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Formulation of a topical anti-acne drug delivery system incorporating essential oils

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FORMULATION OF A TOPICAL ANTI-ACNE DRUG DELIVERY SYSTEM INCORPORATING ESSENTIAL OILS



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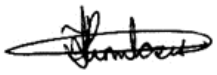
FADILAH KURRIMBOCCUS

A dissertation submitted to the Faculty of Health Sciences, University of Witwatersrand, Johannesburg, in fulfilment of the requirements for the degree of Masters of Pharmacy

2021

DECLARATION

I, Fadilah Kurrimboccus, hereby declare that this dissertation is my own work, with all other sources of information acknowledged by means of a complete reference list. This dissertation is being submitted in fulfilment of the degree of Master of Pharmacy, at the University of the Witwatersrand, Johannesburg. This work has not been submitted before for any degree or examination to this or any other university.



11/08/2021

F Kurrimboccus

Date

ABSTRACT

Acne, a skin disease arising from the pilosebaceous unit, affects approximately 80% of all adults at some point during their lifetime. In the age of emerging antibiotic resistance, researchers are continuously seeking natural alternatives to conventional antibiotics. Essential oils are potent, volatile plant essences which present as a possible alternative. Due to their volatility, water insolubility and thermal instability, the formulation of essential oils into commercial products remains a pharmaceutical challenge. This study aimed to develop a viable anti-acne topical treatment in the form of oil-in-water emulsified lotions as an avenue to overcome these challenges, and optimise the balance between antimicrobial efficacy and physical stability.

The broth microdilution method was used to determine the Minimum Inhibitory Concentration (MIC) of 19 essential oils. The mean MIC across four acne-inducing pathogens (*Cutibacterium acnes*, *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Streptococcus pyogenes*) was calculated for each essential oil to rank them according to their effectiveness. *Vetiver zizanioides* (vetiver) was found to be the most noteworthy essential oil with a mean MIC of 0.14 mg/mL. The second ranked oil was *Santalum austrocaledonicum* (sandalwood), which displayed a mean MIC of 0.23 mg/mL and was the most effective essential oil against *S. aureus*, *S. epidermidis* and *S. pyogenes*. A mean MIC of 0.40 mg/mL for *Thymus vulgaris* (thyme), 0.49 mg/mL for *Syzigium aromaticum* (clove) and 0.50 mg/mL for *Origanum vulgare* (oregano) were obtained, respectively. These five top ranked essential oils were then selected for formulation development.

The Hydrophilic-Lipophilic Balance (HLB) method was used to prepare lotions of optimum physical stability. To determine the HLB of each essential oil, coarse emulsions at HLB values 7-11 were prepared and the emulsion displaying the lowest degree of separation (least creaming) was selected as the required HLB (rHLB) of the essential oil. An HLB value of 7 for *V. zizanioides,* 10 for both *O. vulgare* and *T. vulgaris,* and 11 for both *S. austrocaledonicum* and *S. aromaticum* were obtained. These individual HLB values were then used to calculate the overall rHLB of their respective formulations taking into consideration the HLB of each excipient. Emulsions at this calculated HLB value as well as emulsions extending one HLB unit above and below were prepared. Each emulsion was then assessed and the optimum HLB

was determined by selecting the emulsion with the lowest creaming index (CI) over 28 days, the smallest change in mean droplet diameter (MDD) and polydispersity index (PDI) over 84 days and the lowest difference in turbidity ($\%D_T$) between the top and bottom ends of the emulsion over 28 days. The emulsified lotions of *O. vulgare*, *S. aromaticum* and *V. zizanioides* showed no creaming or phase separation and hence gave no indication of the optimum HLB. The remaining lotions both showed increasing CI with increasing HLB, with the minimum observed at HLB 11 for *S. austrocaledonicum* and HLB 10 for *T. vulgaris*. At tested HLB values of 8, 9 and 10, *V. zizanioides* emulsions displayed maximum stability at HLB 9 with a minimum change in MDD and PDI of 20.61% and 33.33%, and a $\%D_T$ of 0.349%. Emulsions of *T. vulgaris* were prepared at HLB values of 10, 11 and 12 and displayed a change in MDD and PDI of -50.65% and 63.75% respectively and the lowest $\%D_T$ (38.37%) at HLB 10. The *S. austrocaledonicum* emulsions were found to be the most stable at HLB 11 with a change in MDD and PDI of -63.54% and 377.92% respectively and a minimal $\%D_T$ of 8.39%. Similarly, *S. aromaticum* emulsion displayed maximum stability at HLB 11 with a % change in MDD and PDI of 206.06% and 29.57% respectively and a $\%D_T$ of 0.34%. Lastly, peak stability of *O. vulgare* emulsions was seen at HLB 11 with a minimum change in MDD and PDI of 4.57% and 7.65% and $\%D_T$ of 29.19%.

Emulsions of each essential oil at optimum HLB were then selected for further antibacterial studies. Time-kill assays of the formulations were conducted against *S. aureus*, *S. epidermidis* and *S. pyogenes.* Emulsified lotions of *O. vulgare*, *S. aromaticum* and *T. vulgaris* exhibited complete bactericidal activity after 3 hrs (faster than the positive control, ciprofloxacin) while those of *V. zizanioides* and *S. austrocaledonicum* eliminated the bacterial load after 24 hrs. The efficacy of time-kill was found to be dependent on the MDD of the formulations, rather than the individual MIC's of the essential oils themselves. All five formulations displayed bactericidal activity against the selected pathogens after three months of storage, signifying long-term antimicrobial stability.

This study has highlighted the potential of essential oils as viable options in the treatment of acne by producing formulations of optimal stability and long-term antimicrobial activity. Furthermore, this study demonstrates the importance of evaluation of antimicrobial properties in combination with stability studies to develop effective topical preparations in order to deliver consistent and accurate dosing of essential oil containing acne preparation.

DEDICATION

To my parents, Iqbal and Sadia Kurrimboccus, without whom I would not have made it this far. I truly appreciate your countless sacrifices, prayers and your endeavors to give me every comfort which has enabled me to come this far. You have both invested so much to mould me into the person I am today. Thank you for always believing me, especially in the times when I did not believe in myself.

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PUBLICATIONS ARISING FROM THIS STUDY

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LIST OF ABBREVIATIONS

%	Percentage
°C	Degrees Celsius
<	Less than
>	Greater than
µg	Microgram
µg/mL	Microgram per millilitre
µL	Millilitre
α	Alpha
ATCC	American type culture collection
β	Beta
CFU	Colony forming units
CHLB	HLB contribution of each component
CI	Creaming index
g	Gram
GC-MS	Gas-chromatography mass-spectrometry
GMS	Glycerol monostearate
HLB	Hydrophilic-lipophilic balance
HTM	Haemophilus Test Medium
INT	*p*-iodonitrotetrazolium violet
MDD	Mean droplet diameter
Mg	Milligram
mg/mL	Milligram per millilitre
MIC	Minimum inhibitory concentration
min	Minutes
mL	Millilitre
MTCC	Microbial Type Culture Collection (India)
NAD	Nicotinamide adenine dinucleotide
nm	Nanometre
$^{O}/_{W}$	Oil-in-water
OTC	Over-the-counter
PDI	Polydispersity index

PIT	Phase inversion temperature
PTCC	Persian Type Culture Collection
rHLB	Required HLB
rpm	Revolutions per minute
SD	Standard deviation
sec	Seconds
TGB	Thioglycolate broth
TSA	Tryptone Soya agar
TSB	Tryptone Soya broth
UV-vis	Ultraviolet-visible
$^{v}/_{v}$	Volume per volume
$^{W}/_{O}$	Water-in-oil
$^{w}/_{w}$	Weight per weight
WF	Weight fraction

CHAPTER 1 - INTRODUCTION

1.1 Acne vulgaris

Acne is a disease of the skin arising from the pilosebaceous unit (Mahmood and Shipman, 2017). It is the 8th most prevalent disease worldwide (Tan and Bhate, 2015) and affects approximately 80% of all adults at some point during their lifetime (Awan and Lu, 2017). Due to it being considered a common affliction, the severity of the disease and its ensuing consequences are often underestimated. Although it is not a fatal condition, it can lead to permanent scarring and in severe cases, facial disfigurement (Mahmood and Shipman, 2017). As the negative effects of acne are often highlighted as being physical, the profound impact on a person's psychosocial and emotional wellbeing are frequently discounted. Patients with acne are linked to higher rates of unemployment, depression, self-harm and suicide (Kumar *et al.*, 2016; Gallitano and Berson, 2018; Prabhakar *et al.*, 2018). Acne most commonly presents on the face due to its high density of pilosebaceous glands, thereby directly affecting a person's appearance and ultimately their self-esteem. Given that acne most commonly manifests in adolescents, a damaged self-worth from early on in life may lead to impaired social development and hence interfere with an individual's ability to thrive in society (Gallitano and Berson, 2018). Clearly, acne gives rise to many consequences, which can be best resolved through prompt treatment of the actual disease.

1.2 Pathogenesis of acne

Acne vulgaris presents as comedones, nodules, papules, pustules and scarring (Mahmood and Shipman, 2017). These lesions arise from the inflammation and blockage of the pilosebaceous unit (pore), which is composed of the hair follicle with its connected sebaceous gland (Mahto, 2017). An abnormal increase of sebum production and keratinization leads to the formation of sticky keratinocytes, which then proceeds to clog the pore, leading to comedone formation. This provides a moist, enclosed environment that is ideal for commensal pathogens, such as *Cutibacterium acnes* (previously known as *Propionibacterium acnes*) and *Staphylococcus epidermidis*, to proliferate and cause disease. The accumulation of bacteria, combined with increased sebum and keratinocyte production, leads to inflammation and degradation of the

follicular wall. This build-up of debris gives rise to a variety of lesions such as comedones, papules and pustules that often cause pain and tenderness (Mahto, 2017). While mainly presented on the face, acne may also affect the back and chest due to the high density of pilosebaceous glands present in these areas as well (Kapoor and Saraf, 2011).

While endogenous factors such as hormonal changes and sebum production are contributory in the formation of acne, fluctuations in microbial skin flora play a central role (Kumar *et al.*, 2016). The skin is home to a complex ecosystem of micro-organisms which, at low concentrations, serve to protect the human host. However, disruptions in the balance of commensal pathogens may lead to disease. A number of micro-organisms have been isolated as being involved in the pathogenesis of acne with the most common being *C. acnes*, *S. epidermidis*, *Staphylococcus aureus*, and *Streptococcus pyogenes* (Kumar *et al.*, 2016).

1.2.1 Cutibacterium acnes

Cutibacterium acnes (Figure 1.1a) is a Gram-positive, facultative anaerobe primarily known as a major inhabitant of the human skin. Although it is a commensal bacterium, it is an opportunistic pathogen that can cause disease due to its ability to produce multiple virulence factors. Due to its invading and colonizing effects, it is the primary micro-organism involved in the pathogenesis of acne (Toyoda and Morohashi, 2001). Its ability to grow under different oxygen environments further enhances the virulence. It releases secretory enzymes capable of degrading and rupturing the follicular wall. It further targets other cells, such as keratinocytes, to release inflammatory cytokines and pore-forming toxins that result in host tissue degradation (Kumar *et al.*, 2016). If the conditions of microbial flora are such that it favours the growth of *C. acnes*, this microbe may turn from commensal to pathogenic.

1.2.2 Staphylococcus epidermidis

Staphylococcus epidermidis (Figure 1.1b) is a resident of the microbial flora of human skin and is characterised as a Gram-positive, facultative anaerobe (Kumar *et al.*, 2016). Its virulence arises from the ability to produce adhesion factors, such as surface-binding proteins, which allows this pathogen to firmly bind to the skin. This also promotes biofilm formation and facilitates the transfer of resistant genes to other bacteria (Heilmann *et al.*, 1996; Fey and Olson, 2010). The biofilm protects micro-organisms from innate defences, making eradication

difficult and thereby prolonging the treatment of acne. The biofilm also provides a favourable environment for *C. acnes* to grow, making its impact twofold (Kumar *et al.*, 2016). A study found that the colonisation rates of *C. acnes* and *S. epidermidis* was found to increase by 82% and 70% respectively in patients with acne as compared to those without (Pathak *et al.*, 2013).

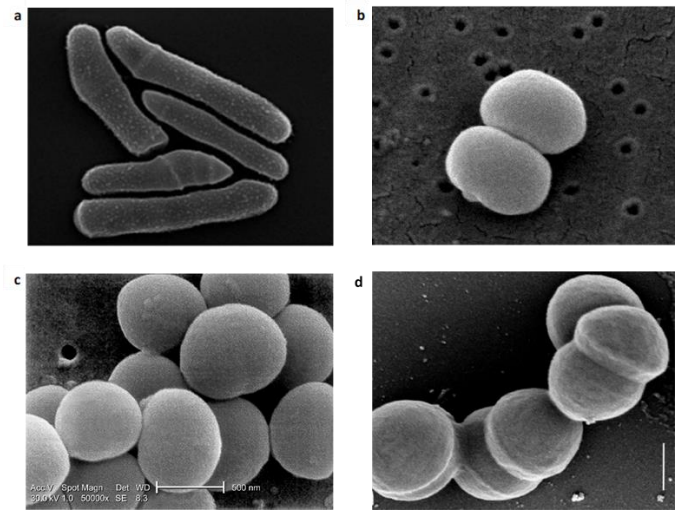


Figure 1.1: Scanning electron microscopy view of a) *C. acnes* (Mak et al., 2013), b) *S. epidermidis* (Photo by Janice Carr, courtesy of Centers for Disease Control and Prevention), c) *S. aureus* (Photo by Janice Carr, courtesy of Centers for Disease Control and Prevention) and d) *S. pyogenes* (Severin et al., 2007).

1.2.3 Staphylococcus aureus

This is the most prominent Gram-positive micro-organism of the skin flora. It is highly adaptable to different environmental conditions which increases its ability to thrive and persist in hosts (Lowy, 2003). *Staphylococcus aureus* (Figure 1.1c) is a major cause of skin and soft tissue infections- from common conditions such as impetigo and folliculitis to more severe conditions (Tong *et al.*, 2015). Its invasive potential allows it to cause a large range of life-threatening diseases such as bacteraemia, sepsis and meningitis (David and Daum, 2017). Once it invades the skin as a pathogen, it releases enzymes such as proteases and lipases to cause tissue damage. The organism is also known to produce exfoliative toxins and cytokines which protect them from defensins, thereby evading detection and establishing disease (Kumar *et al*., 2016). Whilst being the most common nosocomial inhabitant of the skin, it displays a 6-40% mortality rate amongst patients (Frank *et al.*, 2010). There is sufficient evidence to point

towards a relationship of mutualism between *S. aureus* and *C. acnes* (Tyner and Patel, 2016). Lo *et al.* (2011) found that *S. aureus* cultured in the presence of *C. acnes* displayed a greater degree of *β*-haemolysis than that of *S. aureus* alone. Counter wise, *C. acnes* has been found to produce porphyrins which stimulate and promote biofilm formation of *S. aureus* (Wollenberg *et al.*, 2014). One study showed that 43% of its participants with acne were colonized with *S. aureus*, present in either the nose or throat (Fanelli *et al.*, 2011). The mutually beneficial relationship of both pathogens leads to a possible synergistic virulence, which is a noteworthy cause for concern.

1.2.4 Streptococcus pyogenes

Streptococcus pyogenes (Figure 1.1d) is a Gram-positive organism that is present on skin surfaces. It possesses a potential for pathogenicity due to its ability to adhere, colonise and degrade skin tissue. It also produces exotoxins that increase the production of cytokines, leading to inflammation (Kumar *et al.*, 2016). A study conducted by Levy *et al.* (2003) found a higher prevalence and resistance pattern of *S. pyogenes* in the oropharynx of patients presenting with acne than those without. According to Kumar *et al.* (2016), this link may present a direct or indirect association between *S. pyogenes* and the pathogenesis of *C. acnes.*

1.3 Current diagnosis and treatment of acne

Current treatments are aimed at one or more of the causes of acne, i.e. reducing sebum production, decreasing inflammation, regulating keratinocytes or preventing bacterial growth (Orchard *et al.*, 2018a). The mode of treatment of acne vulgaris is dependent on the severity, extent and cause of the disease. The first line treatment for mild acne is usually topical therapy, while moderate to severe acne warrants the use of a combination of oral and topical antibiotics or oral contraceptives. Topical preparations include retinoids, antibiotics such as benzyl peroxide, or azelaic acid; while systemic antibiotics include tetracyclines or macrolides (Zaenglein *et al.*, 2016). In females, contraceptives may be used to counteract the effect of androgen hormones which stimulate the production of excess sebum (Zaenglein *et al.*, 2016). However, the current treatments are not without their side-effects, which may cause poor patient compliance and contribute towards the development of antibiotic resistance (Vora *et al.*, 2018). The commonly prescribed oral isotretinoin, of the drug class retinoids, has been associated with dryness of the skin, photosensitivity and even depression (Mahto, 2017).

Studies have been undertaken to improve the efficacy of prescription drugs by investigating their combinations, such as adapalene and isotretinoin with benzoyl peroxide (Thiboutot *et al.*, 2007; Rosso *et al.*, 2010) which led to superior activity and a quicker onset of action. However, their side-effects also proved to be concentration-dependant leading to burning sensations, erythema and increased irritation (Bikowski, 2005). In the case of moderate to severe acne, long term treatment is required which exposes patients to extended antibiotic therapy, allowing pathogens to adapt and develop resistance to antibiotics such as tetracycline and erythromycin. Evidence of emerging resistance is seen by studies that have identified 50% of *C. acnes* strains being resistant to topical macrolides (Walsh *et al.*, 2016), as well as resistance towards doxycycline, minocycline and trimethoprim-sulphamethoxazole (Moon *et al.*, 2012; Schafer *et al.*, 2013). The daily oral consumption of antibiotics gives rise to multi-drug resistant strains which are much harder to treat. In addition, topical antibiotic formulations are not exempted from contributing to resistance, as shown by Mills *et al.* (1996) where patients exposed to prolonged treatment with 2% erythromycin gel showed a decreased efficacy with time which led to treatment cessation. This highlights the need for alternative herbal therapies that do not contribute to resistance (Sinha *et al.*, 2014).

Cosmeceuticals are products that are intermediary between cosmetics and prescription drugs, and are readily available over the counter (Barros and Zaenglein, 2017). These products, however, are rarely verified by either *in vitro* or *in vivo* tests and there is a constant influx of new over-the-counter (OTC) products being introduced to the market. This combination makes it more difficult for prescribing doctors and dermatologists to keep abreast of these new developments and assist their patients in making informed decisions about suitable treatments (Decker and Graber, 2012). Cosmeceuticals, especially those of a natural origin, are gaining popularity worldwide due to their "green" appeal and ease of access. Due to their natural origin, they are perceived to be safer and therefore less toxic (Winkelman, 2018). A large percentage of people attempt to self-diagnose and self-treat their acne as they cannot afford expensive clinician fees (Decker and Graber, 2012). Approximately 80% of adolescents with acne do not seek medical help, but instead resort to OTC products (Corey *et al.*, 2013). Hence, it is of crucial importance that effective cosmeceuticals backed by supporting scientific evidence be made available.

1.4 Essential oils used to treat acne

The use of essential oils for the treatment of skin conditions date back almost 6000 years, and have been used by ancient civilizations such as the Egyptians and Chinese (Ali *et al.*, 2015). From the late 20th century, there has been a surge in aromatherapeutic literature regarding essential oils as they have garnered increasing attention for their cosmetic, therapeutic and holistic uses (Svoboda and Deans, 1995; Evans, 2009; Esposito *et al.* , 2014). Their popularity has risen internationally, especially in the United States, for their ability to treat a wide range of ailments, particularly those of dermatological concern (Stevensen, 1998; Clarke, 2008; Lee *et al.*, 2012). Numerous aromatherapy websites and layman's literature cite the use of essential oils for the treatment of acne, with the added advantage of anti-aging, anti-inflammatory and wound-healing properties (Carey, 2018; Erickson, 2019; McKay, 2019). Certain essential oils, such as *Salvia sclarea* (clary sage) and *Lavandula angustifolia* (lavender) are claimed to also relieve stress which can contribute to the exacerbation of acne, thereby adding to their holistic appeal (Worwood, 1991; Wilson, 2013).

Aromatherapeutic scientific literature has also played its part in highlighting the potential of essential oils to treat skin diseases. For instance, Orchard and van Vuuren (2017) found that of 98 essential oils recommended for dermatological use, 49 were specifically indicated for the treatment of acne. *In vitro* investigations have identified various essential oils that display noteworthy antimicrobial activity (a minimum inhibitory concentration (MIC) value of less than 1.00 mg/mL) against acne pathogens. For example, *Cinnamomum verum* and *Vetiver zizanioides* both displayed MIC values of 0.25 mg/mL against *S. epidermidis* and values of 0.13 mg/mL and 0.50 mg/mL against *C. acnes* (Orchard *et al.*, 2018a; Orchard *et al.*, 2018b). Combinations of essential oils against the same pathogens have also been investigated, and it was found that in general, combinations inclusive of *Pogostemon patchouli, Vetivera* spp. and *Santalum* spp. promoted noteworthy activity (Orchard *et al.*, 2018a). In another study, the antimicrobial activities of 55 selected essential oils were investigated against *S. aureus*. Several oils, such as *C. verum, Syzigium aromaticum* and the *Santalum* spp., were highlighted as having the most promising activity (Orchard *et al.*, 2018b). In a study by Sfeir *et al.* (2013), 18 essential oils were chosen according to their chemical constituents and tested against *S. pyogenes*, with *C. verum* displaying the most noteworthy activity with an MIC of 0.19% $^{v}/_{v}$.

The antibacterial activity of each essential oil is dependent on the chemical constituents present within. Each oil can contain a mix of anything between a few to over 300 different organic compounds which can be classed according to their chemical functional groups (Dhifi *et al.*, 2016), which in turn directly influences the anti-bacterial activity. Groups such as phenols or aldehydes are reported to have the strongest anti-bacterial effect, hence essential oils containing compounds such as eugenol, thymol and citral make for the most potent antimicrobials. Other groups such as ketones and terpene hydrocarbons may exhibit little to no activity, therefore decreasing the overall antimicrobial activity (Dorman and Deans, 2000; Lambert *et al.*, 2001). The individual compounds within an essential oil may be identified by gas-chromatography mass-spectrometry (GC-MS). Variations in these chemical compounds and their relative concentrations may arise from a number of aspects. Factors such as harvesting, environmental factors and genetic variability as well as the extraction method of the essential oil will affect the chemical composition, which in turn directly impacts the antimicrobial activity (Marotti *et al.*, 1994; Hussain *et al.*, 2008; Anwar *et al.*, 2009). Therefore, it is of utmost importance that the chemical analysis data for essential oil under investigation be reported so as to account for any variations.

At present, a wide selection of natural products incorporating essential oils are available on the market that are used to treat acne and blemished skin. Skin care ranges consisting of cleansers, toners, serums, moisturisers and spot treatments are available which contain *Melaleuca alternifolia* (tea tree), *L. angustifolia* or *Rosmarinus officinalis* (rosemary) or blends thereof. However, these products remain invalidated and often contain minimal amounts of essential oil mainly incorporated for marketing purposes. Additionally, studies have highlighted other essential oils with far more potent antimicrobial activity than those which are commercially very popular, such as *M. alternifolia* (Orchard and Van Vuuren, 2017; Orchard *et al.*, 2018a; Orchard *et al.*, 2018b). For example, Orchard *et al.* (2017) identified *M. alternifolia* as having an MIC value of 2.00 mg/mL and of 4.00 mg/mL against *C. acnes* and *S. epidermidis* respectively, whereas essential oils such as *Pogostemon patchouli* (patchouli), *Santalum album* (sandalwood) and *Syzigium aromaticum* (clove) displayed MIC values of less than 1.00 mg/mL against both pathogens. As such, there is a paucity of studies on the formulation of these less popular essential oils into effective preparations.

1.5 Formulation

Essential oils are highly volatile substances, making their formulation and application a challenge, as they may evaporate before exerting a therapeutic effect (Donsì and Ferrari, 2016; Winkelman, 2018). As oils, their hydrophobicity presents difficulties in formulating a stable delivery form. Currently, there is a gap in the market for an essential oil formulation that is scientifically proven against acne. Preparations containing essential oils require precise formulation methods to produce a system that allows them to be delivered to the cutaneous tissue without compromising their stability or efficacy (Sherry *et al.,* 2013; Castangia *et al.*, 2015). While essential oils are well known for their many topical effects, they can rarely be applied undiluted to the skin (Lahlou, 2004). Due to their natural origin, they are often perceived to be safe and without side-effects. However, undiluted application has been shown to cause skin irritation and contact dermatitis (Trattner *et al.*, 2008). It is thus necessary to formulate an essential oil within a suitable drug delivery system before topical use. As emulsions form the basis of nearly all topical creams, lotions and ointments (Eccleston, 1997), a suitable emulsion system needs to be formulated that is stable and releases the essential oil from the emulsion to elicit its bacterial effect. In most cases, the higher the concentration of emulsifier added to an emulsion, the more physically stable it is (less prone to crack, coalesce, and separate). However, the higher the concentration of the emulsifier in the emulsion, the more it prevents the release of the essential oil. A careful balance between the stability of the emulsion and the release of the essential oil needs to be achieved (Florence and Attwood, 2015; Sanad and Mabrouk, 2016; Han, 2018).

1.5.1 Emulsions and stability

An emulsion is defined as an immiscible liquid dispersed within another liquid in the form of micro or nano-sized droplets. A third constituent, an emulsifier, is utilised to keep the system stable and in equilibrium (Sadtler *et al.*, 2002). Emulsions are frequently used in cosmetic formulations as it is an excellent means of topical administration of hydrophobic active ingredients (Otto and du Plessis, 2015). The advantages of emulsions in skin care are numerous.

They are relatively easy to prepare, inexpensive and their solubilizing capacity for both hydrophilic and lipophilic actives ensure an increased bioavailability (Kogan and Garti, 2006).

Emulsions may be oil-in-water ($^{O}/_{W}$), which are comprised of an oil phase (for example, the essential oil) dispersed uniformly throughout an aqueous phase, or conversely, a water-in-oil ($^{W}/_{O}$) emulsion in which water is dispensed as droplets in an external oil phase (Walstra, 1993). However, emulsions are thermodynamically unstable and will eventually destabilize with time due to creaming, cracking, coalescence, flocculation and phase separation (Figure 1.2) (McClements and Jafari, 2018).

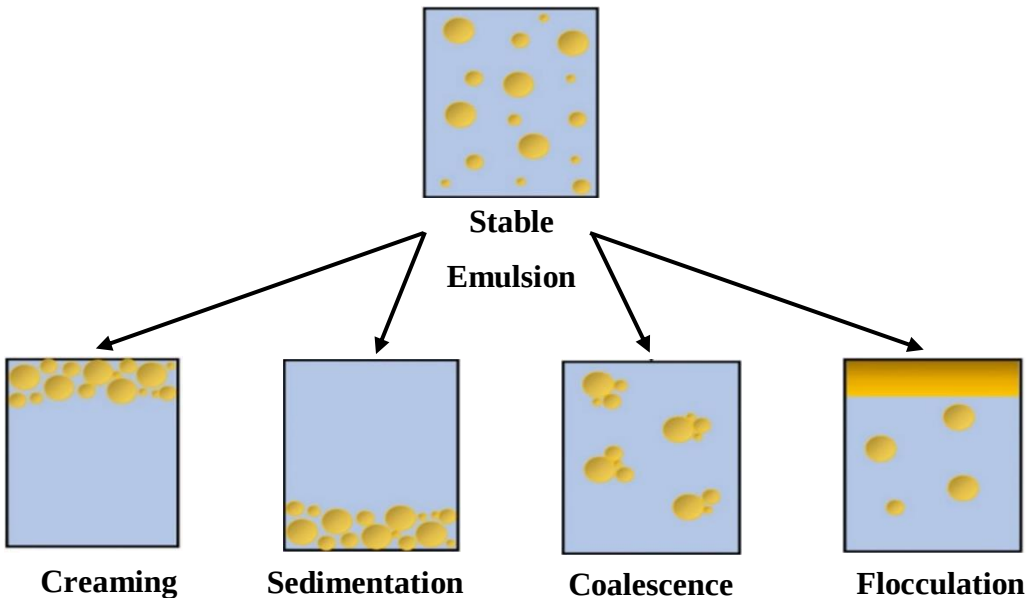


Figure 1.2: Stability mechanisms between oil and aqueous phase. Adapted from Church *et al.* (2019)

Creaming refers to the lifting of essential oil droplets (due to their lower specific gravity) to the surface of the emulsion to form a layer of cream, thereby resulting in the separation of the two phases (Guerra-Rosas *et al.*, 2016; Xiang and Runge, 2016). During coalescence, small oil droplets gradually merge and combine to form larger droplets whereas flocculation refers to the aggregation of oil droplets whilst still retaining their individual integrity (Kulkarni and Shaw, 2015). Interfacial tension, electrostatic repulsion and viscosity of the continuous phase are the biggest factors in the destabilization of emulsions. Emulsifiers play a crucial role as they lower the interfacial tension between individual essential oil droplets, thereby preventing coagulation of smaller droplets to larger ones. A smaller droplet size is desirable as it allows

for the minor phase to remain uniformly distributed throughout and render the phases less likely to separate (Sadtler *et al.*, 2002; Tadros *et al.*, 2004). Therefore, the inclusion of an emulsifier is vital to ensure the stability of an essential oil emulsion and improve the shelf life. The choice of an emulsifier, or blend therefore, is fundamental as not only does it determine how easily the emulsion forms but it also influences the overall properties of the emulsions as well, such as oil droplet size, rate of separation and turbidity (Takamura *et al.*, 1979).

1.5.2 Emulsions and hydrophilic-lipophilic balance (HLB)

The chemical structure of emulsifier molecules typically have a lipophilic end which binds to the oil phase, and a hydrophilic end which binds to the aqueous phase, together keeping the two phases in equilibrium (Figure 1.3). The ratio of the hydrophilic end to the lipophilic end of an emulsifier is referred to as the hydrophilic–lipophilic balance (HLB). A low HLB (10 < X) is characteristic of an emulsifier that is lipophilic, while hydrophilic emulsifiers will have a high HLB (10 > X). A combination of emulsifiers is often recommended as it may provide added stability and enhance the efficacy and functionality of the emulsion (McClements and Jafari, 2018). The required HLB (rHLB) of a system is the HLB of the emulsifier or combination thereof which will allow for the most stable emulsion (Genot *et al.*, 2013). According to Orafidiya and Oladimeji (2002), optimal stability is achieved when the HLB of the emulsifier blend is as close as possible to the rHLB value of the oil phase. The rHLB is calculated from the HLB of each individual ingredient of the oil phase and their relative contribution.

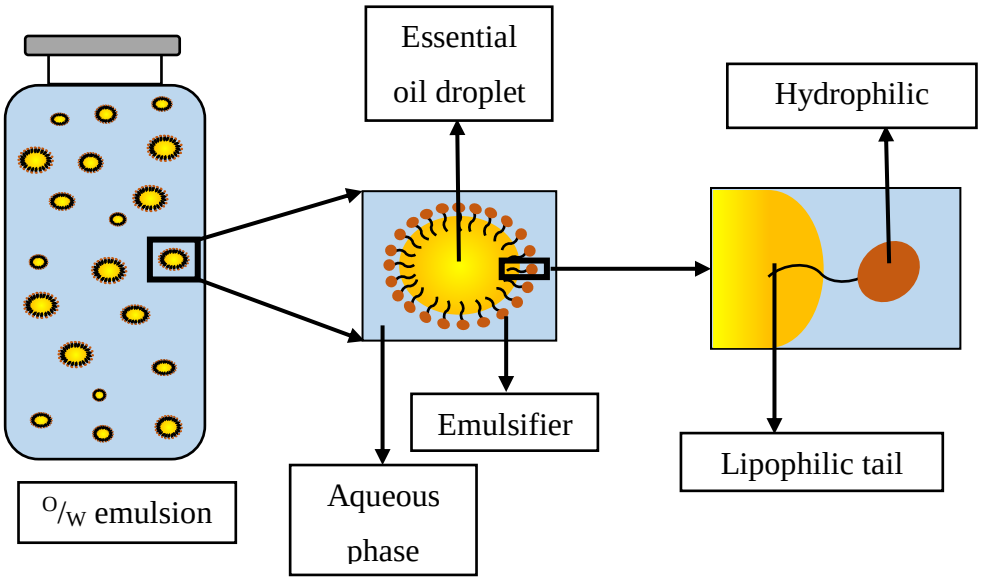


Figure 1.3: Emulsifier mechanism of action. Adapted from Prakash *et al.* (2018)

When formulated at an optimum HLB, the emulsions maintains its droplet size and hence remains stable for longer periods (Mikulcová *et al.*, 2017; Lu *et al.*, 2018). Fortunately, the HLB of many common emulsifiers and excipients such as the Span© series, Tween© series and glycerol stearates are known, and widely reported in literature (Meher *et al.*, 2013). However, the rHLB of many essential oils, including those to be investigated in this study, are not known and hence must be determined experimentally in order to formulate stable emulsions thereof.

In order to determine the HLB of an essential oil, emulsions thereof are prepared at a range of HLB values, and the HLB which produces the most stable emulsion is selected as optimal. Chemical composition of essential oils may affect HLB as the same oil investigated in different studies show different HLBs. For example, Table 1.1 displays two studies that both report the HLB of *Allium sativum* (garlic), each yielding a value of 14.00 and 7.92 respectively. One reason for the major difference could be variations in the percentage composition of chemical constituents giving rise to different lipophilicities, thus affecting the overall HLB. However, the chemical constituents have not been reported in these studies and should always be included in future studies in order to rule out any disparities. Further investigations are required to determine the influence of type of essential oil on the rHLB.

1.5.3 Formulation methods

The method of fabrication of an emulsion is an important aspect for consideration. It plays a decisive role in several emulsion properties such as droplet size and aggregation and hence should be carefully selected and optimized. Emulsions are thermodynamically unstable systems and therefore require energy, either from mechanical input or chemical potential, in order to maintain equilibrium (Walstra, 1993). This principle gives rise to the two main approaches to emulsification: low-energy methods and high-energy methods.

1.5.3.1 Low-energy methods

These methods rely on the chemical energy of components (especially the emulsifier) and phase transitions in order to form emulsions (Sadurní *et al.*, 2005). Low levels of energy in the form of either stirring or heating are additionally required in order to overcome the kinetic barrier (McClements and Jafari, 2018; Pavoni *et al.*, 2020). Spontaneous emulsification method involves the addition of either (i) water to an oil and emulsifier mixture or (ii) oil to a water

and emulsifier mixture with constant stirring until a homogenous product is achieved. The bottle method is a crude method which consists of adding the emulsifier, oil phase and aqueous phase into a screw-cap bottle and vigorously shaking until an emulsion is formed. The phase inversion temperature (PIT) method refers to the conversion of the dispersed phase to the continuous phase and vice versa under the addition of heat (Figure 1.4). It relies on the concept that emulsifiers invert from hydrophilic to lipophilic or vice versa in responses to changes in temperature. In principle, the oil phase and aqueous phase are both heated separately to a temperature above the PIT, combined and then stirred until homogenous dispersions are obtained (Viyoch *et al.*, 2006).

These methods are commonly employed by industries as they utilise low levels of energy, thereby enabling companies to save on high-input machinery. However, they are not compatible with all formulations as they are unsuitable for use with certain oils or emulsifiers such as long chain triglycerides (Pathania *et al.*, 2018; Pavoni *et al.*, 2020). They also require higher concentrations of emulsifier in order to sufficiently lower the interfacial tension of the system to assist with the break-up of oil droplets which may then increase production costs (Pathania *et al.*, 2018; Pavoni *et al.*, 2020). Additionally, thermosensitive compounds such as essential oils may be degraded by the high temperatures employed by PIT, therefore careful monitoring is required.

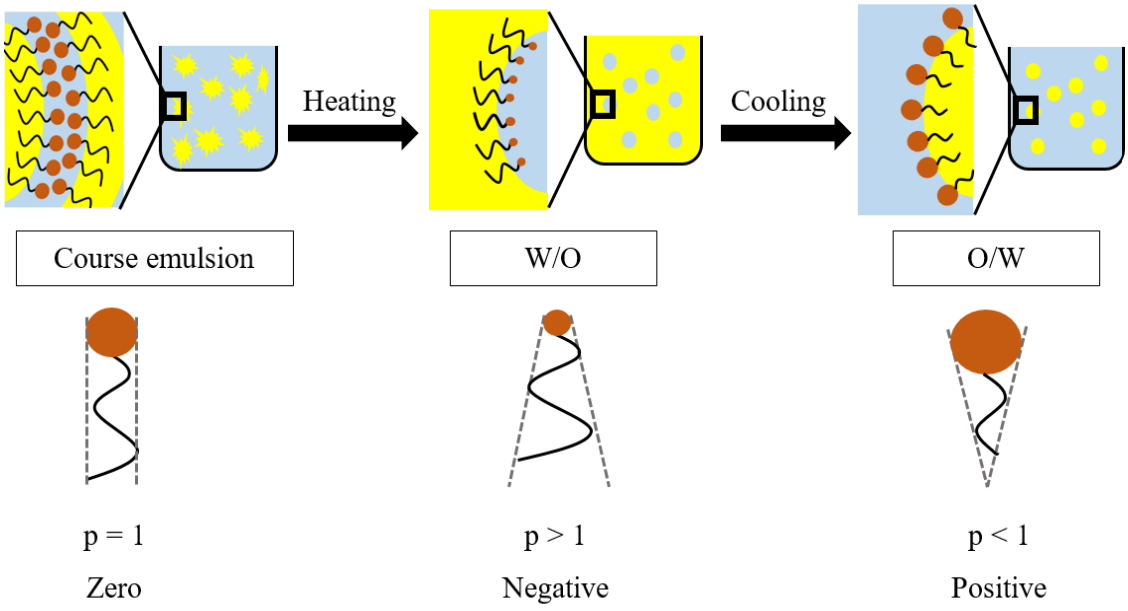


Figure 1.4: Principle of PIT method showing curvature of emulsifier and favoured emulsion type with changes in temperature. Adapted from Gupta *et al.* (2009).

1.5.3.2 High-energy methods

High-energy devices are employed by these methods to mechanically reduce large oil droplets into smaller ones (Donsì and Ferrari, 2016; Pavoni *et al.*, 2020). High-pressure homogenisers contain specialised chambers where high pressures induce a course emulsion to forcibly pass through narrow valves, thereby creating fine oil droplets (Donsì and Ferrari, 2016; McClements and Jafari, 2018). The size of the oil droplets obtained depend on the number of passes, intensity and pressure applied (Donsì and Ferrari, 2016; Pathania *et al.*, 2018). Ultrasonification is another method that is widely employed and makes use of an ultrasonic bath or probe to generate high intensity waves. These, in turn. create disruptive forces which efficiently break-up oil droplets (Donsì and Ferrari, 2016; Pathania *et al.*, 2018; Pavoni *et al.*, 2020). In a similar fashion to homogenisation, increased ultrasonication time leads to smaller oil droplets (Pavoni *et al.*, 2020). These high-energy methods tend to produce very fine emulsions with a uniform droplet size distribution usually of the nano-range (20 – 200 nm) which ensure their stability and prolong shelf-life (Sadurní *et al.*, 2005; Donsì and Ferrari, 2016). However, multiple passes or extended processing times to achieve a desired droplet size may lead to a massive energy consumption, rendering the process inefficient. Furthermore, the intense pockets of pressure created by ultrasonication may disrupt the valuable compounds within essential oils, thereby degrading their antimicrobial activity (Donsì and Ferrari, 2016). Therefore, lengthy processing by sonication is not recommended for the formulations involving essential oils.

It is thus recommended in practice to employ a combination of both high and low-energy methods to create stable, uniform emulsions while retaining the integrity of the essential oils (Sadurní *et al.*, 2005).

1.5.4 Formulation development

Lotions are a form of emulsions that have a high water content, which upon application evaporates to create a cooling effect. They are non-greasy and non-sticky, making them ideal for facial use and their low viscosity provides a pleasant aesthetic feel. They are less comedogenic than creams, which make them superior for the use in the treatment of acne. However, certain lotions may cause allergic reactions or contact dermatitis if formulated with a large solvent concentration, and some lotions offer poor penetration due to large drug particle

size (Garg *et al.*, 2015). Fortunately, a wide array of excipients are available to improve on its limitations and should be carefully selected to create a refined formulation.

The excipients and their relative concentrations within a formulation play a crucial role as they can directly impact the properties of an emulsion. The selection of emulsifier and its concentration directly controls the final droplet size obtained, regardless of the method of emulsification used (Donsì and Ferrari, 2016). The concentration of the emulsifier is also fundamental as demonstrated by Chang *et al*., (2013) where the mean droplet size decreased from 5000 nm to less than 25 nm with an increase of emulsifier (Tween® 80) from 5% to 20%. As emulsifiers decrease the interfacial tension, several mechanisms such as the rate of coalescence and droplet break-up shift to favour smaller droplet sizes (Walstra, 1993). In addition to stability, droplet size affects the antimicrobial activity of emulsified essential oils. Smaller droplet sizes correspond to an increased surface area to volume ratio and facilitates the transfer of active ingredients across biological membranes (Tadros *et al.*, 2004; Salvia-Trujillo *et al.*, 2015), thereby increasing their efficacy by allowing them to effectively penetrate their target sites.

Other than emulsifiers, certain excipients may provide valuable improvements towards stability. Viscosity enhancers, such as acacia gum, tragacanth and methylcellulose, are commonly used in topical formulations to adjust the thickness of the dispersed phase. According to Stokes' Law, creaming may be prevented by decreasing the difference in density between the two phases, i.e. increasing the viscosity of the dispersed phase (Walstra, 1993). A denser aqueous phase prevents the migration of oil droplets to the surface of the emulsion, delaying creaming even further (Pathak, 2017). Other agents, such as cetyl alcohol and stearic acid, may provide benefit as co-emulsifiers, thickeners and emollients (Garg *et al.*, 2015) and serve to improve the long-term stability of formulations.

The selection of excipients is important as the base formulation may affect the release of the drug and hence its activity (Omotosho *et al.*, 1986). While emulsification results in increased stability, it may lead to entrapment of the active essential oil within the formulation and reduce the antimicrobial activity. A strong lipophilic affinity of the drug to the base, as well as a high viscosity, may hinder the release of the drug to its active site (Florence and Attwood, 2015; Sanad and Mabrouk, 2016; Han, 2018). This also makes the assessment of the antimicrobial activity of formulations a challenge as hydrophobic bases do not diffuse easily into hydrophilic

agar and test media, minimising contact between the active components and pathogens to be tested (Orafidiya *et al.*, 2002). The choice of the base is therefore of utmost importance in the design of a topical delivery form in order to ensure maximum bioavailability (Andersson *et al.*, 2013).

1.6 Antimicrobial testing of formulations

While formulation stability and aesthetic appeal are of utmost importance, these factors should not hinder the antimicrobial activity of the preparation. While the standardised methods available for *in vitro* testing of antimicrobial agents are compatible with neat essential oils (CLSI, 2020), their formulations often cannot always be assayed in the same way due to property differences within the formulation. Before selecting an antimicrobial method the viscosity, spreadability and hydrophilicity of the formulation must be taken into account as these factors have the ability to influence the result. Methods devised should also take into consideration the incubation conditions of pathogens to be tested as some methods may not be compatible with anaerobic or fastidious organisms (Isenberg, 2004). Fortunately, the current methods available can be easily adapted to suit individual studies.

1.6.1 Broth microdilution method

Broth microdilution is one of the most basic and widely employed methods available. A 96-well sterile plate is filled with broth and appropriate starting stock concentrations of formulations to be tested are filled into the first well and doubling serial dilutions are performed. Each well is then inoculated with the relevant culture solution and incubated as per respective pathogen conditions. With the use of an indicator, the lowest concentration of the agent required to completely inhibit the growth of the pathogen may be determined. A dye such as *p*-iodonitrotetrazolium violet (INT), is one such suitable indicator. Normally a colourless compound, it undergoes reduction in the presence of biologically active cells to form a coloured formazon dye, which provides an easy colorimetric result (Eloff, 1998; Lall *et al.*, 2013). The lowest concentration of the agent at which the indicator remains colourless deemed the MIC (CLSI, 2003). The advantages of this method is the generation of quantitative MIC values, reproducibility of the test and low volumes of reagents needed. However, the manual labour involved is often considered tedious (Jorgensen and Ferraro, 2009). Formulations to be tested

may also be immiscible with certain test media, thereby reducing contact between the micro-organism and formulation and leading to inaccurate results.

1.6.2 Agar disc diffusion method

This is a well-established technique for determining antimicrobial susceptibility. Agar plates are inoculated with a standardised concentration of the micro-organism. Thereafter, sterile paper discs (usually 6 mm in diameter) are saturated with the formulation and placed upon the seeded agar. This method allows for the test formulation to diffuse out into the agar and inhibit the micro-organism. Plates are then incubated according to respective conditions and the zone of inhibition around each disc is measured (CLSI, 2003). Agar disc diffusion is advantageous as it is simplistic, low cost and provides easy interpretation of results. It yields qualitative data as it allows micro-organisms to be classed as either susceptible, intermediate or resistant and compared to existing guidelines (Jorgensen and Ferraro, 2009). However, no quantitative information such as MIC values can be obtained. Additionally, as this method relies on the principle of growth inhibition, no distinction between bactericidal and bacteriostatic effect can be made (Balouiri *et al.*, 2016). Zones of inhibition are also dependant on the rate of diffusion through the hydrophilic agar, therefore formulations which are lipophilic in nature and are of a high viscosity may yield inaccurate results (Jorgensen and Ferraro, 2009; Sanad and Mabrouk, 2016; Han, 2018).

1.6.3 Time-kill assays

Time-kill allows for the examination of the rate at which a formulation kills a known concentration of micro-organism. This method can be used to determine whether the bactericidal effect exerted by the formulation is time-dependant or concentration-dependant (Isenberg, 2004). Several dilutions containing different concentrations of the formulation are inoculated with culture and incubated. Aliquots are withdrawn from the dilutions at specified time intervals and plated to determine the colony forming units (CFU) per ml, which are then plotted onto a logarithmic curve. Results obtained need to be compared to both a positive and negative control. Despite being labour-intensive and time-consuming, time-kill assay is a powerful tool which has been proven to produce reliable results (Isenberg, 2004; Balouiri *et al.*, 2016; CLSI, 2020).

Different methods of antimicrobial testing provide different data points, however, the selected method should as far as possible eliminate the influence of the emulsion properties on the obtained result. Antibacterial testing should ideally be repeated after a time period to ensure maintenance of activity.

1.7 Review of essential oil formulation studies through the HLB approach

Previous studies have investigated the formulation of various essential oil formulations for application in the treatment of acne (Viyoch *et al.*, 2006; Ansila *et al.*, 2017; Taleb *et al.*, 2018; Misar *et al.*, 2020). However, these studies did not follow the HLB approach, which allows for the selection of the formulation with optimal stability, ensuring minimal destabilisation and prolonged shelf-life. A few studies where the emulsification of essential oils into various delivery systems using the HLB approach is detailed in Table 1.1. To date, the HLB of essential oils still remain largely unreported in literature. While the specific HLB value of an essential oil may vary between batches as a result of the different chemical constituents contained within, the HLB still provides crucial data on the lipophilicity of various essential oils which allows for the facilitation of their emulsification. These studies (Table 1.1) have demonstrated the compatibility of the HLB approach with volatile components such as essential oils, as well as the reliability of the HLB method in producing stable emulsions.

Sanad and Mabrouk (2016) investigated the formulation of two prominent antimicrobial essential oils, *A. sativum* and *Eruca sativa* (rocket) through the HLB approach. A range of emulsions were prepared at HLB 5-15 for each essential oil, and the rHLB was found to be 7.92 for *A. sativum* and 9.76 for *E. sativa* respectively. These values were then used to prepare a stable cream containing a 1:1 ratio of each at various emulsifier concentrations. At an emulsifier blend of 2% $^{v}/_{v}$, the cream was stable for 12 months with no major change in pH or consistency. Additionally, the cream was found to display antimicrobial activity against *S. aureus, Escherichia coli* and antifungal activity against *Malassezia fufur*, comparable to the neat oils themselves. However, Sanad and Mabrouk (2016) found that any increase in the emulsifier blend would increase the viscosity, thereby decreasing the antimicrobial activity. This highlights the importance of considering the impact of the formulation parameters on the antimicrobial activity.

Nirmal *et al.* (2018) investigated the formulation and characterisation of *Backhousia citriodora* (lemon myrtle) and *Syzygium anisatum* (anise myrtle) nano-emulsions and their ensuing antimicrobial activity against food-borne pathogens. A set of emulsions from HLB 8-15 were prepared for each essential oil and the rHLB was determined as 14 for *B. citriodora* and 12 for *S. anisatum.* The emulsions at optimal HLB were then selected for further evaluation. The *S. anisatum* nano-emulsion, as well as the neat *S. anisatum* oil, showed no inhibitory effects against *S. aureus*, *L. monocytogenes* and *E. coli*. However, the *B. citriodora* nano-emulsion at optimal HLB showed an improved MIC of 0.062% $^{v}/_{v}$, 0.031% $^{v}/_{v}$ and 0.25% $^{v}/_{v}$ against *S. aureus*, *L. monocytogenes* and *E. coli* respectively in comparison to the neat essential oil (MICs of 0.16% $^{v}/_{v}$ against *S. aureus* and *Listeria monocytogenes* and 0.63 % $^{v}/_{v}$ against *E. coli*). Therefore, emulsification of the *B. citriodora* essential oil was found to increase its antimicrobial activity.

The development of a stable *Ocimum basilicum* (sweet basil) antiseptic cream through the HLB approach was explored by Yadav *et al.* (2013). The HLB of neat *O. basilicum* was found to be 13.36, which was then used to calculate the rHLB of the cream formulation, taking into account the excipients and their relative concentrations. The prepared cream was found to be stable with consistent viscosity and consistency for a period of three months. The optimal formulation displayed comparable activity to the neat *O. basilicum* oil through similar zones of inhibition against *S. aureus*, *Salmonella typhi*, *Yersinia enterocolitica* and other pathogens. While no major increase in antimicrobial activity was seen through emulsification, the cream formulation still provided the protection of the essential oil from environmental stress, heat and degradation. The study by Yadav *et al.* (2013) has therefore provided valuable insight into the HLB approach and elucidated the formulation approach of a $^{O}/_{W}$ antiseptic cream.

Moghimi *et al.* (2016) explored the antimicrobial properties of *Salvia officinalis* (sage) essential oil against food-borne pathogens, and its formulation into an $^{O}/_{W}$ emulsion was attempted in order to enhance its antimicrobial efficacy A range of HLB 8-15 of *S. officinalis* emulsions were prepared, and HLB 12 was found to produce the most stable emulsion. This optimal *S. officinalis* emulsion was stable for six months, and displayed no signs of creaming or major change in droplet size. Emulsification was found to greatly enhance the antimicrobial activity; the *S. officinalis* emulsion displayed an MIC of 2.00 mg/mL against *E. coli* as opposed to 8.00 mg/mL of the neat oil, 4.00 mg/mL against *Shigella dysentery* as opposed to 8.00 mg/mL of the neat oil and 8.00 mg/mL against *S. typhi* as opposed to 32.00 mg/mL of the neat

oil. Thus, the emulsion produced in the study was both physically stable and antimicrobially effective, highlighting the credibility of the HLB approach.

Satureja khuzestanica (savory) is another essential oil which was investigated for its antimicrobial properties with application in food preservation by Mazarei and Rafati (2019). From an HLB range of 8-15, *S. khuzestanica* emulsion at HLB 10 (containing 3% $^{w}/_{w}$ essential oil and 9% $^{w}/_{w}$ emulsifier blend) was found to be the most stable with minor changes in droplet size over two months. The stable *S. khuzestanica* emulsion displayed comparable antimicrobial acitivity against *E. coli* and *Salmonella enterica* in comparison to the neat oil, however, an improved MIC was obtained against *S. aureus* (0.13% for the emulsion versus 0.25% of the neat oil). This once again demonstrates the advantages of emulsification, and that the selection of the optimal HLB provides for long-term stability.

It is worth noting that various essential oil emulsification studies utilising the HLB approach do not explore the antimicrobial potential of the formulations. For example, in Fernandes *et al.* (2013), the essential oils *Illicium verum* (star-anise) and *R. officinalis* are selected for emulsification on the basis of their bioactivities, yet this aspect remains unexplored. Emulsion of *R. officinalis* at HLB 16.5 was stable for two months with no macroscopic changes while that of *I. verum* at HLB 16.7 showed a small degree of creaming, however, it was the most stable out of the range tested. In view of the promising potential of these formulations, investigation of their antimicrobial activities are certainly warranted.

Orafidiya and Oladimeji (2002) investigated the HLB of essential oils *Eucalyptus globulus* (eucalyptus), *Lippia javanica* (fever tea) and *Mentha piperita* (peppermint) on account of their ectoparasitic, insect-repellent and anti-diarrhoeal properties. The bottle method was used to create emulsions of HLB 4.3 to 15.0. Orafidiya and Oladimeji (2002) worked on the assumption that the emulsion displaying the smallest droplet size would be the optimal HLB, as smaller oil droplets are less prone to coalescence and aggregation. Additionally, the retention of the small droplet size over time would indicate the resistance of the emulsion to change, thereby preventing destabilisation. The study also made use of stability parameters such as degree of creaming and turbidity. The various assays used in Orafidiya and Oladimeji (2002) to determine the HLB of the selected essential oils were found to be in agreement and thus could be used to support each other. As such, the HLB of the essential oils were found to be 9.8 for *E. globulus*, 12.1 for *L. javanica* and 12.3 for *M. piperita.*

Table 1.1: Summary of identified HLB values of essential oils and the antimicrobial activities of their formulations where tested.

EO[a]	EO Chemical composition	HLB	Emulsion type	Method of emulsification	Pathogens tested	Method of testing	Result	Reference
A. sativum	-[b]	7.9	$^{O}/_{W}$[c] micro-emulsion	Bottle method	*S. aureus* (ATCC[d] 29737) *E. coli* (ATCC 25299)	Zone of inhibition	11.80 mm ± 0.80 15.80 mm ± 1.20	(Sanad and Mabrouk, 2016)
A. sativum	-	14	$^{O}/_{W}$ nano-emulsion	Ultrasonification	*Penicillium italicum*	MIC	2.30 mg/mL	(Long *et al*., 2020)
B. citriodora	-	14	$^{O}/_{W}$ nano-emulsion	Ultrasonification	*S. aureus* (ATCC 33591) *L. monocytogenes* (ATCC 19111) *E. coli* (ATCC 11775) *Pseudomonas aeruginosa* (ATCC 926)	MIC	0.62 mg/mL 0.31 mg/mL 2.50 mg/mL No activity	(Nirmal *et al*., 2018)
***C. guianensis* (crabwood)**	Palmitic acid Oleic acid β-Sitosterol Methyl angolensate Gedunin 7-Deacetoxy-7-oxogedunin 6α-Acetoxygedunin	16.7	$^{O}/_{W}$ micro-emulsion	PIT[g]	-	-	-	(Ferreira *et al*., 2010)

EO[a]	EO Chemical composition	HLB	Emulsion type	Method of emulsification	Pathogens tested	Method of testing	Result	Reference
***Cymbopogon* spp. (citronella)**	-	12.6	$^{O}/_{W}$ cream formulation	PIT	-	-	-	(Meher *et al.*, 2013)
E. sativa	-	9.8	$^{O}/_{W}$ micro-emulsion	Bottle method	*S. aureus* (ATCC 29737) *E. coli* (ATCC 25299)	Zone of inhibition	16.20 mm ± 1.60 12.40 mm ± 0.90	(Sanad and Mabrouk, 2016)
E. globulus	-	9.8	$^{O}/_{W}$ micro-emulsion	Bottle method	-	-	-	(Orafidiya and Oladimeji, 2002)
I. verum	E-anethole- 80.1% Shisofuran- 1.03% Foeniculin- 3.3%	16.7	$^{O}/_{W}$ nano-emulsion	PIT	-	-	-	*(Fernandes et al*., 2013)
L. angustifolia	-	10	$^{O}/_{W}$ micro-emulsion	PIT	-	-	-	(Martina, *et al*., 2017)
L. javanica	-	12.1	$^{O}/_{W}$ micro-emulsion	Bottle method	-	-	-	(Orafidiya and Oladimeji, 2002)

EO[a]	EO Chemical composition	HLB	Emulsion type	Method of emulsification	Pathogens tested	Method of testing	Result	Reference
O. basilicum	-	13.4	$^{O}/_{W}$ cream formulation	Phase inversion temperature method	*S. aureus* (MTCC[e] 96) *S. epidermidis* (MTCC 435) *Streptococcus mutans* (MTCC 890) *S. typhi* (MTCC 733) *Y. enterocolitica* (MTCC 861) *Klebsiella pneumoniae* (MTCC 109) *Enterobacter aerogenes* (MTCC 111)	Zone of inhibition	5.60 mm ± 0.60 11.00 mm ± 1.00 5.60 mm ± 0.60 5.00 mm ± 1.00 11.30 mm ± 1.50 7.00 mm ± 1.00 9.00 mm ± 1.00	(Yadav *et al.*, 2013)
M. piperita	-	12.3	$^{O}/_{W}$ micro-emulsion	Bottle method	-	-	-	(Orafidiya and Oladimeji, 2002)
R. officinalis	1,8-cineole- 44.0% β-myrcene- 11.7% α-pinene- 9.4%	16.5	$^{O}/_{W}$ nano-emulsion	PIT	-	-	-	*(Fernandes et al.*, 2013)

EO[a]	EO Chemical composition	HLB	Emulsion type	Method of emulsification	Pathogens tested	Method of testing	Result	Reference
S. officinalis	-	12	$^{O}/_{W}$ nano-emulsion	Ultrasonification	*E. coli* (ATCC 25922) *S. dysentery* (PTCC[f] 1188) *S. typhi* (ATCC 1609)	MIC	2.00 mg/mL 4.00 mg/mL 8.00 mg/mL	(Moghimi *et al.*, 2016)
S. khuzestanica	Carvacrol- 87.16% p-cymene- 6.39% β-myrcene- 0.89%	10	$^{O}/_{W}$ nano-emulsion	PIT + Homegenization/ Ultrasonification	*S. aureus* (ATCC 33591) *S. enterica* (PTCC1639) *E. coli* (ATCC 11775)	MIC	0.13 mg/mL 0.25 mg/mL 0.25 mg/mL	(Mazarei and Rafati, 2019)
S. anisatum	-	12	$^{O}/_{W}$ nano-emulsion	Ultrasonification	*S. aureus* (ATCC 33591) *L. monocytogenes* (ATCC 19111) *E. coli* (ATCC 11775) *P. aeruginosa* (ATCC 926)	MIC	No activity No activity No activity No activity	(Nirmal *et al.*, 2018)

[a]EO ~ essential oil
[b]- ~ data not available
[c] $^{O}/_{W}$ ~ Oil-in-water
[d]ATCC ~ American Type Culture Collection
[e]MTCC ~ Microbial Type Culture Collection (India)
[f]PTCC ~ Persian Type Culture Collection
[g]PIT ~ phase inversion temperature method

While minor deviations in the HLB method exist (such as different evaluation parameters), the principle remains the same. Clearly, the HLB approach with essential oils has been explored and well documented, and produces reliable results in terms of emulsion stability. The emulsification of essential oils has been shown to enhance their antimicrobial activity, in addition to protecting the integrity of the essential oil. Therefore, the HLB method provides a suitable approach for the development of a topical anti-acne drug delivery system incorporating essential oils.

1.8 Aim

The aim of this study is to develop a stable anti-acne topical preparation via the HLB method encapsulating an essential oil as its active ingredient that is effective against the four common pathogens involved in acne.

1.9 Objectives

1.9.1. To identify the most appropriate essential oils that would be effective against pathogens that cause acne from previous literature reviews.

1.9.2. To investigate the antimicrobial activity using the broth microdilution assay to determine the MIC of the selected essential oils against pathogens that cause acne.

1.9.3. To develop an appropriate emulsified lotion base taking into account viscosity and stability

1.9.4. To determine the rHLB of the selected essential oils and formulate them into stable topical emulsion formulations.

1.9.5. To conduct stability testing on the prepared formulations for creaming, mean droplet size, polydispersity index and turbidity.

1.9.6. To determine the antimicrobial activity of the formulations against the acne pathogen reference strains.

Figure 1.5 depicts the structural flow of the project.

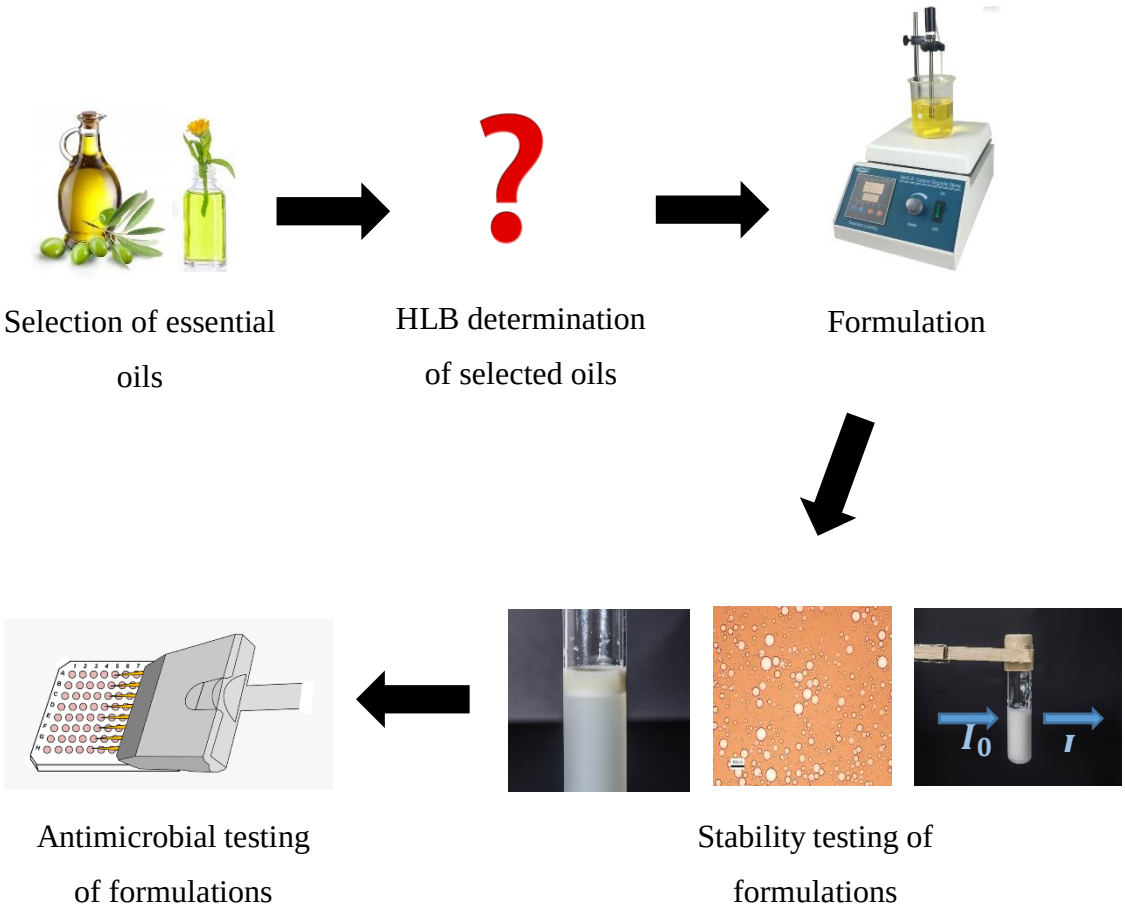


Figure 1.5: Flow diagram of project outline

CHAPTER 2 - ANTIMICROBIAL EFFICACY OF ESSENTIAL OILS AGAINST ACNE-INDUCING PATHOGENS

2.1 Introduction

Scientific studies have shown the *in vitro* benefits of essential oils against a wide array of pathogens (Nazzaro *et al.*, 2013; Swamy *et al.*, 2016; Tariq *et al.*, 2019). In the area of acne specifically, certain essential oils such as *M. alternifolia* (Lee *et al.*, 2013; Hammer, 2015) are touted for their efficacy and have been commercialised as cosmetic products such as toners, serums and creams. However, emerging studies have highlighted other essential oils with stronger antimicrobial activities (Orchard and van Vuuren, 2017; Orchard *et al.*, 2018a; Orchard *et al.*, 2018b; Leigh-de Rapper and van Vuuren, 2020). This chapter explores the essential oils identified from available scientific literature to examine their antimicrobial efficacy against pathogens involved in acne. By determining the antimicrobial activity of various essential oils, those that are the most efficacious will be identified and taken further into the formulation phase.

2.2 Methods and materials

2.2.1 Selection of essential oils and chemical characterization

Based on previous reports of the antimicrobial activity of essential oils (Orchard *et al.*, 2018a; Orchard *et al.*, 2018b; Leigh-de Rapper and van Vuuren, 2020), essential oils displaying noteworthy MIC values of 1.00 mg/mL or less against acne pathogen reference strains were selected. A total of nineteen essential oils were identified and a list thereof is provided in Appendix A. While the antimicrobial activities of these essential oils were previously investigated against *C. acnes*, *S. aureus* and *S. epidermidis* (Orchard *et al*., 2018a; Orchard *et al*., 2018b), the efficacy of certain essential oils against *S. pyogenes* were not explored in van Vuuren and de Rapper (2020). In these cases, the selection criteria was modified to include oils based on noteworthy activity against only three pathogens (*C. acnes*, *S. aureus* and *S. epidermidis*).

2.2.2 Chemical characterization of essential oils

The selected essential oils for antimicrobial analysis and formulation were acquired from Prana Monde (Leuven, Belgium), Scatters Oils (Johannesburg, South Africa), Escentia (Johannesburg, South Africa) or Meadowbank (Durban, South Africa) depending on availability. The GC-MS data of the essential oils were provided by the suppliers. The chemical profiles of each essential oil were analysed and compared to the GC-MS data cited in the baseline literature. Any differences in composition of major chemical constituents were noted in order to account for any differences that may have arisen in antimicrobial activity.

2.2.3 Antimicrobial analysis of essential oils

Antimicrobial assays were performed for the essential oils to identify those which were most effective, in order to proceed to formulation development.

2.2.3.1 Culturing of pathogens

Pathogens were selected based on their degree of involvement in acne, as identified by the literature review of Kumar *et al.* (2016), and were procured from Davies Diagnostics (Johannesburg, South Africa). The facultative anaerobes *S. aureus* (ATCC® 25923™) and *S. epidermidis* (ATCC® 2223 ™) were cultured in Tryptone Soya Broth (TSB) (Oxoid) and incubated at 37°C for 24 hrs. *Streptococcus pyogenes* (ATCC® 12344™) was cultured in Haemophilus Test Medium (HTM) (Oxoid) supplemented with nicotinamide adenine dinucleotide (NAD) (Oxoid). The inoculum was incubated at 37°C for 24 hrs. The aero-tolerant anaerobe *Cutibacterium acnes* (ATCC® 11827 ™) was cultured in Thioglycolate Broth (TGB) (Oxoid) and incubated at 37°C under anaerobic conditions in a CO_2 incubator (5% CO_2) for seven days.

2.2.3.2 Minimum inhibitory concentration assay

The broth microdilution method as per CLSI guidelines (CLSI, 2020) was used to determine the MIC values of the essential oils. Under sterile conditions, 100 µL of the respective broth was added into a prepared 96-well microtitre plate. The essential oils were diluted in acetone to obtain a stock concentration of 32.00 mg/mL, and 100 µL of each was introduced into the

first well of each column so as to obtain a starting concentration was 8.00 mg/mL. Ciprofloxacin (100 µL of a concentration of 0.01 mg/mL), was used as a positive control to ensure microbial susceptibility. Topical macrolides such as erythromycin are usually the antibiotic of choice in the treatment of acne, however, emerging resistance has reduced efficacy of these drugs against certain pathogens associated with acne (Hsueh *et al.*, 1995; Bassetti *et al.*, 2000; Granizo *et al.*, 2000). For this reason, ciprofloxacin was selected as the positive control as it is a broad-spectrum antibiotic, therefore it is expected to show antimicrobial activity against all the acne pathogen reference strains tested within this study. A volume of 100 µL of a 32.00 mg/mL water in acetone preparation was used as the negative control to ensure that antimicrobial activity was not as a result of the solvent. A carrier oil, *Simmondsia chinensis* (jojoba), at 100 µL of a 32.00 mg/mL stock solution was included as a secondary negative control to the essential oils. A 100 µL culture control was included to ensure viable growth of the pathogen. Doubling serial dilutions were then performed on each sample to halve the concentration with each dilution (Figure 2.1).

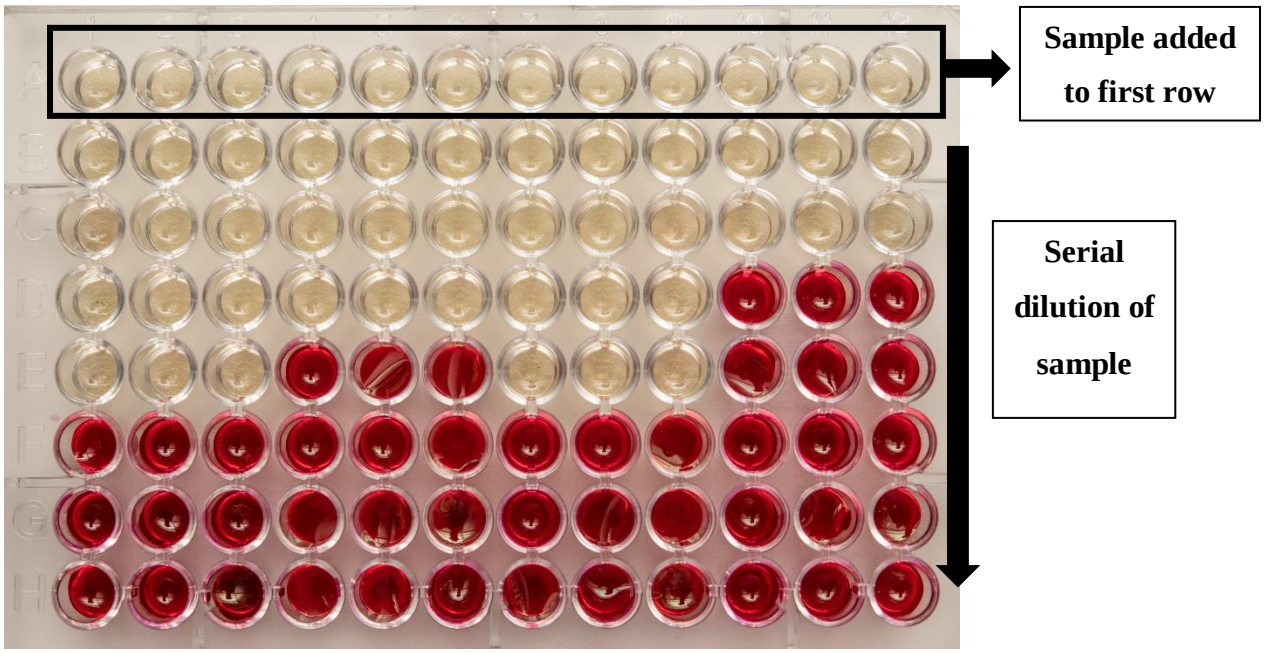


Figure 2.1: Representation of a MIC plate after the addition of INT indicator

A 0.5 McFarland's standard of each pathogen reference strain was prepared to contain an approximate inoculum concentration of 1×10^6 colony forming units (CFU) per mL, and subsequently 100 µL was transferred into each well. The microtitre plates were sealed with sterile adhesive sealing films (to prevent sample evaporation), and incubated as per respective pathogen conditions. After incubation, 40 µL of indicator INT (Sigma-Aldrich®) at 0.04 mg/mL

was added to each well and allowed to react for four to six hours, depending on the response time of the pathogen. A colour change from clear to pink revealed microbial growth, while wells which remained clear were indicative of microbial inhibition. The MIC was interpreted as the first clear well i.e. lowest concentration of sample required to inhibit microbial growth. All tests were performed in triplicate (n = 3) on consecutive days.

2.2.3.3 Data analysis

All MIC results obtained were expected to be noteworthy as all 19 essential oils were selected on the basis that they displayed MICs of ≤ 1.00 mg/mL against the acne pathogen reference strains. Therefore, further distinction between their activities were required. The MIC results were then reclassified as per the categories shown in Table 2.1.

Table 2.1: Reclassification of antimicrobial activity of essential oils (Orchard and van Vuuren, 2017; van Vuuren *et al.*, 2019)

MIC range (mg/mL)	Classification (activity)
≤ 0.50	Outstanding
> 0.50 - ≤ 1.00	Noteworthy
> 1.00 - ≤ 4.00	Moderate
> 4.00 - ≤ 8.00	Poor
> 8.00	Very poor

In order to assess the overall efficacy of each essential oil, the mean MIC value across all four pathogens was calculated. The MIC of *C. acnes* was weighted double to account for its majority influence in the pathogenesis of acne, as shown in Equation 2.1.

$$\frac{(MIC_{C.acnes} \times 2) + MIC_{S.\ aureus} + MIC_{S.epidermidis} + MIC_{S.pyogenes}}{5} = \text{Weighted Mean MIC}$$

Equation 2.1

The weighted mean was then used to rank the essential oils form lowest to highest (i.e. most to least antimicrobially effective). The top five essential oils (displaying the lowest weighted mean MIC values) were then selected for formulation development.

2.3 Results and discussion

2.3.1 Baseline selection of essential oils

From previous studies (Orchard *et al.*, 2018a; Orchard *et al.*, 2018b; Leigh-de Rapper and van Vuuren, 2020), a total of 19 essential oils from these studies were found to display a noteworthy antimicrobial activity against *C. acnes*, *S. epidermidis*, *S. aureus* and *S. pyogenes*. These are shown in more detail in Appendix A.

2.3.2 Chemical analysis of essential oils

A certificate of analysis of the essential oils containing their GC-MS data were provided by the respective companies and the essential oils were analysed to determine chemotype. The major constituents, as determined by GC-MS for each oil, are summarised in Table 2.2. The GC-MS data associated with the MIC values of the essential oils investigated in the baseline literature (Orchard *et al.*, 2018a; Orchard *et al.*, 2018b; Leigh-de Rapper and van Vuuren, 2020) are also included in Table 2.2.

Variations in the GC-MS between the baseline literature (Orchard *et al.*, 2018a; Orchard *et al.*, 2018b; Leigh-de Rapper and van Vuuren, 2020) and the current study were observed. For example, *V. zizanioides* differed in composition with zizanol and *β*-vetirenene previously reported as major constituents, while khusenic acid and khusimol were dominant in the current study. Other oils displayed similar constituents in different concentrations, such as *T. vulgaris* where 46.6% thymol and 17.38% *p*-cymene were obtained in the current study while 18.90% thymol and 41.00% *p*-cymene were previously reported (Orchard *et al*., 2017; Leigh-de Rapper and van Vuuren, 2020). The dominant constituent in the current batch of *R. damascena* was citronella (29.90%) while phenyl ethyl alcohol (63.90%) prevailed in Orchard *et al.* (2018a).

Table 2.2: Chemical composition of essential oils

Essential oil	Current study		Baseline literature	
	Major constituents present	**Supplier**	**Major constituents identified**	**Reference**
***C. verum* J.Presl. (cinnamon bark)**	Cinnamaldehyde (56.97%) β-caryophyllene[a] (1.31%) Cinnamyl Acetate[a] (1.36%) Eugenyl Acetate (8.96%) Linalool[a] (3.17%) Limonene[a] (2.80%)	Scatters	Cinnamaldehyde (61.5%) β-caryophyllene (6.80%) Cinnamyl acetate (6.50%) Eugenol[a] (3.70%)	(Orchard *et al.*, 2018a)
***Cinnamomum zeylanicum* Blume. (cinnamon leaf)**	Eugenol (71.98%) β-caryophyllene[a] (3.31%) Eugenyl acetate[a] (4.79%) Safrole[a] (2.69%)	Scatters	Eugenol (84.7%) β-caryophyllene (6.80%)	(Orchard *et al.*, 2018a)
			Eugenol (78.40%)	(Orchard *et al.*, 2017; Leigh-de Rapper and van Vuuren, 2020)
***Cymbopogon citratus* Stapf. (lemongrass)**	Geranial (42.70%) Neral (28.90%) Limonene (11.70%)	Prana Monde	Geranial (42.70%) Neral (28.90%) Limonene (11.70%)	(Orchard *et al.*, 2017; Leigh-de Rapper and van Vuuren, 2020)
***Cymbopogon martini* Wats. (palmarosa)**	Geraniol (80.70%)	Prana Monde	Geraniol (80.70%)	(Orchard *et al.*, 2017)
***Commiphora myrrha* Engl. (myrrh)**	Furanoeudesma-1,3-diene (46.38%) Lindestrene[a] (4.99%) Curzerene (21.99 %)	Prana Monde	Furanoeusdema-1,3-diene (52.9%) Lindestrene (15.8%)	(Orchard *et al.*, 2017)
***Cymbopogon nardus* Rendle. (citronella)**	Citronellal (42.10%) Geraniol (20.60%) Citronellol (18.50%)	Meadowbank	Citronellal (42.10%) Geraniol (20.60%) Citronellol (18.50%)	(Orchard *et al.*, 2018a)

Essential oil	Current study		Baseline literature	
	Major constituents present	**Supplier**	**Major constituents identified**	**Reference**
***Laurus nobilis* L. (bay)**	Eugenol (54.40%) Myrcene (18.50%) Chavicol (11.50%)	Prana Monde	Eugenol (54.40%) Myrcene (18.50%) Chavicol (11.50%)	(Orchard *et al.*, 2017; Leigh-de Rapper and van Vuuren, 2020)
***Litsea cubeba* Pers. (may chang)**	Geranial (34.10%) Neral (32.80%) Limonene (14.27%) β-pinene[a] (2.32%)	Scatters	Geranial (44.6%) Neral (28.8%)	(Orchard *et al.*, 2017)
***Matricaria recutita* L. (German chamomile)**	Bisabolene oxide A (52.30%) β-farnesene (19.40%) Chamazulene[a] (3.00%)	Prana Monde	Bisabolene oxide A (52.30%) β-farnesene (19.40%) Chamazulene[a] (3.00%)	(Orchard *et al.*, 2018a)
***Nardostachys jatamansi* DC. (spikenard)**	β-gurjunene (9.70%) Ledene (9.10%) α-panasinsen[a] (3.40%) Spirotetemol (5.60%)	Aromatics (Nepal)	β-gurjunene (9.70%) Ledene (9.10%) α-panasinsen[a] (3.40%)	(Orchard *et al.*, 2018a)
***Ocimum tenuiflorum* L. (holy basil aromatics)**	Eugenol (57.30%) β -caryophyllene (30.90%) α-humulene[a] (3.60%)	Aromatics (India)	Eugenol (57.30%) β -caryophyllene (30.90%) α -humulene[a] (3.60%)	(Orchard *et al.*, 2018a)
***Origanum vulgare* L. (oregano)**	Carvacrol (60.40%) Thymol (5.67%) γ-terpinene (7.89%) p-cymene (7.06%)	Prana Monde	Carvacrol (51.7%) Thymol (22.5%) γ-terpinene (10.2%) p-cymene (7.2%)	(Orchard *et al.*, 2018a)
			Carvacrol (67.90%) Thymol (5.50%) λ-terpinene (5.30%) p-cymene (7.60%) β-caryophyllene[a] (3.40%)	(Leigh-de Rapper and van Vuuren, 2020)

Essential oil	Current study		Baseline literature	
	Major constituents present	**Supplier**	**Major constituents identified**	**Reference**
***Pelargonium graveolens* L'Her. (rose geranium)**	Citronellol (26.70%) Eudesmol (12.50%) Citronellyl formate (11.50%) Menthone (7.80%)	Escentia	Citronellol (26.70%) Eudesmol (12.50%) Citronellyl formate (11.50%) Menthone (7.80%)	(Orchard *et al.*, 2018a)
***P. patchouli* Benth. (patchouli)**	α-bulnesene (18.04%) α-guai'ene (14.98%) β-patchoulene[a] (3.20%) Patchouli alcohol (30.80%) α-patchoulene (7.42%) δ-gurjunene (5.03%)	Scatters	α-bulnesene (13.00%) α-guai'ene (11.90%) β-patchoulene (38.30%)	(Orchard *et al.*, 2017)
***Rosa damascena* Mill. (rose otto)**	Citronellol (29.90%) Phenelyl Alcohol (22.40%) Geraniol (20.80%) Nerol (10.20%)	Prana Monde	Citronellol (14.60%) Phenyl ethyl alcohol (63.90%) Geraniol (7.90%) Nerol (6.80%)	(Orchard *et al.*, 2018a)
			Citronellol (29.90%) Phenelyl Alcohol (22.40%) Geraniol (20.80%) Nerol (10.20%)	(Leigh-de Rapper and van Vuuren, 2020)
***Santalum austrocaledonicum* Vieill. (sandalwood)**	α-santalol (52.70%) β-santalol (15.70%) Bergamotol (7.10%) cis-santalol[a] (3.80%)	Prana Monde	α-santalol (52.70%) β-santalol (15.70%) Bergamotol (7.10%) cis-santalol[a] (3.80%)	(Orchard *et al.*, 2018a)
***S. aromaticum* L. (clove)**	Eugenol (71.90%) β-caryophyllene[a] (3.30%) Cinnamaldehyde[a] (1.75%)	Scatters	Eugenol (81.90%) Isoeugenol (13.10%)	(Orchard *et al.*, 2017; Leigh-de Rapper and van Vuuren, 2020)

Essential oil	Current study		Baseline literature	
	Major constituents present	**Supplier**	**Major constituents identified**	**Reference**
***Thymus vulgaris* L. (thyme)**	Thymol (46.6%) p-cymene (17.38%) c-terpinene (9.58%) Carvacrol (5.16%)	Prana Monde	Thymol (18.90%) p-cymene (41.00%) c-terpinene (7.20%)	(Orchard *et al.*, 2017; Leigh-de Rapper and van Vuuren, 2020)
***V. zizanioides* Nash. (vetiver)**	Khusenic acid (15.87%) Khusimol (7.93%) Isovalencenol (7.39%) α-vetivone[a] (3.73%) β-vetivone[a] (2.67%)	Prana Monde	Zizanol (12.80%) β-Vetirenene (8.80%)	(Orchard *et al.*, 2017)

[a]Constituents that are < 5.00% are classified as minor but are still shown for completion.

Essential oils have the ability to inhibit the growth of a wide range of pathogens (Tariq *et al.*, 2019), and the diverse chemical constituents present within the oils are responsible for their wide range of bioactivities (Swamy *et al.*, 2016). The chemical constituents in an oil may differ in concentration due to variations in chemotype, harvesting season and environmental conditions (Nazzaro *et al.*, 2013). Therefore, any variations in antibacterial activity between essential oil batches may often be attributed to these differences (Hussain *et al.*, 2008; Anwar *et al.*, 2009). The chemical profile of procured essential oils should thus always be analysed beforehand in order to explain these variations as well as any trends in antimicrobial activity observed. Furthermore, as established from this study, the chemical constituents of the study oils are mostly consistent with that reported in the literature, apart from the few exceptions shown.

2.3.3 Minimum inhibitory concentration of essential oils

The essentials oils were investigated for antibacterial activity against the four selected pathogen reference strains. The MIC values against each pathogen reference strain are shown in Table 2.3. The essential oils are listed from most to least effective i.e. in order of increasing weighted mean MIC values (Equation 2.1). Certain samples exhibited very poor activity during the assay at the concentrations tested and were denoted as having an MIC > 8.00 mg/mL. Thus, the mean

and weighted mean values could not be calculated for these essential oils as the MIC values remained undefined.

Table 2.3: MIC values (mg/mL) of essential oils against acne pathogen reference strains (n=3)

Essential oil	MIC (mg/mL)				
	***C. acnes*[a]**	***S. epidermidis*[b]**	***S. aureus*[c]**	***S. pyogenes*[d]**	**Weighted Mean MIC[e] ± SD**
***V. zizanioides*[f]**	0.25	0.10	0.17	0.04	0.16 ± 0.09
S. austrocaledonicum	0.75	0.08	0.08	0.02	0.34 ± 0.38
T. vulgaris	0.50	0.50	0.38	0.21	0.42 ± 0.13
S. aromaticum	0.50	0.44	0.63	0.42	0.50 ± 0.08
O. vulgare	1.00	0.30	0.45	0.27	0.60 ± 0.37
P. patchouli	1.00	0.50	0.50	0.13	0.63 ± 0.38
C. verum	0.50	1.00	0.75	0.38	0.63 ± 0.25
C. citratus	1.00	0.35	0.60	0.32	0.65 ± 0.33
M. recutita	1.00	0.35	0.80	0.39	0.71 ± 0.32
L. cubeba	1.00	0.50	0.75	0.50	0.75 ± 0.25
O. tenuiflorum	1.00	0.50	0.75	0.50	0.75 ± 0.25
C. martinii	1.00	0.50	0.90	0.39	0.76 ± 0.29
N. jatamansi	1.00	0.50	1.00	0.50	0.80 ± 0.27
R. damascena	1.00	0.50	1.00	0.50	0.80 ± 0.27
C. zeylanicum	1.00	0.70	0.90	0.46	0.81 ± 0.23
P. graveolens	1.00	0.75	1.00	0.50	0.85 ± 0.22
L. nobilis	2.00	0.50	1.00	1.00	1.30 ± 0.67
C. nardus	2.00	1.28	1.50	0.75	1.51 ± 0.53

Essential oil	MIC (mg/mL)				
	***C. acnes*[a]**	***S. epidermidis*[b]**	***S. aureus*[c]**	***S. pyogenes*[d]**	**Weighted Mean MIC[e] ± SD**
C. myrrha	> 8.00[f]	> 8.00	> 8.00	0.38	N/A[g]
Controls:					
Positive – Ciprofloxacin (µg/mL)	0.04	0.02	0.08	0.31	0.10 ± 0.12
Negative - Acetone	> 8.00	> 8.00	> 8.00	> 8.00	N/A
S. chinensis	6.00	3.00	2.00	2.00	3.80 ± 2.05
Culture control	+[h]	+	+	+	N/A

[a]*C. acnes* ~ ATCC® 11827™
[b]*S. epidermidis* ~ ATCC® 2223™
[c]*S. aureus* ~ ATCC® 25923™
[d]*S. pyogenes* ~ ATCC® 12344™
[e]Weighted mean MIC ~ the mean value calculated with the MIC value of *C. acnes* weighted double
[f]> 8.00 ~ MIC value is above the concentration tested and signifies very poor antibacterial activity
[g]N/A ~ not applicable as values cannot be calculated from current data set
[h]+ ~ bacterial growth present throughout
Shaded areas represent the five essential oils with the mean strongest antimicrobial activity against all four pathogens

The efficacy of the 19 essential oils tested against each pathogen was investigated. The MIC values of each oil were classified as outstanding, noteworthy, moderate, poor or very poor as specified in Table 2.1. A summary of the results presented as the percentage of essential oils within each classification were plotted in order to show resilience of pathogen (Figure 2.2). The results obtained revealed *C. acnes* to be the most resilient reference strain, with 21.05% of essentials oils displaying outstanding MIC values of 0.50 mg/mL and below, compared to *S. pyogenes* (89.47%) and *S. epidermidis* (73.68%). However, a total of 16 out of 19 of the essentials exhibited MIC values of 1.00 mg/mL and below against *C. acnes* and are hence still antimicrobially effective. A previous study by Si *et al.* (2006) found that clove oil (scientific name not provided), as well as thymol and eugenol, displayed reduced efficacy under anaerobic conditions in comparison to aerobic conditions. While the exact mechanism remains unclear, it could explain the weaker inhibition against *C. acnes* in comparison to the aerobic pathogens. However, studies by Luís *et al.* (2016) and Ahirwar *et al.* (2018) found no statistical difference between the efficacy of essential oils against aerobic and anaerobic bacteria. Hence, more conclusive data is required.

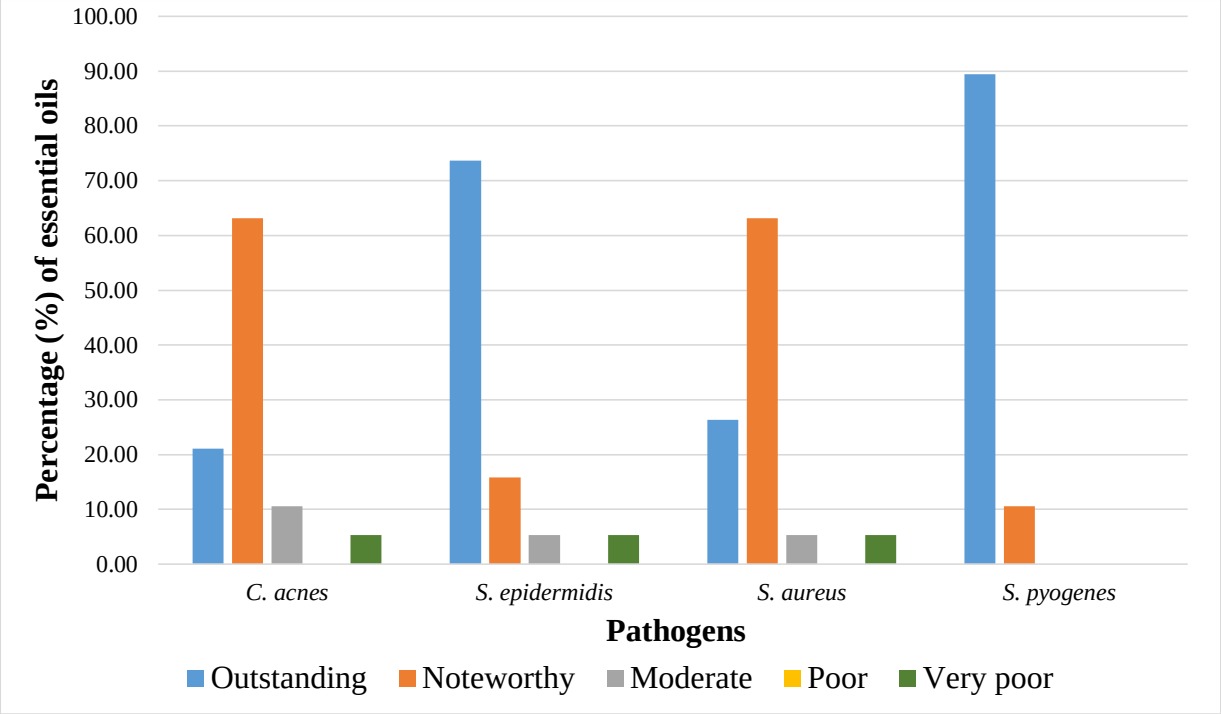


Figure 2.2: Distribution of percentage of essential oils in each classification against pathogens involved in acne

The reference strain *S. epidermidis* was found to be susceptible to the essential oils with 14 out of 19 oils exhibiting outstanding activity (≤ 0.50 mg/mL). In the case of *S. aureus*, 26.32% of essential oils displayed outstanding activity while the antimicrobial activity of the majority of essential oils (63.16%) were classified as noteworthy (MIC values ≤ 1.00 mg/mL), and a minority of the essential oils displayed poor to very poor activity.

Overall, *S. pyogenes* was shown to be the most susceptible micro-organism with 89.47% (17 out of the 19) of essential oils displaying outstanding inhibition and 10.53% of oils displaying noteworthy activity of up to 1.00 mg/mL. A previous study by Inouye *et al.* (2001) found three strains of *Streptococcus*, including *S. pyogenes*, to be more susceptible to essential oils when compared to *S. aureus*. As a Gram-positive microbe, *S. pyogenes* consists of a thick peptidoglycan cell wall which protects it from the invasion of antimicrobial agents. However, certain chemical constituents have been shown to not only penetrate this cell wall, but enhance the permeability and thereby facilitate cell death, as observed in the case of *S. pyogenes* (Wijesundara and Rupasinghe, 2018). It has been previously reported that essential oils containing aldehydes or phenols such as carvacrol, thymol, *p*-cymene, myrcene and γ-terpinene display greater activity against *S. pyogenes* than oils dominant in ketones or oxides (Sfeir *et*

al., 2013; Wijesundara and Rupasinghe, 2018). Thus, it is line with the previous findings that the essential oils dominant in these compounds such as *T. vulgaris* and *O. vulgare* displayed some of the lowest MIC values (0.21 mg/mL and 0.27 mg/mL respectively) against *S. pyogenes*. Essential oils containing phenolic derivatives as opposed to phenols themselves have been proven to be less efficient (Sfeir *et al.*, 2013), which is seen with the eugenol-containing *S. aromaticum* with a slightly weaker MIC of 0.42 mg/mL. However, having noted this, the activity of this oil can still be classified as outstanding.

No essential oils were found to have poor antimicrobial activity against any of the pathogens. Only one essential oil out of the 19 tested (*C. myrrh*) displayed very poor activity against *C. acnes, S. epidermidis* and *S. aureus* with MIC values of greater than 8.00 mg/mL. The top five ranked essential oils obtained from the conducted MIC assay as shown in Table 2.3 were *V. zizanioides, S. austrocaledonicum, T. vulgaris, S. aromaticum* and *O. vulgare* and are discussed further.

The MIC results revealed *V. zizanioides* to be the most noteworthy essential oil with a weighted mean of 0.16 mg/mL. Out of the 19 essential oils tested, the oil obtained the lowest MIC value of 0.25 mg/mL against *C. acnes*. The antimicrobial activity of *V. zizanioides* obtained was 0.25 mg/mL against *C. acnes* and 0.17 mg/mL against *S. aureus*, which was more effective than the 0.50 mg/mL for each previously reported (Orchard *et al.*, 2018a; Orchard *et al.*, 2018b). Analysis of the GC-MS data of the *V. zizanioides* essential oil revealed that a different chemotype from the previous studies was investigated as there were no major chemical constituents in common, therefore discrepancies in antimicrobial activity were observed. The *V. zizanioides* essential oil is distilled from the grass stems of the vetiver plant and previous studies have demonstrated its anti-inflammatory, antiseptic, antifungal and anxiolytic functions (Peng *et al.*, 2014; Han and Parker, 2017; David *et al.*, 2019). It is a popular form of topical aromatherapy and it has been traditionally employed as folk medicine by Asian countries for multiple ailments (Burger *et al.*, 2017; David *et al.*, 2019). Currently, it is widely used as a key ingredient in the perfume and cosmetic industry (Martinez *et al.*, 2004). The *V. zizanioides* essential oil has been used in aromatherapy to regulate sebum production, thereby reducing the formation of comedones and rendering it useful in the treatment of acne in addition to its antimicrobial activities (Chomchalow and Chapman, 2003; Peng *et al.*, 2014; Burger *et al.*, 2017). Additionally, the anti-inflammatory and anti-oxidant benefits may serve to soothe troubled skin, which provides an added benefit as acne often results in a compromised skin

barrier (Chou *et al.*, 2012). Thus, *V. zizanioides* is a versatile oil and qualifies as a strong candidate for the treatment of acne.

The second ranked oil was found to be *S. austrocaledonicum*, which out of the 19 oils tested, displayed the lowest MIC value against *S. aureus*, *S. pyogenes*, and *S. epidermidis*. While Orchard *et al.* (2018a) found the MIC value of *S. austrocaledonicum* against *S. aureus* and *S. epidermidis* to be 0.25 mg/mL each, a lower value of 0.08 mg/mL for both pathogens was obtained in this study. However, both results remain outstanding. Some of the major components found within the oil are *α*-santalol and *β*-santalol upon which the antimicrobial activity is concentration-dependant (Howes *et al.*, 2004; Jirovetz *et al.*, 2006; Roh *et al.*, 2015; Orchard *et al.*, 2018b). The concentrations of the major compounds of the essential oil used within the current study and the baseline study (Orchard *et al.*, 2018a) were found to be identical (52.70% of *α*-santalol and 15.70% of *β*-santalol). Therefore, variations in antimicrobial activity cannot be attributed to chemotype in this instance. As both MIC results obtained were consistently lower than those acquired in the previous studies, the differences may rather be due to different inoculum sizes or resistance to the test strain used during the assay. According to Orchard and van Vuuren (2017), the bacterial inoculum size used within previous studies may range from 5×10^2 CFU/mL to 5×10^8 CFU/mL. The inoculum size directly impacts on the antibacterial activity as a weaker bacterial load will lead to a stronger MIC value and vice versa. Additionally, variations in susceptibility to essential oils within the same microbial strains have been reported and could be an influencing factor (Vel *et al.*, 2017). Multiple studies have already demonstrated the potent antimicrobial, antifungal and cytotoxic effects of *S. austrocaledonicum* (Cho *et al.*, 2016; Choi *et al.*, 2016; Powers *et al.*, 2018), highlighting its potential as an antimicrobial essential oil. The essential oils of the *Santalum* species have demonstrated anti-oxidant and anti-inflammatory effects (Burdock and Carabin, 2008; Saneja *et al.*, 2009; Sharma *et al.*, 2017), thereby proving beneficial to wound healing and for dermatological use (Rybak, 2013). The *S. austrocaledonicum* plant is native to the island of New Caledonia and Ayurvedic practices have recommended its topical use for skin conditions such as itching, inflammation and pruritus (Kumar *et al.*, 2016). Industrially, it is used in the manufacture of pigmented cosmetics, soaps and creams and its unique odour makes it a sought after ingredient in the perfume industry (de Groot and Schmidt, 2017). Due to the supporting evidence of the various bioactivities for *S. austrocaledonicum*, the potential as a topical medicament for acne warrants further exploration.

Analysis of *T. vulgaris* essential oil in this study revealed a mean weighted MIC of 0.42 mg/mL, securing its place as the third ranked essential oil. The chemical analysis revealed that the *T. vulgaris* oil contained 46.61% thymol, when compared to 18.90% reported by Orchard and van Vuuren (2017) and van Vuuren and de Rapper (2020). Thymol is the major constituent responsible for the antimicrobial effects of the essential oil (Borugă *et al.*, 2014; Taleb *et al.*, 2018), therefore a higher concentration thereof will lead to lower MIC values. It is thus congruent that lower MIC values were obtained in this study (0.21 mg/mL – 0.50 mg/mL) when compared to the previous reports (0.50 mg/mL – 1.00 mg/mL) (Orchard *et al.*, 2018a; Orchard *et al.*, 2018b; Leigh-de Rapper and van Vuuren, 2020). The dominant constituent thymol alone was shown by Taleb *et al.* (2018) to have an MIC of 0.70 mg/mL against both *C. acnes* and *S. epidermidis*, however, the minor constituents should not be disregarded as they also contribute towards the overall antimicrobial activity (Taleb *et al.*, 2018). Ács *et al.* (2018) found *T. vulgaris* (containing 46.10% thymol) to display an MIC of 0.42 mg/mL against *S. pyogenes*, marginally weaker than the 0.21 mg/mL achieved in this study. *Thymus vulgaris* essential oil has a history of being traditionally employed for use in various skin conditions such as acne, cellulitis and inflammation (Soković *et al.*, 2009; Tabassum and Hamdani, 2014; Nabavi *et al.*, 2015; Habashy *et al.*, 2018). Zu *et al.*, (2010) found *T. vulgaris* and *C. zeylanicum* to be the most effective out of 10 essential oils against acne with MICs of 0.016% $^{v}/_{v}$ each, however, as the study only involved *C. acnes* as a singular pathogen, further in-depth studies concerning other acne-inducing pathogens are required to be conclusive. This makes *T. vulgaris* a strong contender in the fight against acne, and opens up new avenues for exploration.

The essential oil *S. aromaticum* displayed an outstanding MIC value of 0.50 mg/mL against *C. acnes* and an overall weighted mean MIC of 0.50 mg/mL, with marginally different MIC values against each pathogen. The antibacterial properties of *S. aromaticum* may be attributed to the high concentration of phenolic compounds, such as eugenol, within the essential oil which are obtained through distillation of the flower buds of the clove plant (Lakhan *et al.*, 2020). The antimicrobial properties of *S. aromaticum* have been previously investigated and reported. A previous study by Fu *et al.* (2009), found an MIC value of 0.31 mg/mL against *C. acnes*, which is congruent with the 0.50 mg/mL reported in this study. The *S. aromaticum* sample screened in the current study contained 71.90% eugenol which was a little lower than the 81.90% reported by Orchard and van Vuuren (2017) and van Vuuren and de Rapper (2020). An MIC value of 0.63 mg/mL was obtained against *S. aureus* as compared to 0.88 mg/mL reported by Orchard *et al.* (2018b), and 0.42 mg/L was obtained against *S. pyogenes* as compared to the

2.00 mg/mL reported by van Vuuren and de Rapper (2020). Despite containing a lower concentration of eugenol, the *S. aromaticum* essential oil within this study displayed stronger MIC values. However, minor chemical constituents were present in this sample such as *β*-caryophyllene (3.30%) and cinnamaldehyde (1.75%) which were not reported in the previous studies. Their presence should not be discounted as many of these minor constituents, especially *β*-caryophyllene, have been shown to display antibacterial effects independently (Lamlertthon and Tiyaboonchai, 2007) and may exert synergistic antibacterial effects in combination with the major constituents (Taleb *et al.*, 2018). In certain cases the antimicrobial activity of an essential oil may not be ascribed to any of the major constituents, but rather arises from a specific synergistic combination of constituents which display poor activity individually (Tariq *et al.*, 2019). The *S. aromaticum* essential oil is derived from the clove plant which has been used for centuries as a medicinal source as well as a food preservative due to its potent anti-oxidant and antibacterial properties (Mohammed *et al.*, 2016). The *S. aromaticum* essential oil is also effective in combatting oral pathogens and toothache, making it a popular choice in dental care (El-Maati *et al.*, 2016). Topically, *S. aromaticum* essential oil has been previously cited for the use of healing acne scars, blemishes and comedones (Saeed and Tariq, 2008; Kapoor and Saraf, 2011). However, scientific literature is still lacking and the potential of *S. aromaticum* as a therapeutic treatment for acne warrants further investigation.

Origanum vulgare exhibited outstanding MIC values of 0.30 mg/mL against *S. epidermidis* and 0.27 mg/mL against *S. pyogenes*, however, a considerably higher MIC of 1.00 mg/mL against *C. acnes* increased the weighted mean to 0.60 mg/mL. Both *T. vulgaris* and *O. vulgare* belong to the family Lamiaceae, producing similar aromatic compounds (Taleb *et al.*, 2018). This could in turn explain the similarities in MIC values between the two, for example 0.38 mg/mL and 0.45 mg/mL against *S. aureus* and 0.21 mg/ mL and 0.27 mg/ mL against *S. pyogenes*, respectively. The *O. vulgare* essential oil displayed the fifth strongest antimicrobial activity against *C. acnes* at a noteworthy MIC of 1.00 mg/mL. The essential oil of *O. vulgare* is rich in phenolic compounds, such as carvacrol and rosmarinic acid, which contribute towards the antimicrobial, anti-oxidant and anti-inflammatory effects (Chuang *et al.*, 2018). Investigation of the chemical constituents found the *O. vulgare* essential oil used in this study to contain 60.40% carvacrol, 7.89% of *γ*-terpinene and 5.67% of thymol, while the previous studies conducted by Orchard *et al.* (2018a) and Orchard *et al.* (2018b) reported a lower concentration of 51.0% carvacrol and a higher concentration of 22.5% thymol. Significantly

lower MIC values against *S. aureus*, *S. epidermidis* and *S. pyogenes* were obtained in this study, and maybe attributed to the increased carvacrol. Liolios *et al.* (2009) previously studied the antimicrobial activities of thymol and carvacrol, and found that between the two, carvacrol exhibited superior activity against *S. aureus* and similar activity to *S. epidermidis*. Therefore, it may be postulated that the decreased thymol content does not diminish the influence of the increased carvacrol concentration on the antimicrobial activity. An investigation of seven Mediterranean essential oils against acne by Taleb *et al.* (2018) reported *O. vulgare* to display the strongest antimicrobial activity against *C. acnes* and *S. epidermidis*. Furthermore, *O. vulgare* was the strongest inhibitor of biofilm formation, which is worth noting as *S. epidermidis* biofilm plays a role in the pathogenesis of acne (Kumar *et al.*, 2016).

2.3.4 Other essential oils for acne

The two *Cinnamomum* species tested, *C. verum* (cinnamon bark) and *C. zeylanicum* (cinnamon leaf) displayed different weighted mean MICs of 0.63 mg/mL and 0.81 mg/mL respectively. *Cinnamomum verum* inhibited *C. acnes* with an MIC value of 0.50 mg/mL, which was twofold less than *C. zeylanicum* with an MIC of 1.00 mg/mL. The *C. zeylanicum* oil was superior to *C. verum* only against *S. epidermidis*, with an MIC of 0.70 mg/mL when compared to 1.00 mg/mL. Essential oils extracted from different parts of the same plant have been shown to display different antimicrobial activities due to variations in chemical composition. The GC-MS analysis of *C. verum* showed it to contain major compounds such as cinnamaldehyde (56.97%) and eugenyl acetate (8.96%) while *C. zeylanicum* was found to predominantly contain eugenol (71.98%) and eugenyl acetate (4.79%). Orchard *et al.* (2018a) and Orchard *et al.* (2018b) proposed that cinnamaldehyde exerts a stronger antibacterial effect than eugenol, hence a higher cinnamaldehyde content leads to better antimicrobial activity. It is therefore in line with the previous findings that the cinnamaldehyde-dominant bark oil, *C. verum*, displays better antimicrobial activity than the eugenol-dominant leaf oil, *C. zeylanicum.*

An MIC concentration of 0.13 mg/mL against *S. pyogenes* was obtained by *P. patchouli*, making it the third strongest essential oil against the pathogen. Outstanding noteworthy concentrations of 0.50 mg/mL were recorded for *S. epidermidis* and *S. aureus* and a noteworthy value of 1.00 mg/mL was obtained for *C. acnes.* The predominant chemical compound present was patchouli alcohol at a concentration of 30.44%, which according to Hu *et al.* (2017) is the most critical compound responsible for its bioactivities. Luo *et al.* (2019) reported a patchouli

alcohol concentration of 28.27% in their analysis, however, as zone of inhibition was used to assess the antibacterial activity rather than the MIC broth dilution method, no suitable comparison could be made. Orchard *et al*. (2018a) reported an MIC value of 0.25 mg/mL against *S. epidermidis* while Orchard *et al.* (2018b) reported an MIC value of 0.50 mg/mL against *C. acnes*, both of which were only slightly lower MIC values (i.e. better antimicrobial activity) than the values achieved in this study. These MIC results are comparable to those achieved in the current study and may be deemed congruent.

The essential oil *C. myrrh* displayed very poor activity (> 8.00 mg/mL) against *C. acnes*, *S. epidermidis* and *S. aureus* at the concentrations tested, thus mean MIC values could not be calculated. This is in stark contrast to a projected weighted mean MIC of 0.54 mg/mL that was calculated from MIC values provided in previous studies as shown in Table 2.4 (Orchard *et al.*, 2018a; Orchard, *et al*., 2018b). Comparison of the GC-MS data revealed a lower concentration (4.99%) of the compound lindestrene while the cited literature screened a sample containing 15.8%. The different chemical profile of the *C. myrrh* essential oil tested may account for the reduced antimicrobial activity.

The positive control (ciprofloxacin) demonstrated antimicrobial susceptibility of all the pathogens at the concentrations tested, while the negative control (acetone) showed no inhibition, thus the solvent was inert. The culture control demonstrated growth throughout, therefore the media used was able to support the growth of the pathogens. The carrier oil, *S. chinensis*, was included in the antibacterial assay as a negative control to the essential oils. It displayed a borderline moderate weighted mean MIC of 3.80 mg/mL and therefore does not qualify as an appropriate antimicrobial. Carrier oils in general contain an abundance of fatty compounds and waxes which impart nourishing and soothing properties, however, they lack the potent chemical constituents present in essential oils to have notable antimicrobial effects (Guillaume and Charrouf, 2011) and thus the results obtained are consistent.

The majority of essential oils tested showed noteworthy activity against all four acne pathogen reference strains, and the data obtained highlights multiple options as further avenues for exploration. However, the essential oils *V. zizanioides, S. austrocaledonicum, T. vulgaris, S. aromaticum*, and *O. vulgare* showed the optimum antimicrobial activity and should be considered for further investigation and formulation development.

2.4 Summary

- A total of 19 essential oils were identified from existing literature to display noteworthy activity against *C. acnes*, *S. aureus*, *S. epidermidis* and *S. pyogenes*.
- The chemical profiles of the selected essential oils were confirmed and the major chemical constituents were identified.
- Antimicrobial assays were undertaken on these essential oils to confirm their efficacy against the selected pathogens.
- The most effective oil against *C. acnes* was shown to be *V. zizanioides* with an MIC of 0.25 mg/mL.
- The lowest MIC values against both *S. aureus* and *S. epidermidis* were both achieved by *S. austrocaledonicum* at a concentration of 0.08 mg/mL against both micro-organisms, respectively.
- The weighted mean MIC was calculated to reflect the majority influence of *C. acnes* for each essential oil. Oils were then ranked in increasing order of the weighted mean MIC to determine which were the most effective.
- The five essential oils with the most noteworthy weighted mean MICs were *V. zizanioides* (0.16 mg/mL), *S. austrocaledonicum* (0.34 mg/mL), *T. vulgaris* (0.42 mg/mL), *S. aromaticum* (0.50 mg/mL), and *O. vulgare* (0.60 mg/mL) and were thus selected for further studies.

CHAPTER 3 - FORMULATION

3.1 Introduction

The formulation of essential oil products remain a pharmaceutical challenge, due to their volatility, water insolubility and thermal instability (Lopes *et al.*, 2017; Taleb *et al.*, 2018). Emulsification presents an avenue of opportunity in order to overcome these challenges, and improve on their stability as formulations (Han, 2018). Henceforth, the various factors which must be taken into consideration while designing an emulsion are explored in this chapter without compromising on the integrity of the essential oil.

Each oil-phase component of an emulsion system may be assigned an HLB value as a measure of its polarity, and will contribute towards the overall HLB of the emulsion according to its relative concentration. It is well recognised that optimum stability is achieved when the HLB of the emulsifier is equivalent to the HLB of the oil phase of the emulsion, and this required HLB value is known as the rHLB (Orafidiya *et al.*, 2002). Therefore, if a range of the same emulsion at different HLB values is prepared, the HLB displaying the lowest degree of instability may be deemed as optimal. The degree of instability may be assessed through various parameters such as the presence of creaming, the average droplet size and the turbidity of the emulsion. Hence, this chapter intends to utilise the HLB approach in order to produce stable essential oil formulations. As not only one assay may reveal the correct HLB, this chapter seeks to make use of various stability tests in determining the optimum HLB for the $^{O}/_{W}$ essential oil emulsions. By analysing the results of the various stability tests in combination, this chapter seeks to find an agreement between the methods and consequently conclude an optimum HLB for each essential oil emulsion system.

3.2 Methods and materials

3.2.1 HLB determination of neat essential oils

The HLB approach to formulate a stable emulsified lotion of an essential oil involves prior knowledge of the HLB value of each component of the oil phase in order to determine the

rHLB of the formulations themselves. A preliminary value of the HLB of each individual essential oil was required in order to calculate the approximate rHLB of the lotion and hence were determined using the Griffin method (Orafidiya and Oladimeji, 2002). A series of course essential oil emulsions, ranging from HLB 7 to HLB 11, were prepared using the essential oil as the oil phase, distilled autoclaved water as the aqueous phase and a blend of the emulsifiers sorbitan mono-oleate (Tween® 80) and glycerol monostearate (GMS) as shown in Table 3.1. The lower-spectrum range of HLB values to be tested (7-11) was selected to cater for the lipophilicity of the essential oils.

Table 3.1: Emulsion formula for the determination of the HLB of the essential oils (Meher *et al.*, 2013; Sanad and Mabrouk, 2016)

Phase	Component	Concentration (% $^{w}/_{w}$)	Mass (g) per 10 g of emulsion
Oil	Essential oil	5	0.5
Aqueous	Water	92	9.2
Emulsifier	Tween® 80 / GMS	3	0.3

As a combination of emulsifiers were used, their relative ratios to achieve each specific HLB were determined, taking into account the HLB of Tween® 80 (15.0) and GMS (3.8) (Hauss, 2007). This was calculated using Equation 3.1 and Equation 3.2 (Hauss, 2007; Johansson and Somasundaran, 2007).

$$\%\ \text{of Emulsifier A} = \frac{100\ (\text{Desired HLB} - \text{HLB of Emulsifier B})}{\text{HLB of Emulsifier A} - \text{HLB of Emulsifier B}}$$

Equation 3.1

$$\%\ \text{of Emulsifier B} = 100 - \%\ \text{of Emulsifier A}$$

Equation 3.2

In order to facilitate formulation, an Excel® spreadsheet was then created incorporating Equation 3.1 and 3.2. Additionally, the spreadsheet was designed to automatically calculate the

weight of each emulsifier required to formulate a total mass of 10 g of emulsion as shown in Table 3.2.

Table 3.2: Required emulsifier blend ratios to formulate course emulsions for the determination of the HLB of essential oils

rHLB	% $^{w}/_{w}$ Tween® 80	% $^{w}/_{w}$ GMS	For 10 g	
			Mass of Tween® 80 (g)	Mass of GMS (g)
7	28.57	71.43	0.086	0.214
8	37.50	62.50	0.113	0.188
9	46.43	53.57	0.139	0.161
10	55.36	44.64	0.166	0.134
11	64.29	35.71	0.193	0.107

The PIT method, as described in Chapter 1, Section 1.5.3.1, was used to prepare course emulsions. The lipophilic emulsifier, GMS, was added to the oil phase while the hydrophilic emulsifier, Tween® 80 was dissolved in the aqueous phase. Both phases were heated separately to 55°C in a water bath, whereupon the aqueous phase was added to the oil phase. The solution was then vortexed for 2 min to obtain a homogenous dispersion and then transferred to a 10 mL graduated centrifuge tube. The freshly formed emulsions were stored for 7 days at room temperature (25°C), upon which they were evaluated for physical signs of instability i.e. creaming, cracking or sedimentation by macroscopic analysis (Fernandes *et al.*, 2013). The creaming index (CI) was determined by measuring the height of the cream layer (H_C) and the height of the emulsion layer (H_E) as shown in **Error! Reference source not found.** and calculated as shown in Equation 3.3 (Meher *et al.*, 2013; Sanad and Mabrouk, 2016):

$$CI = \frac{\text{Height of cream layer } (H_C)}{\text{Height of emulsion layer } (H_E)}$$

Equation 3.3

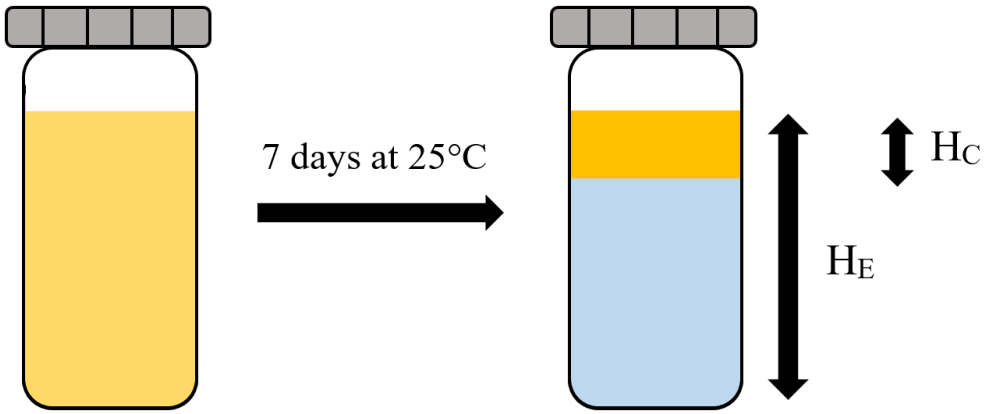


Figure 3.1: Determination of creaming index of emulsion

Course emulsions with a low concentration of emulsifier (3%) and short emulsification time (two min) were deliberately employed in order to facilitate creaming, and thus the differences in CI per emulsion could easily be measured to obtain the optimum HLB. The emulsions were prepared in triplicate (n=3) and the mean CI calculated for each HLB value. The corresponding HLB with the lowest CI was deemed as the optimum HLB value of the neat essential oil and was noted for further investigation. This procedure was repeated for each of the five selected essential oils in order to attain approximate HLB values for each.

3.2.2 Determination of the rHLB of essential oil lotions

Once the HLB values of the neat oils were obtained from the coarse emulsions, the final essential oil lotions were prepared using the HLB approach in order to obtain stable formulations.

3.2.2.1 Formulation of essential oil emulsified lotions

3.2.2.1.1 Selection of excipients and development of base formula

Excipients were selected on the basis that they are non-toxic and non-irritant and possess the ability to impart advantageous qualities to the formulations, and their properties are shown in Table 3.3. Viscosity enhancing agents such as cetyl alcohol and stearic acid were selected due to their role in improving stability as well as texture (Eccleston, 1997; Garg *et al.*, 2015). An increased viscosity leads to increased stability, thereby retarding the coalescence of the

essential oils and extending the shelf-life of the product. However, the use of such lipophilic additives must be exercised with caution. Both the viscosity and the lipophilicity of the base may increase the affinity of the compounds in the essential oil for the base, which may interfere with their release and impair their antimicrobial activity (Han, 2018). Therefore, these ingredients were included at minimal concentrations (2% of cetyl alcohol and 1% of stearic acid).

Table 3.3: Formulation composition

Excipient	% $^{w}/_{w}$	Function	Phase	HLB	Reference
Essential oil/ Carrier oil	10	▪ Active	Oil	Dependent on oil	N/A[a]
Cetyl alcohol	2	▪ Emollient ▪ Co-emulsifier ▪ Viscosity modifying agent	Oil	15	(Garg *et al*., 2015)
Stearic acid	1	▪ Emollient ▪ Co-emulsifier ▪ Viscosity modifying agent	Oil	15	(Eccleston, 1997)
Glycerine	5	▪ Humectant ▪ Protectant ▪ Skin-conditioning agent	Aqueous	N/A	(Patel *et al*., 2010; Becker *et al*., 2019)
Tween® 80	Varied ratio[b]	▪ Hydrophilic emulsifier	Aqueous	15	(Kwon Hong *et al*., 2018)
GMS	Varied ratio	▪ Lipophilic emulsifier ▪ Thickener	Oil	3.8	(Lewis *et al*., 1998; Alam *et al*., 2020)
Water	q.s[c]	▪ Vehicle	Aqueous	N/A	

[a]N/A ~ Not applicable
[b]Ratio of Tween® 80 and GMS to be varied depending on HLB and shown in Table 3.4
[c]q.s ~ quantity sufficient

The selection of the emulsifier and its concentration is one of the most critical steps in the development of an emulsion (McClements and Jafari, 2018) and plays a significant role in

maintaining stability and prolonging shelf-life. An emulsifier exerts its action via multiple mechanism pathways; it reduces the interfacial tension required to break up the oil droplets, aids in the adsorption of particles onto the oil surface and prevents aggregation of the oil droplets (Walstra, 1993; Nesterenko *et al.*, 2014; McClements and Jafari, 2018). Previous studies have found that an increase in emulsifier concentration decreases the oil droplet size, which reduces the risk of coalescence (Jusoh and Othman, 2017; Yang *et al.*, 2019). However, there is a window within which an emulsifier is effective. Low emulsifier concentrations are inadequate to reduce the interfacial tension for emulsification to occur and are unable to prevent coalescence (Chen and Tao, 2005; Jusoh and Othman, 2017). Conversely, too high emulsifier concentrations lead to emulsion breakdown and destabilisation (Nesterenko *et al.*, 2014; Jusoh and Othman, 2017). Therefore, in order to ensure long-term stability, the minimum emulsifier concentration able to achieve emulsification must be adhered to. Additionally, emulsifier concentrations are able to influence the rheology of the formulation (Shtyka and Sęk, 2016). High quantities of emulsifier increase the viscosity of the continuous phase, which is a disadvantage in the manufacture of low viscosity preparations such as lotions (Sanad and Mabrouk, 2016). The viscosity of these preparations were found to be better controlled through the manipulation of viscosity enhancing additives and thickeners, rather than the emulsifier. For this reason, a total emulsifier concentration of 10% was selected for the formulations.

A larger difference between the HLB values of two emulsifiers of a blend may result in increased stability of the emulsion. Emulsifiers with a similar HLB or closer in value may compete for adsorption at the interface, thereby resulting in instability (Devahastin, 2017; Moran-Valero *et al.*, 2017; McClements and Jafari, 2018). The blend of Tween® 80 and GMS provide a feasible option with HLB values of 15 and 3.8 respectively. As an emulsifier, GMS displays multiple advantages. Due to its chemical structure as a saturated monoglyceride, GMS has been shown to increase the orthokinetic stability of an emulsion in comparison to unsaturated monoglycerides by retarding the coalescence rate (Wu *et al.*, 2016). Glycerol monostearate is widely employed in food industry and cosmetic products where spreadable emulsions are required (Wang and Marangoni, 2015). These properties favour its use as an emulsifier and hence the emulsifier blend was finalised as Tween® 80 with GMS.

The ratio of emulsifier blend to the essential oil was set at 1:1. The lotions were kept at a constant concentration of 10% essential oil, which was substituted with *O. vulgare, S. austrocaledonicum, S. aromaticum, T. vulgaris* and *V. zizanioides* accordingly. As the

lipophilicity of the base may alter the release of the essential oil (Han, 2018), the ratio of all excipients were also kept constant so as to not alter the antimicrobial activity of the lotions. The placebo control required for the antimicrobial testing stage was formulated with the carrier oil *S. chinensis* as a substitute for the essential oil at the same concentration of 10%. As shown in Table 2.3 of Chapter 2, *S. chinensis* displays no noteworthy antimicrobial activity hence it is a suitable choice as the negative control.

The total emulsifier blend concentration was set at 10%, however, the ratio of the lipophilic emulsifier (GMS) to the hydrophilic emulsifier (Tween® 80) was varied in order to obtain each particular rHLB. The final weight of each component, including the emulsifiers, to prepare 40g of each emulsified lotion per rHLB are shown in Table 3.4.

Table 3.4: Base formulation for essential oil lotions (40 g)

rHLB	Mass (g)						
	Essential oil	**Cetyl alcohol**	**Stearic acid**	**Glycerine**	**Tween® 80**	**GMS**	**Water**
8	4.00	1.20	0.60	2.00	1.50	2.50	q.s.[a]
9	4.00	1.20	0.60	2.00	1.86	2.14	q.s.
10	4.00	1.20	0.60	2.00	2.21	1.79	q.s.
11	4.00	1.20	0.60	2.00	2.57	1.43	q.s.
12	4.00	1.20	0.60	2.00	2.93	1.07	q.s.
13	4.00	1.20	30.60	2.00	3.29	0.71	q.s.

[a]quantity sufficient

Noticeably, no preservatives were included in the formulation. Preservatives are an essential additive for cosmetic products where lengthy shelf-lives are required, especially with oil-in-water emulsions as the large aqueous phase provides a medium for microbial contamination (Kaczmarczyk *et al.*, 2015). However, many commercial preservatives pose health risks; parabens for example have been linked to increased incidences of breast cancer (Darbre *et al.*, 2004; Pan *et al.*, 2016; Matwiejczuk *et al.*, 2020). Essential oils have been intensely investigated for their preserving effects in both food and cosmetic applications (Donsì and

Ferrari, 2016; Guerra-Rosas *et al.*, 2016; Moghimi *et al.*, 2016; Lu *et al.*, 2018; Martin-Piñero *et al.*, 2019; Nazari *et al.*, 2019; Falleh *et al.*, 2020). Essential oils have been deemed as self-preserving and eliminate the need for synthetic chemicals (Kaczmarczyk *et al.*, 2015). Thus, additional preservatives were not included in the formulation.

3.2.2.1.2 Determination of rHLB of essential oil lotions

The HLB and relative concentrations of each component of the oil phase (namely the essential oil, cetyl alcohol and stearic acid) were used to calculate the overall rHLB of the formulation as shown from Equations 3.4 to 3.6. The HLB contribution of each component (CHLB), as a result of its WF, to the overall rHLB is shown in Equation 3.5. The HLB value for the essential oils was obtained as shown in Section 3.2.1 while the HLB value of the cetyl alcohol and stearic acid were obtained from literature (Eccleston, 1997; Garg *et al.*, 2015). The CHLB of each component was then summed to obtain the overall rHLB of the formulation as shown in Equation 3.6.

$$\text{Weight fraction (WF) of oil component} = \frac{\text{Number of parts of component}}{\text{Number of parts of oil phase}}$$

Equation 3.4

$$\text{CHLB} = \text{WF} \times \text{HLB of oil component}$$

Equation 3.5

$$\text{rHLB} = \text{CHLB}_{\text{Component 1}} + \text{CHLB}_{\text{Component 2}} + ...\text{CHLB}_{\text{Component x}}$$

Equation 3.6

The rHLB value obtained were rounded off to the closest HLB unit. Formulations were then prepared at three different HLBs; the calculated rHLB and at one HLB unit above and below. Using *O. vulgare* and its obtained HLB of 10 as an example, the calculation process is shown in Table 3.5.

Table 3.5: Calculation process for the determination of rHLB of essential oil lotions

Component	HLB	Number of parts	WF[a]	CHLB[b]
O. vulgare	10	10	$\frac{10}{13^c}$	$\frac{10}{13}\times 10 =$ **7.69**
Cetyl alcohol	15	2	$\frac{2}{13}$	$\frac{2}{13}\times 15 =$ **2.31**
Stearic acid	15	1	$\frac{1}{13}$	$\frac{1}{13}\times 15 =$ **1.15**
rHLB[d]	**7.69 + 2.31 + 1.15 = 11.15 ≈ 11**			

[a]Equation 3.4
[b]Equation 3.5
[c]Number of parts of oil phase was 10% (essential oil) + 2% (cetyl alcohol) + 1% (stearic acid) = 13
[d]Equation 3.6

The *O. vulgare* lotions were prepared at the theoretically calculated HLB and one HLB unit above and below. Therefore, as the rHLB was calculated as 11, the *O. vulgare* lotions were prepared at HLB 10, 11 and 12. The process was repeated for all essential oils. The calculated rHLB of each essential oil lotion and the HLB range at which they were prepared and tested at are shown in Table 3.6.

Table 3.6: Determination of HLB range for each essential oil formulation

Essential Oil	HLB of essential oil	rHLB of lotion[a]	HLB Range
O. vulgare	10	11	10-12
S. aromaticum	11	12	11-13
S. austrocaledonicum	11	12	11-13
T. vulgaris	10	11	10-12
V. zizanioides	7	9	8-10

[a]Calculated values were rounded off to the nearest HLB unit
Shaded areas are values as shown in detail in Table 3.7 but included here for completeness

3.2.2.1.3 Preparation of essential oil lotions

The lotions were prepared using a combination of PIT and ultrasonification (Meher *et al.*, 2013; Yadav *et al.*, 2013). The lipophilic emulsifier, GMS, was weighed and added to the oil phase ingredients, namely the cetyl alcohol and stearic acid, while the required amount of Tween®

80 was dissolved in the aqueous phase consisting of autoclaved distilled water and glycerine. The two phases were placed separately in a water bath and heated to 55°C. Once all the lipophilic components of the oil phase were melted, the beaker was removed from heat and allowed to cool to 40°C, whereupon the essential oil was added and mixed thoroughly at 750 rpm on a magnetic stirrer. The aqueous phase, maintained at 55°C, was then added dropwise into the oil phase under constant stirring over a period of 30 sec. The formulation was subjected to further stirring at 1500 rpm for 30 min until emulsification was achieved. To further reduce the mean oil droplet size and remove trapped air bubbles, the formulation was placed in a sonicator bath and ultrasonicated for 120 sec. The freshly formed emulsion was transferred and stored in appropriate sterile containers for further evaluation. Emulsions were prepared in triplicate of each HLB value. The process is depicted in Figure 3.2.

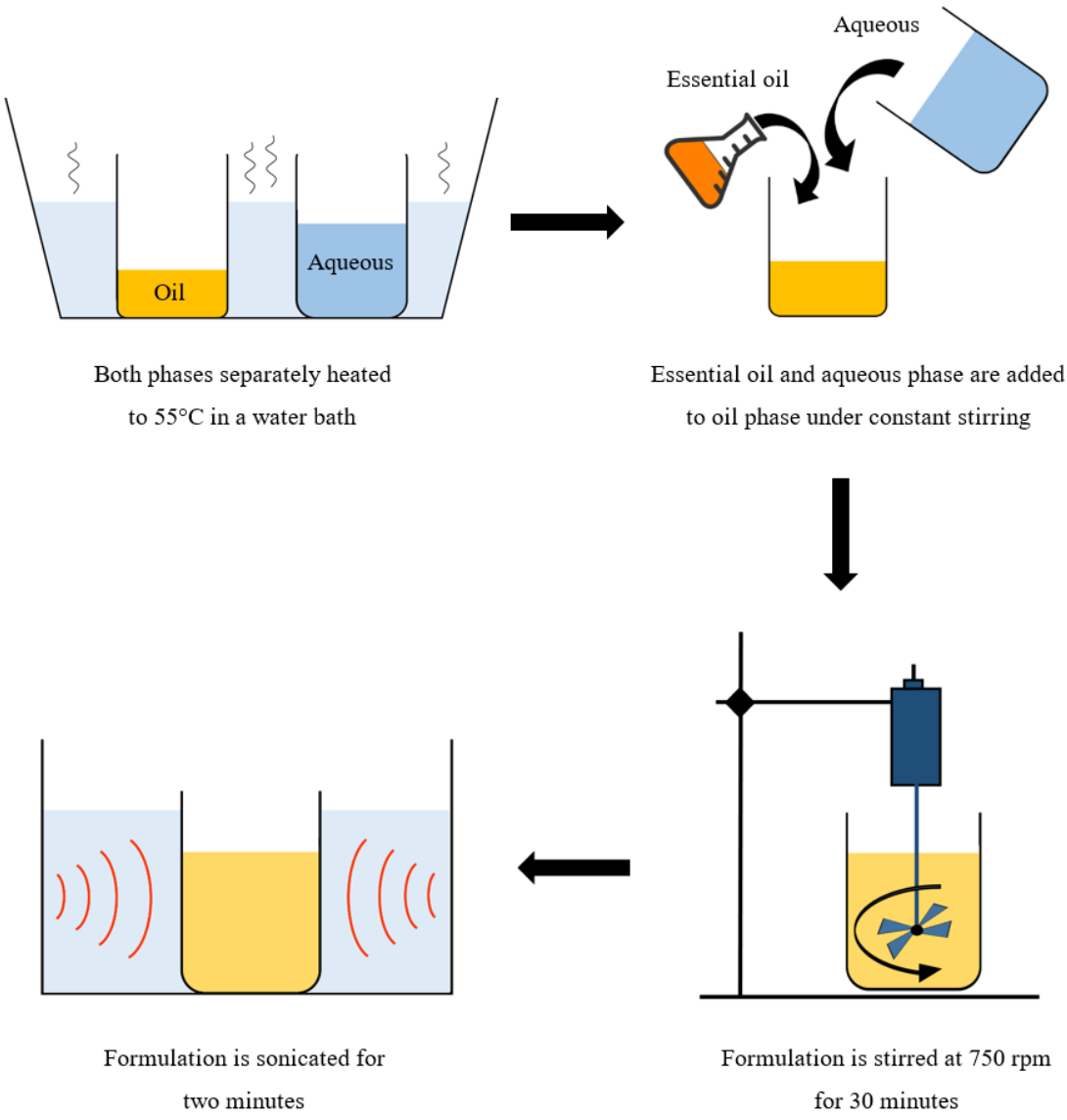


Figure 3.2: Formulation process of essential oil lotions

3.2.2.2 Stability testing of essential oil lotions

3.2.2.2.1 Creaming index and macroscopic analysis

A volume of 10 mL of each emulsified lotion was stored upright at 25°C in a sterile, graduated centrifuge tube. Emulsions were inspected daily for visual signs of instability and breakdown. The state of each emulsion was classified as either creaming, cracking or stable as shown in Figure 3.3.

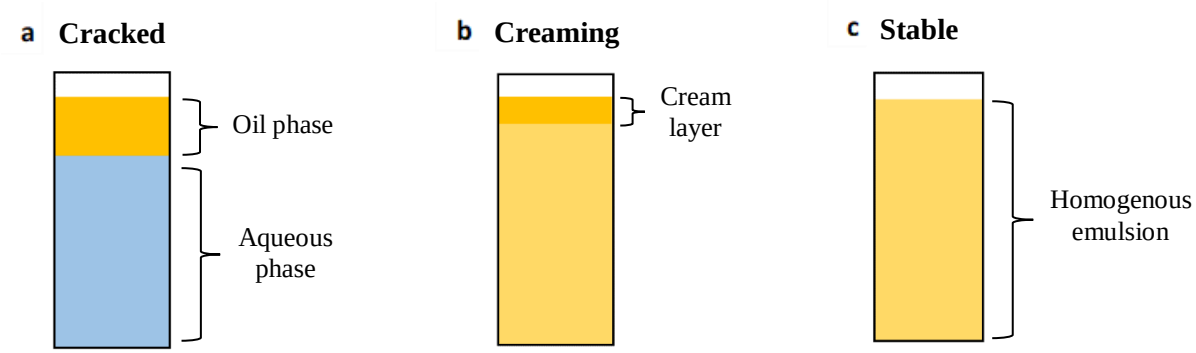


Figure 3.3: Categories of phase separation used to assess stability; a) Complete separation of oil phase and aqueous phase, b) Visible layer of cream as a result of migration of oil droplets to the surface and c) Homogenous emulsion with no evidence of phase separation

In the case of creaming, the height of the cream layer and the height of the emulsion layer of the lotions were measured daily for 28 days and recorded. The CI was then calculated as shown in Equation 3.3 and plotted accordingly.

3.2.2.2.2 Mean droplet diameter (MDD) of emulsified essential oil lotions

The MDD is the most important factor in the determination of HLB, as an emulsion displaying the smallest MDD is closest to the required HLB of a system (Orafidiya and Oladimeji, 2002). The percentage increase in MDD over time is also a useful parameter as optimum HLB will result in a minimal coalescence and hence can be measured by selecting the formulation with the smallest change recorded. The polydispersity index (PDI) is a measure of the standard deviation of the MDD, and shows the degree of distribution of the oil droplets. Generally, PDI

values < 0.5 indicate monodispersed droplets and a homogenous emulsion, while those > 0.5 indicate polydispersity and the presence of instability mechanisms.

The MDD of each emulsified lotion was measured using a Malvern Zetasizer Nanoseries (Malvern, UK) at 25°C and equipped with a 173°C backscatter detector. To avoid multiple scattering effects, each sample was diluted 1:100 using Milli-Q water and mixed thoroughly. The MDD was expressed as a Z-average size and the PDI was also recorded as a measure of homogeneity of the emulsion (Campolo *et al.*, 2020). Data was collected as an average of 13 runs and each measurement was performed in triplicate. The long-term stability of the formulations were observed by measuring the MDD and PDI over a three month period at day 1, 28, and 56 and 84 after preparation.

3.2.2.2.3 Viscosity

The viscosity of the emulsions was measured using a Haake Mars III rheometer (Thermofisher Scientific, Germany). The rheometer was equipped with a cone-and-plate measuring device with a 0.052 mm gap and temperature was set at 25°C. Apparent viscosity (η) and shear stress (τ) were measured as a function of shear rate ($\dot{\gamma}$) and flow curves were obtained with the use of RheoWin® software. Experiments were conducted in triplicate (n=3) and mean values were calculated.

3.2.2.2.4 Turbidity

A volume of 5 mL of each emulsion was stored upright at 25°C in a sterile flat-bottomed polytop and left undisturbed. On the 28th day after preparation, a 1 mL syringe and needle was used to carefully withdraw 0.25 mL from the uppermost top and the bottom of the vial. Each aliquot was diluted in 50 mL of Milli-Q water and the percentage transmission at 600 nm was measured using a Varian Cary® 50 UV-Vis Spectrophotometer (Agilent, Australia). The blank control, Milli-Q water, was set at 100% transmission. Turbidity was calculated as shown in Equation 3.7 (Ferreira *et al.*, 2010; Meher *et al.*, 2013).

$$Turbidity = 100 - \%T$$

Equation 3.7

The percentage difference between the top and bottom turbidity ($\%D_T$) of each emulsion was calculated. A small $\%D_T$ indicates a uniform distribution of oil droplets throughout an emulsion and a minimal degree of gravitational separation. A large $\%D_T$ would signify the presence of coalescence and creaming within an emulsion and can be used as a measure of destabilisation. Thus, $\%D_T$ was calculated as a function of turbidity. The procedure was conducted in triplicate (n=3) and the average % difference was obtained for each HLB value.

The overall formulation design process may thus be summarised as in Figure 3.4.

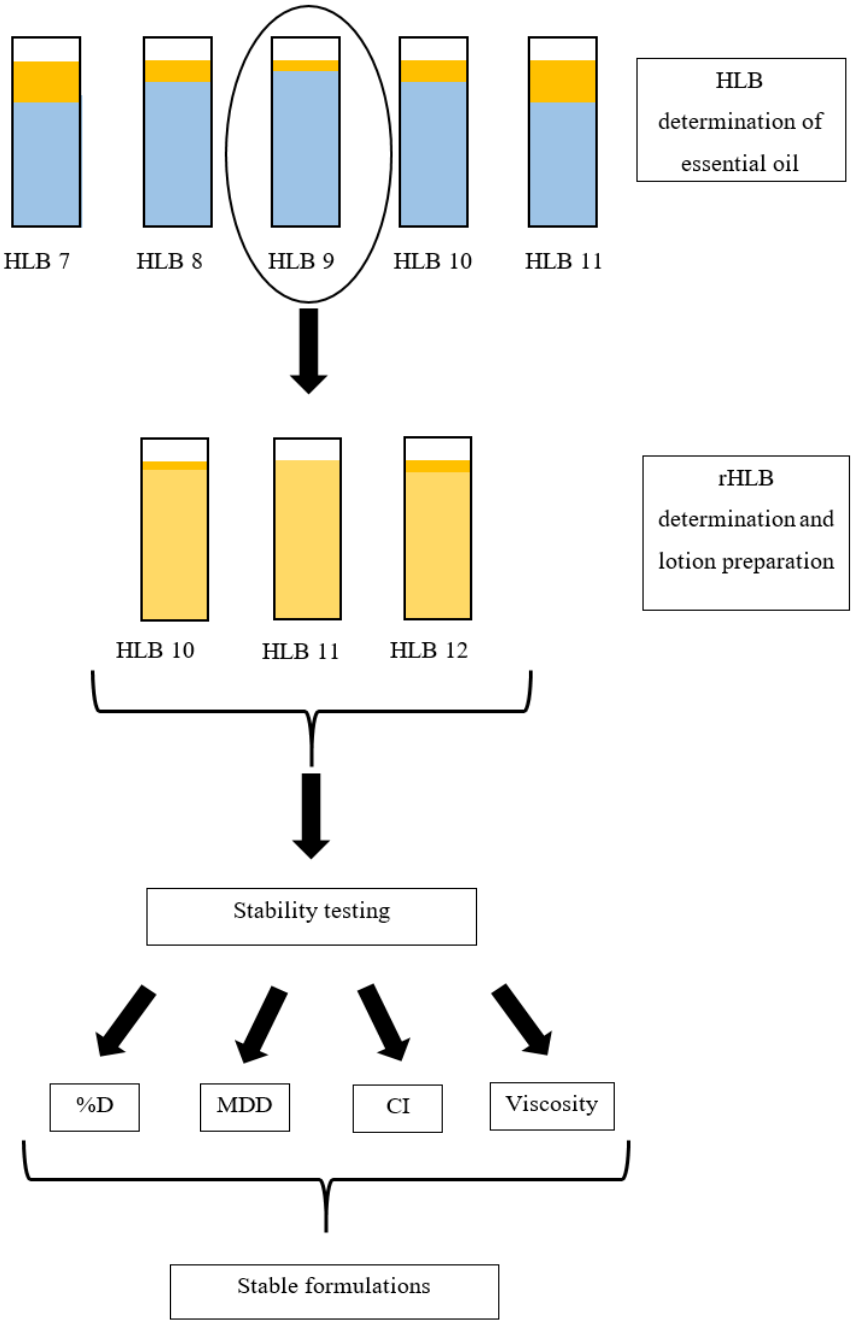


Figure 3.4: Experimental overview for formulation process

3.2.2.2.5 Combined data analysis and selection of optimum HLB

As MDD, viscosity and turbidity are all related via Stoke's equation, their results must be viewed in conjunction in order to obtain the complete picture of an emulsion's stability. The combined method approach is ideal as no one factor may provide a clear answer of a system's rHLB. The HLB of each essential oil lotion displaying the lowest CI, the lowest $\%D_T$ and the smallest % change in MDD and PDI was determined to be the optimum HLB and selected for further antimicrobial testing.

3.3 Results and discussion

3.3.1 The HLB values of selected neat essential oils

The CI of the neat essential oils per HLB are shown in Table 3.7, and the lowest CI (corresponding to the optimum HLB) is reported in bold. With *O. vulgare*, the CI was found to decrease from HLB 7 To HLB 10, then increase again at HLB 11. The lowest CI was therefore obtained at HLB 10 at a mean value of 0.048 ± 0.02. A previous study by Enayatifard *et al.* (2021) found the optimum HLB of *O. vulgare* to be 15, however, mean droplet size was employed as an evaluation parameter rather than macroscopic analysis.

Table 3.7: Creaming index of the neat essential oils measured after 7 days

HLB	Mean Creaming Index ± (SD)[a]				
	O. vulgare	***S. aromaticum***	***S. austrocaledonicum***	***T. vulgaris***	***V. zizanioides***
7	0.127 ± 0.04	0.762 ± 0.02	0.215 ± 0.03	0.087 ± 0.03	**0.098 ± 0.09[c]**
8	0.092 ± 0.03	0.365 ± 0.43	0.205 ± 0.02	0.078 ± 0.02	0.133 ± 0.03
9	0.078 ± 0.02	0.332 ± 0.39	0.182 ± 0.02	0.079 ± 0.01	N/A[b]
10	**0.048 ± 0.02**	0.299 ± 0.46	0.169 ± 0.03	**0.058 ± 0.03**	N/A
11	0.104 ± 0.02	**0.000 ± 0.00**	**0.147 ± 0.02**	0.066 ± 0.03	N/A

[a]Standard deviation (n=3)
[b]Sedimentation present therefore CI could not be determined
[c]Lowest CI value corresponding to the true HLB of each essential oil are reported in bold

An overall decrease in CI with an increase in HLB was observed with *S. aromaticum*, with no creaming found at HLB 11. Creaming formed with *S. aromaticum* emulsions were measured with difficulty as there was no clear delineation between the cream and aqueous layers, due to both components being similarly coloured. However, slight tilting of the emulsion tubes of HLB 11 revealed no visible cream layer or oil film, and were deemed to be homogenous. The optimum HLB of *S. aromaticum* was thus selected as HLB 11. Stable nano-emulsion of *S. aromaticum* has been previously formed at HLB 9 (Shahavi *et al.*, 2019). However, it has been reported that HLB values may differ slightly across studies as a different concentration of the chemical constituents present within the oil may alter its lipophilicity (Rodríguez-Rojo *et al.*, 2012).

The emulsions of *S. austrocaledonicum* formed clear delineated cream layers and the CI was easily measured (Figure 3.5). Similarly to *S. aromaticum*, *S. austrocaledonicum* emulsions displayed a decrease in creaming with an increase HLB, and minimal creaming was found at HLB 11 with a CI value of 0.147 ± 0.02. No past studies could be found investigating the HLB of *S. austrocaledonicum* essential oil.

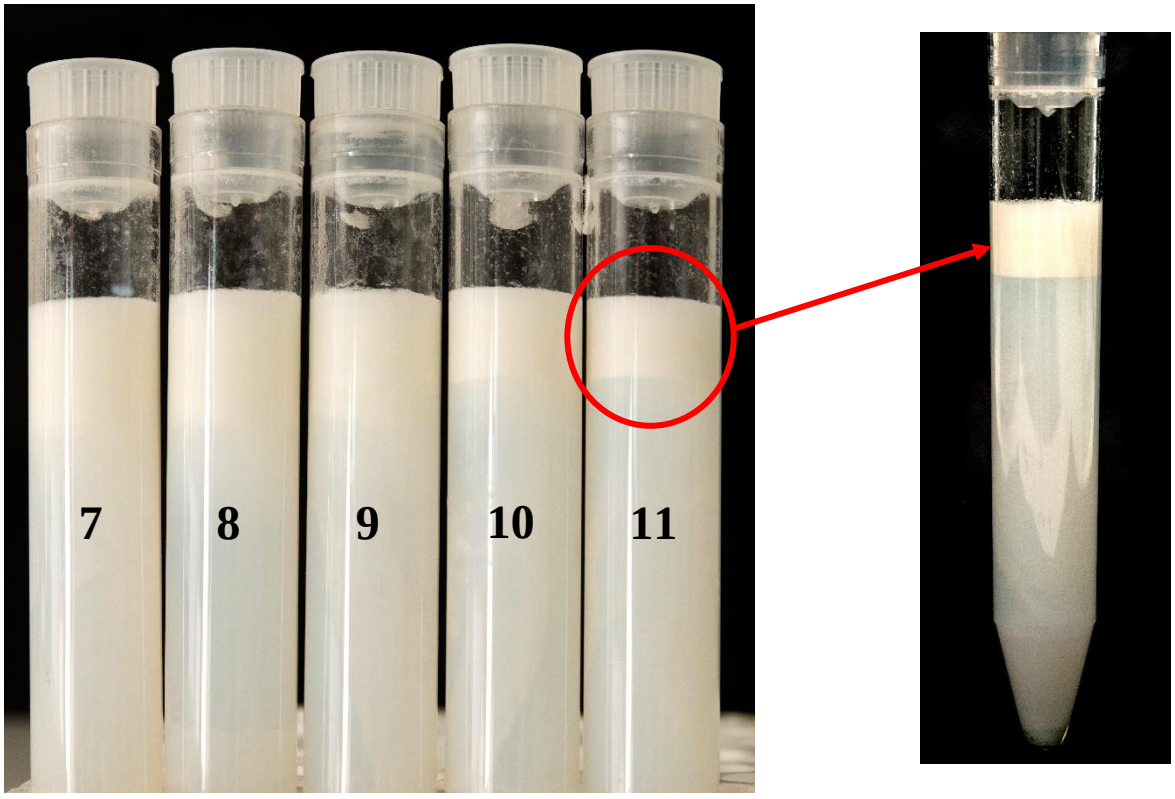


Figure 3.5: CI of *S. austrocaledonicum* essential oil emulsions from HLB 7-11

The lowest CI observed with emulsions of *T. vulgaris* corresponded to an HLB 10 (Figure 3.6). It is worth noting that the CI values obtained were not significantly different across HLB values and could be subject to error. A study by Martin *et al.* (2018) found that emulsifiers of the HLB range 9-9.5 was optimal in stabilising *T. vulgaris* emulsion, and as peak stability is achieved when the HLB of the emulsifier is equivalent to the HLB of the oil (Orafidiya and Oladimeji, 2002), it may be inferred that the HLB of *T. vulgaris* would fall within a similar range.



Figure 3.6: CI of *T. vulgaris* essential oil emulsions from HLB 7-11

Emulsions of *V. zizanioides* displayed increased creaming from HLB 7 to 8. However, emulsions formed at HLB 9 exhibited both creaming and sedimentation while those of HLB 10 and 11 showed sedimentation only (Figure 3.7). As *V. zizanioides* displays a slightly higher density than that of the continuous phase (1.016 g/mL as provided by supplier versus 0.998 g/mL of water at 20ºC), gravitational forces may act upon the oil droplets to hasten density-driven separation, resulting in sedimentation (Preziosi *et al.*, 2013; Hu *et al.*, 2017b; Church *et al.*, 2019). At the incorrect HLB, the emulsifier blend cannot sufficiently reduce interfacial tension to maintain the dispersion of oil droplets throughout the emulsion, resulting in instability phenomena such as settling (Walstra, 1993; Tadros *et al.*, 2004). Therefore, in the case of *V. zizanioides*, an increase in HLB can be attributed to a decrease in stability and thus the low-range HLB of 7 was selected for further investigations.

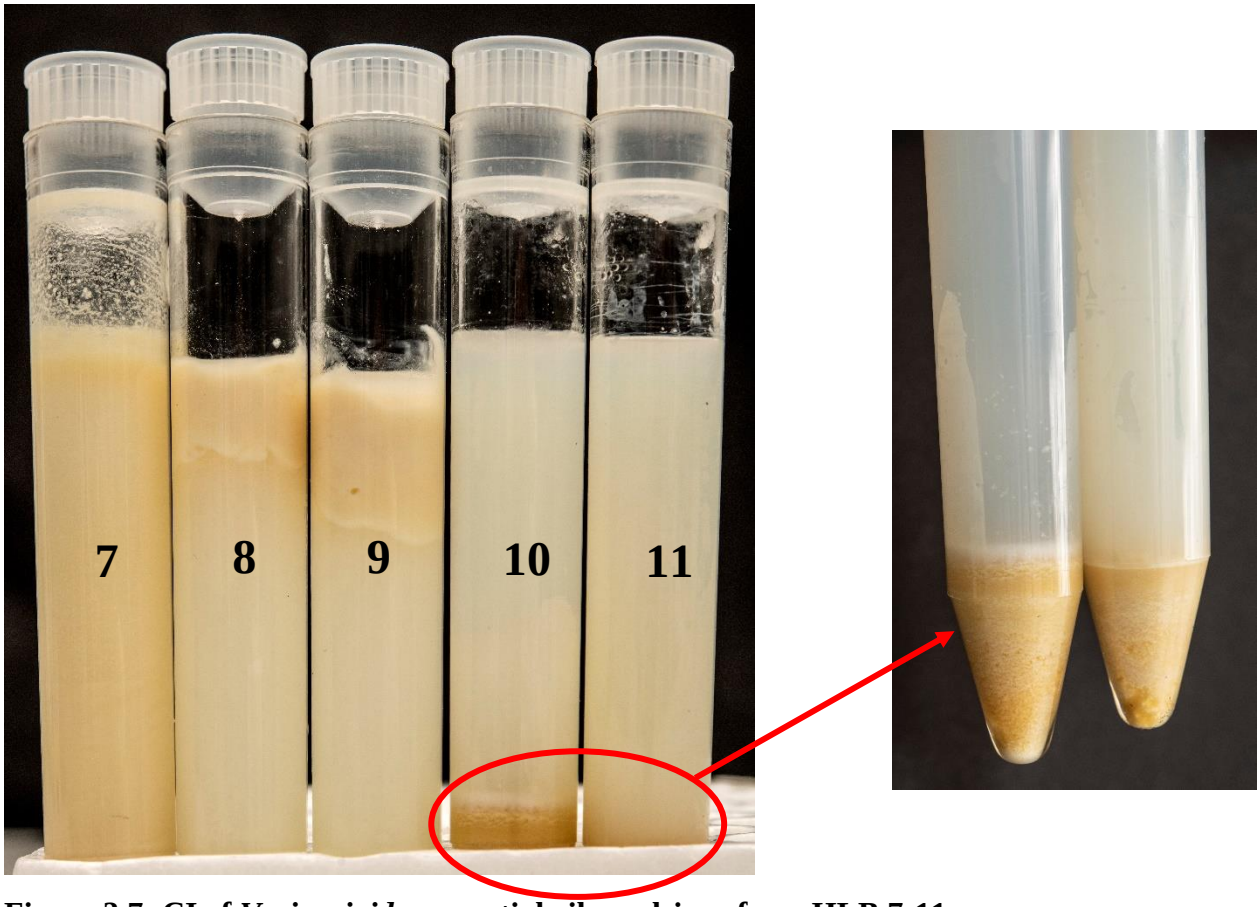


Figure 3.7: CI of *V. zizanioides* essential oil emulsions from HLB 7-11

The HLB of neat essential oils remain widely unreported in literature. While factors such as chemical composition and evaluation parameters may alter the HLB value of an essential oil, it is still a critical step required to ensure maximum stability of their emulsions. As variances in creaming can be seen with changes in HLB of the course emulsions, CI provides critical data that aids in the design of stable emulsions. Determining the approximate HLB values of each essential oil provides ease for the calculation of the rHLB of any custom formulation, with the advantage of ensuring a longer shelf life.

3.3.2 Final formulation and stability testing of essential oil lotions

Once all relative parameters were determined, the essential oil lotions were prepared at the rHLB values specified in Table 3.6. Further evaluation was carried out through stability testing of the relevant parameters.

3.3.2.1 Creaming index

Over a period of 28 days, the emulsions of *O. vulgare*, *S. aromaticum* and *V. zizanioides* displayed no signs of creaming regardless of HLB (Figure 3.8). No colour changes were observed with changes in HLB and homogenous, stable emulsions were formed for each. Therefore, emulsions formed with these oils were stable to creaming and optimum HLB could not be assessed using CI.

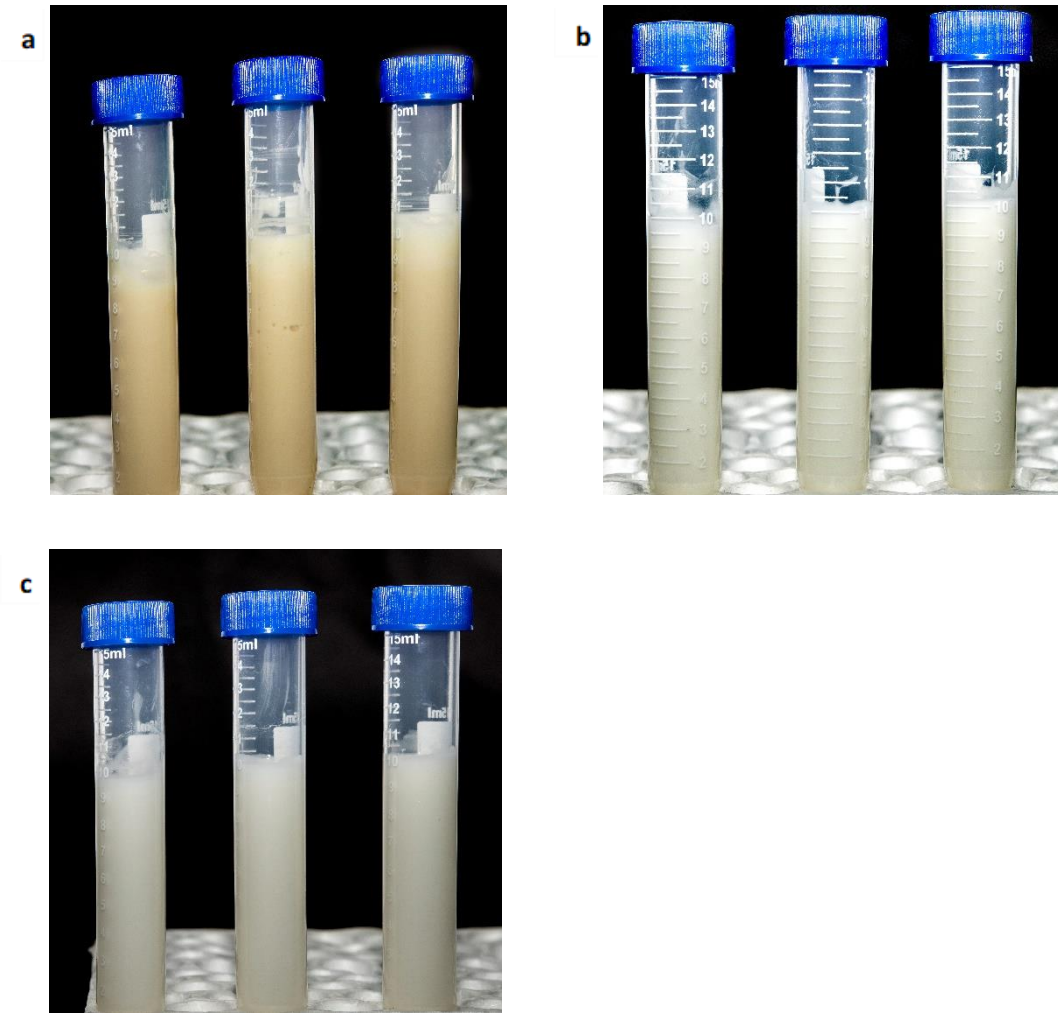


Figure 3.8: Homogenous essential oil emulsions formed at all HLBs for a) *V. zizanioides* lotion, b) *O. vulgare* lotion and c) *S. aromaticum* lotion

At certain HLBs, emulsions of *T. vulgaris* and *S. austrocaledonicum* both exhibited signs of breakdown within minutes of preparation. The degree of instability was observed to increase with HLB for both. With regards to *S. austrocaledonicum*, HLB 11 formed homogenous emulsion (CI = 0) while HLB 12 and HLB 13 displayed CI of 0.697 ± 0.086 and 0.913 ± 0.052

respectively. On the other hand, *T. vulgaris* emulsion at HLB 10 was stable (CI=0), HLB 11 displayed a CI of 0.352 ± 0.085 while HLB 12 presented with the largest CI of 0.514 ± 0.023. Monitoring the CI over 28 days revealed no change in creaming after day 1 of preparation. The emulsions fresh after preparation are generally of a lower viscosity due to the higher temperatures required during the process, and only thicken after the emulsions have returned to room temperature. At this point, the viscosity of the continuous phase is such that it is sufficient to prevent the further migration of oil droplets to the surface (Hu *et al.*, 2017b) and significant creaming ceases. Therefore, while the emulsions at non-optimal HLB exhibited gravitational separation, destabilisation did not progress any further. The emulsions after 28 days could be agitated and the cream layer redispersed to achieve a homogenous distribution. Even though it is a reversible phenomenon, the appearance of creaming may give customers the impression of inferior quality (Loi *et al.*, 2018). However, the tendency towards creaming of these HLB's indicate instability mechanisms (such as coalescence and aggregation) which may compromise on the formulation's ability to penetrate the stratum corneum (Nastiti *et al.*, 2017). This thereby decreases its therapeutic effect and leads to inconsistent dosing. Therefore, CI measurements were indicative of an optimum HLB of 10 for *T. vulgaris* lotion and 11 for *S. austrocaledonicum* lotion. A summary of the CI of each essential oil lotion at the prepared HLBs are shown in Table 3.8.

Table 3.8: Mean CI of essential oil lotions at prepared HLBs measured over 28 days

HLB	Mean CI of essential oil lotions ± (SD)[a]				
	O. vulgare	***S. aromaticum***	***S. austrocaledonicum***	***T. vulgaris***	***V. zizanioides***
8	-[b]	-	-	-	0.000 ± 0.000
9	-	-	-	-	0.000 ± 0.000
10	0.000 ± 0.000	-	-	**0.000 ± 0.000**	0.000 ± 0.000
11	0.000 ± 0.000	0.000 ± 0.000	**0.000 ± 0.000**[c]	0.352 ± 0.085	-
12	0.000 ± 0.000	0.000 ± 0.000	0.697 ± 0.086	0.514 ± 0.023	-
13	-	0.000 ± 0.000	0.913 ± 0.052	-	-

[a]Standard deviation (n=3)
[b] – denotes emulsions were not prepared at these HLB values as they were outside the calculated rHLB range
[c]Lowest CI value of each essential oil lotion is reported in bold where applicable

Several factors influence the creaming behaviour of an emulsion. These could be best described through the use of Stoke's Law, as shown in Equation 3.8 (Meher *et al.*, 2013; Hu *et al.*, 2017b), where V_{Stokes} represents the rate of creaming, g is the acceleration due to gravity, r is the radius of the oil droplet, $\rho_2 - \rho_1$ is the difference in density of the two phases and η is the viscosity of the emulsion.

$$v_{Stokes} = -2gr^2 \ (\rho_2 - \rho_1) / 9\eta_1$$

Equation 3.8

As can be seen, the creaming rate is directly proportional to the radius of the oil droplet and the difference in density between the aqueous and oil phases, and inversely proportional to the viscosity of the emulsion and volume of the aqueous phase (Yadav *et al.*, 2013; Sanad and Mabrouk, 2016). If small enough droplet sizes are formed, the distance between two oil droplets is sufficiently large that it decreases the probability of coalescence. Additionally, the concentration of the emulsifier blend may have been sufficient in reducing the interfacial tension such that equilibrium between the two phases is achieved, regardless of HLB (Mikulcová *et al.*, 2017; McClements and Jafari, 2018). The lipophilic emulsifier GMS displays thickening properties (Lakshminarayan *et al.*, 2006) which may sufficiently increase the viscosity of the emulsion such that may retard the migration of oil droplets to the surface (Mirhosseini *et al.*, 2008; Wang *et al.*, 2012). Additionally, Fredrick *et al.* (2013) demonstrated that saturated monoglycerides such as GMS are capable of slowing down the coalescence rate, therefore the type of emulsifier shows a critical role. This could explain the absence of creaming in the *O. vulgare*, *S. aromaticum* and *V. zizanioides* lotions. By slowing down the gravitational separation of the two phases through control of viscosity, these essential oil lotions were less prone to creaming.

Despite the same base formula being used for all the essential oil lotion, two essential oil lotions out of the five (*S. austrocaledonicum* and *T. vulgaris*) exhibited creaming. This is not unconventional as the efficacy of the emulsifier blend is strongly related to the chemical structure of the active ingredient (in this case, the essential oil) (Paulo *et al.*, 2020). The emulsifier blend employed may more successfully emulsify one oil over another, based on the variation of lipophilicity and ionisable compounds of each (Edris and Malone, 2012).

Therefore, regardless of all factors kept constant and only the essential oil varied, formulations with vastly different properties and stability behaviours were obtained.

While the determination of CI provides a simple and inexpensive method for assessing instability, it is only useful when the degree of creaming is considerable (Hu *et al.*, 2017b). Although the absence of creaming is a positive sign with regards to emulsion stability, it does not provide any data on the optimum HLB, as seen with the lotions of *O. vulgare*, *S. aromaticum* and *V. zizanioides*. Even when creaming is visible to the naked eye, CI has been deemed as more of a preliminary parameter (Yadav *et al.*, 2013) as mechanisms such as agglomeration and Ostwald's ripening cannot be assessed (Hu *et al.*, 2017b). Hence, further analysis with MDD, turbidity and viscosity tests must be carried out in order to provide further clarification.

3.3.2.2 Mean droplet diameter of essential oil emulsified lotions

The change in MDD and PDI over time for each essential oil emulsified lotion are reported below.

3.3.2.2.1 Mean droplet diameter, PDI and intensity of droplet size distribution of *O. vulgare* lotion

The MDD of *O. vulgare* lotion at HLB 10 was recorded as 357 nm one day after preparation, and the total change recorded after 84 days was a mere 4.20% with fluctuations seen in between (Figure 3.9a). The PDI decreased from 0.605 to 0.501 (-17.19%) over the same course of time (Figure 3.9b). The *O. vulgare* lotion at HLB 11 displayed the smallest initial MDD and PDI values with 197 nm and 0.397 respectively. The measurements remained more or less constant with time with less fluctuations observed. The MDD was found to increase by a marginal 4.57% while PDI increased by 7.65% over the 84 day period. The HLB 12 lotion recorded an initial MDD of 235 nm and displayed the largest change in MDD over time (decrease of 38.30%). In a similar fashion, the corresponding PDI decreased by 39.43% to a final value of 0.321. While decreases in MDD and PDI with time are unconventional, it is important to consider that sample preparation techniques such as dilution may alter the structure of the original emulsions (Hu *et al.*, 2017b). Additionally, brief mixing of the emulsions before each measurement in order to evenly redistribute the oil droplets is necessary, but may reduce the

MDD and PDI further. However, truly emulsified oil droplets are thermodynamically stable and should be resistant to any change or deformation (Capek, 2004). For this reason, more emphasis was placed on the % change in MDD and PDI with time (either positive or negative) as markers of instability, rather than choosing the smallest values of each. Thus, from the data presented in Figures 3.9a and 3.9b, it can be seen that *O. vulgare* lotion at HLB 11 showed the least overall changes in MDD and PDI, and was selected as the optimal HLB.

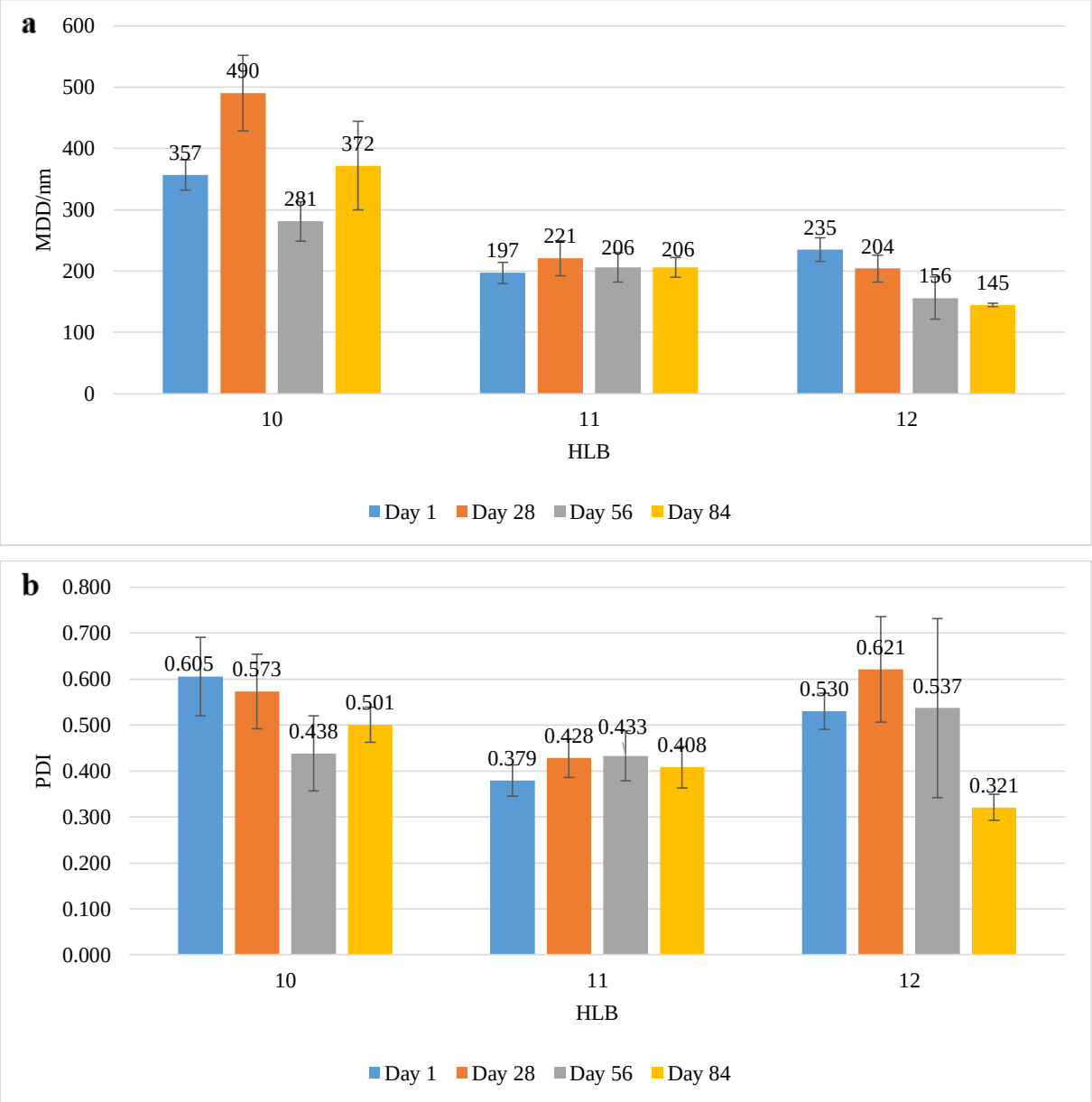
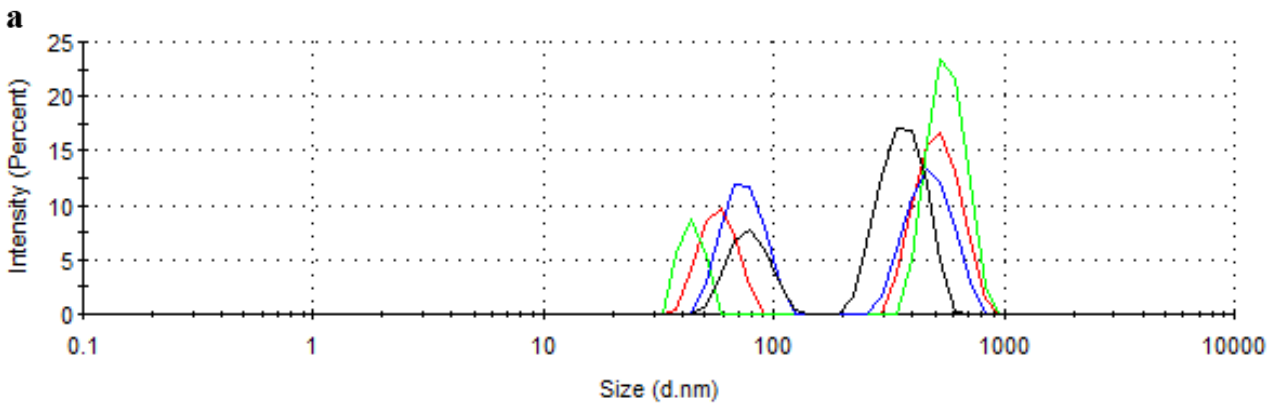


Figure 3.9: Change in a) MDD and b) PDI of *O. vulgare* lotions

The size distribution by intensity for HLB 10, 11 and 12 and their changes over time are shown in Figure 3.10, and provide additional data on the emulsion system dynamics. For all three

HLB values, a bimodal distribution was obtained from day one, possibly indicating a lack of uniformity in droplet size (Kwon Hong *et al.*, 2018). For HLB 10, two completely separate peaks are observed at 62 nm and 543 nm on day one, which would account for its relatively high PDI value of > 0.5. The peaks represent the sizes at which the largest number of oil droplets are present. Minor shifts in intensity and distribution of the peaks are seen with time which reflect the changes in MDD and PDI shown in Figure 3.9. The size distribution of *O. vulgare* lotion at HLB 11 presents with a bimodal peak on day one, which gradually flattens with time due to natural instability mechanisms such as coalescence. However, the overall width and starting intensities of the peaks remain relatively unchanged over the 84 day testing period and thus a slow rate of destabilisation for HLB 11 may be inferred. The *O. vulgare* lotion at HLB 12 initially presented with two separate peaks at 54 nm and 337 nm, and the smaller peak can be seen to gradually grow in intensity with time to mirror the decreasing MDD. However, destabilisation of the emulsion system at HLB 12 can be clearly seen as the peaks progressively become more undefined and distributed over a larger range. Furthermore, the appearance of smaller additional peaks with HLB 12 can be observed as from day 28. While HLB 12 displayed the lowest final values of MDD and PDI, it possessed the overall largest changes in the emulsion system with time which ultimately translates to the largest degree of instability. As such, the emulsion system remaining most resistant to change and destabilisation was thus confirmed to be at HLB 11.



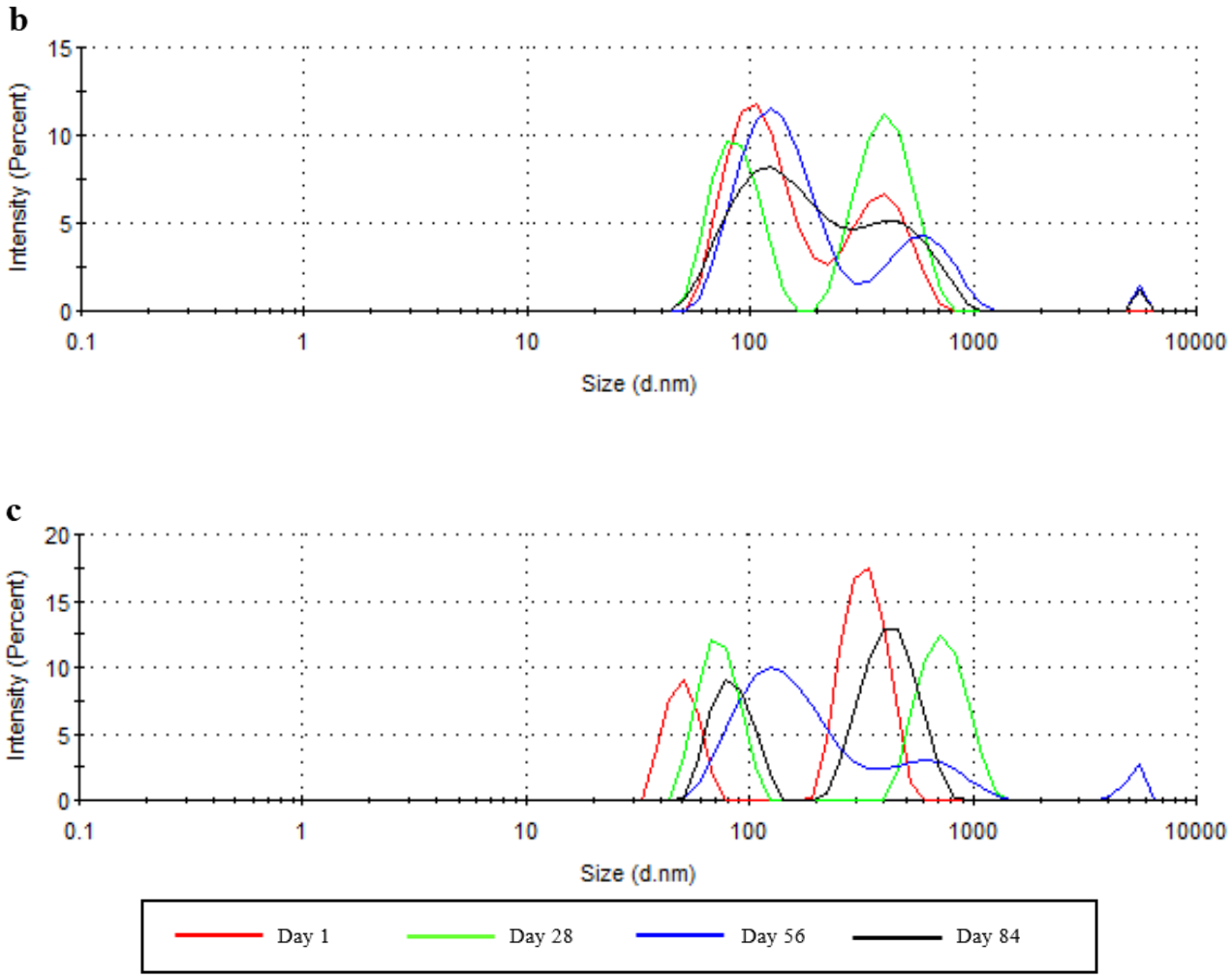


Figure 3.10: Change in size distribution of *O. vulgare* lotion at a) HLB 10, b) HLB 11 and c) HLB 12 with time

3.3.2.2.2 Mean droplet diameter, PDI and intensity of droplet size distribution of *S. austrocaledonicum* lotion

The initial measurements for *S. austrocaledonicum* lotions showed an overall decrease in MDD with an increase in HLB (Figure 3.11a). The smallest MDD was recorded as 1368 nm for HLB 13, while HLB 11 displayed the largest at 1673 nm. Similar PDI values on day one were obtained throughout with 0.154, 0.158 and 0.164 for HLB 11, 12 and 13 respectively and are shown in Figure 3.11b. All emulsions were measured with notable decreases in MDD from day 28 (-63.54%, -53.12% and -78.51% for HLB 11, 12 and 13 respectively) while PDI values increased significantly over the 84 days (377.92%, 203.16% and 306.67% for HLB 11, 12 and 13). It is in agreement with emulsion theory that emulsions destabilise with time to form larger oil droplets in order to reduce their free energy (Capek, 2004; Nursakinah *et al.*, 2013; Martin

et al., 2018; Alam *et al.*, 2020). During sampling, the measurements of the larger oil droplets formed may be "lost" as they fall outside the mean range of oil droplets, resulting in an overall smaller MDD. This theory can be supported by the significantly increase PDI values across all three HLB's, which would indicate the emulsions are now polydisperse and therefore the accuracy of the measurements obtained would be diminished. Additionally, PDI values of above 0.5 indicates the presence of polydisperse oil droplets whereby the accuracy of the measurements cannot be guaranteed (Jiménez *et al.*, 2018), particularly in the case of HLB 11 and HLB 13. The size distribution by intensity graphs were consulted for further clarification.

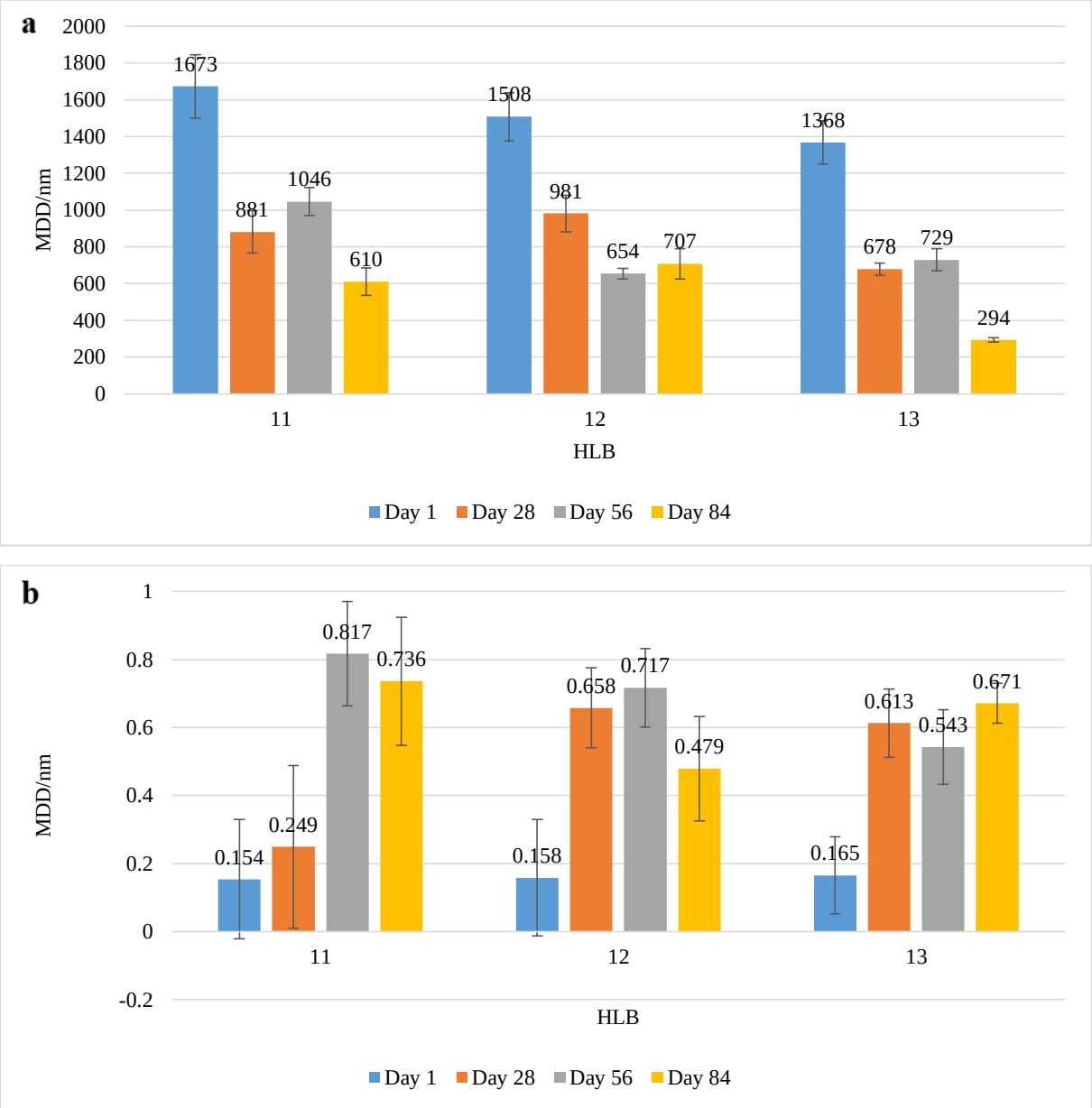
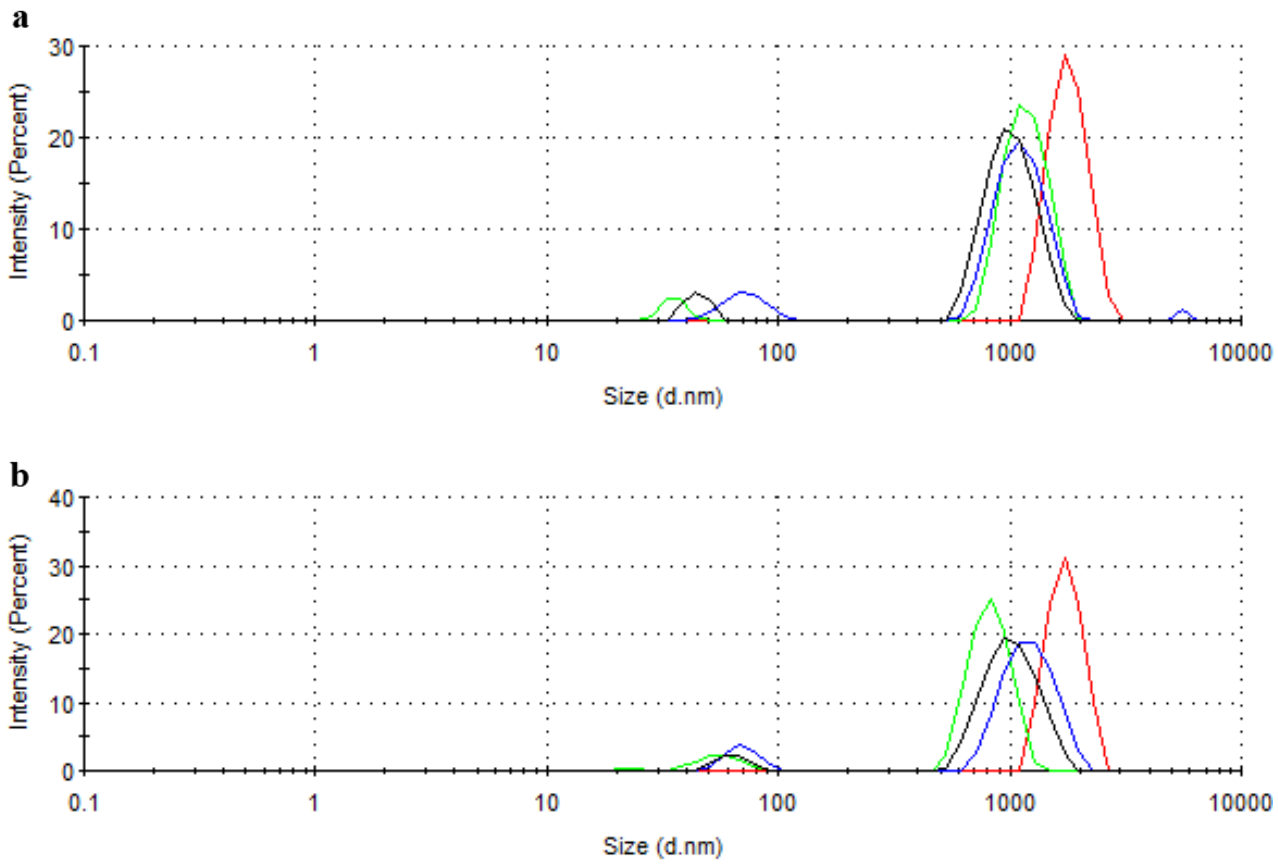


Figure 3.11: Change in a) MDD and b) PDI of *S. austrocaledonicum* lotions

As can be seen in Figure 3.12, monomodal intensity peaks were observed for all three HLBs on day one. The maximum intensity was noted for HLB 11 at 1734 nm, HLB 12 at 1743nm and HLB 13 at 1794 nm, which were closely related. While the peaks for HLB 11 and 12 were nearly identical, the distribution curve of HLB 13 was broader to reflect its slightly larger PDI of 0.165. As from day 28, the appearance of smaller, secondary peaks in the range of 40-500 nm at all three HLB's can be seen in Figures 3.12-3.14 which signify minor emulsion system disruption. Furthermore, the primary peaks for all three HLB's are observed to flatten with time, as a reflection of the large increases in PDI shown in Figure 3.11b. From the size distribution by intensity graphs (Figure 3.12), it can be seen that steady destabilisation occurs at all three HLB's. However, the lowest % change in MDD and PDI (-53.12% and 203.16% respectively) were both recorded at HLB 12 and was thus deemed as optimal according to this assay.



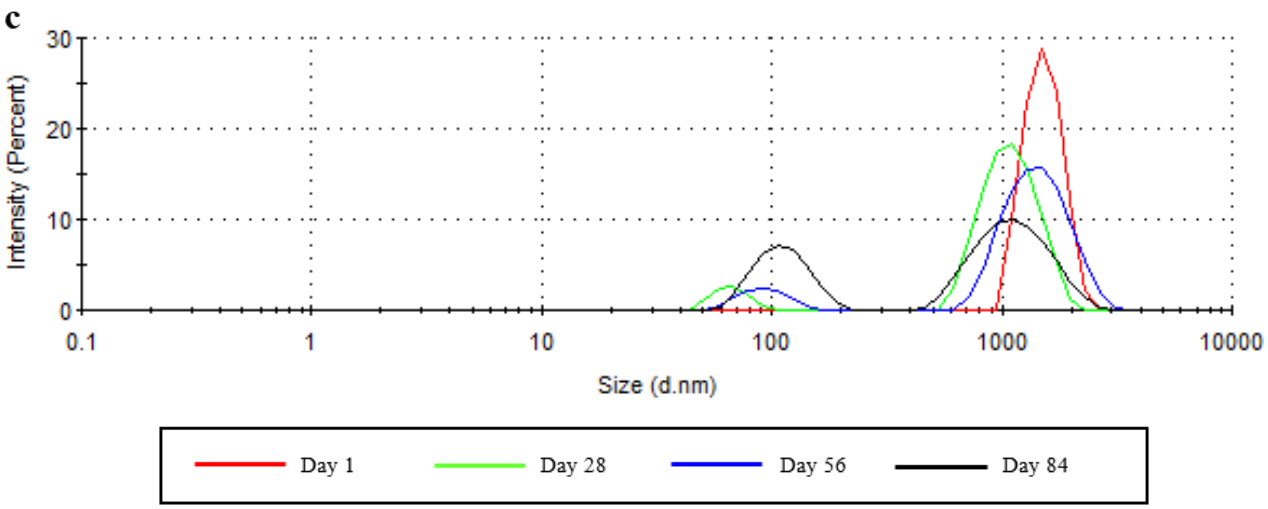


Figure 3.12: Change in size distribution for *S. austrocaledonicum* lotion at a) HLB 11, b) HLB 12 and c) HLB 13 with time

3.3.2.2.3 Mean droplet diameter, PDI and intensity of droplet size distribution of *S. aromaticum* lotions

The emulsions of *S. aromaticum* displayed increasing MDD with an increase in HLB, as shown in Figure 3.13a. On day one, the emulsions of HLB 11 and 12 displayed similar MDDs of 213 and 229 nm respectively while HLB 13 formed an MDD of 321 nm. With a similar trend, the PDI also increased with an increase in HLB, however all PDI's at day one after preparation were recorded as < 0.5, which indicated acceptable polydispersity throughout (Figure 3.13b). The emulsions were found to coalesce with time as expected, and both MDD and PDI recorded as from 28 days revealed larger increases. The MDD of the HLB 11 emulsion increased by 276.06% while the MDD of HLB 12 and 13 increased by 339.74% and 293.77% respectively. The final PDI at 84 days of HLB 11 and 12 were found to be < 0.5 with increases of 29.57% and 54.85% respectively and were thus acceptable. However, the final PDI of HLB 13 was relatively large (0.676) with an increase of 74.23% and thus the emulsion was polydisperse, most likely due to the presence of excessive coalescence. Over the specified time period, the smallest MDD and PDI of *S. aromaticum* emulsions and change thereof both corresponded to HLB 11, which could therefore be deduced as optimal.

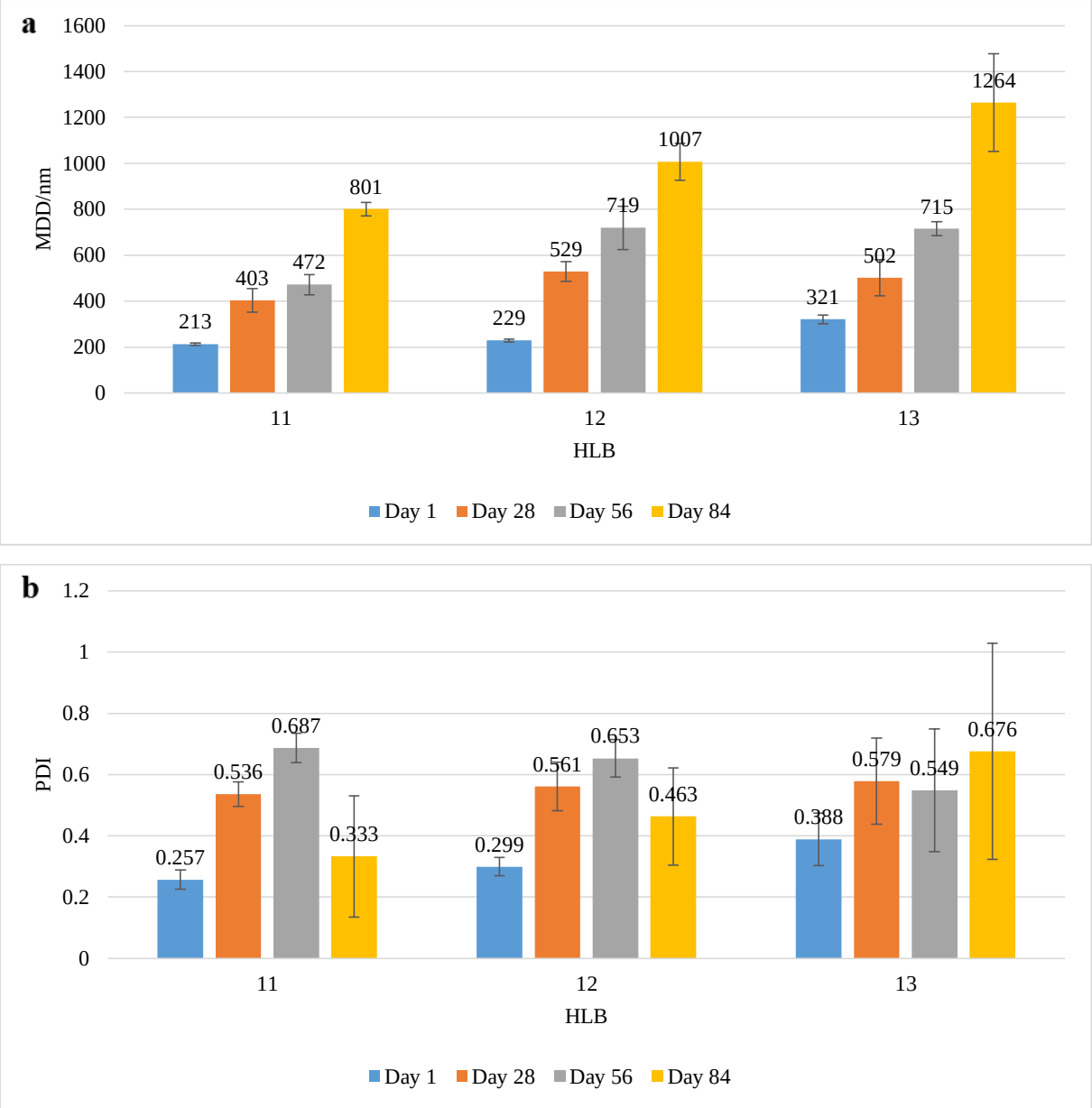


Figure 3.13: Change in a) MDD and b) PDI of *S. aromaticum* lotions

The destabilisation dynamics of the *S. aromaticum* lotions at HLB 11, 12 and 13 can be seen in the droplet size intensity graphs as shown in Figure 3.14. On day one, smooth and monomodal curves could be seen for HLB 11 and 12 corresponding with their smaller PDI values of 0.257 and 0.299 respectively. Two weaker peaks were observed with the distribution curve of HLB 13 which was a reflection of the larger PDI of 0.388 obtained. At 28 days, the initial monomodal peak of both HLB 11 and 12 could be observed to split into two peaks, and the median value of these peaks could be seen to shift right with time, as a reflection of the increasing MDD and PDI of both. As from the second measurement on day 28, the curve of

HLB 13 was flattened and could be seen with multiple curves, indicating widened particle size distribution and flocculation (Jiménez *et al.*, 2018).

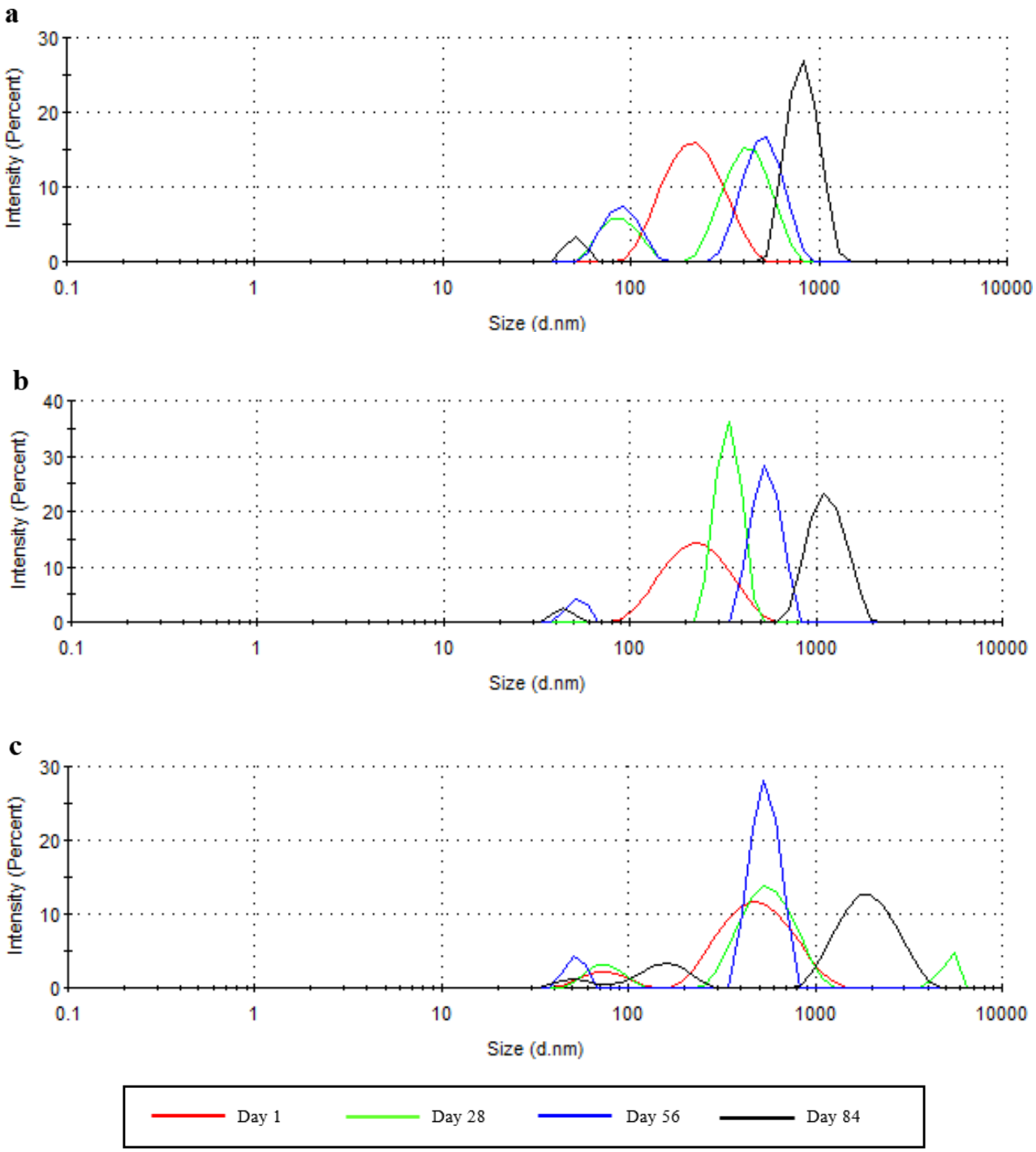


Figure 3.14: Change in size distribution for *S. aromaticum* lotion at a) HLB 11, b) HLB 12 and c) HLB 13 with time

3.3.2.2.4 Mean droplet diameter, PDI and intensity of droplet size distribution of *T. vulgaris* lotion

The change in MDD and PDI with time for *T. vulgaris* lotions are shown in Figure 3.15. After preparation, HLB 10 and 11 were found to display similar MDD's of 764 nm and 848 nm while HLB 12 formed emulsions with a significantly lower MDD of 209 nm. The measured PDI of lotions at HLB 10 and 11 were < 0.5, indicating a narrow distribution. Despite having the smallest MDD, the PDI at HLB 12 was > 0.5, signifying that the emulsions were prone to destabilisation mechanisms such as coalescence and agglomeration. Observation over 84 days showed a decrease in MDD for HLB 10 and 11 (-50.65% and -75.94% respectively) concurrent with a significant increase in PDI (63.75% and 42.39% respectively).

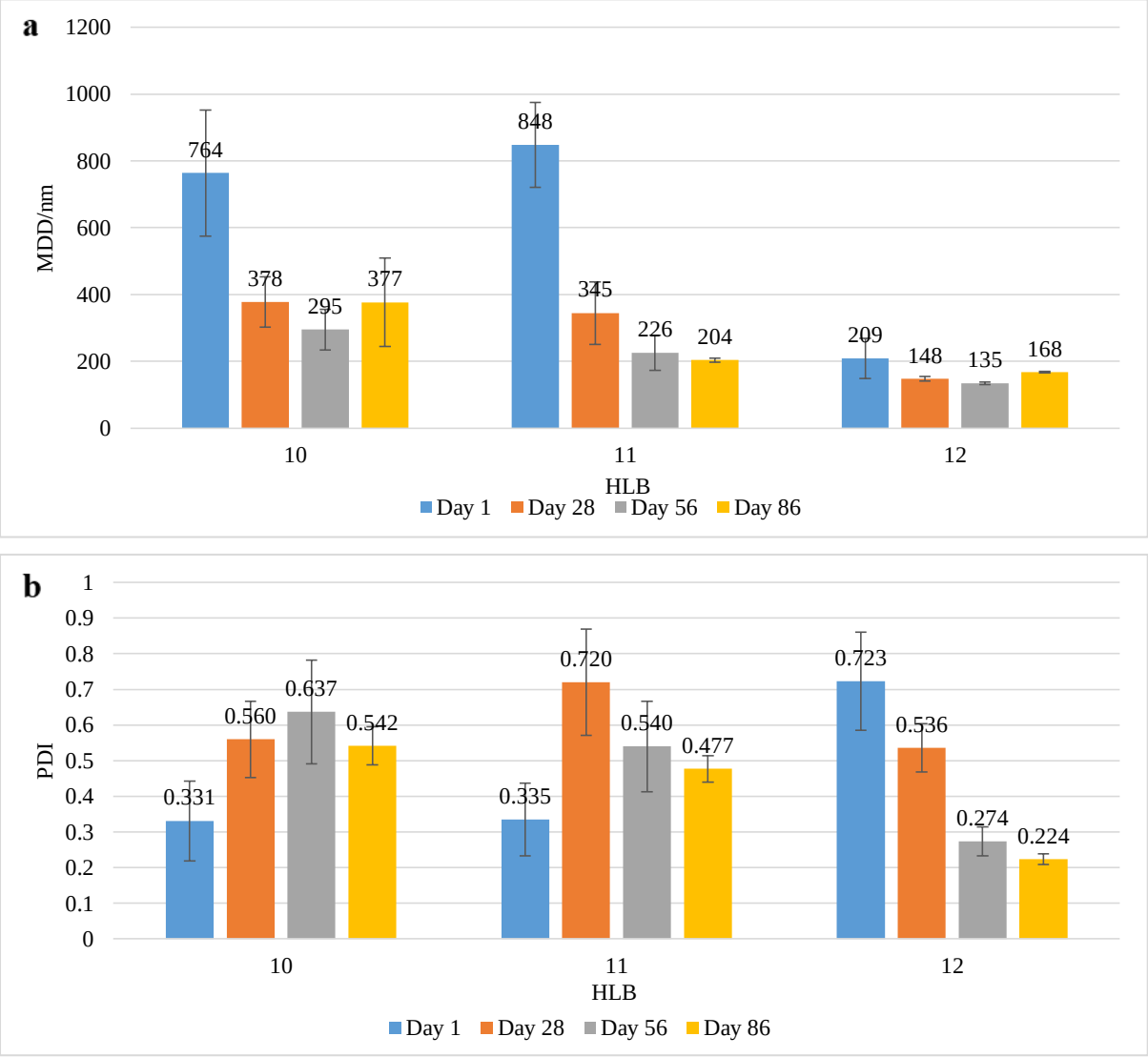
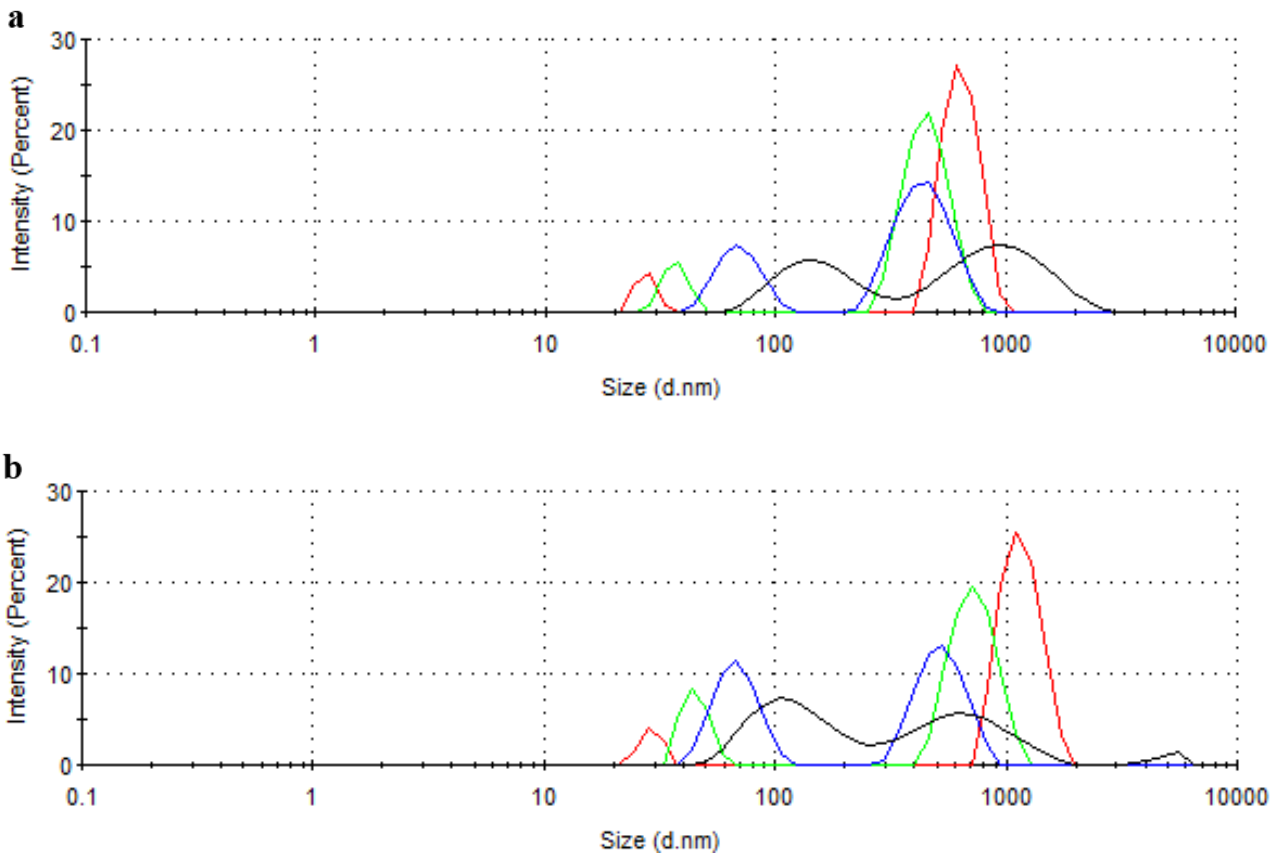


Figure 3.15: Change in a) MDD and b) PDI of *T. vulgaris* lotions

In the case of HLB 12, there was a 19.62% decrease in MDD from 209 nm to 168 nm, with a simultaneous decrease in PDI from 0.723 to 0.224.

The droplet size intensity curves of MDD of *T. vulgaris* lotions showed the distribution of oil droplets at HLB 10, 11 and 12 over time, as shown in Figure 3.16. On day 1, both HLB 10 and HLB 11 were bimodal with one dominant peak and a smaller, secondary peak at 27.14 nm and 29.03 nm respectively. With time, the peaks of *T. vulgaris* lotion at HLB 10 slowly flattened and decreased in intensity to form two approximately equal peaks, shown in Figure 3.16a. At HLB 11, an irregular, uneven distribution was obtained after the three-month period, signifying an unstable system and insufficient emulsification (Figure 3.16b). Despite system disruption being apparent with the irregular curves obtained, the PDI particularly did not reflect this as a value of < 0.5 was obtained on day 84. With regards to HLB 12, interestingly the emulsion appeared to improve in MDD, PDI and size distribution with time as peaks appeared to become sharper and higher in intensity. The resulting measurements revealed HLB 12 to display the smallest MDD and PDI, as well as in the overall change of these parameters. A smooth, monomodal size distribution was observed on day 84.



c

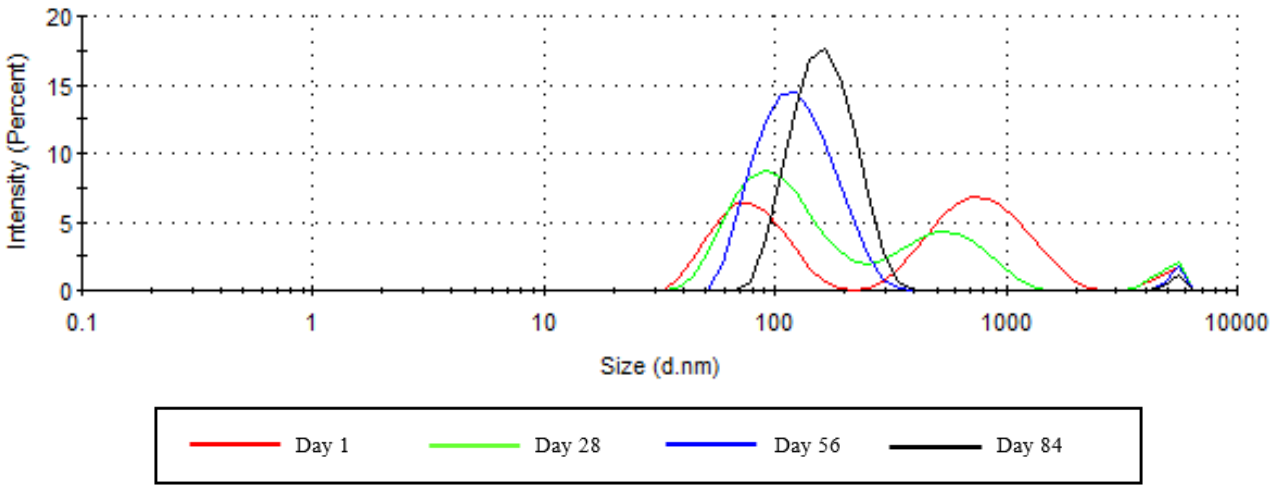


Figure 3.16: Change in size intensity of *T. vulgaris* lotion at a) HLB 10, b) HLB 11 and c) HLB 12 with time

Thus, from the MDD assay, *T. vulgaris* lotion at HLB 12 could be concluded as optimal.

3.3.2.2.5 Mean droplet diameter of *V. zizanioides* lotion

Initial measurement of MDD of *V. zizanioides* lotions showed a trend of increasing MDD with HLB, as shown in Figure 3.17a. The lowest MDD was 1358 nm for HLB 8 while the highest obtained was 1745 nm for HLB 10. This is not as significant of a variation with HLB as seen with the other essential oil formulations. The initial PDI for all three HLB's were acceptable (< 0.5) and indicated uniformly sized droplets had been formed with each (Figure 3.17b). After 84 days, similar increases in MDD was seen with all of the HLBs (19.81%, 20.61% and 20.57% for HLB 8, 9 and 10 respectively). The PDI values indicated an increase of 42.03% for HLB 8 (0.188 to 0.267) and a 33.33% increase in HLB 9 (from 0.279 to 0.372). A sharp increase of 140.23% was observed for HLB 10, indicating that *V. zizanioides* lotion at this HLB might present with a limited shelf-life. However, all PDI values after 84 days remained < 0.5, signifying that the emulsions possessed a minimal level of coalescence and could be deemed relatively stable. While HLB 8 possessed the smallest values of MDD and PDI (1627 nm and 0.267 respectively) after the 84 days, minimal % change in PDI was noted at HLB 9, signifying it to be the most resistant to fluctuations and internal system changes. The *V. zizanioides* lotion at HLB 9 could therefore be predicted to retain its original droplet size for an extended period of time in comparison and was selected as optimal.

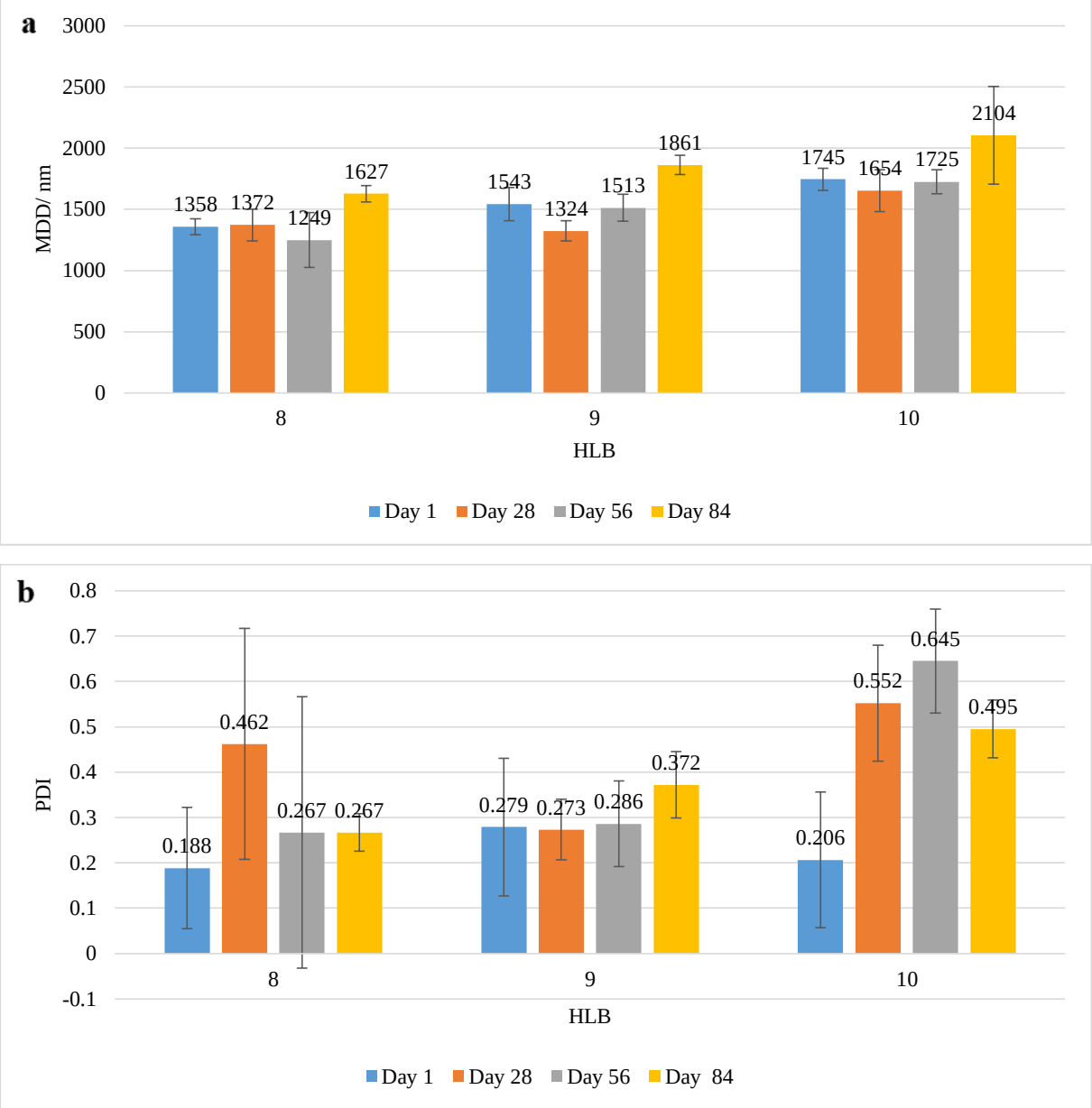


Figure 3.17: Change in a) MDD and b) PDI of *V. zizanioides* lotions

Inspection of the intensity distribution curves after preparation for *V. zizanioides* revealed smooth, monomodal curves for all HLBs as shown in Figure 3.18. Similar curves of distribution were obtained across all HLB's with time and are reflective of the comparable changes in MDD as previously shown in Figure 3.17a. Reviewing the data obtained, HLB 9 is a suitable candidate for the rHLB of *V. zizanioides* emulsion.

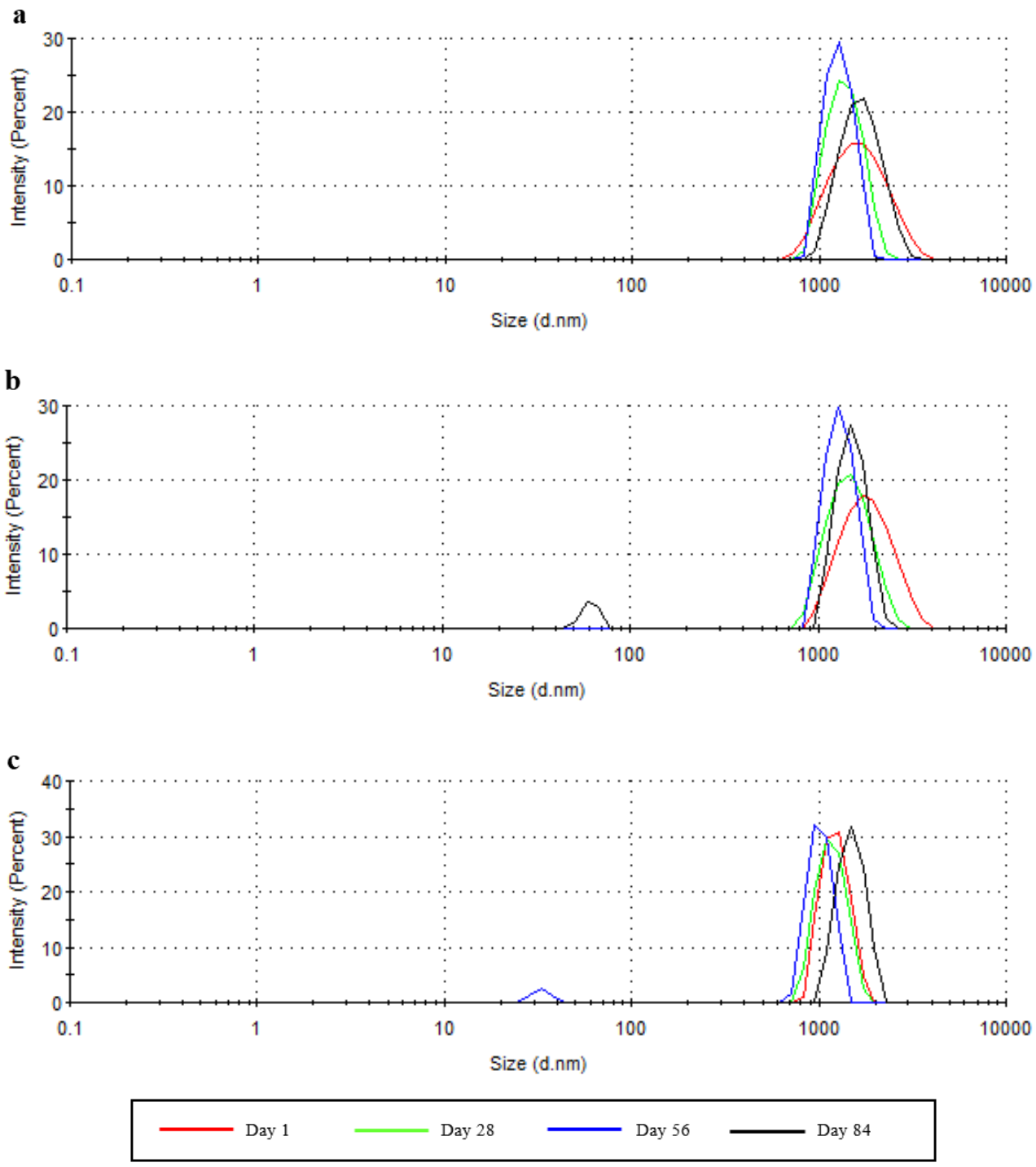


Figure 3.18: Change in size intensity of *V. zizanioides* lotion at a) HLB 8, b) HLB 9 and c) HLB 10 with time

Fluctuations in droplet size may occur, especially in the initial days after preparation as the two phases are in dynamic equilibrium and the oil droplets are continuously being disrupted and reformed (Jiménez *et al.*, 2018). As previously mentioned, is also important to consider that the preparation of samples for the MDD measurement may negatively affect the readings (Hu *et al.*, 2017b). Additionally, nano-emulsion droplets may become encapsulated in a water film

when diluting the samples before measurement (Wang *et al.*, 2021). Unbound essential oil droplets in the formulation, occurring either as a result of inadequate emulsifier or the aforementioned dynamic changes in equilibrium, may coalesce upon diluting with the solvent (distilled water) and impact on the readings. Hence, the MDD and PDI values obtained on a single measurement day may not be a true representation of the formulation properties. While the initial measurement is important, the droplet size distribution and the change of these two parameters with time is a crucial indicator of the long-term stability of a formulation. This highlights the importance of measuring MDD over an extended phase and the ensuing time-dependant measurements should be more heavily considered.

Small droplet sizes are desired as they are able to more efficiently penetrate the site of application and reach the target area. For the skin specifically, compounds need to be relatively small, lipophilic and water soluble in order to penetrate the stratum corneum (Nastiti *et al.*, 2017). Martí *et al.* (2014) found that microspheres of 2000 nm and larger were unable to pass the stratum corneum and deliver its encapsulated contents to its target site. Su *et al.* (2017) recommends that particle sizes should be kept to lower than 500 nm for dermal drug delivery. On the other hand, nanocapsules of 200 nm have been shown to successfully diffuse across the stratum corneum and exert their therapeutic action (Badri *et al.*, 2018). Hence theoretically, based off the MDD results obtained thus far, maximum penetration and antibacterial effect would be achieved by *S. aromaticum*, *O. vulgare* and *T. vulgaris* lotions at optimum HLB. Clearly, every effort should be made to obtain the smallest MDD possible as such a characteristic is advantageous in terms of both stability and efficacy.

3.3.2.3 Viscosity

The viscosity for each essential oil lotion was measured and shown in Table 3.9: Viscosity of essential oil lotions at tested HLBs. The flow curves obtained revealed the viscosity of all formulations to behave as non-Newtonian fluids. They were found to display shear-thinning behaviour, as a linear decrease with an increase in shear rate was seen with each. Lotions of *O. vulgare* and *T. vulgaris* were observed to be the most viscous in general and displayed similar values at each HLB. For *O. vulgare*, decreasing viscosities of 7861 ± 861 mPa/s, 3202 ± 318 mPa/s and 2491 ± 747 mPa/s were obtained while similar values of 7342 ± 1385 mPa/s, 2926 ± 1224 mPa/s and 1922 ± 268 mPa/s were seen with *T. vulgaris* for HLB 10, 11 and 12 respectively. With regards to the *V. zizanioides* lotions, no major difference in viscosity was

seen with a change in HLB. For example, HLB 8 and HLB 10 produced emulsions of viscosity 2610 ± 681 mPa/s and 2637 ± 694 mPa/s respectively while HLB 9 formed a slightly higher viscosity of 3144 ± 351 mPa/s. Lotions of *S. austrocaledonicum* and *S. aromaticum* were the least viscous and both were found to show no statistically different values for emulsions at HLB 11 and 12 (t-tests revealed $p>0.05$). However, a decrease in viscosity for HLB 13 was observed with both.

Table 3.9: Viscosity of essential oil lotions at tested HLBs

Essential oil lotion	HLB	Mean η ± SD (mPa/s) (n =3)
O. vulgare	10	7861 ± 861
	11	3202 ± 318
	12	2491 ± 747
S. austrocaledonicum	11	1377 ± 298
	12	1371 ± 187
	13	1085 ± 215
S. aromaticum	11	1785 ± 485
	12	1751 ± 379
	13	908 ± 65
T. vulgaris	10	7342 ± 1385
	11	2926 ± 1224
	12	1922 ± 268
V. zizanioides	8	2610 ± 681
	9	3144 ± 351
	10	2637 ± 694

The shear-thinning behaviour observed with all the formulations is typical for most emulsions, and has been found to be influenced by the volume fraction of oil in the emulsion. Pal (2000) found that emulsions with a low volume fraction displayed Newtonian behaviour (constant viscosity regardless of stress applied) and with higher volume fractions, emulsions exhibited shear-thinning behaviour. It is thought to be a result of the structural arrangement of the particles (dos Santos *et al.*, 2014). When a shear force is applied, hydrogen bonds and other

interparticle forces are broken (Brewer *et al.*, 2016; Danila *et al.*, 2021). As a result, the particles rearrange themselves in the direction of the flow to minimise the frictional effects and prevent deformation (Danila *et al.*, 2021). These structural changes cause the break up and disruption of multiple oil droplets and lead to a reduction in the total oil volume fraction (Vasiljević *et al.*, 2009). Therefore, a decrease in viscosity of the emulsion is seen with an increase in shear rate.

An apparent decrease in viscosity was seen with an increase in HLB for four of the five formulations. The lipophilic emulsifier used, GMS, is known for its thickening effects in addition to its emulsifying ability, and is commonly employed in food products to improve their rheology and texture (Lakshminarayan *et al.*, 2006; Ali and Hasnain, 2013). At lower HLBs, higher proportions of the lipophilic emulsifier must be employed in order to achieve the desired HLB, thus the higher viscosity of formulations at a lower HLB may be explained by the higher concentration of GMS. A study by Djuris *et al.* (2014) found that both GMS and stearic acid exert a significant influence on determining the viscosity of a formulation. The same study found that the thickening effects of cetyl alcohol, another excipient used in the current study's formulations, were negligible in comparison to the aforementioned ingredients. Additionally, Tween® 80 has been shown to have no effect on viscosity of $^{O}/_{W}$ emulsions (Koocheki and Kadkhodaee, 2011), hence any changes in viscosity is unlikely to be directly attributed to a change in its concentration as a result of varying HLB. Previous studies by Kennedy and Kennedy (2007) and Alam *et al.* (2010) found that an increase in the emulsifier concentration led to an increase in the overall viscosity of the formulation, hence the large 1:1 ratio of emulsifier to essential oil used as compared to other studies (Meher *et al.*, 2013; Wang and Marangoni, 2015; Sanad and Mabrouk, 2016; Campolo *et al.*, 2020) may have successfully increased the viscosity of the emulsions past the threshold of major destabilisation.

Another important factor that impacts the viscosity of an emulsion is the droplet size of the oil phase (Pal, 2000; dos Santos *et al.*, 2011; Kwon Hong *et al.*, 2018). A general trend was observed between the MDD of the formulations and their viscosity at respective HLBs. With the exception of *V. zizanioides*, formulations with overall lower droplet size ranged displayed higher viscosities and vice versa. For example, *O. vulgare* lotions displaying low MDD values of 197.4 nm – 357.2 nm corresponded with the larger viscosities of 2491 mPa/s – 7861 mPa/s and conversely, the relatively large MDD of *S. austrocaledonicum* lotions (1368 nm - 1673 nm) corresponded with the lower range of viscosities obtained (1085 mPa/s – 1377 mPa/s).

Excluding the lotions of *V. zizanioides*, the general trend of MDD from smallest to largest was *S. aromaticum* < *O. vulgare* < *T. vulgare* < *S. austrocaledonicum* while the trend of viscosity from largest to smallest was *O. vulgare* > *T. vulgare* > *S. austrocaledonicum* > *S. aromaticum*. With larger oil droplets within an emulsion, the distance between two given droplets is relatively small (Figure 3.19). As the droplet sizes decrease, the distance between them increases which leads to stronger hydrodynamic forces between them, resulting in a higher viscosity (Pal, 2000). Additionally, smaller droplet sizes present with a larger surface area of dispersion with the aqueous phase. This affects the fluidity of the emulsion such that it results in an increase in the viscosity (Kwon Hong *et al.*, 2018).

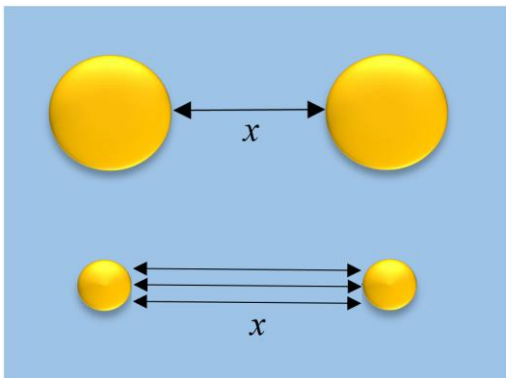


Figure 3.19: Increase in distance between two oil droplets with a decrease in droplet size results in stronger hydrodynamic forces in between

In the case of *S. aromaticum* lotions, a trend of decreasing viscosity with an increase in MDD and HLB were seen however the overall viscosity values obtained were the lowest out of the five essential oil lotions, despite the MDD values being intermediary. Past studies have shown that the viscosity of the dispersed oil also affects the overall viscosity of the formulation (McClements, 2005; Danila *et al.*, 2021), and the relatively low viscosity of neat *S. aromaticum* essential oil could have been a deciding feature. Similarly, *V. zizanioides* essential oil is highly viscous (information provided by supplier) which may have been the overriding factor in similar viscosities being obtained throughout, regardless of HLB and emulsifier ratio.

Overall, the viscosities obtained were within the acceptable range of a lotion. Generally, a viscosity of < 5000 mPa/s is defined as low while that of > 18500 mPa/s is considered high. The viscosity is a critical parameter as it is directly associated with the acceptability of the formulation. If the viscosity is too high, it will reduce the spreadability of the lotion, while a

viscosity too low may increase spillage and make its application a challenge (Safriani *et al.*, 2017). Additionally, the shear-thinning behaviour previously described is ideal as the lotion would display a high viscosity during pouring (low shear) and a low viscosity during application (high shear) (IFSCC, 1997). All five formulations at their varied HLBs were within a satisfactory viscosity, and hence would not impede patient compliance.

The rheology of a formulation plays an important role in determining its shelf life, efficiency and packaging requirements such as pump ratings (Danila *et al.*, 2021). Various factors were found to influence the rheological characteristics of a formulation and these include emulsifier type and concentration, volume of the aqueous phase and oil droplet characteristics such as size, viscosity and interparticle forces (McClements, 2005; Pal, 2011; Vianna-Filho *et al.*, 2013; Hu *et al.*, 2017b). The viscosity plays a significant role in the stability behaviour of an emulsion, as it is inversely proportional to the rate of creaming, as governed by Stoke's Law (Equation 3.8). Therefore, through the measurement of viscosity, the probability of breakdown can be assessed and minimised (Li *et al.*, 2020).

3.3.2.4 Turbidity

The $\%D_T$ of the lotions between the top and bottom of the lotions per HLB for the lotions are shown in Table 3.10. With the *O. vulgare* lotions, $\%D_T$ was found to increase from HLB 10 to 11 with differences of 13.632% ± 2.433 and 29.192% ± 20.217 respectively. The lowest $\%D_T$ was found at HLB 12 with a small 0.418% difference, which indicates the emulsion was homogenous and presented with minimal separation. With regards to *S, austrocaledonicum*, an increase in $\%D_T$ with an increase in HLB was noted, with a minimum $\%D_T$ of 8.389 ± 4.301 at HLB 11 and a maximum of 88.892 ± 4.975 at HLB 13. The larger $\%D_T$ values obtained for HLB 11 and 12 corresponded accordingly with the increasing CI with HLB as seen in Section 3.3.2.1, as a higher CI and a larger $\%D_T$ together would both imply a greater degree of separation and vice versa. The emulsions of *S. aromaticum* exhibited increasing $\%D_T$ with increasing HLB. Emulsions at HLB 13 exhibited the highest degree of separation with a 16.195% ± 21.682 difference, while those of HLB 11 and 12 were similar to each other at 0.343% ± 0.610 and 0.587% ± 6.060 respectively. Hence, optimum HLB could be concluded to be in the range of 11-12 for *S. aromaticum* lotion. It is worth noting that the SD for each of the results is relatively large, as varying $\%D_T$'s during triplicate testing were obtained for each HLB. Still, a clear trend could be observed between the results obtained. The lotions of *T.*

vulgaris showed increasing $\%D_T$ with an increase in HLB, with values of 38.373 ± 0.993, 66.222 ± 0.376 and 76.493 ± 5.130 for HLB 10, 11 and 12. In similar fashion to the *S. austrocaledonicum* lotions, the increasing $\%D_T$ with HLB corresponded to the trend observed with the CI obtained, hence the two results are in agreement. With regards to *V. zizanioides*, minor values of $\%D_T$ (< 0.349%) were obtained throughout, showing that minimal separation was obtained for the emulsions regardless of HLB. For HLB 8, a negative value of -0.451% ± 0.868 was found. This suggests that the density of droplets at the bottom of the emulsion was higher than that of the top, which would imply settling. However, as the SD was again relatively high, no definitive conclusion can be formed. By selection of the minimum $\%D_T$, an optimum HLB of 10 for the *V. zizanioides* could be assumed.

Table 3.10: $\%D_T$ of essential oil lotions

Essential Oil Formulation	HLB	$\%D_T$ ± SD (n=3)	Observation
O. vulgare	**10**	**13.632 ± 2.433**[a]	**Creaming**
	11	29.192 ± 20.217	Creaming
	12	0.418 ± 0.934	Creaming
S. austrocaledonicum	11	8.389 ± 4.301	Creaming
	12	**65.228 ± 25.504**	**Creaming**
	13	**88.892 ± 4.975**	**Creaming**
S. aromaticum	11	0.343 ± 0.610	Creaming
	12	0.587 ± 6.060	Creaming
	13	16.195 ± 21.682	Creaming
T. vulgaris	**10**	**38.373 ± 0.993**	**Creaming**
	11	**66.222 ± 0.376**	**Creaming**
	12	**76.493 ± 5.130**	**Creaming**
V. zizanioides	8	-0.451 ± 0.868	Settling
	9	0.349 ± 0.695	Creaming
	10	0.122 ± 0.157	Creaming

[a]values reported in bold where p-value of results obtained are <0.05 and are therefore significant

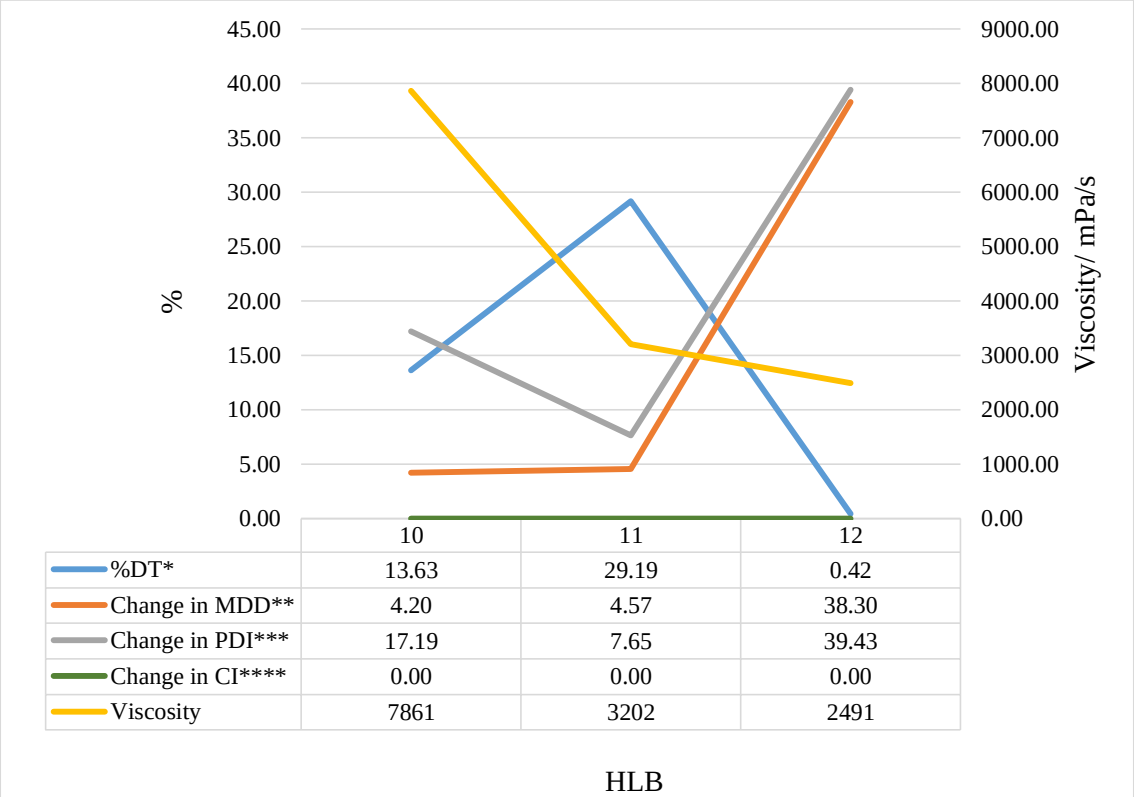
Measurement of turbidity of an emulsion determines the angle of scattering of light upon encountering the oil droplets within. The degree of scattering is highly influenced by the MDD and PDI, as well as the concentration of the oil phase, therefore any change in turbidity is indicative of a change within these factors (Mirhosseini *et al.*, 2008). Previous studies measured solely the difference in turbidity of the bottom of the emulsion as an indication of gravitational separation (Orafidiya *et al.*, 2002; Fernandes *et al.*, 2013; Meher *et al.*, 2013; Sanad and Mabrouk, 2016; Danila *et al.*, 2021). However, the emulsions prepared in this study exhibited starting different turbidity values as the varying emulsifier blend formed lotions of different properties for each HLB. Hence, the percentage difference between the top and the bottom turbidity for each HLB was measured, rather than the value of the turbidity itself. Therefore, $\%D_T$ not only indicates the optimum HLB, but also provides valuable insight in predicting the shelf-life and stability of the lotions formed.

3.3.2.5 Combined data analysis

The stability profile of each essential oil formulation is discussed in further detail, by consideration of the assessed factors in conjunction in order to determine the optimum HLB. In the graphs shown in Figures 3.20-3.24, the $\%D_T$ and the % change in CI, MDD and PDI with HLB are plotted on the left (primary axis) while the viscosity is plotted on the right (secondary axis). As far as possible, the HLB of each essential oil lotion showing the minimal change in the assessed factors was selected as the optimum HLB. All values are stated as positive to avoid false minimums. However, where the results were observed to not be in agreement, the assessment criteria was refined. The MDD has been cited as the single most important indicator of emulsion stability as it directly governs the rate of creaming, as shown by Stoke's Law (Equation 3.8) (Mirhosseini *et al.*, 2008; Wang *et al.*, 2012). A large change in the MDD can therefore be viewed as a precursor of physical instability mechanisms (such as creaming and cracking). Creaming within an emulsion would be sufficient cause for exclusion as the optimum HLB as the ideal emulsion should present with a CI of 0% (Loi *et al.*, 2018). Therefore, in emulsions where physical instabilities were already visible, the CI was given more significance as an assessment parameter as opposed to the MDD and PDI. Where CI was observed to be zero, MDD and PDI were then used as the defining factors of stability. While viscosity was not an assessment criteria, it is still related via Stoke's Law and thus included for completion's sake.

3.3.2.5.1 *Origanum vulgare* lotion

As previously stated, *O. vulgare* lotions displayed no visible creaming, regardless of HLB. Therefore, the MDD, PDI and $\%D_T$ were analysed for further clarification. A minimum % change in MDD and PDI with time was seen at HLB 11 (4.57% and 7.65% respectively), visible with a clear dip as shown in Figure 3.20. However, the $\%D_T$ was at a maximum at HLB 11, which is conflicting with the MDD and PDI results obtained. It is worth noting that the results of $\%D_T$ were not statistically different amongst HLBs (p-value > 0.05), as previously stated. Therefore, the trend observed is subject to a margin of error. Additionally, the $\%D_T$ at HLB 11 was only 29.19%, and as it was not sufficiently large to translate into visible creaming, it could still be deemed as acceptable. A noteworthy observation of decrease in viscosity with an increase in HLB was also seen. As explained by Stoke's Law (Equation 3.8), the viscosity of an emulsion is theoretically inversely proportional to its rate of creaming, which is a function



	10	11	12
%DT*	13.63	29.19	0.42
Change in MDD**	4.20	4.57	38.30
Change in PDI***	17.19	7.65	39.43
Change in CI****	0.00	0.00	0.00
Viscosity	7861	3202	2491

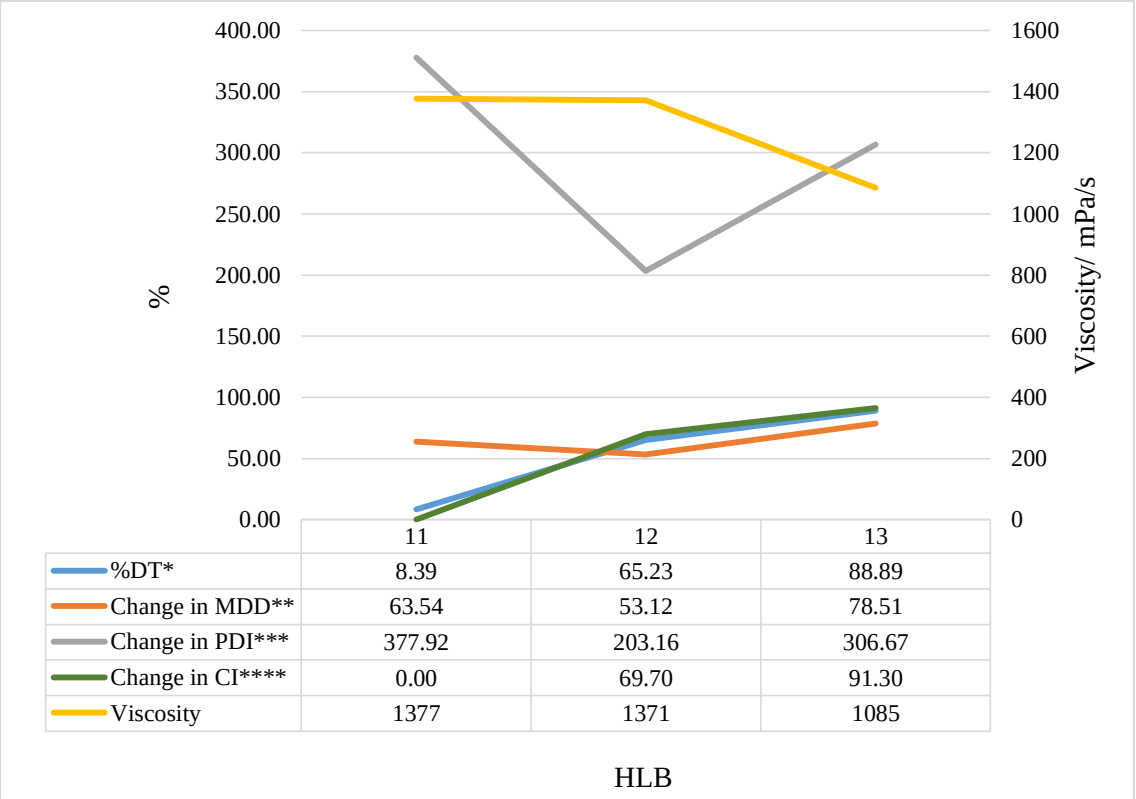
* %DT measured over 28 days
** % Change in MDD measured over 84 days
*** % Change in PDI measured over 84 days
**** % Change in CI measured over 28 days

Figure 3.20: Combined stability results of *O. vulgare* lotions as a function of HLB

of $\%D_T$. Therefore, by consideration of all the concerned parameters, the minimum change in MDD and PDI was taken as a measure of rHLB and HLB 11 was selected as the optimum for the *O. vulgare* lotion.

3.3.2.5.2 *Santalum austrocaledonicum* lotion

The combined stability results for *S. austrocaledonicum* lotion are shown in Figure 3.21. The % change in MDD over time showed comparable results per HLB, and while the minimum was observed at HLB 12, the statistical difference between the results was too small to form a definitive conclusion. A clear minimum of % change in PDI was also seen at HLB 12, hence it could be inferred that this HLB was optimal. However, as previously mentioned, the CI, when present, should take preference as the deciding factor. A clear trend was observed as both the CI and $\%D_T$ increased with an increase in HLB. The *S. austrocaledonicum* lotion only showed homogenous emulsion HLB 11, while those at HLB 12 and 13 showed considerable



	11	12	13
%DT*	8.39	65.23	88.89
Change in MDD**	63.54	53.12	78.51
Change in PDI***	377.92	203.16	306.67
Change in CI****	0.00	69.70	91.30
Viscosity	1377	1371	1085

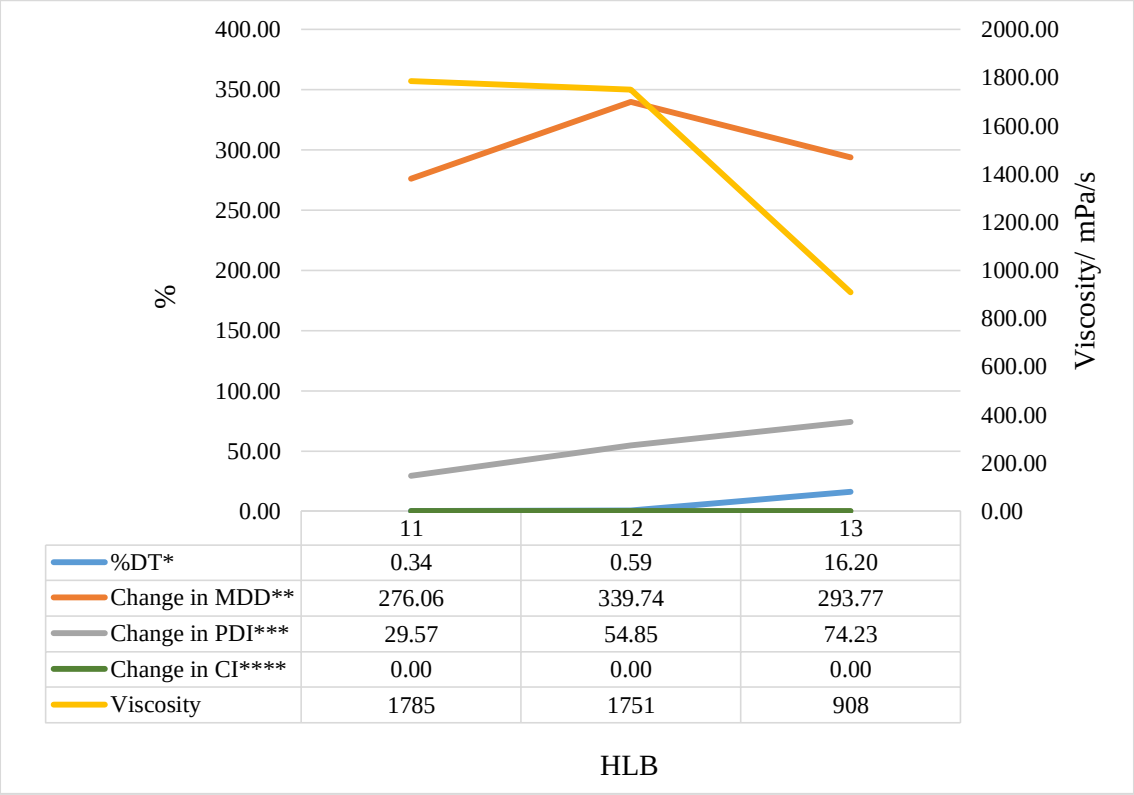
* %DT measured over 28 days
** % Change in MDD measured over 84 days
*** % Change in PDI measured over 84 days
**** % Change in CI measured over 28 days

Figure 3.21: Combined stability results of *S. austrocaledonicum* lotions as a function of HLB

creaming (69.7% and 91.3% respectively). A minimal $\%D_T$ value of 8.39% was observed at HLB 11, while HLB 12 and 13 presented with significantly large values of 65.23% and 88.89%. Thus, it can be concluded that HLB 11 is the rHLB, regardless of the results obtained with MDD and PDI. From Figure 3.21, it can be seen that the lotions of *S. austrocaledonicum* showed increasing CI and $\%D_T$ with decreasing viscosity, which is in agreement with Stoke's Law. The more viscous an emulsion, the harder it is for oil droplets to rise to the surface and form an uneven distribution. Thus, the viscosity may in fact have been a controlling factor in the stability of *S. austrocaledonicum* lotion. As the maximum viscosity, minimum CI and minimum $\%D_T$ were all in agreement, HLB 11 was selected as the rHLB of *S. austrocaledonicum* lotion.

3.3.2.5.3 *Syzigium aromaticum* lotion

As no creaming was observed with the *S. aromaticum* lotions, CI was excluded as a factor in the determination of the rHLB. The remaining factors ($\%D_T$, MDD and PDI) (Figure 3.22)



	11	12	13
%DT*	0.34	0.59	16.20
Change in MDD**	276.06	339.74	293.77
Change in PDI***	29.57	54.85	74.23
Change in CI****	0.00	0.00	0.00
Viscosity	1785	1751	908

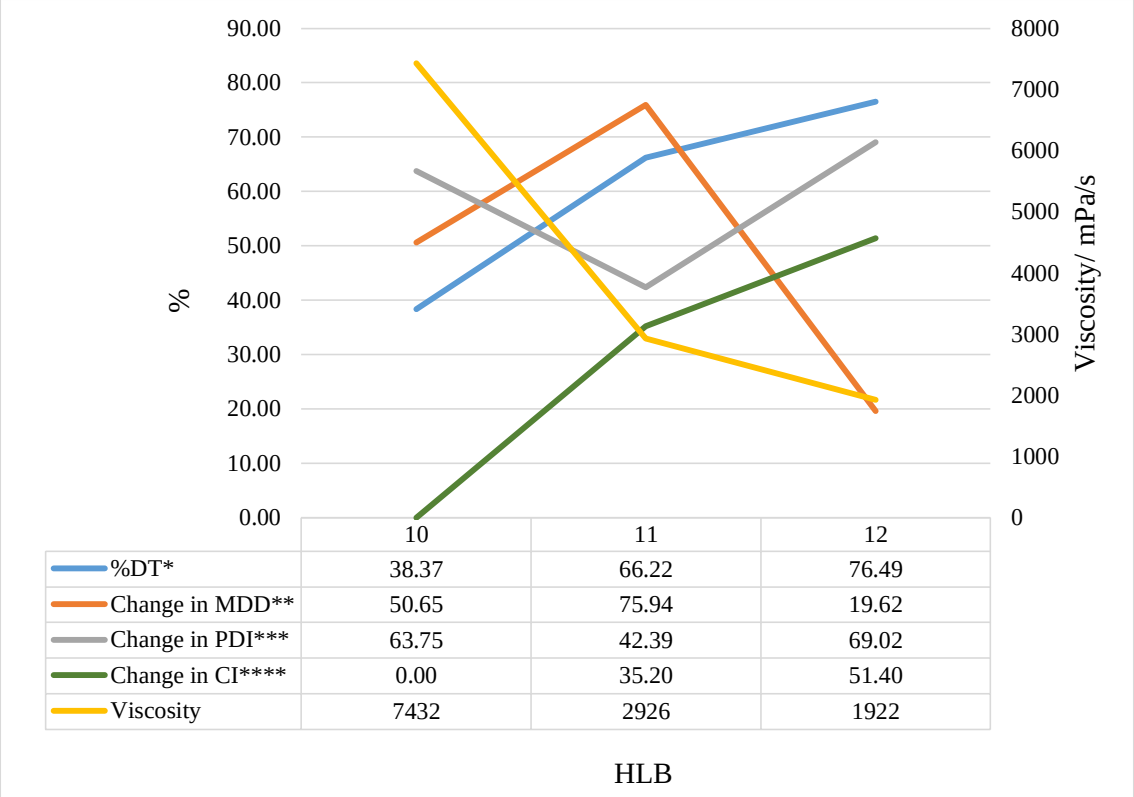
*%DT measured over 28 days
** % Change in MDD measured over 84 days
*** % Change in PDI measured over 84 days
**** % Change in CI measured over 28 days

Figure 3.22: Combined stability results of *S. aromaticum* lotions as a function of HLB

were relied upon to provide insight into the rHLB. In similarly fashion to the *S. austrocaledonicum* lotions, a decrease in viscosity resulted in an increase in $\%D_T$ for *S. aromaticum* lotion. This ultimately translates to a decrease in stability with an increase in HLB, and thus it may be inferred that a lower-spectrum HLB would be preferred for *S. aromaticum* lotions. Additionally, the % change in MDD and PDI both showed a minimum at the lowest tested HLB of 11, with values of 276.06% and 29.57% respectively. Furthermore, a distinct positive correlation between PDI and $\%D_T$ was visible. Higher PDI values indicate the presence of larger oil droplets, which tend to gravitate towards the surface of the emulsion with time. This then leads to a larger difference in turbidity between the top and the bottom of the emulsion. Thus, the results of the PDI and $\%D_T$ obtained are in accordance. As HLB 11 displayed the lowest % change in MDD, PDI and $\%D_T$ simultaneously, it was selected as the rHLB of the *S. aromaticum* lotion.

3.3.2.5.4 *Thymus vulgare* lotion

The combined stability data of *T. vulgaris* lotions for HLB 10 to 12 are shown in Figure 3.23. A strong positive correlation was again observed between $\%D_T$ and CI for *T. vulgaris* lotions, with both factors increasing with HLB. This is in accordance as increased creaming will result in a larger degree of separation. Similarly to *S. austrocaledonicum* lotions, these increases in $\%D_T$ and CI can be attributed to the simultaneous decrease in viscosity observed. The % change in MDD was observed to increase from HLB 10 (50.65%) to a maximum at HLB 11 (75.94%) and decrease sharply to HLB 12 (19.62%). The % change in PDI was seen to follow the opposite trend, with a minimum of 42.39% obtained at HLB 11. Despite the *T. vulgaris* lotion at HLB 12 displaying the smallest % change in MDD, the emulsion showed macro signs of destabilisation day one after preparation, resulting in a large CI and $\%D_T$. It is likely that inadequate emulsification would leave the disrupted or unbound essential oil droplets to slowly evaporate with time due to their volatile nature, leaving the smaller emulsified droplets behind and resulting in a smaller net MDD measurement. As the % change in MDD and PDI results may have been conflicting, the CI and $\%D_T$ still provides an excellent indication of the true stability of the emulsion system. As the lowest CI and lowest $\%D_T$ both corresponded to HLB 10, this could be concluded as optimal for the *T. vulgaris* lotion.



	10	11	12
%DT*	38.37	66.22	76.49
Change in MDD**	50.65	75.94	19.62
Change in PDI***	63.75	42.39	69.02
Change in CI****	0.00	35.20	51.40
Viscosity	7432	2926	1922

* %DT measured over 28 days
** % Change in MDD measured over 84 days
*** % Change in PDI measured over 84 days
**** % Change in CI measured over 28 days

Figure 3.23: Combined stability results of *T. vulgare* lotions as a function of HLB

3.3.2.5.5 *Vetiver zizanioides* lotion

A summary of the combined stability results for *V. zizanioides* lotions at HLB 8-10 are shown in Figure 3.24. With the exception of % change in PDI, the lotions of *V. zizanioides* essential oil were all noted to display minor changes in properties with HLB. No significant change in $\%D_T$ or MDD was seen with a change in HLB. However, as previously discussed in Figure 3.17b, the emulsions of HLB 9 displayed the lowest change in PDI over time (33.33%), which did not translate to a noticeably larger $\%D_T$ due to its slighter higher viscosity out of all three HLB's. While any one of the tested HLBs may provide an acceptable formulation with regards to MDD, CI and $\%D_T$, the PDI turned out to be the only defining indicator of true stability. Thus, HLB 9 was selected as the optimum HLB for *V. zizanioides* lotion.



	8	9	10
%DT*	0.451	0.349	0.122
Change in MDD**	19.81	20.61	20.57
Change in PDI***	42.02	33.33	140.29
Change in CI****	0.00	0.00	0.00
Viscosity	2610	3144	2637

* %DT measured over 28 days
** % Change in MDD measured over 84 days
*** % Change in PDI measured over 84 days
**** % Change in CI measured over 28 days

Figure 3.24: Combined stability results of *V. zizanioides* lotions as a function of HLB

The rHLB of the five essential oil lotions were successfully determined through the analysis of the CI, MDD, PDI and $\%D_T$ for each. At the rHLB, the emulsified lotions are guaranteed to display minimal destabilisation and a therefore a longer shelf-life. Thus, the physical stability of the essential oil emulsified lotions prepared in this study were validated. Consequently, the essential oil lotions at optimal HLB were selected for further antimicrobial testing against acne pathogen reference strains.

3.4 Summary

- For the selected neat essential oils, the approximate HLB was determined as HLB 7 for *V. zizanioides*, HLB 10 for *O. vulgare* and *T. vulgaris* and HLB 11 for *S. austrocaledonicum* and *S. aromaticum.*
- The base formula was developed taking into consideration the desirable properties of a lotion.

- Essential oil lotions were prepared at the calculated rHLB and one HLB unit above and below.
- Stability testing (MDD, CI, $\%D_T$ and viscosity) was conducted on each essential oil emulsified lotion at the various HLBs prepared and correlations between the different assays were observed.
- The lotions of *O. vulgare*, *S. aromaticum* and *V. zizanioides* exhibited no creaming regardless of HLB, while those of *S. austrocaledonicum* and *T. vulgare* were found to display increased creaming with an increase in HLB.
- The lotions of *O. vulgare*, *S. aromaticum* and *T. vulgaris* at optimal HLB were found to display sufficiently small MDD's suitable for dermal application and absorption.
- All essential oil emulsified lotions were found to exhibit shear-thinning behavior, which is ideal in the handling and application of a lotion product.
- All viscosities obtained for the essential oil emulsified lotions were in the range 908-7861 mPa/s, which is acceptable for a lotion.
- The optimum HLB was found at 9 for the *V. zizanioides* lotion, 10 for the *T. vulgare* lotion and 11 for *O. vulgare*, *S. aromaticum* and *S. austrocaledonicum.*

CHAPTER 4 - ANTIMICROBIAL EFFICACY OF ESSENTIAL OIL LOTIONS AGAINST ACNE-INDUCING PATHOGENS

4.1 Introduction

The encapsulation of essential oils into an emulsion provides a suitable mode of transport of the bioactive ingredients (essential oil compounds) to the target site. While pure essential oils have been thoroughly investigated for their antimicrobial potential against various pathogens, the consequent development into stable formulations is not often investigated and to a lesser degree, the antimicrobial efficacy of these formulations validated. Chapter 2 identified the five most antimicrobially active essential oils against the pathogens involved in acne, and Chapter 3 focused on the development of stable lotions incorporating each of these oils as the active ingredient. Optimum stability of the lotions was achieved at HLB 9 for *V. zizanioides*, HLB 10 for *T. vulgaris,* and HLB 11 for *S. aromaticum, S. austrocaledonicum* and *O. vulgare.* Therefore, these lotions were assessed for antimicrobial efficacy as the next step. This chapter thus sought to identify the retention of activity of these essential oils post-formulation against the same pathogens reference strains, and to determine if persistence of efficacy is present over a three month period.

Various vehicles have been investigated for the encapsulation of essential oils, such as micro-emulsions, nano-emulsions and liposomes. The type of vehicle is an important point for consideration as the nature of the base will directly affect the release of the essential oil to its target site (Orafidiya *et al.*, 2002; Tadele *et al.*, 2009). While successful encapsulation will guarantee the stability of the formulations, an excessively strong bond between the emulsifier and the essential oil will prevent the necessary contact between the oil and the pathogen, resulting in a reduced bactericidal activity (Florence and Attwood, 2015). Therefore, a fine line exists between stability and antimicrobial efficacy and the middle road between the two should be sought by ensuring an acceptable level of each.

The method of determining the antimicrobial efficacy is also an important point for consideration. A lipophilic substance may not diffuse adequately throughout an aqueous test

media and vice versa (Tadele *et al.*, 2009), hence an accurate result may be compromised depending on the method. With regards to emulsions, dilution with a solvent other than the aqueous phase may result in a change of the physiochemical properties of the emulsion, such as droplet size (Solè *et al.*, 2012; Hodoroaba *et al.*, 2019). Hence, this chapter further sought to develop a specific test method in order to maximise contact between the essential oils and pathogen, and to minimise disruption of the emulsion system.

4.2 Methods

4.2.1 Culturing of pathogens

Staphylococcus aureus (ATCC® 25923™) and *S. epidermidis* (ATCC® 2223™) were cultured in TSB and incubated at 37°C for 24 hrs. *Streptococcus pyogenes* (ATCC® 12344™) was cultured in HTM with supplement NAD added. The inoculum was incubated at 37°C for 24 hrs. These constitute the facultative anaerobic test organisms. The aero-tolerant anaerobic pathogen, *C. acnes* (ATCC® 11827™) was cultured in TGB and incubated at 37°C under anaerobic conditions in a CO_2 incubator (5% CO_2) for 72 hrs.

4.2.2 Time-kill assay of essential oil lotions against facultative anaerobe pathogens

The broth microdilution method as per CLSI guidelines (CLSI, 2020) was adapted to protect the integrity of the emulsion systems, as shown in Figure 4.1. Under sterile conditions, 100 µL of the required test medium was added into the first row of a 48-well microtitre plate and the remaining rows were filled with 900 µL of sterile 0.90% sodium chloride (NaCl) solution. A 0.5 McFarland's standard of each pathogen was prepared to contain an approximate inoculum concentration of $1x10^6$ CFU/mL, and 100 µL thereof was transferred into the first row of the microtitre plate. A volume of 100 µL of each freshly prepared essential oil lotion at optimum HLB was introduced into the first well of each column. Thereafter, the contents of each well of the first row (containing broth, formulation and inoculum) were thoroughly mixed and 100 µL was transferred to the subsequent well containing 0.90% NaCl. Successive dilutions were then performed into 0.90% NaCl three times. A preliminary test run was carried out beforehand to determine how many dilutions were required to obtain a suitably low concentration of quantifiable colonies. The microtitre plates were sealed to prevent evaporation and then incubated as per the respective pathogen's optimal incubation requirements. A 50 µL sample

was withdrawn from the last dilution of each column at 0, 3, 6 and 24 hrs. Samples were spread onto previously prepared agar plates with the aid of a sterile spreader and again incubated as per respective conditions, after which the number of colonies were counted.

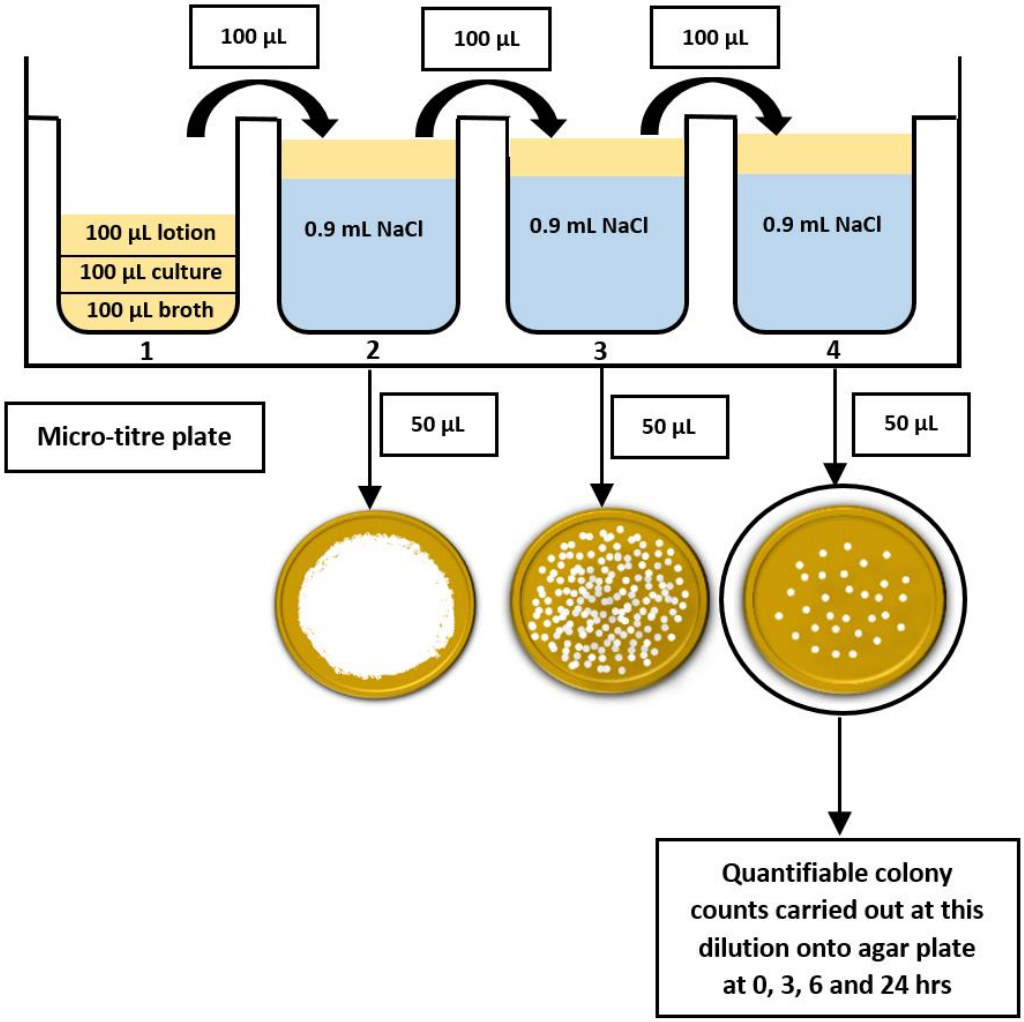


Figure 4.1: Adapted broth microdilution method for the time-kill assay of essential oil lotions against aerobic pathogens

The logarithm of CFU/mL present in the original well with time were calculated using equation 4.1 and were plotted onto a graph.

$$\text{Log (CFU/mL)} = \log_{10} \text{Colony count} \times 1 \times 10^{9}$$

Equation 4.1

A commercial antibiotic, ciprofloxacin (at a concentration of 0.01 mg/mL) was used as a positive control to ensure microbial susceptibility while the negative control was a *S. chinensis* formulation prepared as detailed in Chapter 3, Section 3.2.2.2. As *S. chinensis* is a carrier oil, it is expected to show no noteworthy antimicrobial activity (as confirmed in Table 2.3 of Chapter 2), it may be used as a suitable replacement for the essential oil in the formulation in order to identify whether the excipients employed are antimicrobially inert. A culture control of 100 µL of the prepared 0.5 McFarland's standard of the relevant pathogen reference strain in 200 µL growth media was included to ensure viability and to obtain a growth curve of each pathogen. All measurements were conducted in triplicate.

4.2.3 Antimicrobial efficacy of essential oil lotions against aero-tolerant anaerobe C. acnes

Due to inconsistencies observed in growth of *C. acnes* on aerobic agar plates, the colony count method as describe in Section 4.2.2 could not be utilised to determine the antimicrobial efficacy of the formulations against this anaerobic pathogen. Therefore, broth turbidity (same principle but using broth instead of agar) was used as a measure of microbial growth and is shown in Figure 4.2. In a 48-well microtitre plate, 100 µL of each formulation was diluted with 100 µL of TGB in separate wells under sterile conditions. A volume of 100 µL of a 0.5 McFarland's standard of *C. acnes* (containing an approximate inoculum concentration of $1x10^6$ CFU/mL) was added to each formulation and mixed thoroughly. The microtitre plate was sealed to prevent evaporation and incubated anaerobically for 72 hrs. Thereafter, a 100 µL aliquot of each well was removed and added to 9.9 mL of TGB in a sterile test tube. The resulting dilution was incubated for 72 hrs anaerobically, upon which the test tubes were visually inspected. Visual inspection was the preferred measure of assessment as incubation over the 72 hrs test period resulted in colour changes of the formulation making UV measurements (standard microbial methods) redundant. The absence of turbidity would indicate complete inhibition of *C. acnes* while turbid broth would indicate growth. Ciprofloxacin at 0.01 mg/mL (100 µL) was used as the positive control while 100 µL of *S. chinensis* lotion was used as the negative control. A culture control was also included to ensure microbial viability. All measurements were conducted in triplicate.

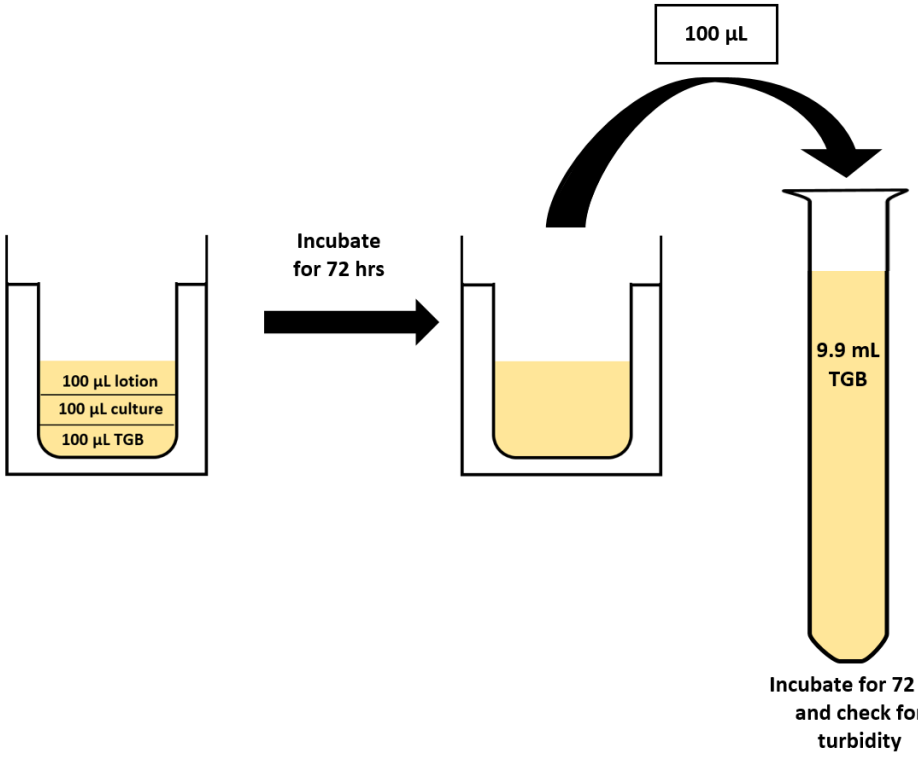


Figure 4.2: Broth turbidity method for antimicrobial activity of essential oil lotions against *C. acnes*

4.2.4 Preservation of antimicrobial efficacy of formulations

Essential oil lotions were stored at room temperature (25°C) for three months, upon which they were reassessed for antimicrobial efficacy. As this assay was only concerned with retention of antimicrobial efficacy, time-dependant aliquots were not removed as in Section 4.2.2, but rather inhibition of microbial growth was assessed at the end of the incubation period of each pathogen reference strain. In a 48-well microtitre plate, 100 µL of broth, 100 µL of aged formulation (three months) and 100 µL of prepared 0.5 McFarland's standard of inoculum was mixed in a well. The plate was sealed with a sterile seal and incubated as per the respective pathogen reference strain's requirements (24 hrs for *S. aureus*, *S. epidermidis* and *S. pyogenes* and 72 hrs for *C. acnes*). Thereafter, a 50 µL sample was removed from each well. For the facultative anaerobes, the sample was spread onto an agar plate and incubated accordingly. A clear plate would indicate total inhibition and therefore the preservation of antimicrobial activity while the presence of colonies would signify loss of efficacy. For the aero-tolerant anaerobe *C. acnes*, the sample was transferred into 9.9 mL of TGB and incubated for 72 hrs.

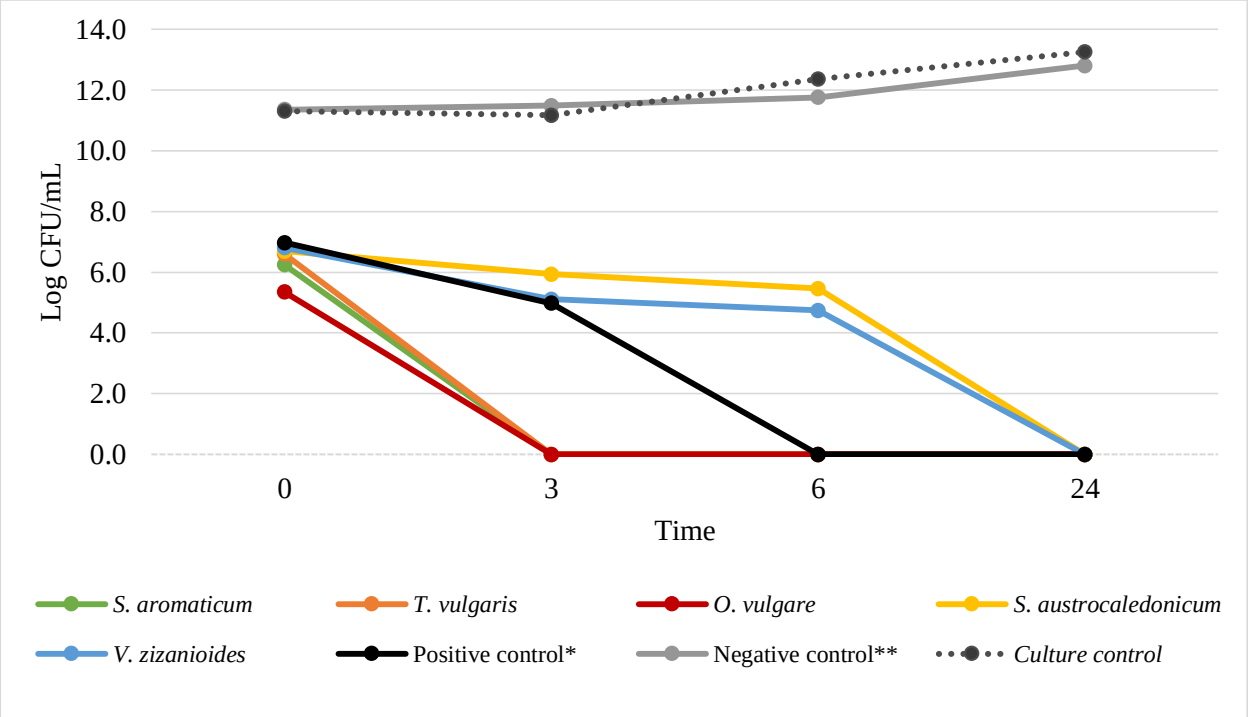
The absence or presence of broth turbidity was recorded for each essential oil formulation in an identical manner to the initial assay, and comparisons between the two were drawn.

4.3 Results and discussion

4.3.1 Time-kill assay of formulations against facultative anaerobic pathogens

4.1.1.1 Efficacy of essential oil lotions against S. aureus

The time-kill assay revealed all five essential oil formulations to display total bactericidal activity against *S. aureus*. The time-kill plots are shown in Figure 4.3. The lotions of *O. vulgare*, *S. aromaticum* and *T. vulgaris* completely eliminated *S. aureus* within 3 hrs of incubation (Figure 4.4). Lotions of *S. austrocaledonicum* and *V. zizanioides* displayed a slower killing dynamic and only showed total elimination at 24 hrs. In comparison, the positive control ciprofloxacin eliminated *S. aureus* within 6 hrs. As expected, the negative control, *S. chinensis* lotion showed no activity and displayed comparable growth to the culture control. This shows that none of the excipients possess or contribute towards antimicrobial activity, and therefore the efficacy of the essential oil lotions can be solely attributed to the essential oils themselves.



* Positive control is ciprofloxacin 0.01 mg/mL
**Negative control is *S. chinensis* lotion

Figure 4.3: Time-kill assay of essential oil lotions against *S. aureus*

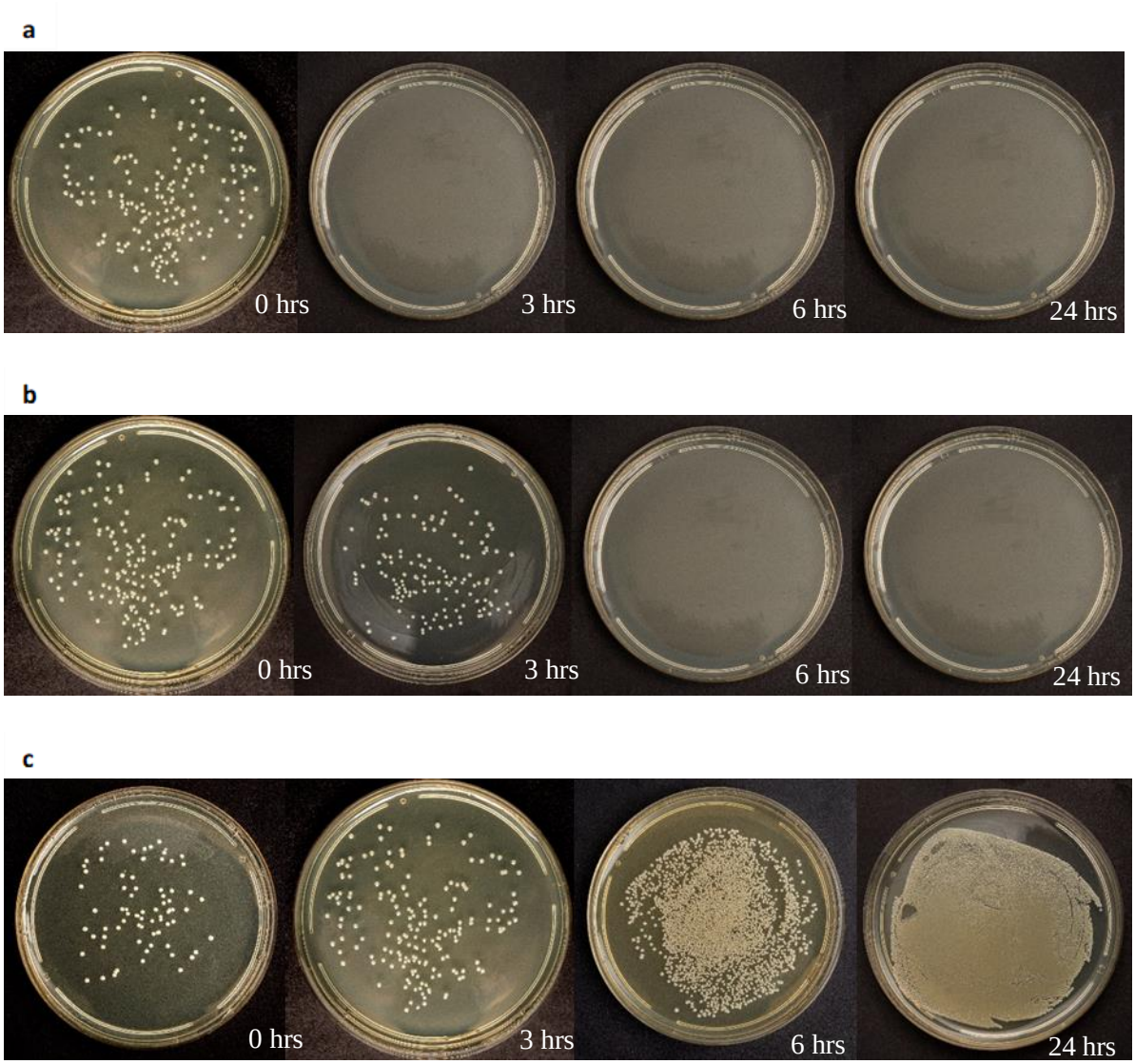
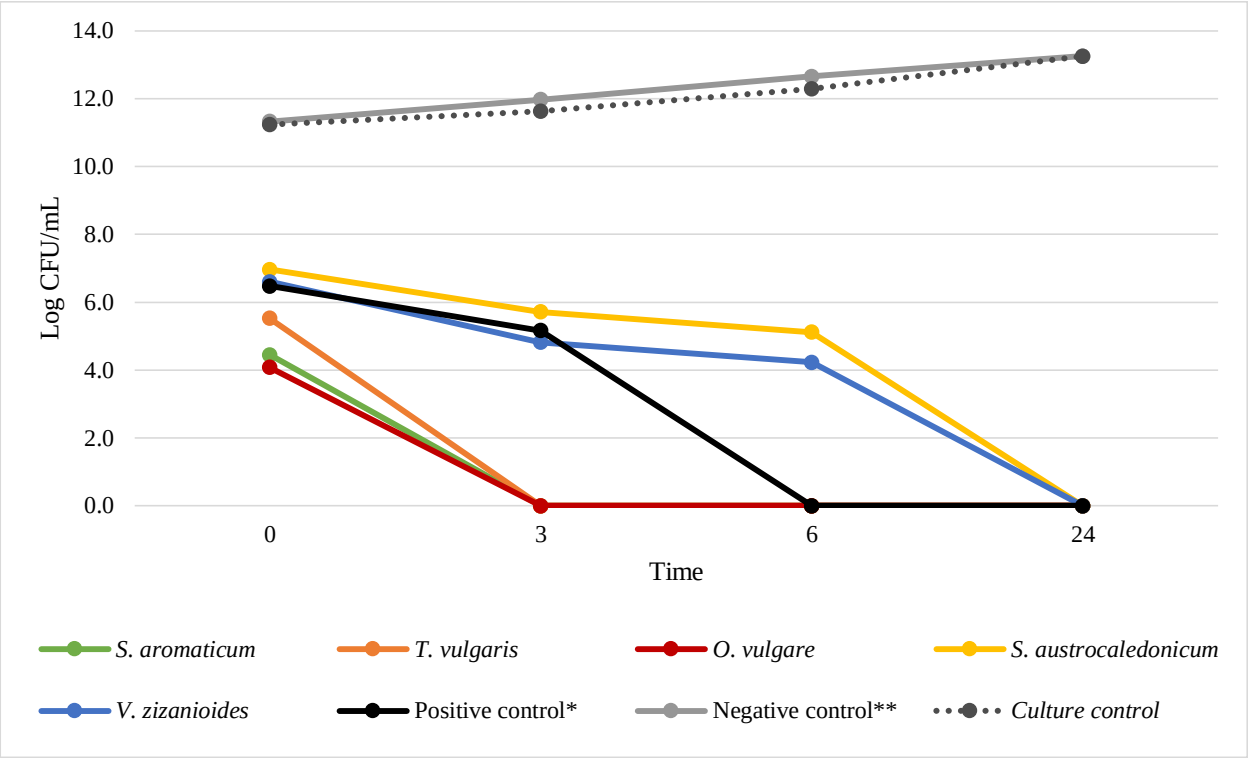


Figure 4.4: Colony counts of a) *O. vulgare* lotion, b) positive control (ciprofloxacin 0.01 mg/mL) and c) culture control (*S. aureus*) at 0, 3, 6 and 24 hrs

4.1.1.2 Efficacy of essential oil lotions against S. epidermidis

The essential oil lotions displayed bactericidal activity against *S. epidermidis*. Within 3 hrs, the culture had been completely eliminated by lotions containing *O. vulgare*, *S. aromaticum* and *T. vulgaris*. The *S. austrocaledonicum* lotion displayed a slightly faster killing dynamic than the *V. zizanioides* lotion, however, the activity for both was gradual and total elimination of the bacterial load was only seen at 24 hrs. All essential oil lotions showed a lower starting bacterial load at zero hrs in comparison to the culture control, suggesting that the bactericidal effect commenced immediately. The time kill rates are shown in Figure 4.5. The negative control, *S.*

chinensis, displayed an increase in colonies with time at a similar rate to the culture control, confirming no antimicrobial activity for the base formulation excipients.



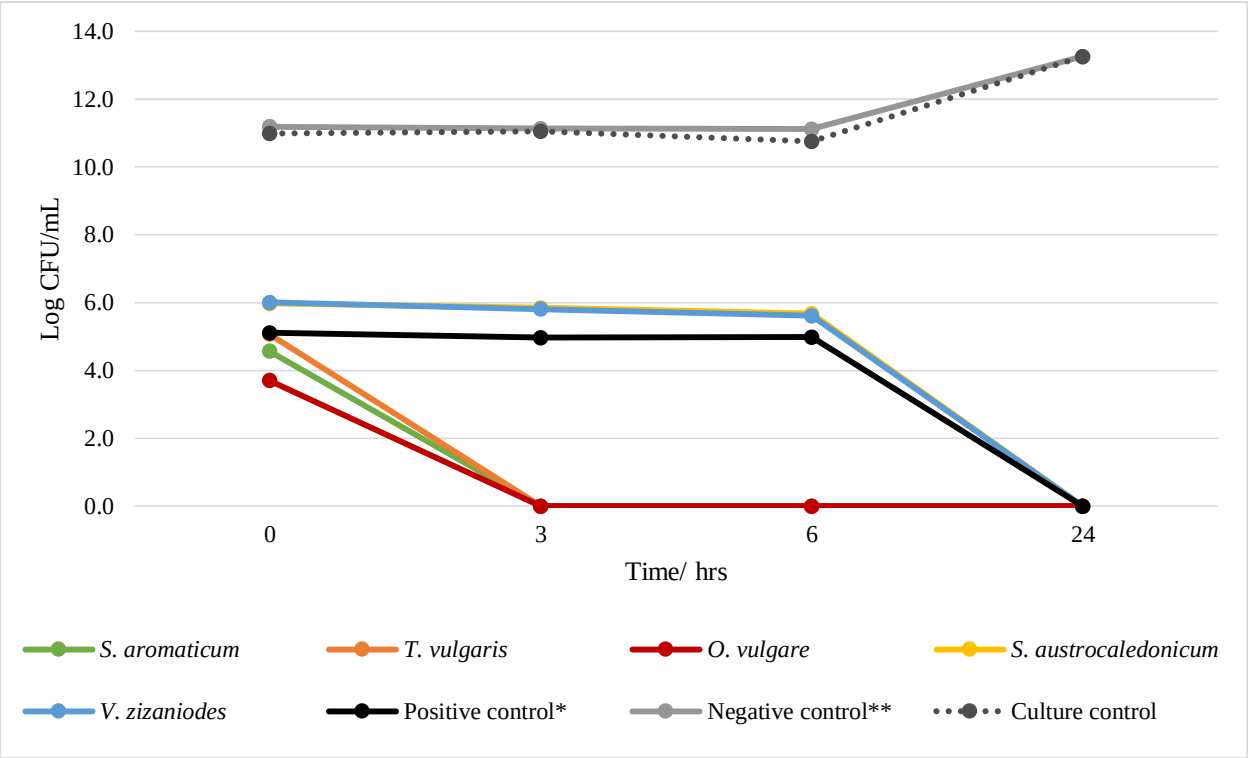
* Positive control is ciprofloxacin 0.01 mg/mL
**Negative control is *S. chinensis* lotion

Figure 4.5: Time-kill assay of essential oil formulations against *S. epidermidis*

4.1.1.3 Efficacy of essential oil lotions against S. pyogenes

In a similar fashion, the essential oil lotions displayed complete inhibition of *S. pyogenes* as shown in Figure 4.6. For lotions containing *O. vulgare*, *S. aromaticum* and *T. vulgaris*, total elimination of culture was achieved within 3 hrs. The essential oil lotions possessed a slightly greater killing capacity against *S. pyogenes* in comparison to *S. aureus* and *S. epidermidis*, as can be seen with a lower starting log (CFU/mL) for all formulations. This is in accordance with the results obtained in Chapter 2, where *S. pyogenes* proved to be the most susceptible pathogen to the pure essential oils. The lotions of *S. austrocaledonicum* and *V. zizanioides* displayed bacteriostatic action from zero to 6 hrs, and bactericidal activity with total elimination was only observed at 24 hrs, showing a slower onset of action in comparison to lotions of *O. vulgare*, *S. aromaticum* and *T. vulgaris*. Comparable antimicrobial activity of the *S. austrocaledonicum* and *V. zizanioides* lotions to the positive control (ciprofloxacin) was displayed against *S. pyogenes*. Both the culture control and negative control displayed a lag phase of six hrs and

then increased rapidly thereafter, thereby providing a smaller bacterial load for the lotions and making elimination an easier task. An extended lag phase is often seen with *S. pyogenes* in certain media due to its strict requirements for growth, and limited nutrients may impede the rate of proliferation (Gera and McIver, 2013).



* Positive control is ciprofloxacin 0.01 mg/mL
**Negative control is *S. chinensis* lotion

Figure 4.6: Time-kill assay of essential oil formulations against *S. pyogenes*

4.1.1.4 Analysis of antimicrobial efficacy of essential oil lotions against facultative anaerobic pathogens

Each essential oil lotions displayed similar elimination times, regardless of the pathogen reference strain it was tested against. Of the five essential oil formulations tested, the lotions of *O. vulgare*, *S. aromaticum* and *T. vulgaris* eliminated the bacterial load within 3 hrs, while the positive control ciprofloxacin only showed total elimination at 6 hrs for *S. aureus* and *S. epidermidis* and 24 hrs for *S. pyogenes*. This is incredibly noteworthy as this study has produced three formulations which display faster time-kill dynamics than a commercial broad-spectrum antibiotic. While the remaining two lotions (*S. austrocaledonicum* and *V. zizanioides*) were slower in eliminating the tested pathogen reference strains, they still showed total inhibition at 24 hrs and should not be disregarded.

The time-kill dynamics of the essential oil lotions were compared to the MIC values of the pure oils as obtained in Chapter 2. The concentration of essential oil was kept constant at an excess of 10% for each lotion, regardless of MIC values. It would thus follow that the rank of antimicrobial efficacy of the lotions would follow the same trend as the activity of the pure oils themselves. However, this was not observed to be the case. The weighted mean MIC values as calculated in Table 2.3 of Chapter 2 revealed the order of essential oils from most to least antimicrobially effective to be *V. zizanioides* > *S. austrocaledonicum* > *T. vulgaris* > *S. aromaticum* > *O. vulgare*. Yet, the lotions of these essential oils were found to display killing rates in the exact same reverse order, i.e. *V. zizanioides* lotion was the least effective while *O. vulgare* lotion was the strongest. Therefore, MIC results could not be used to explain this trend. It is well known that emulsification increases the antimicrobial activity of essential oils due to the large contact surface with the pathogen that emulsified oil droplets provide (Buranasuksombat *et al.*, 2011). Previous studies have noted that the antimicrobial efficacies of nano-emulsions were highly influenced by their microstructure and droplet size (Lee *et al.*, 2011; Majeed *et al.*, 2016; Wang *et al.*, 2021). However, there is a paucity of studies concluding that the antimicrobial activities of a range of essential oil emulsions were dependant purely on the droplet size, as opposed to the corresponding MIC of the neat oils. The stability studies conducted in the current study were thus referred to in order to analyse these discrepancies.

Upon investigation of the droplet sizes of the essential oil formulations one day after preparation (Chapter 3, Section 3.3.4.2), a strong correlation was observed between the MDD of the lotions and their relative efficacy. At the HLB's tested, the MDD of the lotions increased in a similar order to which their killing dynamics decreased. This is shown in more detail in Table 4.1, where lotions are listed in order of increasing kill time. For example, at optimum HLB, *O. vulgare* lotion displayed the smallest overall MDD of 197 nm and simultaneously the fastest killing rate. Conversely, lotions of *S. austrocaledonicum* and *V. zizanioides* displayed larger droplet sizes of 1673 nm and 1543 nm respectively and were the slowest in eliminating the tested pathogens. This observation is in line with the previously discussed notion that smaller droplets sizes are preferred as they lead to increased antimicrobial activity (Terjung *et al.*, 2012; Wang *et al.*, 2021). This may be as a result of two phenomena. Firstly, a smaller oil droplet can more easily penetrate a bacterial cell and lead to cell wall damage and ultimately death (Moghimi *et al.*, 2015; David *et al.*, 2019). Secondly, smaller droplets dispersed within an emulsion provide a higher surface-to-volume ratio for interaction between the bioactive oil

and the pathogen, leading to a quicker onset of action and therefore a faster bactericidal effect (Terjung *et al.*, 2012; Moghimi *et al.*, 2016).

Table 4.1: Essential oil lotions time-kill dynamics and selected stability results

Essential oil lotion	Killing time[a] (hrs)	MIC of pure oil[b] (mg/mL)	MDD[c] (nm)	Viscosity[c] (mPa/s)
O. vulgare	3	0.50	197	3202
S. aromaticum	3	0.49	213	1785
T. vulgaris	3	0.40	764	7432
S. austrocaledonicum	24	0.23	1673	1377
V. zizanioides	24	0.14	1543	3144

[a]calculated as an average against three pathogens- *S. aureus, S. epidermidis and S. pyogenes*
[b]weighted mean calculated against four pathogens as shown in Table 2.3
[c]measured at optimum HLB
Shaded values were reported in previous chapters but are shown again for comparative purposes

Another possible explanation may arise from the chemical structure of the essential oils themselves, and how the structure directly influences the ease of emulsification of each oil. During emulsification, surfactants reduce the tension between the immiscible oil and water phases by forming an interfacial layer. Co-surfactants are amphiphilic molecules capable of partitioning between both phases and usually accumulate at this interfacial layer, as shown in Figure 4.7 (Sharma *et al.*, 2015). Short or medium chain length alcohols, such as cetyl alcohol used in this study, are preferred as their shorter structure "pulls" the interfacial structure closer, enhancing the curvature and encouraging the formation of smaller droplets (Terjung *et al.*, 2012). They also lend flexibility to the interfacial film, allowing it to deform more easily around oil droplets (Pavoni *et al.*, 2020).

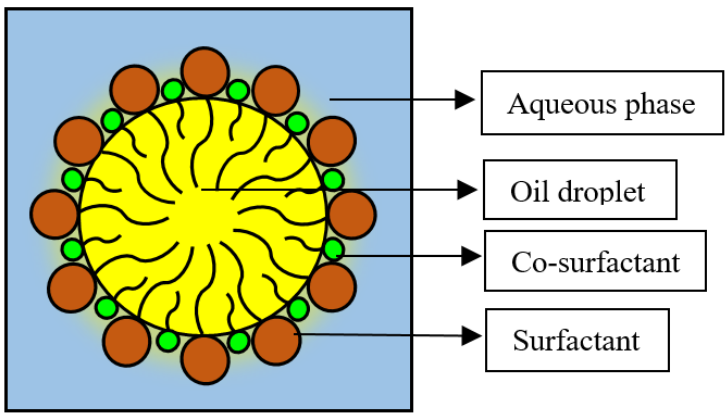


Figure 4.7: Surfactant and co-surfactant placement at interfacial layer

Remarkably, certain chemical constituents present in essential oils, such as monoterpene alcohols, are amphiphilic and display co-surfactant-like behaviour (Stubenrauch *et al.*, 1997; Kim *et al.*, 2009; Pavoni *et al.*, 2020). The GC-MS data of the essential oils as shown in Table 2.2 of Chapter 2 revealed carvacrol (60.40%) to be the dominant constituent in *O. vulgare*, thymol (46.60%) in *T. vulgaris* and eugenol (71.90%) in *S. aromaticum*. Due to its small molecular volume, eugenol is capable of adsorbing onto the interfacial layer and enhancing the curvature of the interfacial film by behaving as a co-surfactant (Tchakalova *et al.*, 2008a; Tchakalova *et al.*, 2008b). Carvacrol and thymol are terpenic hydrocarbons displaying similar structures to eugenol, therefore it may be proposed that they display similar behaviours and also promote the ease of emulsification in their respective formulations (Edris and Malone, 2012). For this reason, much smaller MDD's are obtained for these essential oil lotions compared to *S. austrocaledonicum* and *V. zizanioides.* However, droplet size may not be the only pathway by which the microstructure of the emulsion affects the antimicrobial activity. Several studies have reported that emulsification of certain essential oils has in fact lead to a decrease in their activity (Terjung *et al.*, 2012; Chang *et al.*, 2013; Xue *et al.*, 2015; Moghimi *et al.*, 2016; Bazzaz *et al.*, 2018; Zhou *et al.*, 2018). This arises possibly from entrapment of the oil within the micellar structure, thereby impairing its release (Orafidiya *et al.*, 2002; Florence and Attwood, 2015). Therefore, the presence of antimicrobial constituents at the interface, rather than entrapped within the micelle, may increase contact with the microbial cells and result in quicker death. The location of these constituents therefore plays a critical role in the antimicrobial activity of the lotions. Depending on where they are placed, the emulsion system may act as either a source or simply as a storage of these chemical constituents (Terjung *et al.*, 2012). In this manner, consideration of the microstructure of *O. vulgare*, *S. aromaticum* and *T. vulgaris* lotions provides valuable insight into their antimicrobial behaviour.

It is worth noting that the *S. austrocaledonicum* lotion was observed to display a marginally faster killing capacity of the three tested pathogen reference strains in comparison to *V. zizanioides*, however, the MDD was slightly larger. This could be explained by the considerably higher viscosity of the *V. zizanioides* lotion in comparison to *S. austrocaledonicum* (3144 mPa/s and 1377 mPa/s respectively). The increased viscosity could affect the diffusion of the *V. zizanioides* lotion through the aqueous media, and result in a poor distribution of oil droplets throughout the well (Orafidiya and Oladimeji, 2002; Viyoch *et al.*,

2006; Moghimi *et al.*, 2016). This could thus potentially limit contact between the essential oil and the pathogen and lead to an overall slower killing time.

It can be deduced that with a constant ratio of essential oil, the efficacy is dependent on physical factors such as MDD, viscosity and microstructure rather than MIC values of the pure oil. Therefore, this highlights the importance of utilising physical stability studies in formulation design, as the physiochemical properties may play a decisive role in the antimicrobial activity of the formulation.

Previous studies have explored the antimicrobial efficacy of the neat essential oils investigated in this study and their emulsions. Taleb *et al.* (2018) investigated the use of *O. vulgare* as an anti-acne topical nano-emulsion and found total elimination of *S. epidermidis* within 8 hrs and *C. acnes* at 12 hrs, achieved at 2.68 mg/mL of the neat oil. The nano-emulsion formed was only tested *in vivo* but found to significantly reduce the bacterial load from 1×10^8 to 4.3×10^1 CFU/mL at 0.672 mg/mL, effectively more than the commercial antibiotic erythromycin. Another study by Enayatifard *et al.* (2021) found that nano-emulsion of *O. vulgare* produced significant zones of inhibition against *S. aureus* at 2.5 mg/mL. However, as the agar well diffusion method was used in this study the results may not be reliable as the lipophilicity of the formulation may affect its diffusion through aqueous media (Orafidiya *et al.*, 2002; Florence and Attwood, 2015). A 5% $^{w}/_{w}$ micro-emulsion of *O. vulgare* intended for transdermal use found that the bioactive constituents could successfully cross the skin barrier and exert enhanced anti-inflammatory effects in comparison to the pure essential oil (Laothaweerungsawat *et al.*, 2020). Visibly, the use of *O. vulgare* as a topical treatment for acne has been previously explored. However, the development of a *O. vulgare* lotion that is antimicrobially effective against four major causative pathogen reference strain of acne is novel.

The development of *T. vulgaris* emulsions have been previously investigated by various studies, however, the major focus is kept on its application in the food preservation industry and pathogens involved in food spoilage. A 10% $^{v}/_{v}$ *T. vulgaris* nano-emulsion tested at a concentration of 3% was found to effectively kill *S. aureus* within 75 min, and was found to enhance the shelf-life of fermented milk products by up to six weeks if included as a preservative (El-Sayed and El-Sayed, 2021). Chitosan nanoparticles loaded with *T. vulgaris* essential oil have been found to significantly reduce the population of *S. aureus* in beef burgers

over eight days of storage (Ghaderi-Ghahfarokhi *et al.*, 2016). In terms of dermal application, Tadele *et al.* (2009) prepared various 1% $^{v}/_{w}$ *T. vulgaris* semisolid formulations in different bases intended to combat skin diseases. They were found to produce zones of inhibition comparable to commercial antibiotics against *S. aureus* and *S. pyogenes* (24 and 29 mm respectively). However, the agar diffusion method was used in this study which, as previously discussed, may produce results which are influenced by the affinity of the base for the media (Tadele *et al.*, 2009). Additionally, during formulation, the *T. vulgaris* essential oil was simply incorporated into the various bases without proper encapsulation methods. Hence, the activity observed may be due to the presence of free oil within the formulation rather than emulsified oil. The long-term efficacy of such a formulation would not be guaranteed as the absence of encapsulation would offers no protection of the essential oil towards volatility, oxidation and thermo-degradation (Hosseini *et al.*, 2013; Carpena *et al.*, 2021). Ghaderi-Ghahfarokhi *et al.* (2016) also found that the efficacy of free *T. vulgaris* oil against *S. aureus* decreased with time, while encapsulated *T. vulgaris* oil retained its functional properties. Thus, emulsification prolongs the antimicrobial activity of the essential oil by protecting it from degradation. The emulsified *T. vulgaris* lotion produced in this study would hence be superior in terms of long-term stability and prolonged shelf life.

The essential oil of *S. aromaticum* has garnered interest for its wide range of applications, from food preservation and anti-oxidant effects to insect repellent activities (Shahavi *et al.*, 2019). Previous studies have investigated the formulation of *S. aromaticum* into various encapsulated forms such as $^{O}/_{W}$ and $^{W}/_{O}$ creams, loaded liposomes, chitosan nanoparticles, silver nanoparticles and nano-emulsions (Sebaaly *et al.*, 2015; Safriani *et al.*, 2017; Hasheminejad *et al.*, 2019; Shahavi *et al.*, 2019; Lakhan *et al.*, 2020). However, the majority of studies have focused on the physiochemical properties and characterisation of these formulations while the antimicrobial activity remains a future consideration. In one study, Bazzaz *et al.* (2018) found that *S. aromaticum* solid lipid nanoparticles displayed weak time-kill activity against Gram-positive pathogens such as *S. aureus* while the neat oil was effective. The authors propose that encapsulating this oil may reduce its contact with the pathogen and thus result in decreased activity. The *S. aromaticum* lotion formulated in this study successfully released its content and rapidly eliminated the concerned pathogens in under 3 hrs, signifying its potency and addressing the concerns and limitations raised by previous studies.

In terms of *V. zizanioides* and *S. austrocaledonicum*, their formulation into topical antibacterial emulsions remains largely unexplored. Several articles have reported on the various bioactivities of the pure oils (Kim *et al.*, 2005; Jirovetz *et al.*, 2006; Luqman *et al.*, 2009; Chou *et al.*, 2012; Roh *et al.*, 2015; David *et al.*, 2019). However, *S. austrocaledonicum* has been cited as one of the most precious essential oils in the world (i.e. of great value) and the plant species from which the oil is sourced is endangered (Subasinghe *et al.*, 2013). Over-exploitation, habitat destruction and parasites are some of the problems that have led to diminishing resources, and the growing global demand simply cannot be met (Tennakoon *et al.*, 2000). For this reason, its commercialisation remains a challenge, especially without extensive data on its applications. While *V. zizanioides* is not a threatened species, it is a fairly expensive oil on the market (Wang *et al.*, 2009). Moreover, it is likely that it has not been intensively studied due to its complex structure with over 170 chemical constituents (Kim *et al.*, 2005). Therefore, this study sheds light and reports on a novel application on the use of these oils. As emulsification lowers the concentration of active oil required to exert its effects, the quantity of essential oil required to formulate these lotions would not pose a threat to the *S. austrocaledonicum* and *V. zizanioides* essential oils and endanger them further. Therefore, this study still provides a feasible motive for the use of these essential oils.

4.3.2 Antimicrobial assay of formulations against the aero-tolerant anaerobe C. acnes

All five essential oil formulations were observed to inhibit *C. acnes*. No signs of turbidity were seen after 72 hrs of incubation, signifying that lotions of *O. vulgare*, *S. austrocaledonicum*, *S. aromaticum*, *T. vulgaris* and *V. zizanioides* had completely eliminated the bacterial load. The positive control, ciprofloxacin, also presented with clear broth, while the negative control (*S. chinensis* lotion) and the culture control (*C. acnes*) presented with turbid broth indicating microbial growth. Thus, the essential oil lotions were comparable in activity to the commercial antibiotic ciprofloxacin against *C. acnes*, and as the base formulation excipients showed no activity, the antimicrobial efficacy of the lotions could be attributed solely to the essential oils themselves.

While this method allowed for the assessment of activity of the formulations against *C. acnes*, the order of efficacy could not be ranked among them as the end outcome could only be classified as either positive (clear broth) or negative (turbid broth). However, as *C. acnes* is the

most important pathogen involved in acne (Kumar *et al.*, 2016), the prevention of its growth still provides an excellent marker of the potential of these lotions as a suitable treatment.

While the antimicrobial efficacy of various essential oils against *C. acnes* has been previously studied (Lamlertthon and Tiyaboonchai, 2007; Fu *et al.*, 2009; Daud *et al.*, 2013; Hammer, 2015; Nasri *et al.*, 2015; Orchard *et al.*, 2018a; de Canha *et al.*, 2020; Esmael *et al.*, 2020), data on their subsequent development into formulations and validation of their antimicrobial efficacy is limited. As emulsification may alter the bioactivity of essential oils either positively or negatively, it should not be assumed that an essential oil with noteworthy activity against *C. acnes* will form an emulsion suitable for treatment. While literature is abound on the antimicrobial efficacy of essential oil emulsions against the other pathogens in this study (*S. aureus, S. epidermidis* and *S. pyogenes*), the most significant microbe in the pathogenesis of acne, *C. acnes*, seems to have been neglected. Therefore, the current study sheds important light on the anti-acne potential of selected essential oil lotions by validating the essential oil lotions against *C. acnes* as a final step.

A few studies have, however, attempted the formulation essential oils into anti-acne formulations. Viyoch *et al.* (2006) formulated micro-emulsions containing *O. basilicum* and *Ocimum sanctum* (holy basil) as a topical treatment for the disease, and *in vitro* studies indicated promising activity against *C. acnes*. The micro-emulsions were stable and retained their activity after five heat-cool cycles. Interestingly, Viyoch *et al.* (2006) also found a decrease in antimicrobial activity with an increase in viscosity of the emulsions. This provides additional evidence to support the claim by Tadele *et al.* (2009) that the affinity of the base for the media influences the antimicrobial activity. With the results obtained, Viyoch *et al.* (2006) concluded that $^{O}/_{W}$ emulsions proved a suitable delivery vehicle for essential oils in the treatment of acne.

Athikomkulchai *et al.* (2008) investigated the development of *Eucalyptus globulus* (eucalyptus) and *Psidium guajava* (guava) oil into stable $^{O}/_{W}$ creams. The formulations exhibited comparable activity against *C. acnes* to the control, 5% benzoyl peroxide. However, the basis of the selection of *E. globulus* and *P. guajava* essential oils for formulation is unclear, as the antimicrobial activity of these oils were weak to begin with (MIC against *C. acnes* was 9.38 mg/mL for both as compared to 1.00 mg/mL of the weakest oil in the current study, *O. vulgare*). Therefore, the choice of oils by Athikomkulchai and coauthors (2008), were not

optimal as a selection of oils were not investigated for noteworthy activity, and thus the formulations may not be as effective or beneficial in comparison. Additionally, the agar well diffusion method was used to assess antimicrobial efficacy of the formulations, which provides more of a qualitative assessment. Therefore, the study herein provides a more in-depth selection process and review of essential oil formulations against acne reference strains.

4.3.3 Long-term antimicrobial efficacy of essential oil lotions

All five aged essential oil lotions (three months) displayed total elimination of *S. aureus*, *S. epidermidis* and *S. pyogenes* after incubation. Clear spread plates were obtained for all inoculated essential oil lotion samples after 24 hrs. The positive control, ciprofloxacin, remained at 100% inhibition at 24 hrs while the negative control, *S. chinensis* lotion, and the culture controls, *S. aureus, S. epidermidis* and *S. pyogenes* showed saturated growth on their respective agar plates. Therefore, regardless of fluctuations in MDD over time and changes in emulsion stability, the essential oil lotions remained antimicrobially effective against *S. aureus*, *S. epidermidis* and *S. pyogenes.*

All five essential oil lotions (*O. vulgare*, *S. austrocaledonicum*, *S. aromaticum*, *T. vulgaris* and *V. zizanioides*) displayed complete inhibition of *C. acnes* three months after preparation. No signs of turbidity were seen after the incubation period. The positive control, ciprofloxacin, again presented with clear broth as expected while the negative control (*S. chinensis* lotion) and the culture control (*C. acnes*) produced turbid broth. Thus, all the essential oil lotions retained their antimicrobial efficacy against *C. acnes* over a three-month period.

The antimicrobial efficacy of the essential oil lotions against acne pathogen reference strains was investigated over the same time frame as the stability studies to ensure acceptability in both fields. Just as the stability and optimal HLB of the essential oil lotions were confirmed over a three-month period (Chapter 3), so too was the antimicrobial efficacy. Changes in the emulsion system of the essential oil lotions were seen with fluctuations in droplet size as discussed in Chapter 3, Section 3.3.2.2. At optimum HLB, the MDD of *O. vulgare* lotion changed by 4.57%, *S. austrocaledonicum* lotion by -63.54%, *S. aromaticum* lotion by 276.06%, *T. vulgaris* lotion by -50.65% and *V. zizanioides* lotion by 20.61% over 84 days. However, these changes in MDD clearly did not impact heavily on the antimicrobial efficacy of the formulations as total time-kill at 24 hrs was observed. Emulsion destabilisation is an expected

natural phenomenon, however, as no disproportionate change in MDD or decrease in antimicrobial activity is present, it may be concluded that this destabilisation occurs at a minimal rate for the essential oil lotions formed.

As essential oil products are often prone to degradation due to the volatility of the essential oils themselves, the retention of antimicrobial activity over an extended period is a worthy point for consideration. While previous studies investigated the physical stability of essential oil emulsions over a long-term period, concurrent antimicrobial studies were conducted as once-off and long-term data is lacking (Terjung *et al.*, 2012; Kaczmarczyk *et al.*, 2015; Sanad and Mabrouk, 2016; Jiménez *et al.*, 2018; Prakash Nirmal *et al.*, 2018; Clavijo-Romero *et al.*, 2019; Wadhwa *et al.*, 2019; Danila *et al.*, 2021; Enayatifard *et al.*, 2021). Only two studies could be found with extended antimicrobial activity studies (Cheng *et al.*, 2020; Feng *et al.*, 2020), however, these were conducted over a significantly shorter time period (42 days and 20 days respectively) than the current study (84 days). As the maintenance of activity is absolutely required for the application of essential oil emulsions, this critical step seems to have been overlooked in the past. This thus adds to the novelty of the current study, as the full antimicrobial assessment was carried out to ensure efficacy after three months of storage.

The essential oil emulsified lotions were found to be antimicrobially effective against four acne reference strains over a three-month period. Furthermore, the natural changes in the emulsion system, such as increased MDD and PDI, did not decrease the antimicrobial activity of the formulations. As the time-kill activity of three formulations (*O. vulgare*, *S. aromaticum* and *T. vulgaris* lotions) were stronger than the commercial antibiotic ciprofloxacin, they provide an attractive option for the treatment of acne.

4.4 Summary

- Time-kill assays were undertaken to determine the antimicrobial efficacy of the essential oil lotions against *S. aureus*, *S. epidermidis* and *S. pyogenes.*
- The order of time-kill efficiency of the lotions was observed to be *O. vulgare* > *S. aromaticum* > *T. vulgaris* > *S. austrocaledonicum* > *V. zizanioides.*

- The lotions of *O. vulgare*, *S. aromaticum* and *T. vulgaris* totally eliminated *S. aureus*, *S. epidermidis* and *S. pyogenes* within 3 hrs while *S. austrocaledonicum and V. zizanioides* lotions caused total bacterial death within 24 hrs.
- Three of the lotions (*O. vulgare*, *S. aromaticum* and *T. vulgaris*) displayed faster time-kill rates against the tested pathogens than the commercial antibiotic, ciprofloxacin.
- All five essential oil lotions fresh after preparation were found to inhibit the growth of *C. acnes*.
- All five aged essential oil lotions (three months) were effective in eliminating *S. aureus*, *S. epidermidis*, *S. pyogenes* and *C. acnes*.
- To the best of the author's knowledge, this is the first study to report on the antimicrobial efficacy of *S. austrocaledonicum* and *V. zizanioides* formulations.
- Despite minor changes in the emulsion systems reported in Chapter 3, all the essential oil lotions maintained both their physical and antimicrobial stability over a three-month period.

CONCLUSION

5.1 Summary

The pathogenesis of acne occurs via multiple pathways, however the proliferation of microbes such as *C. acnes, S. pyogenes, S. epidermidis* and *S. aureus* play a central role. Current anti-acne treatments on the market often consist of lengthy antibiotic regimens, which contribute to the antibiotic resistance crisis experienced worldwide. As such, essential oils provide a feasible alternative to antibiotics with a range of bioactivities, extending from antimicrobial to anti-oxidant and anti-inflammatory properties. However, due the volatility and sensitive nature of the essential oils, their consequent development into stable and antimicrobially effective formulations is lacking. Previous studies have identified essential oils which display noteworthy antimicrobial activity against the causative pathogens in acne (Orchard *et al.*, 2018a; Orchard *et al.*, 2018b; Leigh-de Rapper and van Vuuren, 2020). Hence, this study sought to develop viable essential oil lotions which were both physically and antimicrobially stable. A breakdown of the objectives and various outcomes of the study are given as follows;

Objective 1: To identify the most appropriate essential oils that would be effective against pathogens that cause acne from previous literature reviews.

A total of 19 essential oils were found which exhibited MIC values of 1.00 mg/mL or less against the relevant pathogens (Orchard *et al.*, 2018a; Orchard *et al.*, 2018b; Leigh-de Rapper and van Vuuren, 2020). These essential oils were then procured and their chemical profiles were analysed to identify the major chemical constituents of each.

Objective 2: To investigate the antimicrobial activity using the broth microdilution assay against pathogens that cause acne.

The broth microdilution method was carried out on each essential oil and the average MIC across all four pathogen reference strains was calculated for each in order to rank them in order of efficacy. The most susceptible pathogen was *S pyogenes* while the most resilient was found to be *C. acnes.* The strongest essential oil was found to be *V. zizanioides* with a weighted mean MIC of 0.16 mg/mL. The second-ranked essential oil *S. austrocaledonicum* displayed a

weighted mean MIC of 0.34 mg/mL. It was also found to display the lowest MIC values against *S. epidermidis* and *S. pyogenes* from all the essential oil tested, at 0.08 mg/mL for each. Following, *T. vulgaris*, *S. aromaticum* and *O. vulgare* displayed weighted mean MICs of 0.42 mg/mL, 0.50 mg/mL and 0.60 mg/mL respectively. These oils were then selected for further development into stable lotions.

Objective 3: To develop an appropriate lotion base formulation taking into account viscosity and stability.

A lotion base was developed that would allow for the easy incorporation of the essential oils and promote a stable formulation. Tween® 80 was selected as the hydrophilic emulsifier while GMS was chosen as the lipophilic emulsifier. The total emulsifier blend concentration was set at 10% and the essential oil concentration was set at 10%. Excipients such as cetyl alcohol, stearic acid and glycerine were included for their viscosity-enhancing and moisturising properties.

Objective 4: To determine the rHLB of the selected essential oils and thereafter prepare stable topical emulsion formulations.

The HLB approach was used to prepare stable essential oil emulsified lotions. Orafidiya and Oladimeji (2002) found that maximum stability of an emulsion is achieved when the HLB of the emulsifier is close or equal to the HLB of the oil phase. Thus, the HLB of the essential oils was required and subsequently determined by the use of CI. As expected, the HLB of each essential oil was found to vary as the chemical constituents contained in each affect the overall lipophilicity of the oil. The HLB of *V. zizanioides* was found to be 7, *T. vulgaris* and *O. vulgare* were 10 while *S. aromaticum* and *S. austrocaledonicum* were found to be 11. The HLB values of essential oil are not widely reported in literature, hence to the authors' knowledge, this is the first study to report on the HLB of *S. austrocaledonicum* and *V. zizanioides* essential oils.

Using the experimentally determined HLB value of the selected essential oils, the rHLB of each essential oil lotion was calculated taking into account the HLB of the oil phase excipients. Lotions were prepared at one HLB unit above and below the rHLB using a combination of the PIT method and ultrasonification. Stability testing was then conducted on all the lotions prepared in order to determine the optimum formulation for each.

Objective 5: To conduct stability testing for creaming, mean droplet size and turbidity on the prepared formulations over a 12-week period.

The essential oil lotions were assessed for CI and $\%D_T$ over 28 days and change in MDD and PDI over 84 days. The lotions of *O. vulgare*, *S. aromaticum* and *V. zizanioides* showed no creaming or phase separation and hence gave no indication of the optimum HLB. The remaining lotions both showed increasing CI with increasing HLB, with the minimum observed at HLB 11 for *S. austrocaledonicum* and HLB 10 for *T. vulgaris*. At tested HLB values of 8, 9 and 10, *V. zizanioides* emulsions displayed maximum stability at HLB 9 with a minimum change in MDD and PDI of 20.61% and 33.33%, and a $\%D_T$ of 0.349%. Emulsions of *T. vulgaris* were prepared at HLB values of 10, 11 and 12 and displayed a change in MDD and PDI of -50.65% and 63.75% respectively and the lowest $\%D_T$ (38.37%) at HLB 10. The *S. austrocaledonicum* emulsions were found to be most stable at HLB 11 with a change in MDD and PDI of -63.54% and 377.92% respectively and a minimal $\%D_T$ of 8.39%. Similarly, *S. aromaticum* emulsion displayed maximum stability at HLB 11 with a % change in MDD and PDI of 206.06% and 29.57% respectively and a $\%D_T$ of 0.34%. Lastly, peak stability of *O. vulgare* emulsions was seen at HLB 11 with a minimum change in MDD and PDI of 4.57% and 7.65% and $\%D_T$ of 29.19%.

These two assays (CI and $\%D_T$) were found to relate closely with the measured viscosity of the lotions. The viscosity of four out of the five formulations was found to decrease with HLB as the amount of GMS (an emulsifier and viscosity–enhancing agent) employed was decreased to produce higher HLB's. Generally, a lower viscosity of an emulsion allows for a faster rate of gravitational separation as there is less hindrance for oil droplets to rise to the surface. As a decrease in viscosity with an increase in HLB was observed, it could possibly be an instigating factor behind higher states of instability with higher HLB values. The viscosity of the lotions was also found to be influenced by the droplet size of the emulsions. Essential oil lotions of lower MDD were found to display larger viscosities and vice versa. All lotions were found to display shear-thinning behaviour i.e. decreasing viscosity with increased shear rate.

All the tested factors i.e. CI, $\%D_T$, MDD and PDI were considered in conjunction as no one factor may reveal the most stable emulsion. The optimum HLB of the essential oil lotions was

therefore selected as HLB 9 for *V. zizanioides*, HLB 10 for *T. vulgaris* and HLB 11 for *O. vulgare*, *S. austrocaledonicum* and *S. aromaticum.*

Objective 6: To determine the antimicrobial activity of the formulations against the acne pathogen reference strains.

The lotions of *O. vulgare*, *S. aromaticum* and *T. vulgaris* displayed total time-kill against *S. aureus*, *S. epidermidis* and *S. pyogenes* within 3 hrs, which was more effective than the positive control, ciprofloxacin (at 0.01 mg/mL). The lotions of *S. austrocaledonicum* and *V. zizanioides* were bactericidal within 24 hrs. All formulations successfully inhibited the growth of *C. acnes*. All five essential oil lotions showed total inhibition of the acne pathogen reference strains after three months of storage, confirming the retention of antimicrobial activity with time.

A summary of the antimicrobial and physical stability findings amongst all the formulations are shown in Table 5.1. In terms of rank of antimicrobial efficacy, *O. vulgare* lotion displayed the fastest time-kill with the lowest starting log (CFU/mL) against all three facultative anaerobes. Concurrently, with regards to physical stability, the *O. vulgare* lotion also possessed the minimal changes in the emulsion system at optimal HLB (overall smallest % change in MDD, PDI, CI and D_T). Additionally, topical formulations are required to possess droplet sizes of < 500 nm in order to penetrate the subcutaneous skin barrier. Therefore, the obtained MDD of 357 nm for the *O. vulgare* lotion at optimal HLB would be sufficiently small to penetrate the subcutaneous skin barrier and exert its bioactivity. In terms of economic feasibility, neat *O. vulgare* essential oil is relatively inexpensive and costs R30.00 per mL (retrieved from www.pranamonde.co.za on 12/04/2021). Therefore, in light of the data obtained, *O. vulgare* lotion at HLB 11 presents an attractive alternative topical treatment for acne that offers numerous advantages in terms of antimicrobial efficacy, physical stability and economic feasibility.

Table 5.1: Summary of antimicrobial efficacy and physical stability of essential oil lotions

	Antimicrobial efficacy[a]					Physical stability			
Formulation	**HLB[b]**	***S. aureus*[c]**	***S. epidermidis*[c]**	***S. pyogenes*[c]**	***C. acnes*[d]**	**MDD[e]**	**%MDD[f]**	**CI[g]**	**%D_T[h]**
O. vulgare	11	3	3	3	+[i]	357	4.20	0.00	29.19
S. austrocaledonicum	11	24	24	24	+	1673	-63.54	0.00	8.39
S. aromaticum	11	3	3	3	+	213	276.06	0.00	0.34
T. vulgaris	10	3	3	3	+	764	-50.65	0.00	38.37
V. zizanioides	9	24	24	24	+	1543	20.61	0.00	0.349
Ciprofloxacin 0.01mg/mL (positive control)	N/A	6	6	24	+	N/A	N/A	N/A	N/A
***S. chinensis* (negative control)**	N/A	-[i]	-	-	-	N/A	N/A	N/A	N/A

[a]Antimicrobial activity on Day 1
[b] Optimum HLB of essential oil lotion
[c]Time to total kill in hrs
[d] + denotes clear broth i.e. the presence of antimicrobial activity
[e] MDD measured at Day 1 after preparation
[f]% change in MDD over 84 days
[g] CI measured over 28 days
[h]%D_T measured over 28 days
[i] - denotes the absence of antimicrobial activity
Shaded area represents the optimal formulation in terms of both antimicrobial efficacy and physical stability

5.2 Limitations

Essential oils may differ in their chemical constituents depending on their origin, environmental conditions and harvesting. As these chemical constituents are responsible for the observed antimicrobial activity of the essential oils, any variation in the source would lead to a difference in bioactivity. Thus, in terms of the reproducibility of such a formulation, quality assurance procedures would have to be undertaken on every batch to ensure that consistent results are obtained and dosing remains unaffected.

Additionally, the concentration of essential oil used to prepare the lotions were relatively high (10%) in comparison the low MIC's obtained for the selected essential oils (< 0.1%). While this was necessary to maintain the oil volume fraction required, this excess may prove costly on a large scale. Furthermore, certain essential oils used in this study are considered high value due to their high demand and low supply. For example, *S. austrocaledonicum* essential oil costs R882.90 per 5mL while *V. zizanioides* essential oil retails for R324.20 per 5mL (retrieved from

www.pranamonde.co.za on 12/04/2021). At the concentrations used in this study, these products would thus be considerably expensive. A motivation of this study was to prepare accessible OTC treatments for acne, specifically as South Africa contains a large population of economically disadvantaged people who may not be able to afford specialised dermatological care. Unless created specifically for a higher-end market, the cost of such products would defy its purpose. However, the remaining three oils investigated (*O. vulgare*, *S. aromaticum* and *T. vulgaris*) are economically feasible. Out of these three essential oil formulations, *O. vulgare* lotion exhibited the fastest microbial elimination and was the most resistant to destabilisation and should thus should be considered as a first-line option for commercialisation.

5.3 Future recommendations

This study has established the baseline and showed feasibility of the HLB concept for the preparation of essential oil lotions. Further developments, which were not within the scope of this study, are certainly worthwhile in order to optimise the formulations to achieve the best possible outcome.

Essential oils are generally perceived as safe and non-toxic due to their natural origins, however this has been found to not be the case. Essential oils have been linked to incidences of irritation, contact dermatitis and photosensitivity (Bleasel *et al.*, 2002; Pazyar *et al.*, 2013) especially during application in undiluted form. Orchard *et al.* (2019) carried out a baseline toxicity study of essential oils and found that after 24 hrs, *S. aromaticum, T. vulgaris* and *V. zizanioides* displayed a toxicity of 98.88%, 100.00% and 11.37% at a concentration of 1.00 mg/mL respectively. However, a more elaborate cell-line toxicity study of the selected essential oils is warranted. Carrier oils have been previously investigated for their ability to reduce the toxicity of essential oils and in some cases, simultaneously enhancing their bioactivity. The *S. chinensis* carrier oil used as a negative control in the current study, has been shown to reduce to overall toxicity of essential oils by 87.5% (Orchard *et al.*, 2019). Thus, the incorporation of a carrier oil into the formulations may serve to reduce any potential side effects, while providing additional moisturising and soothing properties that carrier oils are renowned for. Additionally, this may allow for the reduction of the concentration of essential oil required to maintain the oil volume fraction, which would further minimise the incidence of adverse effects and reduce the overall cost of the formulations.

Certain essential oils in combination have been observed to show synergistic antimicrobial activity against the pathogens involved in acne (Orchard *et al.*, 2018a). Specifically, *C. myrrha* with *Cananga odorata* (ylang ylang), *L. angustifolia* with *Salvia lavandulifolia* (sage) and *L. nobilis* with *Juniperus virginiana* (juniper) are three combinations which demonstrated synergistic activity against *C. acnes*. The essential oils combinations with the lowest MIC values against *C. acnes* (0.25 mg/mL) arose from *V. zizanioides* with *C. verum* and *S. aromaticum* with *C. verum.* These combinations are certainly worthy of further investigation amongst all four pathogen reference strains examined in the current study to in order to achieve an enhanced antibacterial outcome. Additionally, combinations with commercial antibiotics, such as the ciprofloxacin used in this study, are a worthwhile avenue of investigation. A combination treatment may provide a multimodal targeted treatment by which acne pathogens are eradicated faster, leading to a reduced treatment time. However, the stability of such formulations should always be explored concurrently.

Further stability testing is another important point for consideration. While a three-month test period provides an acceptable indication of stability, a full shelf-life investigation should always be considered. Not only do products remain stocked on shelves for extended periods of time (up to two years), but they may also be exposed to various temperatures and storage conditions which may prove harmful to the emulsions and the essential oils contained within. Accelerated stability testing is an attractive option that involves exposing the lotions to extremes of temperature, light and humidity for a shorter period of time to measure the degradation of the product. This provides a reasonable estimate of shelf-life and saves valuable time- for example, a six month accelerated stability testing study may correspond to a two year study under real life conditions (Waterman *et al.*, 2007; Waterman, 2011). Therefore, the full extent of the shelf-life of the essential oil lotions should be determined to confirm quality.

This study explored the *in vitro* antimicrobial efficacy of the essential oil lotions, however further *in vivo* animal studies and clinical trials are recommended. Theoretically, while small droplet sizes (< 500 nm) are able to transverse the subcutaneous skin barrier and exert their antimicrobial action, *in vivo* studies are certainly required in order to confirm bioavailability. Previous clinical trials have been carried out on essential oils for topical use, and have shown promising results (Sharma *et al.*, 2017; Ahirwar *et al.*, 2018; Deyno *et al.*, 2019). Various commercial essential oil products, especially those of *M. alternifolia*, have flooded the market aimed at the treatment of acne. However, the majority of these products and their claims remain

unverified, and emerging studies have shone the spotlight on the potential of far stronger essential oils. The presence of proven clinical data would allow consumers to make an informed choice by purchasing validated treatments. Further *in vivo* studies are certainly worthwhile in order to fully ascertain the potential of these formulations.

5.4 Final conclusion

This study successfully prepared anti-acne essential oil lotions which demonstrated optimum stability and antimicrobial activity. In the pathogenesis of acne, *C. acnes* is the primary micro-organism responsible for the disease. It was found to be the most resilient out of all the pathogens tested, signifying the difficulty faced in achieving its eradication. However, the selected essential oils in this study all displayed noteworthy activity (< 1.00 mg/mL) against *C. acnes* and the remaining pathogens, highlighting their potential as potent anti-acne treatments.

Emulsification may either enhance or hinder the antimicrobial activity of essential oils. This study successfully formulated three essential oil lotions which displayed improved bactericidal activity in comparison to the commercial antibiotic, ciprofloxacin. The antimicrobial activity of the formulations was found to be dependent on the droplet size and microstructure of the emulsions, rather than the MIC value of the essential oils themselves.

Despite the fact that essential oils and their products are prone to degradation, the essential oil lotions formulated in this study maintained their bioactivity for a minimum of three months. This demonstrates the long-term viability of the formulations and provides proof of the practicality of this concept. This study has shown the importance of undertaking stability studies in tandem with antimicrobial studies as both fields are critical for drug formulation development and performance.

As growing antibiotic resistance threatens the world, there is a dire need for alternatives to conventional antibiotics. The availability of topical essential oil formulations against acne could potentially minimise the use of such medications and reduce the burden of extensive antibiotic therapy. Therefore, this study has provided a feasible alternative to one of the biggest threats to global health.

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APPENDIX A

Table A: MIC values and calculated mean of essential oils identified from literature reviews (Orchard *et al.*, 2018a; Orchard *et al.*, 2018b; Leigh-de Rapper and van Vuuren, 2020)

Essential oil	MIC (mg/mL)			
	C. acnes **(ATCC 11827)**	***S. epidermidis*** **(ATCC 2223)**	***S. aureus*** **(ATCC 25923)**	***S. pyogenes*** **(ATCC 12344)**
C. verum	0.13	0.25	0.25	N/A[a]
S. austrocaledonicum	0.50	0.25	0.25	N/A
V. zizanioides	0.50	0.13	0.50	N/A
P. patchouli	0.50	0.25	0.50	N/A
R. damascena	0.38	1.00	N/A	0.13
C. myrrha	0.50	0.50	1.00	0.19
C. zeylanicum	0.38	0.75	1.00	0.25
M. recutita	0.50	1.00	0.50	N/A
O. tenuiflorum	0.50	1.00	0.50	N/A
O. vulgare	0.25	0.50	1.00	1.00
C. citratus	0.50	1.00	1.00	0.38
T. vulgaris	1.00	0.75	1.00	0.50
C. martini	1.00	1.00	0.50	N/A
P. graveolens	0.50	1.00	1.00	N/A
L. cubeba	0.88	0.75	1.00	N/A
C. nardus	1.00	1.00	N/A	N/A
N. jatamansi	0.19	1.00	2.00	N/A
S. aromaticum	0.50	1.00	0.88	2.00
L. nobilis	1.00	1.00	1.00	3.00

[a]N/A ~ Not available

APPENDIX B – ETHICS WAIVER

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG	HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

Office of the Deputy Vice-Chancellor (Research & Post Graduate Affairs)

TO: Ms F Kurrimboccus
School: Therapeutic Sciences
Department: Pharmancy and Pharmacology
Medical School
University
E-mail: fad_kurr@yahoo.com

CC: Supervisor: Professor S van Vuuren <Sandy.vanVuuren@wits.ac.za>
and <HREC-Medical.ResearchOffice@wits.ac.za>

FROM: Iain Burns
Human Research Ethics Committee (Medical)
Tel: 011 717 1252

E-mail: Iain.Burns@wits.ac.za

DATE: 26/08/2019

REF: *R14/49*

PROTOCOL NO: W-CBP-190926-04 (This is your ethics application study reference number. Please quote this reference number in all correspondence relating to this study)

PROJECT TITLE: *Formulation of a topical anti-acne drug delivery system incorporating essential oils*

Please find attached the Ethics Waiver Certificate for the above project. I hope it goes well and that an article in a recognized publication comes out of it. This will reflect well on your professional standing and contribute to the Government funding of the University.

MSWorks2000/Iain0007/ClearScanWaiver.wps

APPENDIX C – TURNITIN REPORT

FORMULATION OF A TOPICAL ANTI-ACNE DRUG DELIVERY SYSTEM INCORPORATING ESSENTIAL OILS

ORIGINALITY REPORT

12%	8%	9%	2%
SIMILARITY INDEX	INTERNET SOURCES	PUBLICATIONS	STUDENT PAPERS

PRIMARY SOURCES

1	onlinelibrary.wiley.com Internet Source	<1%
2	A Orchard, S.F. van Vuuren, A. Viljoen, Guy Kamatou. " The antimicrobial evaluation of commercially essential oils and their combinations against acne ", International Journal of Cosmetic Science, 2018 Publication	<1%
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