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The Transdermal Permeability of Terpenoids as an Insect Repellent

Chiliza, Unathi Nothando

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The Transdermal Permeability of Terpenoids as an Insect Repellent

UNATHI NOTHANDO CHILIZA



UNIVERSITY OF THE
WITWATERSRAND,
JOHANNESBURG

A dissertation submitted to the Faculty of Health Sciences, University of the Witwatersrand, in fulfilment of the requirements for the degree of Master of Science in Medicine (Pharmacology)

Johannesburg, South Africa

2025

DECLARATION

I, Unathi Nothando Chiliza , declare that this dissertation is my own work. It is being submitted for the degree of Master of Science in Medicine at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other university.



Unathi Nothando Chiliza

Signed at on this day of 31 March 2025.

DEDICATIONS

In loving memory of

Bulelwa Winifred Chiliza

28 January 1944 -16 March 2025

I will always love you

ABSTRACT

Malaria is a communicable disease caused by the multiphase Plasmodium parasite. The disease is characterized as a significant global health concern that presents as a cyclical fever. The rapid development of resistance to antimalarial drugs and insecticides underscores the need to enhance the efficacy of repellents as a tool in vector control.

Terpenoids, a large and diverse class of secondary plant metabolites have shown significant potential as an insect repellent, thus highlighting the need to further investigate the compounds. A method that is often used to accurately measure the terpenoids and assesses their effect on the skin barrier as well as their permeation through the skin layers is High Performance Liquid Chromatography (HPLC) methods. HPLC methods were developed to optimise detection of the terpenoids. The HPLC validation was conducted according to The International Council for Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) guidelines. The parameters include linearity, lowest detectable (LOD), and quantifiable (LOQ) amounts, precision, accuracy, stability, and robustness. Acceptance criteria were expressed as relative standard deviation (%RSD). Linearity was established from the coefficient of determination (R^2) of the line of regression from a calibration curve. *Ex vivo* diffusion and skin retention studies were performed using porcine skin samples mounted into the flow cells of a 7-in-line flow-through diffusion system. Skin integrity after the application of the terpenoids was assessed using the Transepithelial electrical resistance (TEER) measurements. The repellency was evaluated employing a WHO repellency bioassay at 1 mg/mL, 5 mg/mL, and 10 mg/mL.

Reproducible and rapid HPLC methods were developed to detect the terpenoids accurately. The calibration curve for all the terpenoids were linear (R^2>0.9991). Precision, accuracy, and robustness exhibited a %RSD <2.0% across all compounds. The positive control (N, N-diethyl-meta-toluamide) DEET diffused through the skin and the average steady-state flux (Jss) was calculated to be 632 ± 42µg/cm^2/min. The terpenoids did not diffuse across the skin into the acceptor chambers, they however accumulated within the skin layers. The compounds demonstrated significant repellency with *(-)-Beta* pinene*, (-)-alpha* pinene and linalyl acetate producing a 100% repellency percentage. The described methods can be successfully applied for routine analysis of the terpenoids due to them being simple, specific, precise, accurate, and robust. The skin integrity after the application of DEET showed a 51% decrease whereas the terpenoids produced a negligible effect on skin integrity. At the termination of the *ex vivo* permeation studies the terpenoids were not detected, indicating very low possibility of a

systemic effect. The terpenoids have repellency potential with low risk of systemic adverse effects and warrant further investigation

Key words: HPLC validation, permeation, insect repellent, terpenoids

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CHAPTER ONE: INTRODUCTION

1.1. Background of the study

Malaria is a communicable disease caused by the multiphase *Plasmodium* parasite, it is characterised as a significant global health concern that presents as a cyclical fever (Buck and Finnigan, 2023). The female *Anopheles* mosquito transmits the parasitic infection amongst humans; however, it cannot spread from person to person, in addition, blood transfusions and contaminated needles can transmit malaria (Djihinto *et al.*, 2022). Moreover, in the initial stages, malaria symptoms are mild, but if untreated, *P. falciparum* malaria can progress into a severe illness that may result in death (White, 2022). Fortunately, one can prevent malaria by avoiding indoor and outdoor mosquito bites, using insect repellents such as N, N-diethyl-meta-toluamide (DEET) or citronella oil (Nararak *et al.*, 2020). Furthermore, the utilisation of prophylactic medications like atovaquone-proguanil, doxycycline, and mefloquine before travelling to a malaria-endemic area can aid in malaria prevention (World Health Organisation, 2023; Centres for Disease Control and Prevention, 2024).

According to the World Health Organisation (WHO), 263 million malaria cases occurred worldwide in 2023 across eighty-three malaria-endemic countries – a 5.3% increase from 2022. Nigeria, the Democratic Republic of Congo, Uganda, and Mozambique accounted for half of these cases as shown in Figure 1.1 (WHO, 2024). Furthermore, South Africa recorded 9 785 malaria cases and reported 106 malaria deaths with 10% of the population being at risk for contracting the disease (National department of Health, 2024). One of the challenges South Africa faces in eradicating malaria is the importation of the disease from neighbouring countries, particularly from epicentres such as Mozambique (National Institute for Communicable Diseases, 2024). For example, KwaZulu-Natal, shares borders with Eswatini and Mozambique, these neighbouring countries contribute approximately 77% of the country's annual malaria cases (Maharaj *et al.*, 2019). This geographical proximity underscores the need for coordinated efforts in malaria control and prevention strategies.

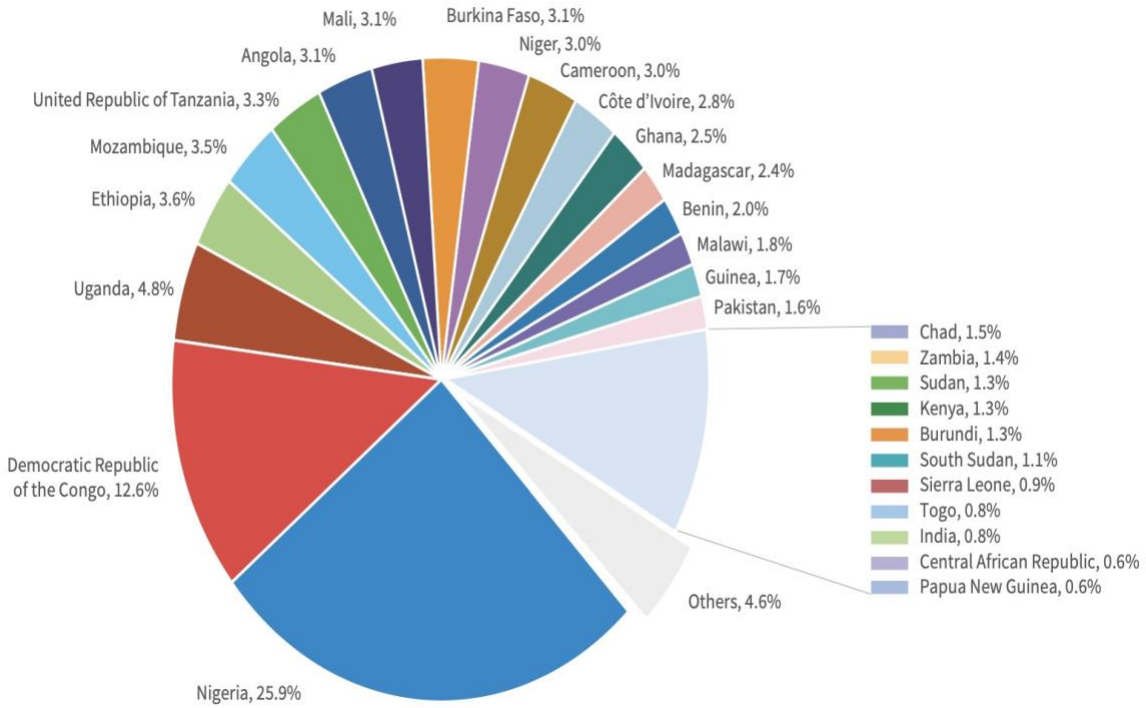


Figure 1.1 *: Malaria cases across Africa (WHO, 2024)*

In addition to the importation of malaria into the country's borders, climate change influences the distribution of malaria-carrying mosquitoes. Similarly, the drug-resistant malaria Plasmodium parasite as well as insecticide-resistant mosquitoes are the causative agents of malaria (National institute for communicable diseases, 2023). However, despite the multitude of advancements within this therapeutic area, challenges persist. Therefore, there is a need for innovative novel compounds such as terpenoids which can be used as a malaria prevention strategy.

Terpenoids represent the most widespread class of natural compounds, found prolifically across all forms of life (Zeng *et al.*, 2022). They influence the scent, flavour, and colour of plant leaves, fruits, and flowers, and are well-known for their substantial pharmacological properties. Furthermore, terpenoids are a modified class of terpenes with various functional groups including; monoterpenes, sesquiterpenes, diterpenes, triterpenes, tetraterpenes and ploy terpenes (Maysita, 2022). Terpenoids exhibit anti-malarial, anti-fungal and anti-cancer activities, qualifying them as phytochemicals. A study by Vainer *et al.* (2024), concluded that borneol, a terpenoid derivative, demonstrated significant mosquito-repelling activities against human host-seeking female *Aedes aegypti*. Beyond their repellent properties, terpenoids also demonstrated potent larvicidal effects, with compounds such as *cis*-nerolidol, *cis-trans*

nerolidol, and (*R)-(+)*-limonene showing bio-insecticidal characteristics against malaria vectors (Rants'o *et al.*, 2023). This highlights the potential of terpenoids as a strategy for malaria control when used as a topical insect repellent.

The principle behind transdermal drug permeability is based on the ability of active compounds to pass into and through the various skin layers and into the systemic circulation (Sivadasan, 2024). The outer layer of the skin (stratum corneum), has effective barrier properties, thus refuses entry of external products in an aid to protect the body (Elias, 2010). The stratum corneum is the main hinderance in transdermal delivery of topically-applied products (Yu *et al.*, 2021). Therefore, other mechanisms are required to improve permeation across the skin (Yu *et al.*, 2021). There are numerous factors that limit the substances from entering and diffusing across the skin; these factors include high potency, lipophilicity, large molecular size, inability to pass through the epidermis, and a melting point above 250 °C (Kovacik *et al.,* 2020; Khan and Sharman, 2023). Lastly, it is noteworthy to mention that the high lipophilicity of the terpenoids influences the kinetics of permeability (Liu *et al.,* 2020). For instance, highly lipophilic terpenoids (Log p value> 4) exhibit low penetration, whereas less lipophilic substances demonstrate high penetration (Cal, 2006). Therefore, the low permeability of highly lipophilic terpenoids proves to be advantageous when employed as an insect repellent.

1.2. Rationale and motivation of the study

Mosquitoes serve as a major vector of various mosquito-borne diseases across the globe (WHO, 2023). Moreover, the prevalence of insecticide resistance in several malaria-endemic Sub-Saharan African countries has become increasingly pronounced since the 1950s (Suh *et al.,* 2023). Climate change and urbanisation, has contributed to the proliferation of vector-borne diseases. An increase in vector borne disease is amplified by facilitating the larval development of mosquitoes, enhanced reproductive fitness, prolonged vector survivorship, and increased blood feeding frequency (Afrane *et al.,* 2012). Similarly, climate change results in a change in rainfall patterns, which, combined with deforestation, causes persistent mosquito breeding sites (Zhang, 2024). In turn, these factors result in an increase in the number of adult *Anopheles* mosquitoes and their ability to transmit diseases such as malaria (Afrane *et al.,* 2012; Burke *et al.,* 2017).

DEET, a commercially accepted synthetic insect repellent, has an acceptable safety profile with an extensive protective time against mosquitoes (Peng *et al.,* 2022). Despite the above, in recent years there have been reports of DEET exhibiting toxicity and ecological risks due to improper use or overuse. Furthermore, there have been numerous reports stating that DEET

causes neurotoxic as well as cardiotoxic effects resulting in encephalopathy, respiratory depression, seizures, and hypotension (Tavares *et al.,* 2018). In addition, the Chang Jiang River was shown to contain elevated levels of DEET which brought to light a severe pollution-related issue resulting from the use of synthetic repellents (Lui *et al.*, 2015). In other cases, Thornson *et al.* (2020) reported DEET's ability to dissolve plastics, making it detrimental to the skin barrier. A preliminary study conducted by the Harvard Medical school indicated that DEET-containing products affect the reproductive fitness of egg cells during meiosis, thus causing a myriad of issues during pregnancy, including birth defects and miscarriage (Duchen, 2024). To conclude, DEET's major limitations are its rapid permeability through the skin into the systemic circulation, as well as its adverse effects (Wang *et al.,* 2013) thus, emphasising the need for an alternative repellent.

Terpenoids demonstrate repellent activities against adult *Anopheles* mosquitoes while exhibiting toxicity against insects in the developmental stages (Sharma *et al.*, 2022; Opoku-Bamfoh *et al*., 2024). Moreover, the evolution of plants has given rise to a diverse array of naturally occurring plants that exhibit defensive properties against insects and unfavourable microbes (Sengul-Demirak and Canpolat, 2022).Terpenes (limonene, myrcene, and pinene terpinene) are referred to as simple hydrocarbons, whereas terpenoids (citronellal, geraniol, linalool, linalyl acetate, and carvacrol) are hydrocarbons that include an oxidised methyl group. In conclusion terpenoids and terpenes illustrate prominent repellent properties. (Masyita *et al.*, 2022; Nerio *et al*., 2010).

This study will conduct *ex vivo* permeation studies on porcine skin as the tissue is comparable to human tissue i.e. this includes haemodynamic values and their size and overall thickness (Lelovas *et al.,*2014). Preliminary studies on porcine tissue will investigate the permeation of terpenoids across porcine skin and the feasibility of the model to predict the permeation across human tissue, as a topical insect repellent. In addition, this study will evaluate the repellence potential of terpenoids at varying concentrations while comparing them to the industry standard DEET. Currently, there is limited information available pertaining to the efficacy of natural chemicals as insect repellents, as well as their safety when applied topically (Commonwealth of Massachusetts, 2024). Despite this, consumers continue to seek natural alternatives, driven by concerns over the safety of synthetic repellents. These concerns are recurring, fuelling the demand for natural repellents, as the belief persists that natural ingredients are superior and contribute to better human health and environmental sustainability (Luker, 2024).

1.3. Problem Statement

The rapid development of resistance to antimalarial drugs and insecticides continue to pose a threat in vector control. Widely used synthetic repellents such as DEET have been reported to show insensitivity amongst the *Aedes aegypti* mosquito population caused by non-genetic factors *(Stanczyk et al., 2013).* Moreover, concerns about DEET's negative effect on marine life and ability to melt synthetic fabrics has driven interest in a safer alternative. Hence, the use of terpenoids have emerged as prospective insect repellents (Sharma *et al.*, 2017). Despite this, there is insubstantial research investigating the repellence potential of terpenoids when tested against *An. arabiensis* mosquitoes, a prevalent malaria vector in South Africa. Additionally, few studies report on the concentrations at which terpenoids exhibit repellent properties. Lastly, there is paucity in studies elucidating the *ex vivo* pharmacokinetics of terpenoids across skin, as it would indicate the potential of reaching the systemic circulation and causing systemic side effects. Therefore, this study aims to investigate the effectiveness of terpenoids as an insect repellent and their ability to permeate across the skin.

1.4. Aims and Objectives

The aim of the study is to ascertain whether select terpenoids possess mosquito repellent properties when used alone or in conjunction with DEET or other terpenoids and to determine transdermal absorption and preliminary toxicological properties.

1.5. Objectives

1.5.1. To develop and validate a Reverse-Phase High-performance liquid chromatography method for detection of select terpenoids.

1.5.2. To determine the *ex vivo* transdermal permeability of the terpenoids

1.5.3. To determine the percentage of topically-applied terpenoids retained in porcine skin after the *ex vivo* experiments.

1.5.4. To determine the skin barrier integrity after topically-applied terpenoids, by measuring skin resistance (Transepithelial Electrical resistance).

1.5.5. To assess the efficacy of select terpenoids as mosquito repellents using the WHO repellency assay.

1.6. Outline of study

To achieve the aim and objectives of this study, the dissertation has been set out as follows:

1.6.1. **Chapter one** provides a brief background to the study leading into the aim of the study and the objectives used to achieve this aim.

1.6.2. **Chapter two** is the introduction to *Plasmodium* and *Anopheles* in South Africa. Chapter two explores the various insect repellents and their origin as well as the various terpenes and terpenoids as well as their roles as permeation enhancers

1.6.3. **Chapter three**, includes the details of the research design, and the various methods, ethics, analysis and statistics used.

1.6.4. **Chapter four**, outlines the findings of this study in various graphs and tables

1.6.5. **Chapter five** is a discussion of results obtained from the study and integration of data.

1.6.6. **Chapter six** concludes the study indicating some limitations encountered in the study; along with recommendations arising from the study for future investigation.

CHAPTER TWO: LITERATURE REVIEW

2.1. Epidemiology of malaria in South Africa

Significant breakthroughs since the early 2000s have resulted in a decline in malaria infections (Balmith *et al*., 2024). Key advancements include the use of indoor residual spraying (IRS) including dichloro-diphenyl trichloroethane (DDT) to counteract the pyrethroid resistance in *Anopheles funestus* (Maharaj *et al.*, 2005; Kleinschmidt *et al.*, 2018). Moreover, the introduction of artemisinin combination therapy and the Lebombo Spatial Initiative, a cross-border collaboration targeting malaria in eastern Eswatini, southern Mozambique, and northern KwaZulu-Natal (Figure 2.1a) (Blumberg and Frean, 2007) have contributed to the decline in malaria infections. More recently, the development of the RTS/AS01 malaria vaccine has marked a significant stride towards malaria eradication (El-Moamyl and El-Sweify, 2023). Regardless of the innovative advancements, challenges persist, including the ongoing threat of malaria importation from neighbouring countries (Balmith *et al*., 2024).

According to the National Institute for Communicable Diseases (NICD) there has been a surge in the number of malaria cases in malaria endemic provinces as well as in Gauteng, a non-endemic region (NICD, 2023; Public Health Bulletin South Africa, 2024). Over 90% of malaria infections in South Africa arise from *P. falciparum,* and prevalent in malaria endemic regions such as Limpopo, KwaZulu-Natal, and Mpumalanga during rainy and humid months (Figure 2.1b) (National Department of Health, 2019). Five species known to affect humans include: *Plasmodium vivax, P. ovale, P. falciparum., P. malariae,* and *P. knowlesi*, with *P. falciparum* being the most fatal (Zhang *et al.,* 2016; Tseha, 2021).

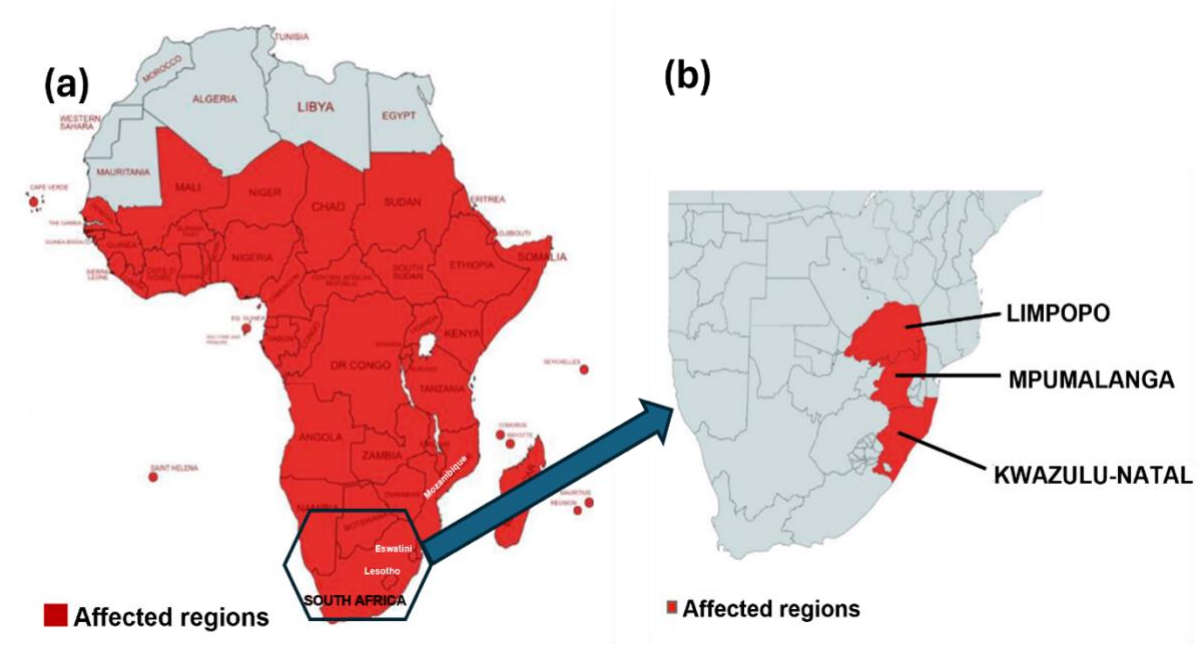


Figure 2.1 (a) Malaria distribution in sub-Saharan Africa and (b) the malaria endemic regions in South Africa (Balmitht et al., 2024).

2.1.1. Malaria life cycle in human and *Anopheles* host

The malaria life cycle involves five distinct stages in two hosts (Figure 2.2) (NICD, 2023). The *Plasmodium* parasite is transmitted by the bite of an infected female *Anopheles* mosquito (Markwalter *et al.*, 2024). When a mosquito bites its human host, infective sporozoites enter the bloodstream from the mosquito's saliva and quickly invade liver cells (Singh *et al.*, 2021). The parasite matures within a hepatocyte into a schizont containing thousands of merozoites; these released merozoites infect red blood cells and undergo further asexual replication resulting in the re-infection of further red blood cells or the differentiation into sexual gametocytes (Roques *et al.*, 2023). When a mosquito takes a blood meal from an infected individual, these gametocytes are ingested, and sexual reproduction continues in the insect's midgut.

2.1.2. Signs and symptoms

Malaria infections present with symptoms such as fever and flu-like manifestations, including chills, headaches, muscular pains, fatigue, as well as nausea, vomiting, and diarrhoea (Srivastav *et al.*, 2023). The loss of red blood cells due to malaria can lead to complications such as anaemia and jaundice (Naser *et al.,* 2024). As the infection progresses, severe malaria may result in renal failure, convulsions, mental disorientation, coma, and even death

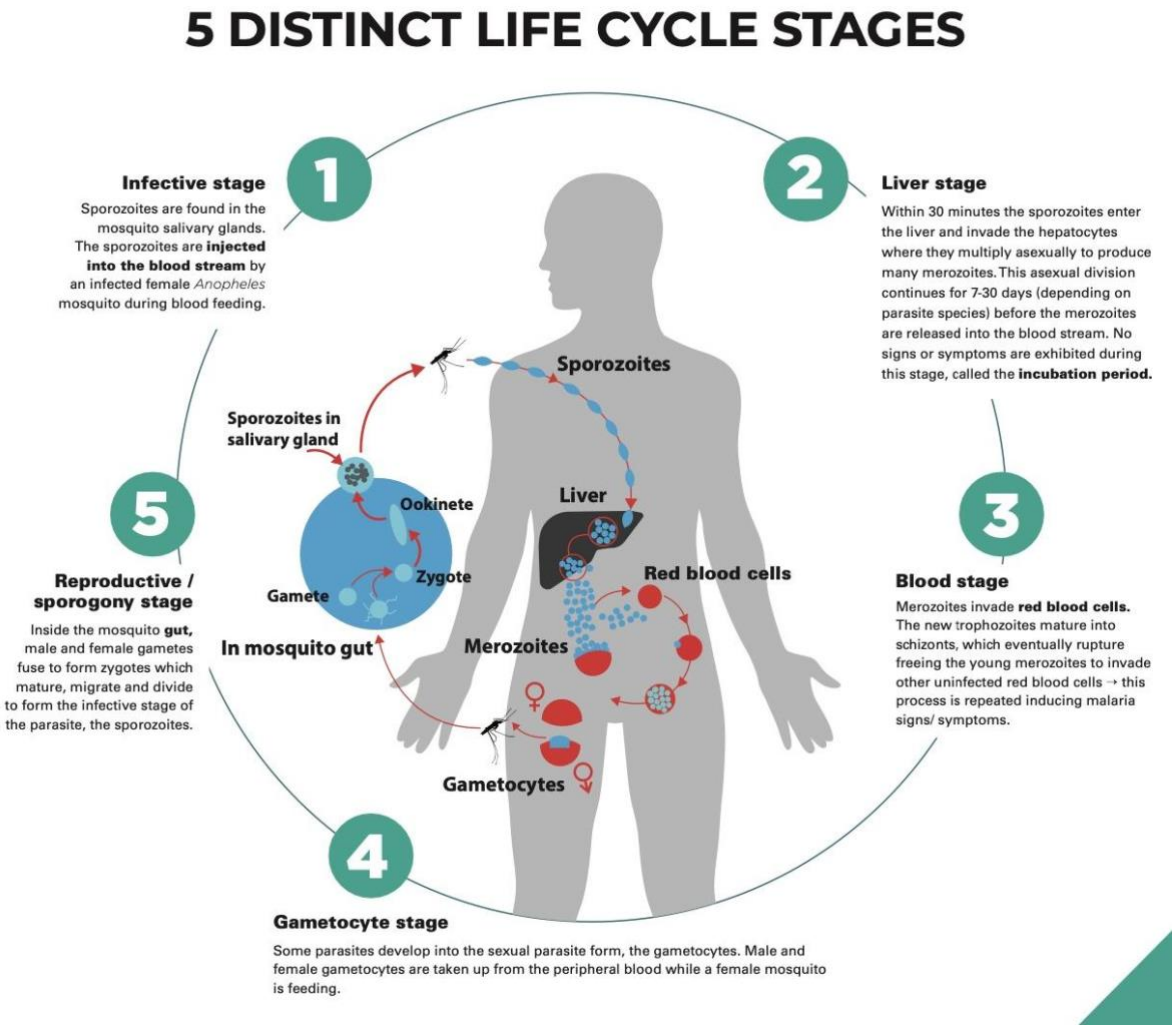


Figure 2.2: The two-host Malaria life cycle (NICD, 2023) involves the female Anopheles mosquito and human host. Stage one involves the transfer of sporozoites by the Anopheles mosquito. Stage two is the incubation period where sporozoites multiply into merozoites for 7-30 days. Signs and symptoms appear during the stage three where merozoites invade red blood cells. During stage 4 gametocytes are formed. The final stage is stage 5 whereby the gametocytes fuse to form zygotes which eventually make up the infective stage.

(Centre Disease Control and Prevention, 2024). When suspecting malaria, the WHO recommends prompt detection using microscopy or rapid diagnostic testing (WHO, 2023). This enables healthcare professionals to differentiate between malarial and non-malarial fevers, thus facilitating optimal treatment decisions with the use of parasite-based diagnostic tests (Ubieko and Ubieko, 2024). These measures not only reduce disease and mortality, but also mitigate the development of antimalarial resistance (WHO, 2023). Lastly, advancements in molecular methods over the past three decades have led to increased utilisation of nucleic acid amplification-based assays (NAATs) in diagnosing *Plasmodium* infections (Cunningham *et al.,* 2020).

2.1.3. Malaria prevention and control

Between the 19th and early 20th century researchers became aware of the correlation between arthropods and the transmission of diseases to humans (WHO, 2023). As such, vector control is a key component in the eradication of malaria. However, due to the lack of effective treatment plans and vaccinations, managing transmissions relies on controlling the *Anopheles* vectors (Manikandan *et al.,* 2023). Vector control consists of using mosquito nets, window/door screens, draining marshes and other bodies of water where insects thrive and treating breeding areas with oil or Paris green (Vythilingam, 2021). These methods reduced mosquito borne diseases but if not consistently and optimally employed, were not able to prevent transmission, but the development of treatment plans and vaccinations have had a positive effect on transmission rates (WHO, 2023). The advances in science and technology has resulted in more sophisticated vector control strategies resulting in significant progress in public health efforts against malaria. (WHO, 2023). A scoping review conducted by Nalinya *et al.* (2022) identified over 10,112 studies that contributed to the conclusion that spatial repellents acted as a sufficient malaria prevention strategy against the transmission of indoor and outdoor malaria. Thus warranting further research into such repellents.

2.1.4. Factors affecting malaria control

2.1.4.1 Resistance to insecticides

According to the World Health Organisation (WHO, 2023) Pyrethroids have been the only effective class of insecticides for insecticide treated nets (ITNs) and indoor residual spraying (IRS). However, resistance to the insecticides has been rampant, consequentially threatening malaria control (WHO, 2023). The large-scale use of pyrethroids has led to the development of resistance mechanisms amongst mosquito populations despite geographical location (Black *et al.,* 2021). A study by Du *et al.*, (2016) confirmed that mutations in the voltage-gated sodium channels, the target site of insecticides are responsible for insecticide resistance. These channels are responsible for the generation and propagation of action potentials and the increased activity of the cytochrome P450 contributes to insecticide resistance (Pu and Chung, 2024). These changes play a key role in resistance development (Pu and Chung, 2024). Furthermore, a frequently cited example of insecticide resistance contributing to malaria control failure is in KwaZulu-Natal, South Africa in the 1990s (Maharaj *et al*., 2005). Despite adequate IRS, there was a significant decrease in malaria cases when pyrethroid was replaced with dichloro-diphenyl trichloroethane (DDT) spraying in response to *Anopheles funestus* pyrethroid resistanc; Kleinschmidt *et al*., 2018)

2.1.4.2. Resistance to antimalarials

Up until the late 1990s, chloroquine (CQ) was considered the first line of malaria treatment in Sub Saharan Africa (Niba *et al.*, 2021). However, it was subsequently replaced by sulfadoxine–pyrimethamine (SP), regrettably, the malaria parasites developed resistance to SP (Lusingu and Von Seidlein, 2008). Mutations in the dihydrofolate reductase (dhfr 51I, 59R and 108N) and mutants of dihydropteroate synthase (dhps436A, 437G, 540E) are linked to SP resistance (Mohamed *et al.*, 2020). *P. falciparum* CQ-resistant transporter (pfcrt) gene and the *P.falciparum* multidrug-resistance gene 1 (pfmdr-1) have been linked to CQ resistance (CQR) (Kayode *et al.,* 2020). Antimalarial resistance has extended to nearly all classes of antimalarial drugs with the exception being the artemisinins. Artemisinins play a vital role in the treatment of multidrug-resistant *P.falciparum* malaria (White, 2004). However, there has been a surge in Artemisinin partial resistance in *P. falciparum* in Rwanda and Uganda, thus resistance to artemisinin poses a great treat to malaria control (WHO, 2023).

2.1.5. Preventing infections using insect repellents

Health officials advocate for the application of topical repellents to aid in the prevention of mosquito-borne diseases (Lo *et al.*, 2018). Additionally, the extensive use of synthetic insecticides and repellents has led to concerns regarding their potential impact on human health and the environment (Smith *et al.,* 2017), prompting a surge in natural alternatives such as plant-derived essential oils derived from plants (Azevedo *et al.*, 2019). Citronella and lemon grass are widely used as mosquito repellents against specifically mosquitoes, prompting further research into plant-derived volatile repellents (Lovinella *et al.,* 2022). Terpenoids and terpenes, known for their potent insecticidal and repellent properties, are emerging as promising substitutes for synthetic insecticides and repellents (Arnault *et al.*, 2018; Bhagat *et al.,* 2020). These terpenoids have demonstrated efficacy against various insect species, including mosquitoes, ticks, and flies (Saric *et al.,* 2016).

Terpenoids can be applied topically to ward off bites from mosquitoes, mites, ticks, and lice, or - less frequently - they can be used to keep ants and cockroaches out of a certain location, such as in packaging to keep stored goods from being infested (Peterson and Coats, 2001). Given that these repellents are frequently used outside during the early hours of the night, they may play a crucial role in places where malaria vectors are most active. As such, they may be used in conjunction with the indoor-based vector control approach (Shibeshi *et al.*, 2024). Terpenoids, including citronellal, citronellol, and limonene, have been reported to exhibit repellent activity against a range of insects (Callahan & Roop, 2011). Among the

various classes of insect repellents, terpenoids have been identified as potential candidates due to their strong odour and volatile nature (Anand *et al.*, 2012).

Arthropod-borne viruses (arboviruses) and parasite infections cannot be effectively prevented by vaccination, hence the development of efficient and secure safe repellents against them is crucial to prevent transmission (Lee, 2018; Onen *et al.*, 2023). Arthropods, particularly mosquitoes, are the only arthropods linked to dengue, malaria, and yellow fever (Shinde *et al.,* 2022). Arboviruses and parasites are transferred to people from these arthropods (Lee, 2018). Arthropod repellents have been the target of intense research and development has been undertaken to develop arthropod repellents with, and synthetic repellents widely have so far gained widespread use. However, their usage of synthetic repellents has given rise to various concerns regarding the safety and risk to the environment and to human health. To replace synthetic repellents, plant essential oils (EOs) and their terpenoids have become quite popular alternative (Lee, 2018).

2.1.5.1. Discovery of Synthetic Repellents

Pyrethrum, a naturally occurring plant oil that originates from two kinds of pyrethrum daisies, namely *Chrysanthemum coccineum* and *Chrysanthemum cinerariifolium*, gained widespread use in the 19th century in China, the Middle East, and throughout Europe. In 1907 however, a Japanese merchant subsequently modified it into a mosquito stick burning spiral coil and marketed it in 1907 (Coats *et al.,* 2021; Debboun and Moore, 2006). Furthermore, the United States Department of Agriculture investigated more than 7,000 chemicals that possess repellent properties with the goal of creating insecticides, miticides, and repellents (Debboun and Moore, 2006; DeGennaro, 2015). Dimethyl phthalate (DMP; patented in 1929 as a fly repellent) was among the first of the chemical repellents to be developed (Debboun and Moore, 2006). Subsequently, ethyl hexanediol (2-ethyl-1,3-hexanediol), also known as Rutgers 612, and Indalo (butyl-3,3-dihydro-2,2-dimethyl-4-oxo-2H-pyran-6-carboxylate), were patented in 1937 (Debboun and Moore, 2006).

Subsequently, researchers produced 33 derivatives of N, N-diethyl benzamide (DeGennaro, 2015). Ultimately, the year 1953 marked the evolution of insect repellents with leading to the discovery of and use of repellents such as N, N-diethyl-3-methylbenzamide (DEET) (Debboun and Strickman, 2013; DeGennaro, 2015). Currently there are several insecticides used to target *Anopheles* mosquitoes in South Africa (Table 2.1).

2.1.5.2. Mechanism of action of Insect Repellents

There are three peripheral olfactory organs present in the *Anopheles* mosquitoes: the labella on the proboscis, the maxillary palps, and the antennae (Figure 2.3.A) (Zwiebel, 2019).These appendages have sensory hairs called sensilla covering them. These hairs are innervated by olfactory neuron dendrites that express different combinations of olfactory receptors (Figure 2.5.B,C) (Konopka *et al.,* 2021). Neurons fire in response to odorant molecules binding to these receptors, transmitting an olfactory signal to the brain's primary processing centre or centres (Xiang *et al.,* 2024). Supporting cells at the base of the sensilla release lymph, the fluid that fills the sensillum, and create proteins called odorant binding proteins (OBPs). It is believed that OBPs, which are among the most prevalent transcripts in antennal tissues, contribute to odour perception by binding to odour molecules and transferring them to odorant receptors (Rihani *et al.*, 2021).

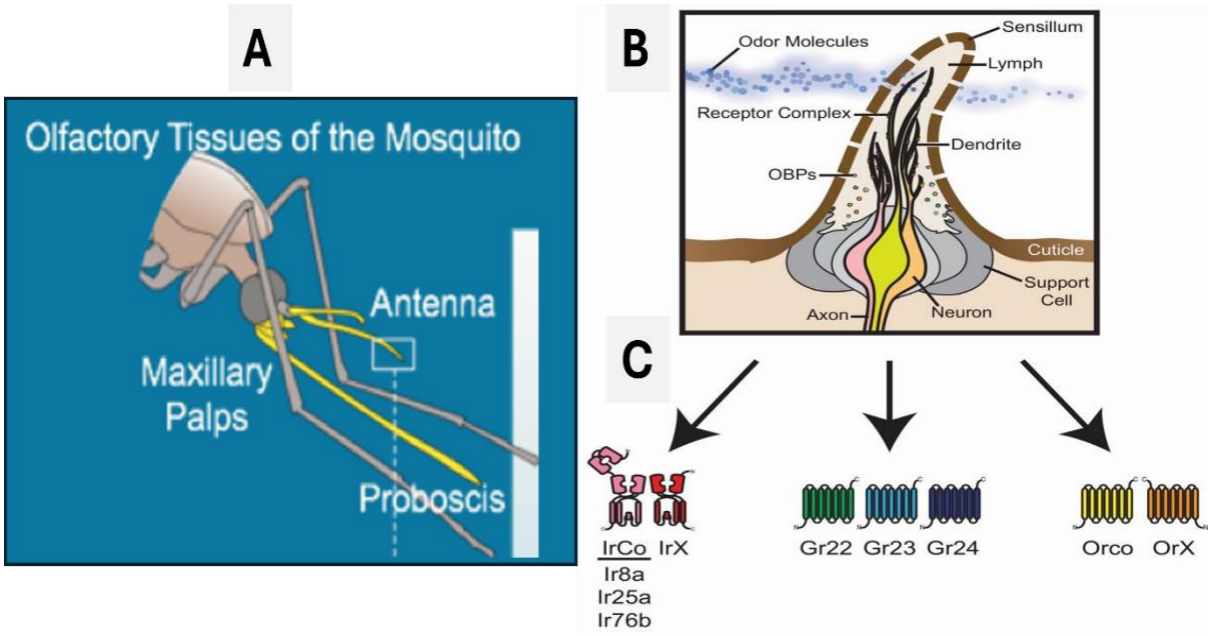


Figure 2.3: Olfaction in Anopheles mosquitoes (Zwiebel, 2019; Konopka et al., 2021). (A) The mouth and olfactory sensory organs and cross-section of a sensillum and olfactory receptors in Anopheles mosquitoes. (B) In Anopheles mosquitoes, each sensillum contains between 2 and 5 olfactory sensory neurons or olfactory sensory neurons (OSNs). (C) Olfactory receptor complexes consist of subunits from 3 broad classes or gene families: ionotropic receptors (IRs), gustatory receptors (GRs), and odorant receptor (ORs).

2.1.5.3. Topical Insect repellent goals

Insect repellents belong to a specific class of compounds whereby they are routinely employed by individuals to deter insects (Lazzari *et al.*, 2024). Repellents work by deterring bites and stings from various arthropods, unlike insecticides, repellents do not possess lethal killing properties against insects (Roy *et al.,* 2017). An insect repellent should demonstrate certain

characteristics to qualify as an ideal insect repellent; these include providing protection against a broad range of arthropods without causing skin irritation upon topical application. Preferably, repellents should not cause any damage to clothing items; they should be odourless or possess a tolerable scent. Repellents should not be easily removed by any form of bathing, wiping, or sweating. In addition to this, repellents should possess chemically stable properties with little to no harm to the skin. The effectiveness of a satisfactory repellent should provide protection against arthropods for up to 8 hours without permeating the skin and entering the systemic circulation (Lee *et al.,* 2018). Table 2.1 summarises the various topical insect repellents found in the South African market with their duration of action, mechanism of action and the various adverse effects.

2.2. Terpenoids

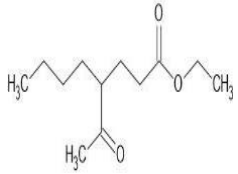
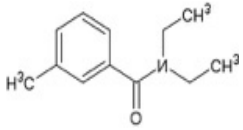
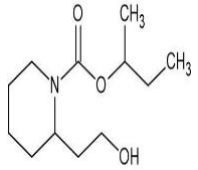
2.2.1. Historical usage and modern applications of insecticidal plants in traditional African societies

Historically, traditional African societies have relied on plants with insecticidal properties to protect themselves against haematophagous insects that transmit infections and parasites leading to illnesses (Adelaja *et al.,* 2021). In the Shamva (100%) and Mazowe (99%) districts in Zimbabwe, it was reported that plants were used to repel mosquitoes, as well as smoke generated from burning animal excrement, egg crates, tissue paper, and elephant excrement as alternative non-traditional insect repellents (Nyasvisvo *et al.,* 2024). The rich indigenous knowledge and African traditional medicines have facilitated the development of modern-day pharmaceuticals (Ali *et al.,* 2023). The benefit of this practice is directly linked to the plant's secondary metabolite composition, as a result medicinal plants have shown to be a great source of bioactive substances.

2.2.2. Role of terpenoids

Terpenoids are found in plants, fungi, and even some marine organisms, playing roles in defence, signalling, and metabolism (Zhao *et al.,* 2022). They are a diverse class of naturally occurring compounds that are the major constituents in essential oils, comprising multiple isoprene units (Maysita *et al.,* 2022). They are either classified as terpenes (simple hydrocarbons) or terpenoids (modified terpenes that contain additional functional groups, usually oxygen-containing groups). The overarching class that contains both terpenes and terpenoids are the isoprenoids or terpenoids (Baser, 2007). A confusing terminology, where terpenoids has been used as the overarching class in this study. They are primarily classified based on number of carbon units, with key categories being mono- (C10), sesqui- (C15), di- (C20), tri- (C30), tetra- (C40) and poly- containing over 40 carbon atoms (Zhang *et al.,* 2022).

Table 2.1: Summarised information on the various synthetic repellents in South African market.

Synthetic Repellents	Chemical Structure	Mechanism of action	Adverse effects	Duration of action (hours)	Products in South African Market	Source
Insect repellent 3535		IR3535, induces intracellular calcium rise through novel cellular mechanisms involving orthostatic/allosteric sites of the M1-muscarinic receptor subtype (M1-mAChR), G protein αβγ subunits, background potassium channels and voltage-gated calcium channels.	Irritability to eyes	4-6	REPEL® FAMILY INSECT REPELLENT	Moreau *et al.*, 2020 Reynoso *et al.,* 2017
DEET		Reports have shown that DEET is an acetylcholinesterase inhibitor that results in neurotoxicity.	Dermal effects: numbness and redness	2-8	Peaceful Sleep® Tabard	Swale *et al.,* 2014
Picaridin		Picaridin stimulates sensory hairs on the mosquito's antennae, and this appears to prevent the mosquito from recognizing its host's cues.	Nausea, vomiting, headache and fatigue	8-11	Repeltec®	National Pesticide information centre, 2011 Heng *et al.,* 2017

2.2.3. Terpenoids as insect repellents

2.2.3.1. Nerolidol

Nerolidol is a naturally occurring sesquiterpene alcohol that exists as two geometric isomers (*cis*- and a *trans*- forms) with notable therapeutic effects (Chan *et al.,* 2016). Nerolidol is found in a variety of plants, particularly those known for their aromatic properties associated with the essential oils of many flowers, herbs, and trees, such as lemongrass (*Cymbopogon citratus*), jasmine (*Jasminum spp.*) and cannabis (*Cannabis sativa*), tea tree (*Melaleuca alternifolia*), ginger (*Zingiber officinale*) and lavender (*Lavandula spp*)(Akingbasote *et al.,* 2022). Nerolidol contributes to the sweet, floral, and woody aroma of these plants and is known for its sedative, antimicrobial, anti-inflammatory, anti-cancer, antiparasitic and insect repellent properties (Chan *et al.*, 2016).

2.2.3.2. Limonene

With the exception of α-pinene, limonene is the most commonly found monoterpene, and exists as a (R)- and/or (S) isomer. It is derived from the rinds of citrus fruit peels (oranges, *Citrus sinensis*), herbs (peppermint, *Mentha* × *piperita* and rosemary, *Rosmarinus officinalis*) and pine trees (*Pinus spp.*) (González-Mas et al., 2019; Kvittingen *et al.,* 2021). Limonene is valued for its uplifting scent, antioxidant properties, and potential therapeutic benefits, such as anti-inflammatory, stress-relieving effects, as well as repellent properties (refs). Lauric acid, an active constituent in vegetables, accounts for the repellent properties of limonene; where lauric acid is reported to discourage the biting or landing of mosquitos, while still remaining harmless towards humans (Mishra *et al.*, 2023). Limonene has been poorly investigated as an arthropod repellent, however it is well studied as an insecticide (Mishra *et al.*, 2023).

2.2.3.3. Alpha pinene

Alpha pinene is a monoterpene and occurs in coniferous plants such as pine (*Pinus spp.*) and fir (*Abies spp.*) trees, and has an enantiomer profile of (+)- and (-)--pinene (Allensoach and Steuer, 2021). This compound is abundant in essential oils such as rosemary (*Rosmarinus officinalis*), basil (*Ocimum basilicum*), eucalyptus (*Eucalyptus spp.*), sage (*Salvia officinalis*) and orange (*Citrus sinensis*) peel oil (Khan *et al.,* 2023). It has a fresh, pine-like aroma and is known for its anti-inflammatory, bronchodilatory, neuroprotective and cognitive-boosting properties (Salehi *et al.,* 2019). This was further exemplified in a study by where alpha pinene (isomer not specified) was reported to have a 100% repellency after 4 hours of exposure, which decreased to 76.92% after 24h when tested on *Diaphorina citri,* a sap sucking hemipteran bug (Li *et al*., 2024).

2.2.3.4. Linalyl acetate

Linalyl acetate is a monoterpenoid ester commonly found in floral and citrus essential oils. Linalyl acetate contributes to the smooth, relaxing scent of these plants and is widely used in perfumes, aromatherapy, and skincare. It has a sweet, fruity, and floral aroma and is known for its calming, anti-inflammatory, and antimicrobial properties (Shin and Soel, 2023). It is found in lavender (*Lavandula angustifolia*), bergamot (*Citrus bergamia*), sweet orange (*Citrus sinensis*), lemon (*Citrus limon*) and clary sage (*Salvia sclarea)* (Shin and Soel, 2023). Few studies have however investigated the repellent properties of linalyl acetate, which is the acetate ester of linalool, known to possess various pharmacological properties including effective (93%) mosquito repellent properties (Muller *et al.*, 2009; Mishra *et al.,* 2023).

Given the growing interest in natural and eco-friendly alternatives to synthetic repellents, essential oils and their terpenoid constituents present a promising avenue for further research. Their diverse chemical structures and bioactive properties suggest significant potential in repelling mosquitoes, yet comprehensive studies are needed to optimize their efficacy, safety, and application methods. Continued investigation into these natural compounds could lead to the development of more sustainable and effective repellents for both public health and agricultural use. As such this study has undertaken to investigate seven terpenoids for their transdermal permeability properties for topical application and repellent effect against *Anopheles* female mosquitoes.

2.3. Transdermal drug delivery systems

The term “Transdermal drug delivery systems” (TDDS) refers to a method of administering and delivering bioactive compounds into cells, tissues, and organs; thus, ensuring optimal therapeutic effects (Vargason *et al.,* 2021). During TDDS bioactive compounds permeate through the stratum corneum, the outer most layer of the skin followed by diffusion thorough the epidermis, dermis thus being released into the capillary networks of the systemic circulation (Vargason *et al.,* 2021).The systems ensure the topical delivery of active pharmaceuticals ingredients of discrete dosages through the patient’s intact skin, this is achieved through penetrating the patients skin barriers.

Transdermal drug delivery systems are widely known for their ability to administer consistent a dosage of a drug and their non-invasive nature-thereby achieving steady plasma concentrations thus improving patient compliance (Sivadasan and Madkhali, 2024). Furthermore, Transdermal drug delivery systems circumvent first pass metabolism alongside and avoid corrosive acidic environments in the stomach which typically occurs with oral intake

of drugs (Phatale *et al.,* 2022). However, despite its promise the system faces challenges as the skin's main function (primarily the stratum corneum) is to defend the body against external threats, thus limiting the functionality of the TDDS (Bocheva *et al.,* 2023).

2.3.1. Skin structure

The skin is the body's largest organ with a surface area of an average 1.7 m^2; this provides a large surface area for easy and accessible drug delivery (Menon, 2002). Topical products such as sunscreens, antiseptics, and insect repellents are created to stay on the surface of the skin, while in contrast, other transdermal medications are designed to penetrate the deeper layers of the skin (Supe and Takudage, 2021). The application of topical medicinal products benefits from the skin's practicality and accessibility; therefore, the evolution of TDDS strives to establish a safe and effective technique of dispensing pharmaceuticals through the skin (Alkilani *et al.,* 2015). Furthermore, skin consists of multiple layers, namely the epidermis, dermis, and hypodermis. The main function of these layers is to shield the body from external damage (Jeckson *et al.,* 2021).

2.3.1.1. The epidermis

The epidermis consists of a superimposed structure encompassing the stratum corneum, stratum basale, stratum spinosum, stratum granulosum, and stratum lucidum (Figure 2.4) (Brown and Krishnamurthy, 2022). The stratum basale houses columnar stem cells and cuboidal cells responsible to produce melanocytes and keratinocytes (Dabbagh Moghaddam *et al.*, 2023). The stratum spinosum houses over eight layers of irregular polyhedral cells adorned with cytoplasmic processes (He *et al.*, 2021). The stratum granulosum consists of over three layers of lamellar and keratohyalin granules (He *et al.,* 2021). Extra stratum lucidum can be found in regions where the skin is thicker, such as the palms and soles. Lastly, the stratum corneum presents itself has twenty to thirty layers of keratinocytes and keratin in the form of horny scales (Yousef *et al.,* 2024). Not only does the skin's structure vary across species, but it also differs within the same species and between bodily sections of the same species (Bartosova and Bajgar, 2012). The stratum corneum resists the absorption of low permeable or larger size (> 500 Da) molecules or substances (El-Sonbaty *et al.,* 2022). Nonetheless, repellents such as DEET are known to have the ability to permeate through the layers of the epidermis by entering the stratum corneum, and eventually the systemic circulation (Feduik *et al.,* 2010)

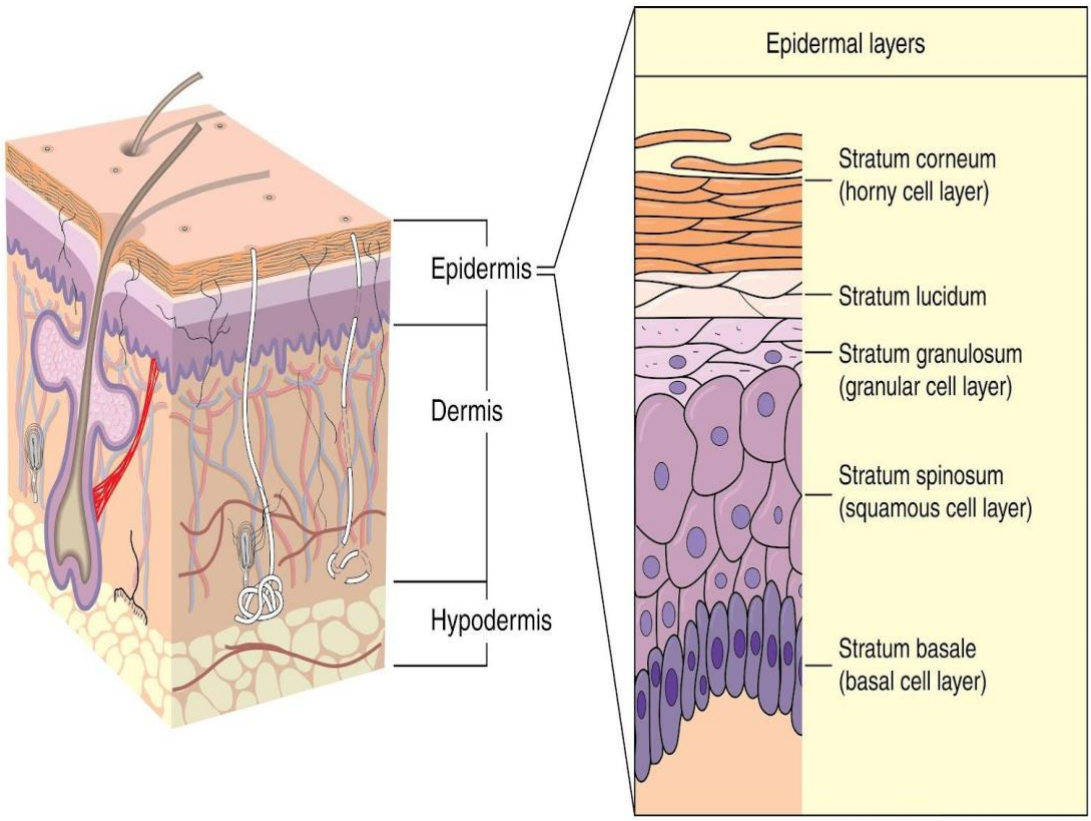


Figure 2.4. The skin consist of three main layers; the epidermis, dermis and hypodermis with the epidermal layer housing the stratum corneum, stratum lucidum, stratum granulosum, stratum spinosum and stratum basale (Ellia , 2019).

2.3.1.2. Dermis

The dermis is divided into two compartments; the thin upper layer referred to as the papillary dermis and the thick lower region, the reticular dermis (Vestita *et al*., 2022). It is located beneath the epidermis and consists mainly of connective tissue, blood vessels, oil and sweat glands, hair follicles and other structures (Lopez-Ojeda *et al.,* 2022).

2.3.1.3. Hypodermis

The hypodermis lies between the underlying organs and the dermis where its fat storage ability enables additional cushioning and insulation. This layer is responsible for connecting the skin to the various muscles (Kim and Dao, 2023).

2.4. Permeation factors

For the efficient passage of compounds through the skin, the compounds should be soluble in both water and oil, due to the stratum corneum's nonpolar lipid nature (N'Da, 2014). In addition, the log partition co-efficient (Log P) of the substance should be between 1.0-3.0; moreover, TDDS employs passive diffusion of active substances into the skin; optimal

absorption is then achieved at a molecular size less than 500 Da (Liu *et al.*, 2024). Furthermore, the application of oil-based formulations on the skin increases skin hydration; this in turn increases the absorption of non-polar fat-soluble molecules (Ogorzalek *et al.,* 2024). Temperature plays a vital role in TDDS. Since the penetration of active substances requires energy, an increase in temperature increases dermal vasodilation that in turn increases blood flow at temperatures between 27ºC and 37ºC (Ghaferi *et al.,* 2024). In addition, as the diffusion co-efficient of the drug decreases, there is an increase in its solubilisation and subsequent penetration. The skin area and skin condition influences drug permeability, an intact stratum corneum reduces permeability, while the permeability of the various sites in the body varies. The permeability of abdominal skin exhibits the greatest thickness at 12 µm, making it suitable for transdermal permeability studies (Yu *et al.,* 2021). Lastly, the stratum corneum regulates the absorption of active substances across the skin where the routes of absorption involve the transcellular and intercellular (paracellular) pathways (Figure 2.5) (CD-Bioparticles, 2021; Yu *et al.,* 2021).

2.4.1. Trans epidermal pathway

The trans epidermal permeation pathway is divided into the transcellular (absorption into the cells) and the intercellular (paracellular, absorption between cells) routes (Yu *et al.,* 2021). The transcellular route involves the movement of hydrophobic drugs through the lipid bilayer of the membrane, whereas the intercellular pathway, the most dominant pathway involves the movement of small hydrophilic drugs and lipophilic molecules into the lipid matrix (Ramadon *et al.,* 2022). However, studies have shown that the intercellular route presents as an obstacle during TDDS (He *et al.,* 2023).

2.4.2. Trans appendageal pathway

The second, trans appendageal pathway (Figure 2.5) facilitates drug delivery through the skin appendages such as the sweat glands or hair follicles and accommodates the passage of large ionisable molecules (Benaouda *et al.,* 2022). Nonetheless, this route presents restrictions for maximal drug absorption due to its smaller absorption area (0.1% of total skin area) compared to the epidermal pathway (Alkilani *et al.,* 2015). This limited absorption through the trans appendageal pathway prompted researchers to develop strategies to

enhance permeation across this pathway, such strategies include disrupting the stratum corneum either chemically or physically (Maher *et al.*, 2022).

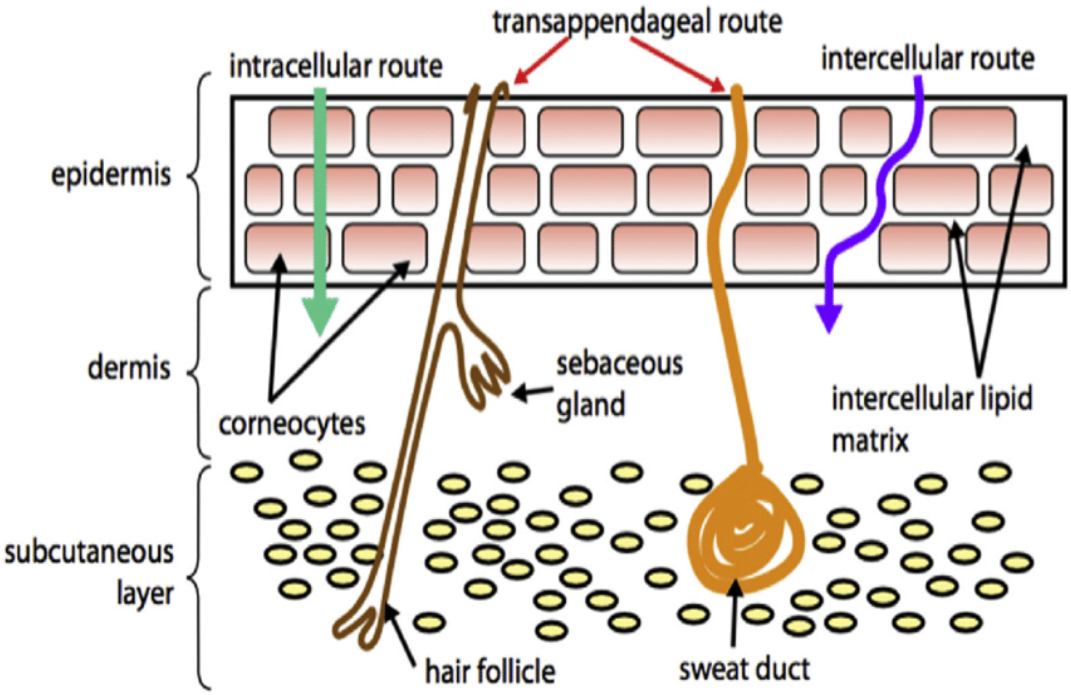


Figure 2.5: The various pathways within the skin, mainly the intracellular and intercellular route.

2.5. Terpenoids as permeation enhancers

Penetration enhancers increase the skin penetration of drug molecules by altering the conformation of keratinised proteins, improving the drug's stratum corneum portioning or acting on the stratum corneum intercellular lipids through fluidisation or extraction (Hmingthansanga *et al.,* 2022). Terpenoids, even at low concentrations, have strong penetration enhancing activity of both hydrophilic and lipophilic drugs (Yu *et al.,* 2024). Terpenoids interact with the stratum corneum compounds through various mechanisms, however their interactions with stratum corneum intercellular lipids have proven to be the most significant (Schafer *et al.,* 2023). The terpenes have high Log P values, which are representative of their lipophilic characteristics, determining their solubility in the stratum corneum intercellular lipids (Gomes *et al.,* 2022). Furthermore, terpenes have the ability to improve the permeation of hydrophilic and lipophilic drugs due to the presence of their polar and nonpolar groups (Hmingthansanga *et al.,* 2022).

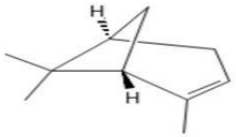
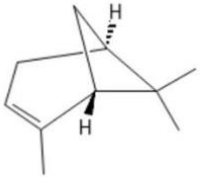
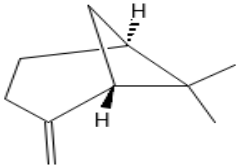
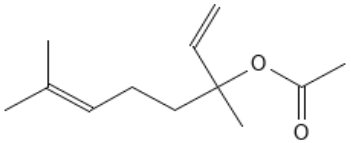
Turning now to the experimental evidence based on histology and diffusion studies where the permeation enhancement ability of various terpenes were investigated, it was shown that terpenes did not significantly increase the drug's solubility (Prosanthi and Lakshini, 2020).

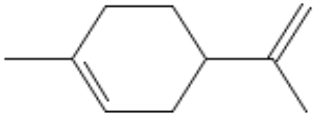
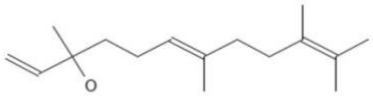
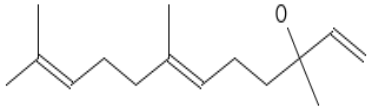
However the terpenes enabled the penetration of the drug into the skin by disrupting the lipid bilayer, thus aiding the drugs diffusion and portioning into the skin. Nerolidol (Log P = 5.36 ± 0.38) showed the highest enhancement ability with its rank of order of nerolidol > farnesol > limonene > linalool > geraniol > carvone > fenchone > menthol in facilitating transdermal delivery of alfuzosin hydrochloride. This was further supported by the findings that terpenes such as nerolidol (isomer not specified) and limonene (isomer not specified) with high Log P values (Table 2.2) enhanced the penetration of lipophilic drugs by disrupting the lipid bilayer of the stratum corneum by forming hydrogen bonds with stratum corneum lipids (Longgos *et al.*, 2024). As such, the terpenes' molecular weight, chemical structure and lipophilicity facilitate the transdermal passage of drugs, thus making them efficient penetration enhancers (Salvoni *et al.*, 2022).

2.6. Terpenoid safety

Literature supports the hypothesis that skin barrier changes caused by essential oils are negligible (Caliskan and Karabus, 2020) as determined by Fourier-transform-infrared spectroscopy that assessed the molecular and conformational changes in the stratum corneum's proteins and lipids (Caliskan and Karabus, 2020). Turpentine oils have been reported to increase the uptake of ketoconazole without causing permanent changes in the skin barrier (Caliskan and Karabus, 2020); whilst 4% nerolidol (isomer not specified) topically applied for 48 hours did not cause any skin irritation, however undiluted nerolidol resulted in erythema or slight oedema (Lopcznski *et al*., 2008).

Table 2.2 *:* The physicochemical properties of miscible terpenoids (Salehi *et al.,*2019, Cavanagh and Wilkinson, 2002, National Center for Biotechnology Information, 2025)

Terpenoids	Chemical structures	Chemical class	Molecular weight (g/mol)	Log P value	Density (g/mL)	Pharmacological properties
(+) Alpha-Pinene		Monoterpene	136.24	4.44	0.858	Anti-Inflammatory Anxiolytic Neuroprotective
(-) Alpha-Pinene		Monoterpene	136.238	4.48	0.858	Anti-Inflammatory Anxiolytic Neuroprotective
(-) Beta-Pinene		Monoterpene	136.23	3.1t	0.872	Anti-inflammatory Anticoagulant Antimalarial
Linalyl Acetate		Monoterpenoid	196.29	4.14	0.901	Local anaesthetic effect Anti-stress

Terpenoids	Chemical structures	Chemical class	Molecular weight (g/mol)	Log P value	Density (g/mL)	Pharmacological properties
(*R*)-Limonene		Monoterpene	136.23	4.57	0.842	Anti-inflammatory Antioxidant Immune regulator
Cis-Nerolidol		Sesquiterpenoid	222.37	5.33	0.875	Antibacterial Anti-inflammatory Anti-cancer Skin repellent
Cis, trans-Nerolidol		Sesquiterpenoid	222.37	5.36	0.875	Anti-bacterial Anti-cancer Anti-nociceptive

CHAPTER THREE: METHODOLOGY

3.1. Introduction

3.1.1. High-performance liquid chromatography

High-performance liquid chromatography (HPLC) is a well sought after analytical tool applied in the pharmaceutical industry. Its main function is to identify, separate and quantify compounds within mixtures. Since its onset in the late 1960s, HPLC has transformed analytical chemistry by providing high resolution, sensitivity and speed in the analysis of a wide range of compounds (Moldovean and David, 2022). The mechanics of chromatographic separation are governed by the interactions between the solute, stationary phase (absorbent), and mobile phase (solvent). These interactions, including adsorption and partitioning, determine the chromatographic environment and influence the separation process. (Engelhart *et al.,* 1980; Saha *et al.*, 2023). Over the past twenty years, the instantaneous evolution of HPLC has transformed scientific research, thus enabling the quantification and identification of organic molecules including pharmaceutical products and pure compounds intended for therapeutic use (Saha *et al.*, 2023).

Reverse Phase-High-performance liquid chromatography (RP-HPLC) has proven to be a vital analytical tool because of its versatility when handling test samples with varying polarity and molecular weight. This has enabled researchers to develop new methods, accelerating investigations aimed at optimizing research and development processes in drug discovery. (Saha *et al.*, 2023). The application of rigorous validation steps and adherence to rigid system suitability standards, such as the United States Pharmacopeia (USP) and the International Conference on Harmonization (ICH), provides enhanced reliability. This stringent validation process is vital for producing accurate data thus underpinning the trustworthy and well-established methodologies in pharmaceutical research (Gumustas *et al.*, 2013; Saha *et al.,* 2023).

3.1.1.1. Instrumentation

A PerkinElmer Flexar™ HPLC system containing a Flexear™UV-Vis detector (U.S.A.), a column oven, a LC binary pump and an auto-sampler was used to conduct all the analyses (Figure 3.1). The column used was a PerkinElmer Brownlee™ Spheri-5 RP-18 (C18) cartridge column (25 x 4.6mm, 5µm) attached to a Brownlee™ NewGuard, RP-18 cartridge (7 µm, 15 mm).

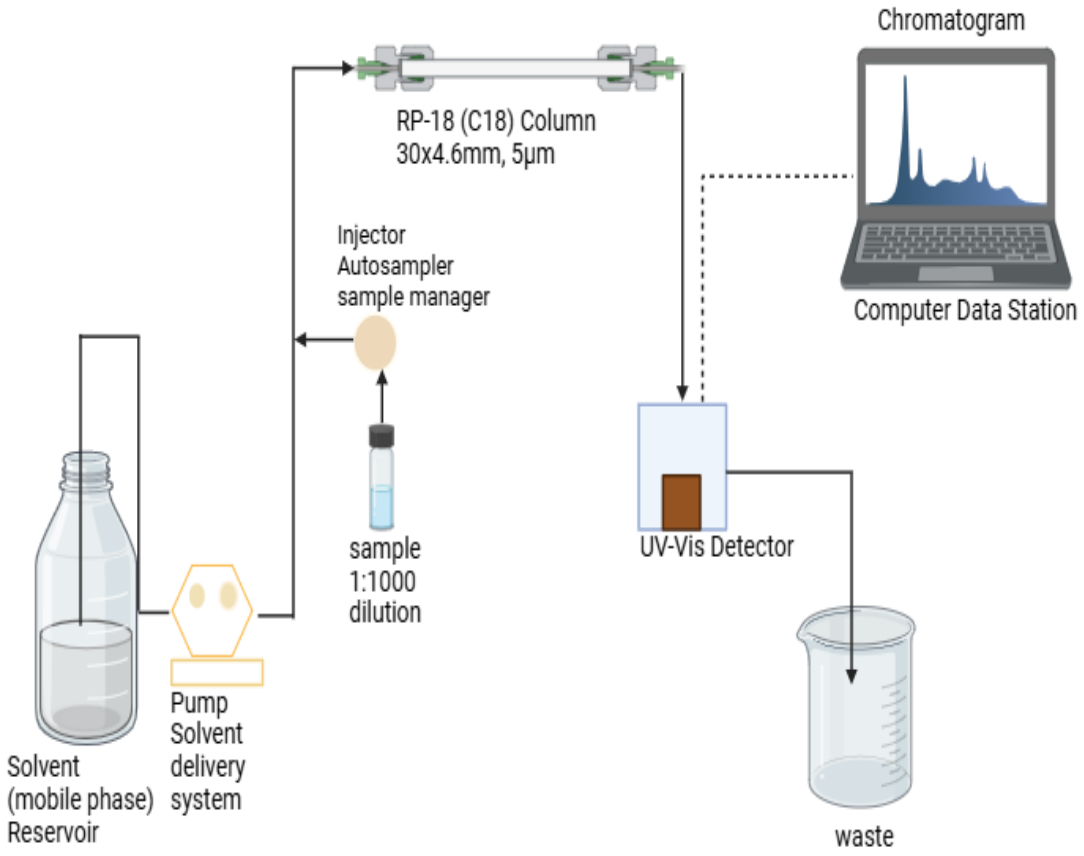


Figure 3.1 : Schematic diagram of the PerkinElmer® Flexar™ HPLC system (created using bio-render).

3.1.2. Materials and Methods

3.1.2.1. Materials

Select terpenoids, including linalyl acetate, cis-Nerolidol, cis trans-nerolidol, (+)-alpha pinene, (-)-alpha Pinene, (-)-Beta pinene and (R)-(+)-limonene were procured from Sigma Aldrich Inc. (St. Louis, Missouri, United States). The standard Phyto-repellent, citronella, was purchased from Burgess & Finch (South Africa), a reputable essential oil company. The reference standards, including caffeine and N, N-Diethyl-meta-toluamide (DEET) were purchased from Sigma-Aldrich. High-performance liquid chromatography (HPLC) grade solutions (methanol, ethanol, acetonitrile and formic acid) for the mobile phases were obtained from Merck & Co., Inc. (New Jersey, USA). All reagents and compounds used for HPLC analyses were filtered (0.45 µm) before use. In addition, Milli-Q® (Merck Millipore Milli-Q™ Advantage water purification) water was utilised. Table 3.1 displays the chemical or agents utilised in the study and the respective supplier.

Table 3.1: List of chemicals reagents used in the study

Chemical/reagent	Supplier	Purity (%)
Methanol (HPLC gradient grade)	Sigma-Aldrich (Germany)	>99
Acetonitrile (HPLC gradient grade)	Sigma-Aldrich (Germany)	>99
Formic acid	Sigma-Aldrich (UK)	-
Dipotassium hydrogen orthophosphate	Sigma-Aldrich (Germany)	-
Potassium dihydrogen phosphate	Labchem (SA)	-
Sodium 1-heptanesulfonate	Sigma-Aldrich (Germany)	-
Sodium hydroxide	Sigma-Aldrich (Germany	-
potassium chloride	Sigma-Aldrich (Germany	-
Hydrochloric acid	Labchem (SA)	-
(-)alpha pinene	Fluka	99
(+)alpha pinene	Sigma-Aldrich (USA)	98
(R)-(+)-Limonene	Fluka	96
Linalyl acetate	Sigma-Aldrich (Germany)	97
Cis-nerolidol	Fluka	96
Cis trans-nerolidol	Sigma-Aldrich (Germany)	98
(-)-beta pinene	Fluka	99
DEET	Sigma-Aldrich (Germany)	99
Caffeine	Sigma-Aldrich (USA)	98.9

3.1.3. Method Validation (Chapter one: objective 1.5.1.)

3.1.3.1. Linearity, Limit of quantification (LOQ) and Limit of detection (LOD)

The analytical method of linearity is defined as the ability of the method to obtain test results that are directly proportional to the analyte concentration, within a preselected range. The calculation of linearity was done using a set of standards with varying concentrations (Migliari *et al.,* 2022). Linearity was evaluated by visual inspection of a plot of signals as a function of analyte concentration or content. Furthermore, If there was a linear relationship, test results were evaluated by appropriate statistical methods, for example, by calculation of a regression line by the method of least squares.

The mentioned concentrations were prepared to produce calibration curves. During analysis of each analyte, each concentration was injected in triplicate (n=3) into the HPLC system.

Three calibration curves were generated separately by plotting the mean peak area values against the aforementioned concentrations (µg/ml) and linear regression performed to generate the linear regression equation (Equation 3.1).

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$$y = m\,x + c$$

Equation 3.1: Where: y=Peak area of analyte; m = Slope; x = Concentration of analyte (µg/ml); c =y-intercept

Furthermore, the LOQ is considered the lowest amount of analyte that gives a signal-to-noise ratio of at least 10:1. This translates as the lowest concentration at which an analyte may be quantified with a satisfactory precision (Migliari *et al.*, 2022), while the LOD is the lowest amount which can be detected but not necessarily quantified (Le *et al.*, 2019), LOD (equation 3.2A) and LOQ (equation 3.2B) can be calculated using equation adopted from the International council for harmonisation (ICH) guidelines (International council for harmonisation, 2023).

$$LOD = 3.3\sigma/S \quad [A]$$
$$LOQ = 10\sigma/S \quad [B]$$

Equation 3.2: Where ,σ is the SD of the data response (y-intercepts of the regression line from multiple calibration curves); S is the slope of the calibration curve.

3.1.3.2. Precision

The precision of the proposed analytical method represents its reproducibility and repeatability, moreover, precision involves two components: Intraday and interday precision (Gupta *et al.,* 2023). Three different concentrations (low, middle and high) of the terpenoids were assessed on the same day (within-day) to determine intraday precision. Whereas, interday precision involved analysing three different standards on three consecutive days (between-day) under similar experimental conditions, both interday and intraday precision were performed in triplicate (n=3). The percentage relative standard deviation (% RSD) (equation 3.3) should be less than 2.

$$\%RSD = \frac{Standard\ deviation}{Mean} \times 100$$

Equation 3.3: The percentage relative standard deviation

3.1.3.3. Accuracy

Accuracy demonstrates the closeness between the measured value and the true or accepted value (Pabale *et al.,* 2024). In this study accuracy was determined by choosing three different concentrations within the calibration curve and injecting them into the HPLC system six times (n=6). The percentage recovery of the response factor (area and concentration) was assessed using equation 3.4.

$$\%\text{Recovery} = \frac{Recovered\ concentration}{Injected\ concentration} \text{ x } 100$$

Equation 3.4: The percentage recovery of the response factor (area and concentration).

3.1.3.4. Robustness

The method's robustness was determined by scrutinising the method's validation parameters; this was achieved by causing deliberate changes to the experimental running conditions (Le *et al.,* 2019). In this study, deliberate changes in the flow rate (± 0.2 ml/min), temperature (± 5 ℃), and mobile phase ratio (± 5%) were made to the method. Three different concentrations were utilised (low, middle, and highest concentration).

3.1.3.5. Stability

During validation of the analytical method, the stability of two standards (lowest and highest concentration) were examined under various conditions in order to evaluate the suitable storage conditions. It is important to note that the storage conditions of the analyte relay the reliability and integrity of the analytical results over time (Mehta, 2023). In this study, the two standards were tested under cold room temperature conditions (4°C) for two weeks and at room temperature (25°C) for one week.

The International Conference on Harmonisation provides procedures and guidelines which should be considered when validating an analytical method (ICH, 2006;European medicines agency). Table 3.2 shows the various validation parameters as well as the acceptance criteria.

Table 3.2: Acceptance criteria for validation parameters (ICH, 2005; Lobrutto and Patel, 2007:461).

Parameter	Criteria
% RSD (Interday & Intraday)	≤ 2%
% Recovery (Accuracy)	85 - 115%
% stability	± 1.5%
Linear recovery	>0.99

3.1.4. Method Development

3.1.4.1. DEET and Caffeine

The method used for the validation of DEET was adapted from a study by Vilar *et al.* (2018). The mobile phase was altered from acetonitrile to methanol because methanol offered better elution strength, the detection wavelength was subsequently adjusted to 205 nm to enable increased UV detection. Lastly the flow rate was altered from 0.3 ml/min to 1 ml/min to accommodate for a better peak shape. The caffeine method (control) was an already validated method obtained from literature (Aminu *et al.,* 2019). Table 3.3 shows the running conditions of the controls DEET and caffeine.

Table 3.3*:* Validation parameters of DEET and Caffeine.

Parameter	DEET	Caffeine
Range	25 – 300 µg/ml	5 – 100 µg/ml
Initial stock	998 µg/ml	1000 µg/ml
Mobile phase	Organic phase (A) Methanol and Aqueous phase (B) Milli-Q® Mobile phase was filtered using a vacuum pump through a 0.45 µm membrane Pump across HPLC at a ratio of A:B, 80:20 v/v ratio	Organic phase (A) Methanol and Aqueous phase (B) Milli-Q® Mobile phase was filtered using a vacuum pump through a 0.45 µm membrane Pump across HPLC at a ratio of A:B, 40:60 v/v ratio
Flow rate	1 ml/min	
Injection volume	20 µL	
Temperature	25°C	
Elution technique	Isocratic	
Detection wavelength	205 nm	

3.1.4.2. Linalyl Acetate, (R)-(+)-limonene. and (-)-beta pinene

In order to optimise the separation of the essential oils using the prescribed HPLC apparatus, high solvent concentration was utilised to ensure the rapid elution. The initial stages of method development signalled that both acetonitrile and methanol were effective solvents across all essential oils chosen for this study. However, during analysis, oils such as linalyl acetate, (R)-(+)-limonene. and (-)-beta pinene produced pronounced peak tailing and broad peaks. To address this, 0.1% v/v formic acid was added. The addition of formic acid resulted in sharp symmetrical peaks. The optimised chromatographic conditions provided reproducible results for the analysis of (R)-(+)-limonene. Table 3.4 displays the running conditions for the routine analysis of Linalyl acetate, (-)-beta pinene and (R)-(+)-limonene.

Table 3.4: Running conditions of Linalyl acetate, (-)-Beta pinene and (R)- (+)-Limonene

Validation Parameters	(R)- (+)-Limonene	-(-)-Beta pinene	Linalyl Acetate
Range	75 - 600 μg/mL	25 – 400 µg/mL	50 - 450 µg/mL
Initial stock	842 µg/mL	859 µg/mL	901 µg/mL
Mobile phase	Organic phase (A): methanol Aqueous phase (B): 0.1% (v/v) formic acid in Milli-Q® water Mobile phase ratio: A:B (92:8 v/v) Mobile phase was filtered using a vacuum pump through a 0.45 µm membrane		
Flow rate	1 ml/min		
Detection wavelength	190 nm		
Injection volume	20 µL		
Column temperature	25°C		20°C
Elution technique	Isocratic		

3.1.4.3. *(-)*-Alpha pinene and (+)-Alpha pinene

An isocratic elution using acetonitrile and methanol in a 70:30 v/v conformation as the mobile phase with a wavelength of detection at 225 nm was the method used (Bielejewska, Duszczyk and Sybilska, 2001) (Table 3.5).

Table 3.5*:* Running condition of (-)-alpha pinene and (+)-alpha pinene.

Validation Parameters	(-)-Alpha pinene	(+)-Alpha pinene
Range	50µg/ml - 800µg/ml	50 µg/ml – 800 µg/ml
Initial stock	858µg/ml	858µg/ml
Mobile phase	Organic phase (A): methanol Aqueous phase (B): 0.1% (v/v) formic acid in Milli-Q® water Mobile phase was filtered using a vacuum pump through a 0.45 µm membrane Mobile phase ratio: A:B (70:30 v/v)	Organic phase (A) Methanol an aqueous phase (B) Milli-Q® Mobile phase was filtered using a vacuum pump through a 0.45 µm membrane Pump across HPLC at a ratio of A:B, 95:5 v/v ratio
Flow rate	1 ml/min	1 ml/min
Detection wavelength	195 nm	225 nm
Injection volume	20 µL	20 µL
Temperature	20°C	25°C
Elution technique	Isocratic	Isocratic

3.1.4.4. *Cis, Trans*-Nerolidol

The isocratic elution of cis, cis trans-nerolidol was developed inhouse, employing a mobile phase content of mainly Milli- Q® and acetonitrile. The table below states the various validation parameters (Table 3.6).

Table 3.6: Validation parameters of cis, trans-Nerolidol and Cis-Nerolidol.

Parameter	cis, trans-nerolidol	Cis-nerolidol
Range	50 - 800µg/ml	6000 – 16000 µg/m
Initial stock concentration	875 µg/ml	875000 µg/ml
Mobile phase	Organic phase (A) Acetonitrile Aqueous phase (B) Milli-Q® Mobile phase was filtered using a vacuum pump through a 0.45 µm membrane Pump across HPLC at a ratio of A:B, 75:25 v/v ratio	Organic phase (A) Aqueous phase (B) Milli-Q® Mobile phase was filtered using a vacuum pump through a 0.45 µm membrane Pump across HPLC at a ratio of A:B, 95:5 v/v ratio
Flow rate	1 ml/min	
Detection wavelength	210 nm	225 nm
Injection volume	20 µL	1 µL
Temperature	25°C	
Elution technique	Isocratic	

3.2. *Ex Vivo* permeation studies

3.2.1. Collection and storage of porcine skin

The decision to opt for porcine skin was supported by various literature sources which state that porcine abdomen skin is comparable to human skin than other skin models (Khiao In *et al.*, 2019; Hwang *et al.*, 2021; Gallagher *et al.,* 2022). An ethics waiver for the approval of the use of porcine skin was obtained from the University of the Witwatersrand Animal Research Ethics Committee (waiver number: 21-09-2022-O) (Appendix A.2). The porcine skin samples were harvested from animals euthanized for other purposes at the central animal unit at the University of the Witwatersrand Medical School. After collection, porcine skin was immediately placed into cold phosphate-buffered saline (PBS)(pH 7.4). Thereafter, excess fat and connective tissue were removed, and specimens were cut into 4x4 cm strips. The tissue strips were then stored inside cryovials, snap-frozen in liquid nitrogen (-195°C), and stored at -70°C. Specimens with any signs of perforation or damage were discarded as they may affect the permeability characteristics of the skin. Cryopreservation is a vital process in long-term tissue storage. It is a process which enables the storage of biological material in a stable state at cryogenic temperature, thus preserving the original cell structures, (Sambu, 2015). It is also the preferred method for preserving skin samples during *ex vivo* permeation studies.

3.2.2. Cream Formulation Preparation

The decision to choose a cream formulation was backed by a study conducted by (Maburutse 2024, unpublished) a member from the same research group, which involved a survey of 447 individuals from the Vaal, Gauteng region. Supplementary information was obtained from surveys conducted by a member of Wits Research Institute of Malaria, in the study community perceptions on the insect repellents were gathered. One of the survey questions investigating the preferred insect repellent formulation the study found that a cream was the preferred formulation for a skin repellent. In the survey, 47% of the participants opted for a cream over the aerosol spray and gel stick formulations (Maburutse, 2024 unpublished).

A cream formulation was prepared according to the method reported by Sonia *et al.* (2017), with minor modifications to the volumes, so as to result in a more homogenous and manageable consistency. The formulation included bleached beeswax (0.5 g), Milli-Q® water (4 ml), olive oil (6 ml), and essential oil (1 ml). The beeswax, Milli-Q® water, and olive oil were transferred into a sterile 15 ml centrifuge tube and heated in a water bath at 70°C separately in their respective centrifuge tubes. Once the beeswax had melted, the olive oil was added and the mixture was stirred vigorously using a vortex mixer. Water was then incorporated until

the mixture transitioned from a liquid to a cream. The cream was allowed to cool to room temperature before the essential oil was added. After cooling, 1 ml of essential oil was mixed in thoroughly using the vortex mixer.

According to literature, industry essential oil repellents are concentrated between 50 mg/ml and 100 mg/ml. For this study, 100 mg/ml was chosen as it produced a longer protection time (Fradin *et al.*, 2002; Khater *et al.*, 2019). To address the volatility of the essential oils and prevent stability issues, the cream was prepared on the day of the experiment. To preserve the integrity of the formulation process, the cream was kept sealed with parafilm, avoiding repeated opening and closing during preparation.

3.2.3. Terpenoids *ex vivo* skin diffusion

For this study, frozen porcine skin was thawed in PBS for 10 mins. Once thawed, the skin was sectioned into seven 2 cm^2 sections. The prepared skin sections were mounted into the 7 in-line flow-through diffusion cells (orifice area of 0.632 cm^2) of the PermeGear 7-In-line flow-through diffusion system (PermeGear INC, Hellertown, PA, USA). Prior to the commencement of the experiment, the cells with tissue were equilibrated at 32°C in PBS (pH 7.4) for 10 mins. The cream formulation containing the active compounds (1 ml) (linalyl acetate, *cis*-nerolidol, *cis*-trans-nerolidol, (+)-alpha pinene, (-)-alpha pinene, -(-)-beta pinene, DEET or -(R)-(+)-limonene) was aliquoted into each of the seven donor compartments of the flow-through cells. PBS (pH 7.4) was pumped through the system at 1.5 ml/h at 32°C and the eluates collected at 2-hour intervals for 16 hours in 20 ml scintillation vials mounted in a Glison FC 204 fraction collector (Glison, Middleton, USA) (Figure 3.2) . After 16 hours, the eluents were analysed on the HPLC system using the already validated methods for the various compounds. Cumulative graphs were constructed of cumulative amounts that diffused (µg/cm^2) vs time (min) and the steady state flux rates (Jss µg/cm2/min) (Equation 3.5.A) and the lag times (min) were obtained from the regression of the linear portion of the cumulative amount per area versus time graph and the x-axis intercepts, respectively. The apparent permeability coefficient (Papp) was calculated from the steady-state flux rates and the change in concentration (C_{Donor}-$C_{Acceptor}$ ≈ C_0) of the active compounds in the donor compartments of the flow cells (Equation 3.5B):

$$Jss = \frac{Q}{A\ x\ t} \qquad (3.5.A)$$

$$Papp = \frac{Jss}{C\ (Donor - Acceptor)} \qquad (3.5B)$$

Equation 3.5: Where: Jss represents the steady-state flux, Q is the quantity of the substance crossing the membrane (µg); A is the exposed membrane area (cm^2), t denotes time exposure (min). Papp is the apparent permeability coefficient (cm/min), C_{Donor}: is the concentration in the donor compartment of the flow cell at t=0 (C_0), $C_{Acceptor}$ is the concentration in the acceptor compartment of the flow cell at t=16 hours. The experiment was conducted under sink and infinite dosing conditions, thus the C_{Donor} (0 h) ≈ C_{Donor} (t=16 h). Sink condition is when the concentration of the permanent in the acceptor compartment is less than 10% of that in the donor compartment. Infinite dosing is an experimental condition that assumes that there is minimal change in the concentrations of the donor (permeant) compartments throughout the experiment, thus ensuring little changes occur within the initial concentration of the formulation over the 16-hour period.

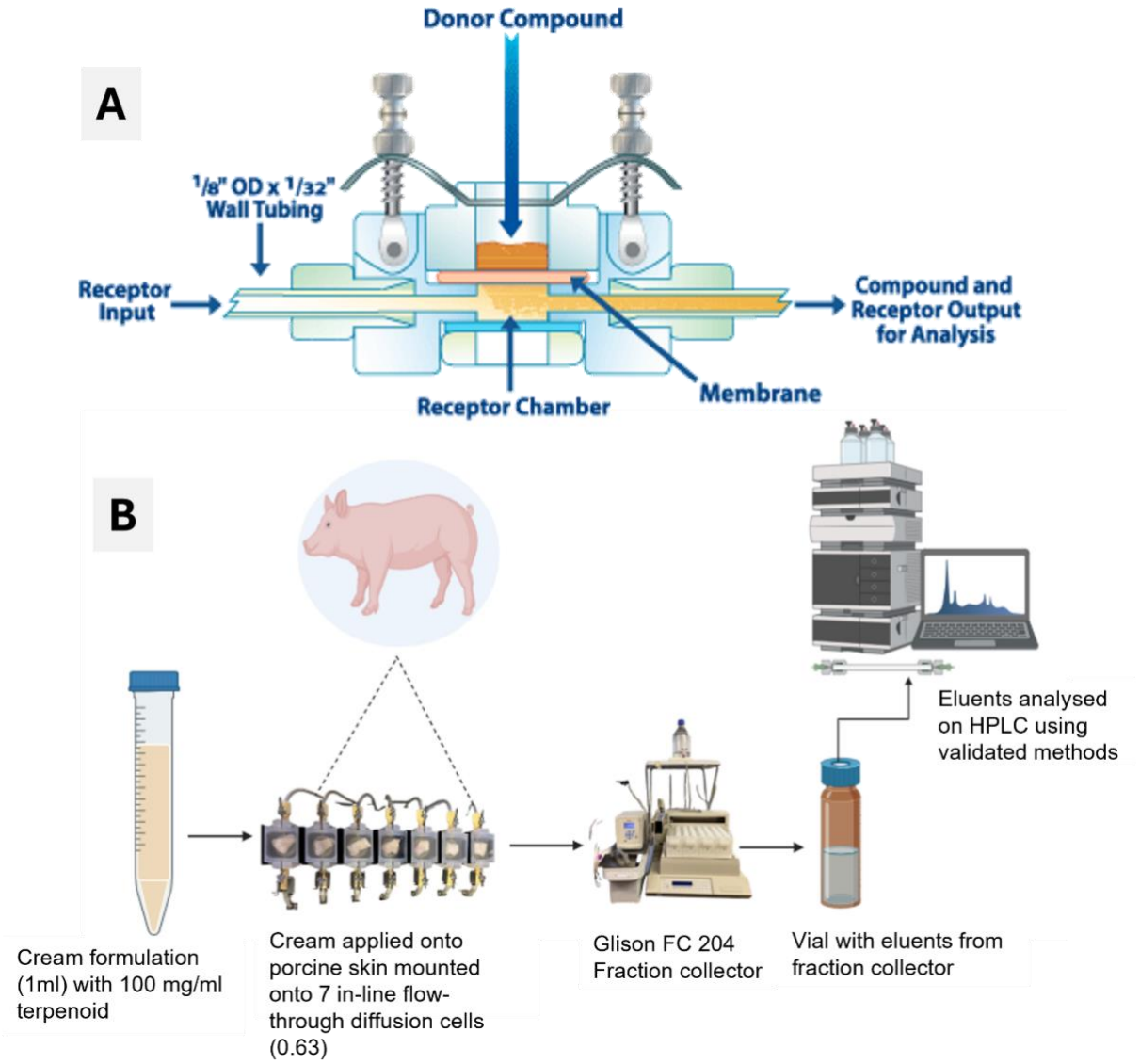


Figure 3.2 The *ex vivo* diffusion assay. (A): An exploded view of the 7-in-line flow-through diffusion cell (B): The diffusion studies design (created using BioRender.com).

3.2.4. Skin retention studies

At the termination of the *ex vivo* diffusion experiment (16 hours), the excised porcine skin discs were removed from the donor chambers and the excess formulation wiped off. Thereafter, the porcine skin discs were placed into sterile 15 ml centrifuge tubes containing 2 ml of HPLC-grade methanol each. After 24 hours the skin discs were homogenised (10 min) and centrifuged at 2000 rpm (10 min)(Zhang *et al.*, 2022)(Figure 3.3). The supernatants were removed and filtered (0.45um). The filtered supernatants were analysed on the HPLC-UV using the validated methods mentioned in Tables 3.3 – 3.6. The amounts of the active compounds accumulated within the skin layers were quantified using the equation below (Equation 3.6).

$$Qt = \frac{CtV}{A}$$

Equation 3.6: The skin retention Where: Qt is the quantity accumulated in the skin after time (t); Ct is concentration extracted in 2 ml of methanol after time (t); V represents the volume of methanol (2 ml); A is the skin area (cm^2).

Equation 3.7 below was utilised to calculate the percentage of the initial amount of the compound within the formulation which accumulated within the skin.

$$Qt\ (\%) = \frac{Qt}{Q0} \text{ x100}$$

Equation 3.7: The skin retention percentage of initial Where: Q0 is the initial amount of essential oil (µg) added to the donor compartment and Qt is the amount accumulated in the skin after time (t).

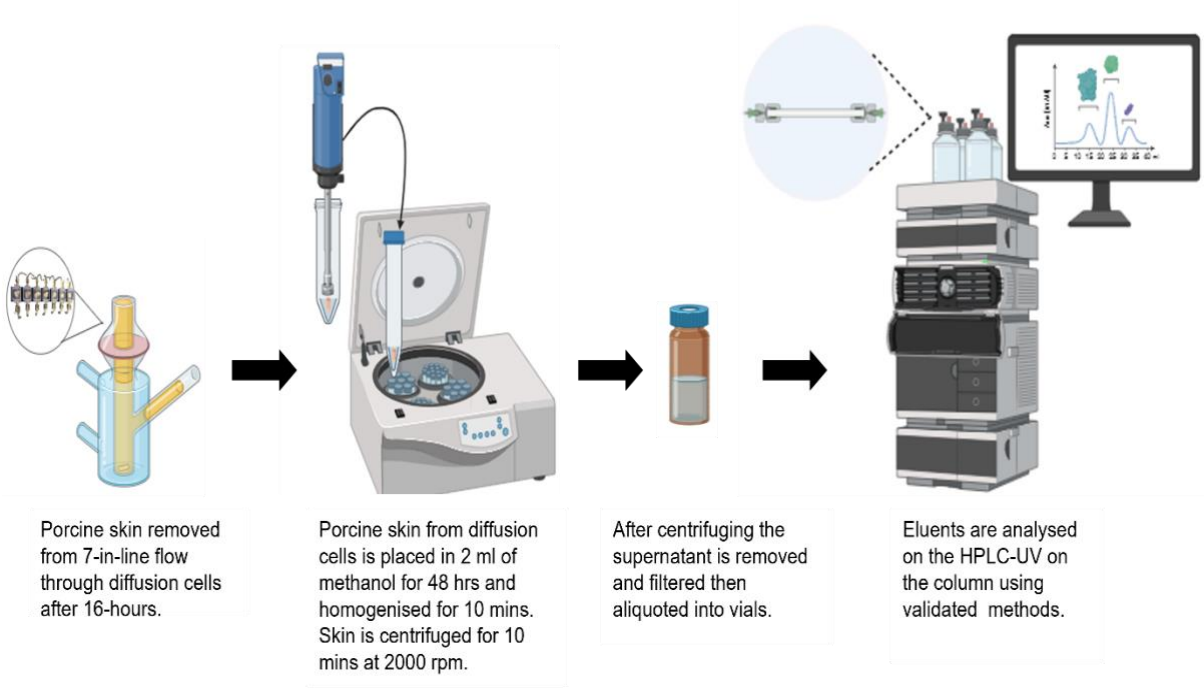


Figure 3.3. The experimental design of the skin retention studies (created using BioRender.com).

3.2.5. Skin integrity (Trans-epithelial electrical resistance) measurements

Trans-epithelial electrical resistance (TEER) is a validated technique that enables the measurement of resistance (**Ω**) over a 24-hour period (0, 2, 4, 6 ,8…18 and 24 hours) at 32°C using a Millipore Millicell® ERS-2 hand-held resistivity meter. During TEER studies low frequency current is applied, and the skin barrier resistance is measured. The effortless flow of current between cells results in lower TEER values, consequently, a reduction in TEER values indicates a loss in skin barrier function .

The setup of the experiment involved mounting excised porcine skin discs ($1 cm^2$) within cell culture inserts in a 12-well transport plate (Corning Costar®, Corning Inc., USA; 12 mm semipermeable membranes pore size: 0.4 µm) and adding the cream formulation (1ml) containing the active compounds (linalyl acetate, cis-nerolidol, cis-trans nerolidol, (+)-alpha pinene, (-)-alpha pinene, -(-)-beta pinene, DEET or (R)-(+)-limonene) in the apical chambers. PBS (pH 7.4, 1 ml) was added in the lower chambers of the transport plates. Two electrodes were employed for the measurement of electrical resistance across the skin, one in the apical chamber and the other in the basolateral chamber. Both electrodes were separated by the semipermeable membrane containing the mounted skin discs (Figure 3.4). TEER was performed in triplicate (n=3) for all compounds and controls on a 12 well plate ($1.1 cm^2$). Trans-epithelial electrical resistance was measured using the equation below (Equation 3.8).

$$\text{TEER} = R_{Sample} - R_{Blank} \times A$$

Equation 3.8: Transepithelial skin resistance measurement Where: Blank is the resistance value of the semipermeable membrane insert without skin (Ω); Sample is the resistance value of the porcine skin model (Ω), A represents area (cm^2).

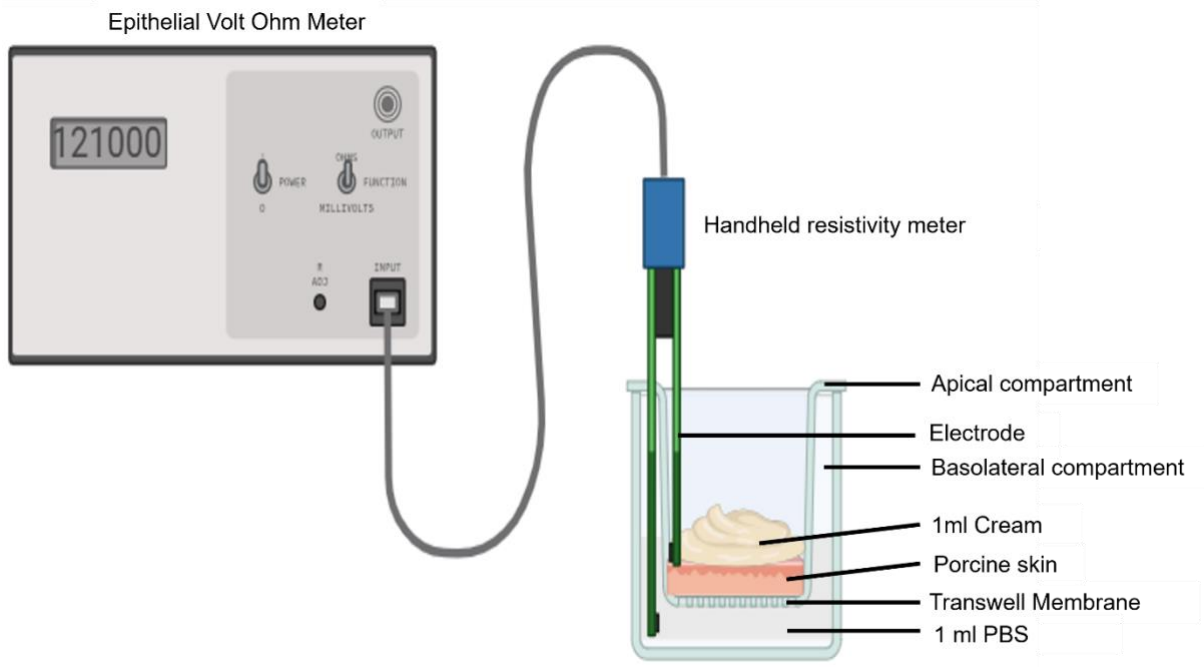


Figure 3.4: *TEER experimental set up (created using BioRender.com)*

3.3. Repellence Bioassay

3.3.1. Biological material

Adult female *Anopheles arabiensis* (5-7 days post emergence KWAG colony) mosquitoes were reared and maintained in the Maureen Coetzee insectary at 27 ± 2 °C and 80 ± 10 relative humidity. Standardised mosquito rearing and testing conditions for mosquitoes were observed. The mosquitoes were fed 10% glucose solution and changed every 3-days (Kaiser *et al.*, 2021). To eliminate possible confounding effects of visual stimuli during the repellence assays, experiments were conducted in the dark (Haselton *et al.*, 2015).

3.3.2. Repellency bioassay

To effectively assess the repellence of the various terpenoids, a repellency bioassay was conducted. The WHO developed and optimised a contact repellency assay for the evaluation of potential repellents against mosquitoes (adapted from WHO, 2013). The compounds were tested on four human volunteers of different race (African, Indian, Coloured and White), age (ages 21-51) and gender (male and female), this was to account for different body chemistries (Okumu *et al.*, 2009). Unfortunately, due to ethical considerations the assay was amended from contact to non-contact. Instead of the application of the active ingredient directly on the arm, the compounds were applied on a wristband ($12cm^2$) (Figure 3.5A).

3.3.2.1. Optimisation of the study

During the optimisation and validation of the non-contact repellency assay, various small scale pilot studies were undertaken. During the initial stages, a pilot study which evaluated the efficacy of 20% DEET against starved *An. arabiensis* using non-contact repellency assay was conducted. The aim of the study was to ascertain whether the adapted WHO assay was a suitable experimental design. However, during the study the mosquitoes exhibited a tendency to naturally congregate on the outer fringes of the cage suggesting a potential spatial preference influenced by structural factors. In an effort to prevent a potential bias during observations a quadrant was created thus delineating a distinct and defined area in which tests were conducted (Figure 3.5B). Concerns surrounding the colour of the quadrant prompted the investigation of the colour perception of the anopheline mosquito, in terms of attraction or repulsion thus these factors were evaluated (Appendix B). Literature suggests that orange promotes attraction towards feeding whereas cyan blue repels (Alonso San Alberto *et al.,* 2022), (Figure 3.5B).

Moreover, a small scale preliminary study was undertaken to assess the effect of colour on anopheline feeding behaviour. The aim of the study was to investigate the influence of cyan and orange on Anopheline behaviour. The results of the study showed p values of the

experiment were above 0.05, signalling a statistical significance. The repellency percentage of DEET when using an orange quadrant (orange paper was sued to section the quadrants) was significantly higher than the repellency percentage obtained from using a cyan quadrant. Therefore, this motivated the use of an orange colour quadrant (Appendix B).

Moreover, the mosquitoes were starved 12 hours prior to the commencement of the study. Prior to the study volunteers were informed to avoid fragranced lotions, deodorants and perfumes as it may influence the results of the experiment. Three concentrations of the terpenoids were examined for repellence 1, 50 and 100 mg/ml; 5 µl. Subsequently, 350µl of the diluted terpenoids were aliquoted onto a 12cm^2 filter paper attached to the wristband. The wristband was placed over the volunteer's hand (Figure 3.5A) the hand was then placed 1 cm away from the BugDorm-4E2222 insect Rearing Cage to prevent biting.

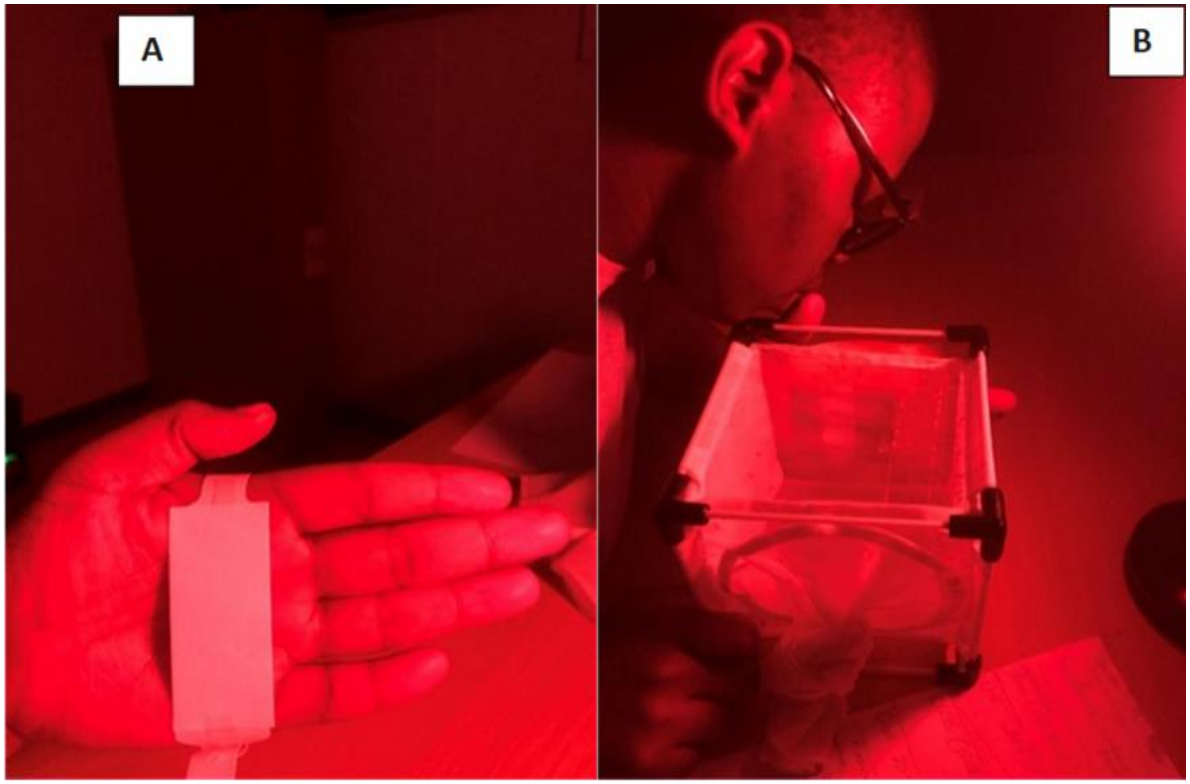


Figure 3.5: Mosquito repellency set up. A: The palm was used as the attractant with the repellent applied on the armband. B: Volunteer evaluating the repellent products against 40 anopheline

3.3.2.2. Repellence Test procedure

Prior to the experiment 40 adult *An. arabiensis* mosquitoes were fed 10% glucose, thereafter the mosquitoes were starved for 12 hours. After 12 hours, the mosquitoes were sexed, once sexed, the female mosquitoes were transferred into a BugDorm-4S2222 (24.5 × 24.5 × 24.5-

cm) rearing cage, using an aspirator. The laboratory in which the study is conducted is conditioned to accommodate the mosquitoes therefore, the room temperature and humidity is set at 27 ± 2 °C and 80 ± 10 relative humidity. The lights were switched off to ensure there is no light perception bias (Fyie *et al.*, 2021). The mosquitoes were transferred in the rearing cages from the insectary to the laboratory and allocated 20-30 mins to acclimate to the laboratory conditions. Furthermore, the sample preparation of the essential oils is done in a separate room to circumvent the push-pull effect, which refers to the overestimation of a spatial repellent (WHO, 2013). The rearing cages are placed on a table, 1 cm away from the volunteers left arm.

During the study, readiness was evaluated to ensure mosquitoes are ready to land/probe, this was assessed for 30 seconds. If 10% of the mosquito's land/probe on the net, experiment was continued, if not the experiment is then terminated (WHO, 2013). Once the readiness test was passed, an armband with filter paper is placed around the volunteer's palm without any compound, this is used as a negative control, thereafter ethanol without compound is applied on the filter paper and used as a negative control. Lastly the terpenes and terpenoids are diluted in ethanol at three concentrations 1, 5 and 10 mg/mL. All active compounds and controls were tested for 3 mins.

3.4. Data Analysis and statistics

The data obtained from the Chromera software was analysed on Microsoft Excel. The statistical parameters, such as the mean, standard deviation, and relative standard deviation (RSD), were calculated to assess the precision and accuracy of the method. Excel was also used to calculate the limits of detection (LOD) and quantification (LOQ) based on the standard deviation of the response and the slope of the calibration curve. Further investigation to determine the linear regression of the peaks was determined using GraphPad Prism version 5.0 (intuitive software for science, San Diego, CA, USA). The ANOVA statistical method was utilised to compare inter- and intraday precision using Graph Prism Pad version 5.0 (intuitive software for science, San Diego, CA, USA).

3.5. Ethical Consideration

An ethics waiver was obtained from the University of the Witwatersrand Animal Research Ethics Committee for the use of porcine skin (waiver number: 21-09-2022-O) (Appendix A.2). The porcine abdominal skin samples were collected from animals euthanised for other purposes at the Wits Research Animal Facility. An additional waiver was granted by the Animal

Research Ethics Committee for the use of *Anopheles* mosquitoes waiver reference: 17-02-2023. Human Research Ethics Committee waiver number: W-CBP-230201-01 (Appendix A.3). Lastly a biosafety waiver was applied for under Prof van Zyl (supervisor) and granted (Appendix A.4)

CHAPTER FOUR: RESULTS

4.1. Method development and linearity

4.1.1. DEET and Caffeine

The adopted DEET and caffeine methods proved to be successful on the PerkinElmer® Flexar™ HPLC system, consisting of the PerkinElmer® Brownlee™ Spheri-5 RP-18 (C18) cartridge column (25 x 4.6mm, 5µm) attached to a Brownlee™ NewGuard, RP-18 cartridge (7 µm, 15 mm). The peaks observed were narrow and symmetrical with retention times of 4.3 min and 4.5 min for DEET and caffeine, respectively. The linear regression obtained from the calibration curves for both compounds, yielded $R^2 > 0.999$ (Figure 4.1A & 4.1 B). For the best elution, the mobile phase ratio of A:B (80:20 (v/v)) for DEET was utilised (containing methanol (A) and Milli-Q® water (B)). Furthermore, caffeine demonstrated the best elution at a mobile phase ratio of A:B (40:60 (v/v)) consisting of methanol (A) and Milli-Q® water (B).

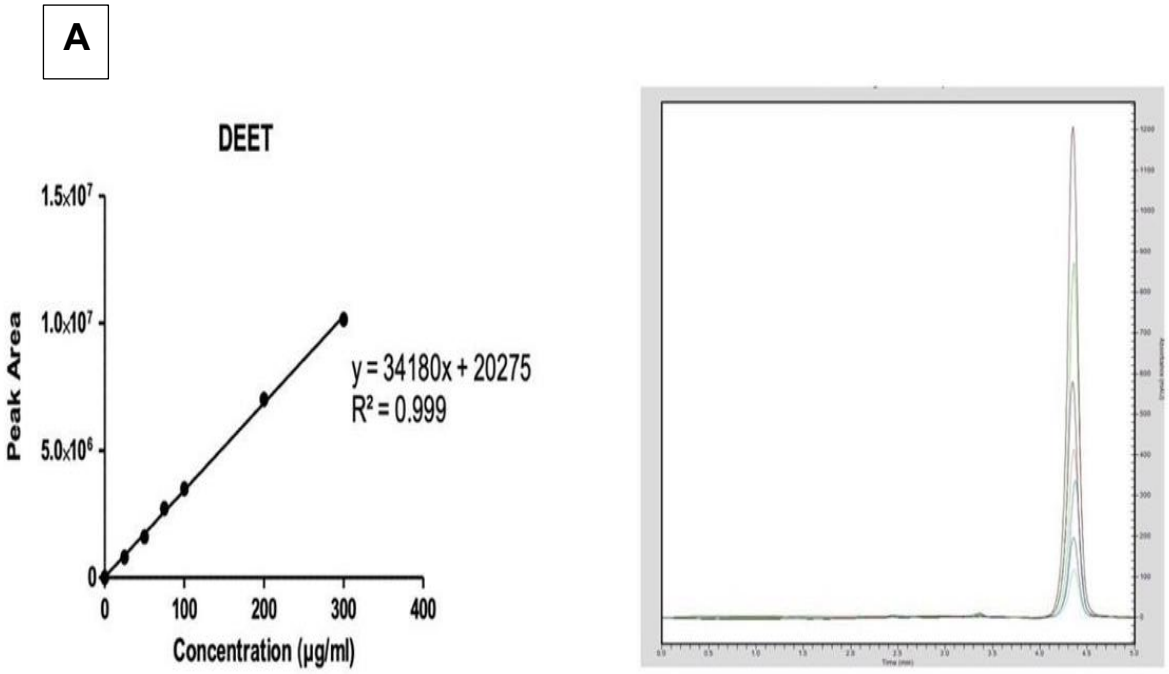


Figure 4.1 A: DEET RP-HPLC calibration curve (n=3) and overlay with a run time of 4.3min and 4.5min and a range of 25-300 µg/ml.

B

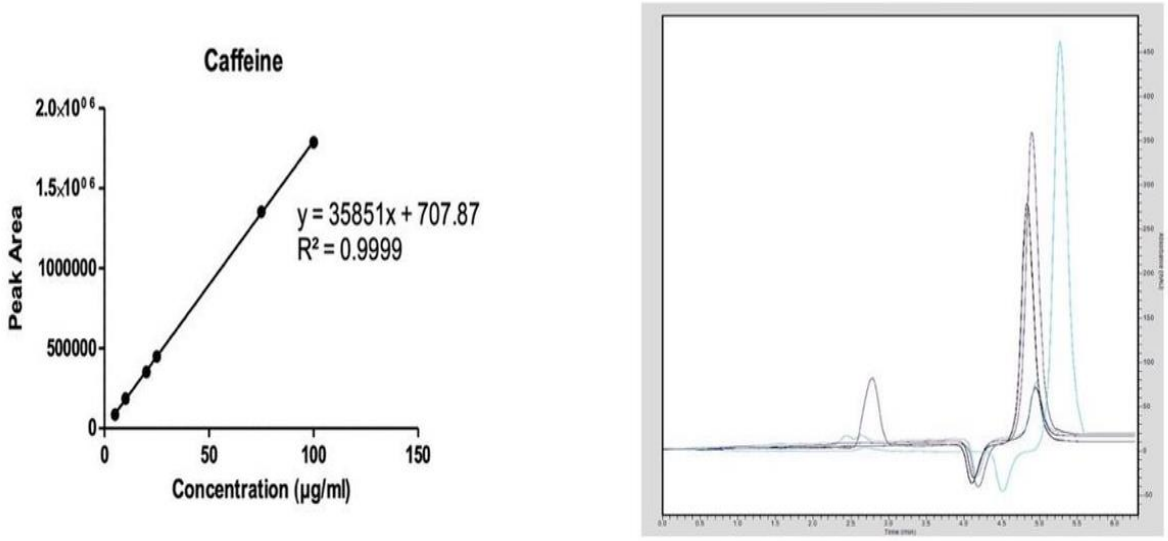
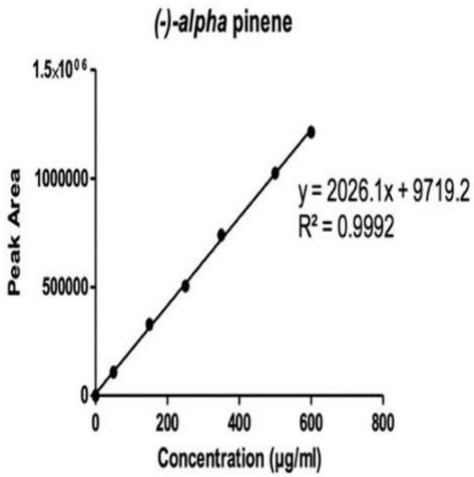


Figure 4.1 B: Caffeine RP-HPLC calibration curve and caffeine plot overlay. Caffeine range (5-100 µg/ml), respectively, using a mobile phase of 80:20 v/v (methanol and Milli-Q® water) for DEET and 40:60 v/v (methanol and Milli-Q® water) for caffeine.

4.1.2. (+)-alpha pinene and (-)-alpha pinene

The method for *(-)*-alpha pinene and (+)-alpha pinene proved to be effective as the peaks were symmetrical and sharp. Both the calibration curves displayed a linear regression of R^2 >0.99, thus falling within the acceptable criteria (ICH, 2005). For the best elution of (-)-alpha pinene (50-800 $\mu g/ml$), the mobile phase consisting of A:B (70:30 (v/v)) methanol (A) and 0.1% (v/v) formic acid in Milli-Q® water (B) resulted in the compound eluting at 3.7 min, whereas, (+)-alpha pinene (50-600 $\mu g/ml$) showed the best symmetry and peak sharpness at a mobile phase ratio of A:B (95:5 (v/v)) (acetonitrile (A) and Milli-Q® water (B)). The retention time for the compound was 4.45 min.

A



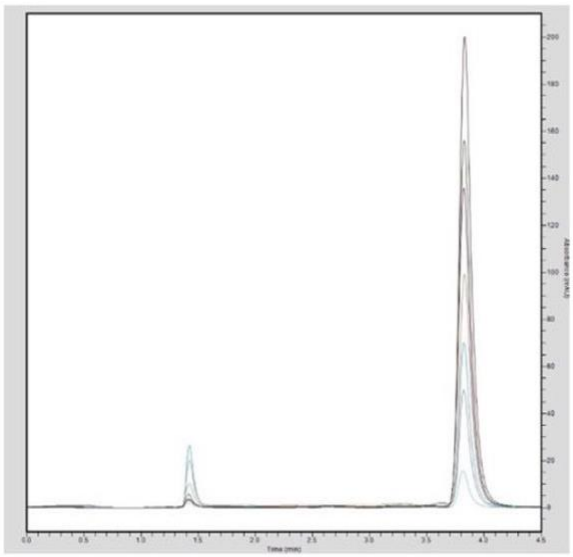
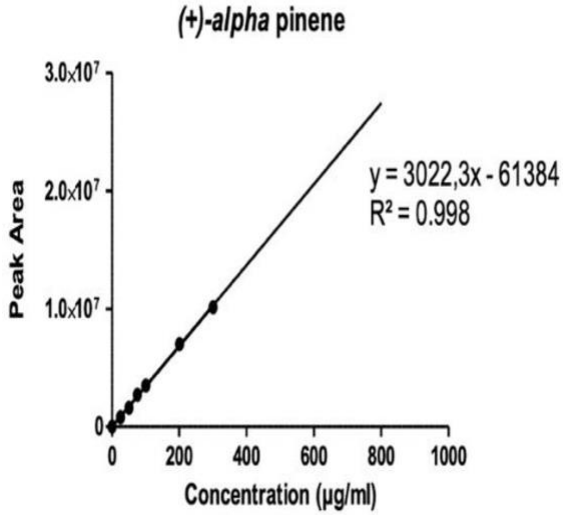


Figure 4.2 A : (-)-Alpha pinene RP-HPLC chromatogram overlay and calibration curve (n=3) with a run time of 3.7min and a range of 50-800 µg/ml, respectively using a mobile phase of 70:30 methanol and acetonitrile.

B



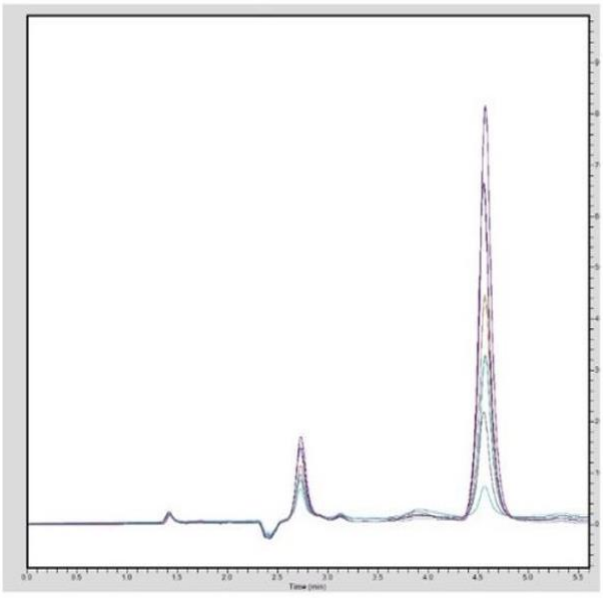


Figure 4.2 B: (+)-*Alpha* pinene (50-600 µg/ml) calibration curve and Chromatic overlay at retention time 4.4mins employing a 95:5 v/v (methanol and Milli-Q® water) mobile phase.

4.1.3. *Cis*-nerolidol and *Cis-Trans* nerolidol

The adopted method for *cis*-nerolidol was deemed successful on the basis of rapid elution of the analyte and narrow peaks. Few *cis*-nerolidol and *cis-trans* nerolidol HPLC methods exist in literature, the available methods are for Gas Chromatography-Mass Spectrometry. The compound, *cis*-nerolidol demonstrated a shorter retention time than current available methods (Scielo technologies, 2025). The retention time was 3.1 min employing a A:B (95:5 (v/v)) mobile phase ratio consisting of acetonitrile (A) and Milli-Q® water (B). However, the isomer *cis-trans*-nerolidol had two peaks that eluted simultaneously. Furthermore, the mobile phase ratio had to be altered to accommodate the separation of the peaks. The initial mobile phase ratio consisted of A:B (95:5 (v/v) (acetonitrile (A) and Milli-Q® water (B)), but this resulted in short retention times with little separation of the co-eluting peaks. Moreover, the addition of 0.1% (v/v) formic acid resulted in a negative effect on peak separation. The best method for the elution of *cis trans*-nerolidol was A:B (75:25 (v/v) acetonitrile (A) and Milli-Q® water (B)). The chosen method proved to be successful compared to other purposed methods and offered more separation between the peaks at a retention time of 7.0 min and a range of 50-800 $\mu g/ml$ (Figure 4.3a & 4.3b)

A

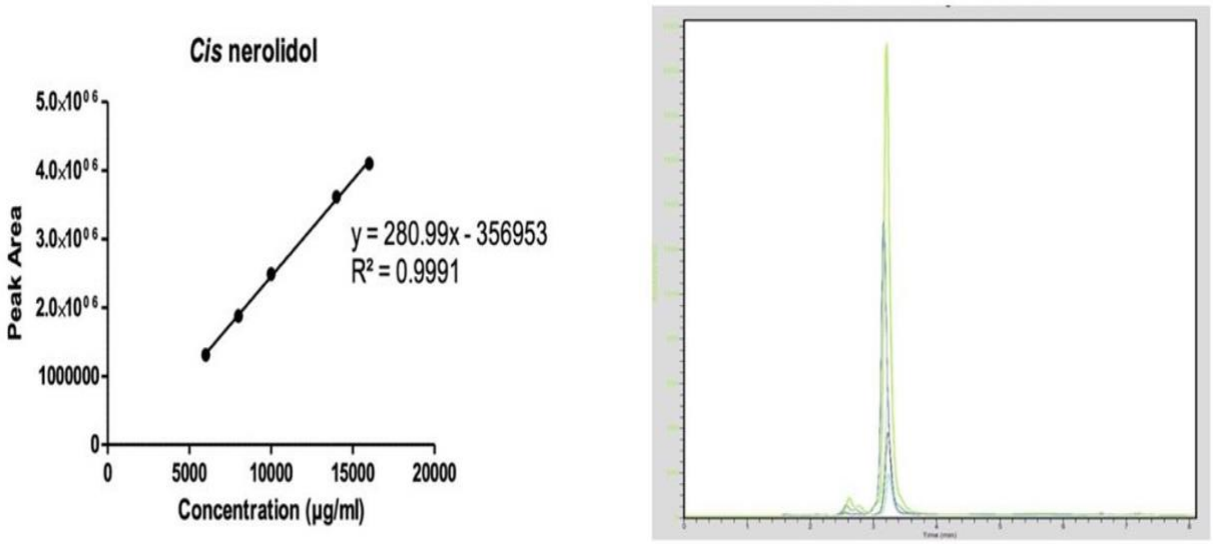
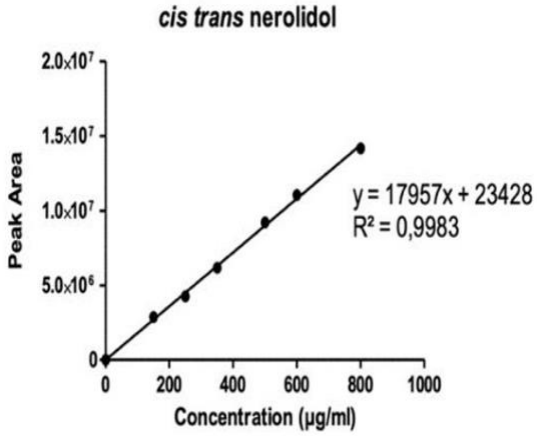


Figure 4.3 A: RP-HPLC chromatogram overlay and calibration curve (n=3) with a run time of 3.1min and 7.1min for (a-b) cis-nerolidol (6000-16000 µg/ml)

B



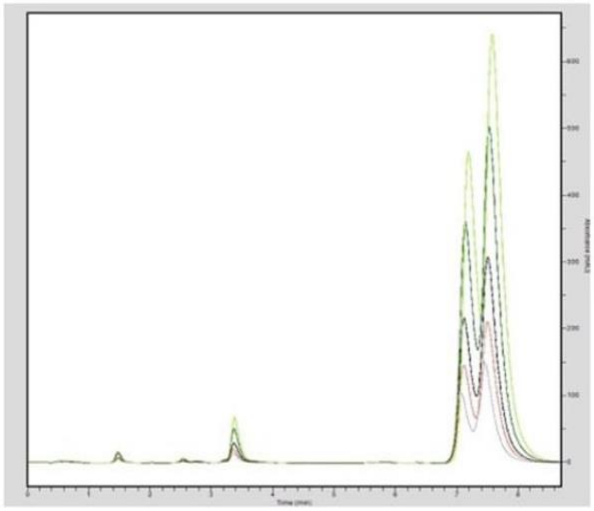
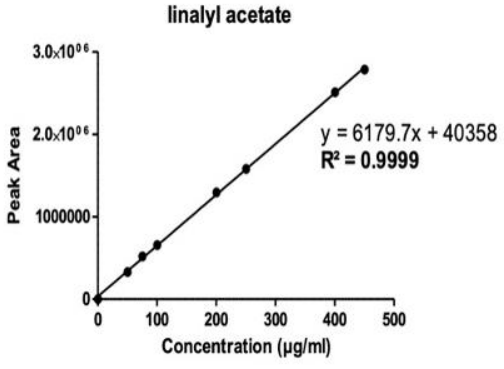


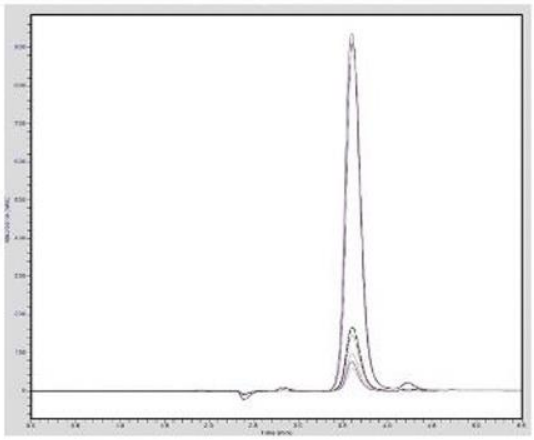
Figure 4.3 B. *Cis trans*-nerolidol (50-800 µg/ml), respectively, using a mobile phase of 95:5 v/v (acetonitrile and Milli-Q® water) for cis-nerolidol and 75:5 v/v (acetonitrile and Milli-Q® water) for cis trans- nerolidol.

4.1.4. Linalyl acetate, (R)-(+)-Limonene and (-)-beta pinene

The implemented method for the routine analysis of the monoterpenoid linalyl acetate and monoterpenes R)-(+)-Limonene and (-)-beta pinene is a mobile phase ratio of A:B (92:8 (v/v) Methanol (A) and Milli-Q® water (B)) with 0.1% (v/v) formic acid. During the initial method development stages the purposed method consisting of a mobile phase ratio of A:B (90:10 (v/v)) (methanol (A) and Milli-Q® water (B)), did not contain formic acid. This method proved to be unsuccessful as it did not resolve the tailing and broad peaks. The addition of 0.1% (v/v) formic acid resulted in symmetrical, rapid and sharp peaks (Figure 4.4). The calibration curves were all linear (R2 >0.99) (Figure 4.4a & b)

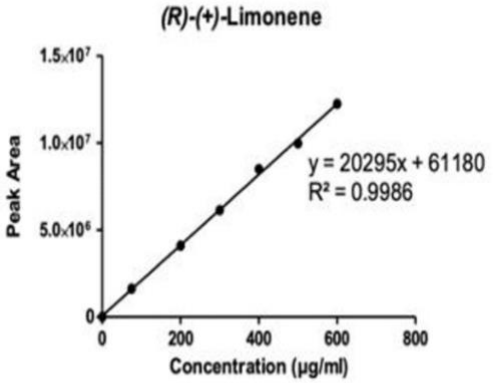
A





curve with a run time of 5.0 mins (a-b) and (A) Limonene (10-500 µg/ml) RP-HPLC chromatogram overlay

B



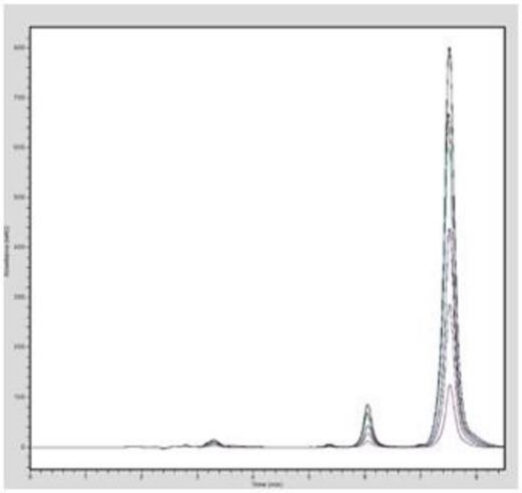
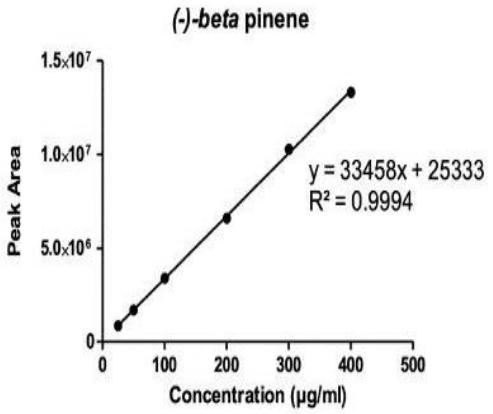


Figure 4.4 B with the calibration curve at a retention time of 7.0 mins and respectively, using a mobile phase of 92:2 v/v and 0.1% v/v formic acid (methanol and Milli-Q® water) for (R-(+))-Limonene

A



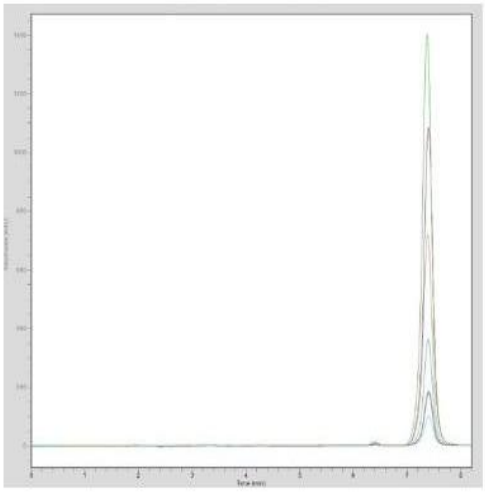


Figure 4.5 A RP-HPLC chromatogram overlay (25-450 µg/ml) (a) and calibration curve with a run time of 7.0mins (f) using a mobile phase of 92:8 v/v (methanol and Milli-Q® water) for (-)-beta pinene

Table 4.1 displays the summarised regression analysis, including the retention times, regression equations, linearity (R^2 values), concentration ranges, and sensitivity parameters (LOD and LOQ) for the terpenes, terpenoids the various controls. The R^2 values ranged from 0.998 to 0.999. The retention times varied across analytes, with Linalyl Acetate eluting at 3.45

Table 4.1: Summary of regression analysis including LOD and LOQ.

Analyte	Retention time (min)	Regression equation	Regression coefficient (R^2)	Range ($\mu g/ml$)	LOD ($\mu g/ml$)	LOQ ($\mu g/ml$)
Caffeine	4.5	y = 35851x+ 707.87	0.999	5-100	9.37	28.40
DEET	4.3	y = 34180x+ 20275	0,999	25-300	27.62	83.69
(-)-Alpha-pinene	3.7	y = 2026,1x + 9719,2	0.999	50-600	35.64	108.01
(+)-Alpha-pinene	4.45	y = 3022.3x- 61384	0.998	50-800	86.42	261.88
(-)-Beta-pinene	7.0	y = 33458x+ 25333	0.999	25-450	21.33	64.63
Cis, Trans-nerolidol	7.0	y = 17957x+ 23428	0.998	150-800	74.52	225.83
Cis-nerolidol	3.1	y = 280.99x- 356953	0.999	6000-16000	2521.54	7641.04
(R)-(+)-Limonene	7.0	y = 20295x+ 61180	0.999	75-600	48.49	146.96
Linalyl acetate	3.34	y = 6123x + 47447	0.999	50-450	6.94	21.04

minutes, while Caffeine eluted at 4.50 minutes. Sensitivity analysis showed that *Cis*-nerolidol had the highest LOD (2521.54 µg/mL) and LOQ (7641.04 µg/mL), suggesting it requires higher concentrations for reliable detection. In contrast, Linalyl Acetate and DEET exhibited lower LOD and LOQ values, making them more detectable at lower concentrations. The table also indicates the concentration ranges within which each compound was analysed, and *cis*-nerolidol requiring a significantly higher range (6000-16000 µg/m).

4.2. Accuracy

The established HPLC method for the various terpenes, terpenoids , and controls (DEET and caffeine) was proved to be efficient. The % recovery ranging from 85-117%, falling within the acceptable limit (85-117%)however, *(+)-a*lpha pinene fell just outside of the acceptable range with the lowest concentration tested at a % recovery of 118 (Table 4.2.)

Table 4.2 : The accuracy of the established HPLC method from three independent standard concentrations. Each analyte concentration was repeated six times (n=6).

Compound	Amount of Analyte Added (µg/mL)	Peak Area Mean ± SD	% Recovery (n=6)	Mean % RSD
Caffeine	15	4747120 ± 3800	86	1.2
	40	142982.17 ± 11159.43	99	0.7
	85	2942794.17 ± 8036.94	97	0.9
DEET	155	5284247.3 ± 20915.35	100.0	0.5
	165	5760136.09 ± ±27417.43	103.0	0.9
	255	8976631.37± 156682.89	102.0	1.2
(+)-Alpha Pinene	300	1009825.11 ± 29762.41	118.0	1.5
	350	1132895 ±22122	102.0	0.8
	400	1235063.79 ±19655.03	97.0	1.3
(-)-Alpha Pinene	525	1121821.47 ± 5400.13	103.0	0.6
	625	1293140.79 ± 13018.44	102.0	1.0
	725	1482296.44 ± 17195.47	99.0	0.9
(-)-Beta Pinene	85	2892019.63 ± 9522.57	100.0	0.5
	300	5552842.93 ± 77809	103.0	2.49
Cis-Trans Nerolidol	200	3109264.04 ± 238020	85.0	2.98
	300	5552842.93 ± 77809	103.0	2.49
Cis - nerolidol	15200	5581815.43 ± 23568	88.0	0.4
	17000	4836626.11 ± 35032.41	86.0	0.6
(R)- (+)- Limonene	85	2121241.72 ± 9431.55	114.0	1.2
	350	8046306.79 ± 22801.43	112.0	1.4
	550	1195457.78 ±79884.74	102.0	0.7
Linalyl Acetate	85	568435.55 ± 1229.43	99.0	1.8
	150	942184.29 ± 16046.12	98.0	2.1
	450	2783367.86 ± 5748.49	100.0	0.9

4.3 Precision

In the present study the precision was evaluated using intraday and interday repeatability studies to assess the reproducibility of the study. During analysis three standards (low, medium and high) were deduced from the calibration curve. During the interday analysis the compounds were injected three times on the same day (n=3), whereas interday studies were carried out on different days. Table 4.3 summarises the precision values obtained for all analytes. Consistent reliability was observed across the various compounds, the reliability is attributed to the %RSD values which fell below 2% during both intraday and interday measurements. The controls DEET and caffeine showed exceptionally low %RSD values, thus reflecting high precision in peak area reproducibility. Furthermore, compounds such as linalyl acetate and cis-nerolidol demonstrated increased %RSD values during intraday measurement compared to the other compounds signalling towards stable chromatographic behaviour over time. The precision values of all compounds fell within the acceptable limit of 2% RSD.

Table 4.3 : Summary of interday and intraday precision values (n=3) of various terpenes and terpenoids.

Compound	Conc. (µg/mL)	Intraday Mean Peak Area ± SD	Intraday %RSD	Inter-day Mean Peak Area ± SD	Interday %RSD
Caffeine	5	199871.152 ± 1839.721	0.924	199871.152 ± 1839.721	0.924
	25	885139.704 ± 235.994	0.026	885139.704 ± 235.994	0.026
	100	3524441.011 ± 5480.510	0.155	3524551.011 ± 5480.510	0.155
DEET	25	958995.533 ± 5529.170	0.577	958985.533 ± 5529.170	0.577
	100	3462696.500 ± 3372.200	0.097	3462796.500 ± 3372.200	0.097
	300	10415364.980 ± 39937.080	0.383	10415364.980 ± 39937.080	0.383
(+)-Alpha Pinene	50	60357.115 ± 164.920	0.273	201414.981 ± 3933.306	1.953
	500	767472.128 ± 12265.230	1.598	3067690.792 ± 65495.930	2.135
	800	1110027.101 ± 15973.060	1.439	4959857.414 ± 55622.236	1.121
(-)-Alpha Pinene	25	1199990.650 ± 20967.166	1.747	1166372.680 ± 3346.857	0.287
	100	4458895.152 ± 15513.829	0.348	4672531.744 ± 23669.725	0.507
	400	18105854.740 ± 68419.952	0.378	17670836.420 ± 106669.407	0.604
***(-)-Beta* Pinene**	25	1199990.650 ± 20967.166	1.747	1166372.680 ± 3346.857	0.287
	100	4458895.152 ± 15513.829	0.348	4672531.744 ± 23669.725	0.507
	400	18105854.740 ± 68419.952	0.378	17670836.420 ± 106669.407	0.604

Compound	Conc. (µg/mL)	Intraday Mean Peak Area ± SD	Intraday %RSD	Inter-day Mean Peak Area ± SD	Inter-day %RSD
***Cis*-Nerolidol**	6000	1522114.060 ± 10615.860	0.697	1111681.435 ± 15229.550	1.370
	10000	2682369.350 ± 9973.960	0.372	2463812.226 ± 18633.910	0.756
	16000	4143610.838 ± 18991.240	0.458	4110163.174 ± 20636.490	0.502
***Cis-Trans*-Nerolidol**	50	853393.144 ± 664.830	0.078	994592.148 ± 664.410	0.067
	350	5270806.891 ± 7386.000	0.140	6418995.430 ± 11487.280	0.179
	800	11739675.940 ± 43721.400	0.372	14186305.130 ± 118924.260	0.839
***(R)-(+)*-Limonene**	75	1786209.870 ± 6274.470	0.351	1520468.175 ± 2339.460	0.154
	200	12098373.140 ± 8155.430	0.067	5717235.644 ± 5436.830	0.095
	300	6438207.594 ± 2071.010	0.032	12556134.540 ± 28979.490	0.231
Linalyl acetate	50	315095.110 ± 4949.100	1.570	1520468.175 ± 2339.460	0.154
	200	1324765.777 ± 24813.170	1.873	5717235.644 ± 5436.830	0.095
	450	2990448.408 ± 5857.770	0.196	12556134.540 ± 28979.490	0.231

4.4. Robustness

The robustness of the proposed HPLC methods was determined by altering chromatic conditions such as: temperature, flow rate and mobile phase component. The robustness results can be observed in Table 4.4 below. The slight adjustment of the validation parameters resulted in a shift in retention time and peak area. The flow rate had a significant impact on the retention time of linalyl acetate and -(-)-Beta pinene. Moreover, the mean % RSD for retention time and peak area fell within the acceptable criteria of <2% for all the established methods. However, Linalyl acetate and -(-)-Beta pinene were notably affected by the adjustment in flow rate with DEET displaying a slightly higher %RSD (2.8%) at a decreased flow rate and temperature suggesting sensitivity to these parameters.

4.5. Stability

Stability was tested at different concentrations using one standard, at week 1 and week 2. HPLC stability is tested at different intervals at lower temperatures and at room temperature to provide evidence on the quality of the product over time under the influence of temperature, light and humidity (ICH, 2003). A retest provides insight on the shelf life of the product (ICH, 2003). In the study stability was evaluated at two different temperatures; at room temperature (Bench top) and at 4°C. The initial standard taken on the day was then compared to the standard taken at week one and week two (Table 4.5).

Table 4.4 : Table demonstrating the robustness of the various established HPLC methods, by altering the temperature, flow rate and mobile phase component. Robustness was performed in triplicates n=3.

Compound	Parameter	Set conditions	Variation	Retention time (mean ± SD)	Area (mean ± SD)	% RSD RT	% RSD area
Linalyl Acetate	Temperature	25°C	22°C	3.450 ± 0.002	1,468,437 ± 5,869	0.05	0.004
			28°C	3.427 ± 0.005	1,506,522 ± 7,864	0.15	0.52
	Flow Rate	1.0 ml/min	0.9 ml/min	3.813 ± 0.003	3,566,879 ± 31,818	0.07	0.89
			1.3 ml/min	2.668 ± 0.004	2,474,606 ± 2,593	0.16	0.10
	Mobile Phase	92:8	90:10	2.668 ± 0.005	6,074,599 ± 21,883	0.19	0.36
(R)-(+)-Limonene	Temperature	20°C	22°C	8.63 ± 0.02	1,535,025 ± 19,001	0.25	1.24
			19°C	8.67 ± 0.02	1,565,924 ± 5,869	0.09	0.70
	Flow Rate	1 ml/min	1.2 ml/min	7.33 ± 0.00	1,242,265 ± 19,039	0.05	1.5
			0.9 ml/min	9.67 ± 0.01	1,700,960 ± 11,841	0.1	0.70
	Mobile Phase	90:10	90:10	10.43 ± 0.01	1,508,759 ± 1,307	0.12	0.09
DEET	Temperature	20°C	19°C	4.63 ± 0.03	1,218,976.78 ± 34,969.74	0.68	2.87
			22°C	4.63 ± 0.03	1,408,218.39 ± 27,633.55	0.68	1.96
	Flow Rate	1.0 ml/min	0.8 ml/min	4.31 ± 0.01	1,218,976.78 ± 34,969.74	0.23	2.87
			1.2 ml/min	4.31 ± 0.01	1,408,218.39 ± 27,633.55	0.23	1.96
	Mobile Phase	80:20	75:25	4.63 ± 0.03	1,218,976.78 ± 34,969.74	0.68	2.87

Compound	Parameter	Set conditions	Variation	Retention time (mean ± SD)	Area (mean ± SD)	% RSD RT	% RSD area
(-)-Beta pinene	Temperature	20°C	22°C	8.22 ± 0.02	287,618.29 ± 21,542.44	0.20	4.0
			19°C	8.23 ± 0.02	318,098.37 ± 21,542.44	0.20	1.87
	Flow Rate	1.0 ml/min	1.2 ml/min	9.08 ± 0.01	269,472.83 ± 4,065.76	0.02	1.51
			0.9 ml/min	6.87 ± 0.01	209,108.10 ± 306.80	0.04	0.1
	Mobile Phase	90:10	90:10	10.43± 0.01	1,508,759.82 ± 1,307.28	0.12	0.09
(+)-Alpha pinene	Temperature	20°C	19°C	5.051 ± 0.009	729,116.25 ± 17,676.90	0.19	2.43
			22°C	5.327 ± 0.028	751,886.92 ± 9,937.70	0.53	1.32
	Flow Rate	1.0 ml/min	0.9 ml/min	4.32 ± 0.04	124,160.15 ± 11,766.22	0.98	9.48
			1.2 ml/min	4.28 ± 0.08	1,521,470.76 ± 3,377.27	1.86	0.22
	Mobile Phase	95:5	94:6	4.39 ± 0.01	1,508,759.82 ± 21,883.19	1.45	1.45
			96:4	4.39 ± 0.01	1,508,759.82 ± 21,883.19	1.45	1.45
(-)-Alpha pinene	Temperature	25°C	22°C	3.974 ± 0.008	1,398,544 ± 225,420	0.16	0.16
			26°C	3.974 ± 0.008	2,563,027 ± 6,504	0.21	0.26
	Flow Rate	0.9 ml/min	1.2 ml/min	4.36 ± 0.00	1,822,952 ± 6,901	0.07	0.38
			0.9 ml/min	4.36 ± 0.00	1,822,952 ± 6,901	0.07	0.38
	Mobile Phase	70:30	72:28	3.89 ± 0.01	1,735,633 ± 6,901	0.07	0.38
			65:35	3.87 ± 0.01	2,767,084 ± 3,625	0.01	1.39

Compound	Parameter	Set conditions	Variation	Retention time (mean ± SD)	Area (mean ± SD)	% RSD RT	% RSD area
Cis-nerolidol	Temperature	25°C	22°C	3.450 ± 0.002	1,468,437 ± 5,869	0.05	0.004
			28°C	3.427 ± 0.005	1,506,522 ± 7,864	0.15	0.52
	Flow Rate	1.0 ml/min	0.9 ml/min	3.813 ± 0.003	3,566,879 ± 31,818	0.07	0.89
			1.3 ml/min	2.668 ± 0.004	2,474,606 ± 2,593	0.16	0.10
	Mobile Phase	95:5	90:10	2.668 ± 0.005	6,074,599 ± 21,883	0.19	0.36
Cis trans nerolidol	Temperature	20°C	22°C	8.63 ± 0.02	1,535,025 ± 19,001	0.25	1.24
			19°C	8.67 ± 0.02	1,565,924 ± 5,869	0.09	0.70
	Flow Rate	1 ml/min	1.2 ml/min	7.33 ± 0.00	1,242,265 ± 19,039	0.05	1.5
			0.9 ml/min	9.67 ± 0.01	1,700,960 ± 11,841	0.1	0.70
	Mobile Phase	75:10	70:25	10.43 ± 0.01	1,508,759 ± 1,307	0.12	0.09
Caffeine	Temperature	25°C	19°C	4.63 ± 0.03	1,218,976.78 ± 34,969.74	0.68	2.87
			22°C	4.63 ± 0.03	1,408,218.39 ± 27,633.55	0.68	1.96
	Flow Rate	1.0 ml/min	0.8 ml/min	4.31 ± 0.01	1,218,976.78 ± 34,969.74	0.23	2.87
			1.2 ml/min	4.31 ± 0.01	1,408,218.39 ± 27,633.55	0.23	1.96
	Mobile Phase	40:60	35:65	4.63 ± 0.03	1,218,976.78 ± 34,969.74	0.68	2.87

Table 4.5 **:** Table demonstrating the stability of the various established HPLC methods at room temperature and at 4°C

Compound	**Concentration (µg/mL)**	Benchtop (1 Week)	Benchtop (1 Week)
Linalyl Acetate	25	4.00	10.33
(R)-(+)-Limonene	75	3.96	4.00
(-)-Alpha-Pinene	50	1.00	1.22
(+)-Alpha-Pinene	75	2.96	1.54
(-)-Beta-Pinene	25	1.89	1.04
Cis-nerolidol	6000	0.21	1.76
Cis-trans nerolidol	150	4,12	0.90
DEET	75	1.05	4.00

4.5. *Ex vivo* permeation

4.5.1. Permeation of terpenes and terpenoids across porcine skin

Regardless of the extended exposure time of the porcine skin to the compounds (16 hours), the compounds failed to diffuse through the skin layers into the acceptor chambers of the in-line flow-through cells. DEET (10 mg/mL) started diffusing through the skin after two hours. The average study-state fluxes (standard deviation) was calculated to be 632±47 μg/cm^2/min whereas the lag phases were calculated to be at 44.74, 13.71 and 39.50 mins. Figure 4.6b & 4.6c shows the detection of DEET and caffeine across the porcine after diffusion studies whereas figure 4.7 demonstrates the diffusion across the skin.

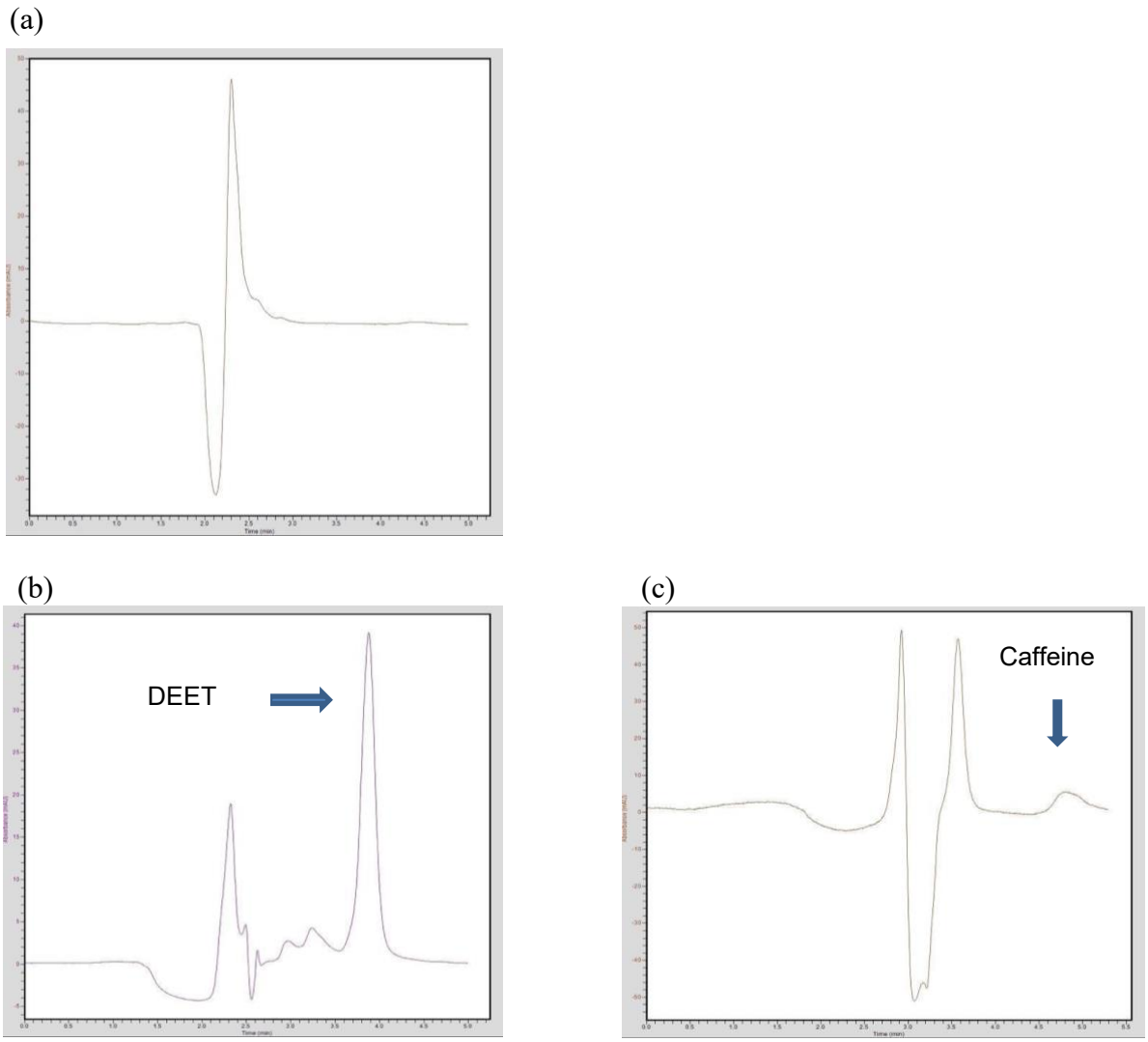


Figure 4.6: RP-HPLC Chromatogram of eluents collected during the *ex vivo* studies across porcine skin for control (PBS) (a); DEET in PBS saline 7.4 eluting at a retention time of 3.5 min (b): and caffeine in PBS saline eluting at 4.6 min(c).

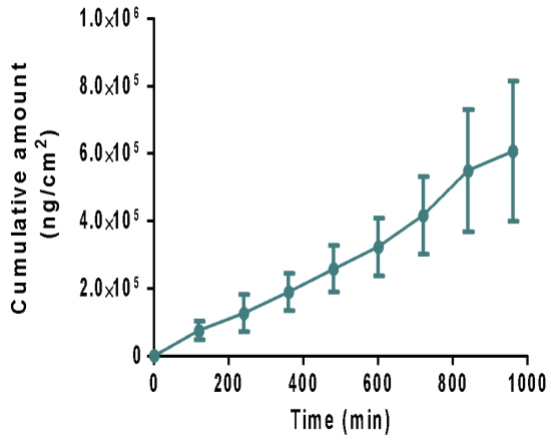


Figure 4.7: *Ex vivo* diffusion of DEET across porcine skin demonstrating cumulative amount vs time graph (n=3), error bars=Standard Error of Mean.

4.6. Skin accumulation studies

The percentage of initial active compounds in the cream formulation, which was extracted from the skin after the *ex vivo* studies varied greatly between the EOCs (Figure 4.8) *(R)-(+)* limonene exhibited the highest % skin retention at 16% with DEET being a close second at 13% and Linalyl acetate at 4.5%. However, (-)-alpha pinene, (+)-alpha pinene,(-)-beta pinene and *cis*-nerolidol displayed very low % accumulation ranging from 0.03-0.2%

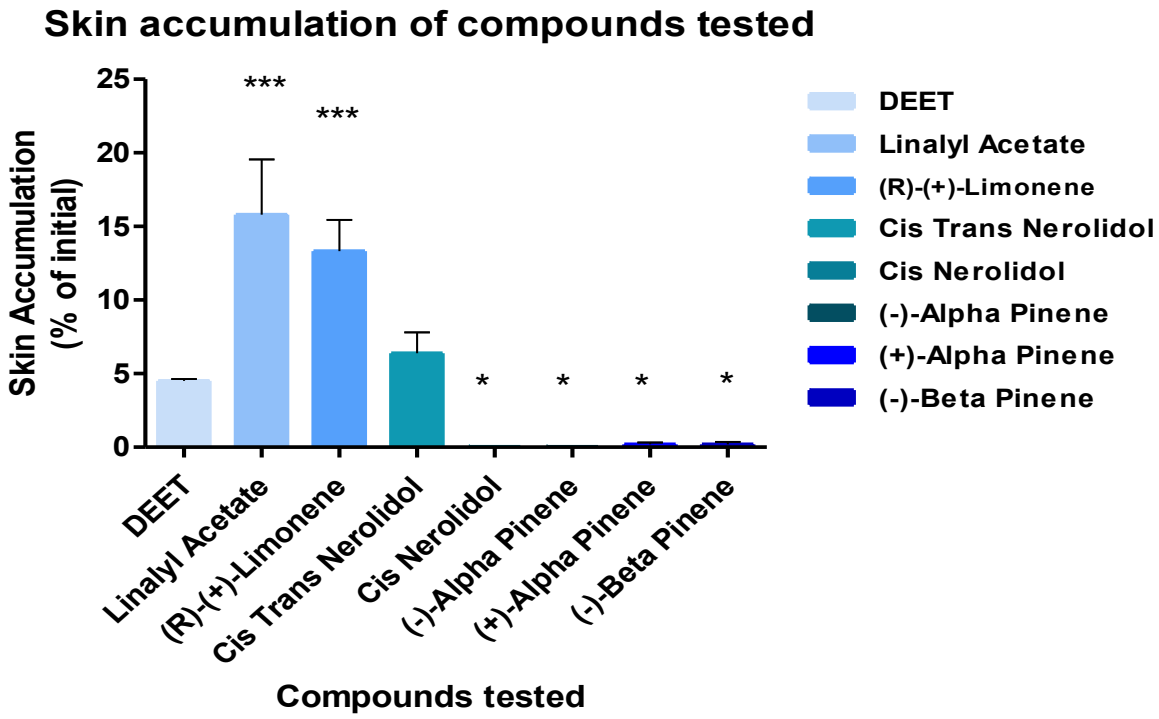


Figure 4.8 : Bar graph illustrating the % skin accumulation of terpenes, terpenoids and DEET within the skin after 16 hours (n=3); ***: Statistically significant $p < 0.0001$; *: $p < 0.05$ compared to DEET.

4.7. Skin integrity (TEER measurements)

A preliminary study was conducted to determine the effect terpenes and terpenoids have on the skin integrity over a period of 24 hours (Figure 4.9).

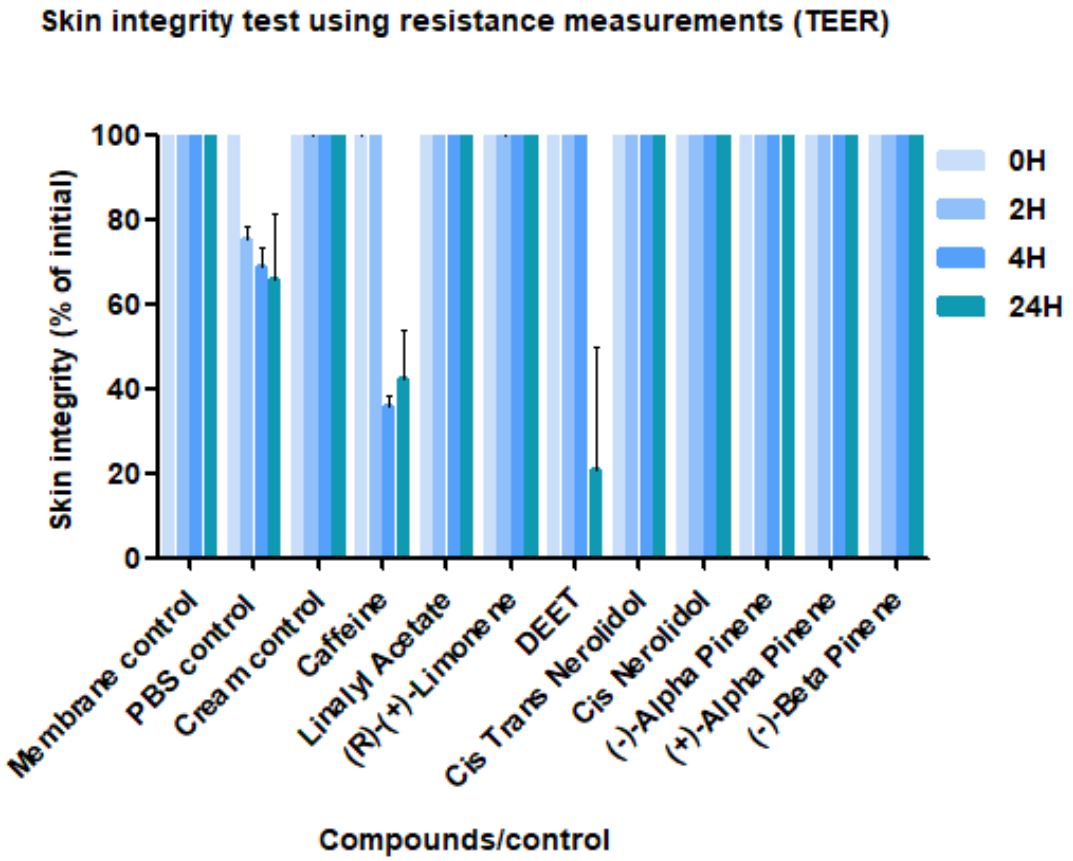


Figure 4.9 : *Bar graph demonstrating the skin integrity (TEER) after 24 hours of skin exposure to terpenes and terpenoids in a cream formulation. The three controls; caffeine, PBS and membrane only are also included (n=3); **: statistically significant p< 0.001 compared to skin integrity at* T_0.

As observed from Figure 4.9 linalyl Acetate, cis trans-nerolidol, cis-nerolidol, (-)-alpha pinene, (+)-alpha pinene, and the cream control exhibit very little, if any alteration in the skin barrier. In contrast, (R)-(+)-limonene exhibits negligible variations, with values slightly decreasing (99.75% to 100%). After 24 hours exposure to DEET, the barrier function of the skin was compromised (20.90% ± 28.91% of initial, p<0.001). Caffeine exposure displays a progressive drop in skin barrier function over the 24-hour time period, with resistance values reaching 42.11 ± 11.83% of initial, p<0.001. Skin exposed to PBS (control) also indicated a progressive loss in barrier function, reaching approximately 65.69± 15.55% of initial (p<0.001) after 24 hours of exposure.

4.8. Repellence Bioassay

Table 4.5 shows the mean percentage repellence (±SD) of various compounds at three different concentrations (1, 5, and 10 mg/ml) across four human volunteers (n=3). The table highlights how each compound performs at three concentrations (low , medium and high), with varying levels of efficacy and consistency. No statistically significant differences in compound repellency was observed when compared to DEET at each concentration level tested, except at 1 mg/ml where Linalyl Acetate and (*R*)-(+)-Limonene indicated negative repellency, $p < 0.001$).

*Table 4.5: Repellence Mean percentage repellence (±SD) of standards at different concentrations (**n=3**).*

		Concentration (mg/ml)		
Compound	**Volunteers (n=3)**	**1**	**5**	**10**
		Mean percentage repellence (%)		
DEET	V1 V2 V3 V4 Mean **±SD**	63.10 21.88 69.44 59.13 53.39±18.56	100 100 100 100 100±0	100 100 100 100 100±0
Linalyl Acetate	V1 V2 V3 V4 Mean **±SD**	-174 -64 26 -0.64 -53.16 ± 77	100 100 94 93.74 98±2.83	100 100 100 100 100±0
(R)- (+)-Limonene	V1 V2 V3 V4 Mean **±SD**	-742.86 -400 -900 -8.33 -512,80±396	85.71 -154.29 -200 100 -42±1.36	100 100 100 83 95.75±7.36
Cis Trans-nerolidol	V1 V2 V3 V4 Mean **±SD**	40 13.75 44.44 11.11 27.33±15.01	85.71 80.00 75.00 -3.33 59±36.39	95.24 93.33 88.9 72.22 87.42±0.09
Cis-nerolidol	V1 V2 V3 V4 Mean **±SD**	100 45.22 -43.22 50.00 38±49.07	100 96.67 -62.22 88.89 56±0.68	100 100 88.89 100 97.22±0.04
(-)-Alpha pinene	V1 V2 V3 V4 Mean **±SD**	66.67 66.67 100 66.67 75±14.43	100 79.49 100 61 85±0.16	100 97.22 100 100 99.31±1.20
(+)-Alpha pinene	V1 V2 V3 V4 Mean **±SD**	100 26.67 69.44 33.33 57.36±30	100 60 100 75 84±0.17	100 100 100 100 100±0
(-)-Beta pinene	V1 V2 V3 V4 Mean **±SD**	77.78 33.33 100.00 46.75 64±0.26	100 34.13 100.00 89.68 81±0.27	100 100 100 100 100±0

CHAPTER FIVE : DISCUSSION

5. HPLC

5.1.1. Method development

Reverse-phase high-performance liquid chromatography (RP-HPLC) is well-suited for analysing lipophilic (hydrophobic) compounds (García-Alvarez-Coque *et al.*, 2016). In RP-HPLC, the stationary phase is nonpolar, typically consisting of alkyl chains bonded to silica particles, whereas *the* mobile phase is polar. The mobile phase is usually a water-organic solvent mixture (García-Alvarez-Coque *et al.,* 2016). This interaction between the stationary phase and mobile phase favours the retention of hydrophobic compounds, which interact strongly with the nonpolar stationary phase, leading to their reduced retention times(Lui *et al.,* 2020).

Linalyl Acetate (Log P value=4.14,molecular weight=196.29 g/mol) and *(R)*-(+)-limonene (Log P value=4.57, molecular weight = 136.23) are both monoterpenoids and terpenes, they form part of the essential oil ingredients in liquid form. The available methods found in literature were deemed to be unsuitable when tested, therefore a unique in-house method was developed. Since these natural compounds are immiscible in a variety of solvents due to their lipophilic nature, they require a particular solvent to dissolve lipophilic compounds (Parafinova-Nemska *et al.*, 2016). Methanol is preferred as a solvent in HPLC due to its ability to interact with silanol groups, consequently mitigating silanophillic effects (Metzger, 2022).

Furthermore, methanol's characteristic as a protic solvent effectively reduces these interactions during the chromatographic processes (Soares *et al.,* 2022). The stationary phase in reverse-phase liquid chromatography (RPLC) is hydrophobic (non-polar), conversely the mobile phase is hydrophilic (polar). Upon introduction of a standard mixture composed of hydrophilic and hydrophobic compounds, hydrophobic molecules adsorb onto the hydrophobic stationary phase. This results in the elution of hydrophilic molecules first. In order to facilitate the elution of analytes, particularly those with higher hydrophobicity, the polarity of the mobile phase can be reduced by increasing the proportion of an organic solvent such as acetonitrile. This adjustment decreases the hydrophobic interactions between the stationary phase and solutes, promoting their elution. In summary, the strategic use of methanol as an organic modifier in HPLC helps to optimize chromatographic separations by minimizing undesirable

silanophilic effects, while modulation of the mobile phase composition enhances the separation of analytes based on their hydrophobic characteristics (Boone and Adamec, 2016). The initial method for *(R)-(+)*-limonene and linalyl acetate resulted in peak tailing, therefore 0.1% (v/v) of formic acid was introduced. Formic acid serves as a crucial pH regulator with a pKa around 3.75 and acts as a buffer component in aqueous mobile phases, typically in combinations such as formic acid-sodium formate or formic acid-ammonium formate solutions. This addition is essential for optimizing the separation of compounds with varying acid-base properties. Interestingly, even at very low concentrations, formic acid has been observed to improve the morphology of chromatographic peaks, underscoring its multifaceted role in enhancing separation quality (Nunez and Lucci, 2014). To address these types of challenges in reversed-phase liquid chromatography (tailing effects), formic acid is frequently added to mobile phases. Symmetrical peaks were subsequently obtained for both (R)-(+)-limonene and linalyl acetate.

Furthermore, a method for *cis*-nerolidol and *cis-trans*-nerolidol, was adapted from SciELO technologies, however this method was altered to accommodate faster elution of cis-nerolidol. The original method for cis-nerolidol was altered from a mobile phase mixture consisting of 80:20 (v/v) acetonitrile and Milli-Q® water, to 95:5 (v/v) acetonitrile and Milli-Q® water. Moreover, two peaks eluted for cis-trans-nerolidol, due to it being an isomer mixture. According to a study by Trindade *et al*. (2020) the first peak to elute is cis-nerolidol, followed by trans-nerolidol. Peaks obtained during *the* cis-trans nerolidol HPLC analysis did not separate completely, this incomplete separation is mainly caused by the various interactions (the electrostatic, hydrophobic interactions, hydrogen bond interactions, dipole-dipole interactions and steric hindrance between the analyte and the stationary phase) (Seilditz, 1996). The molecule of an enantiomer pair which forms the most stable complex with the stationary phase is generally the most retained. Furthermore, in addition to the previously mentioned interactions, the bulkiness of substituents on enantiomer pairs may influence chiral separations, as the bulkiness is closely linked to the resolution of enantiomers (Seilditz, 1996).

5.1.2. Method Validation

The developed HPLC method demonstrated strong specificity, precision and accuracy in resolving the target compounds, as evidenced by the sharp and distinct peaks observed for each analyte. The regression analysis presented in Table 4.1 highlights excellent linearity across the defined concentration ranges, with $R^2 > 0.999$, meeting the acceptance criteria (Table 3.1). This indicates that the method is reliable for quantifying each compound within

the specified range. Analytes such as linalyl acetate, *(R)-(+)*-Limonene, and DEET showed minimal deviation at lower concentrations, further underscoring the method's precision. The calculated limits of detection (LOD) and quantification (LOQ) were well within acceptable thresholds, allowing for efficient detection of even trace amounts of the compounds. (-)-beta pinene exhibited an LOD of 21.33 µg/mL and an LOQ of 64.63 µg/mL, showcasing the method's sensitivity.

The accuracy study confirmed that the method produced recoveries largely within the acceptable range of 85–115% (ICH, 2006), as shown in Table 4.2. Most analytes, such as DEET and (R)-(+)-limonene, demonstrated excellent recovery rates across the tested concentrations, with %RSD values below 2%, highlighting robust reproducibility. However, (+)-alpha pinene displayed a recovery rate of 118% at 300 µg/mL, slightly exceeding the acceptable limit. This anomaly might suggest a matrix effect or experimental variability, requiring further investigation.

Precision analysis (Table 4.3) reinforced the method's reliability, with intra-day and inter-day %RSD values consistently below 2%. DEET exhibited negligible variation across both precision parameters, confirming its consistent performance. Similarly, other compounds such as (-)-beta-Pinene and linalyl acetate also demonstrated strong reproducibility, indicating robust analytical performance.

The robustness evaluation revealed that slight modifications to chromatographic conditions (e.g., temperature, flow rate, and mobile phase composition) generally had minimal impact on the retention times and peak areas, with %RSD values below the acceptable threshold of 2% for most analytes (Table 4.3). However, linalyl acetate and (-)-beta pinene were notably affected by changes in flow rate, as demonstrated by increased %RSD for both retention time and peak area. This sensitivity suggests the need for precise control of flow rate during routine analysis to maintain optimal performance. DEET also displayed a %RSD of 2.8% under altered conditions, indicating slight sensitivity to temperature and flow rate changes (Table 4.3).

Overall, the developed HPLC method exhibited high specificity, accuracy, precision, and robustness, making it suitable for the routine analysis of terpenes, terpenoids, and control compounds. The results also indicate that the method is versatile, capable of quantifying a diverse range of analytes effectively. While certain challenges, such as matrix effects and sensitivity to flow rate variations, were identified, these are manageable through method optimization and proper analytical controls. In conclusion, the method represents a significant

improvement in terms of detection efficiency, reduced retention times, and applicability to multiple compounds, offering a valuable tool for comprehensive compound analysis.

5.1.3. *Ex vivo* permeation

The *ex vivo* study involves the passage of drugs through various biological membranes in an effort to assess drug delivery systems (Wisdom Library, 2024). A relationship exists between lipophilicity and the skin permeation, active compounds with a log P value ≈ 2 suggest efficient permeation through the stratum corneum (Bartsova and Bajgar, 2012). Active compounds with high lipophilicity (Log P > 3) or high hydrophilicity (Log P < 0) demonstrate low passage through the stratum corneum due to their insolubility within the lipid components of the stratum corneum (Hydrophilic compounds) or the accumulation within the lipid components of the stratum corneum (highly lipophilic compounds) (Longgos *et al*., 2024). DEET with a log P value ≈ 2 and a molecular weight of 191.27 g/mol showed permeation through the skin (Colas-Ruiz *et al*., 2023), as was also demonstrated in this study were DEET showed the highest rate of permeation across the skin (Figure 4.7).

The permeation of DEET directly affects its efficacy as an insect repellent due to its ability to form a vapour barrier on the skin which acts as a repellent against mosquitoes (Mapossa *et al*., 2020). The terpenoids permeated through the SC and were detected at their respective retention times (linalyl acetate: 7.51 mins; (R)-(+)-Limonene: 3.44 mins), however detection of the peaks were below the LODs of 146.96µg/ml and 21.04µg/ml for Linalyl acetate and (*R*)-(+)-Limonene, respectively. Both Linalyl acetate and (*R*)-(+)-Limonene have log P values > 4 (4.14 and 4.57) (Table 2.1). It is the lipophilicity (i.e. Log P) of the active component that dictates its passage across the SC (Bartosova and Bajgar, 2012; Bolla *et al,* 2020). (*R*)-(+)-limonene showed the highest permeation rate of all the tested natural compounds with (-)-alpha pinene showing the least *ex vivo* permeation across the skin. These findings were expected with studies indicating that terpenes such as limonene disrupting the lipid membranes thus promoting skin permeation (Yu *et al*., 2024). This is further supported in a study conducted by Hori *et al.* (1991) which reported that limonene enhanced penetration of nicardipine HCL , thus acting as a permeation enhancer across skin.

The non-passage of the active compound is also caused by low aqueous solubility in the viable epidermis layer, the favourable log P value should be between 1 and 3 (Longgos *et al*., 2024). Molecular weight also affects the permeation of the active compound across the skin (Bartosova and Bajgar, *2012*). A molecular weight less than 500 g/mol accommodates passage through the stratum corneum (Fitzpatrick *et al.,* 2017; Lundborg *et al*., 2018).

Previous studies investigated *alpha*-pinene (mixed isomer) where it was applied to rats at 2000mg/kg body weight, provided strong evidence of low acute toxicity and zero systemic effects; where the rat models demonstrated slight reactions such as local erythema and dryness that were reversed on day 13 (OECD TG 402, 2017; Australian Industrial Chemical Introduction Scheme, 2018). Similarly, the application of 33% linalyl acetate on the back of human volunteers for a period of 48 hours resulted in zero signs of irritation (OECD TG 402, 2017). Whilst, limonene has been classified as a dermal irritant at high concentrations; but demonstrated low acute toxicity and is considered safe under the US Food and Drug Association (US Environmental Protection Agency, 1994; Kim *et al.,* 2013). The aforementioned factors make terpenoids a viable option due to their strong repellency combined with a safety profile backed by literature making terpenoids a promising mosquito repellent.

5.1.3. Skin Retention studies

The quantity of the active compound that accumulated within the skin was assessed at the termination of the *ex vivo* permeation experiments. This was done to evaluate the percentage of initial compound that was applied to the skin that remained within the skin (Elonga *et al*, 2023). The cream formulation should result in retainment of the lipophilic compounds within the upper skin layers (e.g. the SC) where the insect repellent will be effective, preventing skin permeation into the deeper skin layers and into the systemic circulation by forming a thin layer covering the skin (Pinto et at., 2017).

The skin retention studies evaluating *(-)*-alpha pinene, *(+)-alpha*- pinene and *(-)-beta*- pinene, revealed a low % skin retention (0.04-0.2% of initial compound applied to the skin) (figure 4.7). These results were similar to those found by Cal and co-workers, where the absorption of various terpenes within the human skin layers was investigated. Results showed a negligible retention of the pinenes (*alpha* and *beta* isomers) (Cal, 2006). This phenomena was attributed to the vast presence of polar groups in pinenes where the polar groups have a tendency of displaying increased affinity towards the polar hydrophilic dermis and polar region of the SC (Cal, 2006). Moreover, the skin retention of *cis*-nerolidol was 0.03% of initial loaded, contrary to the retention of *cis-trans*-nerolidol, which was significantly higher at 6.39% of initial loaded. This difference may be attributed to the molecular weight of the compounds, where *cis*-nerolidol has a molecular weight of 222.37 g/mol compared to *cis-trans*-nerolidol's molecular weight of 444.37 g/mol (Table 2.1). According to Funke *et al.* (2002) a higher molecular weight of a compound is a hindrance during absorption into the skin, compounds with a higher molecular weight tend to accumulate in the top layers of the skin. Furthermore, *(-)-(R)-*

limonene exhibited the highest skin retention percentage at 15.81% and linalyl acetate followed at 13.33% (Funke *et al*., 2002).

5.4. Skin Integrity (TEER values)

Topical insect repellents directly interact with the skin, therefore is it vital to assess the dermal absorption of the active ingredients and estimate their systemic exposure employing *ex vivo* methods (Schafer and Redelmeier, 1996). The skin's outer layer, the SC, consists primarily of dead cells (corneocytes) packed with keratin, embedded within the a lipid matrix, these lipids are referred to as ceramides (Cui *et al.,* 2011). Ceramides are tightly organised within the lipid bilayer, the ceramides acting as the protective barrier by combining the various lipids held together by hydrogen bonds (Cui *et al.,* 2011).

For this study, skin resistance measurements (TEER) was employed to evaluate the effect of various terpenes on the skin barrier function. During *ex vivo* skin permeation studies, increased TEER values (skin resistance measurements) signal a more intact and less permeable skin barrier (Srinivasan *et al.*, 2016). A cold cream formulation was utilised as vehicle, the formulation contained olive oil, a known penetration enhancer, that naturally disrupts the skin barrier function through the disruption of lipids (Haque and Talukder, 2018). The results obtained from the study revealed that the skin barrier function remained consistently at 100% over a 24-hour time period (Figure 4.5).

Furthermore, linalyl acetate, *cis*-trans-nerolidol, *cis*-nerolidol, *(-)-alpha*-Pinene, *(+)-alpha*-pinene, *(-)-beta-pinene* and the cream control (without active compounds) demonstrated negligible changes in skin resistance measurements, thus integrity remaining near 100% throughout the 24-hour period. This indicates that terpenes caused very little, if any change in skin barrier function. However, *(R)-(+)*-limonene showed a minimal decline in skin integrity, decreasing to 99.75% after 6 hours, indicating a slight decline in skin barrier function. Similar terpenes such as d-Limonene ($C_{10}H_{16}$) are known to disrupt the stratum corneum bilayer lipids and/or the keratin domains and tight junctions (Sapra *et al*., 2008), this disruption indicates its potential use as an enhancer (Varman and Singh, 2012).

After 24 hours, there was a drastic drop in skin integrity from 100 to 20.90% after the application of the active ingredient DEET, thus suggesting the decline in skin barrier function. According to literature, DEET is classified as a solvent (Dobson, 2020) that has the ability to melt polymers found in plastics (Guda *et al.,* 2015). Furthermore, caffeine skin integrity remained consistent at 100%, however after 24 hours it dropped to 48,94%, which is consistent with results obtained from a study by Elonga (2023), whereby the % TEER initial

dropped to 50% after 24 hours, exhibiting a decline in skin barrier function. The positive control, PBS without the cream formulation, demonstrated a decline to approximately 60% in skin integrity after the 24-hour period. This drop indicated some loss or degradation of the skin integrity over time.

A decrease in the TEER values indicates a disruption of the skin barrier, TEER is utilised to assess skin integrity and barrier function in response to test materials (Wiese-Rischke *et al*., 2021). In the study, low skin retention was observed, thus indicating the unlikeliness of the test compounds to accumulate within the skin layers, however suggesting that most of the compounds remain on the skin surface. In the context of topical insect repellents, low skin retention suggests a shorter duration of action, therefore necessitating a more frequent reapplication to maintain optimal efficacy. The lower skin retention also implies lower risks of systemic interactions, thus lowering toxicity and making the formulations safer for commercial use.

5.5. Repellency Assay

The increased interest in repellent-treated materials and their testing for efficacy is fuelled by the on-going threat of mosquito-borne diseases (Morimoto *et al.*,2021). At present, various studies opt for the use of simulated laboratory studies (Morimoto *et al.,* 2021). One example is the attractive blood-feeding device (ABFD) that eliminates the element of human volunteers or animal baits to test for repellence (Morimoto *et al.,* 2021). Such studies, however, are unreliable because the WHO recommended a repellent testing procedure that involves the use of adult human volunteers (WHO, 2009). For this study a repellency assay using adult human volunteers was used because it yields results applicable to the actual conditions of use (WHO, 2009).

DEET proved to be the gold standard with a 100% repellence percentage at medium (5 mg/ml) and high (10 mg/ml) concentrations. At 1 mg/ml however, linalyl acetate's mean repellency percentage of -53.16% demonstrated a low repellence and high variability with a SD of 77. Linalyl acetate demonstrated negative repellence values at low concentrations, thus indicating an attractive effect of the terpenoid (Caballero-Gallardo *et al*.,2022). However, it is important to note that at a higher concentration of 10 mg/ml the terpenoid displayed a repellence value of 100% with a mean SD of 0, demonstrating a high repellence with low variability. Furthermore, according to Cosimi *et al.* (2009), Citrus bergamia Risso and Lavandula hybrid essential oils (EOs) rich in linalool and linalyl acetate demonstrated significant repellent activity against *Sitophilus zeamais*. The repellent activity of linalyl acetate was also established for *Rhodnius prolixus* (Sfara *et al.,* 2009). Previous studies investigating the repellence of linalyl

acetate failed to assess the repellence of the monoterpenoid against malaria vectors (Sfara *et al.,* 2009 ;Sayada *et al.,* 2022; Wang *et al.,* 2024).

(R)-(+)-Limonene displayed a mean response of -512.80 ± 396% at a concentration of 1 mg/ml, signalling extreme variability at lower concentrations. Moreover, the volunteer response ranged from -900 to -8.33% at 5 mg/ml, suggesting an inconsistent effect at lower concentrations. At lower concentrations, the repellence percentage was reduced and showed significant variability, suggesting that the essential oil may not offer uniform protection across volunteers at these concentrations. Notably, at higher concentrations (10 mg/ml) the terpene demonstrated a mean response of 95±7.36%. In contrast, evidence from a study conducted by Li *et al.* (2024) that investigated the repellent properties of *(R)-(+)* limonene within a 24h period were between 17.32-12.87% ,thus exhibited attractant effects. Limonene has been poorly investigated as an arthropod repellent, however it is well studied as an insecticide (Mursiti *et al.*, 2019:Feng *et al.*, 2020;Gupta 2025).

The terpene (-)-alpha-pinene exhibited a mean response of 75±14.43% with moderate consistency at 1 mg/ml, conversely, *(+)-alpha*-Pinene showed higher variability with a mean response of 57.36±30 at the same concentration. Furthermore, at 10 mg/ml (-)-alpha pinene's repellency was 99.31±1.20 yet (+)-alpha pinene's repellency was 100±0. An observation made during the study was that the repellence of *(+)-alpha*-pinene was higher than that of *(-)-alpha*-pinene, interestingly, this was also observed in a study by Ndiranga *et al*. (2020). Ndirangu and co-workers investigated the repellent properties of both (-)- and *(+)-alpha*-pinene pure analytical grade standards against adult (5-7 days old) female *Anopheles gambiae* mosquitoes (Ndirangu *et al.,* 2020). The study was conducted using the WHO protocol with human volunteers, and indicated that *(+)*- demonstrated better repellency percentage at 99.38%, whereas (-)-alpha pinene's repellency was 59.12%. Furthermore, a study by Haselton *et al*.(2015) utilizing a contact repellency assay, found that (+)-alpha pinene presented a significantly higher repellence score than (-)-alpha pinene, following the same trend as the results observed in Table 4.3.

The findings of a study by Ramiharimanana *et al.* (2022) concluded that chirality greatly influences molecule repellent properties against *Ae. albopictus*, thus highlighting the influence of chirality on insect repellents. Moreover, Klun *et al.* (2001) assessed the influence of chirality on insect repellents, where the study demonstrated that specific stereoisomers exhibit enhanced efficacy due to their differential interactions with mosquito chemoreceptors. Notably, the (2′S) configuration was more effective in modulating repellent activity, with the (1S,2′S) isomer exhibiting a 2.5-fold increase in efficacy compared to the racemate (Klun *et al.,* 2001).

Extrapolating these findings to (-)-alpha-pinene and (+)-alpha-pinene, it is plausible that their repellence differs as a function of their stereochemical interactions with olfactory receptors. Contrary to Ndirangu et al. 2020 , in the study 10mg/ml of (-)-alpha pinene demonstrated the same efficacy as (+)-alpha pinene. The chirality of terpenoid remains unaffected by concentration. It is the olfactory receptors which are over saturated when exposed to higher dosages thus masking the stereochemical differences between the two enantiomers causing the mosquitoes to react similarly to both. At lower concentrations, only the most sensitive enantiomer specific receptors are activated, allowing the mosquitoes to differentiate between between (+)-alpha pinene and (-)-alpha pinene. The concentrations utilised in a study by Ndirangu *et al.* 2020 were comparatively lower than that of the ones used in the present study. In conclusion similarity at higher doses is a result of receptor saturation not chirality (Sims *et al.,* 2022 ; Hallem and Carlson, 2006). The compound *(-)-b*eta-pinene conveyed the most consistent results with low SD results, offering a stronger protection across human volunteers across all three concentrations. At 1 mg/ml (-)-beta pinene expressed a repellence percentage of 64 ± 0.26, 81± 0.27 at 5mg/ml and 100 ± 0 at 10mg/ml.

The results highlight the differential repellent efficacy of *cis*-nerolidol and cis-trans-nerolidol across various experimental conditions. The repellence of cis-nerolidol shows significant variability, as indicated by the high standard deviation (Mean ± SD: 38 ± 49.07% , 56 ± 0.68%, and 97.22 ± 0.04%). Notably, the first and third data sets exhibit extreme variability, with negative values observed in V3 (-43.22 and -62.22). This suggests that under certain conditions, cis-nerolidol may act as an attractant rather than a repellent (Caballero-Gallardo *et al.,* 2022), an effect potentially linked to enantiomeric interactions with olfactory receptors or volatility changes that influence mosquito behaviour towards the active ingredient (Khemis *et al.,* 2022). Conversely, the final dataset demonstrates a consistent and robust repellent effect (97.22 ± 0.04), suggesting that specific conditions - such as increased concentration—may significantly increase its efficacy.

In contrast, cis-trans-nerolidol exhibits a more stable repellent profile with lower variability (Mean ± SD: 27.33 ± 15.01, 59 ± 36.39, and 87.42 ± 0.09). Although some degree of fluctuation is observed, particularly in the second dataset (V4: -3.33), the overall repellency remains positive, with the highest repellence recorded in the final dataset (87.42 ± 0.09). The consistent repellence observed in cis-trans-nerolidol, particularly in the last dataset, suggests a more predictable mode of action that may make it a superior candidate for vector control strategies. The striking differences in repellence between the two nerolidol isomers indicate that structural conformation plays a pivotal role in receptor binding and olfactory signalling in

mosquitoes (Xiong *et al.,*2025). The observed negative values in specific trials could be attributed to enantioselective interactions with olfactory receptors, where one enantiomer exhibits a repellent effect while the other induces attraction (Dekel *et al.,* 2022). This is exhibited in previous studies where 1-octen-3-ol can have opposing effects on mosquito behaviour, with one enantiomer acting as an attractant and the other as a repellent, demonstrating enantioselectivity in olfactory receptor interactions (Xu *et al,* 2015).

Moreover, differences in volatility, oxidation susceptibility, or metabolic degradation could further modulate these effects, resulting in context-dependent repellence (Xiong *et al.,*2025). A study by Portilla-Pullido *et al.* (2020) evaluated the repellent abilities of nerolidol against female *Aedes aegypti* mosquitoes during an *in vivo* repellent activity bioassay whereby the bites of mosquitoes were observed at various exposure times. The results showed nerolidol at 0.1%, 10% and 25% yielded a total protection percentage of 58%, 100% and 100% (Portilla-Pullido *et al.,* 2020). In the present study, similar results were obtained for nerolidol, whereby *cis*- nerolidol and *cis-trans* nerolidol demonstrated a repellency of 87.42% and 97.22% at a concentration of 10% thus making the results consistent with the findings in the study of Portill-Pullido *et al*. (2020). The repellent capabilities of nerolidol were further highlighted in a study conducted by Haris *et al.* (2022), where the application of 330 µg/cm^2 *cis-trans* nerolidol against female *Aedes aegypti* mosquitoes under laboratory conditions resulted in complete inhibition of mosquito landings for 60 mins.

CHAPTER SIX:
CONCLUSION,RECOMMENDATIONS,LIMITATIONS

6.1. Conclusion

In evaluating any new potential therapy the pharmacokinetics need to be understood for the compound - as such HPLC protocols are required to determine drug concentrations. The HPLC method developed and validated in this study proved to be effective and reliable for the analysis of terpenoids and controls. The protocols exhibited excellent specificity, accuracy, and reproducibility with recoveries and %RSD values largely within the ICH acceptable limits. While minor issues such as flow rate sensitivity and stability were noted, the method's robustness and adaptability demonstrated consistent performance, establishing it as a dependable tool for diverse analytical purposes.

The study demonstrates that lipophilicity (log P) and molecular weight are critical factors influencing skin permeation and skin retention. Formulation of the cream also plays a vital role, this is caused by the presence of penetration enhancers such as olive oil present in the cream formulation. Compounds with log P values around 2, with a molecular weight of 191.274 g/mol such as DEET, permeated through the stratum corneum effectively, while highly lipophilic compounds (log P >4), such as linalyl acetate and *(+)-(R)*-limonene, demonstrated poor permeation. Despite their detection at low levels, these compounds did not permeate effectively, due to their poor solubility in the stratum corneum lipids and the unfavourable log P range for skin absorption. Moreover, lipophilic compounds tend to accumulate in the stratum corneum lipids between the corneocytes, forming a reservoir effect (Tabosa, 2019).

Additionally, compounds with lower molecular weights (<500 g/mol) more readily permeated the stratum corneum, aligning with established guidelines (Colas-Ruiz *et al.*, 2023). However, skin retention was highly variable, with *(+)-(R)*-limonene showing the highest retention (15.81%), suggesting that both moderate lipophilicity and molecular weight contribute to effective retention in the stratum corneum as well as the density of the cream formulation. Overall, these findings emphasise the importance of optimising both lipophilicity and molecular weight for effective topical formulations, particularly in insect repellent creams, to ensure they remain in the upper skin layers without systemic absorption to minimise adverse effects.

Furthermore, molecular weight also played a critical role, with compounds under 500 g/mol that are expected to permeate effectively, such as terpenoids (136-222 g/mol), not permeating

as expected. These findings emphasise the importance of optimising lipophilicity and molecular weight to enhance skin permeation and achieve effective drug delivery.

When the repellency efficacy was tested it was found that there was a concentration dependent effect with some inconsistency. The variation is caused by the individual differences within a person's body chemistry and how each person's body chemistry interacts with the various compounds. The results from the study showed vast repellence across the various terpenoids, thus indicating inconsistency across the different test concentrations. The majority of the terpenes and terpenoids showed variable repellence at 1 mg/ml. However, at higher concentrations, these compounds exhibited stronger repellent activity, although their effectiveness was often inconsistent, as shown by the high standard deviations in their repellency scores. In contrast, (-)-*beta*-pinene demonstrated a more reliable and consistent repellent effect across all tested concentrations, from 1 mg/ml to 10 mg/ml, with low variability in its repellence responses. This suggests that (-)-*beta*-pinene holds potential as a more effective and consistent repellent compared to other terpenoids, making it a promising candidate for use in vector control strategies.

6.2. Limitations

In this study, the main obstacles encountered were operational in nature. A key limitation was the purity of the compounds, as the procurement of 100% pure terpenoids is rarely available. Variations in terpenoid composition can influence both the physicochemical characteristics and biological activity, thus limiting the experimental consistency. Furthermore, the high density and viscous nature of the oils presented significant challenges during sample preparation and chromatographic analysis. The dense oils frequently caused column blockages, leading to interruptions in analysis and necessitating repeated maintenance and recalibration.

Furthermore, ethical restrictions also limited the use of more direct repellency assessment methods. The "arm-in-cage" technique recommended by the WHO, which involves applying a topical repellent to a human volunteer's forearm and exposing the treated skin to mosquitoes while recording the number of bites or landings, could not be conducted due to the absence of human ethics approval. Consequently, repellency testing had to be performed using an alternative setup, which may have influenced the behavioural outcomes. Lastly, owing to time constraints, the study was therefore limited to testing a single formulation rather than evaluating the permeability of multiple formulations across porcine skin.

6.3. Recommendations and future perspectives

For future studies, the procurement of HaCaT cells is recommended to enable cytotoxicity testing and provide deeper insight into the overall safety profile of the essential oils. Further investigations should also explore different formulation types - such as creams, gels, and liquid preparations - to evaluate how formulation affects skin permeability and efficacy. Given the promising results observed with (-)-β-pinene, it would also be valuable to conduct direct application studies on human skin, rather than using a net-based setup. Such an approach would allow for a more accurate assessment of repellent activity by directly quantifying mosquito landings and bites.

Future studies should look into exploring (-)-β-pinene in various formulations, as well as combination studies with the highest performing terpenoids such as linalyl acetate and (-)-β-pinene, the study should investigate whether the terpenoids have a synergist or additive effect.

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Appendix A.1 : Animal ethics waiver for the use of *anopheles* mosquitoes

UNIVERSITY OF THE WITWATERSRAND JOHANNESBURG

ANIMAL RESEARCH ETHICS COMMITTEE
Registration number: AREC-101210-002

Date:17/02/2023

Certificate reference: Waiver 17-02-2023-O
Category: O
Applicant: Unathi Chiliza
Department: Department of Pharmacy and Pharmacology (WITS)
Tel: 011 717 2552; **Email**: **1657318@students.wits.ac.za**

Re: Waiver from the Animal Ethics Research Committee of the University of the Witwatersrand

This letter is to confirm that Unathi Chiliza, MSc student (#1657318) under the supervision of Prof Van Zyl (WITS Department of Pharmacy and Pharmacology), does not require full Animal Ethics Research Committee clearance to undertake the work titled **'Transdermal permeability of terpenoids as an insect repellent'**.

Reason for waiver
The project is using invertebrates: mosquitoes (*Anopheles)* and brine shrimps (*Artemia*).

Details of the study
The aim of this study is to assess *in vivo* repellent efficacy of terpeniods. The compounds will be tested on the various stages of the Anopheles and Artemia under standard internationally recognized protocols and the WHO guidelines. A preliminary toxicological profile of terpenoids on brine shrimps will be undertaken to ensure safety in the aquatic environment. The brine shrimps will be hatched from commercially available eggs, maintained for 72 hours in a designated access-restricted laboratory and euthanised according to international standard practices. The *in vivo* experiments will determine efficacy of the terpenoids as mosquito repellents. The mosquitoes will be maintained under strict conditions in the WRIM Entomology unit and the virgin-uninfected vector will be obtained on the day of the assay. Females will be only fed sugar or commercially available dog food for egg formation. The assay will be undertaken in a designated access-restricted laboratory and will be euthanized according to WHO guidelines

The individuals covered by the waiver are Unathi Chiliza and Prof Van Zyl (WITS).

Please contact me should you require further information.

Yours sincerely

A/Prof Frederic Michel
Chair: Animal Research Ethics Committee
University of the Witwatersrand

Appendix A.2 : Animal ethics for the use of porcine skin

ANIMAL RESEARCH ETHICS COMMITTEE
Registration number: AREC-101210-002

Date: 21/09/2022

Certificate reference: Waiver 21-09-2022-O
Category: O
Applicant: Unathi Chiliza (#1657318)
Department: Department of Pharmacy and Pharmacology (WITS)
Tel: 011 717 2552; **Email**: 1657318@students.wits.ac.za

Re: Waiver from the Animal Ethics Research Committee of the University of the Witwatersrand

This letter is to confirm that Unathi Chiliza (#1657318), Master Student under the supervision of Prof Robyn Van Zyl, Dr Armorel van Eyk and Mr Thankhoe Rants'o (Department of Pharmacy and Pharmacology), does not require full Animal Ethics Research Committee clearance to undertake the work titled ***"Transdermal permeability of terpenes as insect repellents"***.

Reason for waiver
The project is using tissue collected from commercially available animals from local accredited and registered abattoirs in the Gauteng area. (McKenzie Piggery, Butchery & Abattoir, Dreamland Piggery, GOOD Rabbit meat)

Details of the study
The applicant will perform *in vitro* drug diffusion studies on pericardium of *Sus scrofa domesticus* (domestic pig, n=6 to 12). Permeability experiments will be performed on 7 tissue replicates and 6 diffusion experiments will be performed for each drug tested. Equilibration will be allowed in the donor as well the acceptor compartments of the diffusion cells. Thereafter the PBS solution of the donor cell will replaced with the specific drug in PBS solution. Solution will be pumped through the acceptor chambers at a rate of 1.5 ml/h and collected at 2-h intervals for 24 hours. The study will be performed under sink conditions. Drug concentrations will be analyzed by means of RP C18-HPLC.

The individuals covered by the waiver are Unathi Chiliza, Prof Robyn Van Zyl, Dr Armorel van Eyk and Mr Thankhoe Rants'o (Department of Pharmacy and Pharmacology, WITS).

Please contact me should you require further information.

Yours sincerely

Prof Frederic Michel
Chair: Animal Research Ethics Committee
University of the Witwatersrand

Appendix A.3: Human Ethics

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

Office of the Deputy Vice-Chancellor (Research & Innovation)

TO: Ms U Chiliza
School: Therapeutic Sciences
Department: Pharmacy and Pharmacology
Division: Pharmacology
Medical School
University
E-mail: 1657318@students.wits.ac.za

CC: Supervisor: Professor R van Zyl <Robyn.vanZyl@wits.ac.za>
and <HREC-Medical.ResearchOffice@wits.ac.za>

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

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

DATE: 01/02/2023

REF: *R14/49*

PROTOCOL NO: W-CBP-230201-01 (This is your ethics application study reference number. Please quote this reference number in all correspondence relating to this study)

PROJECT TITLE: *Transdermal permeability of terpenoids as an insect repellent*

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

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

Office of the Deputy Vice-Chancellor (Research & Innovation)

01/02/2023

Ref: W-CBP-230201-01

TO WHOM IT MAY CONCERN

Waiver: This certifies that the following research does not require clearance from the Human Research Ethics Committee (Medical)

Investigator: Ms U Chiliza
Student No. (if appropriate): 1657318
Staff No. (if appropriate):

Supervisor: Professor R van Zyl

School: Therapeutic Sciences
Department: Pharmacy and Pharmacology
Pharmacology
Medical School
University

Project title: *Transdermal permeability of terpenoids as an insect repellent*

Reason: In vitro laboratory study
No human participants will be involved in the study

CB Penny

Dr CB Penny
Co-Chairperson: Human Research Ethics Committee (Medical)

Research Office Secretariat:
Third Floor, Phillip Tobias Building, corner of St Andrews and York Roads, Parktown, Johannesburg 2193
Postal address: Private Bag 3, Wits 2050
Tel Nos: +27 (0)11 717 1234/1252/2656/2700
Office E-mail: HREC-Medical.ResearchOffice@wits.ac.za
Website:
https://www.wits.ac.za/research/researcher-support/research-ethics/ethics-committees/

Appendix A.4: Biosafety Clearance

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

Research Office

INSTITUTIONAL BIOSAFETY COMMITTEE (IBC)
(R 14/16)

CLEARANCE CERTIFICATE **PROTOCOL NUMBER: 20190404**

BRIEF DESCRIPTION OF APPLICATION:

The chemotherapeutic properties of novel synthetic and natural compounds

APPLICANT: Prof Robyn Van Zyl

SUPERVISOR: N/A

SCHOOL/DEPARTMENT: Pharmacology

DATE CONSIDERED: 24 February 2025

EXPIRY DATE: 07 March 2030

DECISION OF COMMITTEE: Approved unconditionally

1. This clearance certificate expires on 07 March 2030 and may be renewed on application
2. An annual report must be provided on the anniversary date of this certificate, for as long as the project continues
3. Notification of any proposed modifications must be submitted on the attached form
4. Unreported changes to the application may invalidate the clearance given by the IBC

DATE: 07 March 2025

CHAIRPERSON: 
(Professor J Larkin)

DECLARATION OF APPLICANT:

To be completed and emailed to mmatshepo.taunyane@wits.ac.za and HREC-Medical.ResearchOffice@wits.ac.za.

1. I have read, understood and accepted the approval conditions above
2. I agree to submit a yearly progress report to the Committee and to submit an interim report on the form provided, in the event of any significant unforeseen event, *e.g.* suspension of a drug trial, temporary closure or relocation of my laboratory, etc
3. I note that the University Safety Officer, or his/her representative, may at any reasonable time inspect my laboratory or trial site to ensure compliance with current Health and Safety legislation. I undertake to offer my full co-operation in any such inspection.
4. I have read, understood and will comply with the *recommended standard operating procedures for the handling of biohazardous materials* posted at http://web.wits.ac.za/Academic/Research/Biosafety.htm
5. I declare (delete as appropriate) that:
    a. I have all the approvals required by statute or regulation and by the funding agencies supporting this work, or
    b. that I will not begin work until such approvals are obtained

Signed: _____ **Date:** _____

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES