Evaluation of the *in vitro* antimicrobial activity of the Pheroid®-entrapped plant extract of *Agapanthus africanus* against human pathogens

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Abstract

Antimicrobial resistance is a major concern as existing antimicrobials are becoming less effective due to misuse and overuse (WHO, 2018b). Identifying novel compounds or treatments with a possible antimicrobial activity could facilitate the development of novel treatments, such as a Pheroid® plant extract delivery system. This *Agapanthus africanus* plant extract has proven *in vitro* and *in vivo* activity against fungi affecting crops in South Africa, however, activity against human pathogens have not been determined. In this study, the plant extract's *in vitro* antimicrobial activity was tested against eleven human pathogens, namely *Escherichia coli, Staphylococcus epidermidis, Staphylococcus aureus, Klebsiella pneumoniae, Salmonella enterica, Enterobacter aerogenes, Pseudomonas aeruginosa*, *Trichosporon dermatis, Cryptococcus neoformans, Saccharomyces cerevisiae,* and *Candida albicans.* The plant extract was formulated and entrapped into the Pheroid® drug delivery system, containing different concentrations of the oil phase, *i.e.* 4%, 8%, 10%, 13% and 50%. The *in vitro* antimicrobial activity of these test formulations (Pheroid®-plant extract) was compared to the activity of the plant extract and control formulation (only Pheroid®), to determine if Pheroid® technology influenced the plant extract's activity. The formulations were subjected to accelerated stability testing, after which the minimum bactericidal/fungicidal concentration was determined at month 0, 1, 2, and 3. Improvements were made to this method by adding resazurin to also determine the minimum inhibitory concentration. The formulations were characterised by Malvern Mastersizer (particle size), Malvern Nanosizer (zeta potential) and confocal laser scanning microscopy (morphology) before and during accelerated stability testing.

The characterisation tests showed that optimisation of the test formulations by adjusting the percentage oil phase was possible. Most of the formulations were stable *i.e*. no aggregation, flocculation or creaming was observed during accelerated stability testing. Test formulation PPE 4% proved to be the most promising formulation with the largest initial particle size and remained stable during accelerated stability testing. The *A. africanus* plant extract was identified to have moderate antimicrobial activity against three human pathogens, namely *C. albicans, S. cerevisiae*, and *T. dermatis* with a minimum inhibitory concentration of 1725 µg/ml, 54 µg/ml, and 108 µg/ml, respectively. In conclusion, the addition of Pheroid® drug delivery system increased the inhibitory activity in most cases (excluding *S. cerevisiae*), however, did not seem to have an effect on the fungicidal concentration. The formulation that showed the best initial antimicrobial activity against *C. albicans* was Pro-PPE 50%, however, after accelerated stability testing both Pro-PPE 50% and PPE 4% had the highest antimicrobial activity. Formulation PPE 13% had the highest initial fungicidal activity against both *S. cerevisiae* and *S. epidermidis*, while PPE 8% had the highest fungicidal activity after accelerated stability testing against *S. cerevisiae*. **Keywords:** Antimicrobial susceptibility, *Agapanthus africanus*, plant extract, Pheroid®, accelerated stability testing, human pathogens, formulation.

Uittreksel

Antimikrobiese weerstand is 'n groot probleem, aangesien bestaande antimikrobiese middels minder effektief word weens misbruik (WHO, 2018b). Die identifisering van nuwe verbindings of behandelings met 'n moontlike antimikrobiese werking kan die ontwikkeling van nuwe behandelings, soos 'n Pheroid®-plant ekstrak afleweringsisteem, fasiliteer. Die *Agapanthus africanus* plant ekstrak het *in vitro* en *in vivo* aktiwiteit teen swamme wat gewasse in Suid-Afrika affekteer, maar aktiwiteit teen menslike patogene is nog nie bepaal nie. In hierdie studie is die plant ekstrak se *in vitro* antimikrobiese aktiwiteit getoets teen elf menslike patogene, naamlik *E. coli, S. epidermidis, S. aureus, K. pneumonia, S. enterica, E. aerogenes, P. aeruginosa*, *T. dermatis, C. neoformans, S. cerevisiae,* en *C. albicans*. Die plant ekstrak is saam met die Pheroid® geneesmiddel afleweringssisteem geformuleer, wat verskillende konsentrasies van die oliefase bevat, *i.e.* 4%, 8%, 10%, 13% en 50%. Die *in vitro* antimikrobiese aktiwiteit van hierdie toetsformulerings (Pheroid®-plant ekstrak) is vergelyk met die aktiwiteit van die plant ekstrak en kontroleformulerings (slegs Pheroid®) om te bepaal of Pheroid®-tegnologie die plant ekstrak se aktiwiteit beïnvloed het. Die formulerings was getoets met versnelde stabiliteitstoetsing, waarna die minimum bakteriedodende- / swamdodende konsentrasies by maand 0, 1, 2 en 3 bepaal is. Verbeterings is aan hierdie metode gemaak deur resasurien by te voeg om ook die minimum inhiberende konsentrasie te bepaal. Die formulerings was gekarakteriseer deur Malvern Mastersizer (deeltjiegrootte), Malvern Nanosizer (zeta potensiaal) en konfokale laserskanderingsmikroskopie (morfologie) voor en tydens versnelde stabiliteitstoetsing.

Die karakteriseringstoetse het getoon dat optimalisering van die toetsformulerings deur die persentasie oliefase aan te pas, moontlik was. Meeste van die formulerings was stabiel, d.w.s. geen aggregering, flokkulering of verrooming is waargeneem tydens versnelde stabiliteitstoetsing nie. Toetsformulering PPE 4% was die mees belowende formulering met die grootste aanvanklike deeltjiegrootte en het stabiel gebly tydens versnelde stabiliteitstoetsing. Die *A. africanus* plant ekstrak het matige antimikrobiese aktiwiteit teen drie menslike patogene getoon, naamlik *C. albicans, S. cerevisiae* en *T. dermatis* met 'n minimum inhiberende konsentrasie van 1725 µg/ml, 54 µg/ml en 108 µg/ml, onderskeidelik. Ten slotte het die toevoeging van die Pheroid® afleweringsisteem in die meeste gevalle (met die uitsondering van *S. cerevisiae*) die inhiberings aktiwiteit verhoog, maar het nie 'n effek op die swamdodende konsentrasie gehad nie. Die formulering wat die beste aanvanklike antimikrobiese aktiwiteit teen *C. albicans* getoon het, was Pro-PPE 50%, maar na versnelde stabiliteitstoetsing het beide Pro-PPE 50% en PPE 4% die hoogste antimikrobiese aktiwiteit gehad. Formulering PPE 13% het die hoogste aanvanklike swamdodende aktiwiteit teen beide *S. cerevisiae* en *S. epidermidis* gehad, terwyl PPE 8% die hoogste swamdodende aktiwiteit gehad het ná versnelde stabiliteitstoetsing teen *S. cerevisiae*.
Sleutelwoorde: Antimikrobiese vatbaarheid, *Agapanthus africanus*, plant ekstrak, Pheroid®, versnelde stabiliteitstoetsing, menslike patogene, formulering.

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List of abbreviations

All abbreviations are indicated and explained where they first appear in the text, where after only the abbreviation is used.

%	Percentage
®	Registered trademark
°C	Degrees Celsius
µg/ml	Microgram per millilitre
µl	Microliter
AST	Accelerated stability testing
AB	Antibiotic
AF	Antifungal
AmB	Amphotericin B
AMR	Antimicrobial resistance
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
C.n	*Cryptococcus neoformans*
CFU/ml	Colony forming unit per millilitre
CLSI	Clinical and Laboratory Standards Institute
CLSM	Confocal laser scanning microscopy
dH_2O	Distilled dihydrogen oxide/water
DHA	Docosahexaenoic acid
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
E.a	*Enterobacter aerogenes*
E.c	*Escherichia coli*
EPA	Eicosapentaenoic acid
Ery	Erythromycin
ESBL	Extended-spectrum beta-lactamases
FDA	Food and Drug Administration
g	Gram
GLASS	Global antimicrobial resistance surveillance system
H_2O	Dihydrogen oxide/water
H_2SO_4	Sulfuric acid
K.p	*Klebsiella pneumonia*
MBC	Minimum bactericidal concentration
MDR	Multidrug resistant
MFC	Minimum fungicidal concentration
mg/ml	Milligram per millilitre
MHA	Mueller-Hinton agar
MHB	Mueller–Hinton broth
MIC	Minimum inhibitory concentration

mm	Millimetre
MRSA	Methicillin-resistant Staphylococcus aureus
mV	Millivolt
N_2O	Nitrous oxide
nm	Nanometre
o/w	Oil-in-water
P 10%	10% oil phase Pheroid®
P 13%	13% oil phase Pheroid®
P 4%	4% oil phase Pheroid®
P 8%	8% oil phase Pheroid®
P.a	*Pseudomonas aeruginosa*
PE	Plant extract
PIT	Phase inversion temperature
PPE	Pheroid®-Plant Extract formulation
PPE 10%	10% oil phase Pheroid®-Plant extract
PPE 13%	13% oil phase Pheroid®-Plant extract
PPE 4%	4% oil phase Pheroid®-Plant extract
PPE 8%	8% oil phase Pheroid®-Plant extract
PR	Pathogenesis-related
Pro-P	Pro- Pheroid®
Pro-PPE 50%	50% oil phase pro-Pheroid®-Plant extract
PRSP	Penicillin-resistant Streptococcus pneumoniae
PUFAs	Polyunsaturated fatty acids
RH	Relative humidity
RIIP®/CENQAM®	Research Institute for Industrial Pharmacy, incorporating Centre for Quality Assurance Medicines
ROS	Reactive oxygen species
rpm	Rotations per minute
S.a	*Staphylococcus aureus*
S.e	*Staphylococcus epidermidis*
S.t	*Salmonella enterica*
T.d	*Trichosporon dermatis*
TMP	Trimethoprim
USA	United States of America
v/v	Volume per volume
VRE	Vancomycin-resistant Enterococcus
w/w	Weight per weight
WHO	World Health Organization
ZOI	Zone of inhibition

Chapter 1: Introduction

1.1. Problem statement and background

According to the World Health Organization (WHO) (2018b), a never-ending battle against antimicrobial resistance (AMR) leads to ineffective prevention and treatment of infections caused by bacteria and fungi. The same antimicrobials have been used since the 1940s to treat infectious diseases and have been used for such a long time, causing the infectious pathogens to adapt to them, rendering them less effective (WHO, 2018b). These microorganisms are therefore more difficult and more expensive to treat (WHO, 2018b).

Over the past three years, the Food and Drug Administration (FDA) has approved a total of six new antibacterial agents (Andrei *et al*., 2018). A total of 6.5% antibacterial agents (3 out of 46) were approved in 2017, 9% antibacterial agents (2 out of 22) in 2016 and only 2.2% in 2015 (1 out of 45). Spellberg *et al.* (2004) showed that from 1998 to 2002 versus 1983 to 1987, the amount of FDA approved antimicrobials has decreased by 56% during these 20 years.

In the United States (US), at least 2 million people are annually infected with resistant bacteria of which an estimated 23,000 will die as a direct result of these infections (CDC, 2018a). The global AMR surveillance system (GLASS) report completed for 2016-2017 showed that the most frequently reported resistant bacteria in 17 different countries were *E. coli, K. pneumoniae, S. aureus,* and *S. pneumoniae* followed by Salmonella spp. in 15 different countries (WHO, 2018a). In 2007, the estimated number of infections and deaths in Europe was ~400 000 and 25 000, respectively, due to the above-mentioned resistant bacteria (Festinese, 2013).

There are also several species of *Candida* resistant to antifungals. In the US, *Candida* is the most common cause of healthcare-associated bloodstream infections, also known as candidemia (CDC, 2018b). Some of this *Candida* spp. are becoming progressively resistant to the first-line and second-line candidemia antifungals, such as fluconazole and echinocandins (CDC, 2018b).

AMR renders commonly used antimicrobials less effective, causing us to turn to more expensive treatments. This also contributes to longer hospitalisation and an increased mortality and morbidity. Therefore, there is a need to discover new antimicrobial compounds (Van Wyk and Wink, 2004; CDC, 2018b).

The investigation of traditional medicinal plants to evaluate their potential for pharmaceutical activity, such as the black willow tree leading to discover aspirin, is an important area of research that provides new lead compounds or treatments, especially in the current struggle to identify new antimicrobial agents (Row and Geyer, 2010; Veeresham, 2012).

The crude plant extract (PE) of *Agapanthus africanus* has proven *in vitro* and *in vivo* activity against fungi affecting the crops of South Africa, namely *Botrytis cinerea, Athelia rolfsii, Rhizoctonia solani, Botryosphaeria dothidea, Pythium ultimum,* and *Fusarium oxysporum* (Agrarforum SA (Pty.) Ltd., 2013). The active compounds of the *A. africanus* PE were identified as a triterpene saponin known as agapanthussaponin A, two different flavanones and a chalcone (Agrarforum SA (Pty.) Ltd., 2013). These compounds are discussed in section 2.1.3.

A common method of overcoming resistance and increasing activity of existing compounds is to incorporate an active drug compound into a drug carrier system. Pheroid® is based on a colloidal drug delivery system and is a patented product (Grobler *et al*., 2009). Pheroid® technology has been proven to enhance the absorption, thus increased activity, of orally administered anti-infective drugs (Steyn *et al*., 2009) and topical applications (Saunders *et al*., 1999). Products on the market using the Pheroid® technology include four Pheroid® related topical products and one bio-agricultural product (Saunders *et al*., 1999). The primary components of Pheroid® are ethyl esters of essential fatty acids, pegylated ricinoleic acid, α-dl-tocopherol, and nitrous oxide (N_2O)-saturated water (Saunders *et al*., 1999, Meyer 2002, Grobler, 2008). The details of the Pheroid® technology and the applications thereof will be discussed in section 2.5.

This study will focus on determining the antimicrobial activity of the crude PE from *A. africanus* against human pathogens. Should the crude PE present antimicrobial properties, we would like to determine what the addition of the Pheroid® drug delivery system would have on the antimicrobial activity. This study could be the first step in identifying an alternative treatment to the drugs currently on the market. This study will also contribute to the indigenous knowledge of plants from South-Africa.

1.2. Aims and objectives

General aim:

The general aim of this study was to determine whether a crude PE from *A. africanus* had *in vitro* antimicrobial properties against human pathogens. The crude extract was also formulated and entrapped into the Pheroid® drug delivery system, and then compared with the crude PE to determine if Pheroid® technology influenced the extract's *in vitro* activity. The *in vitro* activity of these different formulations was determined at various time points after they were subjected to accelerated stability testing (AST), to determine if the Pheroid® technology had an effect on the stability of the crude PE.

Specific objectives:

- Determine the *in vitro* antimicrobial activity of the crude PE with a brief screening for activity, against *Staphylococcus aureus, Staphylococcus epidermidis, Escherichia coli, Enterobacter aerogenes, Klebsiella pneumonia, Salmonella enterica, Pseudomonas aeruginosa, Candida albicans* and *Saccharomyces cerevisiae.*
- Optimize the preparation of the Pheroid®-PE formulation by adjusting the percentage of the oil phase present in the Pheroid® formulation (4%, 8%, 10%, 13%, and 50% Pro-Pheroid®). The morphology, particle size and stability will be determined for the above-mentioned formulations before and during AST.
- Determine the *in vitro* antimicrobial activity of the Pheroid®-PE formulations, corresponding Pheroid® control formulations, and the crude PE against *Staphylococcus aureus, Staphylococcus epidermidis, Escherichia coli, Enterobacter aerogenes, Klebsiella pneumonia, Salmonella enterica, Pseudomonas aeruginosa, Candida albicans, Saccharomyces cerevisiae, Trichosporon dermatis,* and *Cryptococcus neoformans* using the microdilution and subculturing method.
- Determine the *in vitro* antimicrobial activity of the Pheroid®-PE formulations, corresponding Pheroid® control formulations, and the crude PE before and after subjecting these formulations to AST.

Chapter 2: Literature review

2.1. Indigenous knowledge

The use of plant material for treating disease and medical conditions has been used since ancient times and even in modern medicine, many pharmaceuticals are produced from plant-derived compounds (Fennel, 2004). Plants have certain compounds in their leaves, stems, flowers, roots and fruit which can be extracted and used as active ingredients in pharmaceutical and agricultural preparations. In South Africa, the use of herbal remedies by traditional healers is a vibrant practice, with around 70% of the population still utilizing the services of traditional healers (Ramgoon *et al.,* 2011). The study of herbal remedies is a useful line of research in order to identify new lead compounds and optimize them for use as pharmaceuticals. The following plants (table 2.1) are well known traditional remedies used in South Africa.

Table 2.1: Examples of traditional remedies used in South Africa.

Common name	Scientific name	Part of plant used in preparation	Indication
Wild garlic	*Tulbaghia violacea*	Bulbs boiled and taken orally Leaves also used.	Tuberculosis and intestinal worms, oesophagus cancer (Agriculture, Forestry and Fisheries, 2013).
River red gum	*Eucalyptus camaldulensis*	Leaves and roots are cooked and taken orally.	Asthma, cough, diarrhoea and sore throat (Abubakar, 2010).
Giant pineapple lily	*Eucomis pallidiflora*	Bulb is boiled in water and taken orally.	Chest complains, mental illness and sexually transmitted infections (Moeng 2010).
Starflower	*Hypoxis hemerocallidea*	Tuber is boiled in water and taken orally.	Arthritis, cold, flu, HIV/AIDS and wounds (Griersonand and Afolayan, 1999).
Lemon	*Citrus lemon*	Leaves are crushed, wrapped in newspaper and smoked.	Cough, flu and fever (Maroyi, 2011).

One plant used as a traditional remedy is *Agapanthus praecox Willd* by the Zulus to treat coughs, colds, chest tightness and pains, heart disease and paralysis. They also believe that wrapping the leaves around the wrist can reduce a fever (Notten, 2004). Another important species of the *Agapanthus* family includes *A. inapertus* and contains a similar saponin to *A. africanus*, known as sapogenin A that causes inhibition of the Cyclic Adenosine Monophosphate (cAMP) phosphodiesterase enzyme causing strong anti-inflammatory effects (Cawood *et al.* 2015).

A. africanus is suspected to cause ulcerations in the mouth when taken orally and haemolytic effects in patients (Stuart, 2016). Medicinal knowledge of *A. africanus* is limited and should be investigated. The *Agapanthus* family is widely used in indigenous medicine in South Africa and it has been demonstrated that the saponins and sapogenins in this group of plants do pose anti-inflammatory, anti-oedema, antitussive and immunoregulatory activity (Stuart, 2016).

2.1.1. Distribution and classification of *Agapanthus africanus*

Agapanthus africanus (family: Agapanthaceae), also known as the cape agapanthus, is indigenous to Western Cape, South Africa and is one of six known species (*A. africanus, A. praecox*, *A. campanulatus, A. caulescens, A. coddii* and *A. inapertus*) (Zonneveld and Duncan, 2003). The name *agapanthus* (flower of love) originates from the Greek words *agape* and *anthos*, both meaning love and *Africanus* is a Latin word describing its African origin (Stuart, 2016).

A. africanus is an evergreen shrub (figure 2.1) with a thick underground stem called a rhizome which is used as a storage organ and is part of the herbaceous perennials genus (van der Una, 1971). Herbaceous plants have no woody stem above ground and perennials are plants that live for more than two years. The white and thick roots grow out of the rhizomes. This plant blooms in the summer creating a long and firm flower stalk (up to 50 cm) surrounded with a cluster of narrow, leathery, basal leaves at the foot of the plant about 2-4 cm wide (van der Una, 1971). The 12 to 30 flowers are typically bright blue-violet and form a flower cluster in which stalks of nearly equal length spring from the main stalk and form a flat or curved surface, this is known as an umbel (Stuart, 2016). The umbel can also be defined as the inflorescence part of the plant which is the reproductive portion of a plant that bears a cluster of flowers in a specific pattern (van der Una, 1971).

Figure 2.1: *A. africanus* plant. Photo taken by Bianca van Lingen.

2.1.2. *Agapanthus africanus* pharmacology

The *Agapanthus* species are widely spread across the eastern parts of South Africa and is used as traditional medicine to induce labour and to treat constipation during pregnancy (Kaido *et al*., 1997; Duncan *et al*., 1999). The *Agapanthus* species have been investigated by various research groups in order to provide scientific evidence for its medicinal uses, described below.

Previous phytochemical and activity studies of *A. africanus* aqueous extract have shown uterotonic activity in mice, which is known to cause smooth muscle contractions in the uterine (Veale *et al*., 1999). In this study, they have proven that the extract presents an agonist effect on the uterine muscarinic receptors and promoted synthesis of prostaglandins in the uterus of oestrogen-treated rats. *A. africanus* is traditionally used to induce labour, this study provided a pharmacological explanation for the ethnic use of this PE (Veale *et al.,* 1999).

It was also demonstrated that *A. africanus* has increased the *in vitro* activity of pathogenesis-related (PR) enzymes in wheat seedlings (Cawood *et al*., 2015). *A. africanus* also had antifungal activity against *T. mentagrophytes* and *Sporothrix schenekii* skin infections in guinea pigs with an MIC of 15.6 µg/ml (Singh *et al*., 2008). The saponin, (25R)-spirost-7-en-2α,3β,5α-triol-3-O-[α-Lrhamnopyranosyl(1→2)-[β-D-galactopyranosyl-(1→3)]-β-D-glucopyranoside, was identified as the active compound in the crude extract (Singh *et al*., 2008). *A. africanus* was found to have antimicrobial properties and caused inhibition of mycelial growth of all tested fungi *(Botrytis cinerea*, *Athelia rolfsii*, *Rhizoctonia solani, Botryosphaeria dothidea, Pythium ultimum* and *Fusarium oxysporum)* at a concentration of 1 mg/ml (Agrarforum SA (Pty.) Ltd., 2013).

A different study investigated the saponin, (25R)-5a-spirostane-2α,3β,5α-triol-3-O-(O-a-L-rhamnopyranosyl-(1→2)-O-(β-D-galactopyranosyl-(1→3))-β-D-glucopyranoside), which is a spirostane with an attached trisaccharide, isolated from aerial parts of *A. africanus* and found that it induced apoplastic peroxidase activity in wheat seedlings and displayed fungicidal properties (Cawood *et al*., 2015). Plants upregulate the activity and gene expression of apoplastic peroxidases when wounded, indicating that the ability of peroxidase to produce and scavenge reactive oxygen species (ROS) is vital to plant wound healing (Minibayeva *et al.*, 2015)

2.1.3. Chemical makeup of *Agapanthus africanus* plant extract

Extracts prepared from combined aerial parts (flower, stem and leaves) of *A. africanus* showed higher anti-fungal efficiency than extracts prepared from single aerial parts (Agrarforum SA (Pty.) Ltd., 2013). This could indicate that there is synergism between the biological processes/compounds and that the extract should be studied as a whole rather than just the individual constituents thereof (Castellano *et al.,* 2012). Although this approach of using extracts have proved beneficial, it is important that the active pharmaceutical ingredients of these mixtures are identified separately. Several of the active compounds (figure 2.2) that have been identified in *A. africanus* are: 5,7,4'-trihydroxy flavanone, 5,7,3',4'-tetra-O-acetylflavanone, trans-4,2',4'-tri-O-acetylchalcone and 3-[{0-β-D-glucopyranosyl-(1→3)-α-L-rhamnosyl-(1→2)}-β-D-glucopyranosyloxy]-agapanthegenin. The first three are phenolic compounds and the last compound is a saponin steroid. According to Cawood *et al.* (2015) the Nuclear Magnetic Resonance (NMR) data of the saponin isolated from *A. africanus*, is identical to agapanthus saponin A, which is isolated from the roots of *A. inapertus* and is a potent inhibitor of cAMP phosphodiesterase. This enzyme is a well-known target for various pharmacological applications (amongst others its anti-inflammatory action) which can act in a synergistic action to fight infection or the adverse effects associated with infections (Nakamura *et al*., 1993).

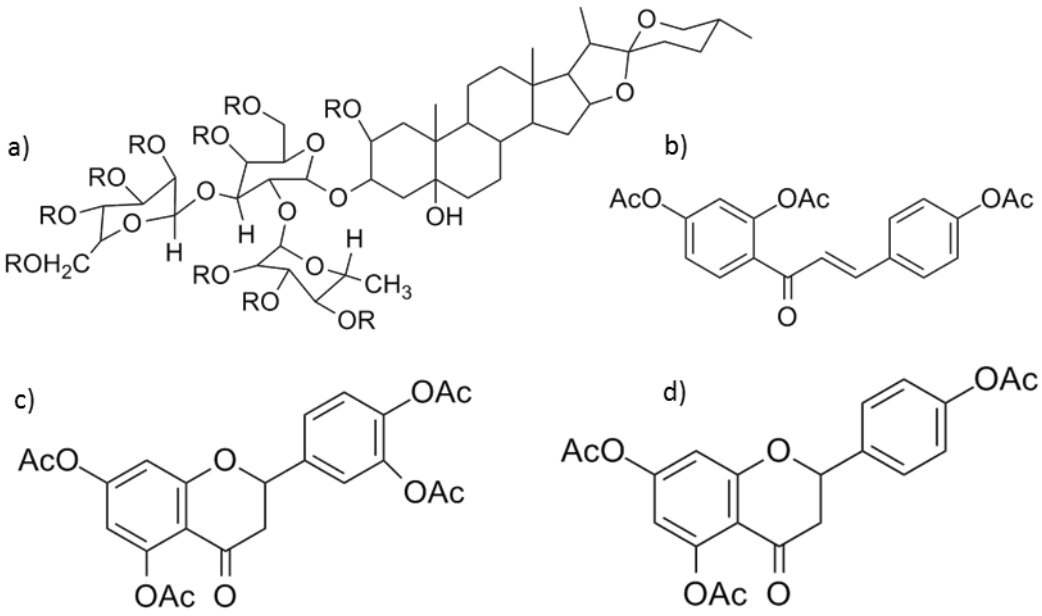


Figure 2.2: The active compounds identified in the crude extract of *A. africanus* are: a) 3-[{0-β-D-glucopyranosyl-(1→3)-α-L-rhamnosyl-(1→2)}-β-D-glucopyranosyloxy]agapanthegenin wherein R = H or acetyl, b) trans-4,2',4'-tri-O-acetylchalcone, c) 5,7,3',4'-tetra-O-acetylflavanone and **d)** 5,7,4'-trihvdroxyflavanone (Chemdraw).

Phenolic compounds, such as flavonoids and chalcones, are found in the water-soluble pigments of plants and have one or more hydroxybenzene groups (Van Wyk and Wink, 2004). Phenolic compounds are planar and electron rich compounds which originate biosynthetically from hydroxycinnamoyl-coenzyme A (Polya, 2003). The planar benzene ring is hydrophobic, but the phenolic hydroxyl group changes the polarity and water solubility and makes hydrogen bonding possible. This allows phenolic-protein interactions and leads to changes in the structural, functional and nutritional properties of both compounds (Ozdal *et al.,* 2013). Phenols, in general, are weak acids and therefore, increase the proton permeability of the mitochondrial inner membrane and uncoupling of the oxidative phosphorylation (OXPHOS) process causing inhibition of adenosine triphosphate (ATP) production (Polya, 2003). This could lead to cellular death since all microorganisms need ATP to survive.

Some phenolic compounds can act as antioxidants through covalent reactions with reactive oxygen species (ROS) (Castellano *et a*l., 2012). In a study done by Proestos *et al*., (2005) a wide variety of PEs was chosen with high phenolic content and the presence of antimicrobial and antioxidant activities was examined. They found that all the PEs showed antioxidant activity, with the highest activity in Rosemary (*Rosmarinus officinalis*) (Proestos *et al.,* 2005). Lin *et al*., (1999) screened twenty-nine crude extracts of traditional Zulu medicinal plants for their anti-inflammatory and antimicrobial activities. They were assayed for prostaglandin synthesis inhibitors and five methanolic PEs showed significant inhibition of cyclooxygenase (COX-1), namely: Idambiso (*Cyphostemma natalitium*), Cobas (*Rhoicissus digitata*), Glossy Forest Grape (*R. rhomboidea*), Cape Grape (*R. tomentosa*) and Bushman's grape (*R. tridentata*). Plants that showed antimicrobial activity were Idambiso (*Cyphostemma natalitium*), Glossy Forest Grape (*R. rhomboidea*) and Cape Grape (*R. tomentosa*). When testing the antimicrobial activity, they found that Gram-positive bacteria were more sensitive to the PEs than Gram-negative bacteria (Proestos *et al*., 2005; Lin *et al*., 1999).

Saponins are terpenoid amphipathic compounds with water-soluble sugar residues and a hydrophobic triterpenoid aglycone part (Polya, 2003). Saponins form a complex with the cell membrane cholesterol of red blood cells, causing haemolysis through pore-forming and is thus known to be a cytotoxic compound (Podolak, Galanty and Sobolewska, 2010). Saponins have detergent properties and phenolic compounds have ATP inhibition, anti-inflammatory and antioxidant properties. Together these compounds could have the potential to break the microorganism membrane and inhibit ATP production, causing death or growth inhibition. As previously mentioned above, studies that have been done on extracts from *A. africanus* have shown significant antimicrobial, anti-fungal and bio-stimulatory activity *in vitro* and *in vivo* on plant and limited animal pathogens (Veale *et al.,* 1999; Singh *et al*., 2008; Cawood *et al*., 2015).

Therefore, there is a need to also investigate possible effects of *A. africanus* extract on human pathogens using *in vitro* antimicrobial susceptibility tests.

2.2. Pathogenic microorganisms

The human body is a nutrient-rich environment with a constant temperature. This makes humans the perfect ecosystem for microorganisms to thrive. Humans have around 10^{13} cells in their body and 10^{14} bacterial, fungal and protozoan cells present in the mouth, large intestine, vagina and on the skin (Mendes *et al.*, 2013). These symbiotic microbes form part of the normal flora of human beings. The normal flora microbes do not cause harm to humans, but they may become harmful in immunocompromised people and in case of an injury they can gain access to a sterile part of the body, such as the abdominal peritoneal cavity (Mendes *et al.*, 2013). Pathogenic organisms have developed specialized mechanisms for crossing biochemical and cellular barriers. They provoke a specific response from the host, such as sneezing and diarrhoea, which contributes to their survival and reproduction (Alberts *et al.,* 2002).

Pathogenic organisms can be defined as an organism with the potential or ability to cause disease in a host. Pathogen`s main goal is to complete its life cycle through successfully infecting the host and to be transferred to another host, thus to reproduce. They achieve this by evading the immune system and reproducing within the host. These microscopic pathogens include bacteria, fungi, viruses, protists, parasitic worms and prions (Bailey, 2018).

Pathogens can be prokaryotic or eukaryotic organisms. Bacteria are prokaryotic and cause illness through producing toxins harmful to the host (Sapp, 2005). Gram-positive and Gram-negative is a term used to classify bacteria into two groups, this is defined through the bacterium's chemical and physical cell wall properties (Gram, 1884). Gram stain is performed on bacteria to determine if a bacterium is Gram-positive (Purple) or Gram-negative (Pink). Gram-positive bacteria have a thick peptidoglycan layer in their cell wall, which retains the staining chemical. Gram-negative bacteria have a thin peptidoglycan layer that is sandwiched between an inner and outer cell membrane (Gram, 1884).

Fungi, protists, and protozoan parasitic worms are eukaryotic pathogens. Humans are also eukaryotic, making it challenging to kill these pathogens without harming the host (Sapp, 2005). Therefore, antifungal and antiparasitic treatments are more toxic to humans and less effective than antibiotics. Fungi and parasites have numerous forms during their life cycles, this makes them difficult to treat due to that the treatment targets a specific form, for example, *Candida albicans* is a dimorphic fungus and can exist as a mold or as a yeast (Pitarch *et al.,* 2002). Yeasts are single celled organisms and reproduce through budding, this is when a bubble forms on the cell surface and eventually separates (Ingraham *et al*., 2004). Molds are filamentous organisms, these individual filaments are called hyphae and are branched, tube-like structures, forming an open mycelium (Ingraham *et al*., 2004).

2.2.1. Classification and characterization of the pathogens used in this study

Organisms are classified and identified down to species level to ease the process of differentiating between organisms and to group them by certain criteria. Subspecies can be identified through their environmental habitat and the disease they cause. The three main tests used to distinguish between species involve phenotypic criteria, biomedical tests, and DNA relatedness (Baron, 1996).

Phenotypic criteria include the Gram reaction of the organism, its motility, whether it is acid-fast, the arrangement of its flagella, the presence of spores, inclusion bodies, capsules, and its shape. Colony characteristics and pigmentation can also be used to determine the phenotype of the organism (Baron, 1996). These characteristics can be used to identify an organism down to a genus level.

Biomedical tests are used to identify an organism`s reaction toward certain chemicals. Some tests are used regularly for many groups of bacteria, for example, oxidase, amino acid degrading enzymes, nitrate reduction, and utilization and fermentation of carbohydrates, while other tests is restricted toward a certain genus or species, such as, pyrrolidinyl arylamides test for Gram-positive cocci and coagulase test for staphylococci (Baron, 1996). Catalase is an enzyme which catalyses the decomposition of hydrogen peroxide to water and oxygen and protects the cell from oxidative damage by ROS (Chelikani *et al*., 2004). Coagulase is an enzyme that converts (soluble) fibrinogen in plasma to (insoluble) fibrin, causing blood to clot. A facultative aerobe is an organism that makes ATP in the presence of oxygen (aerobic respiration) but is able to ferment in the absence of oxygen (anaerobic respiration) (Joubert and Britz, 1987). Lactose fermenters use lactose to lower the pH of the environment by producing acid (Levine, 1941).

A phenotypic group of an organism should be tested for DNA relatedness to determine whether the observed phenotypic homogeneity/heterogeneity is reflected by phylogenetic homogeneity/heterogeneity. Biomedical characteristics should also be re-examined through DNA relatedness to determine biochemical borders between different groups of organisms (Baron, 1996). The organisms present in figure 2.3 and 2.4 have already been classified and identified. The Gram-positive bacteria *Staphylococcus aureus* and *S. epidermidis* are described in table 2.2, Gram-negative *bacteria E. coli, E. aerogenes, K. pneumoniae*, and *S. enteritidis* are described is table 2.3, and yeasts *C. albicans, S. cerevisiae, T. dermatis, C. neoformans,* and *P. aeruginosa* are discussed in table 2.4.

S. aureus (table 2.2, figure 2.3) and *S. epidermidis* (table 2.2, figure 2.3) are Gram-positive bacteria part of the normal human microbiota. *S. aureus* can be found in the nose, axilla, perineum and on the skin and is mostly asymptomatic, but can cause primary and secondary infections, pneumonia, endocarditis, osteomyelitis, septic arthritis and is the leading cause of bloodstream infections, also known as bacteraemia. Wound infection after surgery is commonly caused by this bacterium. There are strains of *S. aureus* that are antibiotic resistant, e.g. methicillin-resistant *S. aureus* (MRSA) and Vancomycin-resistant *S. aureus* (VRSA), and has become a worldwide problem in clinical medicine (Kaufhold *et. al*., 1992). *Staphylococcus epidermidis* is typically found on the skin and has the ability to form biofilms on plastic devices and causes catheter-related and prostheses infections (Vuong and Otto, 2002). Opportunistic pathogens, such as *S. aureus* and *S. epidermidis*, are a major concern to individuals with a human immunodeficiency virus or acquired immunodeficiency syndrome (HIV/AIDS) (Holmes *et al*., 2003).

Table 2.2: Description of the relevant Gram-positive bacteria (Ingraham *et al*., 2004).

Bacteria	Biomedical results	Basic morphology	Disease/syndrome
S. aureus	-Gram-positive -Catalase positive -Coagulase positive -Facultative anaerobic	Cocci in clusters	Primary and secondary infections, pneumonia, endocarditis, osteomyelitis, septic arthritis, bloodstream infections (bacteraemia).
S. epidermidis	-Gram-positive -Catalase positive -Coagulase negative -Facultative anaerobic	Cocci in clusters	Catheter-related and prostheses infections.

E. coli, E. aerogenes, and *K. pneumoniae* (table 2.3, figure 2.3) are Gram-negative bacteria that are part of the normal human microbiota while *S. enteritidis* and *S. aeruginosa* (table 2.3, figure 2.3) are found in nature. *E. coli* is commonly found in the lower intestines of the gut microbiota and is harmless, however, some strains can cause gastroenteritis and food poisoning, which is a common illness even among healthy individuals. *E. aerogenes*is is found on the skin and gastrointestinal tract and can cause opportunistic infections. *E. aerogenes* possess an inducible resistance mechanism, known as lactamase, which causes them to become resistant to standard antibiotics during treatment (Jones *et al.*, 1997). *K. pneumoniae* can cause respiratory tract infections when directly inhaled as well as and gastric infections. *K. pneumoniae* possesses an extended-spectrum beta-lactamase (ESBL) causing them to become resistant to nearly all beta-lactam antibiotics, except carbapenems (Gorrie *et al.*, 2018). *S. enteritidis* can be transferred from animal-to-human and from human-to-human and is known to infect the gastrointestinal tract and cause salmonellosis (Suzuki, 1994). *S. aeruginosa* is found in soil and water and is an opportunistic pathogen, causing infection in individuals with underlining conditions, such as cancer, severe burns and cystic fibrosis. *S. aeruginosa* is a ubiquitous microorganism which has the ability to survive under a variety of environmental conditions (Sapp, 2005).

Table 2.3: Description of the relevant Gram-negative bacteria (Ingraham *et al*., 2004).

Bacteria	Biomedical results	Basic morphology	Disease/syndrome
E. coli	-Gram-negative -Aerobic -lactose fermenters	-Rod-like shaped bacilli	Gastroenteritis, urinary tract infection, neonatal meningitis, food poisoning.
E. aerogenes	-Gram-negative -Facultative anaerobic -Lactose fermenters -β-Lactamase	-Rod-like shaped bacilli -Flagella	Bacteraemia, respiratory tract infections, skin and soft-tissue infections, urinary tract infections, endocarditis, intra-abdominal infections, septic arthritis, osteomyelitis, central nervous system infections, and ophthalmic infections.
K. pneumoniae	-Gram-negative -Aerobic -Lactose fermenters -β-Lactamase	-Rod-like shaped bacilli -Encapsulated	Pneumonia, upper respiratory tract infection, bacterial meningitis, wound infection, osteomyelitis, bacteraemia, septicaemia.
S. enteritidis	-Gram-negative -Facultative anaerobic -Non-lactose fermenters	-Rod-like shaped bacilli	Salmonellosis (acute onset of fever, abdominal pain, diarrhoea, nausea and sometimes vomiting).
P. aeruginosa	-Gram-negative -Facultative anaerobic.	-Rod-like shaped bacilli -Flagella	Pneumonia, infections in immunocompromised hosts, cystic fibrosis, folliculitis.

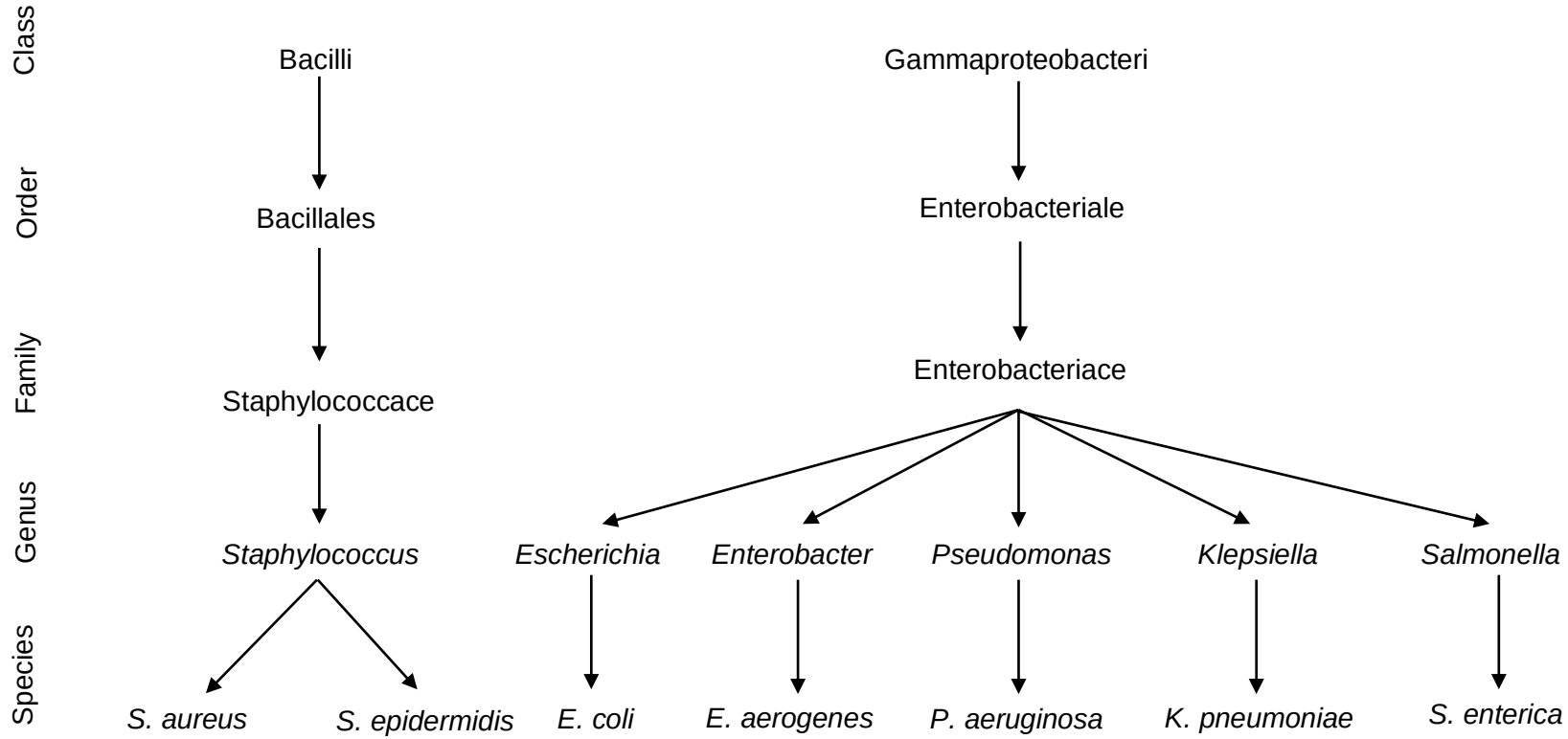


Figure 2.3: Classification of gram positive (Bacilli) and gram negative (Gammaproteobacter) bacteria.

C. albicans, S. cerevisiae, T. dermatis and *C. neoformans* (table 2.4, figure 2.4) are all fungal (yeast) microorganisms. *C. albicans* is normally found on the skin and mucus membranes and is an opportunistic pathogen causing candidiasis and oropharyngeal candidiasis when they overgrow. *C. albicans* has the ability to switch between yeast and hyphal cells, known as filamentation, which contributes to the microorganism's virulence (Azadmanesh *et al.*, 2017). *C. albicans* is a pleomorphic microorganism giving them the ability to alter their shape or size in response to environmental conditions. The germ tube formation test is a screening test which is used to differentiate *C. albicans* from other yeast. *Saccharomyces cerevisiae* (brewer's yeast) is naturally found on grapes and possess the ability to ferment various carbohydrates. *S. cerevisiae* is a single-celled eukaryote and an attractive model organism that is frequently used in scientific research due to the fact that its genome has been sequenced and its genetics are easily manipulated (Galao *et al*., 2007). *Trichosporon dermatis* is found in soil and skin microbiota of humans, causing white piedra in hair when they proliferate. They are opportunistic pathogens causing trichosporonosis in immunocompromised individuals (Colombo *et al.,* 2011). *Cryptococcus neoformans* is typically found in soil, decaying wood and bird droppings. Symptomatic infections such as cryptococcosis, meningitis and lung infections are caused in immunocompromised patients (Kwon-Chung *et al*., 2014).

Table 2.4: Description of the relevant fungi (Ingraham *et al*., 2004).

Fungi	Biomedical results	Basic morphology	Disease/syndrome
C. albicans	-Yeast -Aerobic -Germ tube formation	-Dimorphic -Pleomorphic -Filamentous -Biofilm formation	Candidiasis (yeast infection), oropharyngeal candidiasis
S. *cerevisiae*	Yeast -Aerobic -Fermentation	-Reproduce by budding	Used for brewing.
T. dermatis	-Yeast -None found	-Anamorphic -Biofilm formation	White piedra, trichosporonosis.
C. neoformans	-Yeast -Obligate aerobe -Polysaccharide capsule -Formation of melanin -Urease activity	-Encapsulated -Teleomorph	Cryptococcosis - Central nervous system – meningitis and lung infections.

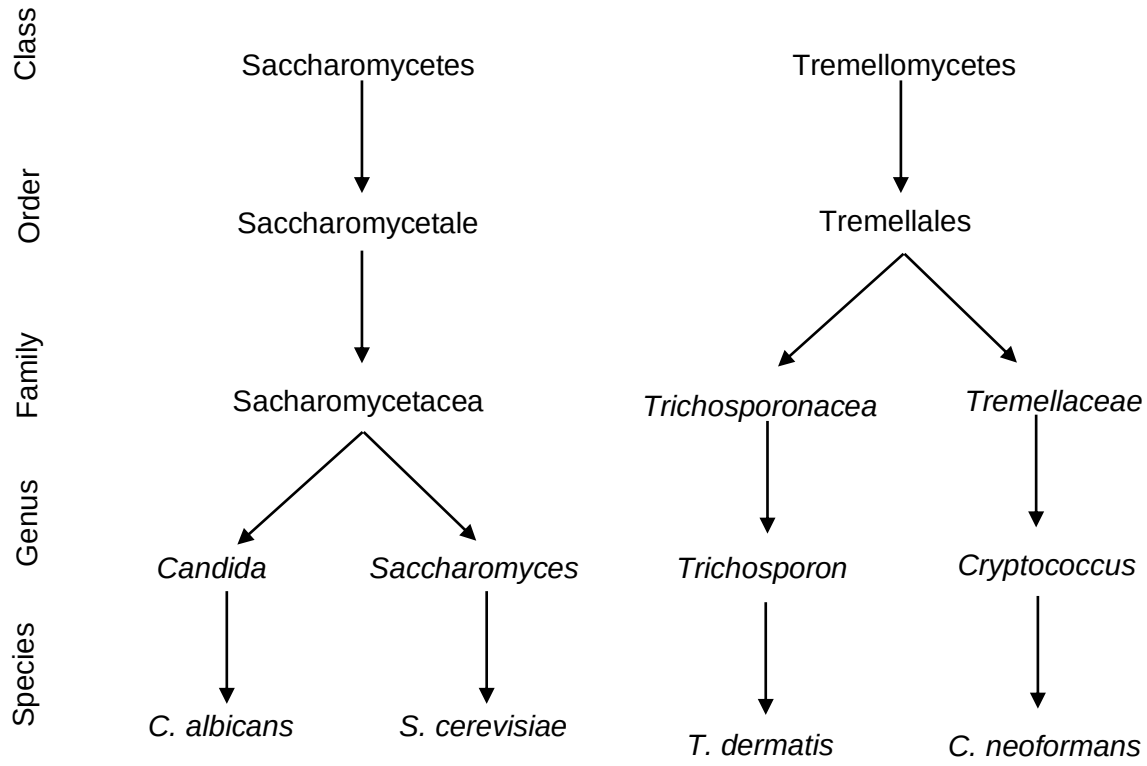


Figure 2.4: Classification of Fungi.

2.3. Antimicrobial susceptibility

The rationale for using antimicrobial susceptibility testing is to confirm the susceptibility of the microorganisms to antimicrobial agents or to identify resistance in individual microorganism isolates. Susceptibility testing is vital for pathogens that may hold acquired resistance mechanisms such as members of the Enterobacteriaceae family, *Pseudomonas* species, *Enterococcus* species, *Staphylococcus* species, and *Streptococcus pneumoniae*. The broth microdilution method is the most commonly used. Some methods provide quantitative results (minimum inhibitory concentration) and other qualitative assessments using categories such as susceptible, intermediate, or resistant (Jorgensen and Ferraro, 2009).

The disk diffusion susceptibility method is practical and simple (Bauer *et al*., 1966). This standardised method is done by applying a microbial inoculum of $1–2\times10^8$ CFU/ml (colony forming units per millilitre) on a Mueller-Hinton agar (MHA) plate with a large surface (150 mm diameter). The paper discs can be commercially obtained and 12 of the discs can be placed on the MHA plate. The plates are incubated for 16-24 h at the appropriate temperature for the microorganism. The zone of growth around each disc is measured in millimetre. The diameter of each zone is related to the antimicrobial susceptibility of the microorganisms and to the diffusion rate of the antimicrobial through the agar medium. The zone diameters for each drug are interpreted using the criteria published by the Clinical and Laboratory Standards Institute (CLSI) (Weinstein, 2018). The results obtained from this method are qualitative with a category of susceptibility, such as susceptible, intermediate, or resistant. The advantages of the disc diffusion method are the easily interpreted results with the categories and the simplicity, while the disadvantage is the lack of automated testing (Jorgensen and Ferraro, 2009).

Broth dilution method tests is one of the earliest antimicrobial susceptibility testing methods and involves preparation of a two-fold dilution of antibiotics or antifungals (eg. 1, 2, 4, 8, and 16 µg/ml) in a liquid growth media (Mueller-Hinton broth) in test tubes (Ericsson *et al.,* 1971). The test tubes containing the antibiotic or antifungal should be inoculated with a standardised microbial suspension of $1–5\times10^5$ CFU/ml and incubated overnight at the appropriate temperature. The test tubes should then be examined for visible microbial growth as shown by turbidity and the lowest concentration where there was no growth visible, represents the minimal inhibitory concentration (MIC). The precision of this method was considered to be ±1 two-fold concentration. This method has the advantage of generating quantitative results, such as the MIC, while the disadvantage was the tedious, manual task of preparing the antibiotic for each test tube, the large quantity of reagents, space required and a larger possibility of error (Jorgensen and Ferraro, 2009).

Microdilution was the next method developed where downsizing of the test tubes by using 96-well plates with each well containing a volume of 0.1 ml. This allows around 8 antimicrobials to be tested on a single plate. Inoculation of the 96-well plates with standardised microorganisms (5×10^5 CFU/ml) using pipets that can transfer 0.01 to 0.05 ml of the standardised microbial solution, following incubation (Jorgensen and Ferraro, 2009). The MICs are determined manually or by automated devices, such as a spectrophotometer, to assess the growth in each well. The advantages include quantitative results, e.g. MICs, reproducibility, the economy of reagent and less space used.

The microdilution method can be improved by adding a colour indicator. Resazurin is used to indicate cell viability by reducing resazurin (blue) to resofurin (pink), indicating that the cells are metabolic active. It uses an oxidation-reduction indicator in cell viability assays for both aerobic and anaerobic respiration (Sarker *et al*., 2007).

For certain infections, it may be important to know the concentration of the antimicrobial that kills the organism rather than just inhibiting its growth. This concentration called the minimal bactericidal concentration (MBC) or minimal fungicidal concentration (MFC), is determined by taking a small sample (0.01 or 0.1 ml) from the tubes/wells used for the MIC assay and spreading the content over the surface MHA plate (Balouiri *et al*., 2016). Any organisms that were inhibited but not killed in the MIC test will now be able to grow, as the antimicrobial agent has been diluted significantly. After a standard incubation, the lowest concentration that has reduced the number of colonies by 99.9% is considered to be the MBC. Bactericidal or fungicidal antibiotics or antifungals usually have an MBC or MFC equal or very similar to the MIC, whereas bacteriostatic of fungistatic antibiotics or antifungals usually have an MBC or MFC significantly higher than the MIC. The definitions of bacteriostatic or fungistatic means the agent prevents the growth of the microorganism and keeps them in a stationary phase, while bactericidal or fungicidal means that the agent kills the microorganisms (Rhee and Gardiner, 2004).

2.4. Antimicrobial resistance

The term antimicrobial drugs consist of a wide range of pharmaceutical agents including antibacterial, antifungal, antiviral and anti-parasitic drugs. AMR occurs when microorganisms change on a genetic level causing the antimicrobials to be ineffective. AMR can occur naturally when the microbes undergo a genetic mutation and may be accelerated through the misuse and overuse of antimicrobials (WHO, 2018b). Despite warnings to take care when using antimicrobials, the WHO reported that there is a serious need for new antibiotics (WHO, 2017). Without effective prevention and treatment of infections, procedures such as surgery, diabetes management, chemotherapy and organ transplants will become high risk to perform and difficult to manage.

Due to careless use of antimicrobials over the years, the penicillin-resistant *Streptococcus pneumoniae* (PRSP), methicillin-resistant *Staphylococcus aureus* (MRSA), and vancomycin-resistant *Enterococcus* (VRE) strains continue to increase (WHO, 2018b).

2.4.1. Approaches to overcome antimicrobial resistance

The global problem of AMR is constantly spreading, increasing morbidity and mortality (Huh and Kwon, 2011). Spreading AMR to different environmental niches and the development of superbugs caused additional complications to effective control strategies. International, national and local approaches have been advised for control and prevention of AMR (WHO, 2018a):

- Rational use of antimicrobials
- Regulation on over-the-counter availability of antibiotics
- Improving infection prevention and control

Thorough understanding of resistance mechanism and innovation in new drugs and vaccines and extensive funding to discover new antimicrobials is needed. Recently antimicrobial nanoparticles (NPs) and nanosized carriers for antimicrobials have proven to be effective in treating AMR (Huh and Kwon, 2011).

2.5. Pheroid® as a drug delivery system

For a drug to be effective, it must reach the desired location in sufficient amounts. The lipid-based Pheroid® delivery system is a nano- and micro-sized particle delivery system designed to act as a biological transporter that can entrap hydrophilic, hydrophobic or amphiphilic compounds for various forms of application (Grobler, 2009). The Pheroid® delivery system enhances absorption of biological and pharmacological compounds. Different delivery systems are used to improve the drug's water solubility, decrease the toxicity, increase the permeability, and increase site specific delivery of the drug (du Plessis *et al.,* 2012, du Plessis *et al.,* 2013, Jacobs *et al*., 2014, Steyn *et al*., 2011). Pheroid® consists of an oil phase (Cremophor RH40, vitamin F ethyl ester CLR, DL-α-tocopherol), water phase and an N_2O gas phase. During a spontaneous reaction, the active pharmaceutical ingredient (API) present is packed into the Pheroid® vesicle (Grobler, 2009).

2.5.1. Classification of Pheroid® and Pro-Pheroid®

Pheroid® is a stable lipid-based submicron and micron-sized structure and is a uniform emulsion containing mostly essential fatty acids. The Pheroid® vesicles that remain in suspension are usually formulated to be between 200 nm and 2 µm in size. The Pheroid® delivery system is a colloidal system that can be manipulated in terms of size, structure, morphology and function. This is done to meet the solubility characteristics (hydrophobic or hydrophilic) of the active ingredient that needs to be entrapped and delivered to the site of action (Gibhard, 2012). Pro-Pheroid® production is similar to that of Pheroid® production, except that no aqueous phase is introduced; instead, the active compounds are dissolved in the oil phase.

Using a colloidal system, such as Pheroid®, as drug carriers can enhance the efficacy and reduce undesirable side effects (Wiechers, 2008). It may also act in synergism with active ingredients and increase the therapeutic effects (Grobler, 2009). During Pheroid® formulation, the rate of delivery and route of administration should be taken into consideration when deciding what type of Pheroid® should be manufactured. There are three main Pheroid® structures (figure 2.5); lipid-bilayer Pheroid® vesicles (80-300 nm), Pheroid® microsponges (0.5-5 µm) which can act as reservoirs for Pro-Pheroids®, and Pro-Pheroids® which is regarded as a precursor for Pheroid® (Grobler, 2009).

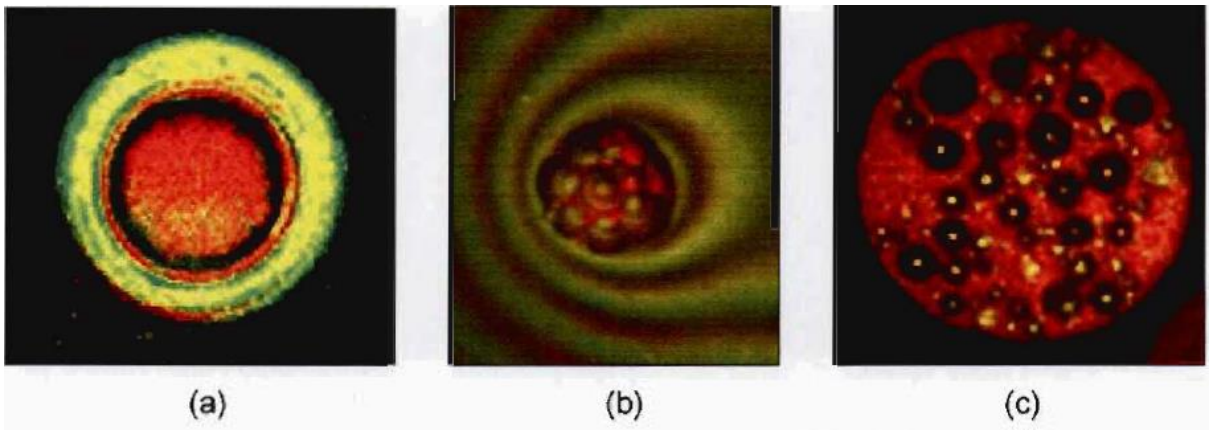


Figure 2.5: Confocal laser scanning micrographs of **(a)** entrapped rifampicin in a Pheroid® vesicle. The fluorescent labelling with Nile red enables us to see the multiple layers of the vesicle. **(b)** Pro-Pheroid® vesicle used in oral drug delivery and **(c)** Pheroid® microsponges with Pro-Pheroid® reservoirs (Reprinted from Grobler, 2009:149 with permission from the author).

The main components of Pheroid® are fatty acids esterified to form ethyl esters, pegylated ricinoleic acid, α-tocopherol and N_2O saturated water. Each component used during formulation manufacturing will be discussed in detail below.

2.5.1.1. Essential fatty acid component

The fatty acid part of Pheroid® and Pro-Pheroid® consists of ethylated and pegylated fatty acids or esters. The fatty acid components used to manufacture Pheroid® and Pro-Pheroid® are Vitamin F ethyl ester CLR, Cremophor RH40 and Incromega E3322.

Vitamin F ethyl ester CLR, also known as linoleic acid ($C_{20}H_{38}O_2$), is a polyunsaturated omega-6 fatty acid and is used in the biosynthesis of prostaglandins and cell membranes (Pubchem, 2018). In a study done by Kabara *et al.,* (1972), they tested the antimicrobial activity of various fatty acids and found that linoleic acid had antimicrobial activity against *C. albicans* (MIC = 0.455 mg/ml).

Cremophor RH40, also known as polyoxyl castor oil, is prepared by reacting 35 moles of ethylene oxide with one mole of castor oil, forming glycerol polyethylene glycol ricinoleate (BASF, 2018). Cremophor RH40 is a non-ionic surfactant used to stabilize emulsions with nonpolar materials in water. Ricinoleic acid and its esters have been described to have antimicrobial activity against lactobacilli and *S. aureus* and *P. aeruginosa* (Narasimhan *et al*., 2007; Black *et al*., 2013).

Incromega E3322 is ethylated esters such as ω-3 polyunsaturated fatty acids (PUFAs), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). These ethylated esters also exhibit antimicrobial activity against *Streptococcus* mutants and *C. albicans* (Huang and Ebersole, 2010).

2.5.1.2. Nitrous oxide component

Nitrous oxide (N_2O) is known as “laughing gas” and has been used as an inhalation anaesthetic and analgesic for many years (Evers *et al*., 2006; Berkowitz *et al*., 1979). N_2O is both water and lipid soluble providing a functional model for transporting hydrophilic and hydrophobic drugs in the N_2O essential fatty acid matrix created through the interactions between the fatty acids and N_2O (Grobler, 2009). N_2O contributes to the miscibility of the essential fatty acids and ensures stability to vesicles.

2.5.1.3. Alpha-Tocopherol component

Vitamin E is a collective term for four types of tocopherols (α-, β-, γ- and δ-) and four tocotrienols (α-, β-, γ- and δ-). Alpha-tocopherol is a lipid-soluble vitamin and is used as a solvent for lipophilic drugs and solubilisation of hydrophobic drugs (Salway, 2006). Alpha-tocopherol also contributes to the anti-oxidative qualities for both the active ingredient and Pheroid®. Alpha-tocopherol also has demonstrated antimicrobial activity (Constantinides *et al.*, 2006).

Liposoluble compounds, such as α-tocopherol, is known to modify the permeability of bacterial cell membranes for several substances, including antibiotics (Pretto *et al.*, 2004; Andrade *et al.*, 2014). Andrade *et al.* (2014) were the first to demonstrate the effect of α-tocopherol as inhibitors of antibiotic efflux systems. Tintino *et al*., (2016) evaluated the inhibitory effect of efflux pumps of α-tocopherol against both *S. aureus* and MDR (multidrug resistant) *S. aureus*. They found that there was a decrease in the MICs which suggest that α-tocopherol presented an inhibitory effect on the efflux pumps (Tintino *et al*., 2016).

2.5.2. Pheroid® technology applications

Pheroid® technology is based on what was previously called Emzaloid® technology. The intellectual property on which Pheroid® technology is based was purchased by the North-West University, South Africa. The application in the cosmetic field is based on the enhancement of absorption of the active ingredient into the epidermis or dermis layers of the skin (Wiechers, 2008).

The Pheroid® delivery system has demonstrated an interesting capability to increase the efficacy of anti-infectives (e.g. rifampicin, ethambutol, isoniazid, pyrazinamide and povidone-iodine) and to enhance the bioavailability of PEs (Moruisi, 2008). The application of Pheroid® for the delivery of anti-infectives is patented (Meyer, 2001) with the system demonstrating an increase in the amount of API the organism is exposed to, leading to shorter treatment time and better adherence by the patient. The Pheroid® system can also combat resistance caused by adaptations of the bacterial cell wall to decrease the amount of drug accumulating in the bacterium, due to an increased permeability of the active ingredient (Grobler, 2009; Meyer, 2001). *In vitro* efficacy of antimalarial drugs (artemisinin, lumefantrine, mefloquine, doxycycline, tetracycline, triclosan, erythromycin and azithromycin) was increased against *Plasmodium falciparum*, with some of the results replicated during *in vivo* evaluations (Steyn, 2009; Langley, 2007; Steyn *et al*., 2011; Grobler *et al*., 2014a; Grobler *et al*., 2014b; Govender, 2012; Du Plessis *et al*., 2015; Van Huysteen, 2010; Van Niekerk; 2010; Du Plessis *et al*., 2012). The efficacy of transdermal application of anti-infectives was also greatly increased by the Pheroid® delivery system, with ketoconazole, isoniazid, rifampicin and acyclovir demonstrating higher transdermal concentrations (Reynecke, 2004; Van der Walt, 2007; Botes, 2007; Benade, 2009; Gerda *et al*., 2014). Currently, Covarex™ (Emzaloid™/Pheroid® entrapped Ketoconazole) is on the market as a superior treatment for topical fungal infections. The bioavailability of orally administered epigallocatechin gallate (obtained from a green tea PE) was increased markedly by entrapment in Pheroid® as was demonstrated in a clinical trial in human volunteers (Moruisi, 2008). It is therefore feasible that the Pheroid® delivery system can be beneficial for the entrapment of an *A. africanus* PE for anti-infective applications.

2.6. Characterisation of formulations

Characterisation of emulsions is important and is usually done to predict the stability and activity of suspension.

2.6.1. Mean particle size and the influence of particle size

The metrics used to describe the particle size, are D-values. The particle size distribution is D10, D50 and D90 which are 10%, 50% and 90% intercepts of the cumulative mass. The mean particle size is the D50 value where 50% of the particles are smaller and 50% are larger than the D50 value (Rawle, 2018).

Oil-in-water (o/w) emulsions are commonly formulated for parenteral and topical administration but also for the oral and ocular administration routes. Each route of administration has to meet its own requirements of formulation, e.g. sterility for parenteral preparations and aesthetic attractiveness for topical products. Another important parameter for emulsions is the size of the oil droplets dispersed in the water. The median size, as well as the distribution of sizes, is very important since they determine the safety of the preparation in the case of intravenous preparations or the release properties of the active ingredient in topical formulations (Roland, *et al*. 2003).

2.6.2. Zeta potential and stability

Stability is related to the zeta potential (mV) of the emulsion which is an indication of the stability of the dispersion, but gives insufficient information of stability over time (Ronald *et al.*, 2003). The zeta potential is defined as the difference in potential (mV) of ions between the surface of the particles and the electroneutral region of the solution. When the zeta potential is more than 25 mV, the repulsive forces are more than the attractive London forces, but if the zeta potential is more than -25 mV (*i.e.* -23mV), the attractive forces will be more than the repulsive forces and flocculation will occur (Lieberman *et al*., 1989).

Other indications of instability in the emulsified system are coalescence, flocculation, creaming, phase separation, and Ostwald ripening. Flocculation is defined as when two droplets become attached to one another while still separated by a thin film of liquid, whereas aggregations when even more droplets are attached (Lieberman *et al*., 1989). If the thin layer of liquid between the droplets are removed, bigger droplets form and coalescence occurs, this is characterized by a wide size distribution with no clusters present (Lieberman *et al*., 1989). When the dispersed particles float on the continuous phase and become creamier, creaming has occurred (Lieberman *et al*., 1989). Ostwald ripening (or molecular diffusion) can cause emulsion instability as long as the droplets are small (0.1 - 0.5 µm) and the dispersed phase (oil phase) has a fixed solubility in

the continuous phase (water phase). Therefore, the small droplets formed initially will dissolve/become smaller and some droplets will grow larger until the smaller droplets are depleted (Welin-Berger and Bergenståhl, 2000). This phenomenon indicates that once the small droplets have dissolved, the system should stabilize.

If the emulsion is exposed to a temperature above the phase inversion temperature (PIT), the oil in water (o/w) emulsion stabilised with non-ionic surfactants tend to invert to water in oil (w/o) emulsions as the surfactant molecules dehydrate and become more lipophilic (Bjerregaard *et al*., 2001).

During AST the products are stored at a temperature higher than room temperature, the increase in temperature causes an increase in the chemical reaction rate (Goldanskii *et al.,* 1973). The nature of the product determines the temperatures that will be used for the AST. Samples are removed at various time intervals and analysed to determine the degree of degradation. Storage of products in atmospheres with a high humidity will accelerate hydrolysis, but the product will only be affected by this if it is stored in an open container. One of the standard AST conditions is 45 °C with a 75% relative humidity (RH) (Baby *et al*., 2007).

2.6.3. Confocal laser scanning microscopy

Confocal laser scanning microscopy (CLSM) assists with visualizing the formed Pheroid® vesicles. This analytical method has the advantage of viewing the fluorescently stained vesicles at varying depths. The samples are marked with a fluorescent marker and excited with a laser that emits photons at a specific wavelength (Park and Kihm, 2006). It is important to have at least one visualization technique to complement quantitative measurements of particle size in order to provide information on the morphology as well.

Chapter 3: Materials and methods

3.1. Materials

3.1.1. Pheroid® formulation

Materials used for Pheroid® and Pro-Pheroid® formulations were vitamin F ethyl ester CLR (Chemimpo, South Africa), Cremophor RH40 (BASF chemicals, South-Africa), DL-α-tocopherol (Chempure Pty Ltd, South-Africa), Incromega E3322 (Croda Chemicals SA Pty Ltd) and medicinal grade N_2O (AFROX, South-Africa). The zeta potential was determined by using ethanol absolute (Sigma-Aldrich®, South-Africa), and distilled water (Merck Millipore Elix® Essential 3). Hydrochloric acid (Sigma-Aldrich®, South-Africa) was used for the dilutions to determine the particle size with Malvern Mastersizer 2000SM. The CLSM used a 1 mg/ml Nile Red fluorescent marker (Sigma-Aldrich®, South-Africa) to observe the vesicles.

3.1.2. *Agapanthus africanus* crude plant extract

The *A. africanus* glycerol PE was obtained from Agrarforum Pty. Ltd., South-Africa. Genetically pure *A. africanus* plants were grown under 40% shade on Bainsvlei soil in Bainsvlei area, Bloemfontein, Free State, South Africa. Ethanol and glycerol were purchased from Lasec SA Pty Ltd, (South-Africa).

3.1.3. Antimicrobial susceptibility testing

Cultures used for antimicrobial susceptibility tests were obtained from the American Type Culture Collection (ATCC), Manassas, Virginia, (United States of America). The Gram-negative cultures are *Enterobacter aerogenes* (ATCC 35029), *Escherichia coli* (ATCC 10536), *Klebsiella pneumoniae* (ATCC 10031), *Pseudomonas aeruginosa* (ATCC 10145) *and Salmonella enterica* (ATCC 9150). The Gram-positive cultures are *Staphylococcus aureus* (ATCC 43300) and *Staphylococcus epidermidis* (ATCC 12228). The fungal culture species are *Saccharomyces cerevisiae* (ATCC 2610), *Candida albicans* (ATCC 14053), *Trichosporon dermatis* (ATCC 201382) and *Cryptococcus neoformans* (ATCC 66031). Mueller–Hinton agar and broth used to grow the cultures and the glucose supplement were obtained from Sigma-Aldrich®, (South-Africa). Erythromycin (Ery), trimethoprim (TMP) and amphotericin B (AmB) antibiotics used as the positive controls were ordered from Industrial Analytical Pty. Ltd. (South Africa). Corning 96 well plates, 25 ml reservoirs and disposable inoculating needles were obtained from Sigma-Aldrich® (South-Africa). Petri dishes 90x15mm and 0.20 µm syringe filters were obtained from Lasec® (South-Africa). Resazurin sodium salt used for cell viability was obtained from Sigma-Aldrich® (South-Africa).

The samples were kept in a KIC fridge at 4°C and incubated in a Labcon incubator at the appropriate temperature. All samples were handled in a level 2 biosafety cabinet (Bioair, s@femate 1.2). A spectrophotometer was used to measure the growth (BioTek PowerWave HT microplate reader).

3.1.4. Accelerated stability testing

The AST was conducted by the Research Institute for Industrial Pharmacy, incorporating Centre for Quality Assurance Medicines (RIIP®/CENQAM®). A Binder incubator was used and temperatures were noted daily 40°C ± 2°C, as well as the relative humidity 75% RH ± 5% RH.

3.2. Study design

Figure 3.1 illustrates a summary of this study`s design. The formulations consist of 5 test formulations containing the PE (4%, 8%, 10%, 13%-Pheroid® and 50% Pro-Pheroid®) and 5 corresponding control formulations (4%, 8%, 10%, 13%-Pheroid® and Pro-Pheroid®), respectively. The crude PE is also included as a test formulation (table 3.2). The characterisation of the test- and control formulations includes determining the particle size, zeta potential and morphology of the vesicles. The test- and control formulations were subjected to AST for 3 months at 40°C ± 75% RH. Each test- and control formulation`s activity were evaluated through the antimicrobial susceptibility tests and were repeated each month for 3 months (*i.e.* month 0, 1, 2, and 3). After the initial results, we identified certain shortcomings and expanded the study by developing an improved method of determining the minimum inhibitory concentration (MIC) of the test- and control formulations using Resazurin as a colour indicator. Using this improved method, the MICs and minimum fungicidal concentrations (MFC) of the test formulations were determined against an updated panel of yeasts.

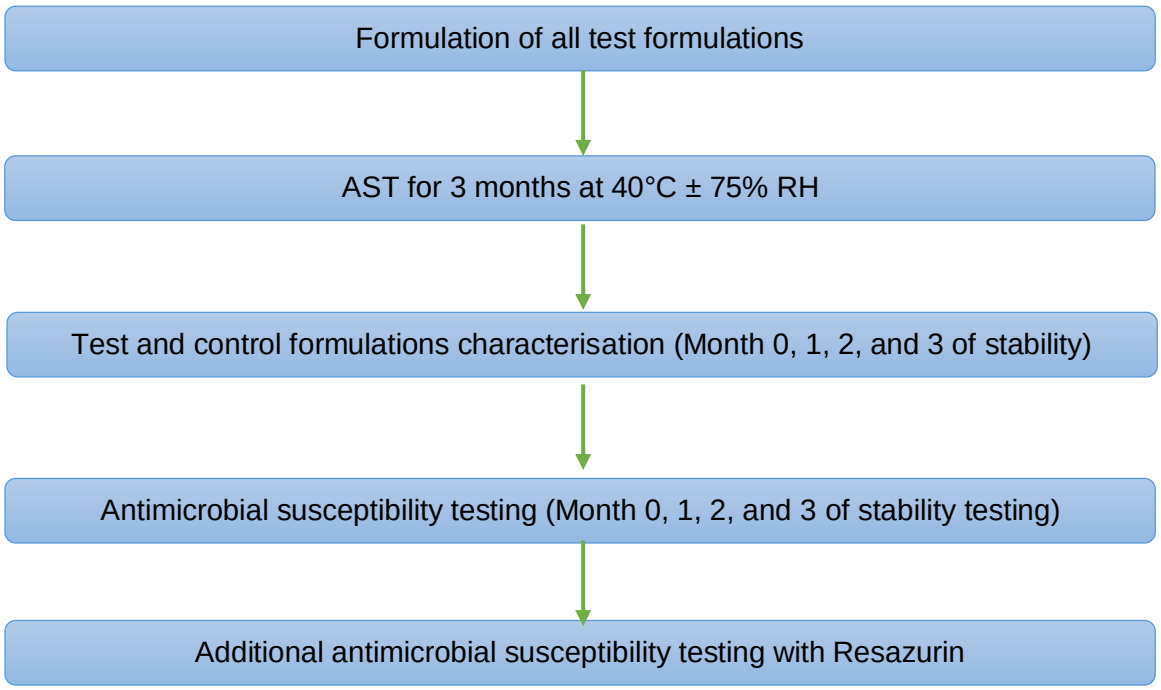


Figure 3.1: Design of the study

3.3. Methods

3.3.1. Plant extract

The *A. africanus* glycerol PE was obtained from Agrarforum Pty. Ltd. (South-Africa) and the preparation (section 3.3.1.1.) and quantification (section 3.3.1.2.) was done by Biopher Pty. Ltd. (Agrarforum Pty. Ltd., South-Africa).

3.3.1.1. Preparation of the crude extract from *Agapanthus africanus*

Leaves, stems and stalks from mature *A. africanus* plants were cut 3 cm from the soil surface. The fresh plant material was dried at 45°C over a period of four days in a wooden cabinet containing a heater and fan. Using a Fritsch laboratory mill (Germany), the dry plant material was ground to a fine powder (± 500 µm).

In a 1 L screw top Schott bottle, 50 g of finely ground *A. africanus* dry material powder was mixed with an ethanol: glycerol mixture (60: 40 v/v) at a volume to mass ratio of 10 ml/g and placed on a mechanical shaker for 16 h. Afterwards, the ethanol glycerol PE was filtered once under vacuum, then again using gravity both times, using a Büchner funnel attached to an Erlenmeyer flask equipped with a sidearm through 3 HW, 125 mm Munktell filter paper. The filtrate was then vacuum distilled to get rid of the ethanol. This was done until the ethanol stopped dripping, with a 1 L round bulb distillation flask using a Buchi R300 Rotavapor. The remaining glycerol extract contained all the components extracted from the dry plant material. Approximately 200 ml of the glycerol fraction was recovered using a bulb pipette and transferred to a glass container. The glycerol fraction was then stored in the refrigerator at 4°C until further use.

The yield of all the components combined in the crude glycerol fraction was determined at 150°C for 8 hours using 1 ml aliquots and spreading them thinly in 9 cm glass Petri dishes. The concentration was determined by weighing these Petri dishes before and after the drying process. The average concentration from four replicas was then used as the final concentration (mg/ml).

3.3.1.2. Quantification of the active compound

Determination of the active compounds in the glycerol fraction was done by using an adapted spectrophotometric method from Baccou *et a*l. (1977). Dioscin (a saponin) was used as an external standard to determine the saponin content in *A. africanus* extracts.

A standard curve was drawn spectrophotometrically by preparing a stock solution of the saponin standard, Dioscin (95%). This is done by dissolving 0.001 g dioscin in 1 ml methanol and preparing a concentration series (table 3.1) in separate tubes.

Table 3.1: Dioscin concentration range used to draw a standard curve spectrophotometrically.

Volume of Dioscin stock solution (µl)	Amount contained in stock solution (µg)	Amount (µg) divided by final Volume(ml) in test tube after reagent addition	Final concentration Reagent (µg/ml)
20	20	20 ÷ 4.05	4.94
30	30	30 ÷ 4.06	7.39
40	40	40 ÷ 4.07	9.83
50	50	50 ÷ 4.08	12.25
60	60	60 ÷ 4.09	14.6
70	70	70 ÷ 4.10	17.07

The following reagents were prepared according to Baccou *et a*l. (1977); reagent A contained 0.2 ml anisaldehyde and 39.8 ml ethyl acetate and reagent C contained 25 ml H_2SO_4 and 25 ml ethyl acetate. The following reagents were added to the test tubes in table 3.1; 2 ml ethyl acetate, 1 ml Reagent A and 1 ml Reagent C and mixed with a Vortex mixer until the glycerol was dissolved completely and placed in a water bath at 60˚C for 20 minutes. The test tubes were then removed and allowed to cool at ambient temperature for 15 min.

A baseline was drawn using a blank solution containing 1 ml of Reagent A, 1 ml of Reagent C, 2 ml of ethyl acetate and 30 µl of glycerol and subsequently, scanned for the optical density of the test samples on a Schimadzu UV-1800 spectrophotometer at a wavelength range of 300 to 550 nm in the spectrum mode. A standard curve (figure 3.2) based on absorption values was executed by linear regression using an NCSS 11 statistical program.

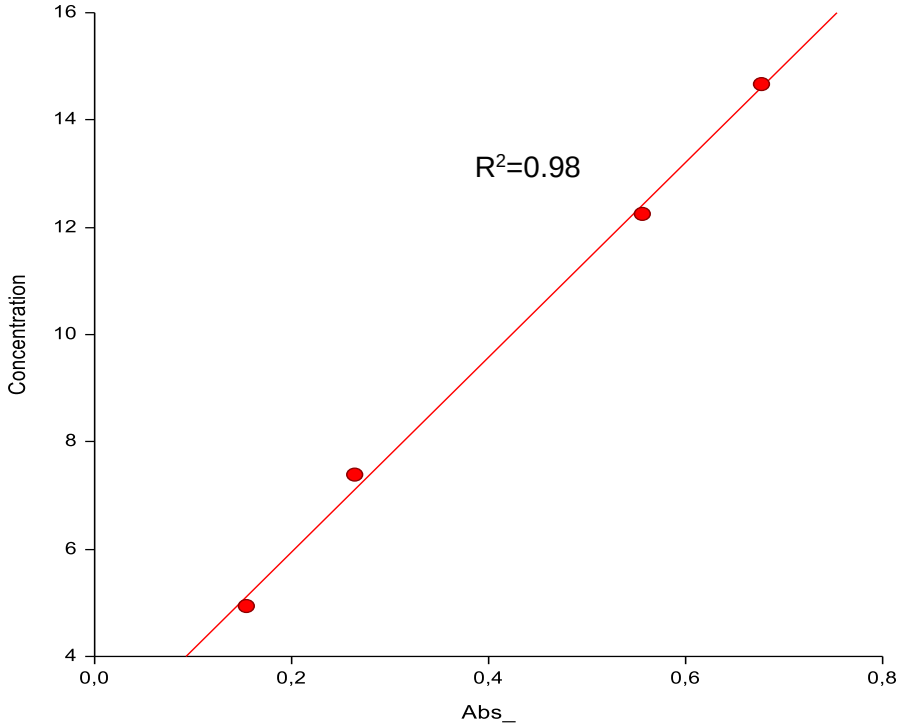


Figure 3.2: Calibration curve of a saponin (Dioscin) standard concentration range for determining saponin content in an *Agapanthus africanus* ethanolic glycerol crude extract.

The estimated mathematical model provided by the statistical program was used to calculate saponin content present in the *A. africanus* extract. The model was applied by multiplying the absorption with 18.13482 and adding 2.32735 and expressing the saponin content as g/L:

Estimated Model: (2.32735) + (18.13482) * (Abs)

The average concentrations of three batches used in this study were 60, 34 and 55 mg/ml respectively. The concentrations of each batch depend on numerous factors, incl. time of year that plants were harvested, average temperatures, and rainfall etc. (Shurson, 2005).

3.3.2. Formulation manufacturing

The percentage oil phase in the Pheroid® formulations, *i.e.* 4%, 8%, 10%, 13% and 50% Pro-Pheroid® (table 3.2) were altered to determine the optimum Pheroid®-PE formulation. To each of the PE test formulations an equal amount of PE and Pheroid® or Pro-Pheroid® was added (a ratio of 1:1 for Pheroid®: PE, w/w), and to each of the corresponding control formulations, no PE was added. Another test formulation was added where the PE alone was diluted with distilled water (1:1 w/w).

The formulations were manufactured in a similar manner as has been described previously by du Plessis and co-workers (2010). Pheroid® vesicles are an oil-in-water emulsion and Pro-Pheroid® is an oil-based mixture. The water phase was gassed for 24 hours with N_2O at 200 kPa. For the 4% test formulation the different raw materials were added in the following ratios: N_2O saturated water (96%), vitamin F ethyl ester (2.8%), Cremophor RH40 (1%), DL-α-tocopherol (0.2%) and Incromega E3322 (0.01%) and for the other test formulations, the oil phase was increased (keeping the different ingredients in the same ratios) and the water phase decreased (table 3.2). The concentration of the PE added to the Pheroid® formulations was in a ratio of 1:1 w/w (table 3.2). In two separate containers, Cremophor RH40 was heated in a microwave to 120°C, and vitamin F ethyl ester and Incromega E3322 was heated to 70°C. After the Cremophor RH40 had cooled down to 70°C, the vitamin F ethyl ester and Incromega E3322 (also 70°C) was mixed. After this mixture had cooled down to 55°C, the DL-α-tocopherol was added, creating the oil fraction of the Pheroid® drug carrier system. The above mentioned gassed water was heated to 70°C, after which the PE and oil fraction were added. The newly formed suspension was then homogenised at 13 500 rpm for 30 seconds, then left to settle for 30 seconds. After settling, the mixture was homogenised again for double the time, *i.e*. 60 seconds, then again left to settle for 30 seconds. This cycle was repeated until the mixture cooled down to at least ≤40°C. The finished Pheroid® emulsion was then gassed with N_2O for 72 hours at 200 kPa. The Pheroid® emulsions and Pro-Pheroid® was stored in glass vials containers in a refrigerator at 4°C.

Table 3.2: The composition of the different test- and control formulations.

Formulations	Vitamin F Ethyl Ester CLR (g)	Cremophor RH40 (g)	DL-α-Tocopherol (g)	Incromega E3322 (g)	Plant extract (PE) (g)	N_2O saturated water (g)	Final amount (g)
PPE 4%	0.44	0.22	0.04	0.10	10	9.20	20
P 4% (Control of PPE 4%)	0.44	0.22	0.04	0.10	0	19.20	20
PPE 8%	0.87	0.45	0.08	0.20	10	8.40	20
P 8% (Control of PPE 8%)	0.87	0.45	0.08	0.20	0	18.40	20
PPE 10%	1.09	0.56	0.10	0.25	10	8	20
P 10% (Control of PPE 10%)	1.09	0.56	0.10	0.25	0	18	20
PPE 13%	1.42	0.73	0.13	0.33	10	7.40	20
P 13% (Control of PPE 13%)	1.42	0.73	0.13	0.33	0	17.40	20
Pro-PPE 50%	5.46	2.79	0.50	1.25	10	0	20
Pro-P (Control of Pro-PPE 50%)	5.46	2.79	0.50	1.25	0	10	20
PE	-	-	-	-	10	10*	20

*The PE was diluted with distilled H_2O.

3.3.3. Formulation characterisation

The formulations were all subjected to AST for three months, after which antimicrobial tests were done at month 0, 1, 2 and 3 to determine if there was a change in activity. For each test- and corresponding control formulation (table 3.2) the morphology, particle size and stability were determined with a CLSM, Malvern Master Sizer and a Nanosizer, respectively during month 0, 1, 2 and 3.

3.3.3.1. Particle size distribution

The Malvern Mastersizer determines the size of Pheroid® vesicles, which is done by measuring the light scatter pattern these particles create. The Pheroid® sample (test- and control formulations) was added to dH_2O in the pump, at 2500 rpm, until an obscuration rate of 10 - 20% was obtained. Background noise and samples were measured for 12 seconds, with a delay of 20 seconds between measurements. Pro-Pheroid® samples were acidified to create Pheroid® vesicles, this is done by diluting 1ml of a Pro-Pheroid® sample in 9ml of 0.1 N HCL. The standard size for Pheroid® vesicle ranges from 2-200nm (Grobler, 2009). This was done to determine if different Pheroid® vesicle sizes have an effect on antimicrobial activity. Each sample was measured three times (Uys, 2006).

3.3.3.2. Zeta potential stability

Zeta potential is the potential difference between the dispersion medium and the stationary layer of fluid attached to the dispersed particle. Pheroid® sample (test- and control formulations) with a 4% fatty acid content was diluted by taking 100µl of the sample and adding it to 10 ml dH_2O, from this first dilution, 200 µl was taken and added in another 10 ml dH_2O resulting in a 1:5000 dilution. Pheroid® sample with a fatty acid content higher than 4%, e.g. 8% was diluted to a 4% fatty acid content. This was calculated as such:

$$C_1V_1 = C_2V_2$$

$$(0.04)(100) = (0.08)\ V_2$$

$$V_2 = 50\mu l \text{ diluted in } 10ml\ dH_2O$$

The apparatus was first rinsed with the sample and 1 ml of the second dilution was added to the apparatus and measured. Each sample was analysed three times by the machine and the average was used as the final zeta potential. The apparatus was rinsed after each measurement with 5 ml absolute ethanol and 5 ml dH_2O. A zeta-potential of ≤ -25 mV is considered beneficial since this provides stronger repulsive forces that overcome attractive forces between particles, resulting in a stable emulsion (Roland *et al*., 2003).

3.3.3.3. Morphology

A CLSM was used to create a visual presentation of the Pheroid® vesicles (Uys, 2006). This provides confirmation that the correct morphology is obtained, *i.e.* if the lipid bilayer is present forming a vesicle. This was achieved by adding 1 µl of 1 mg/ml Nile Red fluorescent marker to 50 µl of samples, which emits light when excited with the laser. The samples were vortexed and incubated at room temperature for 15 minutes in the dark at room temperature. After incubation, 20 µl of the sample was placed on a microscope slide and analysed for morphological and fluorescent characteristics by CLSM. The CLSM has three lasers emitting light at wavelengths of 405, 488 and 543 nm. Fluorescence light emission was detected at wavelengths of 485-545 nm, 540-650 nm and above 650 nm. Combined, these three images were used to confirm the presence of the Pheroid® vesicles.

3.3.4. Accelerated stability testing

The formulations were thermally stressed at 40°C with 75% relative humidity (RH), which is the pharmaceutical standard for PEs. Samples were taken during a 3-month period (month 0, 1, 2, and 3) after which the particle size, zeta potential, morphology, and *in vitro* antimicrobial susceptibility was determined for each sample. Furthermore, visual inspection was also performed to evaluate changes (texture, colour, and odour) that might indicate stability problems (Jacobs *et al*., 2014). Results were compared with month 0, to determine if there were any changes.

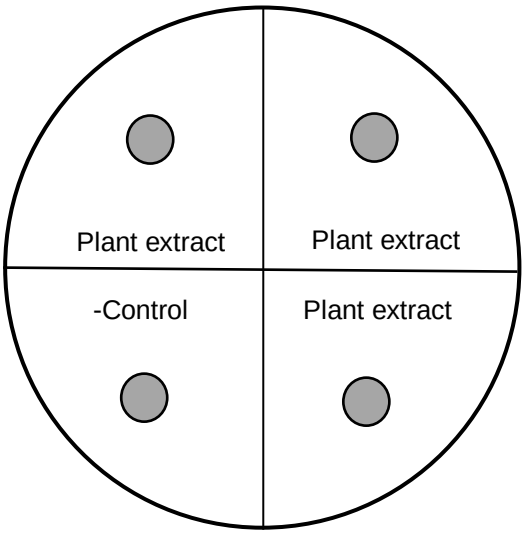
3.3.5. Antimicrobial susceptibility tests

The Kirby Bauer disc diffusion method (section 3.3.5.1.) was used as a quick and inexpensive method of determining if the PE would have any potential activity against a number of known bacteria (Gram-positive and Gram-negative) and fungi. Secondly, the MIC of the test formulations against the susceptible microorganisms was determined by using the broth microdilution method (section 3.3.5.2.). Subsequently, the test formulations were subjected to bacteriostatic/fungistatic or bactericidal/fungicidal testing (section 3.3.5.3.). Antimicrobial susceptibility tests (MIC and MBC/MFC) were conducted during month 0, 1, 2 and 3 of AST. After screening the formulations for potential antimicrobial activity against the test microorganisms, the MIC and MFC were determined again using an updated panel of microorganisms and a different technique incorporating resazurin as an indicator (section 3.3.5.4.). Due to that Pheroid® is an emulsion, the activity could not be expressed as a concentration (mg/ml). Therefore, to compare the Pheroid® and Pro-Pheroid® control formulations to the test formulations containing the PE, we used the dilution factor (DF) as a comparison of activity. The calculations and comparisons can be seen in Annexure B and in Annexure C.

Although some of the individual Pheroid® components have antimicrobial activity on their own, the whole formulation, *i.e*. the Pro-Pheroid® oil phase as well as the water phase, act as the carrier system. To determine what degree of the antimicrobial activity observed for the test formulations can be attributed to the Pheroid® vesicles or the crude PE, each test formulation and their corresponding control formulation were diluted concurrently, after which the antimicrobial activity of each dilution was determined. When comparing the test formulations to one another the concentration of the active saponin was used.

3.3.5.1. Kirby Bauer disc diffusion

The Kirby Bauer disc diffusion method was used to determine the zone of inhibition (ZOI) (Smit *et al*., 2016). MHA plates were inoculated with one of the organisms in figure 3.3, by using the growth method. When using the growth method, a loop is used to touch the top of 3-5 colonies that are morphologically similar and well-isolated on the same agar plate culture. They are then inoculated in 10 ml of Mueller–Hinton broth (MHB) and incubated overnight at ±35°C for the bacteria and ±30°C for fungi with a 2% glucose supplement. After incubation, 100 µl organisms was dispensed and sprayed on the plates, creating a lawn of organisms.



Bacterial strains

Gram-positive

- *Staphylococcus aureus* (S.a)
- *Staphylococcus epidermidis* (S.e)

Gram-negative

- *Escherichia coli* (E.c)
- *Enterobacter aerogenes* (E.a)
- *Klebsiella pneumonia* (K.p)
- *Salmonella enterica* (S.t)
- *Pseudomonas aeruginosa* (P.a)

Fungi strains

- *Candida albicans* (C.a)
- *Saccharomyces cerevisiae* (S.c)

Figure 3.3: Visual presentation of the disk diffusion method and the microorganisms which were used. The negative control was a sterilized filter paper disc.

The PE was diluted with dH_2O to a 50% stock solution (1:1 w/w) and three sterilized filter paper disks were submerged in the stock solution and placed on a lawn of test organisms (described above). After ±16 h of incubation, the plates were examined for inhibition zones. This was done in triplicate and each ZOI was measured with a metric ruler. The diameter of the ZOI determines the sensitivity of the organism to the PE, with a bigger zone indicating greater sensitivity and *vice versa*. The organisms that were inhibited, were subjected to a more thorough method known as the microdilution method (discussed below). The negative control was a sterilized filter paper disc.

3.3.5.2. Determining the minimum inhibitory concentration

To determine the MIC of the test formulations, the broth microdilution method was used. The MIC endpoint is the lowest concentration of the test formulations at which there is no visible microorganism growth. The growth of the organisms mentioned in figure 3.3 was tested against all the formulations in table 3.2. This method is done by using 96-well plates and a serial dilution ratio of 1:2 (v/v). The serial dilution was done by transferring 100 µl with a multichannel pipet from the first column through to the eleventh column and discarding the last 100 µl. Then 20 µl of the relevant organism was added to all wells, except for the sterility control, at the end. Each test formulation was done in triplicate.

The 96 well plates were prepared as illustrated in figure 3.4. Each well in the negative control (C1) row contained 80 µl MHB and 20 µl of the relevant organism, while each well in the sterility control (C3) only had 80 µl MHB. The first well of the positive control (C2), containing the relevant antibiotic or antifungal, was loaded with 157 µl MHB and 23 µl of the relevant antibiotic or antifungal stock solution and serially diluted before adding 20 µl of the relevant organism to each well. The relevant antibiotic or antifungal had a final concentration of 10.22 µg/ml in the first well, calculations can be seen in Annexure A.

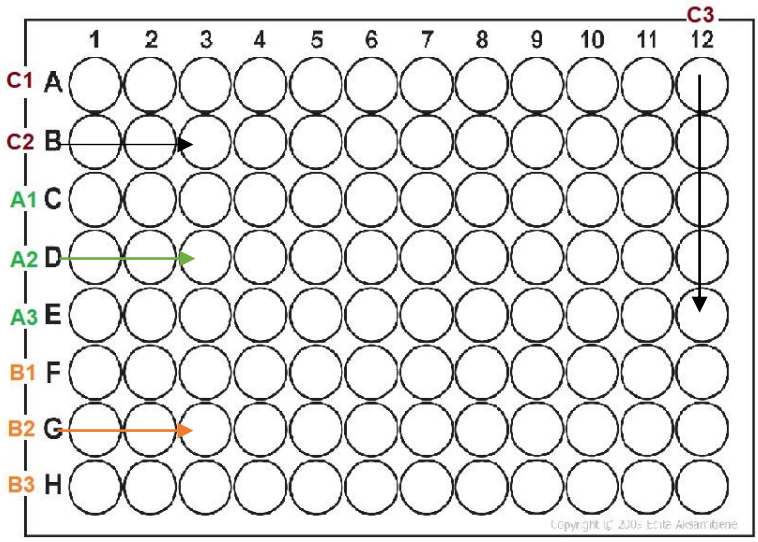


Figure 3.4: Layout of 96-well plates. C1 = negative control, C2 = positive control (antibiotic/fungicide), C3 = sterility control, A1-3 = Pheroid ®-PE formulation (n=3), B1-3 = corresponding Pheroid® control (n=3).

The positive controls were prepared as follows; an AmB solution was used as the positive control for the yeasts and was made by dissolving 10 mg AmB in 10 ml DMSO to obtain a concentration of 1 mg/ml. The solution was filter sterilized with a 0.2 µm filter, and a stock solution with 900 µl sterilized MiliQ H_2O and 100 µl AmB solution was created to give a final concentration of 0.1 mg/ml. Ery and TMP solutions were used as positive controls for the Gram-negative and Gram-positive bacteria, respectively. They were made by separately dissolving 100 mg Ery and 100 mg TMP in 10 ml methanol to obtain a concentration of 10 mg/ml for both controls. Both solutions were filter sterilized with a 0.2 µm filter. A stock solution with 990 µl sterilized MiliQ H_2O and 10 µl Ery and TMP were created to give a final concentration 1 mg/ml. A solvent control was done to determine if the solvent has an effect on the microorganisms.

The PPE test formulations (4%, 8%, 10%, 13% or 50%), marked as A1-3 in figure 3.4, and the corresponding control test formulations, marked as B1-3, were done in triplicate. All of these wells were loaded with 80 µl MHB and 100 µl of the test formulation was added to the first well and serially diluted, before adding 20 µl of the respective organism to each well. The PE formulation

was done in triplicate on a different 96-well plate by loading each well with 80 µl MHB and 100 µl PE in the first well and serially diluted, before adding 20 µl of the relevant organism to each well. Each test formulation and antibiotic (AB) or antifungal (AF) were subjected to a serial dilution ratio of 1:2 (v/v) leaving a final well volume of 100 µl, the exact concentration of each well can be seen in Annexure B.

The 96-well plates with the test organisms underwent incubation for ±16 h at 37°C (bacteria) and ±24 h at 30°C (fungi). The microbial growth was measured manually using a black card to compare turbidity of the wells and electronically with a spectrophotometer at an absorbance of 600nm (Rodríguez-Tudela *et al*., 2017). This was done before and after incubation to determine the MIC.

3.3.5.3. Determining the minimum bactericidal- and minimum fungicidal concentrations

After the MIC of each formulation was determined, the microbial growth in each microwell, as mentioned above in section 3.3.5.2., was evaluated to determine if the relevant test formulation was bacteriostatic/fungistatic or bactericidal/fungicidal. This was done by dividing the petri dishes into small grids, roughly 10 mm × 10 mm, and labelling each square with the corresponding well name. Each well from the 96-well plate was inoculated to MHA, including those showing no growth. The petri dishes were incubated for ±16 h at 37°C for bacteria and ±24 h at 30°C for fungi. After incubation, the petri dishes were examined to determine if there was any microbial growth. If there was no growth, the test formulation was considered to be bactericidal/fungicidal at that concentration. If there was growth at a concentration where the test formulation showed inhibition on the 96-well plate, the test formulation was considered to be bacteriostatic/fungistatic at that concentration. The results were recorded with a plus (+) *i.e.* 100% growth or a minus (-) *i.e.* 0% growth was observed. Each well containing a different dilution, as discussed above in section 3.3.5.2. was inoculated in triplicate and the average was calculated to determine the minimum bactericidal concentration (MBC) or minimum fungicidal concentration (MFC). The concentration containing an average growth of <50% was considered to be the MBC or MFC. The microbial growth was then evaluated to determine if the test formulation was bacteriostatic/fungistatic or bactericidal/fungicidal.

3.3.5.4. Determining minimum inhibitory and minimum fungicidal concentration using resazurin

After the initial antimicrobial tests, the microbial panel was adapted to include only the existing yeasts, as well as *Trichosporon dermatis* and *Cryptococcus neoformans*. The MIC of *C. albicans, S. cerevisiae, T. dermatis* and *C. neoformans* was determined using resazurin as a colour indicator (Sarker *et al.*, 2007). Resazurin is a blue dye that is reduced to a pink colour by metabolic active cells. This method was done similarly to the method discussed in section 3.3.5.2. The organisms were prepared to McFarland standard of 5×10^{-6} CFU/ml. The resazurin was made by dissolving 270 mg in 40 ml sterile dH_2O and filter sterilized with a 0.2 µm filter.

The layout of these plates was similar to that of figure 3.4. The PPE test formulations (4%, 8%, 10%, 13% or 50%), marked as A1-3, and the corresponding control test formulations, marked as B1-3, were done in triplicate. The first wells in A1-3 and B1-3 were loaded with 155 µl MHB, 25 µl formulation, and the rest of the wells were loaded with 85 µl MHB and serially diluted, before adding 5 µl resazurin and 10 µl of the relevant micro-organism. The PE control formulation was done in triplicate on a separate 96-well plate by loading the first wells with 155 µl MHB, 25 µl PE control formulation, and the rest of the wells were loaded with 85 µl MHB and serially diluted, before adding 5 µl resazurin and 10 µl of relevant micro-organism. The final volume of each well was 100 µl.

Each well in the negative control (C1) row contained 85 µl MHB and 10 µl of the relevant organism and 5 µl resazurin. The sterility control (C3) had only 85 µl MHB and 5 µl resazurin. The first well of the positive control (C2), containing AmB, was loaded with 157 µl MHB and 23 µl of AmB stock solution and serially diluted before adding 10 µl of the relevant organism and 5 µl resazurin. AmB had a final concentration of 10.22 µg/ml in the first well, calculations can be seen in Annexure A.

The 96-wells were incubated for ±16-24 h at ±30°C. After incubation, the lowest concentration of colour change is considered to be the MIC (pink=growth, blue=inhibition). The average MIC was calculated and the exact concentration of each well can be seen in Annexure B. After the MIC is calculated, the MFC was determined via the method in section 3.2.5.3. If there was growth where there was shown to be inhibition, the test formulation was considered to be fungistatic at that concentration.

3.3.6. Statistical analysis

The mean particle size and average zeta potential values were evaluated by a one-way analysis of variance (ANOVA), followed by a Tukey and Kruskal-Wallis multiple comparison tests. Pro-PPE 50% and Pro-P were analysed separately from 4%, 8%, 10% and 13% test- and control formulations, respectively. Differences in means were considered significant if $p \leq 0.05$ (*i.e*. at the 5% level of significance), using Statistica statistical software (StatSoft, Inc., 2018).

Chapter 4: Results

4.1. Formulation characterization

The mean particle size, zeta potential and morphology results for each test- and control formulation (table 3.2, section 3.3.2.) are presented below in section 4.1.1, 4.1.2 and 4.2.3, respectively. Firstly, discussed is the effect of the different percentages oil phase on the formulations where we will focus on the difference between each formulation, for example, PPE 4% vs PPE 8%. Secondly, the effect of AST on the formulations is discussed where we focus on the difference between each month, for example, M0 vs. M3.

During the study, three different PE batches were received from the supplier (Biopher) and used at different time points of the study, containing a concentration of 55.2, 34, and 60 mg/ml of the active saponin. The test formulations, containing the PE, were formulated on three different times throughout the study with their corresponding control formulations.

4.1.1. Mean particle size

The mean particle size (µm) of three separate test- and control formulations were determined for 4%, 8%, 10%, 13%, and Pro-Pheroid® according to the method described in section 3.3.3.1. The average d(0.5) value of each formulation was used to compare the different percentage oil phases (w/w) for the Pheroid® and Pro-Pheroid® formulations (figure 4.1, table 4.1). The d(0.5) is the diameter of the particle that represents a value at which 50% of the vesicles are smaller and 50% are larger than the d(0.5) value. The particle size distribution graphs for each formulation can be seen in Annexure H. The test formulations were compared to the control formulations to determine if the PE had an effect on the particle size and this was done before the AST began (month 0), as well as during the AST (month 1, 2, and 3). The control formulations were compared as well.

Before AST (Month 0):

When comparing the test formulations (Pheroid®-PPE) to the corresponding control formulations (Pheroid®), test formulation PPE 10% (0.294 ± 0.067 µm) and PPE 13% (0.295 ± 0.063 µm) had a statistically ($p \leq 0.05$) smaller mean particle size compared to their corresponding control formulations P 10% (0.445 ± 0.117 µm) and P 13% (0.435 ± 0.17 µm), respectively. While most of the test formulations had no statistical difference ($p > 0.05$) in mean particle size compared to the corresponding controls, *i.e.* formulation PPE 4% (1.902 ± 2.413 µm) *vs*. formulation P 4% (0.374 ± 0.119 µm), formulation PPE 8% (0.406 ± 0.149 µm) *vs*. formulation P 8% (1.177 ± 1.234 µm), and formulation Pro-PPE 50% (1.895 ± 1.398 µm) *vs*. formulation Pro-P 50% (3.312 ± 4.581 µm) (figure 4.1, table 4.1). Addition of the PE decreased the mean particle size of the vesicles only when comparing PPE 10% vs. P 10%, and PPE 13% vs. P 13%.

When comparing only the test formulations to one another, formulation PPE 4% was found to have the statistically ($p \leq 0.05$) largest mean particle size compared to both PPE 10% (0.294 ± 0.067 µm) and PPE 13% (0.295 ± 0.063 µm). Formulation PPE 8% (0.406 ± 0.149 µm) did not differ statistically significantly ($p > 0.05$) when compared to PPE 4% (1.902 ± 2.413 µm) (figure 4.1, table 4.1).

When comparing only the control formulations to one another, formulation P 4% (0.374 ± 0.119 µm) had the smallest mean particle size, followed by formulation P 13% (0.435 ± 0.17 µm), formulation P 10% (0.445 ± 0.117 µm), formulation P 8% (1.177 ± 1.234 µm) and formulation Pro-P (3.312 ± 4.581 µm). However, there is no statistical difference between the control formulation's mean particle size ($p > 0.05$) (figure 4.1, table 4.1).

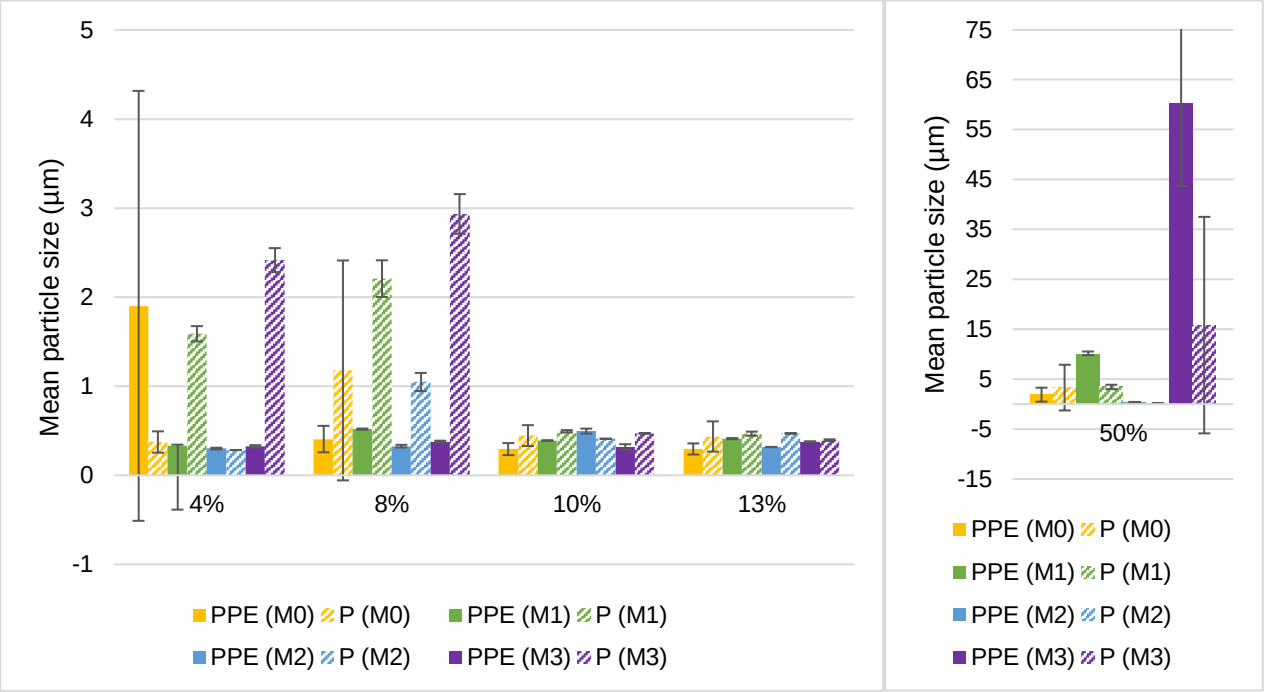


Figure 4.1: Mean particle size for the test- and control formulations during the AST M0 (n=9), M1 (n=3), M2 (n=3), and M3 (n=3).

During AST (Month 0, 1, 2 and 3):

The mean particle size of each formulation at a given month (0, 1, 2 and 3) are summarized in figure 4.1 and table 4.1. Comparing the test formulations to themselves over the course of the AST, neither PPE 4%, *i.e.* month 0 (1.902 ± 2.413 µm), 1 (0.335 ± 0.010 µm), 2 (0.300 ± 0.010 µm) and 3 (0.325 ± 0.013 µm), or PPE 8%, *i.e.* month 0 (0.406 ± 0.149 µm), 1 (0.516 ± 0.01 µm), 2 (0.327 ± 0.015 µm) and 3 (0.377 ± 0.012 µm) showed any statistically significant ($p > 0.05$) change in their mean particle size. Formulation PPE 10% had no statistically different particle sizes comparing month 0 (0.294 ± 0.067 µm), 1 (0.390 ± 0.002 µm) and 3 (0.319 ± 0.031 µm), however month 2 (0.496 ± 0.028 µm) had a statistically larger particle size compared to both month 0 and 3. Formulation PPE 13% had no statistically different particle sizes comparing month 0 (0.295 ± 0.063 µm), 2 (0.317 ± 0.002 µm) and 3 (0.378 ± 0.003 µm), however month 0 and 1 (0.414 ± 0.004 µm) differed statistically ($p < 0.05$) from one another. Formulation Pro-PPE 50% had no statistically different particle sizes comparing month 0 (1.895 ± 1.398 µm), 1 (9.824 ± 0.720 µm) and 2 (0.401 ± 0.036 µm), however all three months had statistically ($p < 0.05$) smaller particle sizes compared to month 3 (60.123 ± 16.408 µm). In general, the mean particle size of PPE 4% and PPE 8% stayed relatively constant over three months of AST. The mean particle size of PPE 10%, PPE 13% and Pro-PPE 50% on the other hand increased when comparing month 0 to either month 1, 2 or 3.

Comparing the control formulations to themselves over the course of the AST (figure 4.1, table 4.1), control formulation P 4% month 2 (0.284 ± 0.001 µm) and 3 (2.416 ± 0.135 µm) differed statistically ($p \leq 0.05$) from one another, while month 0 (0.374 ± 0.119 µm) and 1 (1.589 ± 0.086 µm) did not differ from one another or compared to month 2 and 3. Control formulation Pro-P 50% month 2 (0.137 ± 0.001 µm) and 3 (15.832 ± 21.696 µm) differed statistically ($p \leq 0.05$) from one another, while month 0 (3.312 ± 4.581 µm) and 1 (3.436 ± 0.444 µm) did not differ statistically significant form one another or compared to month 2 and 3. . There was statistically no difference ($p > 0.05$) between month 0 vs. month 1 vs. month 2 vs. month 3 for control formulation P 8%, P 10%, and P 13%. In general, the particle size of formulation P 8%, P 10% and P 13% remained the same over the three months of AST. However, control formulation P 4% and Pro-P 50% showed an increase in the size of their vesicles when comparing month 3 to month 2.

Table 4.1: The mean particle size of each test- and control formulation at month 0, 1, 2 and 3 of stability testing.

Formulations	Month 0		Month 1	Month 2	Month 3
	µm (±SD)	p-value	µm (±SD)	µm (±SD)	µm (±SD)
PPE 4%	1.902 (±2.413) ^{aA}	>0.05	0.335 (±0.009) ^{a}	0.300 (±0.010) ^{a}	0.325 (±0.013) ^{a}
P 4%	0.374 (±0.119)		1.589 (±0.086)	0.284 (±0.001) *	2.416 (±0.135) *
PPE 8%	0.406 (±0.149) ^{aAB}	>0.05	0.516 (±0.009) ^{a}	0.327 (±0.015) ^{a}	0.377 (±0.012) ^{a}
P 8%	1.177 (±1.234)		2.207 (±0.206)	1.047 (±0.102)	2.934 (±0.223)
PPE 10%	0.294 (±0.067) ^{aB}	≤0.05	0.390 (±0.002) ^{ab}	0.496 (±0.028) ^{b}	0.319 (±0.031) ^{a}
P 10%	0.445 (±0.117)		0.494 (±0.014)	0.410 (±0.002)	0.470 (±0.002)
PPE 13%	0.295 (±0.063) ^{aB}	≤0.05	0.414 (±0.004) ^{b}	0.317 (±0.002) ^{ab}	0.378 (±0.003) ^{ab}
P 13%	0.435 (±0.170)		0.467 (±0.023)	0.470 (±0.001)	0.394 (±0.010)
Pro-PPE 50%	1.895 (±1.398) ^{a†}	>0.05	9.824 (±0.720) ^{a}	0.401 (±0.036) ^{a}	60.123 (±16.408) ^{b}
Pro-P	3.312 (±4.581)		3.436 (±0.444)	0.137 (±0.001) *	15.832 (±21.696) *

a/A A value belonging to group A according to the Tukey HSD variability test. This value will differ statistically significant compared to a value belonging to group B.
b/B A value belonging to group B according to the Tukey HSD variability test. This value will differ statistically significant compared to a value belonging to group A.
ab/AB A value belonging to both group A and B. This value does not differ statistically significant from either a value in group A or B according to the Tukey HSD variability test.
*Kruskal-Wallis H-test: Multiple comparisons of p-values differ statistically ($p \leq 0.05$).
† Pro-PPE50% was omitted during the statistical analysis comparing the test formulations to one another.

4.1.2. Zeta potential stability

The average zeta potential (mV) from three readings of three samples/batches were determined according to the method described in section 3.3.3.2. The different formulation's zeta potential was compared to one another and to their corresponding control formulations, to determine not only if the amount of oil phase, *i.e.* 4%, 8%, 10%, 13% and Pro-Pheroid®, but also if the PE had an influence on the zeta potential (figure 4.2 and table 4.2). This was done before the AST began (month 0), as well as during AST at month 1, 2 and 3.

Before AST (Month 0):

When comparing the test formulations to their control counterparts, most of the test formulations had a statistically significant ($p \leq 0.05$) smaller average zeta potential compared to their control counter parts, *i.e.* formulation PPE 4% (-38.5 ± 4.6 mV) *vs*. formulation P 4% (-20.1 ± 5.4 mV), formulation PPE 8% (-33.1 ± 3.1 mV) *vs*. formulation P 8% (-22.0 ± 5.1 mV), formulation PPE 13% (-31.1 ± 6.8 mV) *vs*. formulation P 13% (-18.4 ± 5.3 mV) and formulation Pro-PPE 50% (-23.8 ± 7.1 mV) *vs*. formulation Pro-P 50% (-13.4 ± 10.5 mV). The only exception was observed with formulation PPE 10% (-18.5 ± 14.3mV) *vs*. formulation P 10% (-21.5 ± 7.6 mV) which did not differ statistically significant ($p > 0.05$). This showed that by incorporating the PE into each formulation the zeta potential difference of each formulation was increased, except for the formulation PPE 10% (figure 4.2, table 4.2).

When comparing only the test formulations to one another, it was observed that formulations PPE 4%, PPE 8% and PPE 13% had significantly ($p = \leq 0.05$) smaller Zeta potentials compared to formulation PPE 10%. Formulation Pro-PPE 50% did not differ statistically from either PPE 8%, PPE 13% or PPE 10% (figure 4.2, table 4.2).

When comparing only the control formulations to one another, formulations P 4% (-20.1 ± 5.4 mV), P 8% (-22.0 ± 5.1 mV), P 10% (-21.5 ± 7.6 mV) and P 13% (-18.4 ± 5.3 mV) had a slightly smaller zeta potentials than formulation Pro-P (-13.4 ± 10.5 mV). This indicates that formulation P 4%, P 8%, P 10% and P 13% were slightly more stable than Pro-P 50%. However, statistically there was no difference ($p \leq 0.05$) between the average zeta potentials (figure 4.2, table 4.2).

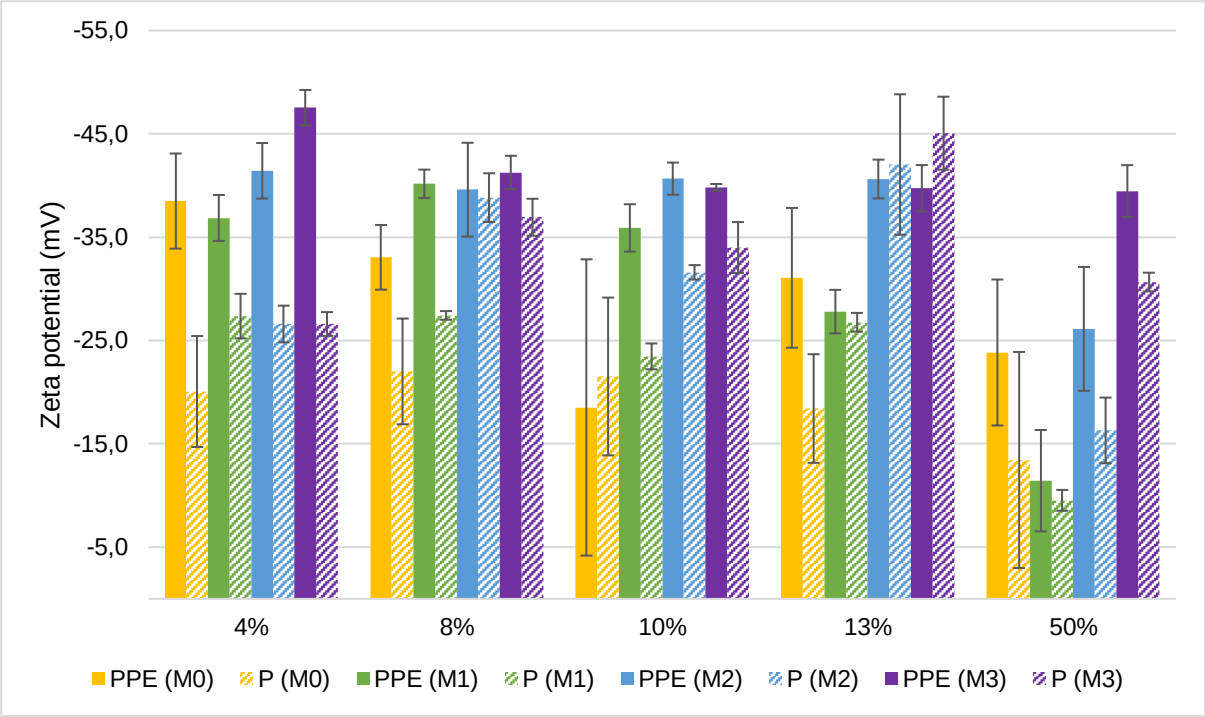


Figure 4.2: Zeta potential of the test- and control formulations during AST. M0 (n=9), M1 (n=3), M2 (n=3), and M3 (n=3).

During AST (Month 0, 1, 2 and 3):

The average zeta potential of each formulation at a given month (0, 1, 2 and 3) are summarized in figure 4.2 and table 4.2. Comparing the test formulations to themselves over the course of AST, formulation PPE 4% showed a statistically significant decrease when comparing month 0 and 1 (-36.9 ± 2.2 mV) to month 3 (-47.5 ± 1.7 mV). Month 2 (-41.4 ± 2.7 mV) did not differ statistically from any other months. Formulation PPE 8% showed a statistically significant decrease when comparing month 0 to 3 (-41.4 ± 1.6 mV), whereas month 1 (-40.2 ± 1.4 mV) and 2 (-39.6 ± 4.5 mV) did not differ statistically significantly from one another or month 0 and 3. Formulation PPE 10% did not have any statistically significant different zeta potentials when comparing month 0, 1 (-35.9 ± 2.3 mV), 2 (-40.7 ± 1.6 mV) and 3 (- 39.8 ± 0.3 mV) to one another. Formulation PPE 13% showed a statistically significant decrease comparing month 1 (-27.8 ± 2.1 mV) to month 2 (-40.6 ± 1.9 mV), however, neither month 0 or 3 (-39.7 ± 2.3 mV) differed statistically from one another or compared to month 1 and 2. Formulation Pro-PPE 50% showed a statistically significant decrease comparing month 1 (-11.4 ± 4.9 mV) and 0 to month 3 (-39.5 ± 2.5 mV).

These results were unexpected as formulations PPE 4%, PPE 8% and Pro-PPE 50% all showed a statistically significant decrease in their zeta potential when comparing month 0 with month 3 at the end of AST, indicating that their vesicles had a greater potential difference between the dispersion medium and the stationary layer thus making them more stable.

Comparing the control formulation to themselves over the course of AST, it can be seen that formulation P 4% did not differ statistically when comparing month 0 (- 20.1 ± 5.4 mV), 1 (-27.4 ± 2.2 mV), 2 (-26.6 ± 1.8 mV) and 3 (-26.6 ± 1.2 mV) to one another. Formulation P 8% showed a statistically significant decrease in zeta potential comparing month 0 (-22 ± 5.1 mV) to month 2 (-38 ± 2.4 mV) and 3 (-36.9 ± 1.8 mV). Month 1 (-27.4 ± 0.4 mV) and 3 did not differ statistically significant form one another. Formulation P 10% had no statistically significant different zeta potentials when comparing month 0, 1 (-23.5 ± 1.3 mV), 2 (-31.6 ± 0.7mV) and 3 (-34 ± 2.5 mV) to one another. Formulation P 13%, on the other hand, showed a statistically significant decrease in zeta potential comparing month 0 and 1 (-26.8 ± 0.9 mV) to month 2 (- 42 ± 6.8mV) and 3 (-45.1 ± 3.5 mV). Formulation Pro-P showed a statistically significant decrease in its zeta potential comparing month 1 (-9.5 ± 1 mV) and 3 (-30.7 ± 0.9 mV), however neither month 0 or 2 (-16.3 ± 3.2 mV) differed statistically significantly from one another or compared to month 1 and 3 (figure 4.2, table 4.2).

Again, these results were unexpected as formulations P 8% and P 13% showed a statistically significant decrease in their zeta potential when comparing month 0 with month 3 at the end of accelerates stability testing, indicating that their vesicles had a greater potential difference between the dispersion medium and the stationary layer thus making them more stable.

Table 4.2: The average zeta potential of each test- and control formulation at month 0, 1, 2 and 3 of stability testing.

Formulation	Month 0		Month 1	Month 2	Month 3
	mV (±SD)	p-value	mV (±SD)	mV (±SD)	mV (±SD)
PPE 4%	-38.5 (±4.6) [aA]	≤0.05	-36.9 (±2.2) [a]	-41.4 (±2.7) [ab]	-47.5 (±1.7) [b]
P 4%	-20.1 (±5.4) [a]		-27.4 (±2.2) [a]	-26.6 (±1.8) [a]	-26.6 (±1.2) [a]
PPE 8%	-33.1 (±3.1) [bAB]	≤0.05	-40.2 (±1.4) [ab]	-39.6 (±4.5) [ab]	-41.3 (±1.6) [a]
P 8%	-22.0 (±5.1) [c]		-27.4 (±0.4) [bc]	-38.8 (±2.4) [a]	-36.9 (±1.8) [ab]
PPE 10%	-18.5 (±14.3) [aC]	>0.05	-35.9 (±2.3) [a]	-40.7 (±1.6) [a]	-39.8 (±0.3) [a]
P 10%	-21.5 (±7.6) [a]		-23.5 (±1.3) [a]	-31.6 (±0.7) [a]	-34 (±2.5) [a]
PPE 13%	-31.1 (±6.8) [abAB]	≤0.05	-27.8 (±2.1) [b]	-40.6 (±1.9) [a]	-39.7 (±2.3) [ab]
P 13%	-18.4 (±5.3) [b]		-26.8 (±0.9) [b]	-42.0 (±6.8) [a]	-45.1 (±3.5) [a]
Pro-PPE 50%	-23.8 (±7.1) [bcBC]	≤0.05	-11.4 (±4.9) [c]	-26.1 (±6) [ab]	-39.5 (±2.5) [a]
Pro-P	-13.4 (±10.5) [ab]		-9.5 (±1.0) [b]	-16.3 (±3.2) [ab]	-30.7 (±0.9) [a]

[a/A] A value belonging to group A according to the Tukey HSD variability test. This value will differ statistically significant compared to a value belonging to group B.

[b/B] A value belonging to group B according to the Tukey HSD variability test. This value will differ statistically significant compared to a value belonging to group A.

[ab/AB] A value belonging to both group A and B. This value does not differ statistically significant from either a value in group A or B according to the Tukey HSD variability test.

[bc/BC] A value belonging to both group B and C. This value does not differ statistically significant from either a value in group B or C according to the Tukey HSD variability test.

4.1.3. Morphology

The CLSM images were obtained according to the method described in section 3.3.3.3. These images are used to confirm the structure of the Pheroid® vesicles in both the test- and control formulations. All of the CLSM images and ImageJ analyses are shown in both Annexure F and Annexure G. The vesicles tend to move while the images are taken, causing some blurriness, *i.e.* seeing aggregation of vesicles where there is none as evident by the zeta potential. This is a qualitative method used to quantify a small amount of each sample.

Before AST (month 0)

Comparing the PPE 4% test- and P 4% control formulation (figure 4.3), it can be seen that the PPE 4% vesicles (figure 4.3.a.) are uniform in shape with a clear spherical shaped vesicle that are evenly dispersed. The corresponding P 4% control (figure 4.3.b.) showed that the vesicles are also spherical in shape and evenly distributed, but there are some larger vesicles visible.

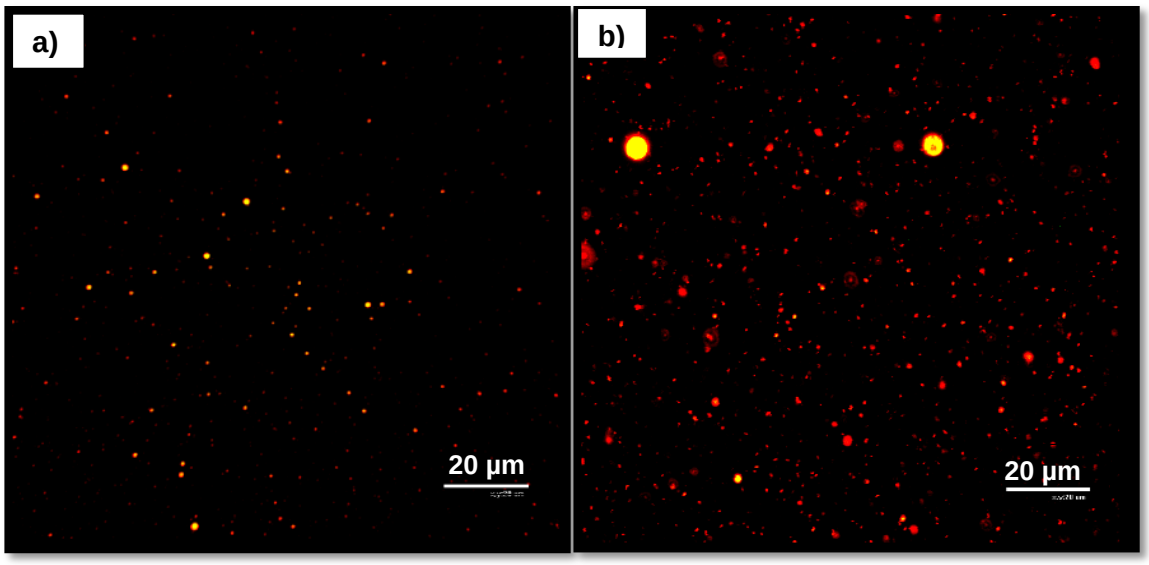


Figure 4.3: CLSM images (n = 5) of **a)** 4% Pheroid®-Plant extract and **b)** 4% Pheroid®.

Comparing the Pro-Pheroid® test- and control formulation, Pro-PPE 50% and Pro-P (figure 4.4), it can be seen that the Pro-PPE 50% vesicles (figure 4.4.a.) are uniform in shape with clear spherical shaped vesicle that are evenly dispersed. The corresponding Pro-P control (figure 4.4.b.) showed that the vesicles are also spherical in shape and evenly distributed, with some larger vesicles visible.

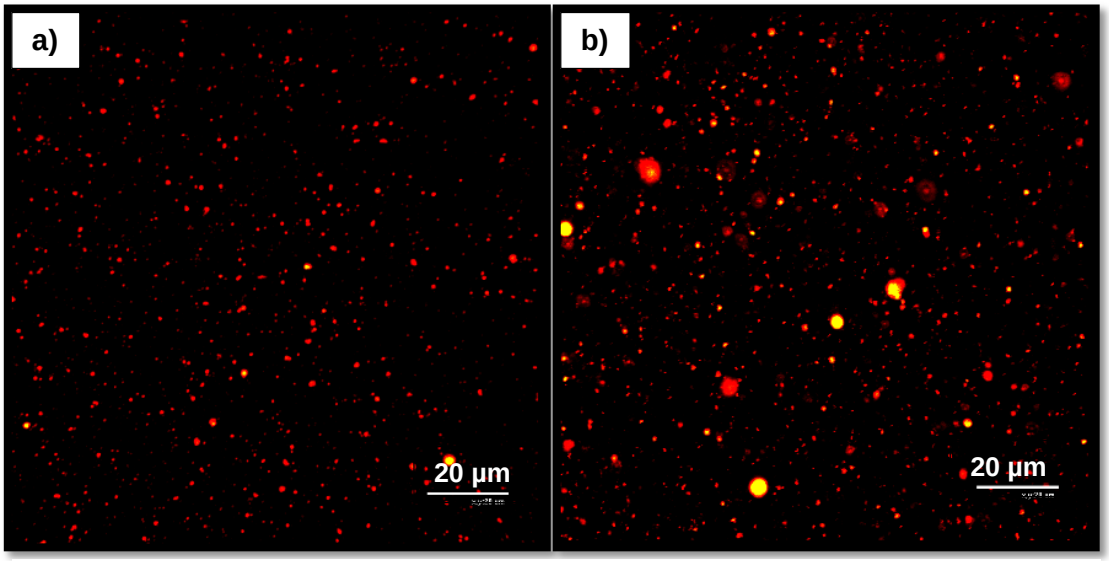


Figure 4.4: CLSM images (n = 5) of **a)** 50% Pro-Pheroid®-Plant extract and **b)** Pro-Pheroid®.

Comparing the PPE 8%, PPE 10%, and PPE 13% test formulations and P 8%, P 10%, and P 13% control formulations (Annexure G), it can be seen that the vesicles are uniform in shape with clear spherical shaped vesicles. In all the images, the test formulations appear to have smaller vesicles than the control formulations.

During AST (month 0, 1, 2, and 3)

Comparing the PPE 4% test formulation for month 0, 1, 2, and 3, (figure 4.5) it can be seen that the PPE 4% vesicles are overall uniform in shape with clear spherical shaped vesicles that are evenly dispersed. During month 1 and 2 some unusually shaped object (indicated with blue arrows) can be observed, which could be plant fibres. During month 3 some larger vesicles were visible.

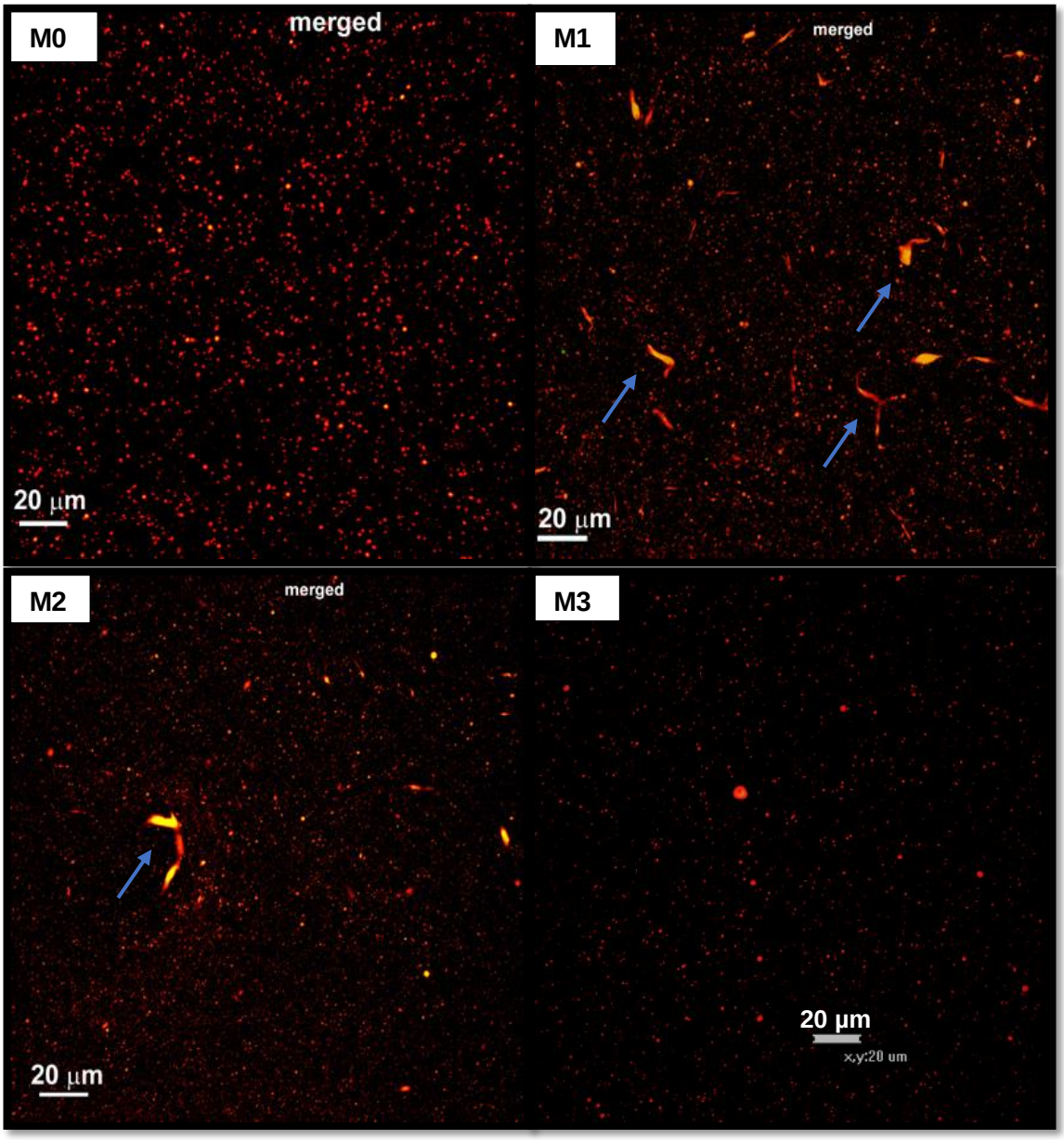


Figure 4.5: CLSM images of the 4% Pheroid® - Plant extract (PPE 4%) test formulation during AST, *i.e.* month 0 (M0), month 1 (M1), month 2 (M2) and month 3 (M3) (n = 5).

The corresponding control formulation P 4% also had spherical vesicles that were evenly distributed, however, some larger vesicles were also visible at month 0, 1 and 2. At month 2 we also observed depot vesicles, indicated with blue arrows (figure 4.6).

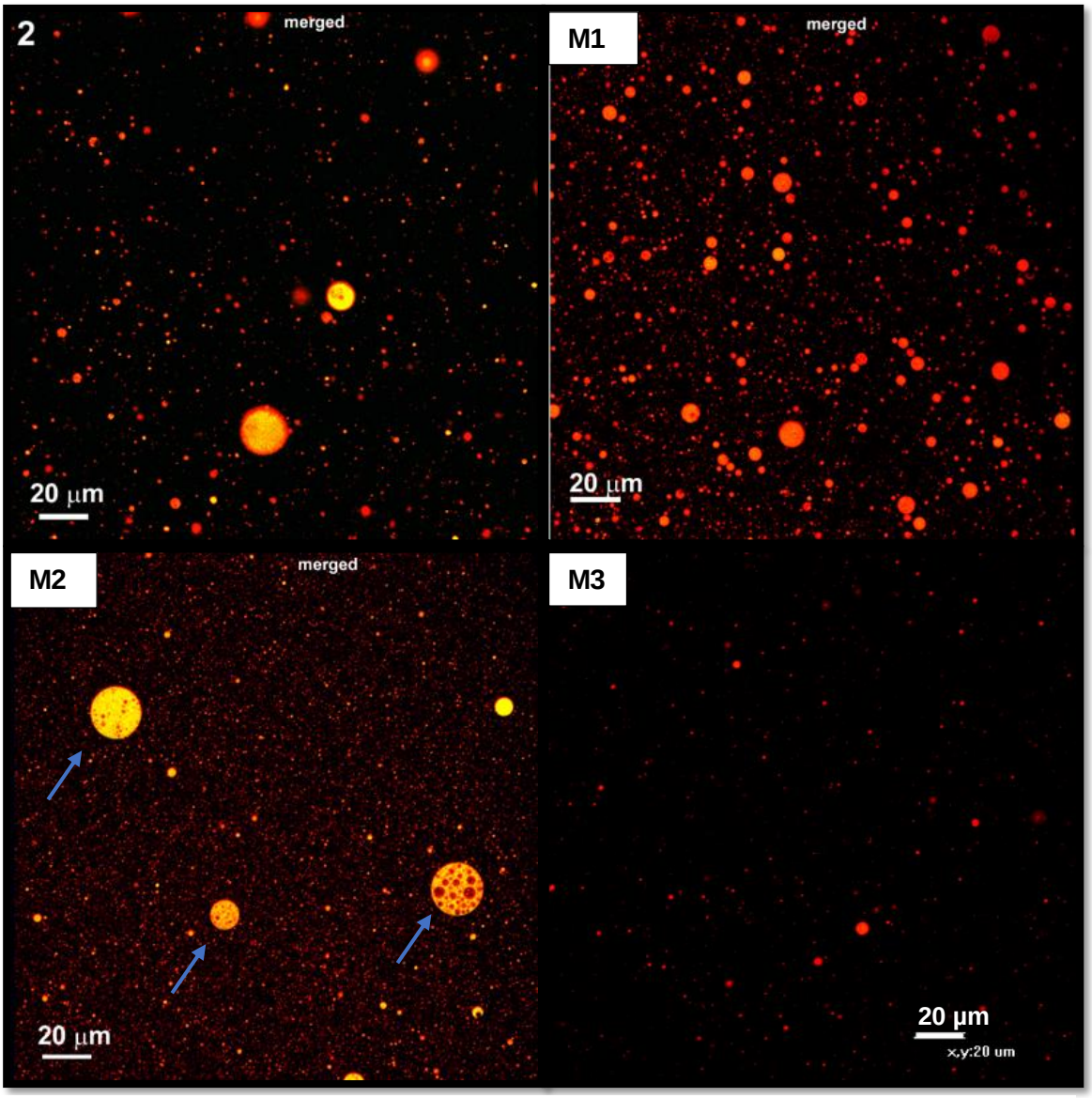


Figure 4.6: CLSM images of the 4% Pheroid® (P 4%) control formulation during AST, *i.e.* month 0 (M0), month 1 (M1), month 2 (M2) and month 3 (M3) (n = 5).

Comparing the PPE 13% test formulation for month 0, 1, 2, and 3, (figure 4.7) it can be seen that the PPE 13% vesicles are overall evenly dispersed and uniform in shape with clear spherical shaped vesicles that are. During month 0 (figure 4.7, M0) there are a lot of vesicles visible, while the number of vesicles seems to decrease over the months (figure 4.7, M1-M3).

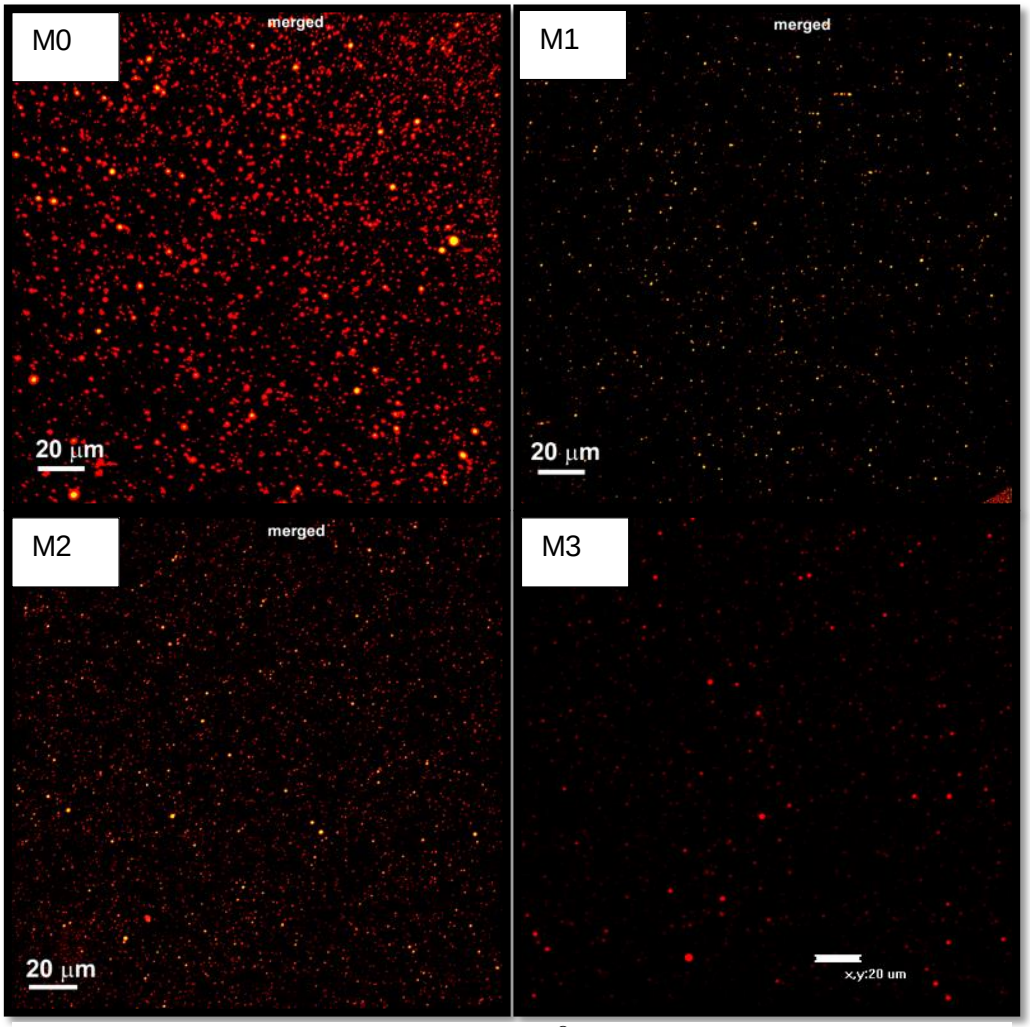


Figure 4.7: CLSM images of the 13% Pheroid® - Plant extract (PPE 13%) test formulation during AST, *i.e.* month 0 (M0), month 1 (M1), month 2 (M2) and month 3 (M3) (n = 5).

The corresponding control formulation P 13% also had spherical vesicles that were evenly distributed, however, some larger vesicles were visible at month 0 (figure 4.8, M0). At month 1 a depot vesicle was observed, indicated with a blue arrow (figure 4.8, M1).

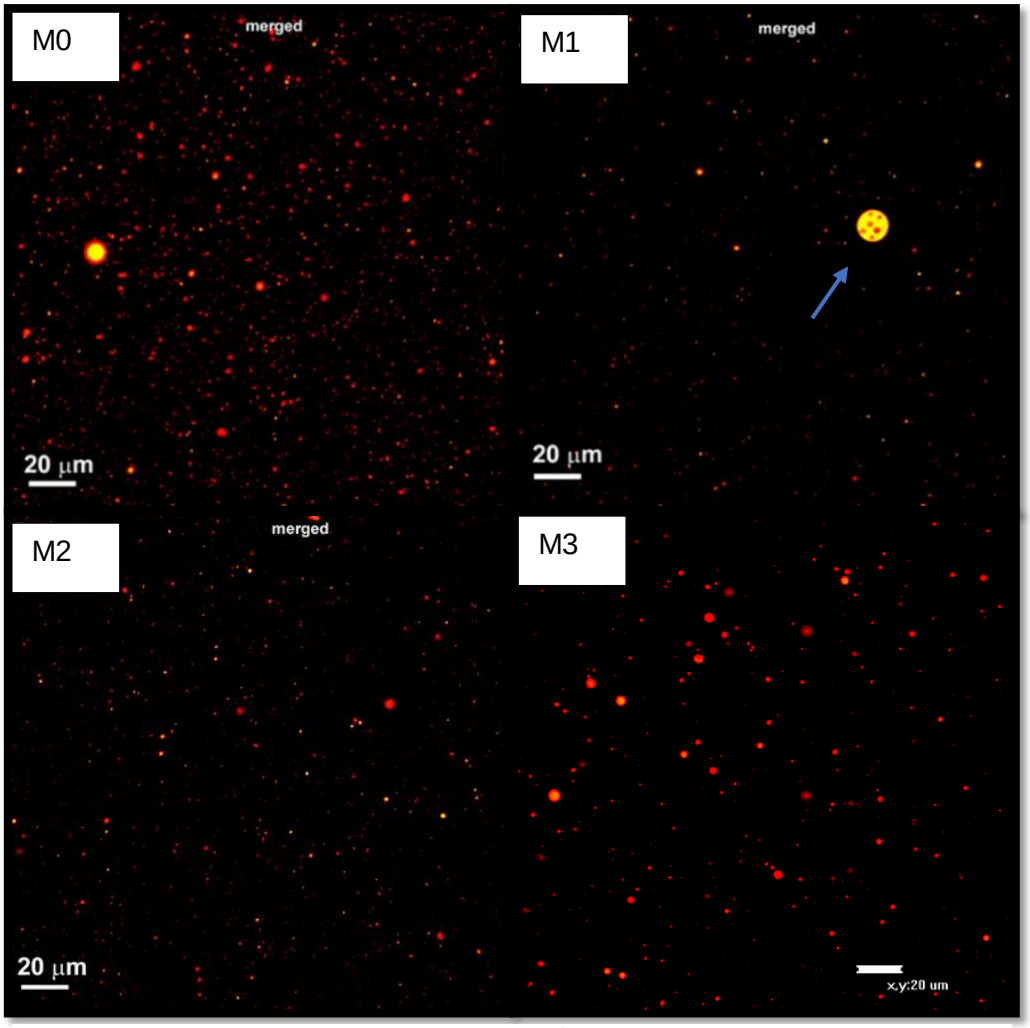


Figure 4.8: CLSM images of the 13% Pheroid® (P 13%) control formulation during AST, *i.e.* month 0 (M0), month 1 (M1), month 2 (M2) and month 3 (M3) (n = 5).

Comparing the PPE 8%, PPE 10%, and Pro-PPE 50% test formulations to the P 8%, P 10%, and Pro-P control formulations (Annexure F), it can be seen that the vesicles are uniform in shape with clear spherical shaped vesicles. The influence of different percentage oil in Pheroid® (w/w) is not clearly noticeable.

4.2. *In vitro* antimicrobial susceptibility tests

The Kirby Bauer disc diffusion, MIC and MBC/MFC results are presented below in section 4.2.1, 4.2.2 and 4.2.3, respectively. The antimicrobial susceptibility test results, using resazurin as a colour indicator, is presented in section 4.2.4.

4.2.1. Kirby Bauer disc diffusion

The initial antimicrobial screening was done according to the method described in section 3.3.5.1. The susceptibility of the mentioned microorganisms was determined against the crude PE.

The PE infused disc had a ZOI of 16.67 ± 1.53 mm, 13.67 ± 0.58 mm, 7 ± 0 mm, 7 ± 0 mm, 6.67 ± 0.58 mm, 5.33 ± 4.62 mm, and 4.67 ± 4.04 mm against *S. cerevisiae, C. albicans, E. coli, S. epidermidis, S. aureus, K. pneumonia* and *S. enterica* respectively, indicating various degrees of activity against these microorganisms (table 4.3, figure 4.7).

Table 4.3: Zone of inhibition (n = 3) of each relevant microorganism obtained using the Kirby Bauer disc diffusion method.

Organism	ZOI diameter (±SD mm)
C. albicans (C.a)	13.67 (± 0.47)
S. cerevisiae (S.c)	16.67 (± 1.53)
S. epidermidis (S.e)	7 (± 0)
S. aureus (S.a)	6.67 (± 0.58)
S. enterica (S.t)	4.67 (± 4.04)
K. pneumoniae (K.p)	5.33 (± 4.62)
E. coli (E.c)	7 (± 0)

Using this method, the PE showed moderate activity against the yeasts *S. cerevisiae, C. albicans*, and only minor activity against *E. coli, K. pneumonia and S. enterica* (Gram-negative bacteria) as well as *S. epidermidis* and *S. aureus* (Gram-positive bacteria). The PE had no activity against *P. aeruginosa* and *E. aerogenes*, both Gram-negative bacteria (data not shown).

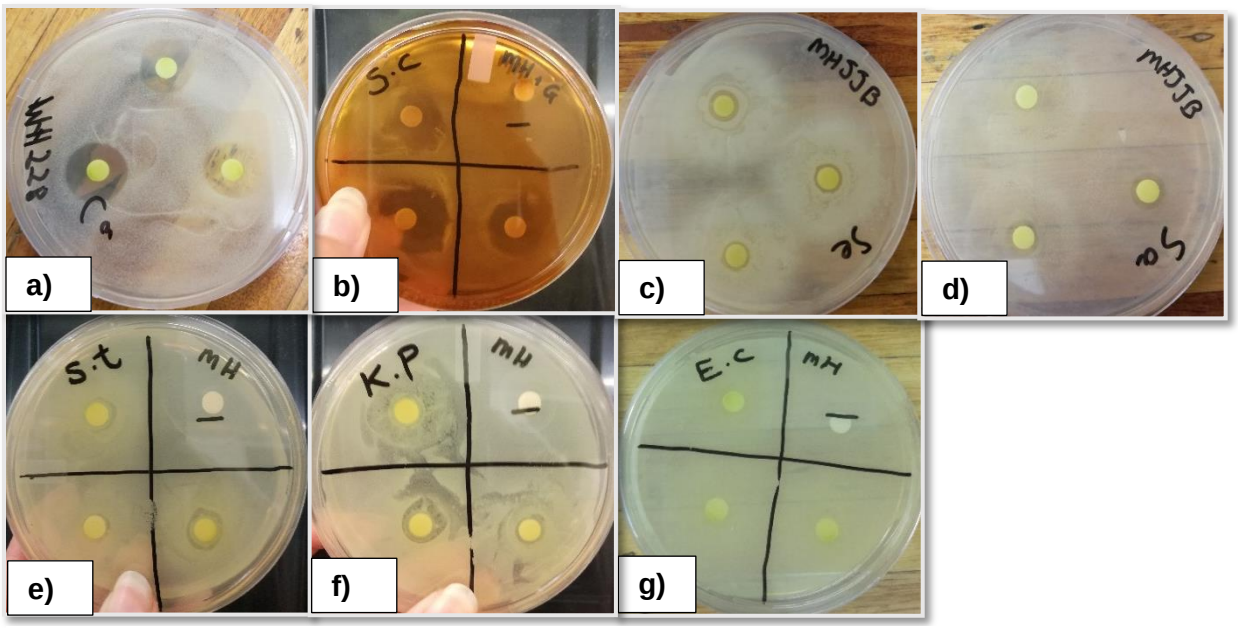


Figure 4.7: ZOI on Mueller Hinton agar for **a)** Ca, **b)** Sc, **c)** Se, **d)** Sa, **e)** St, **f)** Kp, and **g)** Ec

4.2.2. Determining the minimum inhibitory concentration

The method used is described in section 3.3.5.2. The test- and control formulations proved to be too turbid to allow any interpretation of the MIC values during the AST using the broth microdilution method. Neither visual inspection nor spectrophotometric analysis, using a 600 nm wavelength, yielded any accurate results. This was a result of the formation of the Pheroid® vesicles which created a milky suspension, preventing any analysis.

4.2.3. Determining the minimum bactericidal- and fungicidal concentrations

Using the same 96-well plates as prepared in section 3.3.5.2, we determined the MBC/MFC values of both the test- and control formulations as described in section 3.3.5.3. There was no significant antimicrobial activity observed against S*. aureus, E. coli, E. aerogenes, K. pneumonia, S. enterica*, and *P. aeruginosa*, therefore the results are not presented. After the initial bactericidal/fungicidal action of the test- and control formulations ended, some of the formulations again killed the relevant bacteria/yeast at a lower concentration. These MBC/MFC values are indicated in brackets.

C. albicans

Firstly, we determined the antimicrobial activity the test formulations had against *C. albican*s in comparison to the PE and the AmB (positive control) (table 4.4). Comparing the MFC of test formulations to themselves during month 0, we observed the following. The MFCs of the test formulations were 8.5 mg/ml (PPE 4%, PPE 8%, PPE 10%), 4.24 mg/ml (PPE 13%) and 1.6 $x10^{-2}$ mg/ml (Pro-PPE 50%), indicating that the Pro-PPE 50% test formulation had the highest antimicrobial activity against *C. albicans*, followed by the PPE 13% test formulation and then the PPE 4%, PPE 8%, PPE 10% test formulations. Test formulation PPE 8% and PPE 10% however again resumed killing *C. albicans* at MFCs of 2.66 $x10^{-1}$ mg/ml and ≤1.66 $\times10^{-2}$ mg/ml respectively. The crude PE and AmB (positive control) had MFCs of 8.5 mg/ml and 3.99 $x10^{-5}$ mg/ml respectively, after which the PE again resumed killing *C. albicans* at a concentration of ≤1.6 $x10^{-2}$ mg/ml. Thus, against *C. albicans,* test formulations Pro-PPE 50% had the most promising antimicrobial activity compared to the PE. However, none of the test formulations or PE were as potent as AmB.

Secondly, we determined the MFC of each formulation during AST. Comparing the MFC of the test formulations to themselves for *C. albicans* (table 4.4) during month 0, 1, 2, and 3 we observed the following. The crude PE's MFCs were 8.5 mg/ml (month 0), 8.5 mg/ml (month 1), 15 mg/ml (month 2) and 17 mg/ml (month 3). The crude PE's initial antimicrobial activity thus decreased two-fold (by 50%) compared to month 3. The test formulation PPE 4%'s MFCs were 8.5 mg/ml (month 0), 8.5 mg/ml (month 1), 30 mg/ml (month 2) and 8.5 mg/ml (month 3). This was a strange observation as the antimicrobial activity decreased drastically between month 1 and 2, then increased again to its initial activity at month 3. This phenomenon could be due to the PE batch variation as mentioned in section 4.1 as a different batch PE was used during month 2. Except for month 1, both test formulations PPE 8% and PPE 10% had exactly the same MFC values, *i.e.* 8.5 mg/ml (month 0), 4.25 mg/ml and 0.27 mg/ml (month 1), 15 mg/ml (month 2) and 17 mg/ml (month 3), respectively. Their MFC values thus showed a similar trend compared to the PE, where a 50% decrease in activity was observed when comparing month 0 to 3. Test formulation

PPE 13%'s MFCs were 4.24 mg/ml (month 0), 0.27 mg/ml (month 1), 7.5 mg/ml (month 2) and 8.5 mg/ml (month 3), again showing a 50% decrease in activity when comparing month 0 to 3. The test formulation Pro-PPE 50%'s MFCs were ≤1.6x10^{-2} mg/ml (month 0), 8.5 mg/ml (month 1), 15 mg/ml (month 2) and 8.5 mg/ml (month 3). This formulation had the most potent initial antimicrobial activity, which declined to an antimicrobial activity similar to the initial activity of test formulations PE, PPE 4%, PPE 8%, and PPE 10%.

Table 4.4: The MFC values of the different test formulations against *C. albicans* during month 0, 1, 2, and 3 of AST.

Test formulation	Minimum fungicidal concentration, MFC (mg/ml)			
	Month 0	Month 1	Month 2	Month 3
AmB	3.99 x10^{-5}	-*	-*	-*
PE	8.5 (≤1.66 x10^{-2})†	8.5	7.5	17
PPE 4%	8.5	8.5	30	8.5
PPE 8%	8.5 (2.66 ×10^{-1})†	4.25	15	17
PPE 10%	8.5 (≤1.66 ×10^{-2})†	0.27	15	17
PPE 13%	4.24	0.27	3.75	8.5
Pro-PPE 50%	≤1.66 x10^{-2}	8.5	15	8.5

All tests formulations were repeated three times (n=3) and AmB was repeated twenty times (n=20)

*Was not included in the AST.

†Second MFC value after the initial microbial death stopped.

Lastly, we determined the antimicrobial activity the test- and control formulations had on *C. albican*s comparing the MFC DF values of the test- and control formulations to one another (annexure C, table 1). The DF values presented in brackets below is where the formulation started to kill *C. albicans* again after the initial microbial death stopped. The DF of PPE 4% vs. P 4% was 0.5 vs. ≤9.77 x10^{-4}, PPE 8% vs. P 8% was 0.5 (1.56 ×10^{-3}) vs. ≤9.77 x10^{-4}, PPE 10% vs. P 10% was 0.5 (≤9.77 ×10^{-4}) vs. ≤9.77 x10^{-4}, PPE 13% vs. P 13% was 0.25 vs. ≤9.77 x10^{-4}, Pro-PPE 50% vs. Pro-P was ≤9.77 x10^{-4} vs. ≤9.77 x10^{-4}. In all instances, the control formulations displayed higher antimicrobial activity against *C. albicans* than the test formulations. Initially the control formulations were considerably more active than both the test formulations and PE, however, the test formulations had no antimicrobial activity after 3 months of AST. None of the test- or control formulations or PE were as potent as AmB.

S. cerevisiae

Firstly, we determined the antimicrobial activity the test formulations had against *S. cerevisiae* in comparison to the PE and AmB (table 4.5). Comparing the MFC of the test formulations to one another for *S. cerevisiae* during month 0, we observed the following. The MFCs of the test formulations were 8.5 mg/ml (PPE 13%), 17 mg/ml (PPE 8% and PPE 4%), while PPE 10% and Pro-PPE 50% had no antimicrobial activity, indicating that the PPE 13% test formulation had the highest antimicrobial activity against *S. cerevisiae*, followed by the PPE 8% and PPE 4% formulations. Both PPE 4% and PPE 13% resumed killing *S. cerevisiae* at an MFC of 1.33 $\times 10^{-1}$ mg/ml. The PE and AmB (positive control) had MFCs of 8.5 mg/ml and 3.99 $\times 10^{-5}$ mg/ml, respectively, however, the PE resumed killing *S. cerevisiae* at a concentration of ≤1.66 $\times 10^{-2}$ mg/ml. Thus, against *S. cerevisiae,* test formulation PPE 13% had similar antimicrobial activity compared to the PE. None of the test formulations or PE were as potent as AmB.

Secondly discussed, is the MFC of each formulation during the AST. Comparing the MFC of the test formulations to themselves for *S. cerevisiae* (table 4.5) during month 0, 1, 2, and 3 we observed the following. The MFC values of both test formulation PPE 4%, *i.e.* 17 mg/ml (month 0), 4.25 mg/ml (month 1), 30 mg/ml (month 2), ≤0.017 mg/ml (month 3), and PPE 8%, *i.e.* 17 mg/ml (month 0), 4.25 mg/ml (month 1), 7.5 mg/ml (month 2), 0.53 mg/ml (month 3), showed a substantial increase in antimicrobial activity when comparing the initial antimicrobial activity to month 3. At both month 0 and 1 PPE 4% resumed killing *S. cerevisiae* at a concentration of 1.33 $\times 10^{-1}$ mg/ml and ≤1.66 $\times 10^{-2}$ mg/ml respectively Initially test formulation PPE 10% had no antimicrobial activity (month 0), after which the MFCs were 0.27 mg/ml (month 1), 7.5 mg/ml (month 2) and 2.13 mg/ml (month 3). This was a strange observation as the antimicrobial activity increased drastically between month 0 and 3, for the PE, PPE4%, PPE8% and PPE10% formulations. The test formulation PPE 13%'s MFCs were 8.5 mg/ml (month 0), 0.27 mg/ml (month 1), 7.5 mg/ml (month 2) and 4.25 mg/ml (month 3). The MFC at month 1 showed an inexplicable increase in activity, after which the antimicrobial activity declined to 4.25mg/ml which were again higher than month 0. Pro-PPE 50% test formulation initially had no antimicrobial activity, where after the MFCs was 8.5 mg/ml (month 1), 7.5 mg/ml (month 2) and 4.25 mg/ml (month 3). This formulation thus followed a similar trend as compared to the other test formulations where an increase in antimicrobial activity was observed during the progression of the AST. The PE's MFCs were 8.5 mg/ml (month 0), 8.5 mg/ml (month 1), 7.5 mg/ml (month 2) and 2.13 mg/ml (month 3). The PE's initial antimicrobial activity thus increased (by 75%) compared to month 3.

Table 4.5: The MFC values of the different test formulations against *S. cerevisiae* during month 0, 1, 2, and 3 of AST.

Test formulation	Minimum fungicidal concentration, MFC (mg/ml)			
	Month 0	Month 1	Month 2	Month 3
AmB	3.99×10^{-5}	-*	-*	-*
PE	8.5 ($\leq1.66\times10^{-2}$)†	8.5	7.5	2.13
PPE 4%	17 (1.33×10^{-1})†	4.25 ($\leq1.66\times10^{-2}$) †	30	≤0.017
PPE 8%	17	4.25	7.5	0.53
PPE 10%	-	0.27	7.5	2.13
PPE 13%	8.5 (1.33×10^{-1}) †	0.27	7.5	4.25
Pro-PPE 50%	-	8.5	7.5	4.25

All tests formulations were repeated three times (n=3) and AmB was repeated twenty times (n=20)

*Was not included in the AST.

†Second MFC value after the initial microbial death stopped.

Lastly discussed, is the antimicrobial activity the test- and control formulations had on *S. cerevisiae* comparing the MFC DF values of the test formulations to their corresponding control formulations (Annexure C, table 2). The DF values presented in brackets below is where the formulation resumed killing *S. cerevisiae* after the initial microbial death stopped. The DF for test formulations vs. control formulations for PPE 4% vs. P 4% was 1 (7.81×10^{-3}) vs. ≤9.77×10^{-4}, PPE 8% vs. P 8% was 1 vs. ≤9.77×10^{-4}, PPE 10% vs. P 10% was no activity vs. 0.06, PPE 13% vs. P 13% was 0.5 (7.81×10^{-3}) vs. ≤9.77×10^{-4}, Pro-PPE 50% vs. Pro-P was 0 vs. ≤9.77×10^{-4}. In all instances, the control formulations displayed higher antimicrobial activity against *S. cerevisiae* than the test formulations. Initially the control formulations were considerably more active than both the test formulations and PE, however, the control formulations had no antimicrobial activity after 3 months of AST. None of the test- or control formulations or PE were as potent as AmB

S. epidermidis

Firstly, we determined the antimicrobial activity the test formulations had on *S. epidermidis* in comparison to the PE and Ery (positive control) (table 4.6). Comparing the MBC of the test formulations to themselves against *S. epidermidis* during month 0, we observed the following. The MBCs of the test formulations were 8.5 mg/ml (PPE 13%), 17 mg/ml (PPE 4% and PPE 8%), while PPE 10% and Pro-PPE 50% had no antimicrobial activity, indicating that the PPE 13% test formulation had the highest antimicrobial activity against *S. epidermidis*, followed by the PPE 4% and PPE 8% test formulations. Both PPE 8% and PPE 13% resumed killing *S. epidermidis* at a concentration of 1.33 $\times 10^{-1}$mg/ml. The PE had no antimicrobial activity against *S. epidermidis* while Ery (positive control) had an MFC of 1.6 $x10^{-4}$ mg/ml.

Secondly, we determined the MBC of each formulation during the AST. Comparing the MFC of the test formulations to themselves for *S. epidermidis* (table 4.6) during month 0, 1, 2, and 3 we observed the following. The PE had no activity. Both test formulation PPE 4% and PPE 8% had similar MFCs, *i.e.* 17 mg/ml (month 0) and 17 mg/ml (month 1), where after all antimicrobial activity was lost at month 2 and 3. PPE 4% resumed killing *S. epidermidis* at a concentration of 1.33 $\times 10^{-1}$mg/m during month 0. Interestingly, both test formulation PPE 10% and Pro-PPE 50% were only active during month 1, each with an MFC of 17 mg/ml. The test formulation PPE 13%'s MFCs were 8.5 mg/ml (month 0), 17 mg/ml (month 1), while there was no activity at month 2 and 3.

Table 4.6: The MBC values of the different test formulations against *S. epidermidis* during month 0, 1, 2, and 3 of AST.

Test formulation	Minimum fungicidal concentration, MBC (mg/ml)			
	Month 0	Month 1	Month 2	Month 3
Ery	1.6 $x10^{-4}$	-*	-*	-*
PE	-	-	-	-
PPE 4%	17 (1.33 $\times 10^{-1}$)†	17	-	-
PPE 8%	17	17	-	-
PPE 10%	-	17	-	-
PPE 13%	8.5 (1.33 $\times 10^{-1}$) †	17	-	-
Pro-PPE 50%	-	17	-	-

All tests formulations were repeated three times (n=3) and Ery was repeated twenty times (n=20)

*Was not included in the AST.

†Second MFC value after the initial microbial death stopped.

Lastly, we determined the antimicrobial activity the test- and control formulations had on *S. epidermidis,* comparing the MFC DF values of the test formulations to their corresponding control formulations (annexure C, table 3). The dilution factor (DF) for test formulations vs. control formulations PPE 4% vs. P 4% was 1 vs. ≤9.77 $x10^{-4}$, PPE 8% vs. P 8% was 1 (7.81 $\times10^{-3}$) vs. ≤9.77 $x10^{-4}$, PPE 10% vs. P 10% was 0 vs. 0.07, PPE 13% vs. P 13% was 0.5 (7.81 $\times10^{-3}$) vs. ≤9.77 $x10^{-4}$, Pro-PPE 50% vs. Pro-P 50% 0 vs. ≤9.77 $x10^{-4}$. In all instances, the control formulations displayed higher antimicrobial activity against *S. epidermidis* than the test formulations. Initially the control formulations were considerably more active than both the test formulations and PE, however, the test formulations had no antimicrobial activity after 3 months of AST. None of the test- or control formulations or PE were as potent as the Ery.

4.2.4. Determining minimum inhibitory and minimum fungicidal concentration using resazurin

The method used to determine the MIC can be seen in section 3.3.5.4. Using the same 96-well plates as prepared in section 3.3.5.4, we then determined the MFC values of both the test- and control formulations as described in section 3.3.5.3.

C. albicans

Firstly, we determined the antimicrobial activity the test formulations had on *C. albicans* in comparison to the PE and AmB (positive control) (table 4.7). Comparing the test formulations to one another, it was found that the PPE 4%, PPE 8%, PPE 10% and PPE 13% formulations all had an MIC of 108 µg/ml, while Pro-PPE 50% had no activity and the PE`s MIC was 1725 µg/ml. This indicates that Pheroid® increased the antimicrobial activity of the PE. The test formulations, *i.e.* PPE 4%, PPE 8%, PPE 10% and PPE 13%, had a higher antimicrobial activity in comparison to the crude PE, however the PE, test- or control formulations did not have a higher activity than AmB (MIC = 0.16 µg/ml, MFC = 1.28 µg/ml). At these concentrations, none of the tests formulations or PE had an MFC.

Secondly, comparing the test- and control formulations to one another (table 4.7)., it was found that none of the control formulations, *i.e.* P 4%, P 8%, P 10%, P 13% and Pro-P 50%, had antimicrobial activity at these concentrations while the test formulations, *i.e.* PPE 4%, PPE 8%, PPE 10% and PPE 13%, all had MICs of 108 µg/ml, excluding Pro-PPE 50% which had no antimicrobial activity. At these concentrations, there was no MFC activity found for both test- and control formulations.

S. cerevisiae

Firstly, we determined the antimicrobial activity the test formulations had on *S. cerevisiae* in comparison to the PE and AmB (positive control) (table 4.7). Comparing the test formulations, it was found that the PPE 4%, PPE 8%, PPE 10% and PPE 13% test formulations, as well as the PE, all had MICs of 54 µg/ml, while Pro-PPE 50% had no activity. The positive control, AmB, had an MIC of 0.64 µg/ml and an MFC of 0.64 µg/ml.

Secondly, comparing the test- and control formulation to one another (table 4.7), it was found that none of the control formulations, i.e. P 4%, P 8%, P 10%, P 13% and Pro-P 50%, had any antimicrobial activity while the test formulations, *i.e.* PPE 4%, PPE 8%, PPE 10% and PPE 13%, all had MICs of 54 µg/ml, excluding Pro-PPE 50% which had no antimicrobial activity. The MFC for PPE 4%, PPE 8%, PPE 10% and PPE 13% was 54 µg/ml, 108 µg/ml, 108 µg/ml, and 863 µg/ml respectively. The MFC should be higher or the same than the MIC, as it is the concentration where the microorganism is killed.

T. dermatis

Firstly, we determined the antimicrobial activity the test formulations had on *T. dermatis* in comparison to the PE and AmB (positive control) (table 4.7). Comparing the test formulations, it was found that the test formulations PPE 4%, PPE 8%, PPE 10%, PPE 13% and Pro-PPE 50% had an MIC of 27 µg/ml, 27 µg/ml, 54 µg/ml, 27 µg/ml, and 108 µg/ml respectively, and the PE's MIC and MFC were 108 µg/ml and 431 µg/ml, respectively. The test formulations had a higher antimicrobial activity than the PE, excluding Pro-PPE 50% which had the same antimicrobial activity. However, neither the PE, test- or control formulations had a higher activity than AmB (MIC = 0.08 µg/ml, MFC = 1.28 µg/ml).

Secondly, comparing the test- and control formulations (table 4.7), it was found that none of the control formulations (P 4%, P 8%, P 10%, P 13% and Pro-P 50%) had antimicrobial activity. The test formulations PPE 4%, PPE 8%, PPE 10%, PPE 13% and Pro-PPE 50% had an MIC of 27 µg/ml, 27 µg/ml, 54 µg/ml, 27 µg/ml, and 108 µg/ml respectively. The test formulation Pro-PPE 50% had a MFC of 1725 µg/ml.

C. neoformans

Comparing the antimicrobial activity of the test- and control formulation for *C. neoformans*, none of the test- or control formulations had antimicrobial activity, except PPE 4% which had an MIC of 863 µg/ml. There was also no antimicrobial activity for both the PE and AmB (table 4.7).

Table 4.7: The MIC and MFC values of the different test formulations against *C. albicans, S. cerevisiae, T. dermatis*, and *C. neoformans*.

Formulation	*C. albicans*		*S. cerevisiae*		*T. dermatis*		*C. neoformans*	
	MIC (µg/ml)	MFC (µg/ml)	MIC (µg/ml)	MFC (µg/ml)	MIC (µg/ml)	MFC (µg/ml)	MIC (µg/ml)	MFC (µg/ml)
AmB	0,16	1,28	0,64	0,64	0,08	1,28	-	-
PE	1725	-	54	-	108	431	-	-
PPE 4%	108	-	54	54	27	-	863	-
PPE 8%	108	-	54	108	27	-	-	-
PPE 10%	108	-	54	108	54	-	-	-
PPE 13%	108	-	54	863	27	-	-	-
Pro-PPE 50%	-	-	-	-	108	1725	-	-

All tests formulations were tested three times (n=3) and AmB was tested five times (n=5)

The MFCs in table 4.7 differ from the MFCs in table 4.4 and 4.5. This could be because, during the AST, the microorganisms were not diluted to a McFarland standard of 0.6 before being added to the 96-well plates.

Chapter 5: Discussion and conclusion

5.1. Discussion

AMR is a major concern as existing antimicrobials are becoming less effective due to misuse and overuse (WHO, 2016). An estimate of 23,000 individuals die each year in the United States alone as a direct result of AMR related infections (CDC, 2018a). The FDA is approving fewer antimicrobial drugs each year due to more stringent requirements (Spellberg *et al.,* 2004). Identification of compounds or treatments, which could have possible antimicrobial activity is thus urgently needed.

In this study, we determined if an *A. africanus* PE exhibited antimicrobial activity against 11 human pathogens (*S. cerevisiae, C. albicans, E. coli, S. epidermidis, S. aureus, K. pneumonia, S. enterica, E. aerogenes, P. aeruginosa, C. neoformans,* and *T. dermatis*). As well as, evaluating the antimicrobial activity of the Pheroid®-PE formulation to determine if Pheroid®, with varying percentage oil phases (w/w), had an effect on the antimicrobial activity. By using a combination of antimicrobial susceptibility and formulation characterisation tests, the PE was found to have antimicrobial activity against *C. albicans,* S*. cerevisiae, S. epidermidis,* and *T. dermatis*. Characterisation showed that optimisation of the formulations was possible, some were more successful than others, and that most of the formulations were stable *i.e*. no aggregation, flocculation or creaming was observed during AST.

5.1.1. Formulation characterization

Mean particle size

Initially (month 0), both PPE 13% and PPE 10% had statistically smaller mean particle sizes than their corresponding controls, this can also be seen in the CLSM images, while there was no difference between the other test- and control formulation's particle sizes. This indicated that overall the PE did not influence the particle size of the formulations. There was statistically no difference in mean particle size between the control formulations. Formulation PPE 4% had the largest mean particle size, while formulation PPE 10% and PPE 13% had the smallest mean particle size. This showed that overall the mean particle size for the test formulations decreased with an increase of percentage oil phase (w/w). Pro-PPE 50% did not follow this trend, this could be due to that Pro-Pheroid® is not homogenised during formulation (Floury *et al.*, 2000). The release of the PE could be increased by the larger vesicles, therefor PPE 4% and Pro-PPE 50% could have the highest antimicrobial activity (van Ruth *et al.*, 2002). The CLSM images show that all of the formulations are clearly spherical and uniform in shape and evenly dispersed.

During AST, the mean particle size of PPE 10%, PPE 13% and Pro-PPE 50% increased when comparing month 0 to either month 1, 2 or 3, while PPE 4% and PPE 8% stayed relatively

constant. Both PPE 4% and PPE 8% would be the most suitable for further product development, as their particle sizes remained statistically constant during AST. The mean particle size for control formulations P 4% and Pro-P increased from month 2 to month 3. While with the other control formulations, no significant changes were observed indicating that they remained stable. The increase in mean particle size could be explained by aggregation of the oil droplets/micelles formed to create lipid bilayer vesicles, known as Pheroids® (Gaysinsky *et al*., 2007).

Zeta potential stability

Initially (month 0), all of the test formulations had significantly smaller zeta potentials than their corresponding control formulations, except for PPE 10%. This showed that by incorporating the PE into the Pheroid® vesicles, the average zeta potential decreased, theoretically resulting in more stable vesicles. The addition of a PE to an emulsion has been linked to improvements in stability of the vesicles (Abdalla and Roozen, 1999; Acedo-Carrillo *et al*., 2006). Therefore, the *A. africanus* PE could be the reason for the improved zeta potentials. It is possible that the active compounds in the PE could become embedded in the lipid bilayer causing a decrease in zeta potential (figure 5.1 and 5.2). Formulations PPE 4%, PPE 8%, PPE 13% and Pro-PPE 50%, all had smaller average zeta potential, resulting in the more stable formulations, than formulation PPE 10%. No correlation can thus be made between the percentage oil phase and the stability of the vesicles. Contrary to this study's results, Wang *et al*. (2010) found that the zeta potential decreased with an increase in the lipid phase. Further investigation of the zeta potentials should be conducted to provide an explanation. There was statistically no difference between the average zeta potentials for the control formulations. The zeta potential can be directly linked to the formation of stable Pheroid® vesicles. When the zeta potential is less or equal to -25 mV, the repulsive forces are more than the attractive London forces and formation of defined and stable vesicles will form (Lieberman *et al*., 1989).

During AST, the zeta potential of formulations PPE 4%, PPE 8%, P 8%, P 13% and Pro-PPE 50% decreased from month 0 to 3. Formulation PPE 13%'s zeta potential increased until month 1 and decreased at month 2, while Pro-P's zeta potential increased until month 1 and decreased at month 3, resulting in more stable vesicles. This was unexpected, as emulsions tend to become less stable during AST (Gu *et al.,* 2005). The sedimentation potential effect could be a possible explanation for the decrease in zeta potential (figure 5.1). Sedimentation potential occurs when a colloidal system is allowed to settle and generate a potential difference. As the particles move, via temperature or pressure, they are constantly rearranging and establishing a flow of charge into one side and vice versa (figure 5.1) (Hunter, 1981:3).

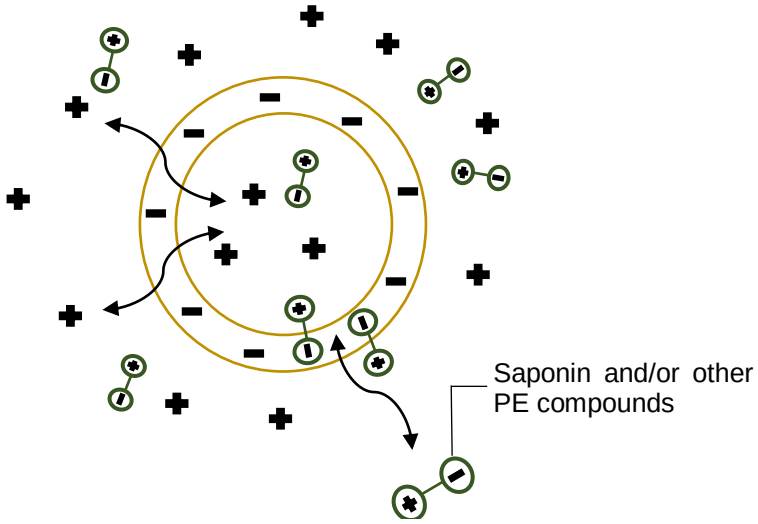


Figure 5.1: A schematic presentation of the current flow generating the sedimentation potential. Adapted from Hunter, 1981:3.

With regards to the particle size, test formulation PPE 4% proved to be the most promising formulation. Not only did this formulation have statistically larger vesicles before AST, but also stayed stable comparing month 1 with month 3 of AST. With regards to the zeta potential, both test formulations PPE 4% and PPE 8% proved promising as both of them not only had statistically lower zeta potentials, but also inexplicably became more stable during AST comparing month 0 to month 3.

5.1.2. *In vitro* antimicrobial susceptibility tests

The MFC/MBC antimicrobial susceptibility tests showed that all the control formulations initially had higher antimicrobial activity than the test formulations, however after AST their fungicidal activity decreased almost completely against *C. albicans,* S*. cerevisiae* and *S. epidermidis*. None of the formulations or PE was as potent as AmB or Ery. The purity of the PE could have had a major effect on the antimicrobial activity, as the PE is a mixture of active compounds (Diekema *et al*., 2003). The addition of Pheroid® increased the antimicrobial activity against some microbes, while against others it decreased the antimicrobial activity of the PE. There are three possible causes for the results seen in this study, namely, sequestering of the PE's active compounds in the oil phase, solubilisation of the PE's active compounds in surfactant micelles, or a reduction of the active compounds present in the PE during AST, as they are sensitive to temperature. These phenomena are discussed in more detail below (figure 5.2).

Initial screening via the Kirby Bauer disc diffusion method determined that the PE had moderate activity against S*. cerevisiae* and *C. albicans,* minor activity against *E. coli, K. pneumonia* and *S. enterica* (Gram-negative bacteria) as well *as S. epidermidis* and *S. aureus* (Gram-positive bacteria). We initially failed to determine the MICs via the standard microdilution method, where after the MBC/MFC was determined with subculturing. It was found that *C. albicans*, S. cerevisiae, and *S. epidermidis* were susceptible to the PE and formulations.

Initially (month 0), it was found that the PE and test formulations PPE 4%, PPE 8%, and PPE 10% all had an initial MFC of 8.5 mg/ml against *C. albicans*. However, PPE 13% (4.25 mg/ml) and Pro-PPE 50% (0.016 mg/ml) showed an increase in antimicrobial activity with Pro-PPE 50% having the most promising antimicrobial activity against *C. albicans*, indicating that an increase in percentage oil phase (w/w) in Pheroid® increased the activity. Pro-PPE 50%'s increased activity can be the result of an increase in the percentage oil phase (w/w) or an increase in the mean particle size of the vesicles. During AST, Pheroid® did not prevent loss of activity as the antimicrobial activity decreased immensely after AST. In general, after AST, test formulation PPE 8%, PPE 10%, PPE 13% and the PE had a 50% decrease in antimicrobial activity, while the activity of PPE 4% stayed constant. The Pro-PPE 50% test formulation had the best activity against *C. albicans*, even better than the PE. At the end of the AST, Pro-PPE 50%'s activity was comparable to the initial MFC of PPE 8%, PPE 10%, PPE 13% and PE. The formulations having larger particle sizes appear to have higher antimicrobial activity against *C. albicans*. Sequestering or solubilisation could be plausible explanations for this phenomenon. The PE is soluble in water and oil, therefore the following explanations could be possible. Sequestering of the PE's active compounds in the oil phase: The active compounds present in the PE could have become embedded in the lipid bilayers of the vesicles, acting as a surfactant (figure 5.2) (Gaysinsky *et al*., 2007; Terjung *et al*., 2012). This will cause the concentration of the PE to decrease in the aqueous

phase along with a decrease in particle size. If the active compounds present in the PE are embedded in the vesicle there would be less available in the water phase to interact with the microorganisms, therefore decreasing the overall antimicrobial activity (Campos *et al*., 2009).

Solubilisation of the PE's active compounds in surfactant micelles is another explanation, as a constant amount of surfactant was used. Therefore, if smaller vesicles should form, more surfactant will be needed to stabilize the vesicles (Lawrence, 1994; Terjung *et al*., 2012). The PE could act as a surfactant, forming smaller vesicles. Formation of smaller vesicles could result in fewer micelles present, whereas with larger vesicles more micelles could be present (figure 5.2). Micelles solubilise the active compounds present in the PE, resulting in fewer active compounds available in the aqueous phase making the formulation less active (Gaysinsky *et al*., 2007). The AST could also have caused a reduction of the active compounds present in the PE, as they are sensitive to temperature (Spigno *et al*., 2007).

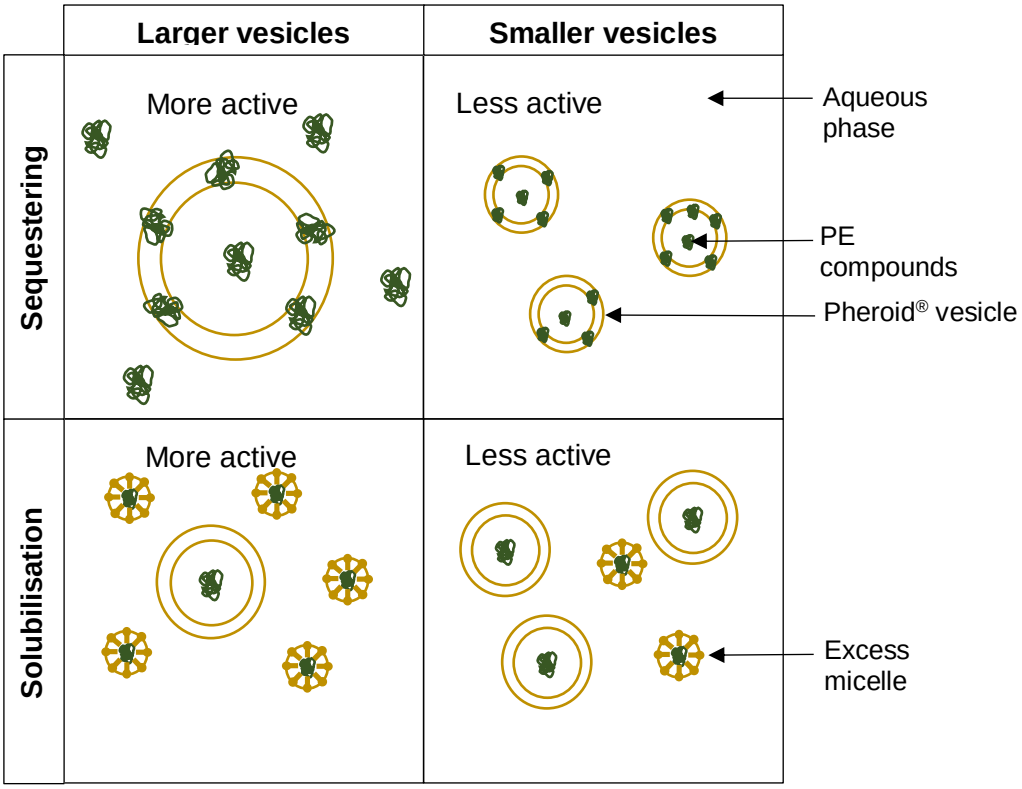


Figure 5.2: This figure explains the difference in sequestering of PE compounds in the lipid phase and solubilisation of PE compounds in surfactant micelles. Adapted from Gaysinsky *et al*., 2007

Initially (month 0), the PE and PPE 13% test formulations both had an MIC of 8.5 mg/ml against *S. cerevisiae,* and PPE 4% and PPE 8% both had an MFC of 17 mg/ml, while PPE 10% and Pro-PPE 50% had no activity. After AST, the activity of all test formulations was increased. Formulation PPE 13% was thus the most promising initial test formulation, while PPE 4% had the highest antimicrobial activity after AST. No conclusion could be drawn between the percentage oil phase (w/w), the mean particle size of the vesicles and the antimicrobial activity against *S. cerevisiae*. It is possible that some of the active compounds present in the PE, which had the most activity against *S. cerevisiae,* became sequestered in the vesicle's lipid bilayer membrane during month 0 and during AST the amount active compounds increased in the water phase, increasing the antimicrobial activity (figure 5.2) (Gaysinsky *et al.*, 2007*)*.

Initially (month 0), test formulation PPE 13% had the most promising MBC against *S. epidermidis* and PPE 4% and PPE 8% had an MBC of 17 mg/ml, while the PE, PPE 10% and Pro-PPE 50% had no fungicidal activity. This indicated that adding Pheroid® increased the antimicrobial activity of the PE against *S. epidermidis*. After AST, test formulation PPE 4%, PPE 8%, and PPE 13% all decreased in activity.

After improvements were made to the initial microdilution method by adding a colour indicator, known as Resazurin. The PE had an MIC of 1725 µg/ml when tested against *C. albicans*, while all of the test formulations had an MIC of 108 µg/ml, except Pro-PPE 50% which had no inhibitory activity. By incorporating the PE into the Pheroid® drug delivery system, the PE's activity was increased roughly 16 times.

While determining the MIC of the PE and test formulations against *S. cerevisiae*, only the Pro-PPE 50% test formulation had no activity. All other test formulations and the PE had an MIC of 54 µg/ml. The PE had no fungicidal activity at these concentrations, while PPE 4% had an MFC of 54 µg/ml. A trend was observed where the MFC values decreased with an increase of percentage oil phase (w/w) in Pheroid®.

The PE had an MIC and MFC of 108 µg/ml and 431 µg/ml against *T. dermatis*. Test formulation PPE 4%, PPE 8% and PPE 13% had an MIC 4 times higher than the PE, while PPE 10%'s MIC was 2 times higher. The Pro-PPE 50% formulation had the same MIC as the PE but the MFC was 4 times lower. The addition of Pheroid® increased the MIC while decreasing the MFC.

5.1.3. Limitations

There were a few limitations found in this study. During the AST, the MIC and MFC/MBC were determined with a method known as the microdilution method. However, the test- and control formulations proved to be too turbid to allow any interpretation of the MIC values. This was a result of the formation of the Pheroid® vesicles which created a milky suspension. The turbidity problem was solved by adding a colour indicator known as resazurin. The MFC obtained for *C. albicans* and *S. cerevisiae* from the AST differs from the MFCs obtained from the resazurin microdilution method. This could be due to the difference in CFU used during the AST. The number of organisms in each broth was not quantified during the AST, while during the resazurin testing, the organisms were diluted to a McFarland standard of 0.6 before being added to the 96-well plates. The accuracy of the MIC and MBC/MFC values are not exact, due to the wide confidence interval of the exponential concentration series *i.e.* 1, 2, 4, 8, 16, 32 and 64 mg/ml (Liu *et al.,* 2003). This means that if the MIC and MBC/MFC is 32 mg/ml, the exact MBC/MFC can be anywhere between 16-32 mg/ml.

5.2. Conclusion

In this study we firstly determined the *in vitro* antimicrobial activity of the PE with the Kirby Bauer disc diffusion method, against *Staphylococcus aureus, Staphylococcus epidermidis, Escherichia coli, Enterobacter aerogenes, Klebsiella pneumonia, Salmonella enterica, Pseudomonas aeruginosa, Candida albicans* and *Saccharomyces cerevisiae.* During this screening process we found that the crude PE showed moderate activity against the yeasts *S. cerevisiae, C. albicans*, and only minor activity against *E. coli, K. pneumonia and S. enterica* (Gram-negative bacteria) as well as *S. epidermidis* and *S. aureus* (Gram-positive bacteria). The PE had no activity against *P. aeruginosa* and *E. aerogenes*, both Gram-negative bacteria.

The morphology, particle size and zeta potential characterisation tests showed that optimisation of the Pheroid®-PE formulations by adjusting the percentage oil phase was possible. Most of the formulations were stable *i.e*. no aggregation, flocculation or creaming was observed during AST. There was no difference in the particle size between the test- and control formulations, except for PPE 10% and PPE 13%, indicating that in most cases the PE did not influence the particle size. Test formulation PPE 4% proved to be the most promising formulation with the largest initial particle size and remained stable during AST. Incorporating the PE into the Pheroid® vesicles resulted in more stable vesicles, as all of the test formulations, expect PPE 10%, had statistically lower zeta potentials as compared to the corresponding control formulations. The test formulations became more stable during AST, as could be seen with PPE 4% and PPE 8% which had the lowest initial zeta potentials and became more stable during AST, indicated by the

significant decrease in their zeta potentials. No statistical correlation could be made between the particle size, zeta potential and percentage oil phase (w/w).

The initial microdilution method used to determine the MICs of the PE, test-, and control formulations did not succeed, as the formulations proved to be too turbid to allow any interpretation of the MIC values before and during the AST. We adapted the microdilution method by using resazurin as a colour indicator. During the adapted method, we excluded the microorganisms against which the PE and test formulation had no activity, and included two yeasts with the other susceptible microorganisms. The *A. africanus* PE was identified to have moderate antimicrobial activity against three human pathogens, namely *C. albicans, S. cerevisiae*, and *T. dermatis* with an MIC of 1725 µg/ml, 54 µg/ml, and 108 µg/ml, respectively. The test formulations had an MIC of 108 µg/ml and 54 µg/ml against *C. albicans and S. cerevisiae*, while the MIC against *T. dermatis* was 27 µg/ml expect for Pro-PPE 50% and PPE 10%. In conclusion, the addition of Pheroid® increased the MIC in most cases, however, did not seem to have an effect on the MFCs. This excludes *S. cerevisiae* where the PE and test formulations had the same MIC, while the PE had no MFC on its own, however by adding Pheroid® the resulting formulation had fungicidal properties.

During AST, the *in vitro* antimicrobial activity of the PE, test- and control formulation was determined. Initially, the control formulations had a higher antimicrobial activity than the test formulations, however, after AST, they had almost no antimicrobial activity. The formulation that showed the best initial antimicrobial activity against *C. albicans* was Pro-PPE 50%, however, after AST both Pro-PPE 50% and PPE 4% had the highest antimicrobial activity. Formulation PPE 13% had the highest initial fungicidal activity against both *S. cerevisiae* and *S. epidermidis*, while PPE 8% had the highest fungicidal activity after AST against *S. cerevisiae*.

The *A. africanus* PE had moderate antimicrobial activity against four human pathogens, namely *S. epidermidis, S. cerevisiae, C. albicans,* and *T. dermatis*. Formulation of the Pheroid® PE delivery system was also determined to be possible, yielding stable vesicles. Overall, the PE and the test formulations did have *in vitro* antimicrobial activity, although the degree of activity was varied between the strains of bacteria/fungi.

Chapter 6: Future prospects

During this study, certain observations were made that suggest follow up studies should be done:

1) A purification method should be developed to extract the different active component from the PE, to determine the activity of the individual compounds in the plant extract against these 11 human pathogens.

2) Develop a spectrophotometric method to determine the difference in turbidity after incubation of the microorganisms with Pheroid® samples. This would be important to be able to determine a concentration-killing curve.

3) Some formulations, *i.e.* PE, PPE 8%, and PPE 10% vs. *C. albicans*, PE, PPE 4%, and PPE 13% vs. *S. cerevisiae* and PPE 8% and PPE 13% vs. *S. epidermidis*, displayed an induced killing effect where the formulations started to kill the microorganisms again after the initial MFC. The reason for this phenomenon is unclear, thus a concentration-killing curve (CKC) will have to be established to determine the exact cause and MBC/MFC (Liu *et al.*, 2004).

4) The micro broth dilution method usually gives an overestimate of the MBC/MFC, as this method uses ranges where the microorganism is killed (Lui *et al.,* 2003). To determine a more accurate estimate of the MBC/MFC, an agar dilution test can be performed where colony formations can be counted on agar plates to determine an exact MBC/MFC (NCCLS, 2002).

5) During the AST, the zeta potentials of numerous formulations decreased from month 0 to 3. Further investigation of the zeta potentials should be conducted to provide a clear explanation. The sedimentation potential theory could be tested to possibly provide an explanation.

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Annexures

Annexure A: Antimicrobial agents' calculations

Calculations to determine the AF concentration in the first well for AmB:

1) 10mg AmB in 10ml DMSO

$$C = \frac{m}{V} \times \frac{1}{M}$$

$$\frac{0.01g}{0.01L} \times \frac{1}{924.079\ g.mol^{-1}}$$

$$= 1082.15\ \mu M$$

2) Concentration in stock solution

$$C_1V_1 = C_2V_2$$

$$(1082.15\ \mu M)(100\mu l) = C_2(1000\mu l)$$

$$C_2 = 108{,}22\ \mu M$$

3) Concentration in 23µl

$$C_1V_1 = C_2V_2$$

$$(108.22\ \mu M)(23\mu l) = C_2(180\mu l)$$

$$C_2 = 13.83\ \mu M$$

4) Concentration in first well

$$C_1V_1 = C_2V_2$$

$$(13.83\ \mu M)(80\mu l) = C_2(100\mu l)$$

$$C_2 = 11.06\ \mu M$$

$$\therefore \mathbf{C_2 = 10.22\ \mu g.ml^{-1}}$$

Calculations to determine the AB concentration in the first well for Ery:

1) 100mg Ery in 10ml Methanol

$$C = \frac{m}{V} \times \frac{1}{M}$$

$$\frac{0.1g}{0.01L} \times \frac{1}{733.93\ g.mol^{-1}}$$

$$= 13625\ \mu M$$

2) Concentration in stock solution

$$C_1V_1 = C_2V_2$$

$$(13625\ \mu M)(10\mu l) = C_2(1000\mu l)$$

$$C_2 = 136.25\ \mu M$$

3) Concentration in 23µl

$$C_1V_1 = C_2V_2$$

$$(136.25\ \mu M)(23\mu l) = C_2(180\mu l)$$

$$C_2 = 17.01\ \mu M$$

4) Concentration in first well

$$C_1V_1 = C_2V_2$$

$$(17.01\ \mu M)(80\mu l) = C_2(100\mu l)$$

$$C_2 = 13.93\ \mu M$$

$$\therefore \mathbf{C_2 = 10.22\ \mu g.ml^{-1}}$$

Calculations to determine the AB concentration in the first well for TMP:

1) 100mg TMP in 10ml Methanol

$$C = \frac{m}{V} \times \frac{1}{M}$$

$$\frac{0.1g}{0.01L} \times \frac{1}{290.32\ g.mol^{-1}}$$

$$= 34444.75\ \mu M$$

2) Concentration in stock solution

$$C_1V_1 = C_2V_2$$

$$(34444.75\ \mu M)(10\mu l) = C_2(1000\mu l)$$

$$C_2 = 344.45\ \mu M$$

3) Concentration in 23µl

$$C_1V_1 = C_2V_2$$

$$(344.45\ \mu M)(23\mu l) = C_2(180\mu l)$$

$$C_2 = 44.01\ \mu M$$

4) Concentration in first well

$$C_1V_1 = C_2V_2$$

$$(44.01\ \mu M)(80\mu l) = C_2(100\mu l)$$

$$C_2 = 35.21\ \mu M$$

$$\therefore \mathbf{C_2 = 10.22\ \mu g.ml^{-1}}$$

Annexure B: Dilutions of the 96-well plates

Table 1: Dilution of each well for all the test formulations including the antimicrobial agents used during the AST and activity tests.

Well number	1	2	3	4	5	6	7	8	9	10	11
Dilution factor	1:1	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024
	0	0.5	0.25	0.125	6.25×10^{-2}	3.13×10^{-2}	1.56×10^{-2}	7.81×10^{-3}	3.91×10^{-3}	1.95×10^{-3}	9.77×10^{-4}
AmB, Ery, Tmp mg/ml	1.02×10^{-2}	5.11×10^{-3}	2.56×10^{-3}	1.28×10^{-3}	6.39×10^{-4}	3.19×10^{-4}	1.60×10^{-4}	7.98×10^{-5}	3.99×10^{-5}	2.00×10^{-5}	9.98×10^{-6}
PE mg/ml [34]	17	8.5	4.25	2.125	1.063	0.531	0.266	0.133	0.066	0.033	0.017
PE mg/ml [60]	30	15	7.5	3.75	1.875	0.938	0.469	0.234	0.117	0.059	0.029
P4%, P8%, P10%, P13%, P50%, Pro-P	1	0.5	0.25	0.125	0.063	0.031	0.016	7.81×10^{-3}	3.91×10^{-3}	1.95×10^{-3}	9.77×10^{-4}

Table 2: Dilution of each well for all the test formulations including the antimicrobial agents used for Resazurin and activity testing.

Well number	1	2	3	4	5	6	7	8	9	10	11
Dilution factor	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	1:2048	1:4096	1:8192	1:16384
	6.25×10^{-2}	3.13×10^{-2}	1.56×10^{-2}	7.81×10^{-3}	1.95×10^{-3}	1.95×10^{-3}	9.77×10^{-4}	4.88×10^{-4}	2.44×10^{-4}	1.22×10^{-4}	6.10×10^{-5}
AmB, Ery, TMP mg/ml	1.02×10^{-2}	5.11×10^{-3}	2.56×10^{-3}	1.28×10^{-3}	6.39×10^{-4}	3.19×10^{-4}	1.60×10^{-4}	7.98×10^{-5}	3.99×10^{-5}	2.00×10^{-5}	9.98×10^{-6}
PE mg/ml [55,2]	1.725	0.863	0.431	0.216	0.108	0.054	0.027	0.013	6.74×10^{-3}	3.37×10^{-3}	1.68×10^{-3}
P4%, P8%, P10%, P13%, P50%, Pro-P	0.063	0.031	0.016	7.81×10^{-3}	3.91×10^{-3}	1.95×10^{-3}	9.77×10^{-4}	4.88×10^{-4}	2.44×10^{-4}	1.22×10^{-4}	6.10×10^{-5}

Annexure C: Summary MBC/MFC tables for relevant organisms during AST.

Table 1: The MFC values and DF of the different test- and control formulations against *C. albicans* during month 0, 1, 2, and 3 of AST

Test formulation	Month 0		Month 1		Month 2		Month 3	
	MFC (mg/ml)	DF	MFC (mg/ml)	DF	MFC (mg/ml)	DF	MFC (mg/ml)	DF
AmB	3.99×10^{-5}	3.91×10^{-3}	-*	-*	-*	-*	-*	-*
PE	8.5 ($\leq 1.66 \times 10^{-2}$) †	0.5 ($\leq 9.77 \times 10^{-4}$) †	8.5	0.5	7.5	0.25	17	1
PPE 4%	8.5	0.5	8.5	0.5	30	0.5	8.5	0.5
P 4%	-	$\leq 9.77 \times 10^{-4}$	-	1	-	-	-	0.5
PPE 8%	8.5 (2.66×10^{-1}) †	0.5 (1.56×10^{-3}) †	4.25	0.25	15	0.25	17	1
P 8%	-	$\leq 9.77 \times 10^{-4}$	-	-	-	0.125	-	1
PPE 10%	8.5 ($\leq 1.66 \times 10^{-2}$) †	0.5 ($\leq 9.77 \times 10^{-4}$) †	0.27	1.56×10^{-2}	15	0.25	17	1
P 10%	-	$\leq 9.77 \times 10^{-4}$	-	0.5	-	-	-	-
PPE 13%	4.24	0.25	0.27	1.56×10^{-2}	3.75	0.125	8.5	0.5
P 13%	-	$\leq 9.77 \times 10^{-4}$	-	-	-	0.125	-	1
Pro-PPE 50%	$\leq 1.6 \times 10^{-2}$	$\leq 9.77 \times 10^{-4}$	8.5	0.5	15	0.25	8.5	0.5
Pro-P	-	$\leq 9.77 \times 10^{-4}$	-	-	-	-	-	1

All tests formulations were repeated three times (n=3) and AmB was repeated twenty times (n=20).

*Was not included in the AST.

† The values in brackets are where the test formulation started to kill the cells/organisms again

Table 2: The MFC values and DF of the different test- and control formulations against *S. cerevisiae* during month 0, 1, 2, and 3 of AST testing

Test formulation	Month 0		Month 1		Month 2		Month 3	
	MFC (mg/ml)	DF	MFC (mg/ml)	DF	MFC (mg/ml)	DF	MFC (mg/ml)	DF
AmB	2×10^{-5}	1.95×10^{-3}	-*	-*	-*	-*	-*	-*
PE	8.5 (≤1.66×10^{-2})†	0.5 (≤9.77×10^{-4})†	8.5	0.5	7.5	0.25	2.13	0.125
PPE 4%	17 (1.33 ×10^{-1})†	1 (7.81 ×10^{-3})†	4.25 (≤1.66×10^{-2})	0.25 (≤9.77×10^{-4})†	30	1	≤0.017	≤9.77×10^{-4}
P 4%	-	≤9.77×10^{-4}	-	≤9.77×10^{-4}	-	1 (1.95×10^{-3})†	-	-
PPE 8%	17	1	4.25	0.25	7.5	0.25	0.53	0.031
P 8%	-	≤9.77×10^{-4}	-	≤9.77×10^{-4}	-	≤9.77×10^{-4}	-	1
PPE 10%	-	-	0.2656	1.56×10^{-2}	7.5	0.25	2.13	0.13
P 10%	-	0.0625	-	6.25 ×10^{-2} (7.81 ×10^{-3})†	-	0.5 (≤9.77×10^{-4})†	-	-
PPE 13%	8.5 (1.33 ×10^{-1})†	0.5 (7.81 ×10^{-3})†	0.2656	1.56×10^{-2}	7.5	0.25	4.25	0.25
P 13%	-	(≤9.77×10^{-4})†	-	7.81×10^{-3}	-	≤9.77×10^{-4}	-	-
Pro-PPE 50%	-	-	8.5	0.5	7.5	0.25	4.25	0.25
Pro-P	-	(≤9.77×10^{-4})†	-	0.125 (9.77 ×10^{-4})†	-	1 (≤9.77×10^{-4})†	-	-

All tests formulations were repeated three times (n=3) and AmB was repeated twenty times (n=20).

*Was not included in the AST.

† The values in brackets are where the test formulation started to kill the cells/organisms again

Table 3: The MBC values and DF of the different test- and control formulations against *S. epidermidis* during month 0, 1, 2, and 3 of AST.

Formulations	Month 0		Month 1		Month 2		Month 3	
	MBC (mg/ml)	DF	MBC (mg/ml)	DF	MBC (mg/ml)	DF	MBC (mg/ml)	DF
Ery	1.6×10^{-4}	1.56×10^{-2}	-*	-*	-*	-*	-8	-*
PE	-	-	-	-	-	-	-	-
PPE 4%	17	1	17	1	-	-	-	-
P 4%	-	$\leq 9.77 \times 10^{-4}$	-	-	-		-	-
PPE 8%	17 (1.33×10^{-1})†	1 (7.81×10^{-3})†	17	1	-	-	-	-
P 8%	-	($\leq 9.77 \times 10^{-4}$)†	-	-	-	0.125	-	-
PPE 10%	-	-	17	1	-	-	-	-
P 10%	-	0.0625	-	-	-		-	-
PPE 13%	8.5 (1.33×10^{-1})†	0.5 (7.81×10^{-3})†	17	1	-	-	-	-
P 13%	-	($\leq 9.77 \times 10^{-4}$)†	-	1	-	0.125	-	-
Pro-PPE 50%	-	-	17	1	-	-	-	-
Pro-P	-	($\leq 9.77 \times 10^{-4}$)†	-	-	-		-	-

All tests formulations were repeated three times (n=3) and Ery was repeated twenty times (n=20).

*Was not included in the AST.

† The values in brackets are where the test formulation started to kill the cells/organisms again.

Annexure D: Summary of the MIC/MFC values obtained during the Resazurin testing

Table 1: The MIC and MFC values of the different test formulations against C. *albicans, S. cerevisiae, T. dermatis*, and *C. neoformans*

Formulation	*C. albicans*		*S. cerevisiae*		*T. dermatis*		*C. neoformans*	
	MIC µg/ml / (DF)	MFC µg/ml / (DF)	MIC µg/ml / (DF)	MFC µg/ml / (DF)	MIC µg/ml / (DF)	MFC µg/ml / (DF)	MIC µg/ml / (DF)	MFC µg/ml / (DF)
AmB	0.16 ($9{,}77 \times 10^{-4}$)*	1,28 ($7{,}81 \times 10^{-3}$)*	0.64 ($3{,}9 \times 10^{-3}$)*	0.64 ($3{,}9 \times 10^{-3}$)*	0.08 ($4{,}88 \times 10^{-4}$)*	1,28 ($7{,}81 \times 10^{-3}$)*	-	-
PE	1725 (0,063)*	-	54 ($1{,}95 \times 10^{-3}$)*	-	108 ($3{,}9 \times 10^{-3}$)*	431 ($1{,}56 \times 10^{-2}$)*	-	-
PPE 4%	108 ($3{,}9 \times 10^{-3}$)*	-	54 ($1{,}95 \times 10^{-3}$)*	54 ($1{,}95 \times 10^{-3}$)*	27 ($9{,}77 \times 10^{-4}$)*	-	1725 (0,063)*	-
P 4%	-	-	-	-	-	-	-	-
PPE 8%	108 ($3{,}9 \times 10^{-3}$)*	-	54 ($1{,}95 \times 10^{-3}$)*	108 ($3{,}9 \times 10^{-3}$)*	27 ($9{,}77 \times 10^{-4}$)*	-	-	-
P 8%	-	-	-	-	-	-	-	-
PPE 10%	108 ($3{,}9 \times 10^{-3}$)*	-	54 ($1{,}95 \times 10^{-3}$)*	108 ($3{,}9 \times 10^{-3}$)*	54 ($1{,}95 \times 10^{-3}$)*	-	-	-
P 10%	-	-	-	-	-	-	-	-
PPE 13%	108 ($3{,}9 \times 10^{-3}$)*	-	54 ($1{,}95 \times 10^{-3}$)*	863 ($1{,}95 \times 10^{-3}$)*	27 ($9{,}77 \times 10^{-4}$)*	-	-	-
P 13%	-	-	-	-	-	-	-	-
Pro-PPE 50%	-	-	-	-	108 ($3{,}9 \times 10^{-3}$)*	1725 (0,063)*	-	-
Pro-P	-	-	-	-	-		-	-

All tests formulations were repeated three times (n=3) and AmB was repeated five times (n=5).

*The values in brackets are the dilution factors (DF)

.

Annexure E: MIC results pictures of 96-well plates

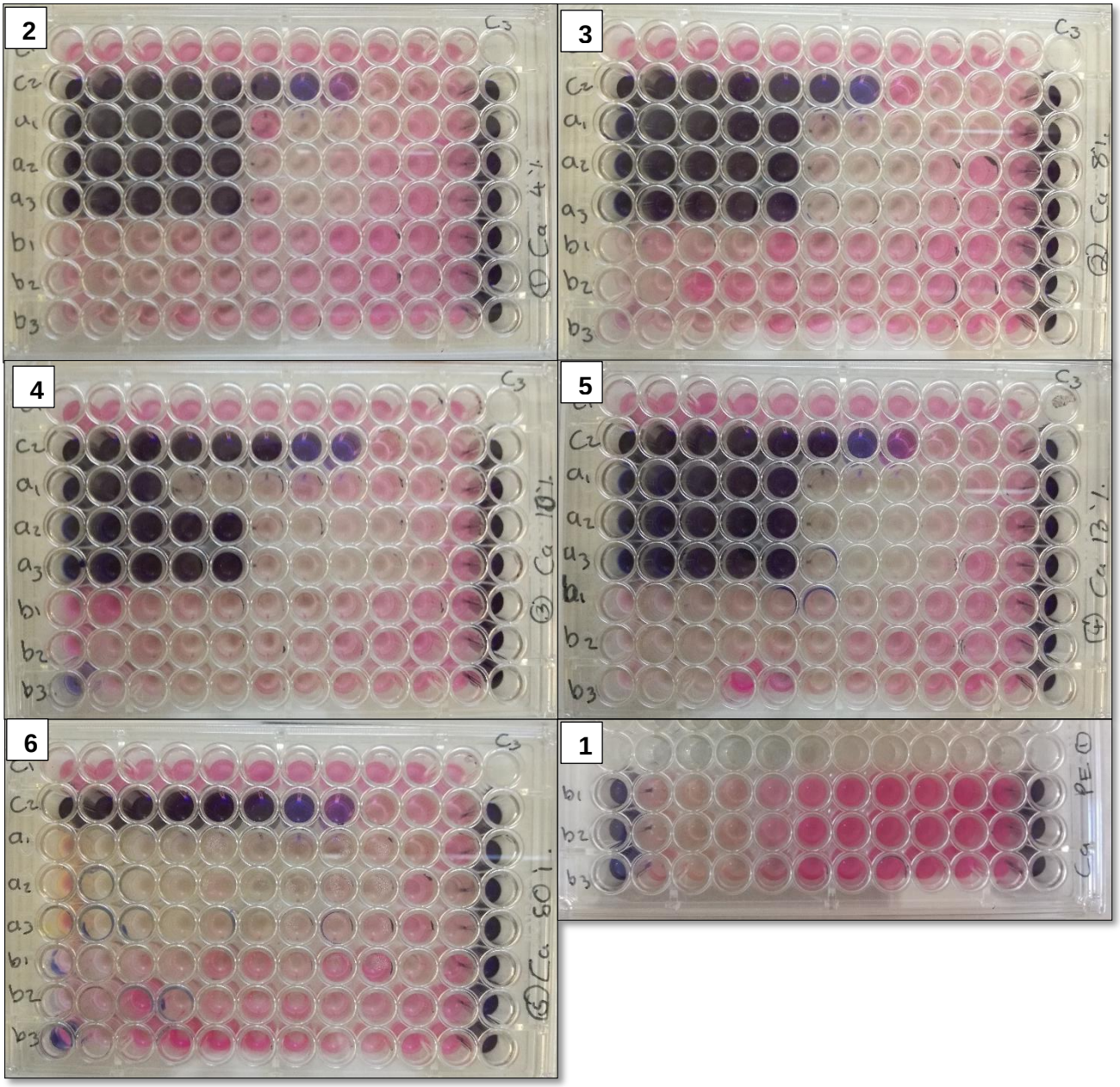

Figure 1: These images display the MIC of 1) PE, 2) PPE 4% and P 4%, 3) PPE 8% and P 8%, 4) PPE 10% and P 10%, 5) PPE 13% and P 13%, 6) pro-PPE 50% and pro-P obtained from using Resazurin as a colour indicator for *Candida albicans*.

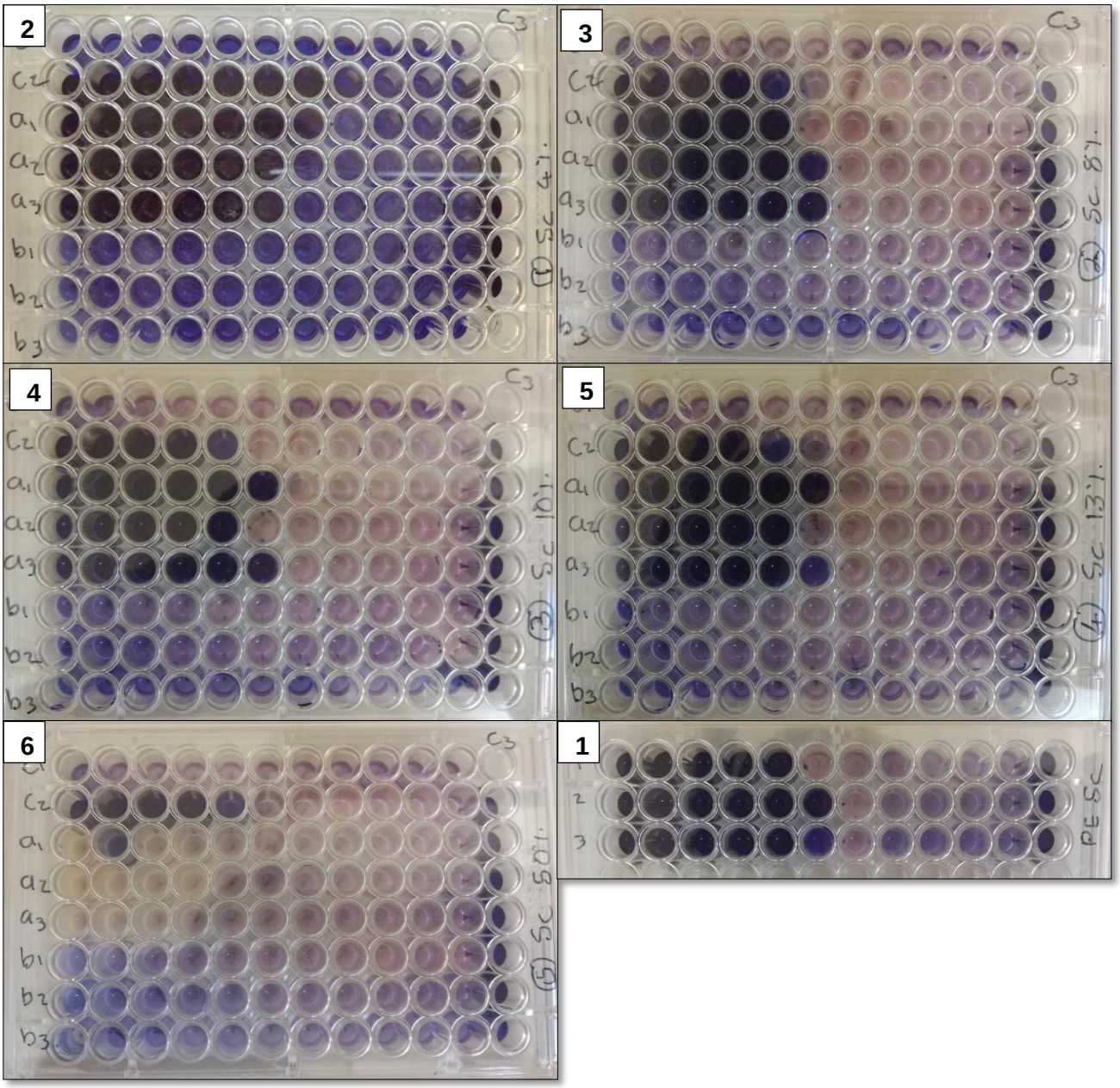

Figure 2: These images display the MIC of 1) PE, 2) PPE 4% and P 4%, 3) PPE 8% and P 8%, 4) PPE 10% and P 10%, 5) PPE 13% and P 13%, 6) pro-PPE 50% and pro-P obtained from using Resazurin as a colour indicator for *Saccharomyces cerevisiae.*

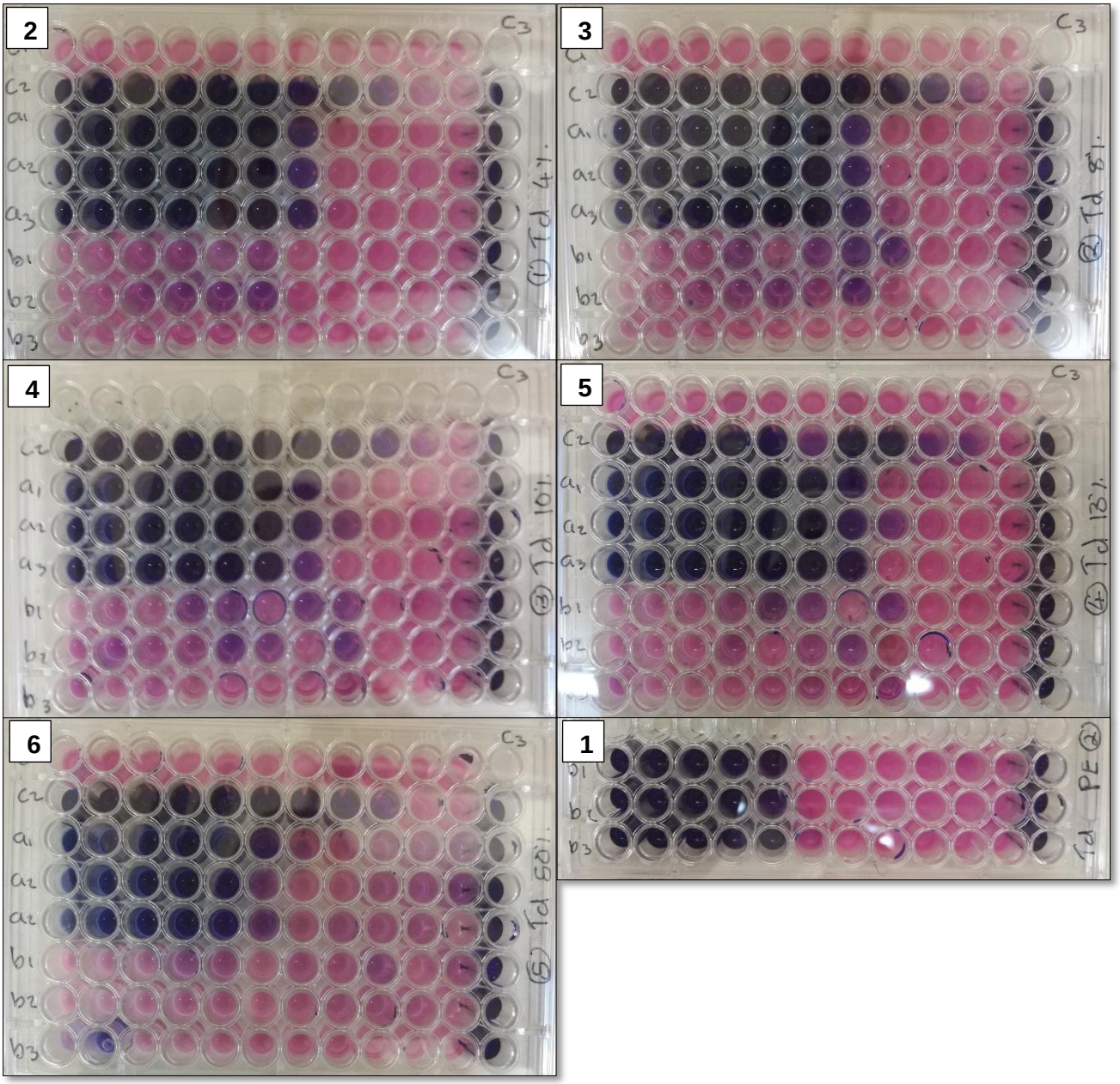

Figure 3: These images display the MIC of 1) PE, 2) PPE 4% and P 4%, 3) PPE 8% and P 8%, 4) PPE 10% and P 10%, 5) PPE 13% and P 13%, 6) pro-PPE 50% and pro-P obtained from using Resazurin as a colour indicator for *Trichosporon dermatis*.

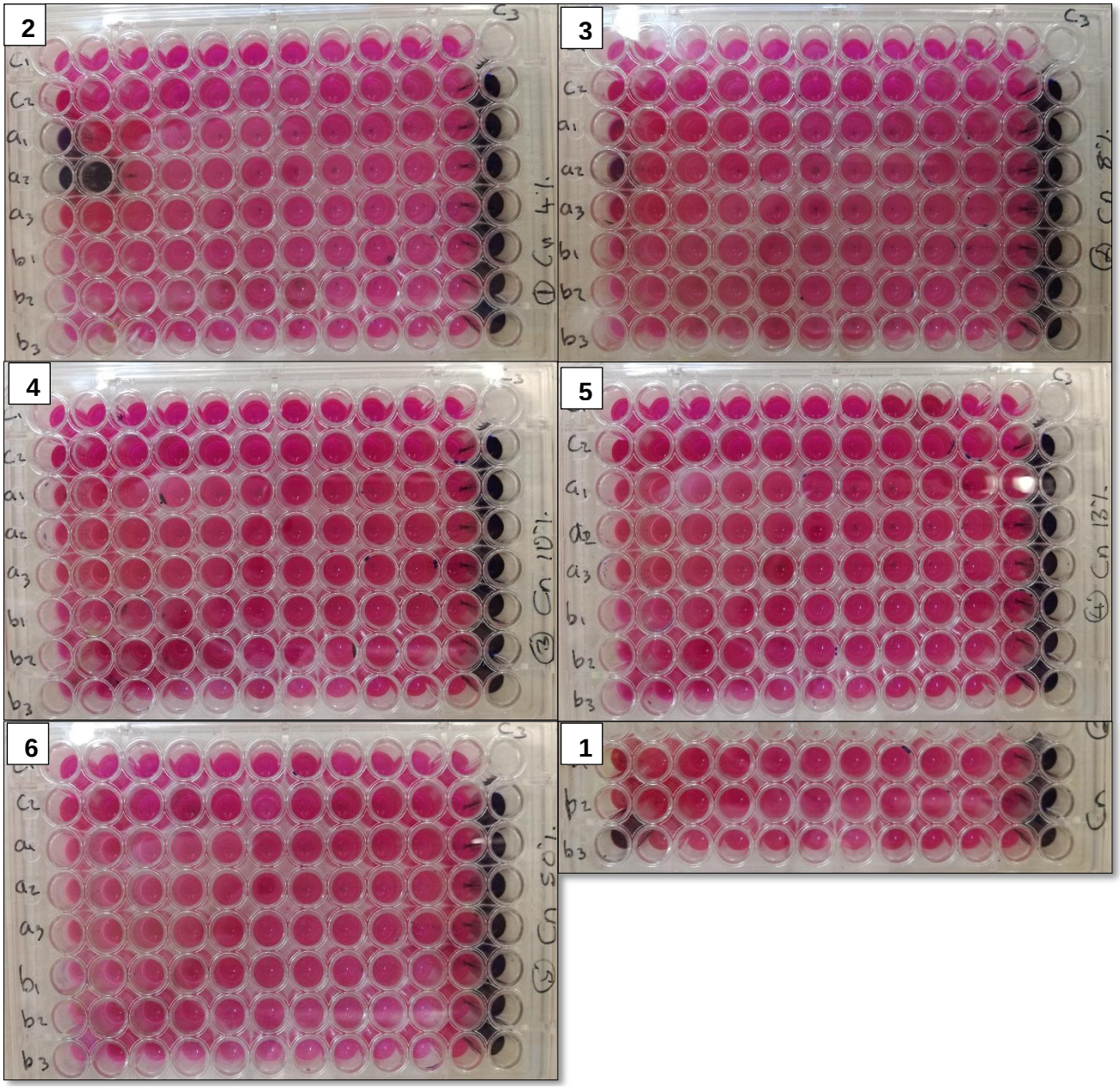

Figure 4: These images display the MIC of 1) PE, 2) PPE 4% and P 4%, 3) PPE 8% and P 8%, 4) PPE 10% and P 10%, 5) PPE 13% and P 13%, 6) pro-PPE 50% and pro-P obtained from using Resazurin as a colour indicator for *Cryptococcus neoformans*.

Annexure F: CLSM images and results of the test formulations during AST.

This is a qualitative method used to quantify a small amount of each sample and is therefore not an exact estimate of average vesicle size for the entire sample, only of these pictures presented below. As well as the particle size is the “mean” data, whereas this is the average.

Table 1: CLSM image analysis (n = 5) of the test- and control formulations during AST.

Formulation	Month 0		Month 1		Month 2		Month 3	
	Average size (µm)	Concentration (vesicles/mL $\times 10^8$)	Average size (µm)	Concentration (vesicles/mL $\times 10^8$)	Average size (µm)	Concentration (vesicles/mL $\times 10^8$)	Average size (µm)	Concentration (vesicles/mL $\times 10^8$)
PPE4%	0.636	766.1	0.699	2636.3	0.494	2082	0.440	240
P4%	0.587	377.1	0.759	2177.9	0.487	1613.5	0.621	61.2
PPE8%	1.183	116.3	0.603	1661.8	0.53	1329	0.181	10.9
P8%	0.703	555.8	0.637	1067	0.606	1182	1.862	8.87
PPE10%	1.555	232	0.753	1808.2	0.57	1549.2	1.332	10.4
P10%	0.685	566.7	0.658	755.2	0.633	925.8	0.903	21.3
PPE13%	1.239	742	0.477	1285.1	0.694	1163	0.660	92
P13%	0.718	717.4	0.655	412.3	0.566	331.5	0.874	27.7
Pro-PPE50%	0.567	1640.4	0.466	723.6	0.659	2156.5	0.471	8.97
Pro-P	0.729	1693.8	0.608	2092.3	0.561	3943.7	1.511	9.26

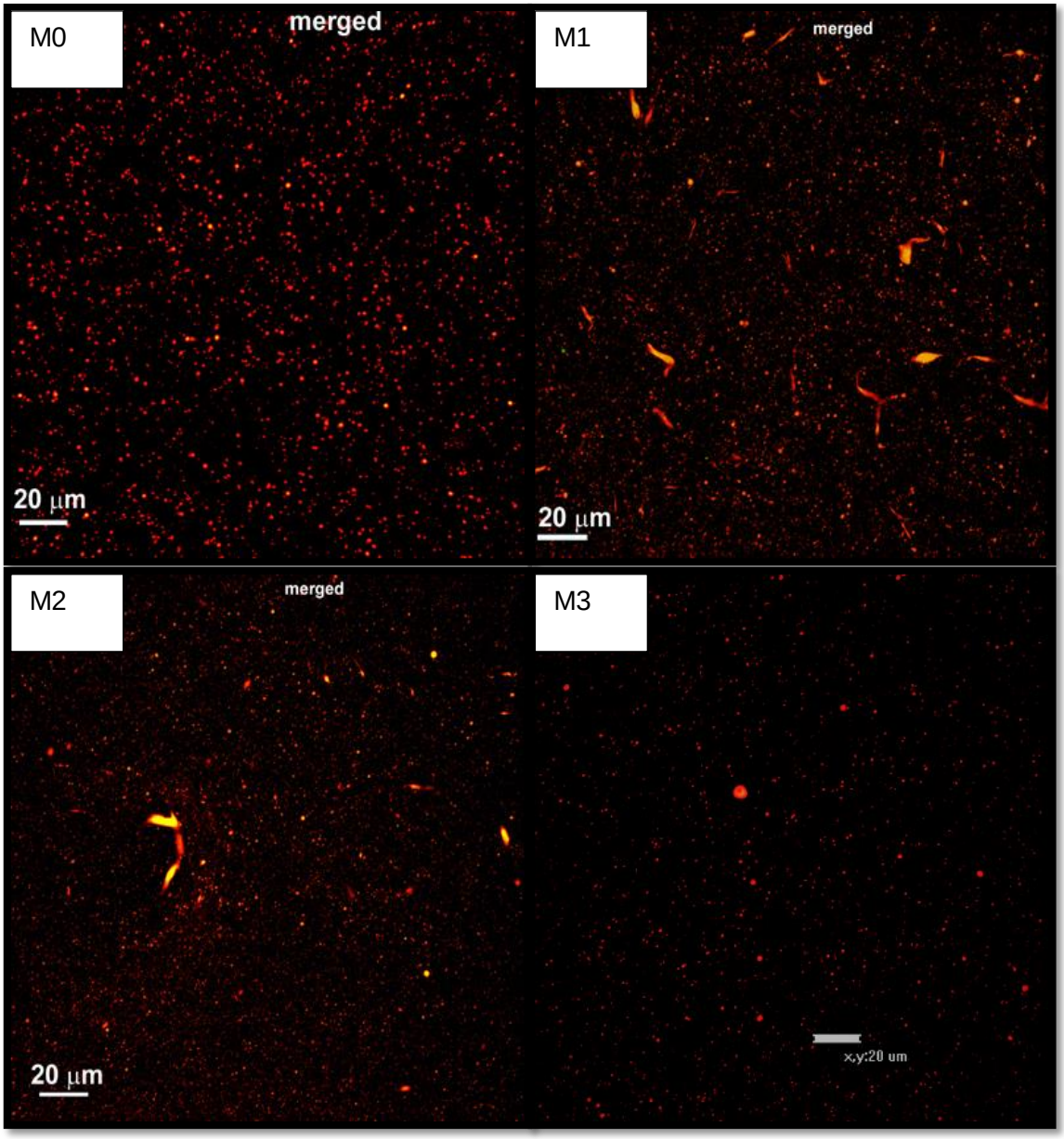

Figure 1: CLSM images of the 4% Pheroid® - Plant extract (**PPE 4%**) test formulation during AST (n = 5). The average confocal and mean particle sizes for these samples during each month were **M0** = 0.636 µm vs. 5.111 ± 0.342 µm, **M1** = 0.699 µm vs. 0.335 ± 0.009 µm, **M2** =0.494 µm vs. 0.300 ± 0.01 µm, **M3** =0.440 µm vs. 0.325 ± 0.013 µm.

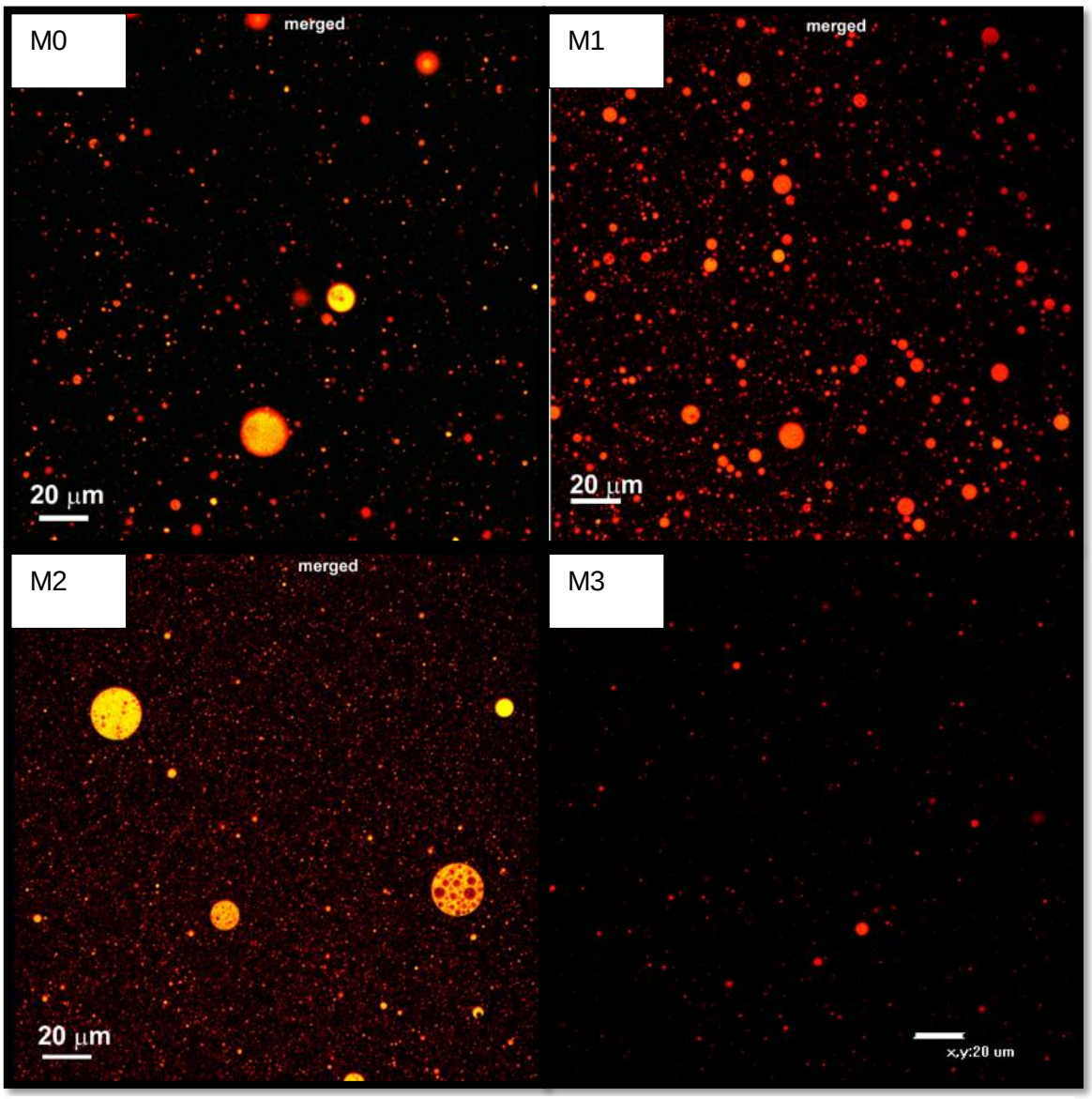

Figure 2: CLSM images of the 4% Pheroid® (**P 4%**) control formulation during the AST (n = 5). The average confocal and mean particle sizes for these samples during each month were **M0** = 0.587 µm vs. 0.305 ± 0.002 µm, **M1** =0.759 µm vs. 1.589 ± 0.086 µm, **M2** = 0.487 µm vs. 0.284 ± 0.001 µm, **M3** = 0.624 µm vs. 2.416 ± 0.135 µm.

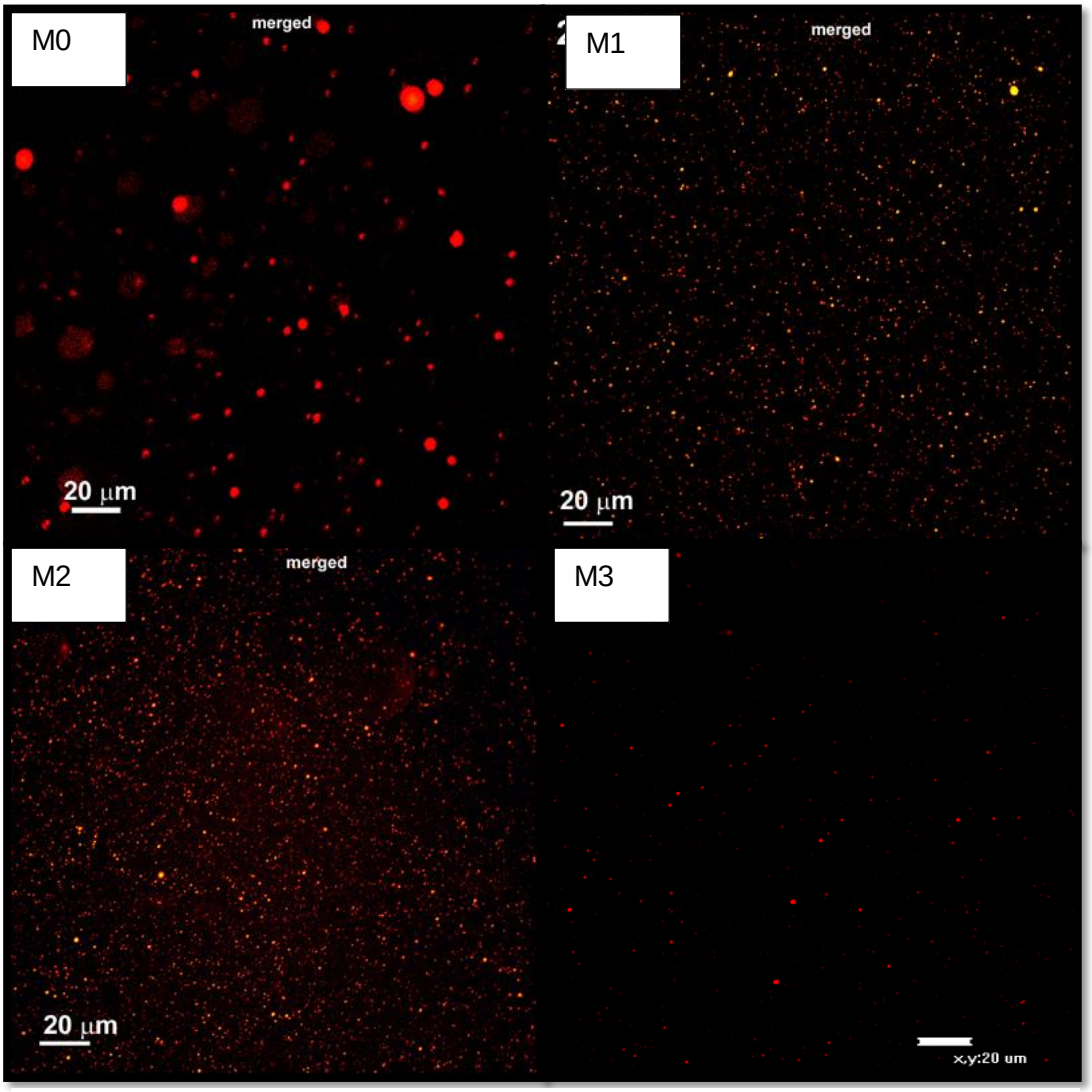

Figure 3: CLSM images of the 8% Pheroid® - Plant extract (**PPE 8%**) test formulation during AST (n = 5). The average confocal and mean particle sizes for these samples during each month were **M0** = 1.183 µm vs. 0.438 ± 0.117 µm, **M1** = 0.603 µm vs. 0.516 ± 0.009 µm, **M2** = 0.53 µm vs. 0.327 ± 0.015 µm, **M3** = 0.181 µm vs. 0.377 ± 0.012 µm.

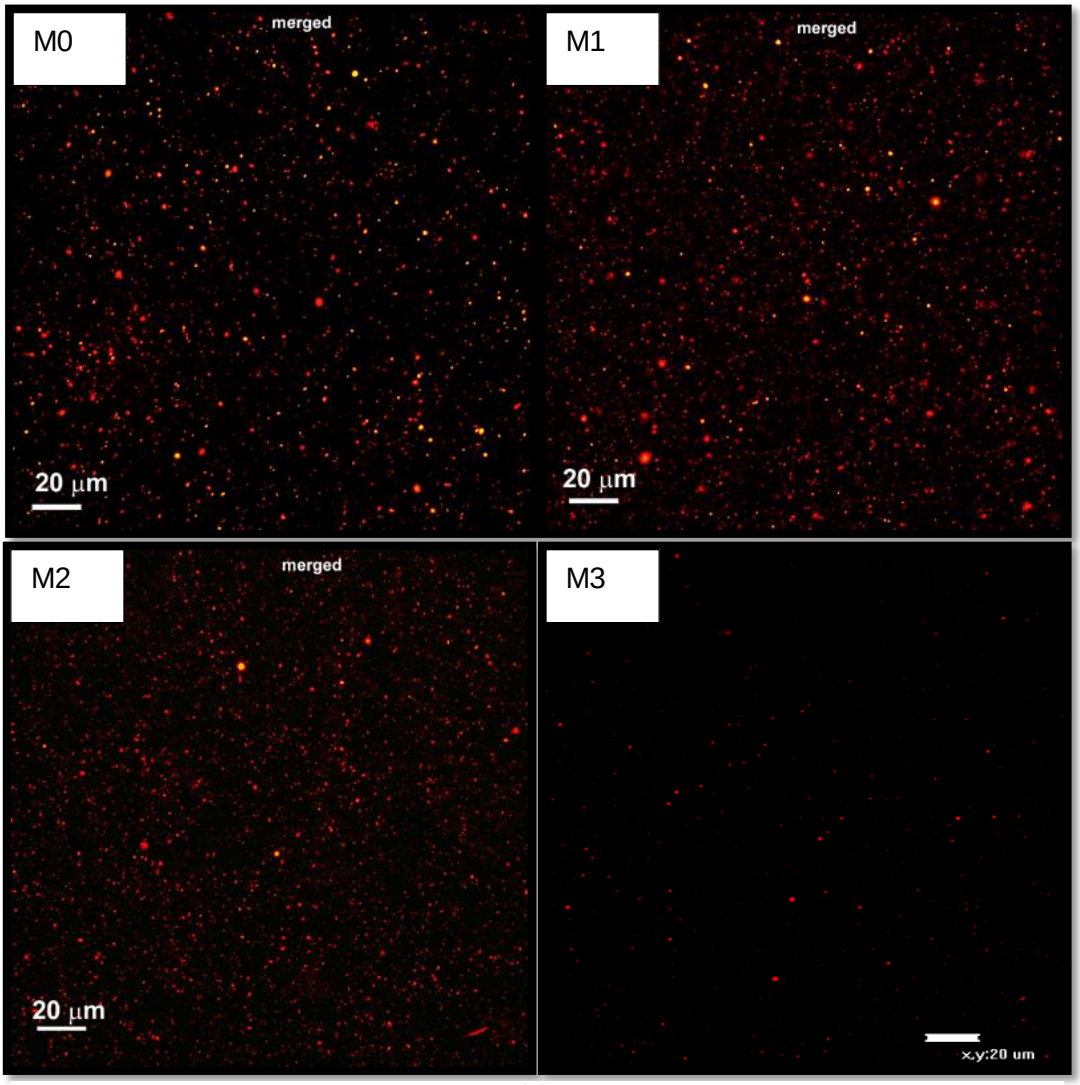

Figure 4: CLSM images of the 8% Pheroid® (**P 8%**) control formulation during AST (n = 5). The average confocal and mean particle sizes for these samples during each month were **M0** = 0.703 µm vs. 0.420 ± 0.021 µm, **M1** = 0.637 µm vs. 2.207 ± 0.206 µm, **M2** = 0.606 µm vs. 1.047 ± 0.102 µm, **M3** = 1.862 µm vs. 2.934 ± 0.223 µm.

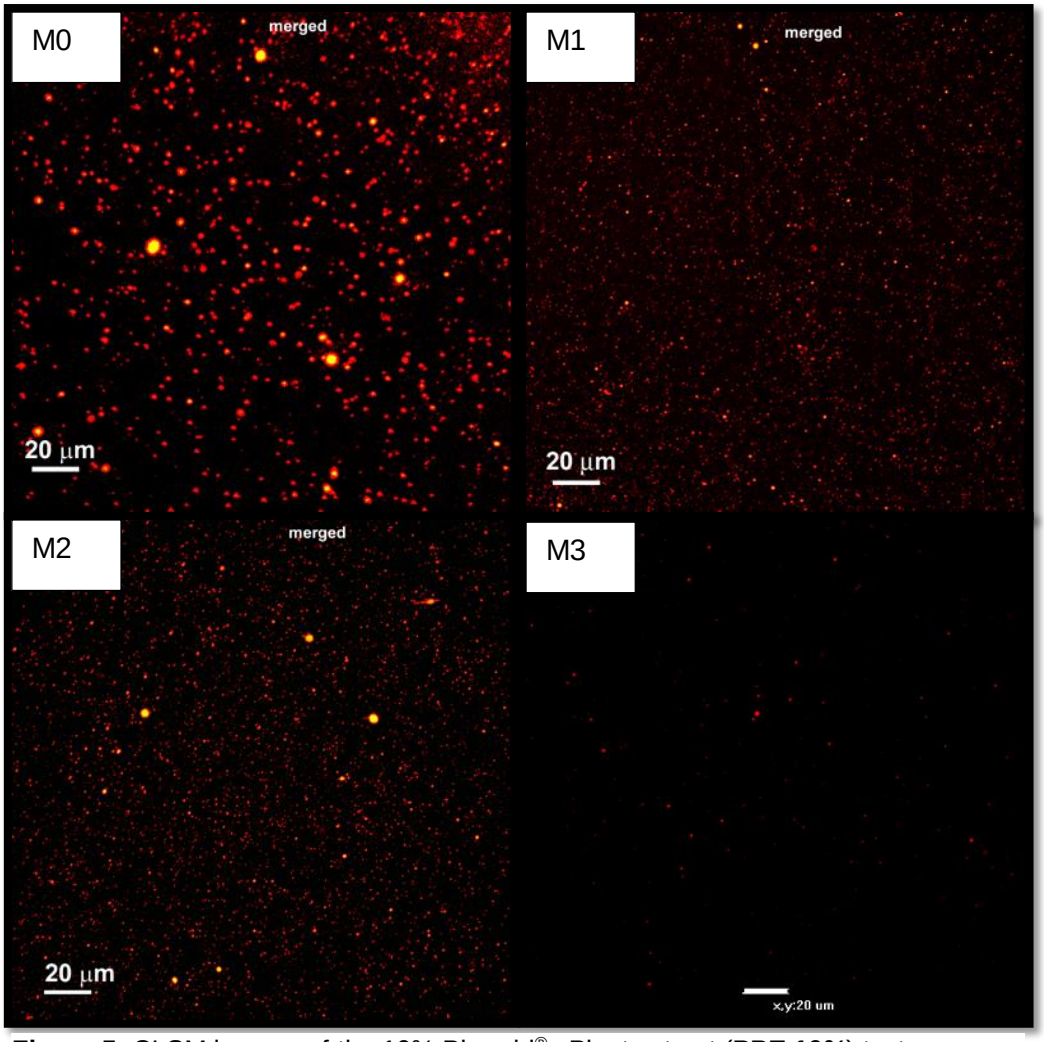

Figure 5: CLSM images of the 10% Pheroid® - Plant extract (**PPE 10%**) test formulation during AST (n = 5). The average confocal and mean particle sizes for these samples during each month were **M0** = 1.555 µm vs. 0.370 ± 0.001 µm, **M1** = 0.753 µm vs. 0.390 ± 0.002 µm, **M2** = 0.57 µm vs. 0.496 ± 0.028 µm, **M3** = 1.332 µm vs. 0.319 ± 0.031 µm.

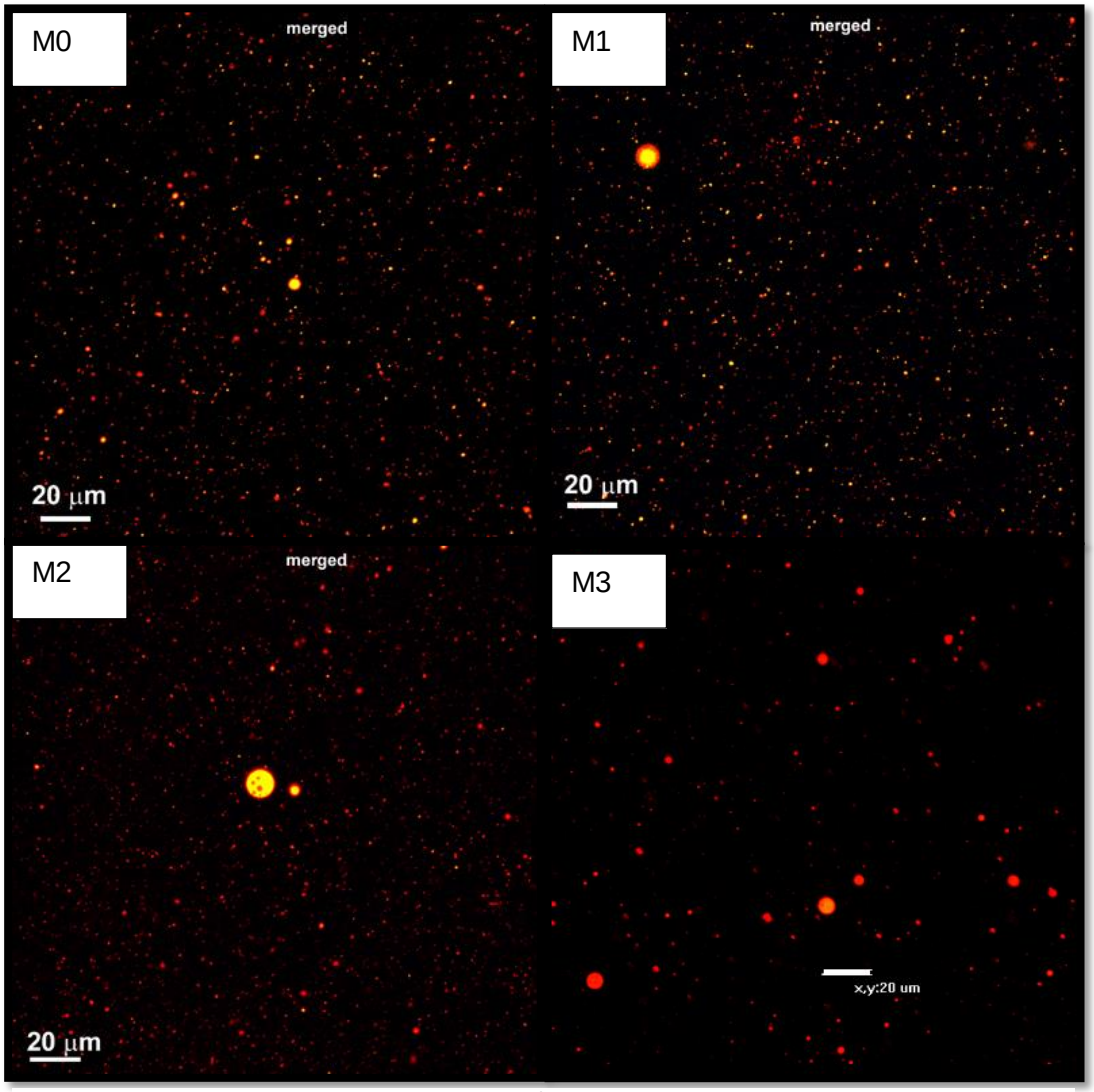

Figure 6: CLSM images of the 10% Pheroid® (**P 10%**) control formulation during AST (n = 5). The average confocal and mean particle sizes for these samples during each month were **M0** = 0.685 µm vs. 0.514 ± 0.034 µm, **M1** = 0.658 µm vs. 0.494 ± 0.014 µm, **M2** = 0.633 µm vs. 0.410 ± 0.002 µm, **M3** = 0.903 µm vs. 0.470 ± 0.002 µm.

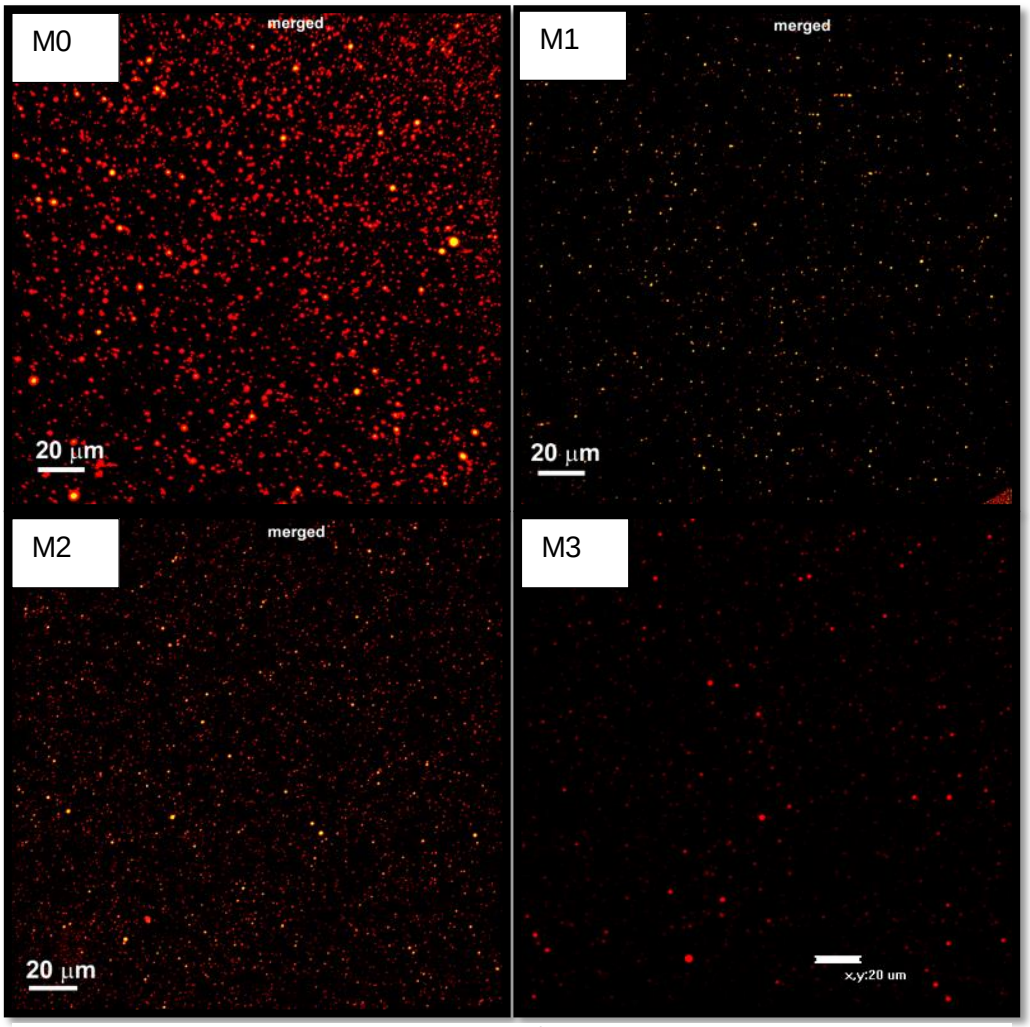

Figure 7: CLSM images of the 13% Pheroid® - Plant extract (**PPE 13%**) test formulation during AST (n = 5). The average confocal and mean particle sizes for these samples during each month were **M0** = 1.239 µm vs. 0.298 ± 0.002 µm, **M1** = 0.477 µm vs. 0.414 ± 0.004 µm, **M2** = 0.694 µm vs. 0.317 ± 0.002 µm, **M3** = 0.660 µm vs. 0.378 ± 0.003 µm.

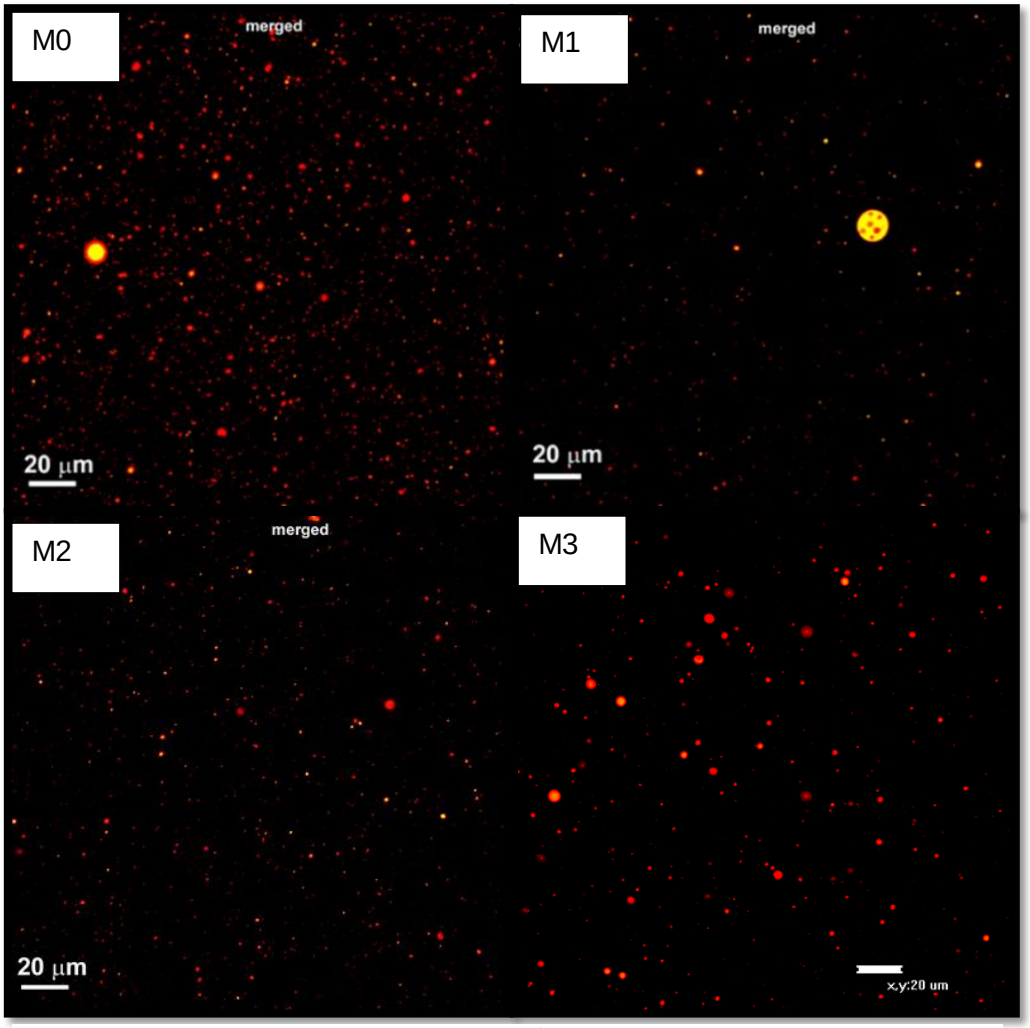

Figure 8: CLSM images of the 13% Pheroid® (**P 13%**) control formulation during the AST (n = 5). The average confocal and mean particle sizes for these samples during each month were **M0** = 0.718 µm vs. 0.619 ± 0.017 µm, **M1** = 0.655 µm vs. 0.467 ± 0.023 µm, **M2** = 0.566 µm vs. 0.470 ± 0.001 µm, **M3** = 0.874 µm vs. 0.394 ± 0.01 µm.

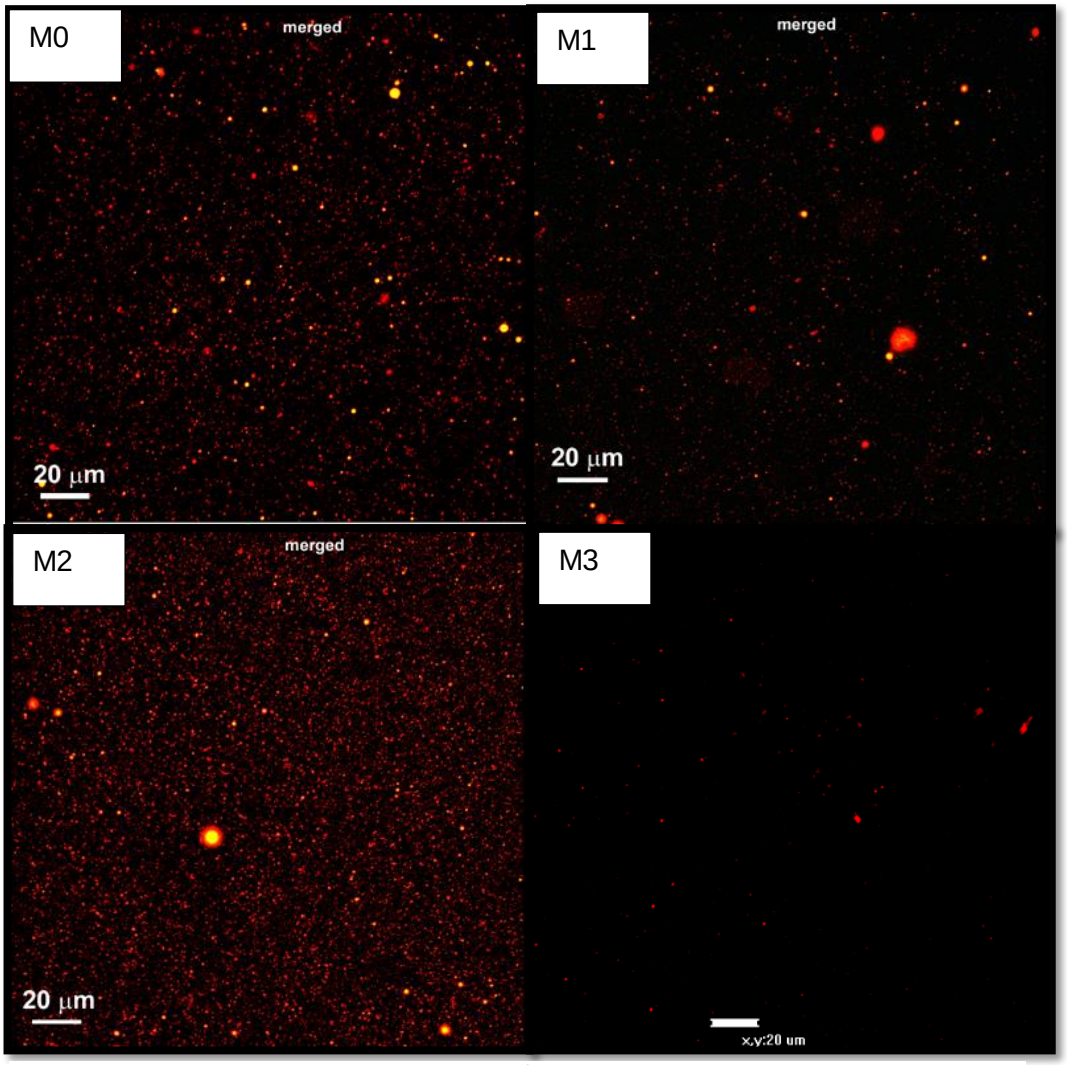

Figure 9: CLSM images of the Pro-Pheroid® Plant extract (**Pro-PPE 50%**) test formulation during AST (n = 5). The average confocal and mean particle sizes for these samples during each month were **M0** = 0.567 µm vs. 3.496 ± 0.25 µm, **M1** = 0.466 µm vs. 9.824 ± 0.72 µm, **M2** = 0.659 µm vs. 0.401 ± 0.036 µm, **M3** = 0.471 µm vs. 60.123 ± 16.408 µm.

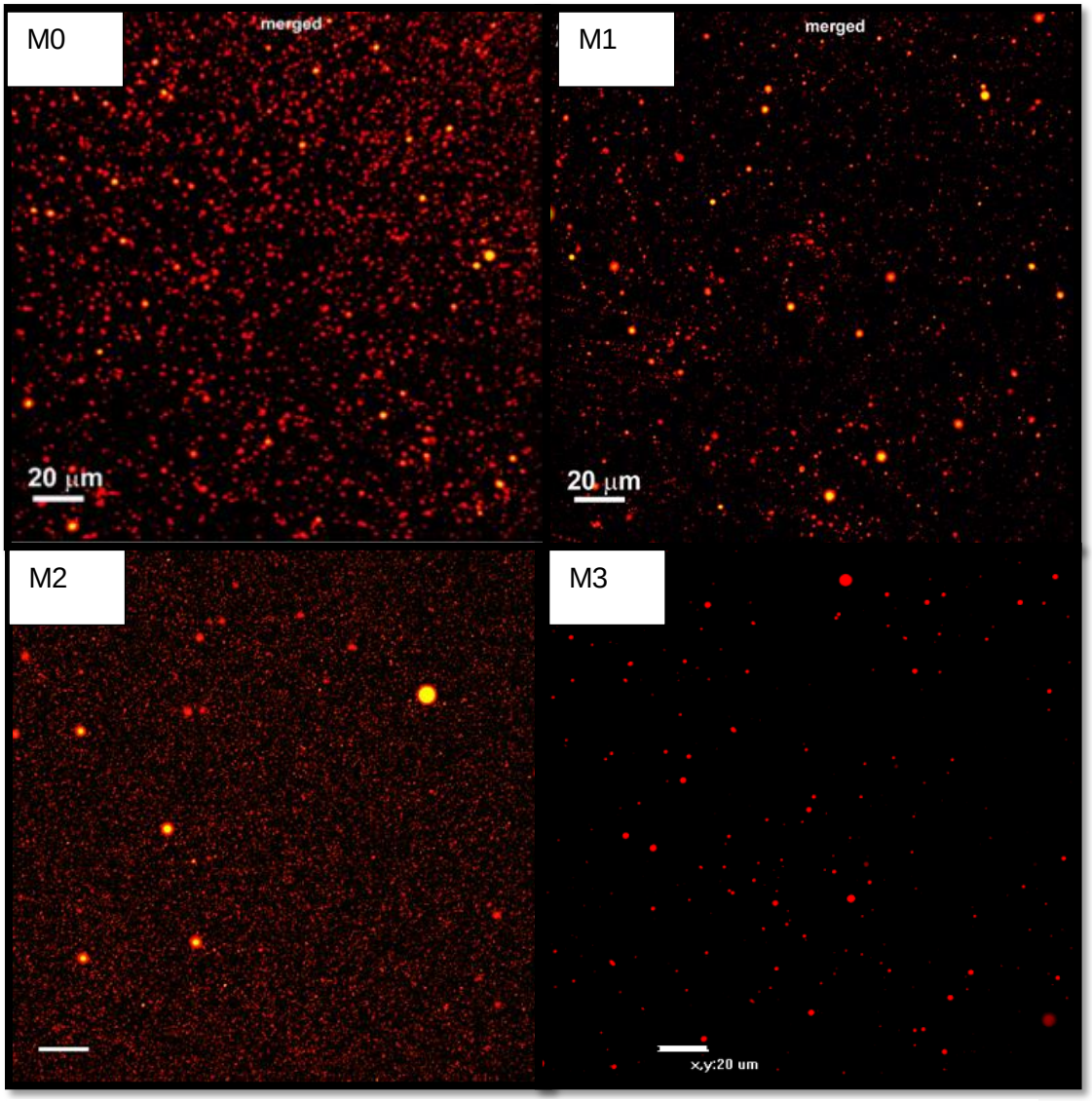

Figure 10: CLSM images of the Pro-Pheroid® (Pro-P) control formulation during AST (n = 5). The average confocal and mean particle sizes for these samples during each month were **M0** = 0.729 µm vs. 0.157 ± 0.001 µm, **M1** = 0.608 µm vs. 3.436 ± 0.444 µm, **M2** = 0.561 µm vs. 0.137 ± 0.001 µm, **M3** = 1.511 µm vs. 15.832 ± 21.696 µm.

Annexure G: CLSM images of the test formulations during Resazurin susceptibility tests

This is a qualitative method used to quantify a small amount of each sample and is therefore not an exact estimate of average vesicle size for the entire sample, only of these pictures presented below. As well as the particle size is the “mean” data, whereas this is the average.

Table 1: Summary of the CLSM image analysis (n = 5) with ImageJ for the formulations used during the Resazurin testing.

Formulation	Average size (µm)	Concentration (vesicles/mL ×10^8)
PPE4%	0.140	278.4
P4%	0.126	199.8
PPE8%	0.159	71.6
P8%	0.150	149.4
PPE10%	0.151	290.1
P10%	0.161	41
PPE13%	0.137	523.8
P13%	0.140	182.6
Pro-PPE50%	0.130	657.7
Pro-P	0.140	313.9

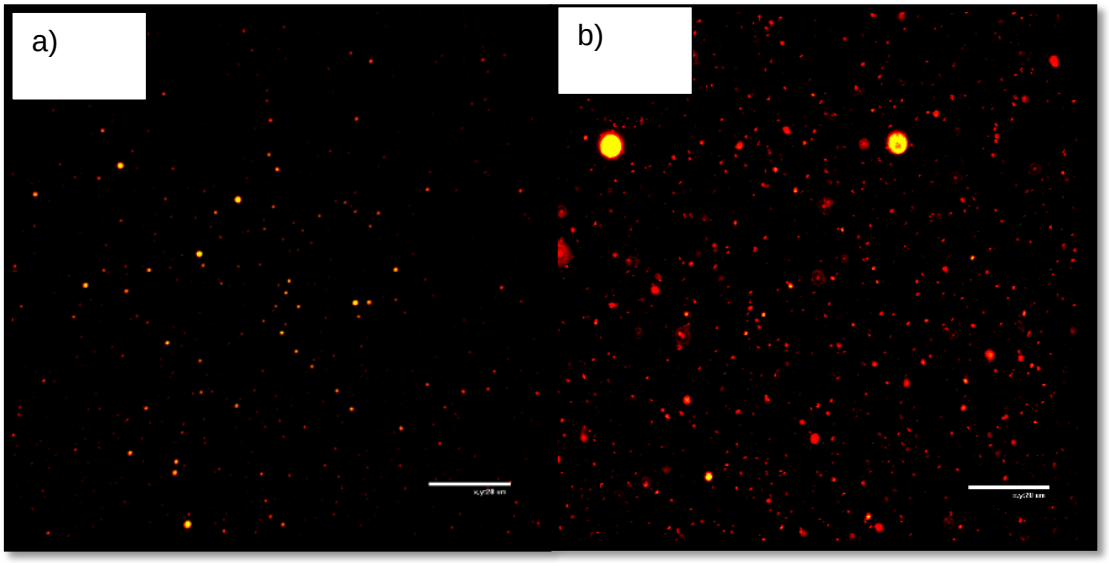

Figure 1: CLSM images of **a)** 4% Pheroid®-Plant extract (0.252 ± 0.001 µm vs. 0.140 µm) and **b)** 4% Pheroid® (0.285 ± 0.015 µm vs. 0.126 µm) used during the resazurin antimicrobial susceptibility tests. The values presented in brackets are the mean particle sizes vs. average vesicle size.

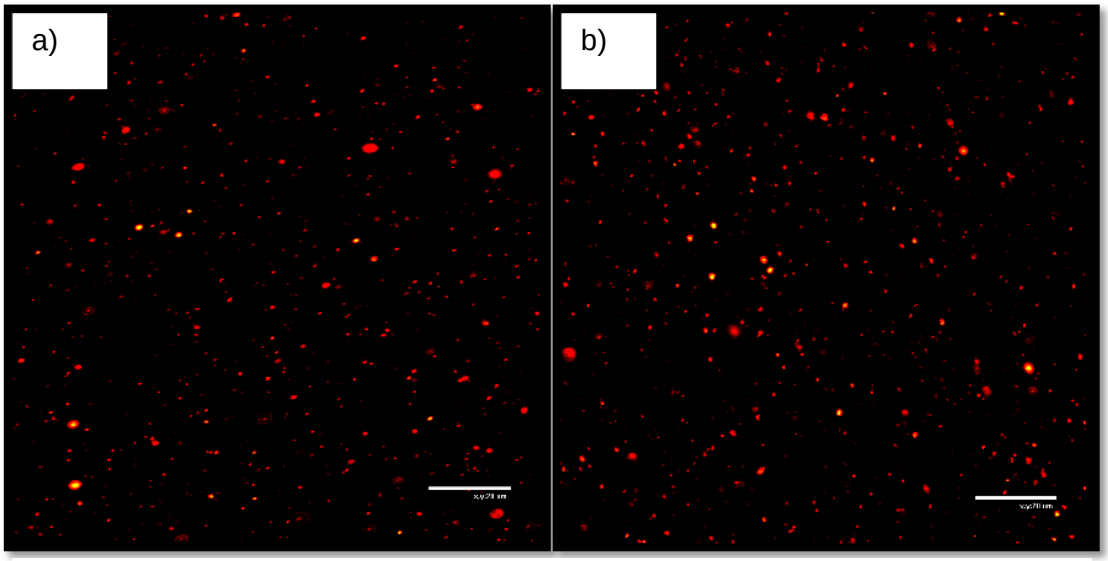

Figure 2: CLSM images of **a)** 8% Pheroid®-Plant extract (0.239 ± 0.002 µm vs. 0.159 µm) and **b)** 8% Pheroid® (0.293 ± 0.001 µm vs. 0.150 µm) used during the resazurin antimicrobial susceptibility tests. The values presented in brackets are the mean particle sizes vs. average vesicle size.

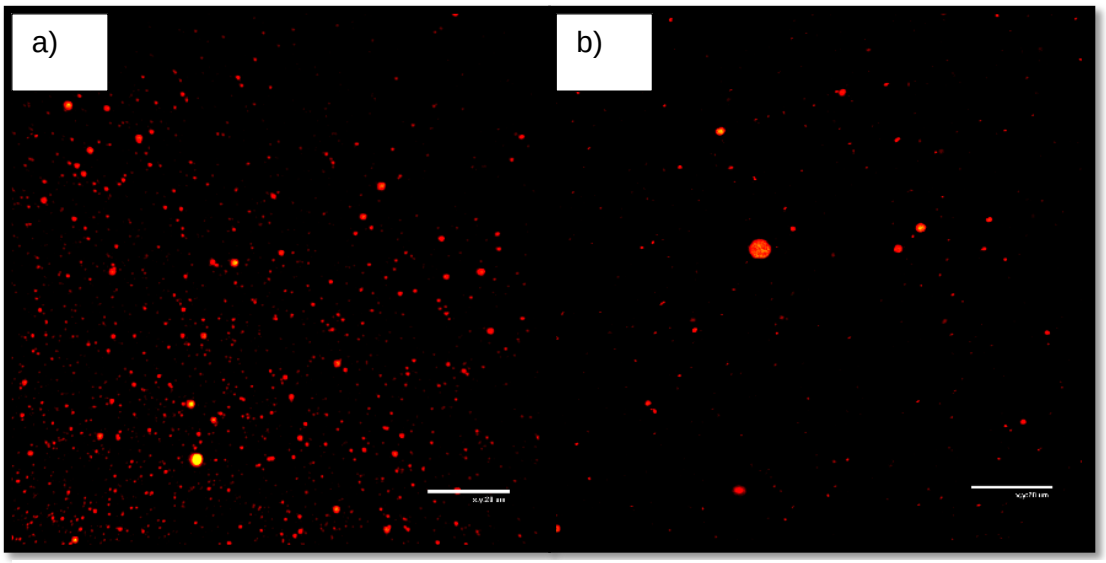

Figure 3: CLSM images of **a)** 10% Pheroid®-Plant extract (0.215 ± 0.013 µm vs. 0.151 µm) and **b)** 10% Pheroid® (0.292 ± 0.005 µm vs. 0.161 µm) used during the resazurin antimicrobial susceptibility tests. The values presented in brackets are the mean particle sizes vs. average vesicle size.

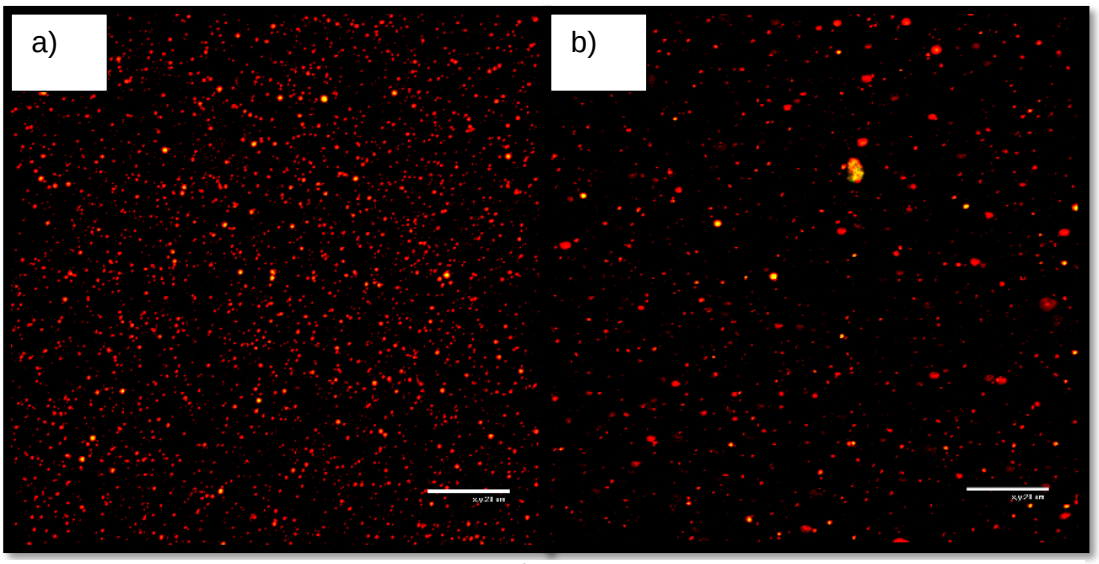

Figure 4: CLSM images of **a)** 13% Pheroid®-Plant extract (0.223 ± 0.003 µm vs. 0.137 µm) and **b)** 13% Pheroid® (0.229 ± 0.003 µm vs. 0.140 µm) used during the resazurin antimicrobial susceptibility tests. The values presented in brackets are the mean particle sizes vs. average vesicle size.

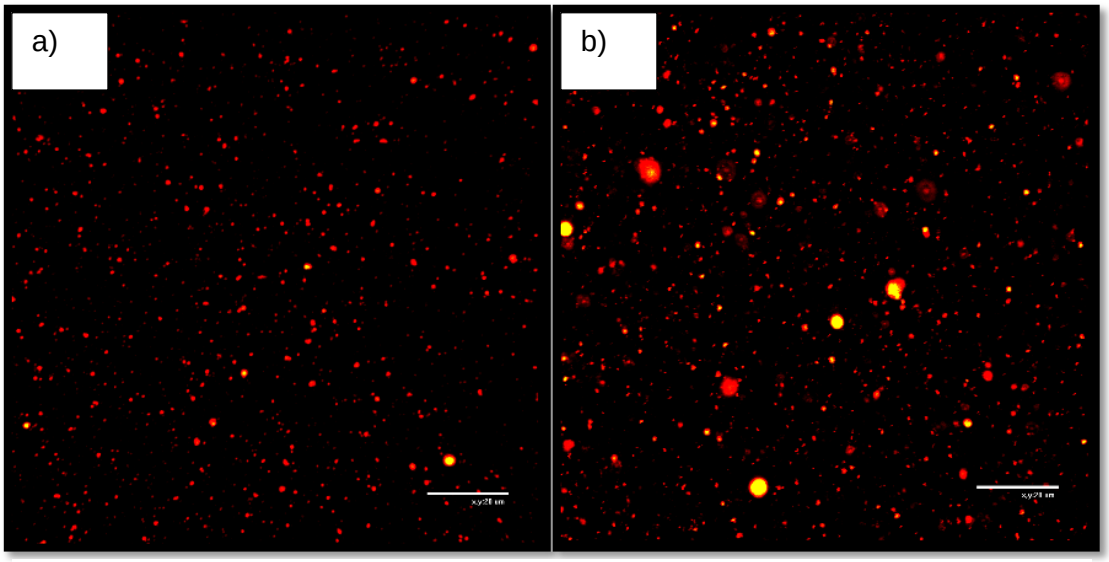

Figure 5: CLSM images of **a)** 50% Pro-Pheroid®-Plant extract (0.282 ± 0.024 µm vs. 0.130 µm) and **b)** Pro-Pheroid® (0.367 ± 0.059 µm vs. 0.140 µm) used during the resazurin antimicrobial susceptibility tests. The values presented in brackets are the mean particle sizes vs. average vesicle size.

Annexure H: Particle size distribution graphs for each formulation and batch.

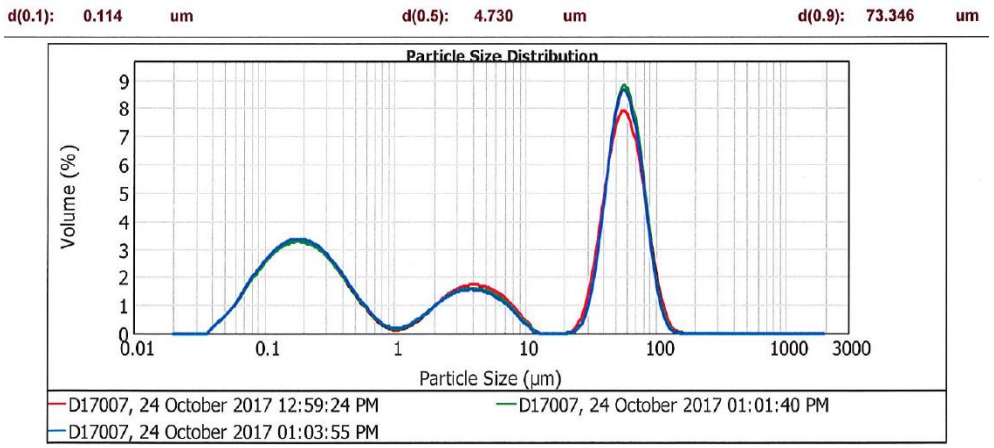

Figure 6: Particle size distribution for **PPE 4%** month 0 (batch 1).

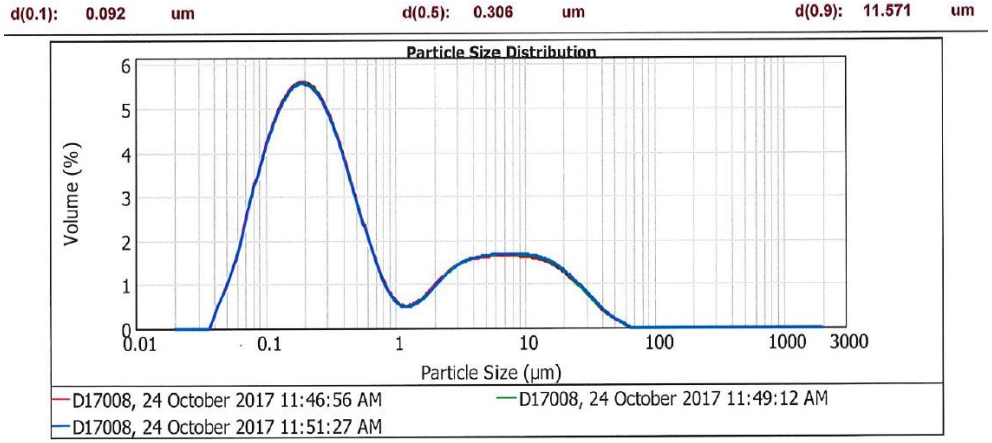

Figure 5: Particle size distribution for **P 4%** month 0 (batch 1).

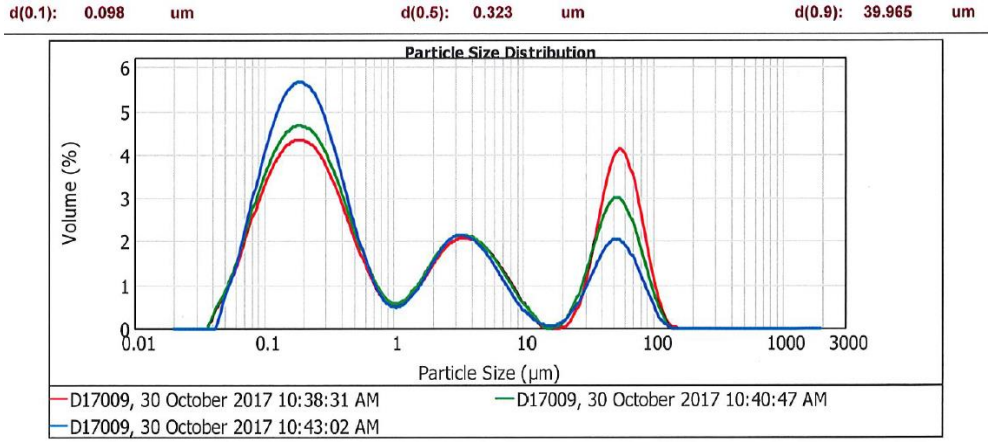

Figure 8: Particle size distribution for **PPE 8%** month 0 (batch 1).

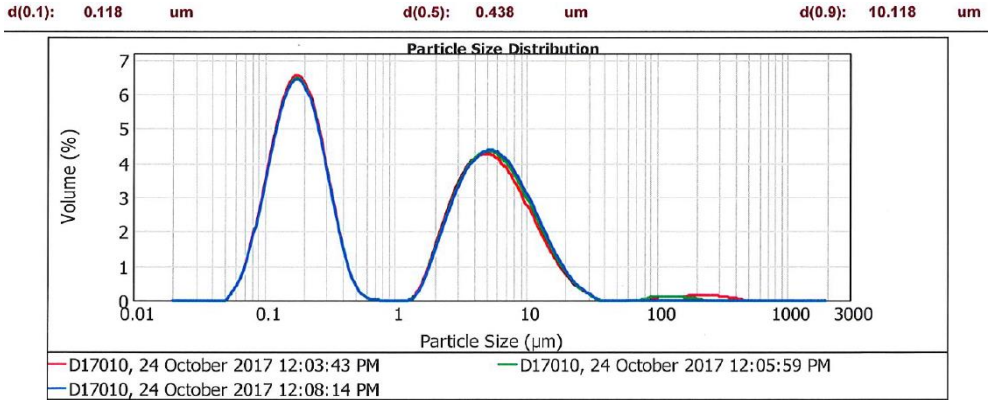

Figure 7: Particle size distribution for **P 8%** month 0 (batch 1).

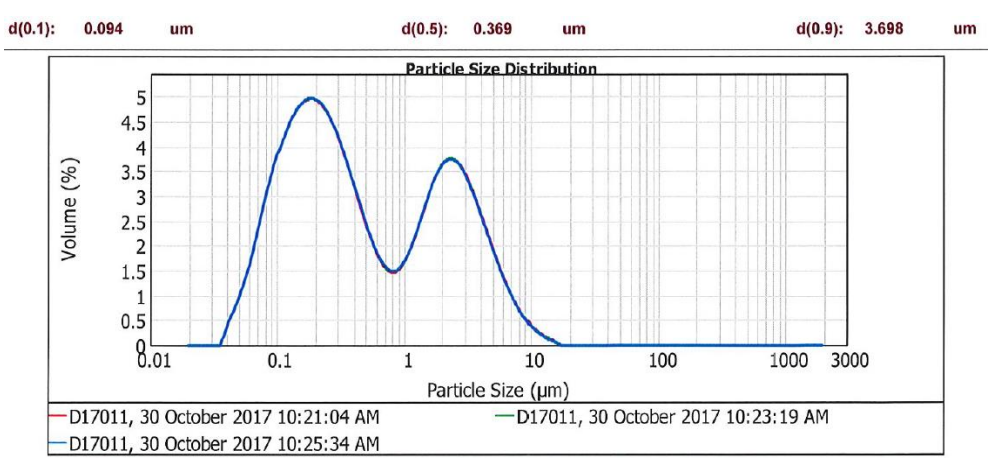

Figure 12: Particle size distribution for **PPE 10%** month 0 (batch 1).

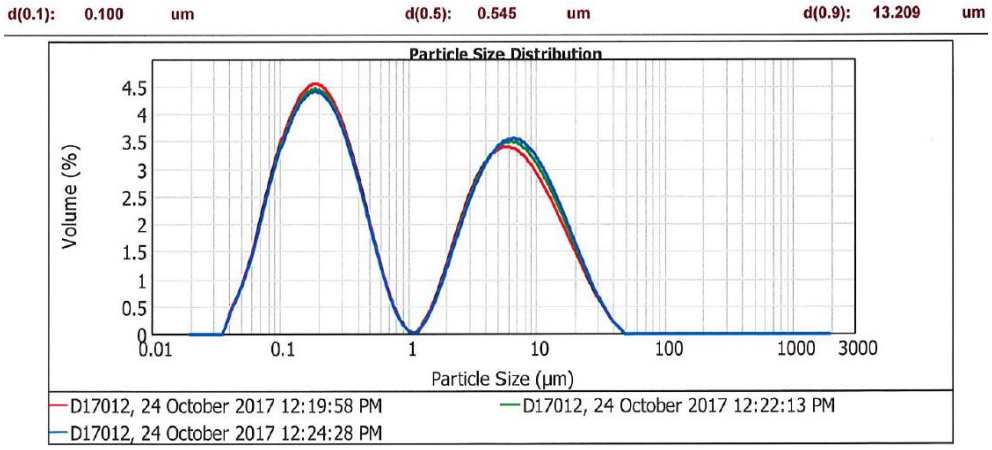

Figure 11: Particle size distribution for **P 8%** month 0 (batch 1).

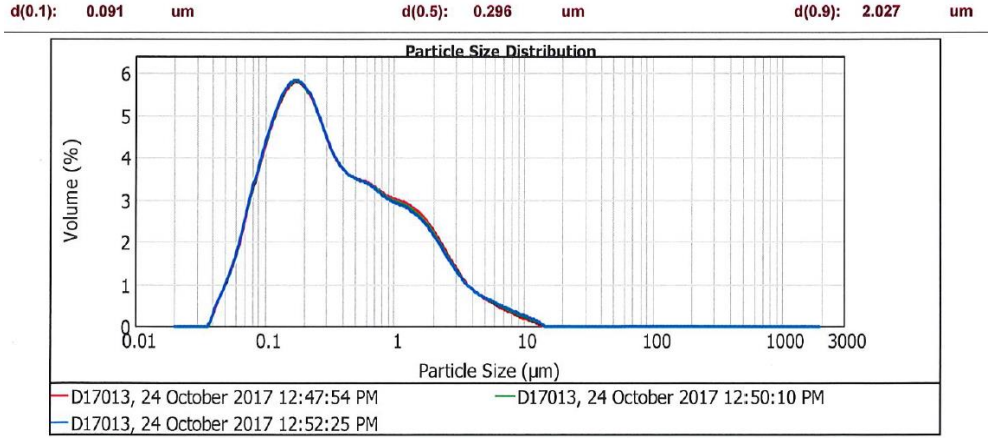

Figure 9: Particle size distribution for **PPE 13%** month 0 (batch 1).

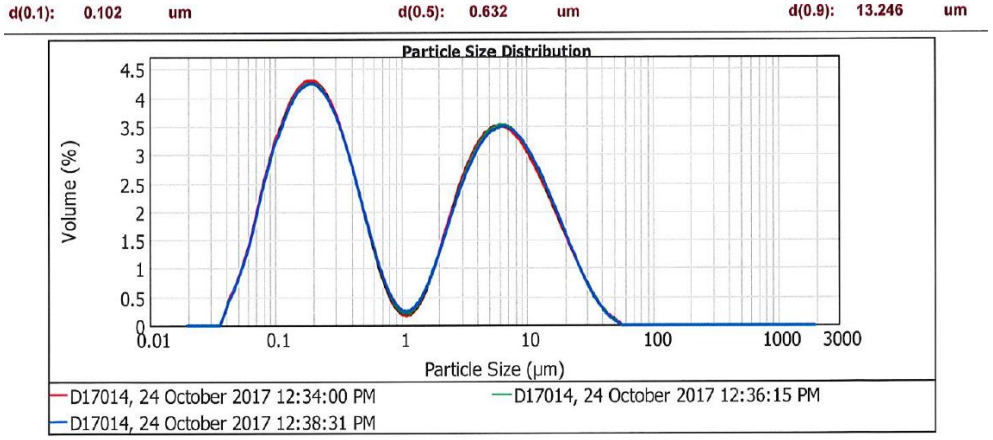

Figure 10: Particle size distribution for **P 13%** month 0 (batch 1).

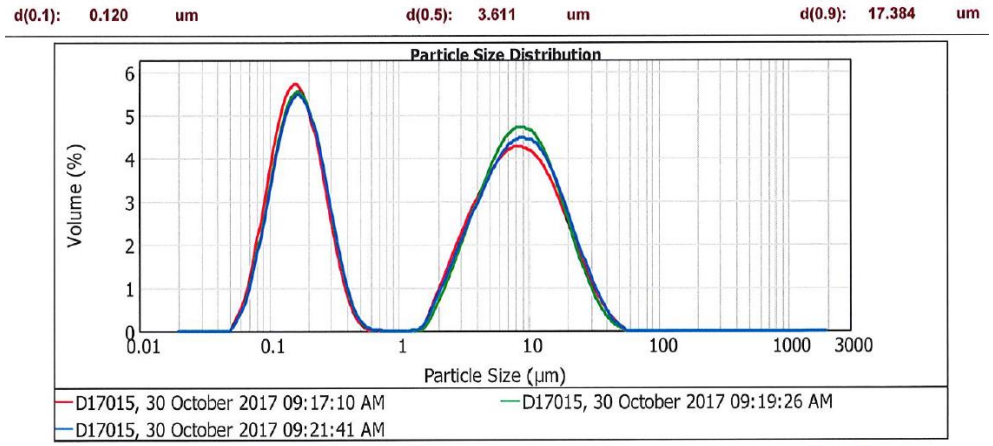

Figure 14: Particle size distribution for **Pro-PPE 50%** month 0 (batch 1).

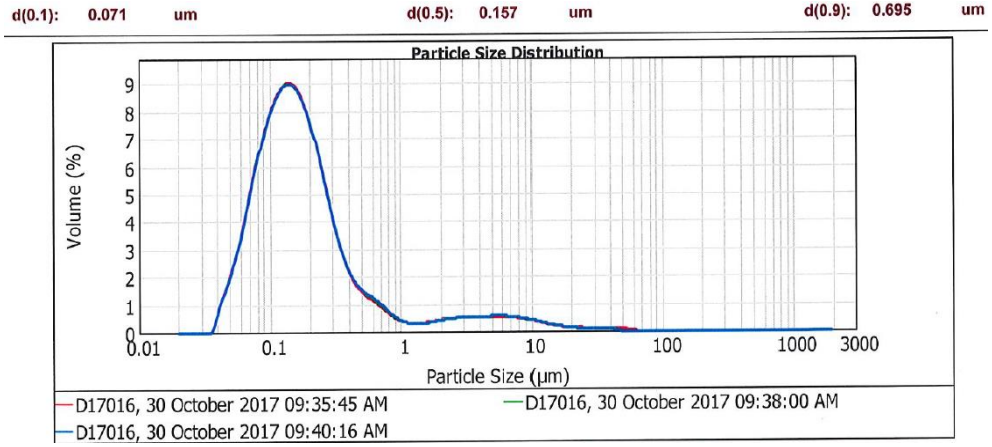

Figure 13: Particle size distribution for **Pro-P** month 0 (batch 1).

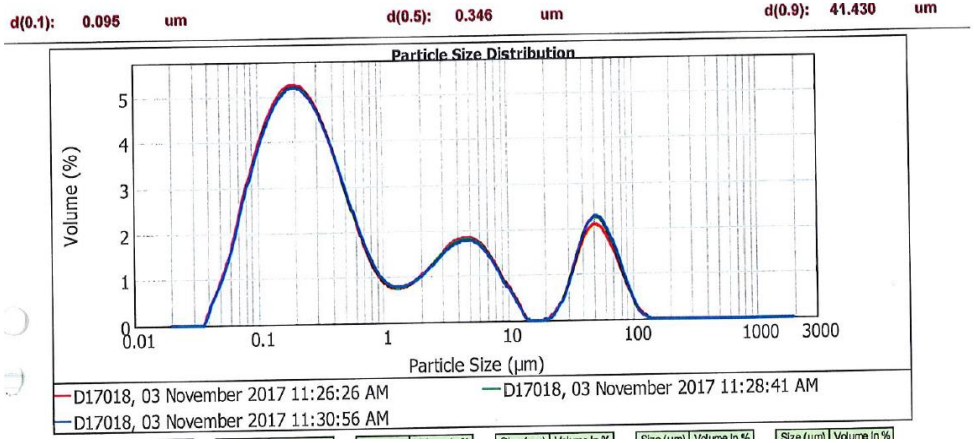

Figure 16: Particle size distribution for **PPE 4%** month 0 (batch 2).

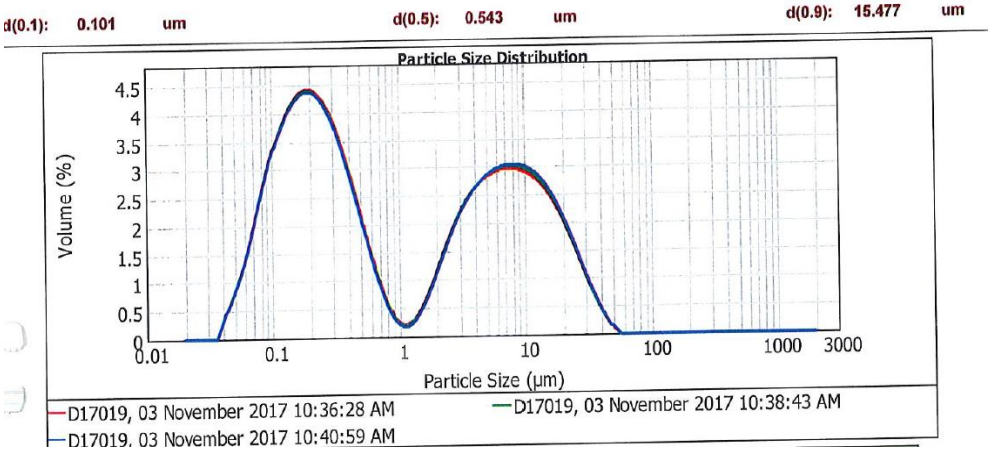

Figure 15: Particle size distribution for **P 4%** month 0 (batch 2).

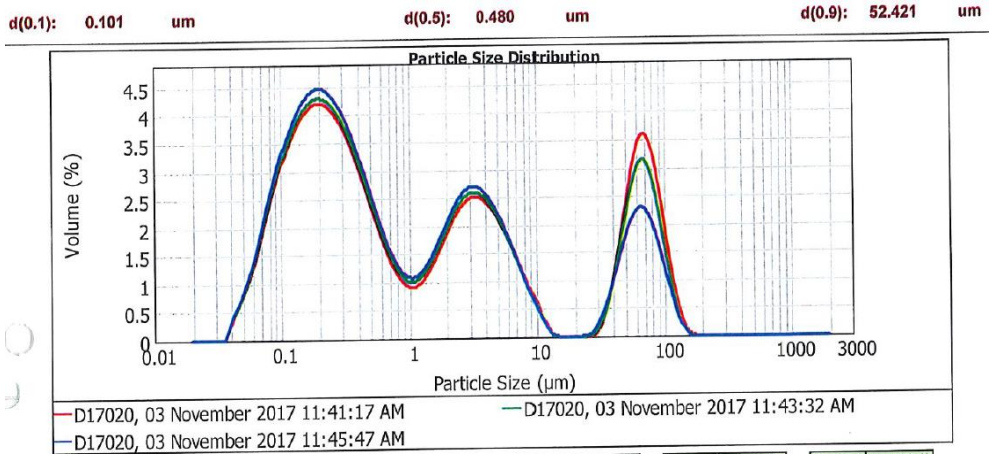

Figure 13: Particle size distribution for **PPE 8%** month 0 (batch 2).

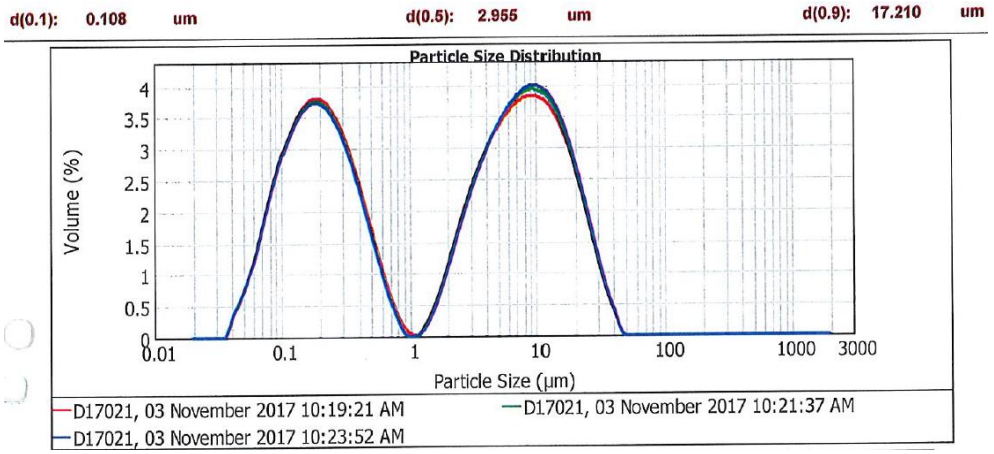

Figure 18: Particle size distribution for **P 8%** month 0 (batch 2).

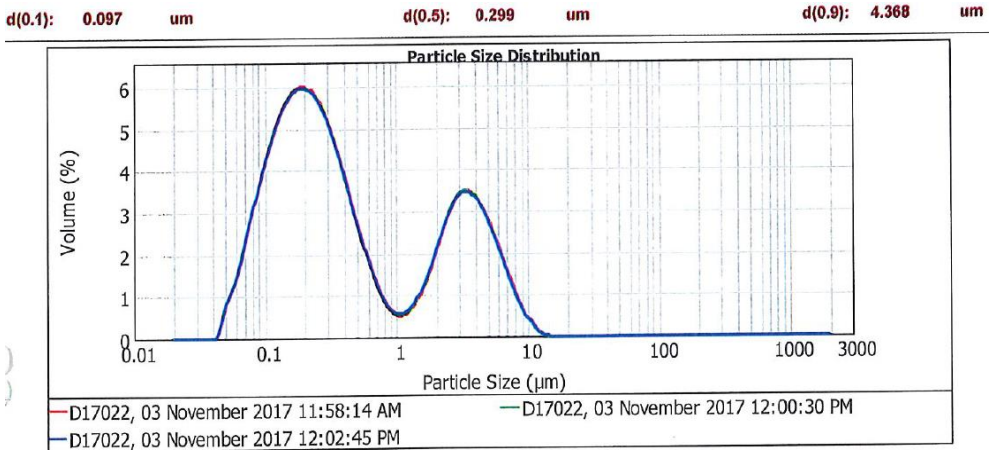

Figure 15: Particle size distribution for **PPE 10%** month 0 (batch 2).

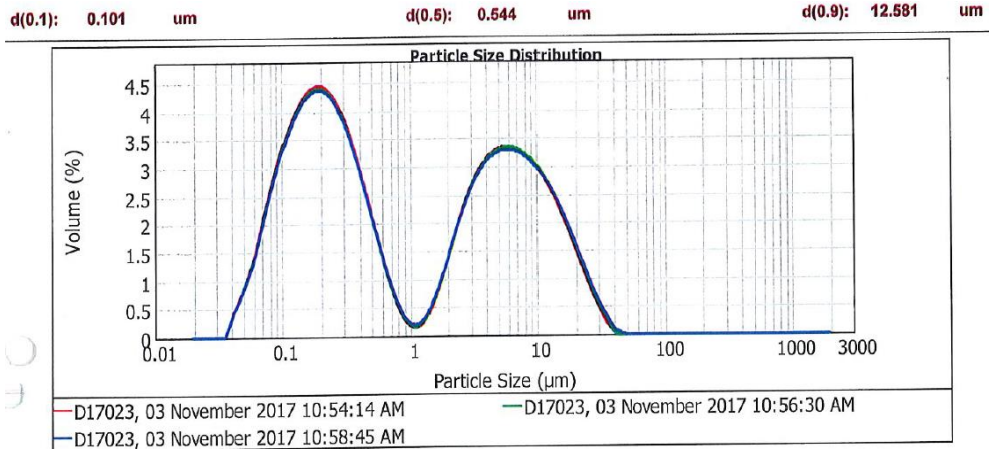

Figure 1617: Particle size distribution for **P 10%** month 0 (batch 2).

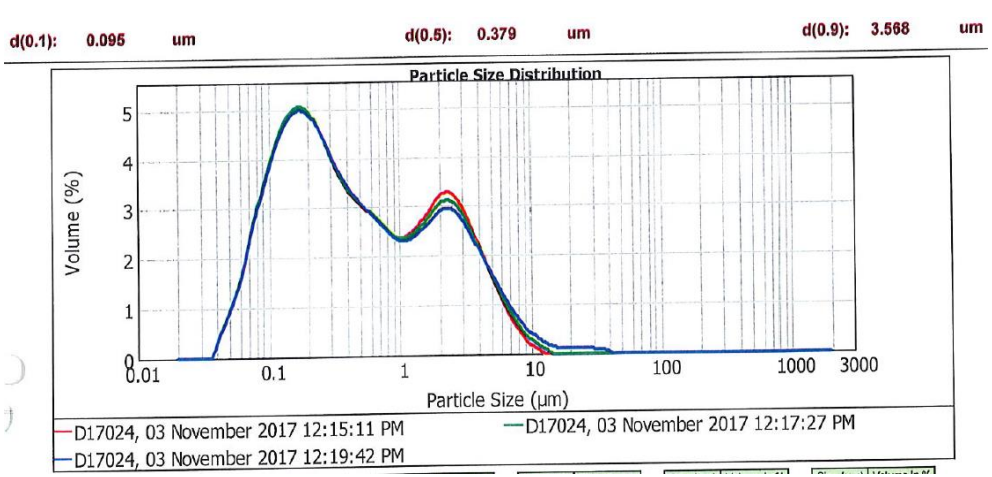

Figure 17: Particle size distribution for **PPE 13%** month 0 (batch 2).

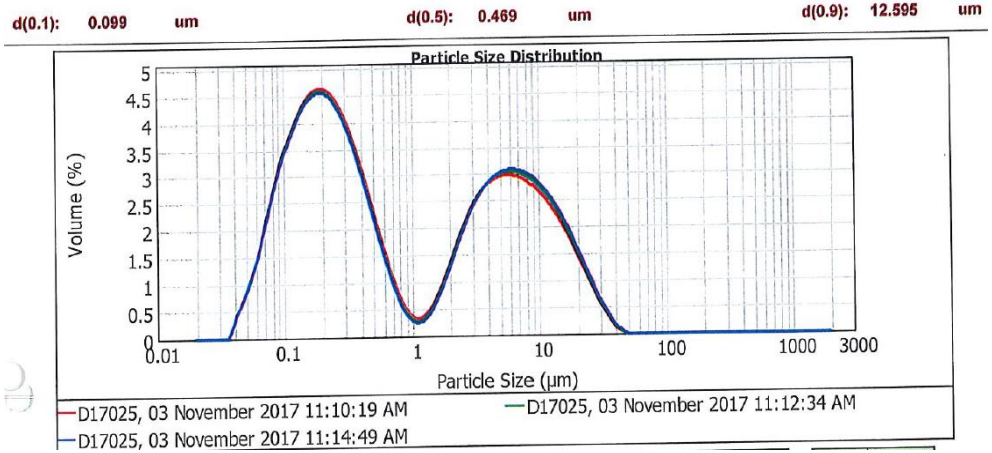

Figure 18: Particle size distribution for **P 13%** month 0 (batch 2).

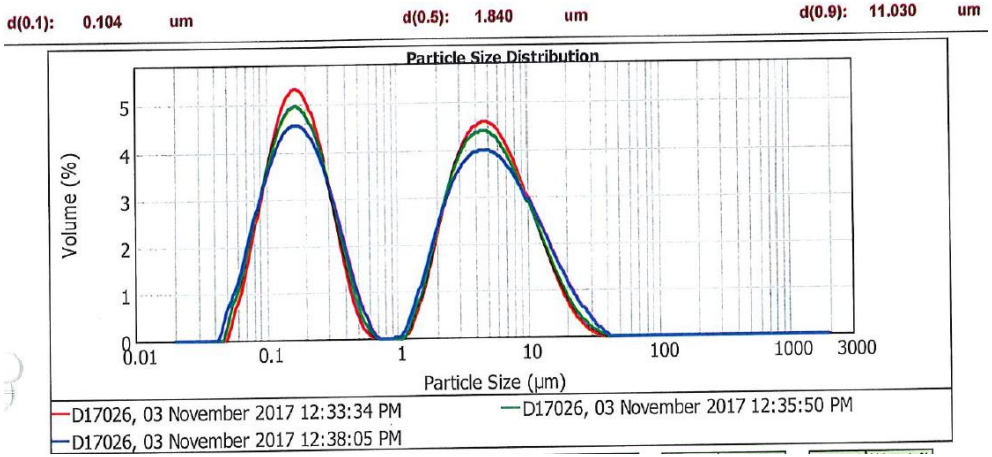

Figure 19: Particle size distribution for **Pro-PPE 50%** month 0 (batch 2).

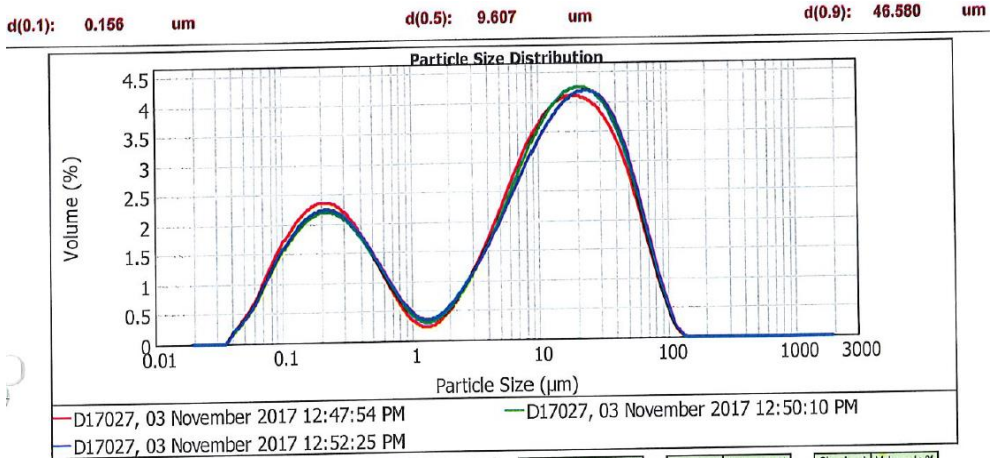

Figure 20: Particle size distribution for **Pro-P** month 0 (batch 2).

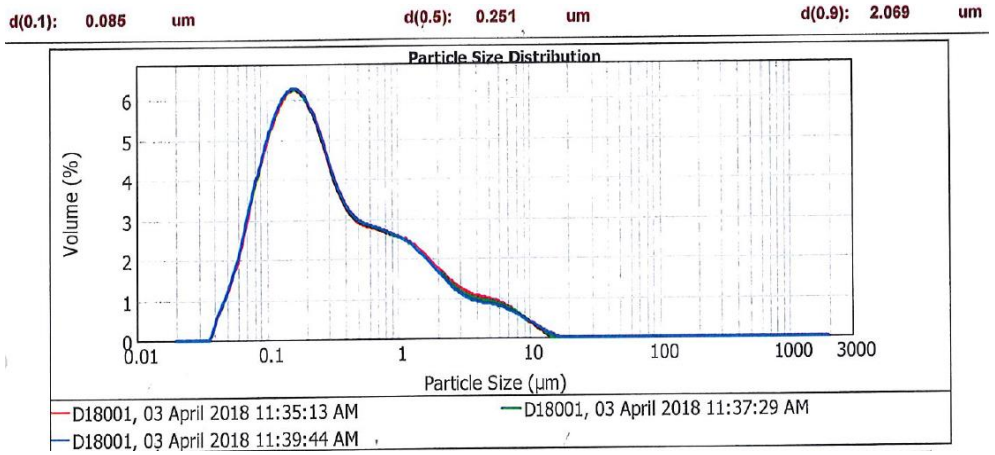

Figure 21: Particle size distribution for **PPE 4%** month 0 (batch 3).

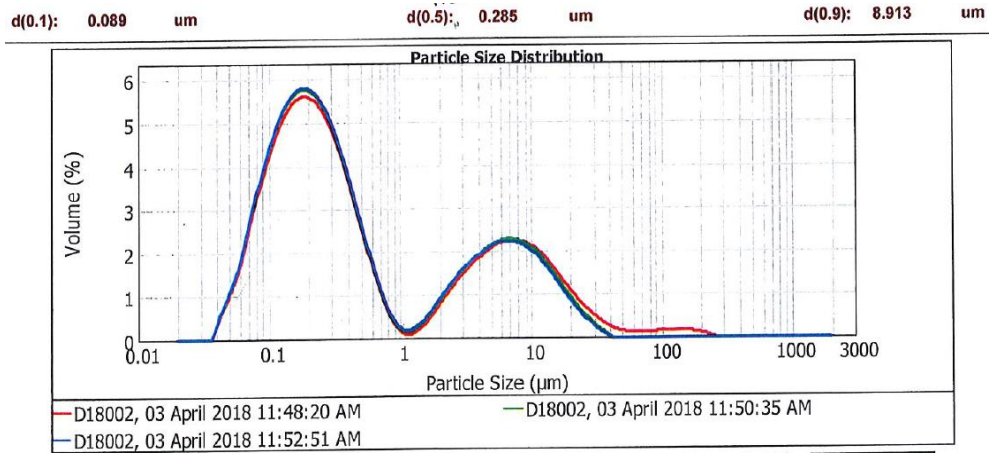

Figure 22: Particle size distribution for **P 4%** month 0 (batch 3).

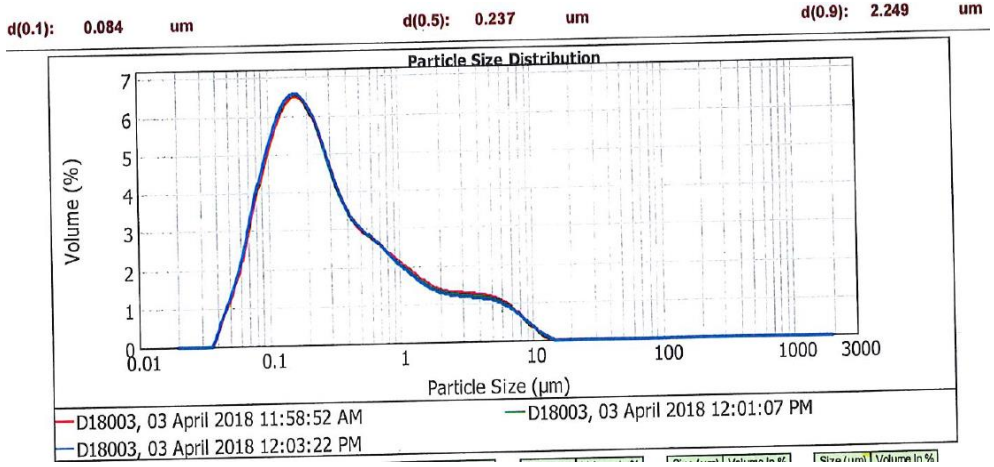

Figure 2320: Particle size distribution for **PPE 8%** month 0 (batch 3).

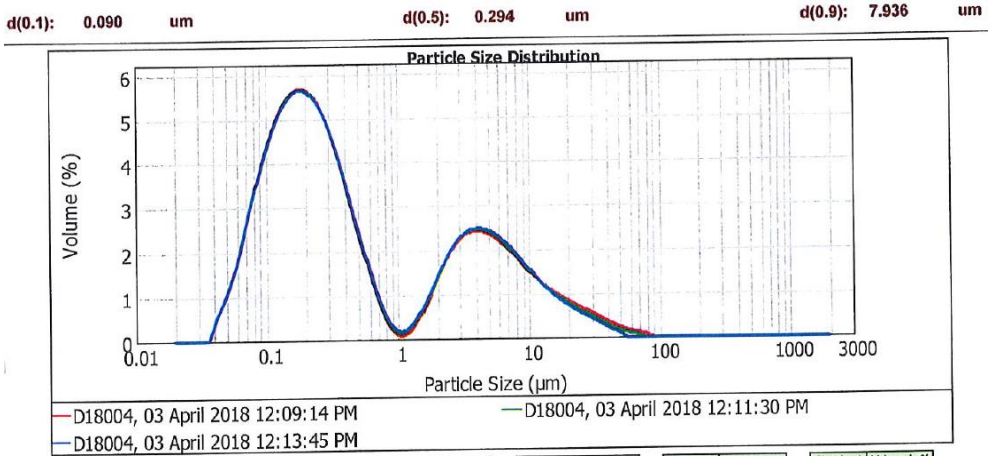

Figure 2419: Particle size distribution for **P 8%** month 0 (batch 3).

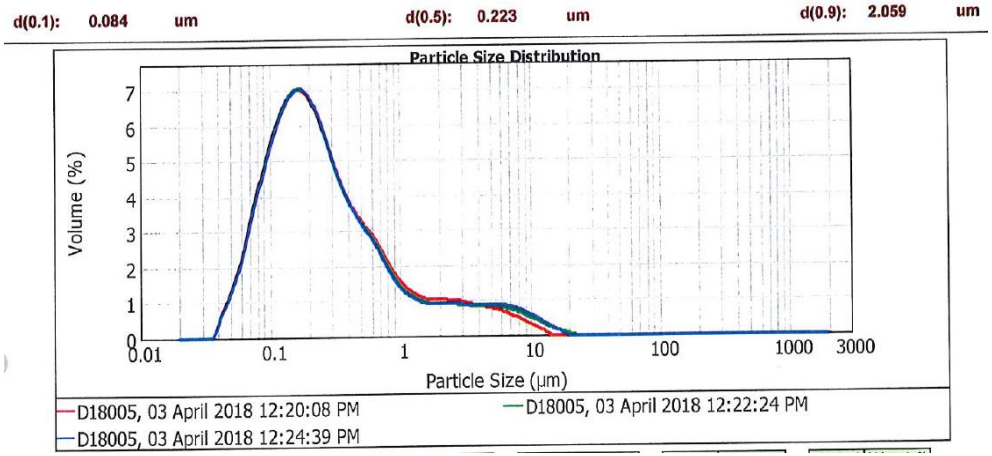

Figure 25: Particle size distribution for **PPE 10%** month 0 (batch 3).

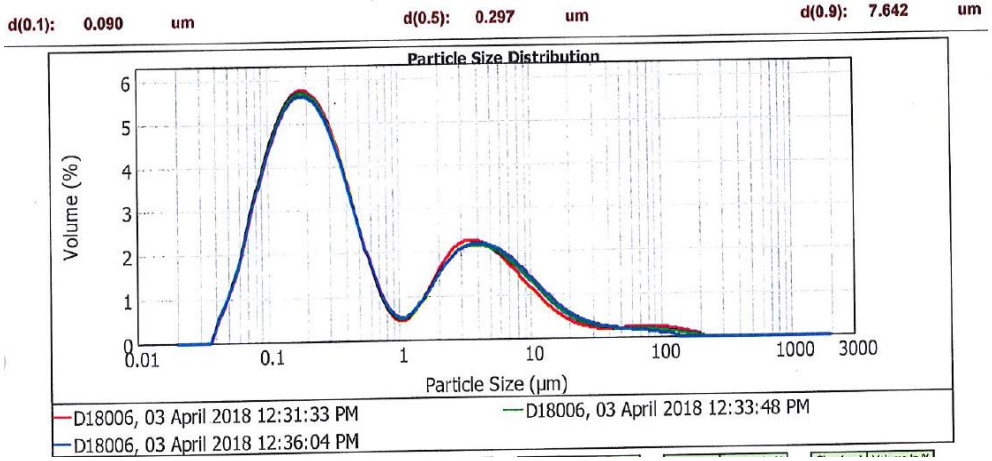

Figure 26: Particle size distribution for **P 10%** month 0 (batch 3).

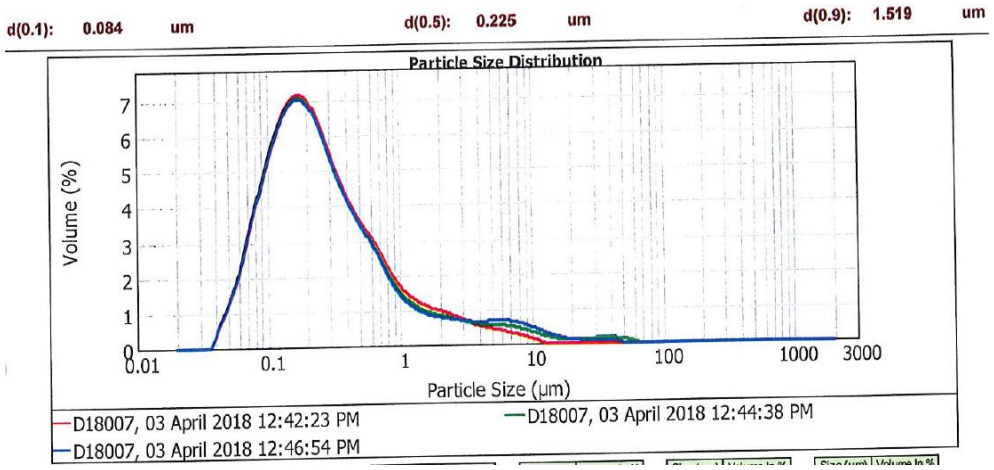

Figure 27: Particle size distribution for **PPE 13%** month 0 (batch 3).

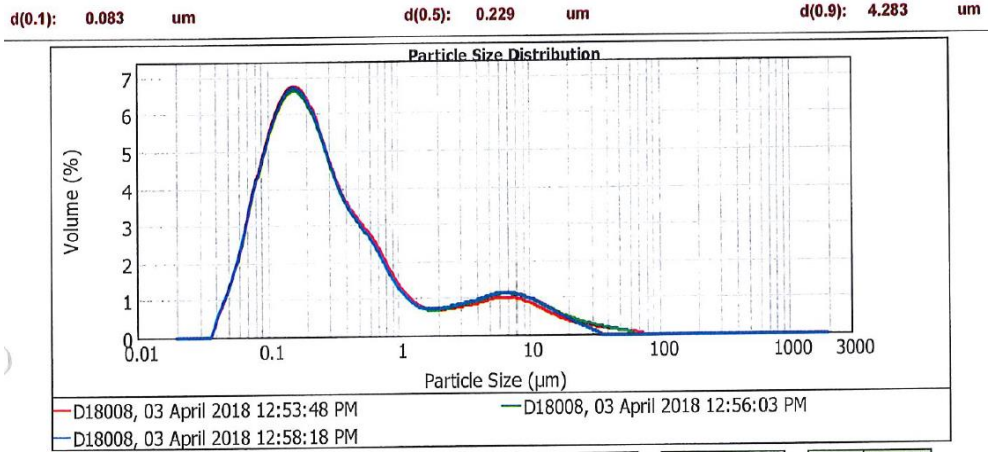

Figure 28: Particle size distribution for **P 13%** month 0 (batch 3).

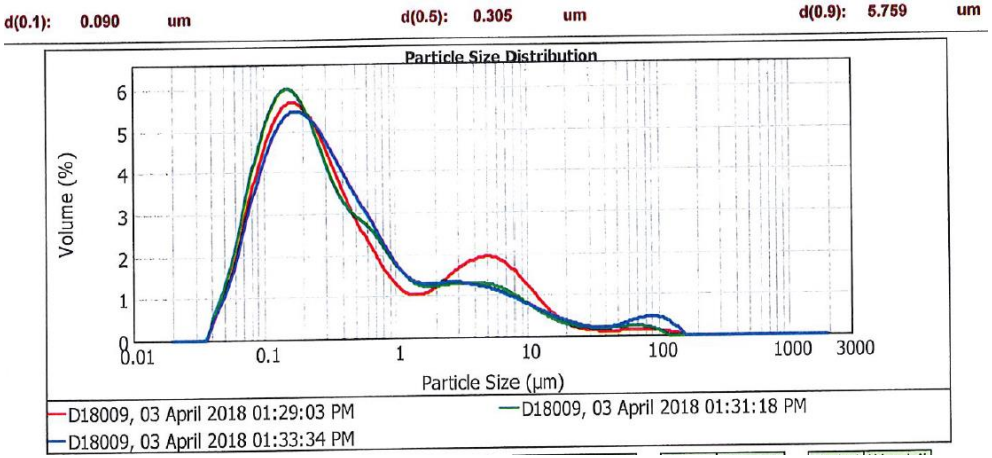

Figure 24: Particle size distribution for **Pro-PPE 50%** month 0 (batch 3).

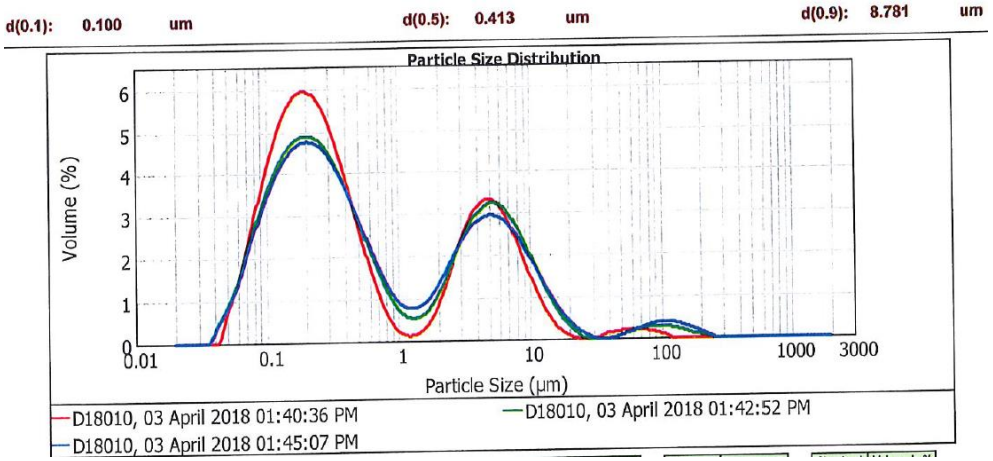

Figure 23: Particle size distribution for **Pro-P** month 0 (batch 3).

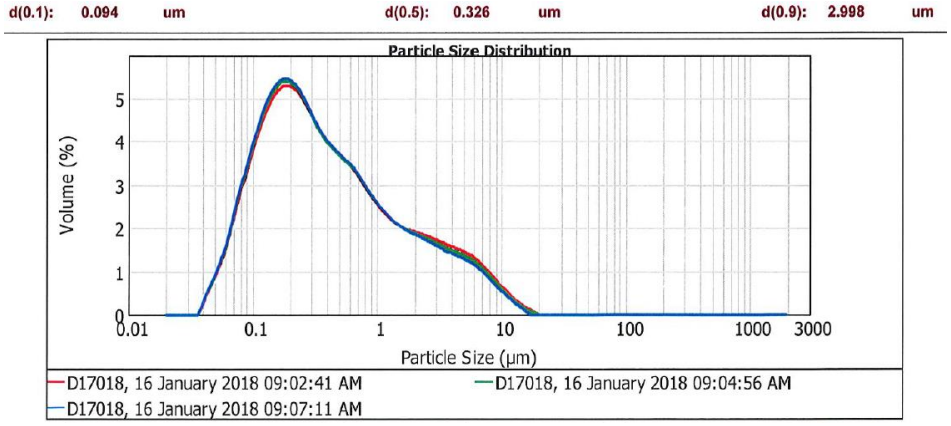

Figure 21: Particle size distribution for **PPE 4%** month 1 (batch 2).

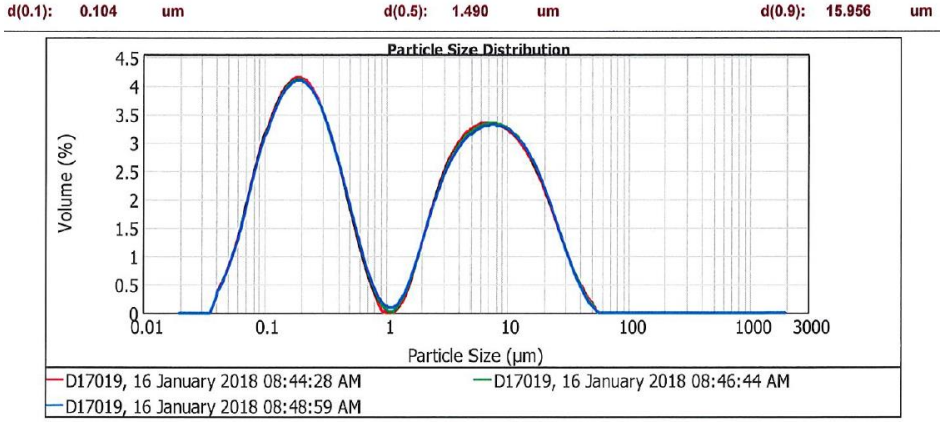

Figure 22: Particle size distribution for **P 4%** month 1 (batch 2).

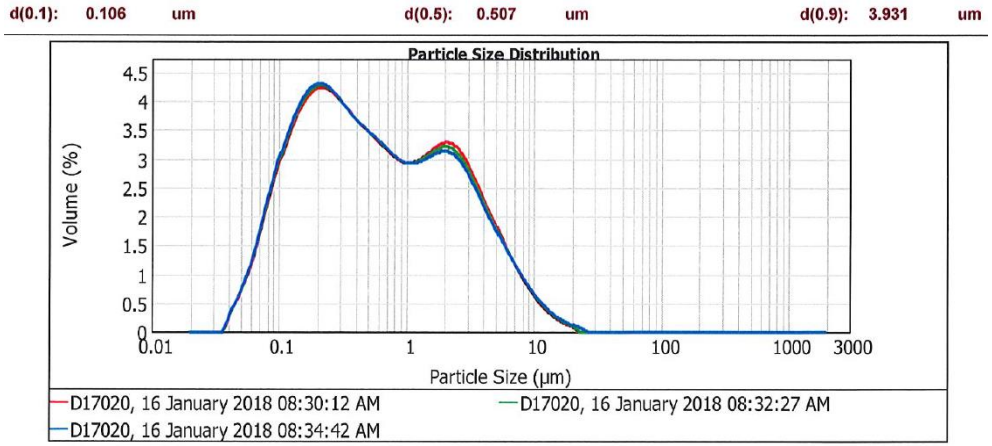

Figure 28: Particle size distribution for **PPE 8%** month 1 (batch 2).

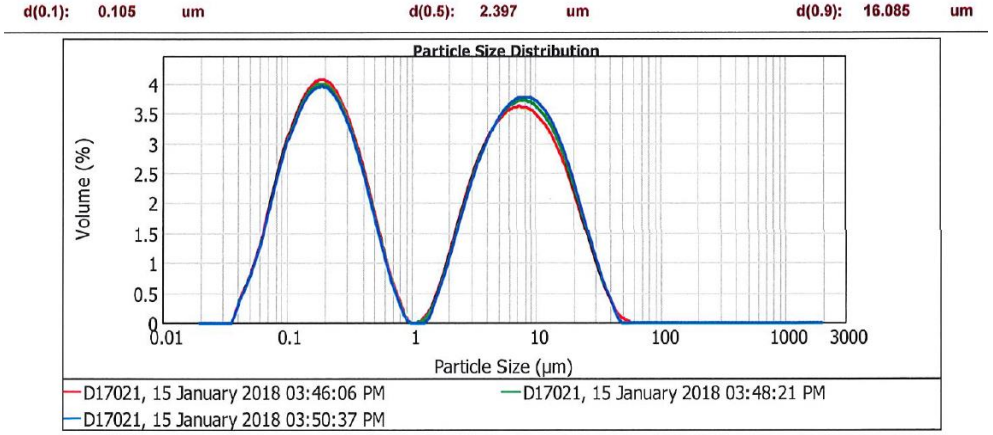

Figure 27: Particle size distribution for **P 8%** month 1 (batch 2).

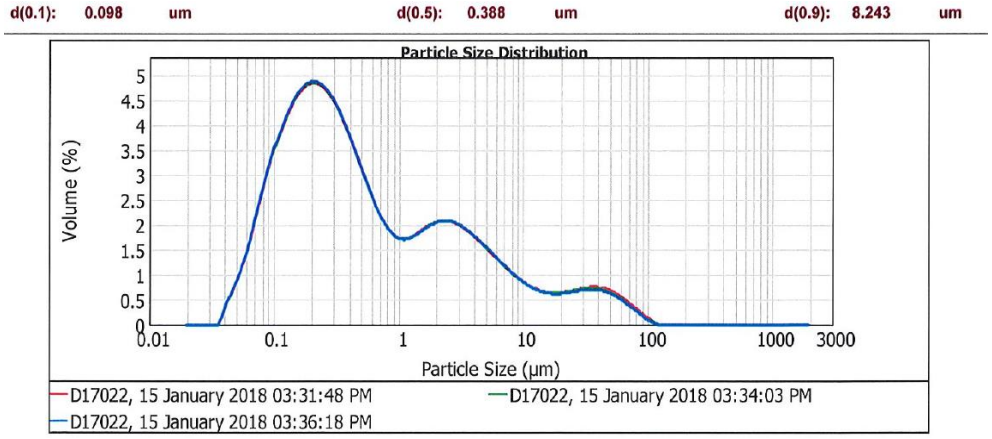

Figure 26: Particle size distribution for **PPE 10%** month 1 (batch 2).

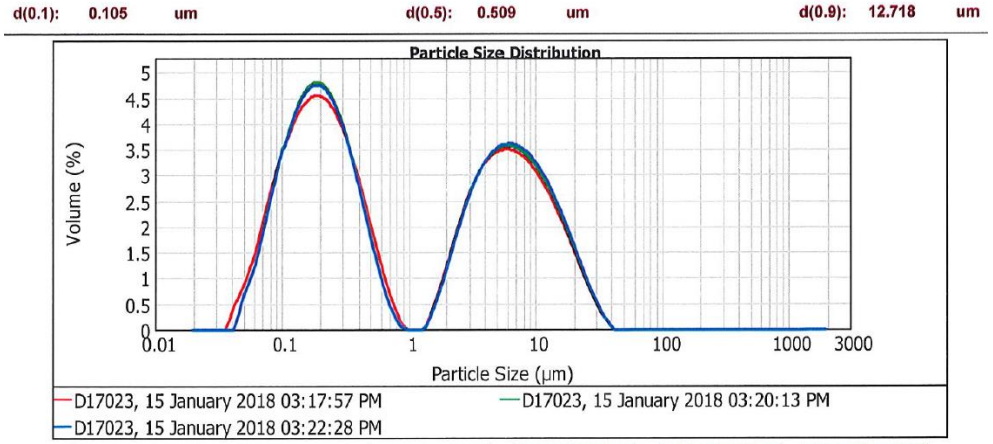

Figure 25: Particle size distribution for **P 10%** month 1 (batch 2).

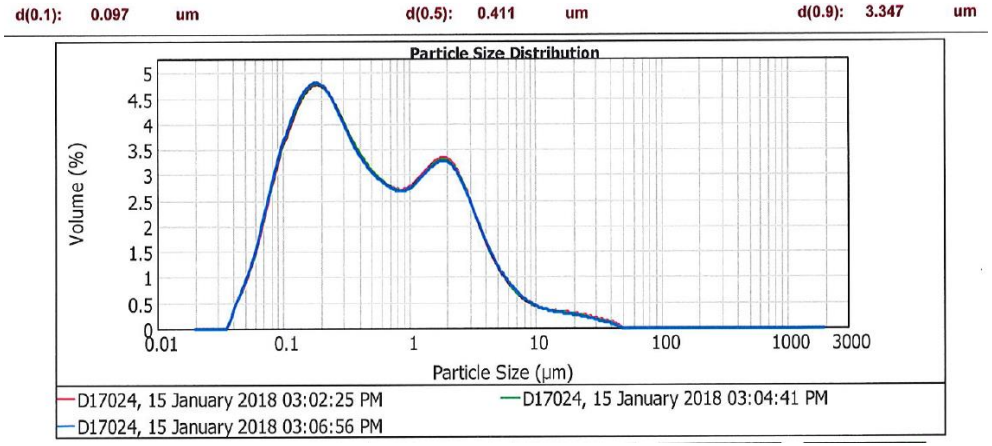

Figure 37: Particle size distribution for **PPE 13%** month 1 (batch 2).

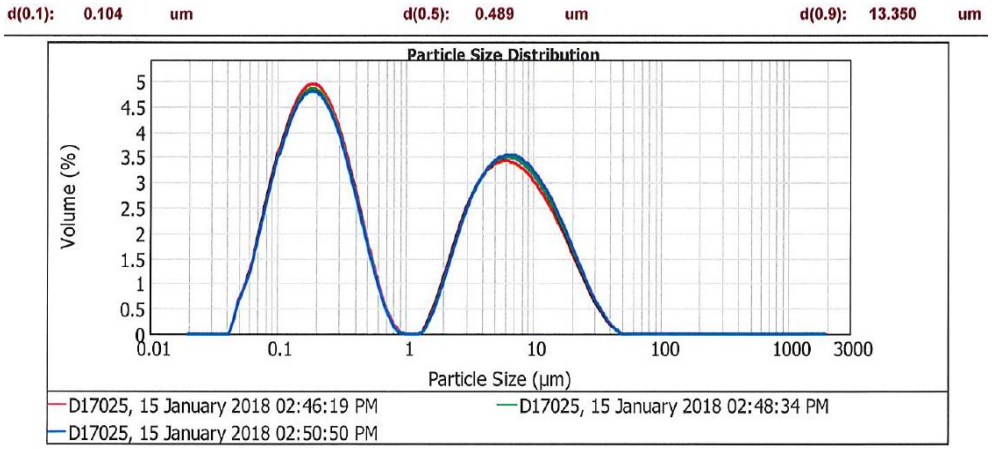

Figure 38: Particle size distribution for **P 13%** month 1 (batch 2).

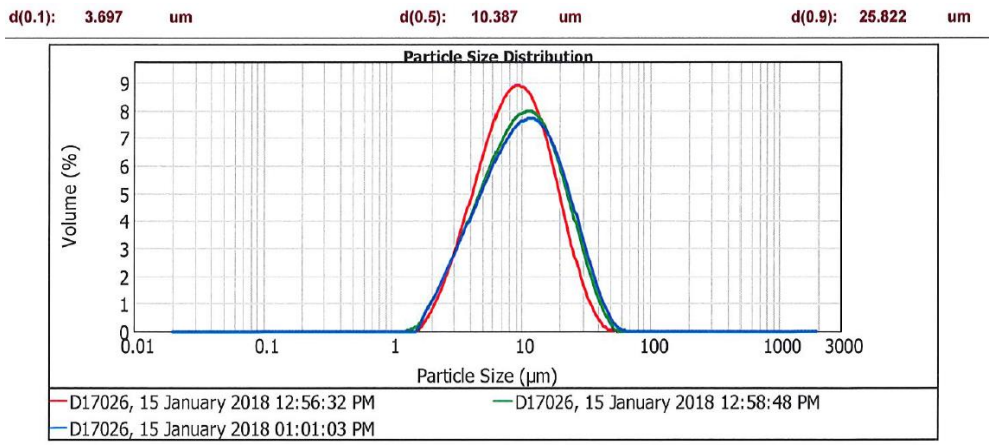

Figure 39: Particle size distribution for **Pro-PPE 50%** month 1 (batch 2).

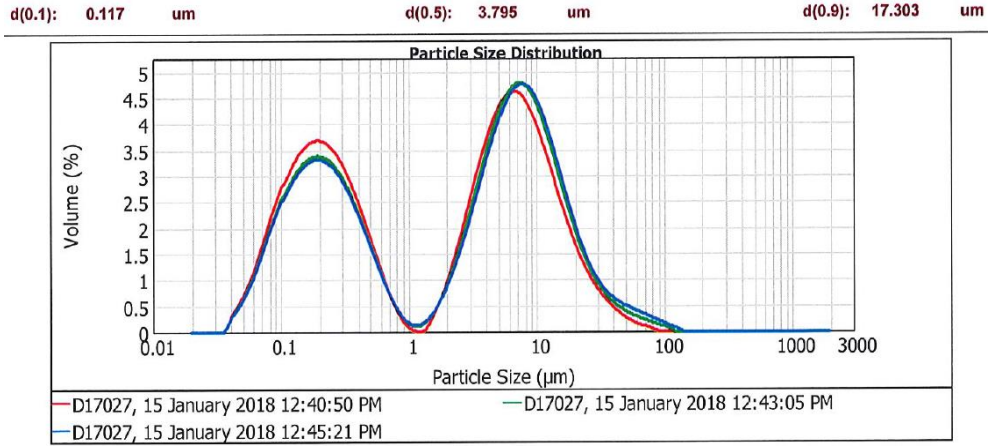

Figure 40: Particle size distribution for **Pro-P** month 1 (batch 2).

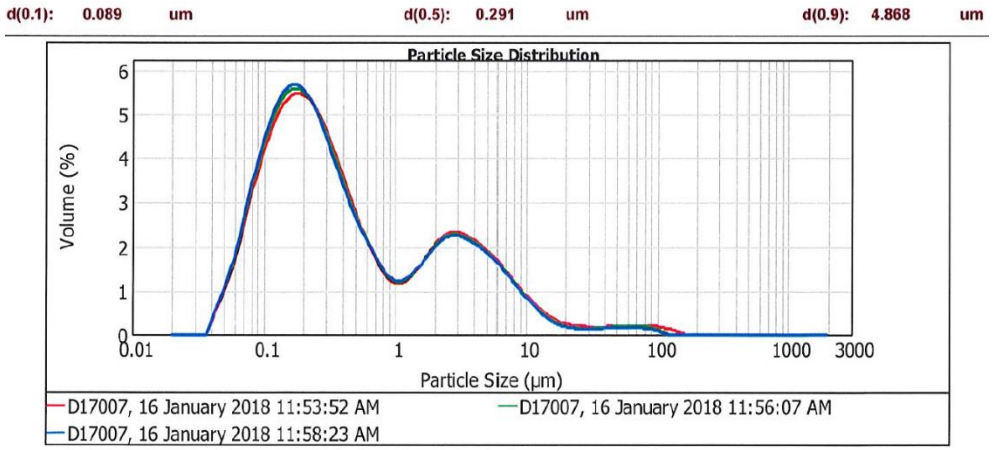

Figure 41: Particle size distribution for **PPE 4%** month 2 (batch 1).

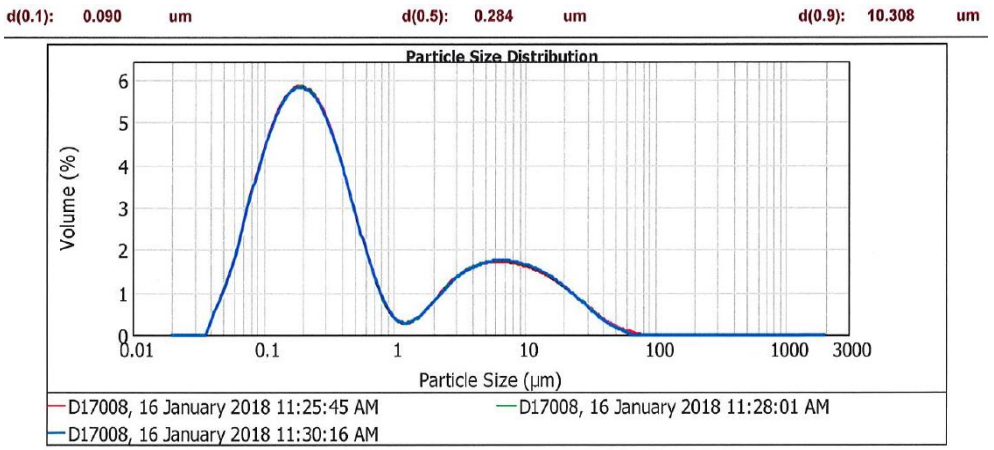

Figure 42: Particle size distribution for **P 4%** month 2 (batch 1).

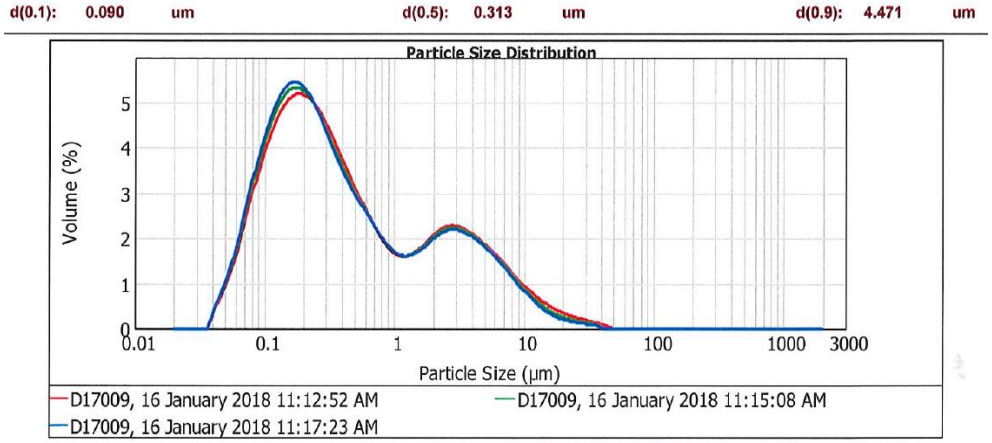

Figure 43: Particle size distribution for **PPE 8%** month 2 (batch 1).

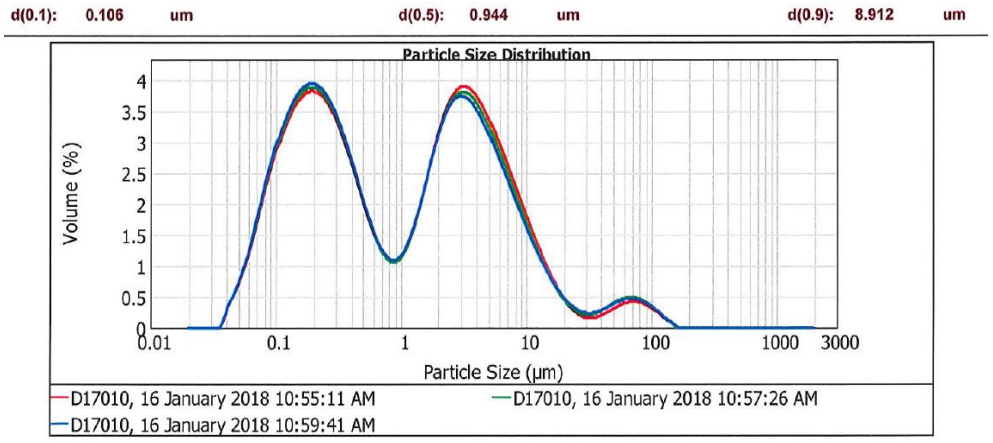

Figure 44: Particle size distribution for **P 8%** month 2 (batch 1).

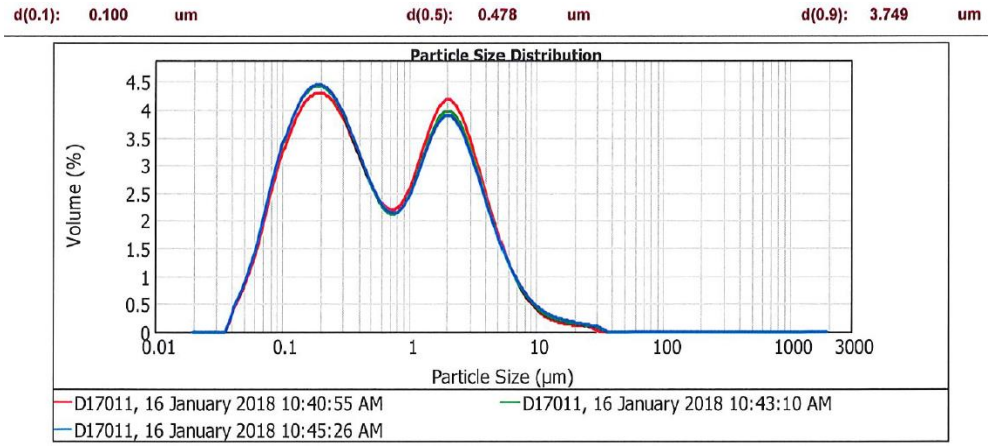

Figure 30: Particle size distribution for **PPE 10%** month 2 (batch 1).

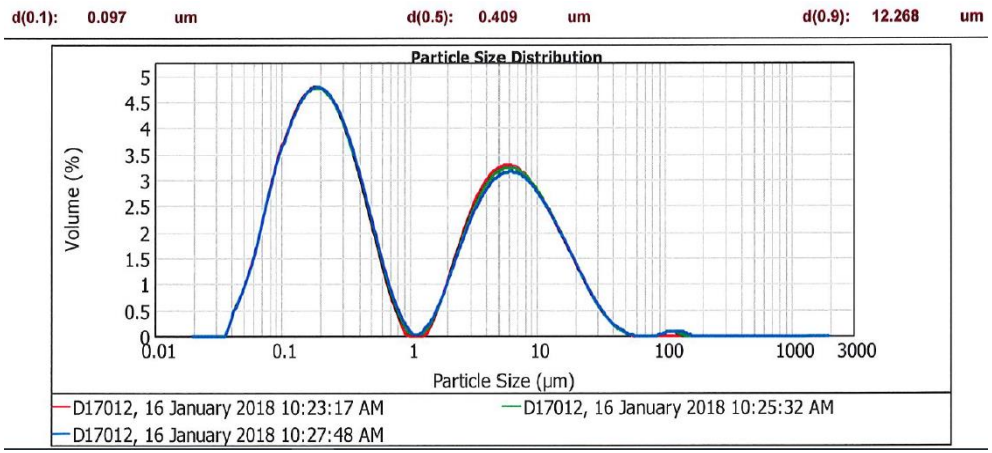

Figure 29: Particle size distribution for **P 10%** month 2 (batch 1).

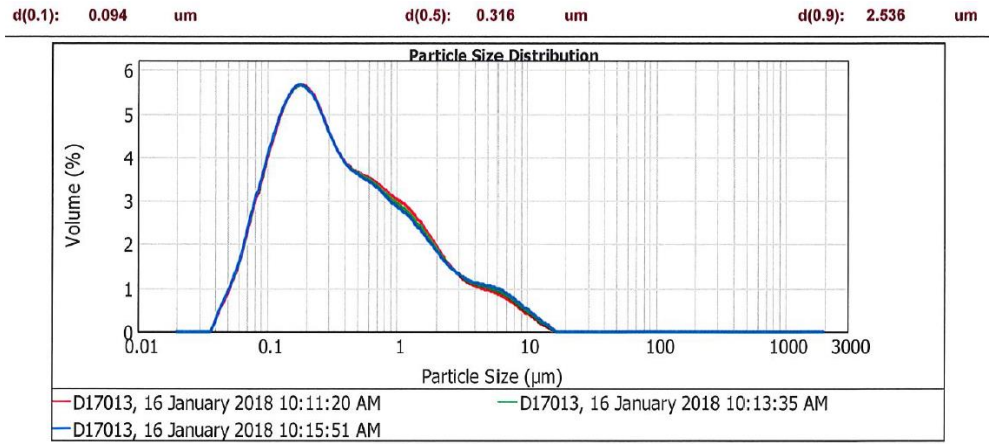

Figure 47: Particle size distribution for **PPE 13%** month 2 (batch 1).

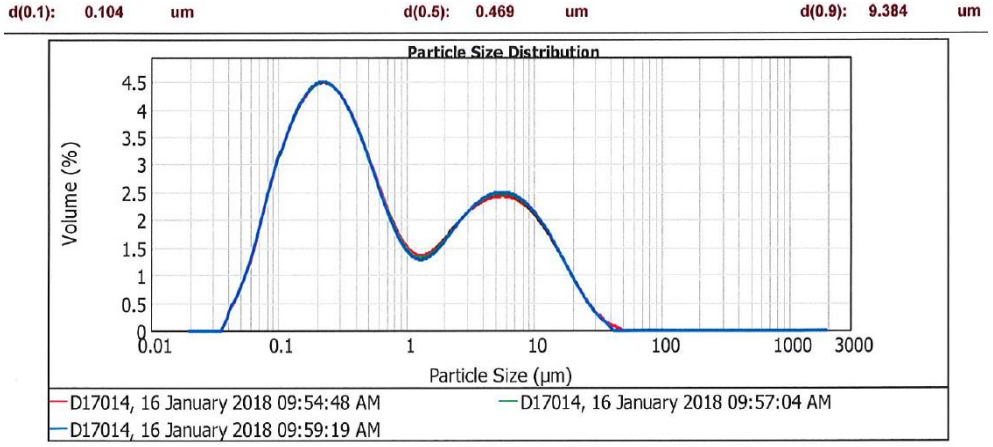

Figure 48: Particle size distribution for **P 13%** month 2 (batch 1).

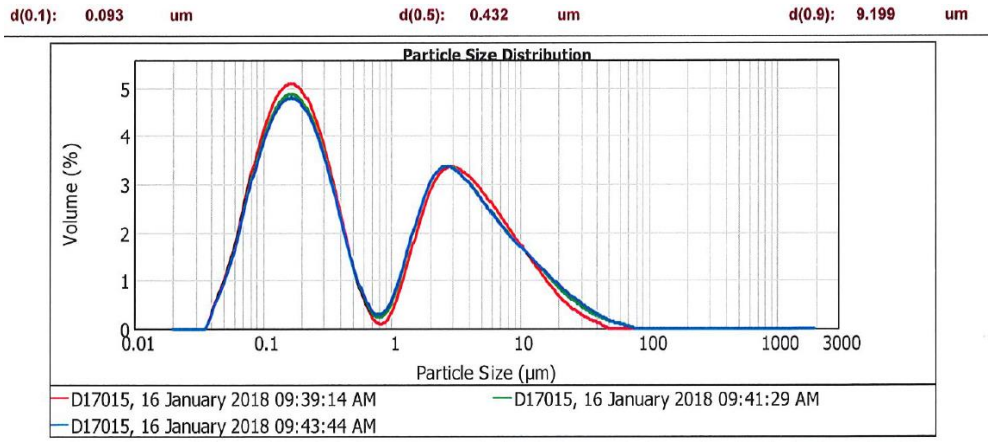

Figure 34: Particle size distribution for **Pro-PPE 50%** month 2 (batch 1).

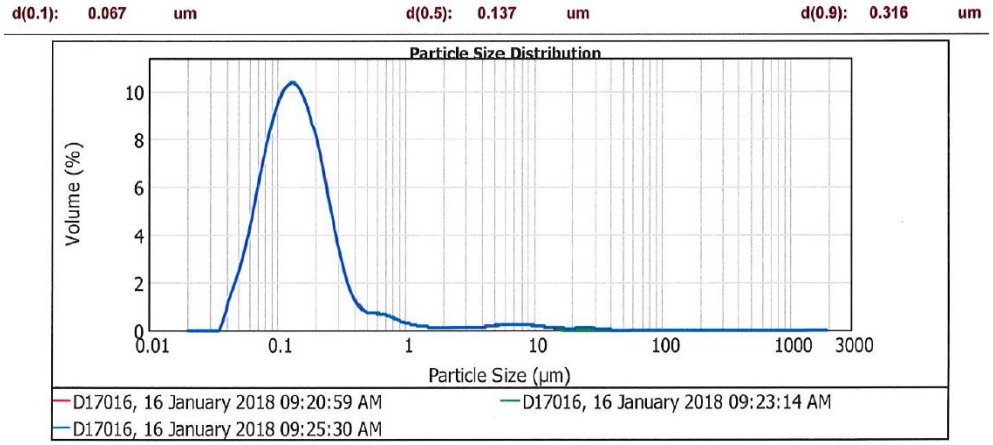

Figure 33: Particle size distribution for **Pro-P** month 2 (batch 1).

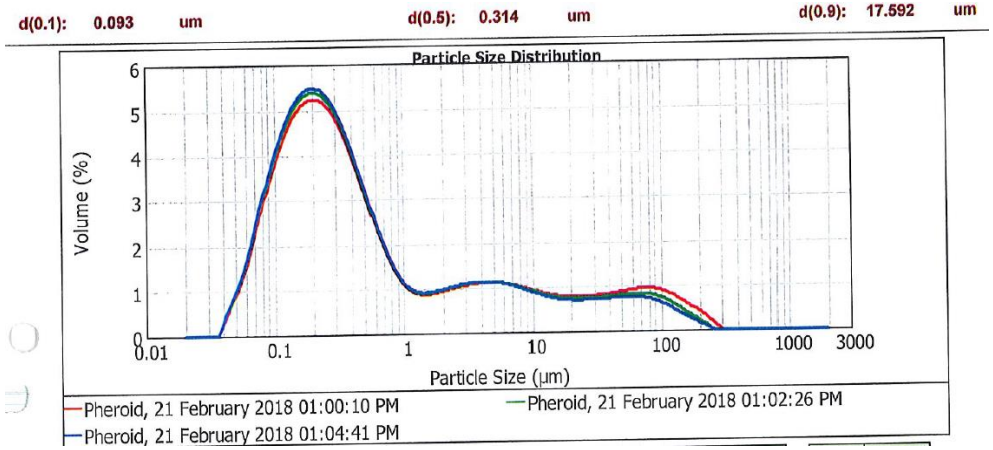

Figure 32: Particle size distribution for **PPE 4%** month 3 (batch 2).

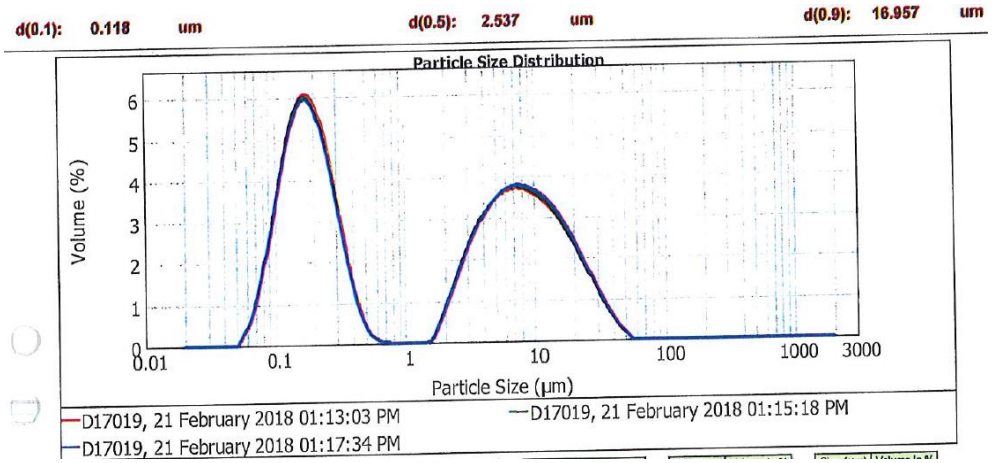

Figure 31: Particle size distribution for **P 4%** month 3 (batch 2).

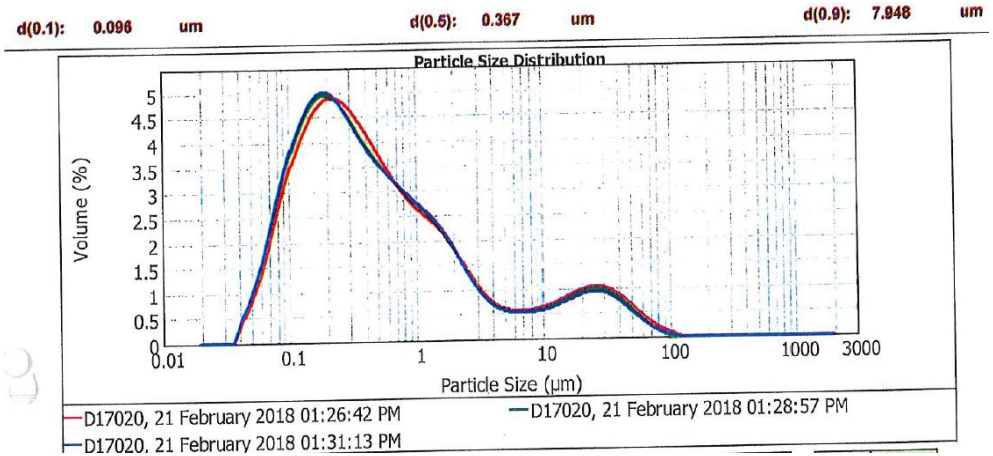

Figure 53: Particle size distribution for **PPE 8%** month 3 (batch 2).

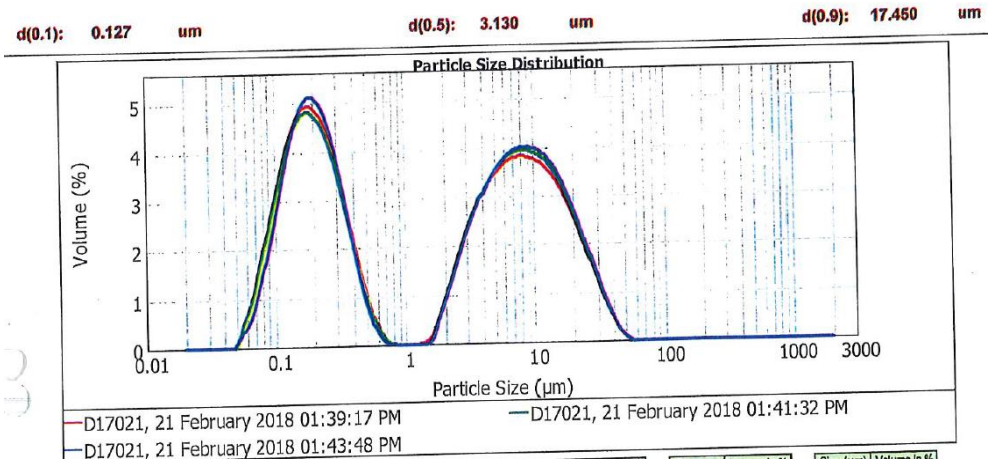

Figure 54: Particle size distribution for **P 8%** month 3 (batch 2).

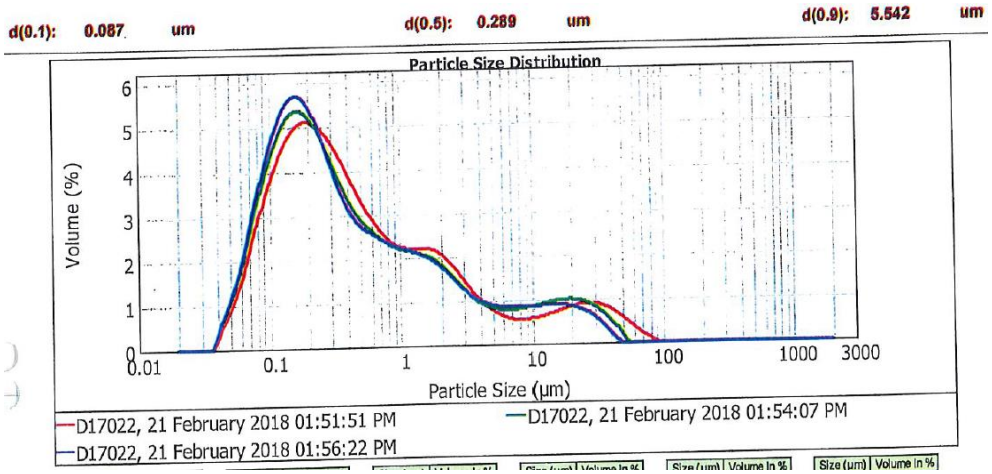

Figure 55: Particle size distribution for **PPE 10%** month 3 (batch 2).

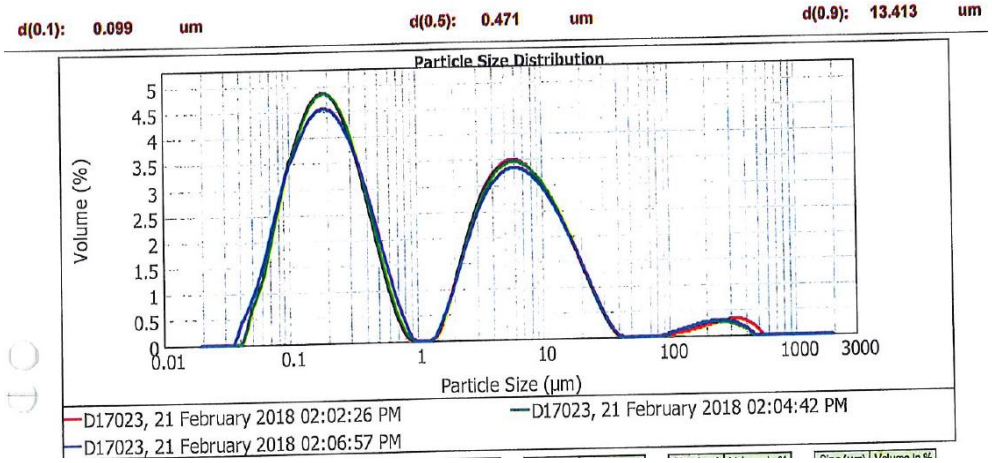

Figure 56: Particle size distribution for **P 10%** month 3 (batch 2).

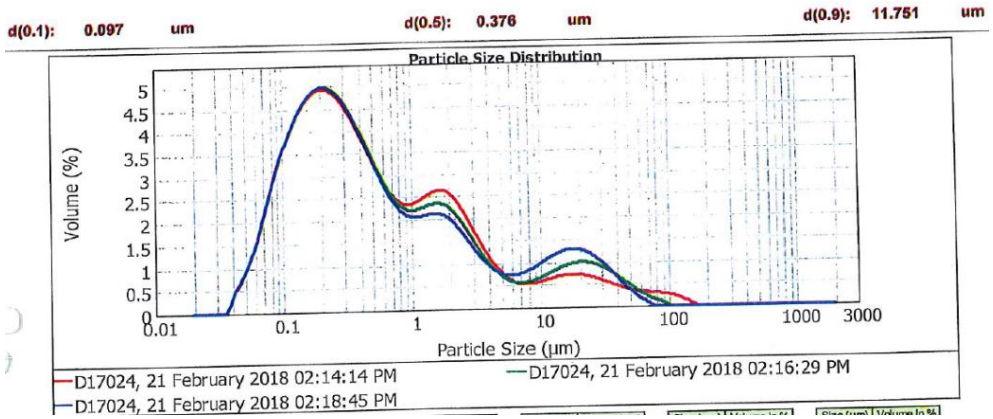

Figure 57: Particle size distribution for **PPE 13%** month 3 (batch 2).

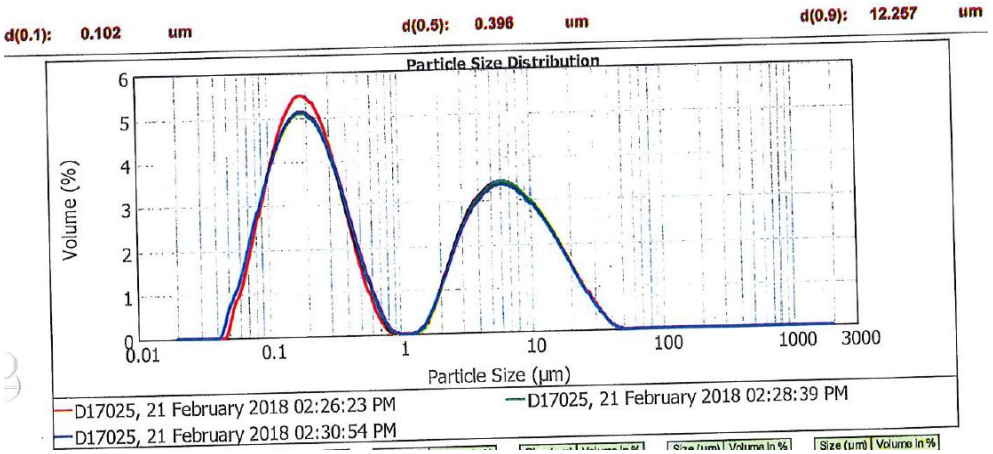

Figure 58: Particle size distribution for **P 13%** month 3 (batch 2).

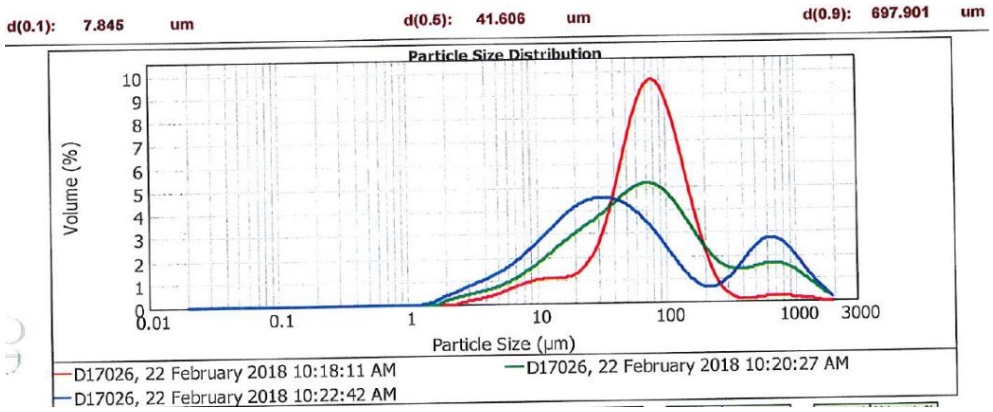

Figure 59: Particle size distribution for **Pro-PPE 50%** month 3 (batch 2).

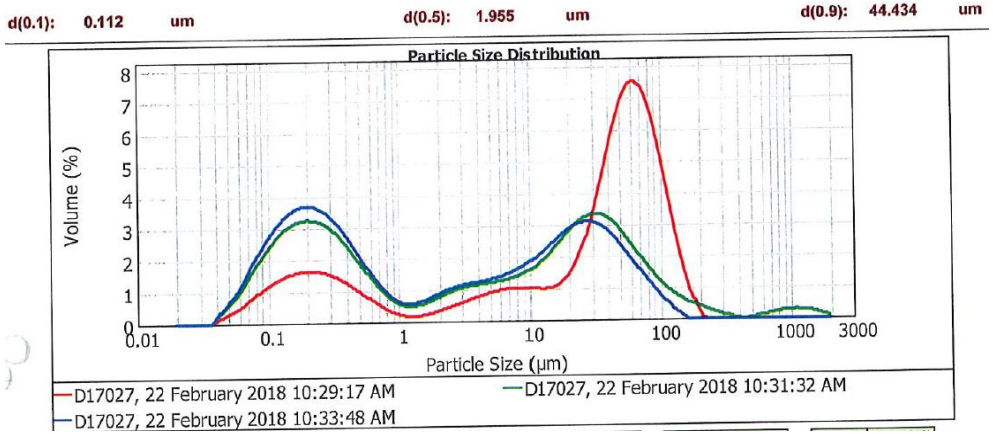

Figure 60: Particle size distribution for **Pro-P** month 3 (batch 2).