral agents.

All previous published studies on the antimicrobial nature of microemulsions have utilized oil-in-water (O/W) microemulsions. The objective of this study was to investigate the antimicrobial activity of a water-in-oil (W/O) microemulsion systems by preparing two microemulsion formulae ME1 (12.6% Tween 80, 56.3% propylene glycol, 28.6% isopropyl myristate, 2.5% sterile distilled water) and ME2 (27.5% Tween 80, 27.5% span80, 40% peanut oil, 5% sterile distilled water) and subsequently, to test the physical stability of these microemulsions, their antimicrobial activity and their effectiveness as self-preserving systems.

Materials and Methods

Chemical reagents. Tween 80, Iso-Propyl myristate (IPM), propylene glycol (PG), Span 80, Peanut oil (Sigma Aldrich, Germany).

Preparation of the microemulsions. Two Microemulsion systems were prepared, **Microemulsion 1 (ME1; Table 1)**. Water in oil (W/O) microemulsion system was prepared by slowly titrating weighed amounts of the three components mixture (surfactant, co-surfactant and oil) with water at room temperature ($20\pm 2^\circ\text{C}$). Mixtures were prepared by mass in sterile screw glass tubes. Aqueous surfactant (3g of Tween 80) and co-surfactant (13.4g of propylene glycol) were dissolved in the oil phase (6.8g of IPM). The mixture was then titrated with double distilled water (0.6g). The titration end point was marked by an overnight persisting turbidity. ME1 is used as a drug delivery system for ophthalmic preparations. We used the formula as it is without addition of the ophthalmic active ingredient.

Table 1. Composition of microemulsion (taken from Radomska & Dobrucki, 2000)

Formula	Composition (g)	% of component (w/w)
Tween 80	3g	12.6
Propylene glycol	13.4g	56.3
Iso-Propyl Myristate	6.8g	28.6
H ₂ O (sterile, double distilled)	0.6g	2.5

Microemulsion 2 (ME2; Table 2). Suitable K_m (surfactant/cosurfactant ratio) values were determined by drop-wise titration of surfactant-oil-water mixture with the related cosurfactant. After each addition, the mixture was vortexed and observed visually. The end point of the titration process was the conversion of the turbid surfactant-oil-water mixture to a transparent microemulsion. The K_m value was calculated when stable microemulsion was obtained and no phase separation was observed upon standing.

A water-in-oil microemulsion (W/O) containing (peanut oil / H₂O / 1:1 Tween 80: Span 80) was prepared by water titration method. Firstly, a Tween80 and span80 mixture of the K_m ratio 1:1 was prepared in a sterile screw glass tube and mixed using a vortexer. Subsequently, the peanut oil was added

to this mixture. Various oil to tween80:span80 (1:1) mixtures were prepared at ratios of (i) 2.6:2.18, (ii) 2:2.75 and (iii) 2.6:2.2 and placed in screw glass tubes. The three components were then titrated with water to give the final stable microemulsion. The titration end point was marked by an overnight persisting turbidity.

Inspection of physical stability of microemulsion. Microemulsion 1 was tested for stability by means of repeated centrifugation for 30 min at 13000 rpm (Z300, Hermle LaborTechnik GmbH, Germany), followed by visual observation for any phase separation. For long term stability studies, suitable volumes of Microemulsion 1 were placed in sterile screw-top glass tubes and stored for long periods at room temperature (6 month) in darkened cupboards and then visually observed for any phase separation [6].

Microemulsion 2. Microemulsion stability was tested by means of centrifugation for 30 minutes at 13000 rpm (Z300, Hermle LaborTechnik GmbH, Germany) followed by visual observation for phase separation. Subsequently, an oil soluble stain (Sudan black) was added to the microemulsion in small amounts and mixed well. The ME2 / stain mix was then centrifuged for 20 min. at 4000 rpm (Z300,

Table 2. The composition of Microemulsion 2 (ME2). 'Initial' indicates the composition of microemulsion with zero % of water and the 'final' indicates the composition of microemulsion mixture with water (after titration)

Sample Number	Initial / Final	H ₂ O (w/w %)	Peanut Oil (w/w %)	Tween 80 (surfactant) (w/w %)	Span 80 (cosurfactant) (w/w %)
1	Initial	0	54.6	22.7	22.7
	Final	4.4	52.2	21.7	21.7
2	Initial	0	42.1	28.95	28.95
	Final	5	40	27.5	27.5
3	Initial	0	36.2	31.9	31.9
	Final	6	34	30	30

Hermle LaborTechnik GmbH, Germany) after which the dispersal of the stain should be uniform in all parts of the microemulsion. For long term stability studies, suitable volumes of Microemulsion 2 were placed in sterile screw-top glass tubes and stored for long periods at room temperature (6 month) in darkened cupboards and then visually observed for any phase separation [6]. ME2 was a novel microemulsion formula for a thermodynamically stable water in oil microemulsion. In order to test that the water phase was properly internalized within the microemulsion, the water used was initially stained with the use of a water soluble stain (methyl orange). The stain disappeared as the microemulsion formed, hence showing that water was the internal phase.

Microorganisms and culture maintenance.

Pseudomonas aeruginosa ATCC 9027, *Escherichia coli* ATCC 8739, *Candida albicans* ATCC 10231, *Staphylococcus aureus* ATCC 6538P and *Aspergillus niger* ATCC 16404 were used in this series of experiments.

Cultures of *C. albicans* ATCC 10231 were cultured on Sabouraud dextrose agar (SDA; Oxoid, England) plates and incubated at 20°C for 48 h after which they were stored until use in a dark cupboard at room temperature (20±2°C).

Cultures of *S. aureus* ATCC 6538P, *P. aeruginosa* ATCC 9027 and *E. coli* ATCC 8739 were grown on tryptic soya agar (TSA; Oxoid, England) plates and incubated at 37 °C for 48h after which they were stored until use in a dark cupboard at room temperature (20±2°C). Fresh cultures of each strain were prepared every month and their morphological characteristics were confirmed by macroscopic and microscopic examination. All culture media were sterilized by autoclaving at 121°C, 15psi for 15 min.

For *Aspergillus niger* ATCC 16404, overnight broth culture (0.2 ml) was spread onto the surface of SDA plate, after which the plate was incubated for five days at 20°C. Fungal culture was harvested

by washing the surface of the culture plate with a small volume of sterile distilled water. The harvested microorganisms (largely spores) were centrifuged at 6000rpm for 15 min (Z300, Hermle LaborTechnik GmbH, Germany), after which the pellet was washed twice with sterile distilled water and then centrifuged at 4000 rpm for 15 min. (Z300, Hermle LaborTechnik GmbH, Germany). The final pellet was re-suspended in sterile distilled water until use. The viable count method [7] was then used to determine the final spore count.

The determination of the kinetics of killing

Overnight broth cultures of *S. aureus*, *P. aeruginosa* and *E. coli* were prepared using nutrient broth and *C. albicans*, was prepared in Tryptic soya broth. The cultures were incubated at 37°C for bacteria and at 20°C for fungi. An inoculum of *A. niger* was prepared by the method mentioned above. Aliquots (1 ml) of these cultures with known inoculum sizes as follows, *P. aeruginosa* (1.45×10^7 CFU ml⁻¹), *E. coli* (1.54×10^7 CFU ml⁻¹), *C. albicans* (8.1×10^7 CFU ml⁻¹), *S. aureus* (1.09×10^6 CFU ml⁻¹) were challenged by addition to a volume (9 ml) of the ME1. Test samples (1 ml) were removed from each challenge test at seven time intervals (15 sec., 30 sec., 45 sec., 1 min., 2 min., 3 min. and 5 min.) for each of the five microorganisms and neutralized by addition to 9 ml of sterile distilled water. The viable count of each neutralized test sample was then determined by performing a serial dilution and plating out aliquots (0.1ml) onto appropriate solid medium (SDA or TSA) plates in triplicates.

For ME2 aliquots (10 µl) of overnight cultures of each of the test species with known inoculum sizes as follows, *P. aeruginosa* (2.8×10^6 CFU ml⁻¹), *E. coli* (1.28×10^6 CFU ml⁻¹), *C. albicans* (8.5×10^6 CFU ml⁻¹) and *S. aureus* (3×10^8 CFU ml⁻¹) were challenged by addition to separate volumes (10 ml) of the microemulsion. Subsequently, test samples (1 ml) were taken at four time intervals (10 min., 30 min., 1 h.,

24 h.) for *E. coli* and *P. aeruginosa* and at seven time intervals (10 min., 30 min., 1 h., 2 h., 3 h., 4 h., 5 h.) for *S. aureus* and *C. albicans* and neutralized by addition to 9 ml of sterile distilled water. The viable count of each neutralized test sample was then determined by performing a serial dilution and plating out aliquots (0.1ml) onto appropriate solid medium (SDA or TSA) plates in triplicates. Positive control was done by replacing the microemulsion with sterile broth.

The neutralization step was performed by taking an aliquot (1 ml) of test sample and adding it to sterile distilled water (9 ml). The mixture became turbid and this is an indication that the structure of microemulsion has ceased to exist and that there should be no further antimicrobial activity.

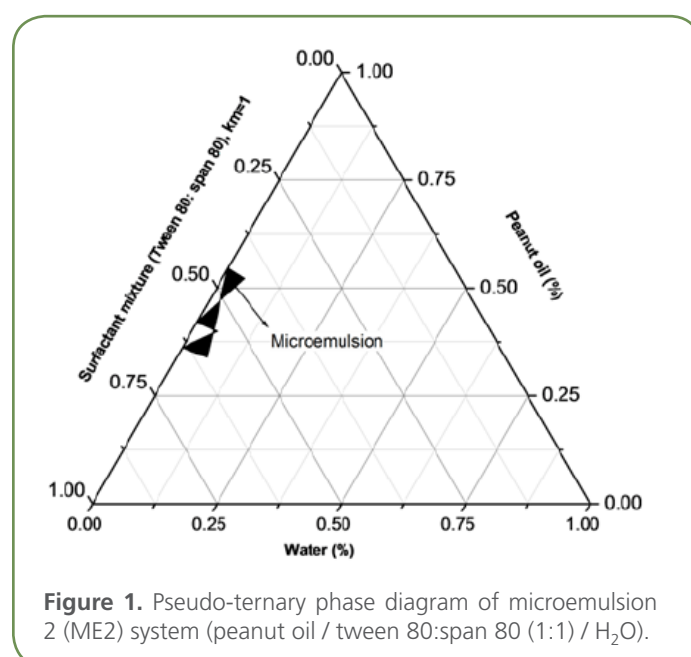
Preservative effectiveness test. Four different microorganisms (*S. aureus*, *P. aeruginosa*, *C. albicans* and *A. niger*) were inoculated onto TSA for bacteria and SDA for fungi. The cultures of bacteria were incubated at 37°C for 18 to 24 h, the culture of *C. albicans* at 20 to 25 °C for 48 h, and the culture of *A. niger* at 20 to 25 °C for 1 week or until good sporulation was observed. The microorganisms were then harvested by washing the surface of the culture with sterile distilled water and centrifugation at 4000 rpm for 15 min. (Z300, Hermle LaborTechnik GmbH, Germany). The pellet was washed three times and finally suspended in sterile normal saline (0.9% w/v). An aliquot (0.1 ml) was removed from each suspension in order to determine the viable count (CFU ml⁻¹) of each suspension. A count of 10⁷ CFU ml⁻¹ was prepared in a final volume of 10 ml for the preservative effectiveness test.

ME1 was added to a series of sterile glass screw-cap containers (20 ml volume), each with a 1 : 10 suspension (1 ml of microbial culture in 9 ml of microemulsion) of one of the microorganisms to be tested, within a final inoculum count of 10⁵ CFU in 10 ml of the microemulsion. The inoculated containers were stored at 20 to 25°C and protected from light.

Aliquots (1 ml) were taken after 2 days, 7 days, 14 days and 28 days and the residual antimicrobial activity of the microemulsion was neutralized by two-fold serial dilution using sterile distilled water. Test samples (0.1ml) were cultured on TSA for bacteria and SDA for fungi and incubated at 37°C for 18 to 24 h for bacteria and 20 to 25°C for 48 h for *C. albicans* and at 20 to 25 °C for 3 days for *A. niger*. The total number of surviving microorganisms for each test treatment was determined.

Results

Microemulsion existence area. Table 2 presents the composition of initial and final points of the titration for ME2. The initial points characterize different weight ratios of peanut oil and tween 80: span 80 mix (1:1) mixtures with zero percentage weight of water. While final points are characterized by the weight ratios of the microemulsion components, peanut oil, tween 80: span 80 mix (1:1) and H₂O, at the end of the titration process. The value of the final points is used in the construction of phase diagram shown in **Figure 1**.



Kinetics of killing of the microemulsion. Kinetics of killing of Microemulsion 1 (ME1).

Decreases in the viability of cultures *P. aeruginosa*, *C. albicans*, *E. coli* and *S. aureus* were observed over a short period of time after exposure to a known concentration of ME1 (Figure 2), which gives clear indication of biocidal activity. The viability decreased rapidly until no viable cells were observed after 1 min. for *P. aeruginosa* and *C. albicans*. The LT90% value (the time taken to kill 90% of all cells present at the beginning of the exposure period) for *P. aeruginosa* was 21 sec. and 36 sec. for *C. albicans*. For *E. coli*, the viability decreased rapidly yielding a LT90% value of 12 sec. The viability for *S.*

aureus decreased by 6 log-cycles (a reduction in viability of greater than 6 logarithmic (base 10) cycles or 1,000,000-fold) over a period of 2 min. and the LT90% value for *S. aureus* was 60 sec.

Kinetics of killing of microemulsion 2 (ME2).

Decreases in the viability of cultures *P. aeruginosa*, *C. albicans*, *E. coli* and *S. aureus* were observed after exposure to a known concentration of ME2 (Figure 3), which shows higher activity against Gram-negative bacteria than that against Gram-positive bacteria or fungi. The viability decreased until no viable cells were observed after a period of 1 h for *P. aeruginosa* and *E. coli*. The LT90% value for *P.*

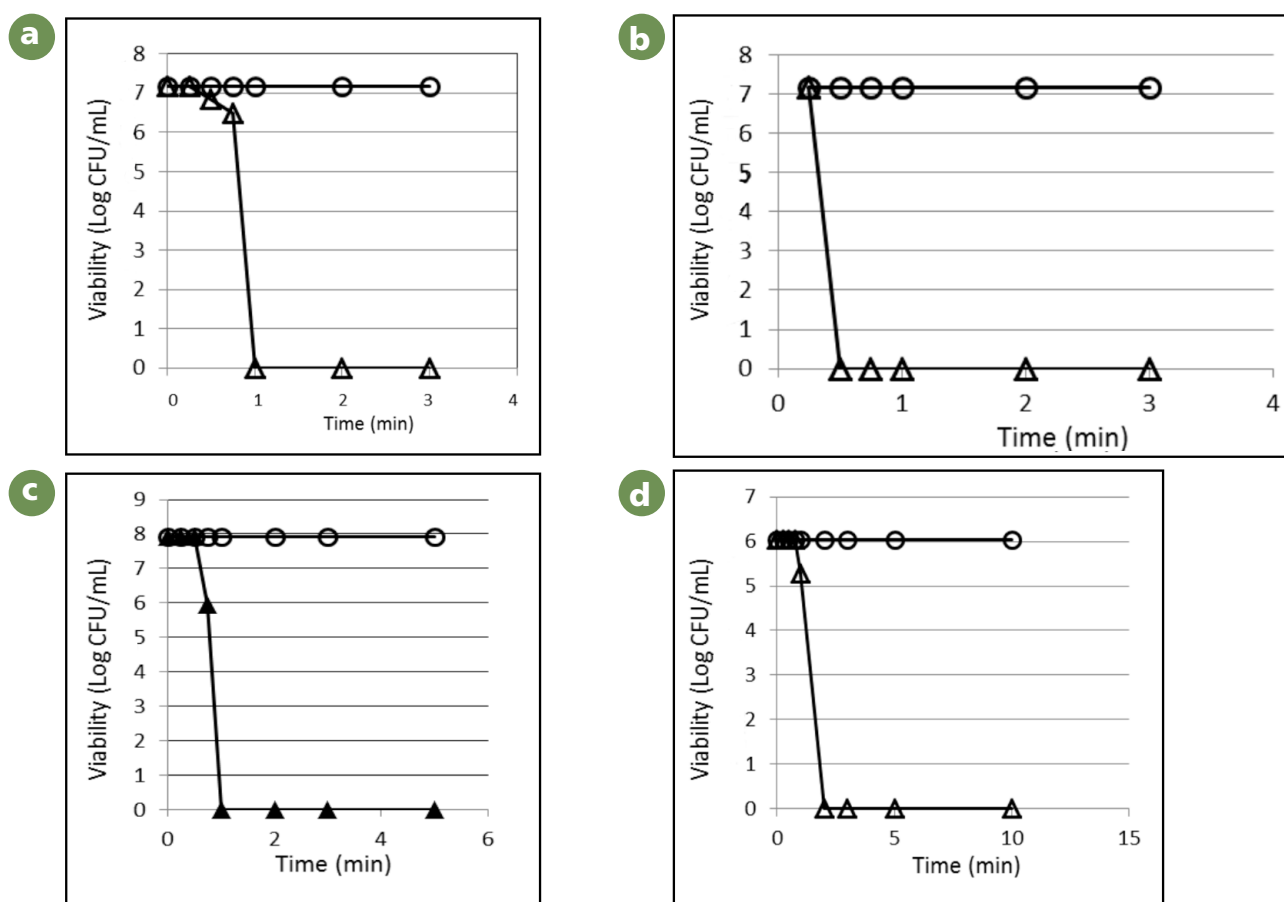


Figure 2. Time exposure viability curves for the addition of microemulsion 1 (ME1) formula to cultures of (a) *Pseudomonas aeruginosa* (viable count 1.45×10^7 CFU ml^{-1}); (b) *Escherichia coli* (viable count 1.54×10^7 CFU ml^{-1}); (c) *Candida albicans* (viable count 8.1×10^7 CFU ml^{-1}); (d) *Staphylococcus aureus* (viable count 1.09×10^6 CFU ml^{-1}). O = Controls cultures. Δ = Test cultures.

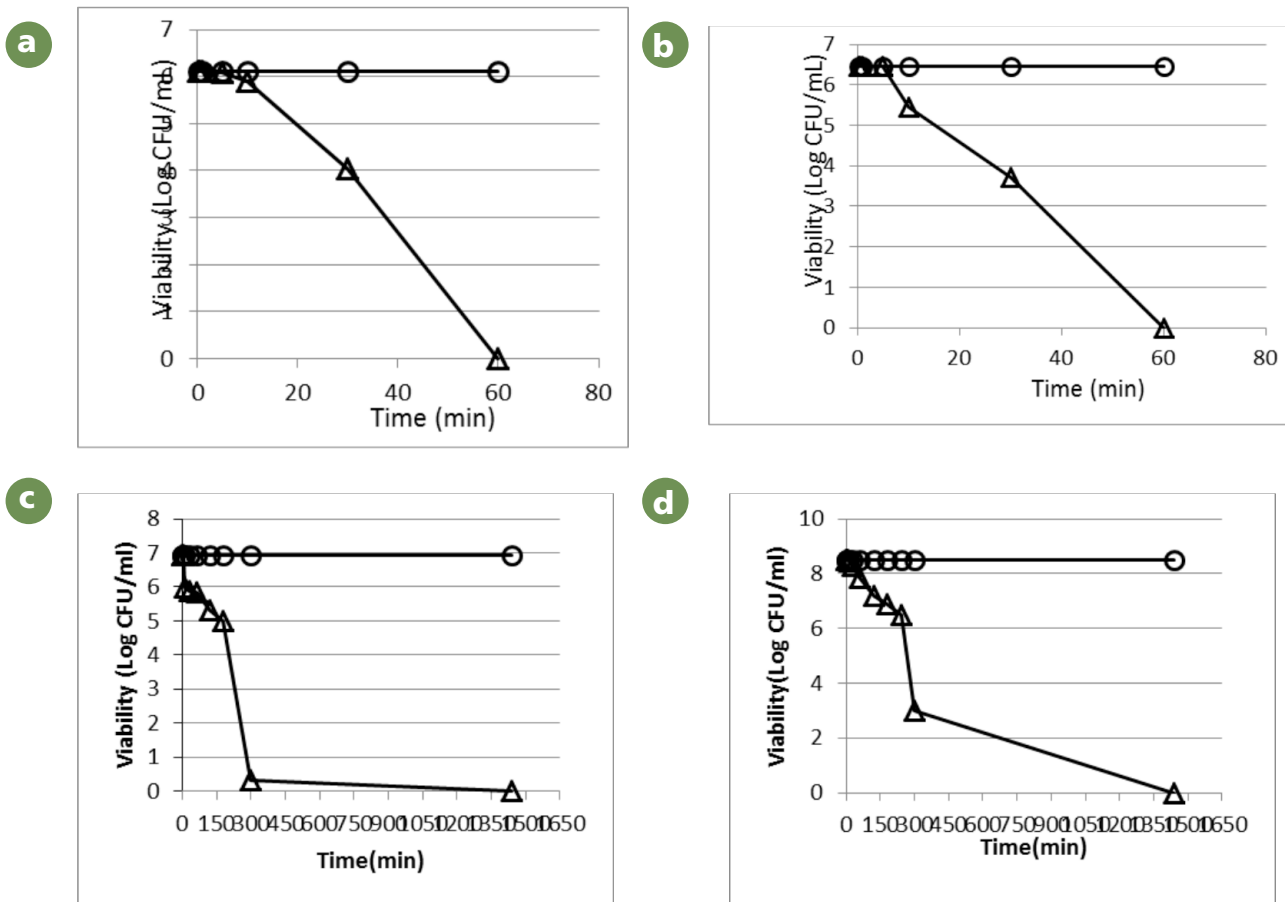


Figure 3. Time exposure viability curves for the addition of microemulsion 2 (ME2) formula to cultures of (a) *Pseudomonas aeruginosa* (viable count 2.8×10^6 CFU ml^{-1}); (b) *Escherichia coli* (viable count 1.28×10^6 CFU ml^{-1}); (c) *Candida albicans* (viable count 8.5×10^6 CFU ml^{-1}); (d) *Staphylococcus aureus* (viable count 3×10^8 CFU ml^{-1}). O = Controls cultures. Δ = Test cultures.

aeruginosa was approx. 19 min. and for *E.coli* was approx. 10 min. While for *C. albicans*, the viability decreased over a period of 300 min. and the LT90% value was approx. 30 min. the viability for *S. aureus* decreased by 5 log-cycles (a reduction in viability of greater than 5 logarithmic (base 10) cycles or 100,000-fold) over a period of 300 min. and the LT90% value for *S. aureus* was approx. 90 min.

Preservative effectiveness test for ME1. Table 3 shows the number of colonies in the tested pharmaceutical samples of the preservative effectiveness test for topical preparations. The results for the first sampling in the preservative effectiveness test is shown in Table 3, the European Pharmaco-

peia requires a 2 log-cycle (a reduction in viability of greater than 2 logarithmic cycles or 100-fold) reduction in bacterial count, and this requirement is achieved. The results for the second stage of preservative effectiveness test are shown in **Table 3**, the European Pharmacopeia requires a 3 log-cycle (a reduction in viability of greater than 3 logarithmic (base 10) cycles or 1000-fold) reduction in bacterial count, and this requirement was achieved. The results for the third sampling of preservative effectiveness test is shown in Table 3, the European Pharmacopeia requires a 2 log-cycle (a reduction in viability of greater than 2 logarithmic cycles (base 10) or 100-fold) reduction in fungal counts, and this requirement was achieved. The results for the

Table 3. Preservative test results for Microemulsion 1 (ME1) showing the number of colonies in the tested pharmaceutical samples of the preservative effectiveness test for topical preparations (Where; T = Test; C= Control; NG = no growth; - = not done).

Microorganism Time interval	Staphylococcus aureus		Pseudomonas aeruginosa		Candida albicans		Aspergillus niger	
	T	C	T	C	T	C	T	C
After 2 days	NG	2.5x10 ⁶	NG	3.6x10 ⁶	-	-	-	-
After 7 days	NG	2.4x10 ⁶	NG	3.2x10 ⁶	-	-	-	-
After 14 days	NG	2x10 ⁶	NG	2.7x10 ⁶	NG	5.5x10 ⁶	NG	8.2x10 ⁶
After 28 days	NG	1.4x10 ⁶	NG	2.3x10 ⁶	NG	5.4x10 ⁶	NG	7.6x10 ⁶

fourth sampling of preservative effectiveness test is shown in Table 3, the European Pharmacopeia requires no increase in bacterial and fungal counts, and this requirement was achieved.

Discussion

The results in **Figure 2** show that the viability of the microorganisms against ME1 was decreased rapidly as follows: (i) For *P. aeruginosa* within one min. and no viable cell was detected which indicate a 7 log-cycle reduction and the LT90% value 21 sec. (ii) For *E. coli* the viability decreased rapidly over a shorter period of time (15 sec.), also exhibiting a 7 log-cycle (a reduction in viability of greater than 7 logarithmic (base 10) cycles or 10,000,000-fold) reduction and LT90% value <12 sec. (iii) *C. albicans* viability decreased in a period similar to that for *P. aeruginosa* of approximately 1 min. and gave a 7 log-cycle reduction and LT90% value of 36 sec. (iv) While *S. aureus* required 2 min. for complete killing, indicating a 6 log-cycle reduction and LT90% value of 60 sec.

The results obviously indicate that the ME1 formula is an effective antimicrobial system and it is clear that it is more effective on Gram-negative bacteria and fungi than on Gram-positive bacteria. The

results of kinetics of killing for *P. aeruginosa*, *E. coli* and *C. albicans* indicate a 7 log-cycle reduction in a period of less than 1 min., while the results of kinetics of killing for *S. aureus* exhibit a 6 log-cycle reduction within 2 min. LT90% values show clearly that *E. coli* is the most sensitive microorganism to ME1, while *S. aureus* is the most resistant. These differences may be due to the differences in the cell wall structure between Gram-positive and Gram-negative bacteria. Gram-negative bacteria possess an outer membrane containing lipopolysaccharide (LPS, which consists of lipid A, core polysaccharide, and O antigen) in its outer leaflet and phospholipids in the inner leaflet [7]. The structure of the W/O microemulsion possesses an external phase which is oily and hence, it diffuses faster into the Gram-negative bacterial cells compared to Gram-positive bacteria which lacking lipids in their cell wall structure.

The results in Figure 3 show the effect of ME2 on the viability of microorganisms. A 6 log-cycle reduction in the viabilities of *P. aeruginosa* and *E. coli* in a period of 1h and the LT90% value was approximately 19 min. for *P. aeruginosa* and approximately 10 min. for *E. coli*. While for *C. albicans* the viability decreased in a period of 5 h, it required between 6 h and 24 h for complete killing and that indicate a 6 log-cycle reduction and LT90% value was approx.

30 min. Similar results were obtained for the effect of ME2 against *S. aureus* (Figure 4d), which shows a 5 log-cycle reduction in 5 h and between 6 h and 24 h until no viable cells were detected and LT90% value was approximately 90 min.

These results clearly indicate that ME1 formula has a greater antimicrobial activity than ME2. This variation between the two formulae may be due to the type of oil used. In ME1 the oil was synthetic (IPM), while in ME2 the oil was natural (Peanut oil). The peanut oil (103 Cst) is 10 times more viscous than IPM (6.5 Cst), therefore peanut oil can diffuse slowly through the cell wall of microorganisms. The two microemulsions used in this paper were W/O microemulsions (i.e.: the external phase is oil). The two oils used were of different viscosities (IPM = 6.5 Cst and peanut oil + 103 Cst). The large difference in viscosities between the two oils clearly affects their interaction with the cell surface and hence, the penetration of the oil to the target site of the microorganisms. Our results are in accordance with Rashid [8, unpublished], in which a W/O microemulsion prepared using a synthetic oil (IPM) exhibited high bactericidal activity. The work of Orhan *et al.* [9] showed that peanut oil itself has an antimicrobial activity against *S. aureus* ATCC25923, *E. coli* ATCC3528, *C. albicans* ATCC10231 and *Candida parapsilosis* ATCC22019. However, their study [9] was performed using extracted free oil. In this study we used a microemulsion formula rather than the free oil. Therefore, the formulation of the peanut oil in a microemulsion might mask its natural antimicrobial activity and this supports the previous suggestion that the "very structure of the microemulsion is responsible for the antimicrobial activity" [2]. The reason that ME2 gave weak antimicrobial activity may be due to the position of the microemulsions within their zones of stability as suggested by Al-Adham *et al.* [10], in which they indicated that there is an intimate relationship between the physico-chemical properties of microemulsions, in particular the position of the microemulsions within zones of stabil-

ity (existence zone) and their observed effects as antimicrobial agents [10]. However, their study was performed using an O/W microemulsion. It is clear that ME2 also has antimicrobial activity on Gram-negative bacteria, which is higher than the observed antimicrobial activity on Gram-positive bacteria, but it needs longer time of exposure.

Al-Adham *et al.* [2], using O/W microemulsion (ethyl oleate, Tween 80 and n-pentanol) showed that the microemulsion gave a 5 log-cycle reduction in the viable numbers of *S. aureus* and *P. aeruginosa* in only 45 sec., and an LT90% of 15 sec. [2]. Later, the same group showed that the microemulsions are active against biofilms of *P. aeruginosa* [3], which was later supported by the work of Teixeira *et al.* [4], who observed high rates of antimicrobial activity of microemulsions and nanoemulsions against *P. aeruginosa*, *E. coli*, *S. typhimurium* and *S. aureus* biofilms, with the exception of the biofilm formed by *L. monocytogenes* [4].

The antifungal mechanism of microemulsions was studied by Zhang *et al.* [11], who evaluated the antifungal activity of a prepared Glycerol monolaurate (GML)-loaded microemulsion against *A. niger*, indicating the disruption and dysfunction of fungal cell walls and biological membranes. Additionally, the antimicrobial activity of microemulsions against fungi, such as *C. albicans*, *A. niger*, *Schizosaccharomyces pombe* and *Rhodotorula* spp. has been studied by Al-Adham *et al.* [10], who indicated that a microemulsion (17.3% Tween-80, 8.5% n-pentanol, 5% IPM and 69.2% sterile distilled water) has true biocidal activity against these fungi.

The results of this work exhibit that W/O microemulsions are effective antimicrobial agents. ME1 was chosen to be challenged for testing self-preservation properties of W/O microemulsions. The test was done according to European Pharmacopeia and four types of microorganisms were used (*S. aureus*, *P. aeruginosa*, *C. albicans* and *A. niger*). The results in **Table 3** shows that ME1 is a self-preserving system as suggested by Friberg [1].

Conclusions

In conclusion, this study indicates that ME1 has high biocidal activity over short time intervals against the five selected microorganisms and that it is an excellent self-preserving system. The zone of stability of W/O microemulsions requires further study. However, it is clear that W/O microemulsions are less effective as antimicrobial agent than O/W microemulsions.

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