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The Antimicrobial Activity of Oil-in-Water Microemulsions is predicted by their position within the Microemulsion Stability Zone

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Abstract

Background: It has been shown previously that thermodynamically stable oil-in-water microemulsions have significant antimicrobial activity against planktonic cells and biofilm cells over short periods of exposure. It was the aim of this study to identify whether the position of the microemulsion within the microemulsion stability zone of the pseudo-ternary phase structure predicts the efficiency of the antimicrobial action of the microemulsion.

Methods: Microemulsions were formulated at different points within the microemulsion stability zone. Experiments were performed to observe the kinetics of killing of these microemulsions against selected test microorganisms (*Pseudomonas aeruginosa* ATCC 9027, *Candida albicans* ATCC 10231, *Staphylococcus aureus* ATCC 6538 and *Aspergillus niger* ATCC 16404).

Results: The results give clear evidence of a good biocidal activity for the microemulsion system against the bacterial species (greater than 6 log cycles of reduction in *P. aeruginosa* viability when exposed to microemulsion Formula 1 in less than 15 s.), but a lesser effect against the fungi. Results also indicate that the antimicrobial activity of the microemulsion is dependant upon its position within the zone of stability and is greater nearer the centre of that zone.

Conclusion: The results indicate that significant antimicrobial activity can be observed at all points within the zone of microemulsion stability, but that maximal activity is to be found at the centre of that area.

Key words: microemulsion, antimicrobial activity, stability zone, biocide, *Pseudomonas aeruginosa*

Running Title: Antimicrobial Activity of Microemulsions

Introduction

It has been suggested that microemulsions are self-preserving antimicrobials in their own right (1) . This suggestion is made on the basis that bacteria cannot survive in pure fat or oil and that water is necessary for their growth and reproduction (2). Therefore, it is important to investigate the relationship between the physico-chemical properties of microemulsions and their observed biological and antimicrobial activity. It has been suggested that the molecular and ionic structure of liposomes is harmful to the bacterial cell and in particular, that they adversely affect the structure and function of the bacterial membrane (3-4). As liposomes and microemulsions have structural and ionic similarities, albeit liposomes are particu-

late and microemulsions are not, it is reasonable to suggest a similar mode of action for microemulsions. Previous work by Al-Adham *et al.* (2) clearly indicates the antimicrobial activity of thermodynamically stable oil-in-water microemulsions against bacterial cells within very short periods (<45 sec). This work has been subsequently supported by the observations of Teixeira *et al.* (5). who observed very high rates of antimicrobial activity of microemulsions and nanoemulsions on *Listeria monocytogenes*, amongst other test bacteria. This high level of inherent antimicrobial activity suggests excellent biocidal activity. Such rapid biocidal activity is indicative of direct attack on the structural integrity of the cell, rather than secondary effects via metabolic inhibition (6). This hypothesis was tested by Al-Adham *et al.* (2) by the use of transmission electron microscopy. Electron micrographs of microemulsion-treated cells of *P. aeruginosa*, (20000x magnification) exhibit both extra- and intra-cellular effects.

Findings from some significant recent studies (7-10). on the use of microemulsions as food-grade antimicrobial agents suggest that there may be a link between the antimicrobial activity of microemulsions and their overall stability. This suggestion has been the focus of experimental studies in our group for some time and we propose that the antimicrobial activity is directly related to the position of the microemulsion within its existence zone (or zone of stability).

This study undertakes the microbiological examination of a physically stable oil-in-water microemulsion system over extended periods and conditions, utilising Tween-80 as the surfactant. Experiments were designed to observe the kinetics of killing of a microemulsion preparation against selected test microorganisms (*Pseudomonas aeruginosa* ATCC 9027, *Candida albicans* ATCC 10231, *Staphylococcus aureus* ATCC 6538 and *Aspergillus niger* ATCC 16404). *P. aeruginosa* is an opportunistic and potentially dangerous pathogen. Biochemically it represents the most versatile organism encountered throughout the cosmetic and pharmaceutical industries (11). *P. aeruginosa* is generally as resistant to hydrophobic molecules as other Gram-negative bacteria due to the presence of LPS molecules (12). However, *P. aeruginosa* also exhibits resistance to small hydrophilic antibiotics. Hence, the outer membrane of *P. aeruginosa* is suspected of having a lower level of permeability than that of other Gram-negative bacteria. *P. aeruginosa* has 12 to 100 fold less outer membrane permeability than that of *Escherichia coli* (13-15).

Additionally, it has been suggested by Al-Adham *et al.,* (2) that the antimicrobial nature of oil-in-water microemulsions may result from the antimicrobial action of the individual components of microemulsion formulations. In particular, many of the surfactant and co-surfactant components of microemulsions have proven antimicrobial action at higher concentrations than those used in microemulsion formulations. Al-Adham *et al.* (2) undertook some simple experiments in order to disprove this theory for the purposes of their project.

It has been shown that microemulsion systems have limited differences in some of the physical properties observed within the microemulsion existence area (**Figure 1**). This may be due to differences in the component ratios. '*The pronounced effect on the area of existence (stability zone) of a microemulsion in the phase diagram caused by changing two surfactants mixture ratio*' (16-17). In order to establish the relationship between the physico-chemical properties of microemulsions and their antimicrobial activity, a formulated microemulsion will be assayed for its antimicrobial activity at the centre-point of the microemulsion stability zone and by testing at different points within the same stability zone. This relationship could be due to their unique structure, especially the presence of water (majority component, v/v) being effectively bounded to the structure of microemulsion and therefore, not freely available to the microorganisms. Hence, these systems may display low water activity (a_W) (2) . The above suggestion is supported by the work of Osborne *et al.* (18) who studied the effect of microemulsion composition upon the *in-vitro* skin transport of water from a microemulsion system. It was conclude that most of the water in microemulsions is bound to the surfactant head groups (18).

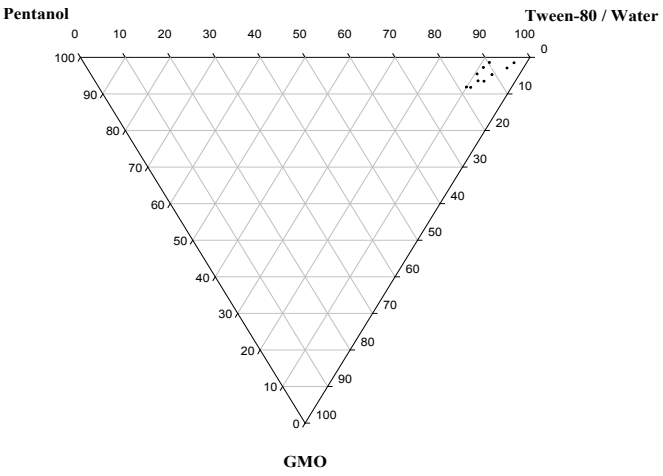

Figure 1. Pseudo-ternary phase diagram, the microemulsion existence area of Formula 1 (Glycerol Mono Oleate, Tween-80, Pentanol and Water) on a 100% scale plot. The marks in the top right hand corner represent the outer limits of the zone of stability for this microemulsion.

In summary, this study aims to undertake microbiological observations on a prepared, physically stable microemulsion system over extended periods and conditions and at different positions within the zone of existence. The general aim of these investigations will be to determine the effects of different microemulsion formulae within single *'zones of microemulsion stability'*.

Materials & methods

Maintenance of Microbial Cultures

Cultures of *Pseudomonas aeruginosa* (ATCC 9027) and *Staphylococcus aureus* (ATCC 6538) were maintained and stored on Nutrient agar (105450) slopes in a darkened cupboard at room temperature (20 ± 2°C) after being incubated at 35°C for 48h. Cultures of *Candida albicans* (ATCC 10231) were maintained and stored on Sabouraud Dextrose agar (107315) slopes and stored in darkened cupboard at room temperature (20 ± 2°C) after being incubated at 20°C for 48h.

Preparation of vegetative cells for challenge studies

An overnight culture of each microorganism was prepared using the appropriate sterile solid agar medium. These cultures were then incubated at either 35°C (bacteria) or 20°C (yeast). Cultures of bacteria and yeast were harvested by washing the surfaces of incubated agar plates with sterile saline TS solution (0.05% w/v Tween-80, 0.9% w/v NaCl) to give a final cell concentration of approximately $1.0x10^8$ cells ml^{-1}. In all cases, the final cell concentration was determined by viable counts of cells.

Preparation of Fungal spores for challenge studies

Culture of *Aspergillus niger* (1ml) was spread onto the surface of pre-dried Sabouraud Dextrose agar (107315) plates. Excess suspension was drained from the plates and removed using a Pasteur pipette. The plates were inverted and incubated at 20°C for three days. The culture was harvested by washing the surface of incubated agar by the aid of sterile glass beads with sterile saline TS solution (0.05% w/v Tween-80, 0.9% w/v NaCl) to give a final cell concentration of approximately $1.0x10^8$ spores ml^{-1}. The final spore concentration was determined by viable counts of cells.

Preparation of Test O/W Microemulsions

All microemulsions used in this investigation were prepared using the protocol described in Al-Adham *et al.* (19). The main test microemulsions used were variants on Formula 1: 17.9% Tween-80, 7.5% Pentanol, 3% GMO and 71.6% water.

Kinetics of killing of O/W microemulsion systems

Overnight cultures of *Pseudomonas aeruginosa* ATCC 9027, *Candida albicans* ATCC 10231, *Staphylococcus aureus* ATCC 6538 and spores from three day cultures of *Aspergillus niger* ATCC 16404 grown in nutrient broth (bacteria) or Sabouraud Dextrose medium (yeast & fungi) were washed twice (with sterile normal saline; centrifugation 4000 g) and used to prepare test cultures in fresh media with a known microbial inocula (*P. aeruginosa,* 7.4 x 10^8 cells ml^{-1}; *S. aureus,* 6.1 x 10^8 cells $ml^{-1;}$ *C. albicans,* 5.8 x 10^8 cells ml^{-1} and *A. niger,* 7.6 x 10^8 spores ml^{-1}). Aliquots (0.1 ml) of these cultures were then challenged by addition to a volume (0.9 ml) of one of the undiluted microemulsion preparations (Formula 1) and incubated at 35°C or 20°C as appropriate for 24 h. Results (**Tables 1 & 2**) indicate that such dilutions (9:1) of the microemulsions were not deleterious to their stability or antimicrobial action. Additionally, subsequent antimicrobial activity assured that the dilution was not deleterious to stability or activity. This was due to the fact that previous experiments by Al-Adham *et al.,* (2) had previously shown that any antimicrobial activity was due to the presence of an active microemulsion and not the component parts of a microemulsion.

Table 1. Calculated % composition (w/w) of the zone of stability of the original microemulsion formulation (Formual 1)

Component	%Composition	
	From	To
Tween 80	14.62	18.8
Glycerol Mono-oleate	1.45	8.04
Pentanol	2.9	10
Water	81.03	63.16

Table 2. Calculated % composition (w/w) of the modified microemulsion formulae (various points within the stability region of Formula 1) after dilution by addition of 0.1ml of microbial inoculum. Results indicate that all such diluted formulae continued to exist within the zone of stability for Formula 1 **(Table 1** above).

Component	% Composition			
	1-A	1-B	1-C	1-D
Tween 80	16.7	16.4	15.9	15.48
Glycerol Mono-oleate	2.25	2.7	3.6	4.5
Pentanol	4.5	5.4	7.2	8.1
Water	76.55	75.5	73.3	71.92

Aliquots (0.1ml) were taken from each tube at time intervals (15 s, 30 s, 1 min, 2 min, 3 min, 4 min, 5 min, 10 min, 15 min and 24 h) for bacteria and *C. albicans* and at a time intervals a 2 min, 15 min, 30 min, 1 h, 2 h, 4 h, 5 h and 24 h for *A. niger*. Further 1:10 serial dilutions (this served as both a dilution and neutralizing step for the antimicrobial action of the microemulsion were made in sterile saline TS solution (0.05% w/v Tween-80, 0.9% w/v NaCl) and viable counts performed upon appropriate dilutions (2). Results were expressed as CFU ml^{-1} against time. This procedure was repeated for each microemulsion system to be tested for the kinetics of killing of microemulsion with new fresh test culture cells.

Kinetics of killing of O/W microemulsion systems for different points within the microemulsion existence area

This experiment was designed to establish a relationship between the physicochemical properties of microemulsions and their potential effects as antimicrobial agents. This was achieved by testing Formula 1 (17.9% w/w Tween-80, 3% w/w GMO, 7.5% w/w Pentanol and 71.6% w/w water) for its antimicrobial activity at different points inside the microemulsion stability zone (**Figure 1**). The microemulsion systems used in this experiment are shown in **Table 3** and were prepared according to the protocols published in our earlier paper. Overnight cultures of *P. aeruginosa* were washed twice (with sterile normal saline; centrifugation at 4000 *g*) and used to prepare test cultures in fresh media with a known microbial inoculum (*P. aeruginosa,* 7.4 x 10^8 cells ml^{-1}). An aliquot (0.1 ml) of this culture was then challenged with known volumes (0.9 ml) of one of the microemulsion preparations (Formula 1-A 18.5% w/w Tween-80, 2.5% w/w Pentanol, 5% w/w GMO and 74% w/w water), and incubated at 35 °C for 24 h. Aliquots (0.1ml) were taken from each tube at time intervals (15 s, 30 s, 1 min, 2 min, 3 min, 4 min, 5 min, 10 min, 15 min and 24 h). **Table 2** indicates the final active % composition (w/w) of the microemulsions at each test point. Further 1:10 serial dilutions were made in sterile saline TS solution (0.05% w/v Tween-80, 0.9% w/v NaCl) and viable counts performed upon appropriate dilutions (this served as both a dilution and neutralizing step for the antimicrobial action of the microemulsion (2). Results were expressed as CFU ml^{-1} against time. This procedure was repeated for each microemulsion system (**Table 1**) to be tested for the kinetics of killing of microemulsion with the same microorganism.

Table 3. Composition (%, w/w) of the component of the microemulsion Formula 1 (Tween-80, Glycerol Mono Oleate (GMO), Pentanol and Water) used to determine the kinetics of killing for different points within the existence area.

System	Tween-80 (%w/w)	GMO (% w/w)	Pentanol (% w/w)	Water (%w/w)
Formula 1	17.9	3.0	7.5	71.6
Formula 1-A	18.5	2.5	5.0	74.0
Formula 1-B	18.2	3.0	6.0	72.8
Formula 1-C	17.6	4.0	8.0	70.4
Formula 1-D	17.2	5.0	9.0	68.8

Results

Kinetics of killing of O/W microemulsion systems

The results show the rate of killing observed treated cultures of *P. aeruginosa*, *S. aureus, C. albicans,* (**Figure 2**) and *A. niger* (**Figure 3**) with microemulsion Formula 1: (17.9% w/w Tween-80, 3% w/w GMO, 10% w/w Pentanol and 71.6% w/w Water). These results give clear evidence of a good biocidal activity for the microemulsion system against the bacterial species, but a lesser effect against the fungi. The viability of *P. aeruginosa* and *S. aureus* cultures (**Figure 2**) decreased rapidly over a period of less than 15 s until no viable cells were observed. This indicates that a more than 6 log reduction in bacterial number was achieved in less than 15 s and that the $LT_{90\%}$ value (time at which 90% of the original population was killed) is < 15 s for both bacteria. $LT_{90\%}$ values of this order suggest that the antimicrobial compound involved is highly and effectively biocidal. The viability of *C. albicans* cultures (**Figure 2**) treated with the same microemulsion system also decreased rapidly over a short period of time, less than 30 s. These results indicate that a more than 4 log reduction in *Candida* numbers was obtained in less than 30 s. The $LT_{90\%}$ value was < 15 s for *C. albicans*. **Figure 3** shows the viability of treated cells of *A. niger* with the same microemulsion system (Formula 1) These results indicate that the viability of *A. niger* also decreases rapidly over a period of 2 h. The $LT_{90\%}$ value was 30 m for *A. niger*.

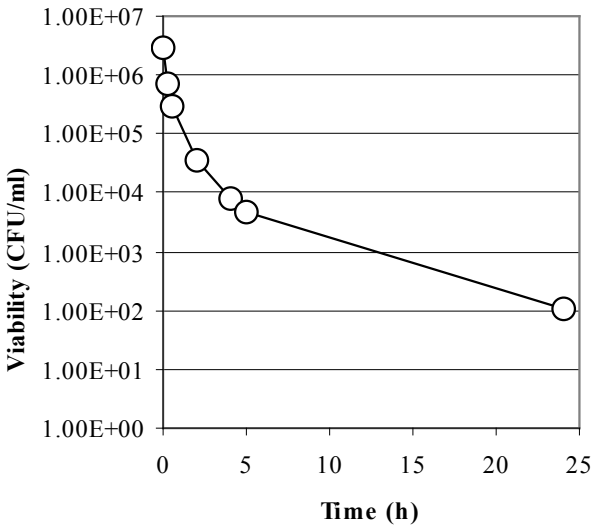

Figure 3. The exposure viability (CFU ml^{-1}) for the addition of microemulsion Formula No.1 (17.9% w/w Tween-80, 3% w/w Glycerol Mono Oleate (GMO), 7.5% w/w Pentanol and 71.6% w/w Water) to a culture of *A. niger* (O). Experimental data presented in this figure represents the mean of duplicate data sets (n = 2). Error bars are not given in this figure due to the use of the logarithmic scale.

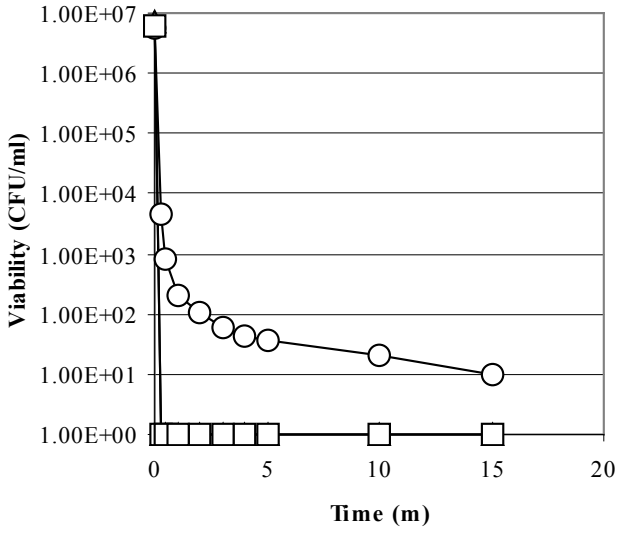

Figure 2. The exposure viability (CFU ml^{-1}) for the addition of microemulsion Formula No.1 (17.9% w/w Tween-80, 3% w/w Glycerol Mono Oleate (GMO), 7.5% w/w Pentanol and 71.6% w/w Water) to cultures of *C. albicans* ATCC 10231 (O), *P. aeruginosa* ATCC 9027 (Δ, masked by *S. aureus* data) and *S. aureus* ATCC 6538 (□). Experimental data presented in this figure represents the mean of duplicate data sets (n = 2). Error bars are not given in this figure due to the use of the logarithmic scale.

Kinetics of killing of O/W microemulsion systems for different points within the microemulsion existence area

Figure 4 shows the rate of killing observed for cultures of *P. aeruginosa* after exposure to aliquots of microemulsions Formulae 1, 1-A, 1-B, 1-C and 1-D. These results clearly indicate that good antimicrobial activity was observed for all of these points within the microemulsion existence area. The viability of bacterial cells decreased by more than 3 log cycles over approximately 15 s. The $LT_{90\%}$ value for all of the tested points from Formula 1 (inside the microemulsion existence area) was < 15 s. $LT_{90\%}$ values of this order for *Ps. aeruginosa* suggest that these microemulsion systems are highly effective biocidal agents.

Figure 4 also indicates more than 6 log cycles of reduction in *P. aeruginosa* viability when exposed to microemulsion Formula 1 (17.9% w/w Tween-80, 3% w/w GMO, 7.5% w/w Pentanol and 71.6% w/w water; the centre of the area of

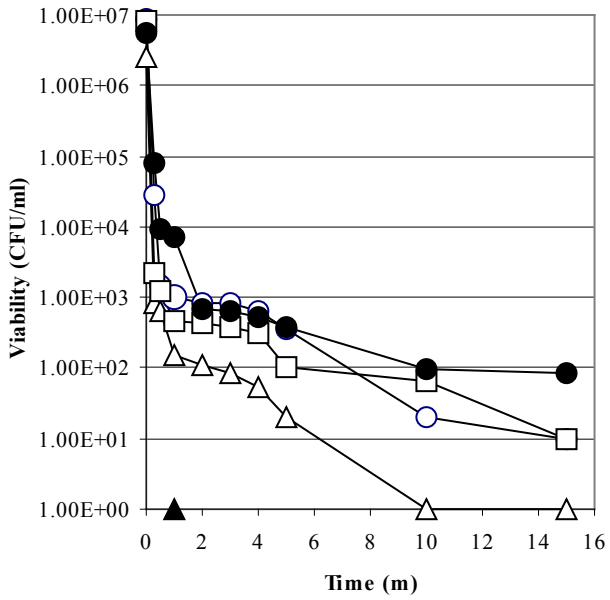

Figure 4. The exposure viability (CFU ml^{-1}) for the addition of microemulsions: Formula No.1: (17.9% w/w Tween-80, 3% w/w Glycerol Mono Oleate (GMO), 7.5% w/w Pentanol and 71.6% w/w Water) (▲); Formula No.1-A: (18.5% w/w Tween-80, 2.5% w/w GMO, 5.0% w/w Pentanol and 74% w/w Water) (O); Formula No.1 B: (18.2% w/w Tween-80, 3.0% w/w Glycerol Mono Oleate (GMO), 6.0% w/w Pentanol and 72.8% w/w Water) (Δ); Formula No.1-C: (17.6% w/w Tween-80, 4.0% w/w Glycerol Mono Oleate (GMO), 8.0% w/w Pentanol and 70.4% w/w Water) (□) and Formula No.1-D: (17.2% w/w Tween-80, 5.0% w/w Glycerol Mono Oleate (GMO), 9.0% w/w Pentanol and 68.8% w/w Water) (●) to cultures of *P. aeruginosa* ATCC 9027. Experimental data presented in this figure represents the mean of duplicate data sets (n = 2). Error bars are not given in this figure due to the use of the logarithmic scale.

microemulsion existence area) in less than 15 s. These results exhibit an $LT_{90\%}$ value of < 15 s. These results are compared with a 3 log cycle reduction in bacterial numbers obtained in approximately 15 s, coupled with $LT_{90\%}$ value of < 15 s, for planktonic cells treated with Formulae 1-A, 1-B, 1-C and 1-D (Figure 4). Figure 4 also indicates that there are slight differences in the rate of killing for the different Formulae (Formula 1-B > Formula 1-C > Formula 1-D > Formula 1-A). These results clearly indicate that the formulae closest to the centre of the microemulsion existence area (Formula 1-B and Formula 1-C) have a higher rate of killing than those near the perimeter of the microemulsion existence area (Formula 1-A and Formula 1-D).

Discussion

The high levels of inherent antimicrobial activity observed in these microemulsions, demonstrated by the results of the kinetics of killing experiments, indicate that these microemulsions are self-preserving systems. The observed 6 log reduction in bacterial cells number and more than 3 log cycle reduction in yeast cells numbers over a relatively short period of time (less than 15 s) coupled with $LT_{90\%}$ value of <15 s for both bacteria and *C. albicans* observed with these microemulsions, suggests excellent biocidal activity. Such rapid biocidal activity is indicative of direct attack on the structural integrity of the cell rather than a secondary effect through metabolic inhibition (6). This suggestion was supported by the work of Al-Adham *et al.* (2), using Transmission Electron Microscopy (TEM) on microemulsion-exposed cells. These results indicated that the microemulsion used was capable of significant anti-membrane activity, resulting in the gross disturbance and dysfunction of membrane structure. The level of dysfunction could potentially result in the death of the cell and may explain the rapid loss of cell viability observed in the kinetics of killing experiment used here.

The results observed for the rate of killing for the different points clearly indicate the significant antibacterial activity of the microemulsions systems at all points within the microemulsion existence area and that the most effective one is that point representing the centre of the microemulsion stability zone (Formula 1). However, points that are near, but not at the centre of the microemulsion stability zone (Formulae 1-B & 1-C) exhibit higher antimicrobial activity than those formulae at the outer perimeter of the microemulsion existence area (Formulae 1-A & 1-D). These results suggest that there is an intimate relationship between the physico-chemical properties of microemulsions and their potential effects as antimicrobial agents. This may be due to the variation in water activity (a_w) at the different points within the microemulsion existence area. That is, the water (majority component, v/v) is effectively bound within the structure of the microemulsions and therefore, it is not freely available for the microorganisms to utilise for growth and reproduction. It is suggested that the water bound to the microemulsion structure is higher in the centre of the microemulsion existence area than the perimeter areas, which may contain more free water molecules.

An alternative hypothesis is that the molecular and ionic structure of the microemulsion is such that it causes catastrophic and irreversible damage to the membranes of the cells challenged. In the case of *P. aeruginosa* this effect would occur initially at the outer membrane and subsequently at the cytoplasmic membrane. In the case of *S. aureus*, *C. albicans* and *A. niger*, the effect occurs at the cytoplasmic membrane only.

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It must be stressed that both the above are suggestions regarding the mode of action of the oil-in-water microemulsions, based upon our observations and published knowledge of the physico-chemical nature of these structures. Work continues to try and determine the true nature of these observed phenomena.

In summary, all the microbiological assessment results clearly indicate that the microemulsions are stable, self-preserving antimicrobial agents, with highly effective killing rates against *S. aureus, C. albicans,* and the particularly resistant bacterial species*, P. aeruginosa*. The $LT_{90\%}$ value was < 15 s for *S. aureus, P. aeruginosa* and *C. albicans*. The prepared microemulsion systems also have highly effective killing rate against *A. niger,* a spore-forming fungi. The viability of *A. niger* decreases rapidly over a period of 2 hr coupled with $LT_{90\%}$ value of 30 min.

Conclusions

The results of this study suggest that there is an intimate relationship between the physico-chemical properties of microemulsions, in particular the position of the microemulsions within their zones of stability (existence zone) and their observed effects as antimicrobial agents. These results also suggest that the high levels of observed antimicrobial activity are due to the unique thermodynamically stable, yet kinetically active structure of O/W microemulsion systems, rather than the chemical activity of their individual components.

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