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The transdermal absorption of tea tree oil and potential anti-inflammatory properties

by

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ABSTRACT

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THE TRANSDERMAL ABSORPTION OF TEA TREE OIL AND POTENTIAL ANTI-INFLAMMATORY PROPERTIES by Kelly Hislop Lennie

Melaleuca alternifolia (Tea tree oil) is commonly used by the general public in the treatment of superficial dermatological conditions. There is a growing body of evidence to support its use as an anti-fungal and anti-bacterial agent. However, there is little evidence of the *in vivo* penetration of components of the oil through the skin, imperative to ensure its use is directed appropriately and safely. Furthermore the extent of TTO's ability as an anti-inflammatory agent and its potential mode of action are not known.

This thesis describes the adaptation and validation of the method *in vivo* dermal microdialysis in order to identify and quantify components of tea tree oil present at the dermal epidermal junction following the topical application of 100% TTO. *In vitro* investigations identified that the addition of hydroxypopyl-β-cyclodextrin to the perfusate, the adjustment of flow rate and the use of cuprophan membranes ensured optimal recovery of components. Furthermore tape stripping was utilized to identify components present within the stratum corneum (SC). These methods were coupled with gas chromatography-mass spectrometry and were successful in the identification and quantification of terpinen-4-ol, 115.64±28.1 (ng±SEM) and 1,8 cineole, 15.05±2.6 at the dermal epidermal junction (n=10). Also the presence of 9 hydrophilic and lipid components (overall subjects) were observed within this top layer of epidermis (n=7).

In addition the potential anti-inflammatory action of TTO and its component T-4-ol is investigated *in vitro* using the HaCaT cell line (model keratinocytes) including exploration of a potential mode of action. An inflammatory action was induced using lipopolysaccharide (LPS) and the cell supernatant analyzed using the MSD™ electrochemiluminesence assay. A statistically significant increase in the release of IL1β was observed when non-stimulated HaCaT cells were incubated with TTO (not T-4-ol alone), compared to control (medium alone). Furthermore a statistically significant increase in IL6 was observed when non-stimulated HaCaT cells were incubated with TTO and T-4-ol compared with the incubation of stimulated HaCaT cells with the oil and its component. Investigation into the effect of TTO and T-4-ol on the transcription factor NFκB demonstrated that the oil and its component did not exert its effect by initiation of this pathway.

The findings of this research have implications for clinical practice, particularly in the use of TTO on areas of dermatological inflammation and its use on 'healthy' skin.

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Academic Thesis: Declaration Of Authorship

I, …….. [Kelly Hislop Lennie]

declare that this thesis and the work presented in it are my own and has been generated by me as the result of my own original research.

The transdermal absorption of tea tree oil and potential anti-inflammatory properties

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Part of the work within this thesis has been presented at the following conferences:

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In Memory of Reg Hislop (my Dad)

List of Abbreviations

AP-1	Activator protein-1
AUP	Area under peak
CAM	Complementary and alternative medicine
CHR	Chronic hypersensitivity reaction
DCM	Dichloromethane
DMEM	Dublecco's modified eagles medium
DNA	Deoxyribonucleac acid
ELISA	Enzyme linked immunosorbent assay
EMLA	Eutectic mixture of local anaesthetics
EMSA	Electro Mobility Shift Assay
FACS	Fluorescence activated cell sorting
FCS	Foetal calf serum
fMLP	N-formyl-methionyl-leucyl-phenylalanine
GC-MS	Gas chromatography-Mass spectrometry
GLP	Good laboratory practice
HPLC	High performance liquid chromatography
HPβCD	Hydroxypropyl-β-cyclodextrin
ICAM	Intercellular adhesion molecule
ID	Internal diameter
IFN	Interferon
IL	Interleukin
IκB	I-kappa-B
IκK	I-kappa kinase
JAK	Janus kinase
JNKs	C-Jun N-terminal kinases
LCSM	Laser scanning confocal microscopy
LLOD	Lower limit of detection
LPS	Lipopolysaccharide
MAPK	Mitogen activated protein kinases
MIC	Minimum inhibition concentration
mRNA	Messenger RNA
MRSA	Methicillin resistant *staphylococcus aureus*
MTT	3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide
MW	Molecular weight

NFκB	Nuclear factor kappa beta
p38	Protein 38
PBMC	Peripheral blood mononuclear cell
PBS	Phosate buffered saline
PKC	Protein kinase C
PMA	Phorbol 12-myristate 13-acetate
RCT	Randomised controledl trial
RIA	Radio immunoassay
ROS	Reactive oxygen species
RR	Recovery rate
RT	Retention time
SC	Stratum corneum
SEM	Standard error of the mean
STAT	Signal transducers and activators of transcription
T-4-ol	Terpinen-4-ol
TEWL	Transepidermal water loss
TLR	Toll like receptor
TNCB	Trinitrichlorobenzen
TNF	Tumour necrosis factor
TTO	Tea tree oil
UVB	Ultraviolet B
VCAM	Vascular cell adhesion molecule

Chapter 1
General Introduction

1 Introduction

The use of complimentary/alternative medicine (CAM) by the general public is increasing. It is estimated that 46% of the population in the UK will use one or more CAM therapies during their lifetime (Bishop et al., 2008). The retail sales of complementary medicines in the UK were estimated to be worth £115 million in 2000. Furthermore, the use of complimentary therapies is not limited to the private sector and has cost the NHS in excess of £1million per year since 1998 (Barnes, 2003).

Prior to the formulation of the first synthetic drug (asprin) in 1899, CAM was the primary treatment for ailments. However, the successful development of synthetic drugs since this time has revolutionised medical care. Despite the benefits, this diversion has been expensive; furthermore, mainstream medicine is not always successful in the treatment of all conditions, thus leading the general public to explore alternatives (House of Lords, 2000).

CAM refers to over 50 complementary therapies including homoeopathy, herbal medicine, acupuncture, essential oil and spiritual healing, some involving the administration of pharmaceutical-type remedies (Barnes 2003). The regulation of CAM and those who provide the therapies is erratic, as is research to support such interventions (Hammer et al., 2006). Such has been the concern that a select committee was commissioned by Parliament to publish a scientific report into Complementary and Alternative Medicine (House of Lords, 2000). This report highlights the lack of rigorous research into CAM therapies and that theories about modes of action are not congruent with current scientific knowledge. The report identifies the need for:

- evidence that the therapy is efficacious above and beyond the placebo effect;
- evidence that the therapy is safe;
- evidence that the therapy is cost-effective;
- evidence concerning the mechanism of action of the therapy (House of Lords, 2000).

Since the publication of this report there has been an increase in research into various CAM therapies (Banes-Marshall et al., 2001; Hart et al., 2000). One such area has been the use of essential oils (Kenia et al., 2008; Shimizu et al., 2008). Distilled oils from the leaves of specific plants termed 'essential oil', including lavender, chamomile, eucalyptus and tea tree, are reported to relieve physical and psychological symptoms (Lahlou 2004; Kenia et al., 2008; Shimizu et al., 2008).

Tea tree oil (TTO) has attracted much attention in recent years; it is easily available to the general public in pharmaceutical and cosmetic preparations and is often recommended for use as a home remedy for superficial wounds and other topical ailments (Hammer et al., 1998). TTO has long been used as a topical agent based on anecdotal evidence for its antibacterial and anti-inflammatory properties. Its use includes application to superficial cuts, acne, dandruff and athletes foot (Tong et al., 1992; Satchell et al., 2001; Basset et al., 1999). It has been documented that the tree itself was therapeutically used by the Bundjalung Aborigines of northern New South Wales, Australia (Hammer et al., 2003). TTO was also included in the first aid kits of Australian troops during World War 1 to treat burns, bites and infections (Satchell et al., 2002b). With such anecdotal support, this essential oil has the potential to provide relief of symptoms of some chronic conditions that current conventional medicine struggles to allay. In addition the safe and efficacious use of this easily available oil should be confirmed by empirical data in order for it to be used within a conventional healthcare setting today. There are frequent calls in the literature for guidelines and further investigation on the use of this oil (European Commission, 2004).

Emerging evidence supports the anti-bacterial and anti-fungal properties of TTO (Carson et al., 2001; Banes-Marshall et al., 2001; Hart 2000). Despite suggestion of the oil's anti-inflammatory actions, empirical support is still growing (Cox et al., 2001; Hart et al., 2000; Brand et al., 2001). Exploration of all potential actions of TTO will respond to calls for research from Parliament (House of Lords, 2000) the European Commission (2004) and current literature (Hammer et al., 1998), to ensure its efficacious and safe use (Hammer et al., 2006; Hart et al., 2000).

1.1 Tea tree oil (TTO)

TTO is a naturally occurring substance distilled from the leaves of *Melaleuca Alternifolia*, a shrub-like tree native to Australia (Satchell et al., 2002b). The oil consists of over one hundred terpene hydrocarbons and alcohols. It is only partly soluble in water and is miscible with nonpolar solvents such as dicholoromethane and chloroform.

As the oil is a natural product containing a complex mixture of components, the composition is subject to batch to batch variation depending on season and regional conditions (Hammer et al., 2006). To limit these variations the International Organisation for Standardisation (ISO) has stipulated the composition percentage for fourteen components of the oil as presented in *table 1.1.*

Table 1.1 International Organisation for Standardisation (ISO4730) for Melaleuca alternifolia (TTO)

Component	Compositon (%)
	ISO4730 range
T-4-ol	>30
λ terpinene	10-28
α terpinene	5-13
1,8 cineole (eucalyptol)	<15
Terpinolene	1.5-5
ρ cymene	0.5-12
α pinene	1-6
α terpineol	1.5-8
Aromadendrene	Traces-7
δ cadinene	Traces-8
Limonene	0.5-4
Sabinene	Traces-3.5
Globulol	Traces-3
Viridiflorol	Traces-1.5

(Hammer et al., 2006)

International standards require that TTO has a maximum level of 1,8 cineole (15%) and a minimum level of Terpinen-4-ol (T-4-ol) (30%) [ISO 465]. It is considered that 1,8 cineole, also known as eucalyptol, is an irritant (Cal and Krzyzaniak, 2006), although this is disputed by others (Carson et al., 2002) and that T-4-ol is the main active ingredient of the oil (Brand et al., 2002a; Hammer et al., 2006). However there is limited knowledge of the synergistic nature of TTO components or if individual components can be separated from the oil to increase its benefit whilst reducing the potential of irritancy (Caldefie-Chezet et al., 2004).

1.2 Literature Review

The focus of current research regarding TTO is the anti-fungal and anti-bacterial potential of this agent, with some support of the anti-inflammatory actions.

1.2.1 Anti-fungal properties

Fungal infections are often opportunistic, recurrent and difficult to treat, requiring repeated systemic use of antifungal drugs such as fluconazole, increasing the likelihood of anti-fungal drug resistance (Jose et al., 2002; Bagg et al., 2006). Fungal infections often colonise the skin leading to common ailments such as interdigital *tinea pedis* and dandruff. The topical application of TTO reduces the need for systemic drugs as the oil can be directed specifically at the site of infection (Banes-Marshall et al., 2001).

Infections such as oral candidiasis are common in immuno-compromised patients, for example, those with cancer, HIV and AIDS. Studies investigating the impact of TTO on oral candidiasis in immunosuppressed patients who are resistant to some conventional treatments indicate that TTO has a positive effect at reducing the colonisation of oropharyngeal candidiasis (Jose et al., 2002; Bagg et al., 2006) and are summarised in *Table 1.2.* Whilst Jose's (2002) *in vivo* study identifies a 28% clinical cure and 32% clinical improvement in patients with oral candidiasis using a TTO based oral solution compared to those using a placebo solution, no concentration is stated, therefore making comparison to other studies difficult. This study demonstrates that side effects were increased in patients using an alcohol based oral solution (placebo or

TTO inclusive) compared to an alcohol free oral solution (placebo or TTO inclusive), suggesting that it is the vehicle rather than the TTO that causes side effects.

Yeast isolates from patients experiencing oral candidiasis often include *Candida.albicans (C.albicans)* and *C.glabrata*. A number of *in vitro* studies have examined the minimum inhibitory concentration $(MIC)_{50}$, the *in vitro* dose sufficient to reduce colonisations of specific yeasts by 50% and MIC_{90}, a reduction of 90% for these yeasts. It has been identified that concentrations between 0.06-1% TTO v/v consistently provided a MIC_{50} (Bagg et al., 2006; Vazquex et al., 2000; D'Auria et al., 2001). A MIC_{50} of *C.albicans* resistant to fluconazole was attained with 0.5% TTO, increasing to 1% to realise MIC_{90}. *C.glabrata* MIC_{50} was achieved when a concentration of TTO between 0.125-0.25% was applied (Bagg et al., 2006; Vazquez et al., 2000). In summary, TTO at doses up to 1% v/v is effective at reducing the colonisation of *C.albicans* and *C.glabrata*, common yeast isolates present during oral candidiasis infection, including strains resistant to conventional treatment, *in vitro*.

In vivo studies examining the action of TTO on the symptoms and mycological effect on *tinea pedis* (athlete's foot) identify a dose dependent reaction. Two *in vivo* randomised controlled trials (RCT) identifying mycological cure and symptom improvement of *tinea pedis* following treatment with TTO produce variable results (Tong et al., 1992; Satchell et al., 2002b). A 10% w/w TTO containing cream provided no mycological cure; yet there was a demonstration of improvement of symptoms, compared to a successful mycological cure following treatment with a conventional treatment (1% Tolfanate). A placebo cream provided no mycological cure or symptom improvement (Tong et al., 1992). However, a 25% w/w TTO cream achieved a mycological cure in 31% of patients and this was increased to 64% following treatment with a 50% w/w TTO cream; symptoms were improved in both cases. In contrast, the placebo cream exhibited no mycological cure or symptomatic improvement (Satchell et al., 2002b).

Toenail onychomycosis is a chronic condition often requiring a long duration of treatment which can incur side effects. An RCT examined the effect of 5% w/w TTO cream identified no mycological cure at 8 and 29 weeks following

treatment but there was a reduction in side effects when compared to conventional treatment. However, despite the increase in side effects, conventional treatment (2% butenafine hydrocholoride) achieved 80% mycological cure following 8 weeks and 100% at 29 weeks: Placebo treatment did not provide a mycological cure or altered side effects (Syed et al., 1999).

The yeast isolate *Plasmodium ovale* (*P.ovale*) is responsible for the common condition known as dandruff. A placebo RCT was undertaken comparing the use of a shampoo containing 5% TTO for 4 weeks with a placebo shampoo on the improvement of symptoms. An improvement of 40% in dandruff symptoms was achieved when using the TTO shampoo compared with an 11% improvement when using the placebo shampoo.

In conclusion, the use of TTO against a range of fungal infections has been investigated. TTO at low concentrations (0.06-1%) achieves successful $MIC_{50/90}$ *in vitro* against common yeast strains occurring in oral candidiasis infections, but not all. Whilst symptoms are reduced following the use of TTO containing gels and creams during *in vivo* investigations at relatively low concentrations (5-10%) in the treatment of *tinea pedis* and toenail onychomycosis it is only when large concentrations of TTO (25-50%) are used that a successful mycological cure can be achieved. Consistently it is shown that mycological cure and symptom reduction are concentration dependent.

Table 1.2 Summary of articles investigating the Anti fungal effects of TTO

	Design of trial	**Condition**	**Outcome measure**	**Treatment and Concentration**	**Effective: ✓ Not effective; x**			
Jose et al., 2002	Single site open labelled *in vivo* trial (n27)	Fluconazole refractory oropharyngeal candidiasis in patients with AIDS	Clinically cured (*in vitro* mycological evaluation) (CC), Clinically improved (CI), Side effects of the oral solution (SE ↑ increase, SE ↓ decreased) Measured at 28 days post start of treatment.		CC	CI	SE↑	SE↓
				TTO alcohol free oral solution (no conc. Indicated)	✓ (28 %)	✓ (32%)	×	✓
				TTO alcohol based solution (no conc. indicated)	✓ (28 %)	✓ (32%)	✓	×
				Placebo alcohol free solution	×	×	×	✓
				Placebo alcohol based solution	×	×	✓	×
Bagg et al., 2005	*In vitro* trial (301 isolates from 199 mouth swabs/washes) A standardized	Oral Candidiasis resistant to fluconazole and itraconazole in patients with advanced cancer	Minimal Inhibitory Concentrations (MIC) were measured in strains of *C.albican* and *C.glabrata* that were (r) and were not		TTO %			
					MIC (50)		MIC (90)	
				C. albicans (r)	0.5%		1%	
				C.albican (nr)	0.125-1%		?	
				C.glabrata (r)	0.25%		1%	

	broth dilution and agar dilution method were used		(nr) resistant to fluconazole and itraconazole after 48 hours of incubation with TTO at varying concentrations.	C.glabrata (nr)	0.125–0.5%	?
Tong et al., 2007	Randomised double blind *in vivo* trial (n104)	Tinea Pedis	Mycological Cure (MC), Symptom improvement		MC	Symptoms improved; ✓ symptoms unaffected; x
				1% Tolfanate (standard treatment)	✓	✓
				10% w/w TTO cream	×	✓
Satchell et al., 2002	Randomised placebo controlled blinded study (n150)	Tinea Pedis	Mycological Cure (MC), Symptom improvement		MC	Symptoms
				25%w/w TTO cream	✓ (31%)	✓
				50% w/w TTO cream	✓ (64%)	✓
				Placebo	×	×

Syed et al., 1999)	Randomised placebo controlled trial (n60)	Toenail onychomycosis	Mycological Cure (MC), Side Effects (SE ↑ increased, SE ↓ decreased), measured at 8 and 29 weeks			8 weeks	29 weeks
				2% butenafine hydrocholoride		✓MC(80%)	✓
				5%w/w TTO cream		×MC SE ↓	×SE ↓
				Placebo		×	×
Satchell et al., 2001	Randomised placebo controlled trial (n126)	*P.ovale* (dandruff)	Reduction in dandruff after 4 weeks, expressed as percentage			Improvement	
				5% TTO shampoo		40%	
				Placebo shampoo		11%	
Banes-Marshall et al., 2001	*In vitro* agar dilution method and broth microdilution method	A range of isolates from specimens of leg ulcers (incl. 11 Candida *Spp*.)	MIC and minimum cidal concentration (MCC) of TTO v/v (%). Agar Dilution method (ADM). Broth Microdilution Method (BMM)			MIC	MCC
				Candida Spp.	ADM	0.5-1%	-
					BMM	3%	4%

D'Auria et al., 2001	*In vitro* broth microdilution test	A range of yeasts including *C.albicans*	MIC of TTO % v/v	All yeasts isolated including *C.alb*	0.12-0.5%
Vazquez et al., 2000	*In vitro* broth microdiltution test	Yeast isolates from mouth swabs of patients with HIV and AIDS incl. *C albicans, C.parapsilosis, C.kefyr, C.galbarata* and *Asperigillus species*	MIC of TTO% v/v	C.albicans	0.06-0.25%
				C.parapsilosis	0.06-0.25%
				C.kefyr	0.06-0.25%
				C.galbarata	0.25%
				Asperigillus species	No effect
Mondello et al., 2006	Animal model and *in vitro* broth microdilution method	C.Albicans	MIC 90	T-4-ol,	0.06% v/v
				1,8 Cineole	4% v/v

Throughout the literature there is little explanation for the amount of TTO chosen for each experiment. In addition there is little discussion regarding the vehicle that is used for the TTO, with the one exception of Jose's (2002) study which identifies that an alcohol free oral solution reduces side effects of oropharyngeal candidiasis in patient with AIDS when compared to an alcohol based oral solution, although again, there is no mention of the concentration of TTO that was used, making the results difficult to compare. Choice of vehicle will affect the amount of TTO that penetrates the Stratum Corneum (SC) and therefore have an effect on the condition being treated.

1.2.2 Anti-bacterial effects of TTO

Bacterial infections such as acne vulgaris and methicillin-resistant *staphylococcus aureas* (MRSA) can swiftly become chronic and resistant to current antibiotics (Dryden et al., 2004). *In vivo* studies investigating the effect of TTO on these conditions have been undertaken. A 5% TTO (v/v) gel was compared to the conventional treatment, 5% benzyl peroxide gel and placebo *in vivo*. At the end of the study there were no significant differences between the benzyl peroxide and TTO groups, yet there was a significant improvement between the two groups compared with placebo. Despite this, TTO required a longer time period to reduce the acne lesions but incurred less side effects when compared to benzyl peroxide (Bassett et al., 1990). The results are supported by a subsequent study when a 5% TTO (v/v) gel was successful in the reduction of severity and number of lesions compared with placebo after six weeks, although, this study did not compare TTO with a conventional treatment (Enshaieh et al., 2007).

MRSA has been described as a 'public health threat' (LaPlante, 2007). Whilst its effect in healthy humans is minimal, individuals with suppressed immune systems can be quickly overcome by this infection, leading to increased morbidity and possible death (Dryden et al., 2004). MRSA colonizes superficial skin sites and lesions as well as nasal sites. Conventional treatment entails the patient washing in a chlorohexidine body wash. A 10% TTO containing cream and 5% TTO body wash was compared to standard treatment. It was identified that there were no significant differences between the clinical effectiveness of both. However, closer investigation observed standard treatment was significantly better at clearing the nasal site of MRSA than TTO and TTO was

significantly better at clearing MRSA in skin lesions and on superficial skin sites (Dryden et al., 2004). The results of this study suggest TTO acts directly at the site of application rather than exerting a systemic effect. The effect of TTO and T-4-ol was further investigated *in vitro*. T-4-ol exhibited a MIC_{50} of 0.125% against MRSA and 0.25% achieved MIC_{90}. TTO however demonstrated a MIC_{50} at concentrations of 0.25% and MIC_{90} at 0.5%. The study also observed the effect of T-4-ol and TTO against common clinical bacteria isolates. It was demonstrated T-4-ol to have a MIC_{50} at 0.125% and MIC_{90} at 0.25%. TTO achieved MIC_{50} at a concentration of 0.5%, the same concentration achieved MIC_{90}. The author suggests that this study demonstrates T-4-ol's superior ability to inhibit MRSA and clinical skin isolates and should therefore be considered as a treatment for these infections. It is suggested that the hydrophilic nature of T-4-ol allows for permeation of the skin and cell membrane and incorporation into the cytoplasm of the bacteria in order to exert its effect (Loughlin et al., 2008). In contrast an *in vitro* study investigating the effect of TTO upon MRSA isolates compared with gentamycin and vancomycin (conventional treatment) observed TTO to be significantly less active than conventional treatment, however, the author does suggested that if MRSA becomes resistant to gentamycin and vancomycin in the future, there may be a place for treatment with TTO. This study further highlights that whilst some combinations of treatment enhance the anti-bacterial effect against MRSA, there was no synergy observed between TTO and other treatments included within this study (LaPlante, 2007).

The effect of TTO on dental plaque was investigated and revealed no difference between reduction of plaque following treatment with TTO compared with placebo. However, dental plaque was significantly reduced following conventional treatment with chlorohexidine (Arweiler et al., 2000). Whilst in a subsequent study no difference was found in the reduction of dental plaque following treatment with TTO compared with placebo, there was a significant reduction of inflammation of gingival tissues following TTO treatment compared with placebo (Soukoulis et al., 2006). The results support the former study, observing no effect on dental plaque following TTO treatment, however, the latter study suggests a potential anti-inflammatory property of the oil. A further *in vitro* study investigating the effect of TTO at 0.05%, chlorohexidine (conventional treatment), alpha bisabolol (an alternative treatment) and control

upon *Staphylococcus moorei* (*S. moorei*, an oral bacteria causing halitosis) observed the inhibition of the bacteria in an agar plate following incubaion for 1 and 10 minutes with each treatment. It was observed that *S. moorei* was particularly susceptible to 0.05% TTO, this effect was not observed with alpha bisabolol. However, TTO acted synergistically with alpha bisabolol, enhancing bactericidal activity when applied in conjunction with each other. The author concludes that both TTO and alpha bisabolol should be considered in the treatment of halitosis caused by *S. moorei* (Farrer et al., 2013).

There have been attempts to identify a potential mode of action. It has been observed that the oil does not cause cell lysis, as some anti-bacterial treatments do. However, it was identified that TTO acts upon the cell wall causing permeability. It is suggested that TTO acts in much the same way as a membrane active disinfectant, causing leakage of potassium thus inhibiting respiration (Gustafson et al., 1998, Cox et al., 1998, Hammer et al., 2003b). A more recent study identified T-4-ol as having an inhibitory effect upon *Staphylococcus aureas* (*S. aureas)* and *Staphylococcus epidermidis* (*S. epidermidis*). The potential of essential oil components including T-4-ol and 1,8 cineole to effect the polarity (thus effecting membrane potential) and permeability of the cell wall were assessed by flow cytometry. Despite 1,8 cineole being one of the least active components tested against *S. aureas* and *S. epidermidis,* the component was demonstrated to have a relatively high impact upon polarity and permeation. It is suggested that 1,8 cineole effects the cell membrane and activity at levels below that to have an impact upon cell viability. In contrast, T-4-ol was observed to have an effect upon the above bacterial strains and exhibited a particular effect upon membrane depolarization. TTO exhibited a similar effect (Hammer et al., 2012).

In summary, TTO and T-4-ol appear to exert an anti-bacterial effect, particularly upon clinical bacterial skin isolates such as *S. aureas* and *S.epidermidis.* However, T-4-ol has demonstrated a stronger anti-bacterial effect against some strains compared with TTO, this is attributed to its hydrophilic nature and the ability to penetrate the bacterial cell membrane and incorporate into the cell cytoplasm. The majority of studies are consistent with the methods used (e.g. agar plates) and so are able to be compared. Low concentrations of TTO and T-4-ol (*in vivo* 5-10%, *in vitro* 0.125-0.5%) are

required to exert an anti-bacterial effect. However the effect of TTO upon MRSA is in contrast to previous studies suggesting TTO not to be as active as previously thought (LaPlante, 2007). The mode of action is suggested to be upon the membrane of the bacterial cell causing membrane permeability and enhanced polarity, thus inhibiting cell respiration (Gustafson et al., 1998; Cox et al., 1998; Hammer et al., 2003b; Hammer et al., 2012).

1.2.3 Anti-inflammatory properties

The inflammatory (hypersensitivity) response is divided into 4 categories;
Type I – Immediate hypersensitivity (e.g. allergy);

- Immediate within minutes of contact (release of histamine, vasoactive response).
- Late phase reaction 2-4 hours after exposure (involving the release of cytokines).

Type II – Cytotoxic, antibody dependent hypersensitivity (e.g. thrombocytopenia)
Type III – Immune complex disease (e.g. systemic lupus erythamatosis)
Type IV – Delayed type hypersensitivity response (e.g. contact dermatitis, multiple sclerosis)

Each response is controlled by different aspects of the immune system leading to the various outcomes. The majority of research into the topical application of TTO in the reduction of local inflammation is focused upon Type I (allergy) and Type IV (delayed type hypersensitivity, contact dermatitis) reactions.

Immediate phase hypersensitivity response is characterised by the induction of a weal and flare response. Upon contact with an allergen, histamine and prostaglandins are released leading to vasodilation and increased permeability of vessel walls allowing for the influx of immune active cells, causing itch.

Topical application of TTO has been demonstrated to significantly reduce mean wheal area following intradermal histamine injection *in vivo* measured at 40, 50 and 60min. post injection. However mean itch score and flare area did not reduce significantly compared to control (Khalil et al., 2004). In another study using a murine model, the application of 100% TTO following injection with histamine at 10 and 20min. post injection significantly decreased mean

wheal and flare area (Brand et al., 2002b). In contrast, the application of 100% TTO 30min prior to intradermal injection of histamine significantly increased oedema. Subsequently individual components of TTO, Terpinen-4-ol (T-4-ol), 1,8 cineole and α-terpineol were tested and found that T-4-ol alone reduced swelling, the latter two components exhibited no activity. The swelling associated with the application of TTO to a non-inflamed area may be associated the lipid components of the oil (Brand et al., 2002b). Individual components were applied revealing only T-4-ol to significantly reduce the wheal and flare response. Reduction of flare by TTO suggested here is not supported by other similar studies (Koh et al., 2002; Khalil et al., 2004). However, the time of application differs in each study, which may account for the attenuation of the flare response when TTO is applied at an earlier time point. Results are summarised in *table 1.3*.

Suggested modes of action for this response include the use of a rat physiological model to determine potential pre and post-terminal sensory nerve regulation. To evaluate pre-terminal events a blister was raised on the hind footpad of a denervated rat, the base of which was perfused with 0.125% TTO or water soluble components of TTO either 10min prior to, during or 20min after electrical stimulation. Electrical stimulation causes release of neuropeptides such as Substance P, causing an increased microvascular blood flow. There was a significant decrease in the vasodilation response (approx. 50%) following application of 0.125% TTO, 0.0025% 1,8 cineole and 0.038% α-terpineol but no effect was exhibited following application of 0.053% T-4-ol (dilutions were estimated that following 100% TTO topical application, a concentration of one thousandth of this would penetrate the epidermis). Post terminal sensory nerve regulation was demonstrated in sensory nerve intact rats following application of Substance P. Micro vascular flow was reduced by approximately 30% following application of 0.125% TTO, α-terpineol reduced vasodilation following substance P induced inflammation but 1,8 Cineole and T-4-ol showed no effect. T-4-ol did, however, reduce vasodilation at a higher concentration of 0.2%. The author suggests that pre-terminal anti-inflammatory effects are due to the components 1,8 cineole and α-terpineol, and the post terminal effects due to T-4-ol alone (Khalil et al., 2004).

A murine ear model was used to investigate TTO's effect on histamine induced oedema. 100% TTO was applied either 30min prior to or immediately after injection of histamine. Neuropeptide activity of the c-fibres was depleted so the mice were unable to release neuropeptides such as substance P (a post-terminal mediator of inflammation causing vasodilation as a result of an inflammatory insult). The author suggests this allows for the specific mode of action to be identified, whether by action on sensory neurons or not. Saline was injected into the murine ear, then TTO was applied, this led to an increase in oedema. In contrast, following a histamine injection and TTO application oedema decreased. Compound 48/80 (a substance which degranulates mast cells causing histamine release) was injected into the murine ears and saline was injected as control. Results of application of TTO were similar to that following histamine injection. The individual water soluble components of TTO were then tested. T-4-ol reduced swelling but 1,8 cineole and α-terpineol had no effect. T-4-ol did not increase swelling following injection of saline, unlike TTO (Brand et al., 2002a).

In summary, following intradermal injection of histamine, mimicking a type I hypersensitivity response to allergen, topical application of 100% TTO significantly reduces mean wheal area following application from 10min-60min post injection. However, mean flare and itch score were not significantly reduced when measured from 40min post injection but was demonstrated following application of 100% TTO 10min post injection. T-4-ol was demonstrated *in vivo* to have the ability to reduce mean wheal and flare area following *in vivo* application similar to that seen after application of 100% TTO. Application of TTO to non-inflamed skin resulted in an increase in oedema which is possibly due to the interaction of lipids within the oil and the SC. Potential mode of action include that 1,8 cineole and α-terpineol regulate pre-terminal sensory nerve activity, whilst T-4-ol regulates post terminal sensory nerve activity.

Table 1.3 Investigations into the anti-inflammatory potential of TTO

Author	Study population	Challenge	Treatment	Effect
Khalil et al., 2004	*In vivo*	Intradermal histamine injection	100% TTO 40, 50 and 60min post injection	Mean wheal area sig ↓ Mean itch and flare no difference
Brand et al., 2002b	Murine model	Intradermal histamine injection	100% TTO 10 and 20min post injection	Mean wheal and flare area sig ↓
			Pre-treatment 100% TTO 30min prior to injection	Mean wheal area sig ↑
			T-4-ol 1,8 Cineole A-terpineol	Wheal and flare Sig ↓ No effect No effect
Pearce et al., 2005	*In vivo* – nickel sensitised and non-sensitised subjects	Nickel challenge	100% TTO 3 days post challenge Nickel sensitised subjects Non-sensitized subjects	5 days post challenge Erythema and flare sig ↓ No effect
	In vitro proliferation of PBMCs (monocytes)	Nickel challenge	0.125% TTO Nickel sensitised Non-sensitised	Cell proliferation sig ↓ No effect

Brand et al., 2002a	Murine – TNCB sensitised and non-sensitised mice	TNCB	100% TTO applied 30min prior to or 2, 4 or 7 hours post challenge TNCB sensitised Non-sensitised	 Oedema sig ↓ No effect
			10% TTO gel	No effect
			Control gel	No effect
			5% TTO ointment	Oedema sig ↓
	Immunohistochemistry	TNCB	100% TTO measured 24 hours post challenge TNCB sensitised	Oedema ↓ No effect on infiltration of inflammatory cells

Hart et al., 2000	Monocytes – modulation of pro inflammatory mediators TNFα, IL1β, IL10 and PGE^2 and IL8	500ng/ml LPS for 20 hours	0.125% TTO for 20 hours Activated cells Non-activated cells	 TNFα, IL1β, IL10 and PGE^2 (not IL8) sig ↓ No effect
			0.052% T-4-ol Activated cells Non activated cells	After 40 hours pro inflammatory mediators sig ↓ No effect
			0.0025% 1,8 cineole Activated cells Non activated cells	 No effect No effect
			0.004% α-terpineol Activated cells Non-activated cells	 No effect No effect

Caldefie-Chezet et al., 2004	Monocytes – Production of reactive oxygen species (ROS)	Phyohemagglutin A	0.1% TTO Activated Cells Non activated cells	 ROS production sig↓ ROS production sig ↑
	Leucocytes (including neutrophils) Production of ROS and modulation of anti-inflammatory mediators IL2 and IL4		0.1% TTO Activated cells Non activated cells	 ROS production sig↓, IL2 and IL4 sig ↑ ROS production sig↓, IL2 and IL4 sig ↑
			0.01% TTO Activated cells Non activated cells	 ROS production sig ↓, IL2 and IL4 sig ↑ ROS production sig ↓, IL2 and IL4 no effect
Brand et al., 2002b	Murine model	UVB irradiation	100% TTO Applied 30min prior to exposure Applied 24 hours post exposure	 Oedema sig ↑ No effect on oedema

Brand et al., 2001	Monocytes and neutrophils Production of superoxide and TNFα	LPS 100ng/ml, fMLP, PMA	Monocytes Stimulated: PMA	Water soluble fraction of TTO min. % active ≥ 0.008% Superoxide production sig ↓ (very potent)
			fMLP	≥ 0.031% Superoxide production sig ↓ (dose dependent)
			LPS	≥ 0.031% Superoxide production sig ↓ (dose dependent)
			Non-stimulated	≥0.125% Superoxide production sig ↓
			Stimulated; PMA	T-4-ol No effect
			fMLP	≥ 0.013% superoxide production sig ↓
			LPS	≥ 0.013% superoxide

				production sig ↓
			Non-stimulated	No effect
			Stimulated; PMA	A-terpineol ≥ 0.00025% superoxide production sig ↓ (not in a dose dependent manner)
			fMLP	≥ 0.001% superoxide production sig ↓ (dose dependent manner)
			LPS	≥ 0.001% superoxide production sig ↓ (dose dependent manner)
			Non-stimulated	No effect
			Stimulated; PMA	1,8 cineole No effect
			fMLP	No effect
			LPS	No effect
			Non-stimulated	No effect

In contrast, type IV hypersensitivity reaction (delayed hypersensitivity reaction), including contact hypersensitivity response (CHR), is regulated by B and T-cells. These have many functions including activation and regulation of immune cells, protecting against viruses and preventing autoimmunity. T-cells can become memory T-cells, allowing the body to recognize a particular allergen it is sensitized to in order to illicit an inflammatory response.

The topical application of 100% TTO to an area challenged with nickel on nickel sensitive individuals measured for size of reaction, flare and erythema 3, 5 and 7 days post application, demonstrated a significant reduction in erythema and flare on day 5 compared with areas treated with 5% TTO lotion, placebo lotion (no TTO) and 100% macadamia oil (control) (Pearce et al., 2005). The potential of TTO to effect the proliferative response of peripheral blood monocyte cells (PBMCs) following challenge with nickel in nickel sensitive and non-nickel sensitive subjects was investigated and identified the proliferative response of PBMCs from nickel sensitive subjects was significantly reduced following incubation with 0.125% TTO (v/v). However, TTO had no effect on mitogens (which do not require processing by antigen presenting cells). Pearce suggests that this demonstrates the ability of TTO to affect the antigen presenting cells or the antigen presenting process.

The effect of TTO application to murine skin challenged with a model hapten (2, 4, 6-trinitrocholorobenzene:TNCB) in mice previously sensitized and non-sensitised demonstrates that following re-application of TNCB there is no difference in oedema during the initial 7 hours, but after this time, oedema is rapidly reduced in non-sensitised mice yet remains high in those sensitized. Application of 100% TTO 30min prior to challenge had no effect on oedema exhibited by non-sensitised mice during 7 hours post challenge however sensitized mice demonstrated a significant reduction in oedema during this time following pre-treatment with 100% TTO or application of 100% oil at 2, 4 or 7 hours post challenge. It is also demonstrated that when applied individually T-4-ol could reduce oedema (not significantly) but 1,8 Cineole and α-terpineol had no effect. However, when applied together, T-4-ol and α-terpineol significantly reduced swelling to an extent similar to that of 100% TTO (Brand et al., 2002a). Application of 10% TTO gel and control gel 7 hours

post challenge had minimal effect on oedema, although, in sensitized mice, 5% TTO ointment significantly suppressed TNCB swelling by 35%.

Immunohistology investigations 24 hours post challenge demonstrated that, following TNCB challenge and 100% TTO application, although oedema was reduced, it did not prevent infiltration of inflammatory cells (Brand et al., 2002a).

In summary there are limited studies investigating the potential of TTO to attenuate the CHR, however those available support the use of 100% TTO and 5% TTO ointment in the treatment of oedema as a result of CHR in previously sensitized subjects. In contrast to the treatment of early phase type I allergen response, the pre-treatment of previously sensitized mice with 100% TTO prior to challenge significantly decreased oedema. The TTO component, T-4-ol is demonstrated to exert a positive effect on oedema when used alone, however when used in combination with α-terpineol the effect is strengthened. Brand's study further identifies that TTO did not reduce the infiltration of inflammatory cells. The investigation of the effect of TTO on the modulation of inflammatory mediators released from the infiltrating inflammatory cells may lead to further opportunity to confirm TTO's mode of action. Limitations of these studies include a lack of justification for amounts of TTO and its individual components applied.

Late stage type I hypersensitivity reactions occur 2-4 hours post insult and are characterised by the up-regulation of pro-inflammatory cytokines and recruitment of cells of the immune system including neutrophils and monocytes. Pro-inflammatory and anti-inflammatory cytokines are small mediators released by most cells, including those with specific immunological function. It is hypothesised by a number of authors that the ability of TTO to reduce production of pro-inflammatory cytokines and up-regulate anti-inflammatory cytokines will support the use of TTO as an anti-inflammatory agent (Hart et al., 2000; Caldefie-Chezet et al., 2004).

Monocytes are part of the innate immune system, which, during the inflammatory response, migrate to the site of infection in the tissue and divide into macrophages and dendritic cells. Cytokines produced by monocytes

recruit further inflammatory cells to destroy foreign bodies and infectious agents and to regulate the inflammatory response to ensure the site returns to a non-inflamed condition once the external insult has been resolved.

The effect of TTO and its individual components on the production of cytokines produced by monocytes *in vitro* was examined. Cells were challenged with 500ng/ml of LPS to induce an inflammatory response for 20 hours. Next they were incubated with the water soluble components of TTO (0.125%) and the individual components T-4-ol (0.052%), 1,8 Cineole (0.0025%), α-terpineol (0.004%). These concentrations were determined by those equivalent to the individual components in 0.125% TTO. TTO significantly suppressed the production of the pro-inflammatory mediators TNFα, IL1β, IL10 and PGE_2 but not IL8 by stimulated cells after 20 hours. TTO exhibited no modulatory effect on pro-inflammatory mediators produced by non-activated monocytes. T-4-ol was demonstrated to have the ability to reduce the production of the pro-inflammatory mediators which at 40 hours post incubation was to a similar extent as TTO. However, 1,8 cineole and α-terpineol had no effect (Hart et al., 2000). In addition to the production of cytokines, monocytes produce reactive oxygen species (ROS). In response to stress they can become produced in an uncontrolled manner, becoming cytotoxic and causing tissue damage. The *in vitro* production of ROS by peripheral blood mononuclear cells (PBMCs), including monocytes, following incubation with TTO for 30min at various quantities was examined (Caldefie-Chezet et al., 2004). Concentrations of 0.1% TTO and above were able to reduce intracellular production by PBMCs following stimulation with phyohemagglutinin A (an inflammatory stimulator), however incubation of 0.1% TTO with non-stimulated cells significantly increased ROS production. This study further investigated the effect of 0.01% and 0.1% TTO on leucocytes, a group of inflammatory cells including neutrophils, eosinophils and basophils which protect against bacteria, fungus and allergens respectively. The production of ROS and the anti-inflammatory cytokines IL2 and IL4 was measured in stimulated and non-stimulated cells following incubation with TTO. ROS production was reduced and that of IL2 and IL4 was increased in simulated and non-stimulated cells following incubation with 0.01% TTO and 0.01% TTO. However, increase of IL2 and IL4 was not evident following incubation of non-stimulated cells with 0.01% TTO. The reduction of ROS

production by monocytes is supported by Brand (et al., 2001), however only the water soluble components of TTO were studied.

Following exposure to UVB irradiation, pro-inflammatory mediators are released including IL10, TNFα, prostaglandin, histamine and ROS, causing local oedema. A study by the same group investigated the effect of the water soluble components of TTO on superoxide production (a type of ROS) in monocytes and neutorphils following stimulation with LPS, *N*-formyl-methionyl-leucyl-phenylalanine (fMLP - often employed to activate the signal transduction protein kinase C) and phorbol 12-myristate 13-acetate (PMA), which exhibits a large role in chemo attractants. Results are displayed in *table 1.3*.

A murine model was used to examine the effect of TTO on the reduction of oedema induced by UVB irradiation. Results show that application of 100% TTO 24 hours post UVB exposure did not reduce oedema. When applied 30min prior to UVB exposure, oedema was significantly increased compared to UVB alone (Brand et al., 2002a). The author suggests this is due to the interaction of oil components and UVB wavelengths or enhancement of UVB penetration of skin by the TTO application.

In summary, the limited research examining the potential of TTO to modulate anti-inflammatory cell mediator production supports its use. It is evident that incubation of TTO with stimulated cells produce an anti-inflammatory effect and incubation of TTO in some circumstances with non-stimulated cells induces inflammation. This may have implications for the use of TTO *in vivo*. Oedema is increased following exposure of murine skin to UVB that has been pre-treated with TTO. This may also have implications for TTOs use *in vivo*, however, it is suspected that the increase in oedema in this case is due to the lipophilic properties of the oil and not the action on inflammatory mediators. Limitations of these studies include that they are not *in vivo* and further investigation is required to ensure the findings are transferable. The amount of TTO utilized in the *in vitro* experiments are estimates and commonly determined by cytotoxicity investigations, there are calls within these articles for further *in vivo* research to be undertaken in order to confirm the identity and quantification of TTO components beneath the SC.

1.2.4 The Skin

Previously considered as only an aesthetic covering for the body, current research continually identifies the multi-faceted functions of the skin. It not only presents a physical barrier to the external environment but also possess immunological properties (Thody and Friedmann, 1986).

Comprising of three layers, the epidermis, dermis and sub cutis (*fig 1.1*), the skin is the largest organ of the human body and has a surface are of around $2m^2$ (Tortora 2003). The penetration of TTO through the epidermis is key to realising its full potential *in vivo*. The epidermis consists of four layers (five in certain areas of the body) and includes the stratum basale (at the dermal epidermal junction), stratum granulosum, stratum lucidum, stratum spinosum and stratum corneum (SC). The most abundant cell within the epidermis, the keratinocyte, changes form and function as it migrates through the layers of the epidermis (Totora 2003). Beginning as columnar nucleated cells in the stratum basale, it progressively loses nuclei and organelles as lipids are released into the extra cellular matrix. When the keratinocyte has migrated to the SC they are known as corneocytes and are flattened, dead cells with a thickened cell envelope enclosing a matrix of keratin tonofibrils. The corneocytes are shed from the epidermal surface (Tortora 2003).

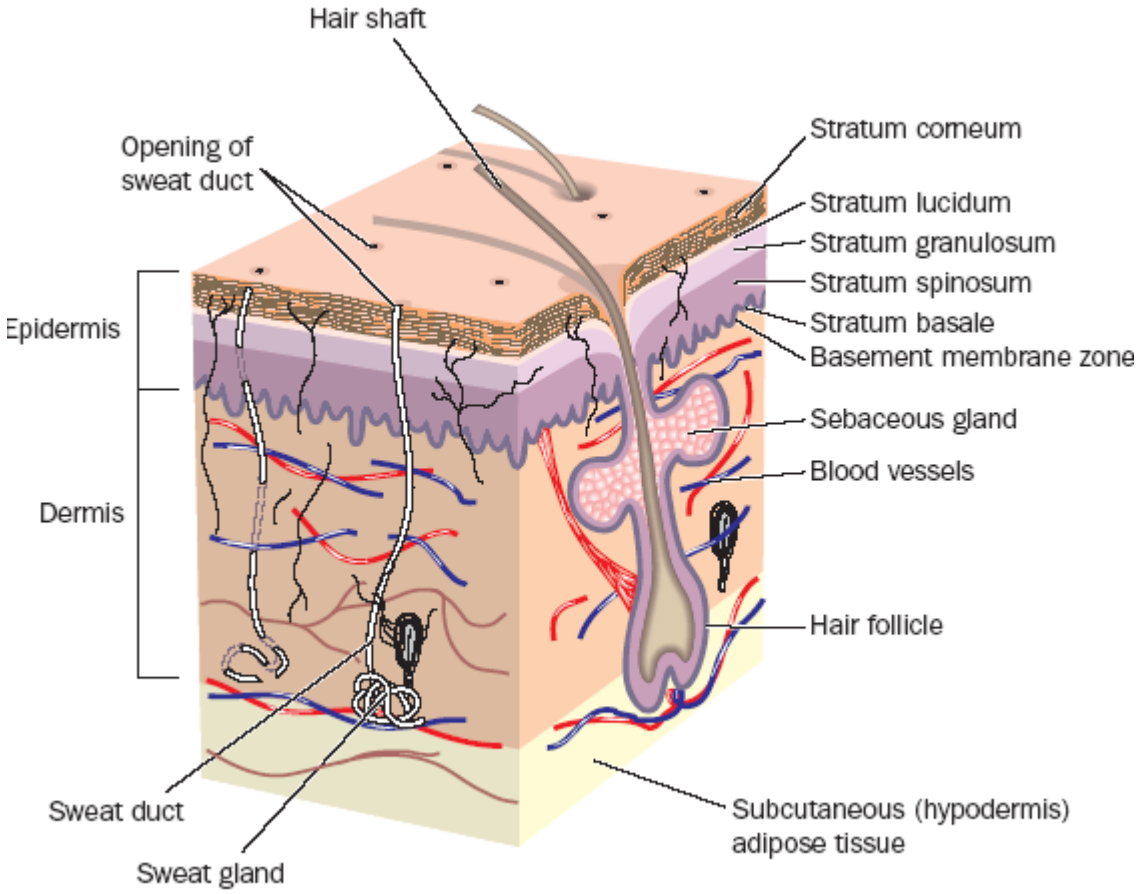
The SC forms the main barrier of the skin (Bronaugh and Maibach 1990). Despite this property, the skin is permeable to some hydrophilic and lipohilic substances (Williams and Barry, 2004). The formation of the skin barrier and the subsequent penetration of substances through the skin is the subject of much debate (Kitson et al., 2000; Kasting et al., 2002).

1.2.5 Skin Penetration Studies involving TTO

Commonly the presence and quantification of components of TTO that penetrate the skin barrier is based upon presumption (Caldefie-Chezet et al., 2004). Evidence of components such as T-4-ol as a penetration enhancer for other substances (William and Barry, 1991) suggests the likelihood of its presence at the dermal epidermal junction. However, there is little knowledge regarding the presence of other components here (Reichling et al., 2006; Cross et al., 2008). Despite demonstration of the active nature of T-4-ol (Brand et al., 2001) evidence in the literature suggests this component may act on

particular pathways and that other components such as α-terpineol and 1,8 cineole may exert effects in a different manner (Brand et al., 2002a). There are calls within the literature for the accurate identification and quantification of the presence of all components of TTO within the epidermis *in vivo* (Hart et al., 2000; Caldefie-Chezet et al., 2004). This will support and direct its use more effectively.

The investigation of penetration of TTO through the skin has been attempted within the literature. Limitations of current research include that many studies are *in vitro* or analyse for only one or two components (Reichling et al., 2006; Cross et al., 2008; Cal et al., 2006; Cal et al., 2008; Nielson et al., 2006; Biju et al., 2005). The penetration of T-4-ol was measured in the receptor fluid (ethanol/water mixture) following absorption through excised abdominal epidermis mounted in a static Franz diffusion cell, n=3-4 (Reichling et al., 2006). Following application of 5% TTO in a semi-solid emulsion T-4-ol was measured as 0.067μl/cm^2/h, application of 5% TTO in cream and ointment resulted in 0.022 and 0.51μl/cm^2/h present in the receptor fluid. Following the application of 100% TTO, 0.2μl/cm^2/h (2.28mg/cm^2/24h) was measured. A similar study undertaken by Cross (et al., 2008), n=6 demonstrated under non-occluded conditions that T-4-ol was able to be recovered, however at a much lower mean amount, 206.4μl/cm^2/24h of T-4-ol following application of 100% TTO



(Voegeli 2001)

Figure 1.1 Sectional View of Human Skin

All layers of full thickness skin are shown including all levels of the epidermis.

(representing approx. 3.6-8% neat TTO). Cross also identified α-terpineol 21.9μg/cm²/24h, but found evidence of no other TTO component. Following occlusion T-4-ol concentrations increased to 500μg/cm²/24h, α-terpineol 44.7μg/cm²/24h (approx. 7% of 100% TTO) and 1,8 cineole could also be identified 19.8μg/cm²/24h. The difference in results between these two studies is large: This may be due to the acceptor fluid in experiments, Reichling using an ethanol/water mix whilst Cross uses phosphate buffered saline (PBS) and 4% bovine serum albumin, a solution closer to physiological conditions. Both authors conclude that it is likely that all components of TTO are present within the epidermis but that only the most hydrophilic penetrate to the dermis.

In contrast to this, a brief commentary describes the penetration of T-4-ol under non-occluded conditions through excised full thickness skin mounted in a Franz diffusion cell following application of 100% TTO (Cal 2008). Analysis of the acceptor fluid revealed no presence of T-4-ol. The skin samples were subsequently cleaned and tape stripped. Analysis of skin cells removed following this revealed the presence of T-4-ol throughout the epidermis (910μg/cm²) and dermis (1500μg/ml). The commentary of this study is very brief revealing little detail of the methods used or number of replications leading to comparisons with other studies to be difficult.

It was hypothesised that bacteria causing acne are present in follicular casts and therefore the presence of TTO within this area may enhance the anti-bacterial effects (Biju et al., 2005). A Franz diffusion cell model was once again utilised investigating bovine udder skin. 5% TTO (w/w) was applied in various formulations and the acceptor fluid analysed identifying only T-4-ol to be present; the author concludes that as this is the component present in the largest amount in TTO it could be presumed that its presence would represent existence of the whole oil. Follicular biopsies were then taken and the amount of T-4-ol quantified for each formulation, the largest recovered amount being 0.43% micro-emulsion, the smallest recovered amount being 0.16% following application with a colloidal bed formulation. The author concludes that following application of 5% TTO in a micro-emulsion, T-4-ol is present in the follicles of the skin at levels of clinically beneficial amounts for patients with acne vulgaris (Biju et al., 2005). This study supports previous evidence of the

importance of the composition of the vehicle applied (Reichling et al., 2006). However the transferability of the results of this study is difficult as the study is undertaken using bovine skin, furthermore, the presence of T-4-ol has been shown to not represent the presence of the whole oil as demonstrated by subsequent studies (Cross et al., 2008; Cal, 2008).

An *in vivo* study, n=8 (Cal 2006), measured the absorption of T-4-ol and linalool following application of both terpenes at 5% in grape seed oil or carbomeric hydrogel for 1 hour. Following analysis of tape stripping of the SC, the amount of T-4-ol present following application with the carbomeric hydrogel was 44μg/cm^2 compared with 24μg/cm^2 following application with the grape seed oil. The author summarises that T-4-ol is eliminated by evaporation from the SC and drainage into the 'reservoir' of the dermis.

A study examining the penetration enhancing effect of benzoic acid and methiocarb, two drugs with different solubility, 3.0g/l and 0.03g/l respectively (Nielson et al., 2006), revealed the presence of T-4-ol, α-terpineol and cineole to be present in the receptor fluid after 48 hours. Franz diffusion cells were used, with excised full thickness human skin. TTO was mixed with 1% Tween and 0.9% NaCl in concentrations of 0.1,1, 5 and 10%. The amounts are not quantified as this was not part of the main experiment, but it is highlighted that these are the least lipophillic components of the oil. The study observed the effect of the above concentrations of TTO mixed with a solution of benzoic acid and methiocarb on the absorption of these drugs. It was found that at concentrations of >5%, TTO significantly reduced the penetration of both drugs. The barrier integrity of the skin was studied following application of TTO by the study of the penetration of tritiated water, the concentration of T-4-ol reduced by 50% and the lag time was prolonged by approximately 40% as concentrations of TTO rose from 1-5%. TTO was also found to reduce the absorption of tritiated water by reducing flux and prolonging lag time. It is suggested that this demonstrates that TTO reversibly effects skin barrier function at concentrations of greater than 5%.

Review of the literature reveals little conclusion as to the penetration of components of TTO through the epidermis following topical application. Methods of experiment and analysis vary and *in vivo* evidence is limited. There

are frequent calls within the literature for identification and quantification of TTO components below the SC, evidence of which is currently lacking (Hammer et al., 2006; Caldefie-Chezet et al., 2004).

In conclusion, there is evidence of the anti-bacterial, anti-fungal and, to an extent, anti-inflammatory properties of TTO and it has been identified that individual components such as T-4-ol, α-terpineol and 1,8 cineole are particularly active. Various modes of action have been suggested which identify the possibility of different components acting upon different pathways. Whilst there are calls for TTO to be produced with high levels of T-4-ol, evidence suggests that components of the oil act in synergy and therefore the composition of the oil should not be altered. There is a need for the identification and quantification of TTO components beneath the SC *in vivo* as this will link *in vitro* evidence with potential *in vivo* effectiveness. Furthermore, the examination of the effects of TTO on keratinocytes has not been previously investigated. Keratinocytes have many functions including immunological activity. Detection of modulatory effects of TTO on pro-inflammatory mediators produced by these specific cells and the identification of potential mode of action will add to the growing evidence regarding this oil.

1.3 Aim of this thesis:

1.3.1 Research Questions

1. Are components of TTO present at the dermal epidermal junction following topical application of 100% TTO?

2. Are components of TTO present within the SC following topical application of 100% TTO?

3. Does TTO possess an anti-inflammatory action when applied to keratinocytes?

4. What is the potential mode of action for any modulation of pro-inflammatory cytokines by TTO within keratinocytes?

1.3.2 Objectives

I. Identify and validate a method to identify components of TTO at the dermal epidermal junction and within the SC.

II. Identify and validate a method of analysis to quantify any components identified as present at the dermal epidermal junction.

III. Observe the effects of TTO on pro-inflammatory cytokines released by stimulated and non-stimulated keratinocytes *in vitro*.

IV. Observe the effects of TTO on the phosphorylation of the transcription factor NFkB within keratinocytes *in vitro*.

Chapter 2
Methods

2 Introduction

To date, there have been few attempts to identify the transdermal absorption of TTO following topical application *in vivo*, as discussed in *Chapter 1 (section 1.2.5*). This is due in part to challenges regarding appropriate methods to measure transdermal absorption and the subsequent analysis. *Chapter 2* will discuss the methods available to measure transdermal absorption and those chosen for these investigations. Methods of analysis are also considered. *Chapter 3* will describe adaptations and validation of these methods thus ensuring optimal conditions for the subsequent investigations in this thesis and accuracy of results.

2.1 Methods to measure topical drug absorption

Traditionally, drug absorption from oral or topical administration is measured using blood serum and, in the case of topical administration, an estimation of topical presence of the drug made (Incecayir et al., 2011). This is not always an appropriate method to utilise when measuring the bioequivalence or pharmacokinetic properties of a topically applied drug (Holmgaard et al., 2010). Often the amount of drug present in blood serum following topical application is low and does not correlate with the amount present in the skin, which may be the target organ for the medication (Holmgaard et al., 2010).

A number of methods have emerged over recent years to determine the bioequivalence of a substance following topical application. These include tape stripping, confocal microscopy, skin blisters, skin biopsies and dermal microdialysis (Lau et al., 2010; Hathout et al., 2011; Benfeldt et al., 2007). While these methods have benefits there are also limitations. Not only does the invasive nature and acceptability of the method require consideration, furthermore the complexity of drug transport through the skin, (for example, tortuosity and protein binding) the nature of the penetrating substance and method of analysis also needs deliberation.

2.1.1 Tape Stripping

The tape stripping method is attracting increasing attention as a method to assess not only the dermatopharmacokinetics of topically applied substances but also the SC distribution profile (Padula et al., 2010). It has been used *in*

vivo (Padula et al., 2010; van der Molen et al., 1997), it is minimally invasive, well tolerated and does not scar (Cal and Krzyzaniak, 2006). The method can be undertaken thus, following the application of a topical drug for a specified time, it is removed and small pieces of adhesive tape are then applied and removed sequentially. The first one or two pieces are discarded to ensure results are not contaminated with residual applied drug. The remaining tape is treated with a solvent to separate the components of interest. The solvent with any components collected is then analysed (Incecayir et al., 2011). However limitations of this method include the influence of variables caused by inter-individual and inter-seasonal differences in the SC structure and the type of adhesive tape used and has led to validated and endorsed guidelines produced by the US Food and Drug Administration (FDA 1998) being withdrawn in 2002 due to unreliable results (Parfitt et al., 2011). Nevertheless, since this time, there has been increasing efforts attempting to limit these variables and therefore increase the reliability of this inexpensive, minimally-invasive method (Lau et al., 2010; Caussin et al., 2009; Jacobi et al., 2005; Incecayir et al., 2011).

2.1.2 Skin Biopsies

Skin biopsies are a validated method for the diagnosis of clinical disorders including autonomic disease (Cetin et al., 2013) and malignant skin lesions (Brehmer et al, 2012) and can also be utilized in research (Dearman et al., 2004). The method requires the selected area to be anaesthetized with an intradermal local anaesthetic agent. A biopsy of the required size (e.g. 2mm, 4mm) is then taken with the punch biopsy instrument. The area is then sutured or covered (if less than 2mm) with a dressing. The sample is sent for histological analysis or can be homogenized prior to analysis (McArdle et al., 2004). Whilst this method allows for the assessment of full thickness skin and can be easily analysed (McArdle et al., 2004), limitations include the acceptability of the method to the participant as the biopsy will leave a scar and the injection may cause discomfort.

2.1.3 Laser Scanning Confocal Microscopy

Visualization of the disposition of an applied substance through the skin has been successfully achieved using laser scanning confocal microscopy (LSCM) (Hathout et al., 2011). The method involves attaching a component of the

substance of interest to a fluorescent label. The substance is applied to the skin for a specified time; the area is scanned using the LSCM which will then allow for the visualization of the labelled component in the skin (Hathout et al., 2011). This method has been used to ascertain the skin barrier enhancing qualities of a lipid emulsion (Rieger et al., 2007). LCSM is well tolerated by the participant and is non-invasive. However, whilst it allows for the demonstration of penetration of a known substance through the skin it is not absolute and is often used in conjunction with other methods to confirm results (Hathout et al., 2011; Rieger et al., 2007). Furthermore, only one or two components can be fluorescently labelled therefore limiting its potential (Rieger et al., 2007; Hathout et al., 2011; Semper et al., 2003).

2.1.4 Skin Suction Blisters

Skin suction blisters can be utilized to measure substances within the epidermis as well as those that have penetrated it (Semper et al., 2003). The method can be undertaken thus; following topical application of the substance of interest for a specified time, suction blisters are generated using suction blister cups maintained at a vacuum pressure for a period of time (e.g. 90min). After the vacuum is released, the blister fluid can be aspirated using a sterile needle and syringe and then the blister top is removed using sterile scissors. The wounds are then covered with an occlusive dressing (Semper et al., 2003). This method will give information regarding substances within the interstitial fluid as well as those within the epidermal layers. Skin suction blisters are minimally invasive, causing some discomfort and the resulting area of inflammation may take a few weeks to heal (Benfeldt et al., 1999). Studies include the successful demonstration of the presence of paracetamol in blister fluid comparable with blood serum following oral ingestion of the drug (Brunner et al., 1999) and a study to examine the expression of Langerhan's cells within the epidermal layers (blister tops) (Semper et al., 2003).However, there is concern that due to the nature of the induction of the blister there may be an increase of protein levels in the interstitial fluid (blister fluid) as a result of inflammation that may not represent normal interstitial fluid. This can also led to an overestimation of protein bound drug such as paracetamol. In addition, the inflammation induced will cause release of inflammatory mediators which may mask the process being studied (Andersson et al., 1994).

2.1.5 Dermal Microdialysis

The use of dermal microdialysis to measure topical drug availability has been frequently discussed within the literature over recent years (Benfeldt 2007; Morgan et al., 2003; Tegeder et al., 1999; Tegeder et al., 2002; Muller et al., 1998). The technique involves the insertion of a small semi-permeable membrane superficially parallel to the skin surface via a guide cannula into the dermal epidermal junction. The membrane has pores of specific sizes, known as a 'cut off', for example, 10kDa cut off, 2kDa cut off (dependent on the size of analyte to be collected). The membrane is then perfused with a tissue compatible fluid (e.g. saline) and passive diffusion allows for the collection of any endogenous substances present to be collected in the dialysate (Benfeldt et al., 2007). This method has been successfully used to recover water soluble nicotine and lidocaine (Muller et al., 1998; Benfeldt et al., 2007). However, the hydrophilic membranes prevent recovery of lipophilic substances as demonstrated by Muller et al., (1998) when using dermal microdialysis to attempt to recover topically applied estradiol, in addition to this the amount of dialysate fluid recovered is small, thus limiting the choice of method of analysis (Russell et al., 2009). Furthermore, due to the small pore size of the membrane, protein bound analytes will not be recovered, therefore potentially providing misleading data. Nevertheless the benefits of dermal microdialysis include the ability to establish the pharmacokinetics of a drug, as dialysate can be collected over time (up to many hours) (Benfeldt et al., 2007). In addition, microdialysis allows for the sampling of several sites simultaneously in the same participant (Benfeldt et al., 2007). Furthermore, despite the limitations of the pore size within the membrane, the benefit is that this limits the inclusion of large molecules thus providing a relatively 'clean' sample (Russell et al., 2009). Dermal microdialysis is well tolerated by participants, local anaesthetic cream is applied therefore reducing any discomfort caused by insertion of the guide cannula and there is no scarring.

2.1.6 Summary

Clearly all methods presented above have benefits and limitations. Whilst tape stripping is well tolerated by participants, the results would be limited to the components of TTO present in the SC. In contrast, skin biopsies provide a sample of full thickness skin allowing for identification of TTO components throughout these layers, but the method is less well tolerated by participants.

In comparison, laser scanning confocal microscopy is non-invasive and will allow for the visualization of labelled analyte within full thickness skin. Yet as, the process of labeling components of interest is expensive this will only allow for the identification of one or two components present within the skin. Furthermore this method is not absolute and has often been used in conjunction with other methods. Again, suction blisters, whilst providing access to the epidermal layers of the skin and the exogenous fluid *in vivo*, is an invasive procedure and less well tolerated by participants when compared to other methods. Moreover, results may not be a true representation of what would occur in non-inflamed skin due to the inflammation caused by the generation of the blisters. In contrast, dermal microdialysis allows for the sampling of exogenous substances over time at the dermal epidermal junction thus providing evidence regarding the potential of TTO. It is a minimally invasive method which is well tolerated by participants. Despite the difficulty in recovering lipids, recent studies have discussed methods to enhance the recovery of lipophilic substances, for example, with the addition of beta cyclodextrins (Ao et al., 2003).

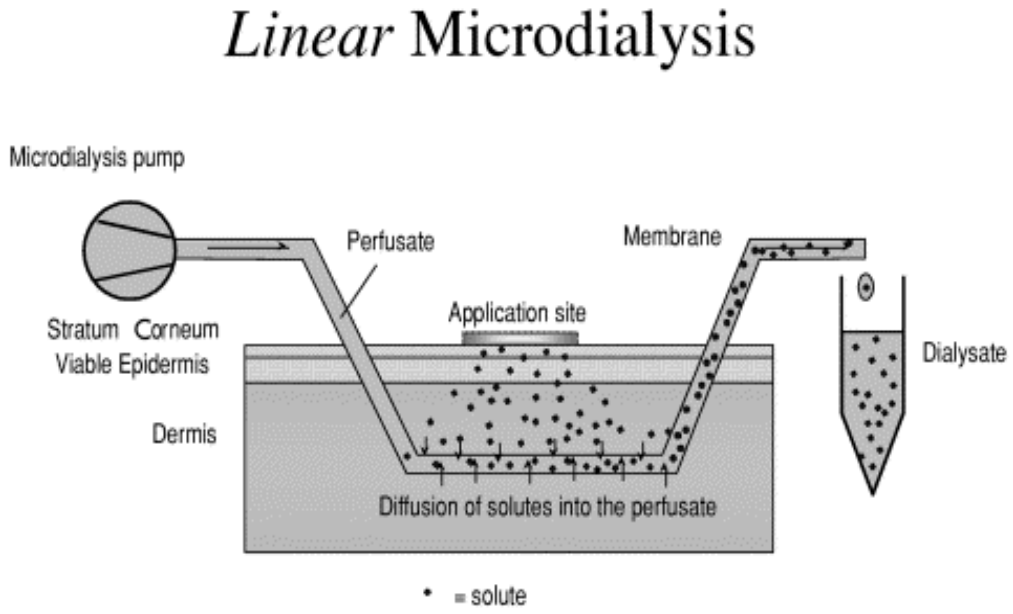
In summary, dermal microdialysis will be utilized to provide evidence of the bioavailability and pharmacokinetics of TTO *in vivo* at the dermal epidermal junction. This has not been previously demonstrated. In addition to this, the tape stripping method will also be employed in order to identify components of the oil within the SC. Adaptations to the original dermal microdialysis method will be required to ensure all components, hydrophilic and lipophilic, will be recovered. Furthermore an appropriate method of analysis will be sought for both methods.

2.2 Microdialysis

Microdialysis is a minimally invasive local sampling method and can be used to sample endogenous and exogenous molecules within the skin (Benfeldt et al., 2007). The small semi-permeable membrane is inserted into the tissue at the dermal epidermal junction and acts as a small blood vessel. The membrane is attached at one end to a microinfusion pump which perfuses the membrane with a tissue compatible fluid, the perfusate. The pores within the membrane allow for the exchange of molecules across the membrane by passive diffusion. The perfusate is then collected by the outlet and is now called dialysate

(Holmgaard et al., 2010). *Figure 2.1* demonstrates linear microdialysis diagrammatically.

Microdialysis is an established method of sampling molecules *in vivo* (Russell et al., 2009; Incecayir et al., 2011). However, to ensure *in vivo* conditions are optimal for the recovery of TTO components, *in vitro* investigations will be undertaken (Cano-Cebrian et al., 2005).



(Herkenne et al., 2007)

Fig 2.1. Diagrammatically demonstrating the process of microdialysis
The microdialysis membrane is inserted superficially parallel to the skin surface via a guide cannula into the dermal epidermal junction. The membrane is continually perfused with a biocompatible solution (e.g. saline) at a very low rate (e.g. 3-5µl/min). The substance of interest is applied topically above the inserted microdialysis membrane. A concentration gradient is established allowing passive diffusion of substances in the perfusate to enter the interstitial fluid and substances surrounding the membrane in the interstitial fluid to diffuse into the perfusate to be collected as dialysate.

The microdialysis system consists of a microinfusion pump, a syringe, tubing, the microdialysis membrane and vials for collection of dialysate. All parts are commercially available; however the microdialysis membrane can be constructed within the laboratory. *In vitro* microdialysis investigations comprise the same components, but the membrane is placed in a bath of the

substance of interest. Conditions can be altered and compared in order to identify optimal conditions for recovery of the components. Results require adjustment to account for transport through the skin *in vivo* including tortuosity and protein binding.

2.2.1 *In vitro* Dialysis efficiency

In order to quantify the amount of TTO components present at the dermal epidermal junction following dermal microdialysis it is necessary to ascertain the efficiency of the microdialysis membrane. This can be calculated by either measuring the loss from and gain into the membrane (of substance of interest) or calculating Relative Recovery (RR) or loss.

Passive diffusion determines the process of dialysis and is similar in principle to that of the exchange of small hydrophilic substances across the microvascular wall and can be described by Fick's First Law of Diffusion as described by *equation 1*.

$$J = -DA\, dC \,/\, dx \qquad \textbf{(1)}$$

J = the flux across the membrane
D = the diffusion coefficient of the solute
A = the area of diffusion
dC/dx = the concentration gradient

The solute flux of the membrane J, is proportional to the diffusion coefficient of the solute, D, the area of diffusion, A and the concentration gradient dC/dx. The concentration gradient is determined by the concentration of solute in the perfusate (Cperfusate) and that in the tissure (Ctissue). Ctissue is then determined by the rate of removal of the solute in the dialysate and its clearance from the tissue space by factors including local blood flow and metabolism.

Dialysis efficiency of the microdialysis probe for TTO components can be measured by relative loss or delivery of the solute expressed as a percentage and can be calculated using the *equation 2*.

$$(Cdialysate - Cperfusate)/(Ctissue - Cperfusate) \quad \textbf{(2)}$$

In theory, recovery and loss are equal, however, in practice, this is not always the case. *In vitro* and *in vivo* probe efficiencies may also not be the same due to factors influencing *in vivo* experiments (e.g. tortuosity, microvasculature). Kreilgaard (2002) suggests *in vitro* dialysis efficiency may be calculated as the ratio Cdialysate/Cmedium, where Cperfusate = 0. Groth (1996) further investigates and (following *in vitro* and *in vivo* semi-quantitative studies) have demonstrated that relative loss or recovery of a wide range of substances is independent of the external concentration of the solute. Moreover, if the experimental protocol is standardized and parameters such as probe length and perfusion rate are kept constant, the efficiency of dialysis should also remain constant and therefore the concentrations of solutes recovered in dialysate reliably reflect those in the tissue space.

As membrane length, perfusate rate and type will be standardized across *in vitro* and *in vivo* studies, this will enable the amount of TTO component present in tissue to be estimated following *in vivo* microdialysis after topical application.

However, due to the lipid nature of TTO, the components adhere to the plastic of the syringe used to hold the perfusate, making the estimation of RR using this method not possible. Alternative methods to estimate actual amounts *in vivo* include the 'low flow rate' method and measurement of 'absolute amount' (Kreilgaard, 2002). The 'low flow rate' method assesses recovery rate of a component at various flow rates (generally <1µl/min). This indicates the most appropriate flow rate to use *in vivo*. A limitation of the 'low flow rate' method includes the time it would take to collect the amount of dialysate required for analysis. In contrast, 'absolute amount' measures the total amount of component recovered during a specific time period. The optimal perfusate flow rate will be used following *in vitro* investigations and the absolute amount of component of interest recovered over a 90 minute period will be calculated. Despite this method accounting for tortuosity and protein binding within the epidermis, it will provide a lower limit of the concentration present at the dermal-epidermal junction. Therefore the quantification of TTO component

recovered at the dermal-epidermal junction will be assessed utilizing this method.

2.2.2 Construction of microdialysis membrane

Microdialysis membranes used in the investigations described in this thesis were constructed in the laboratory under clean conditions. For comparison two commercially available haemodialysis membranes of different material were purchased, Haemophan dialysis membranes (GFE 18 haemodialysis cartridge, Gambro Gmbh, Germany) and Cuprophan dialysis membranes (Membrana). The membranes had an outside diameter of 216μm, a wall thickness of 8μm and a molecular weight cut off of 2kDa. The membranes were cut into 7cm lengths and strengthened by inserting cut and straightened stainless steel wire, diameter 0.1mm ± 10% (Goodfellow Cambridge
Ltd.). Insertion of the wire into the membrane was carefully undertaken to avoid the risk of damage to the fragile dialysis membrane. The components were attached to 30cm length of fine bore polyethene tubing ID 0.28mm, OD 0.61mm (Portex, Kent), the join was secured with a small amount of medical line instant adhesive (Loctite 4061, BSL, UK). The glue was allowed to dry for 24 hours. Membranes for use in *in vitro* studies were then stored. Membranes for *in vivo* use were sealed into autoclave envelopes in groups of five and sterilised by ethylene oxide gas (In Health Decontamination Services, University Hospital of Wales, Cardiff).

2.2.3 Design of Bath for *in vitro* microidalysis studies

TTO contains both lipophilic and hydrophilic components (ISO4730). The interaction of TTO with glass and plastic has previously been investigated (Hart et al., 2000). It was demonstrated that plastic interacted with the TTO components, causing adhesion of lipids and thus removal of the lipids from the oil. In contrast, glass had little effect on the TTO components. Traditionally polystyrene 30ml universal containers have been used as the bath in *in vitro* microdialysis studies. However, as TTO contains both lipophilic and hydrophilic components, it was anticipated that the lipid components of the essential oil would adhere to the sides of the polystyrene container. This was confirmed in an in-house study, a 30ml universal plastic container was used as the bath: Not only did components adhere to the bath, furthermore the silicone sealant used to attach the membranes to the bath was dissolved causing leakage and

reduced dialysate collection volume. Therefore an alternative was sought. Glass has been demonstrated to interact less with TTO components than plastic (Hart et al., 2000) thus a glass bath was designed (*figure 2.2)*.

The membranes can be inserted through the holes, ensuring that a 2cm length of membrane is exposed on the inside of the bath, secured with silicone sealant (B&Q) and left to dry for 24 hours. Silicone is inert and will not affect the TTO or perfusate. After experiments the membranes and silicone can be removed with silicone remover (B&Q).

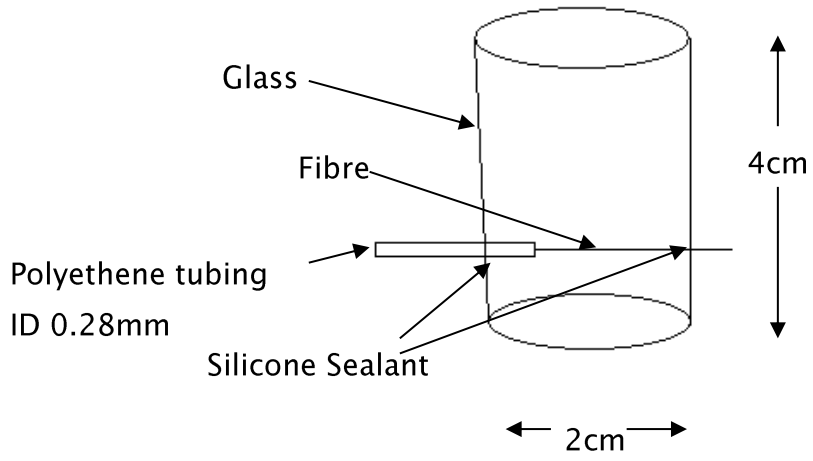


Fig. 2.2 Diagram to demonstrate glass bath with membrane inserted.
The bath was designed to be reusable, the membranes able to be replaced. The bath can be thoroughly cleaned between experiments.
The bath was also economical, reducing the amount of essential oil needed (2ml when previously 7ml was required).

The University Glass Blower was commissioned to make a bath to the following specifications.
The bath is cylindrical, 4cm deep and 2cm wide.
Two holes were created 0.3mm in diameter, 0.5mm above the base of the container, on opposite sides of bath.

2.2.4 Collection containers

Dialysate collections were made into amber glass vials (Fisher Scientific, UK). The dark glass reduces the effect of light on the photosensitive oil and glass reduces adherence of oil to the vial (Hart et al., 2000).

2.2.5 Assembly of equipment for *in vitro* microdialysis investigations

A 1ml syringe (Portex, UK) was connected to 10cm of polyethene tubing ID 0.35mm (Portex UK) using a 16 gauge epidural catheter connector (Southern Syringe Services, Enfield). The syringe contained 1ml of chosen perfusate fluid and the polythene tubing was gently purged until perfusate fluid reached the end. This was then attached by inserting one tube inside the other for 1cm, to the constructed microdialysis membrane/glass bath unit as previously described (*section 2.2.3*). The end of the microdialysis membrane was placed inside the amber glass vial. The test fluid (2ml of TTO) was gently poured into the glass bath. Photographs of the assembled equipment are presented in *figure 2.3*.

2.2.6 Calibration of pump

The microinfusion pump (CMA 400 syringe pump, CMA, Sweden) was calibrated by collecting phosphate buffered saline solution (Tayside Pharmaceuticals, Dundee) at a rate of 5µl/min into weighed glass vials covered using parafilm (Fisher Scientific, UK) to reduce risk of sample loss by evaporation, and the amount delivered over 30 minutes calculated. The weight of fluid collected in milligrams (mg) is equivalent to the amount in micro litres (µl) thus allowing for testing of the accuracy of the pumps. The pumps were demonstrated to be accurate using this method. The amount collected over 30min was as expected. A sample of results is shown in *table 2.1*. The samples were taken at 30min intervals over a period of 90min for each membrane (A and B). The pumps were calibrated for 10 minutes prior to the commencement of each study.

Table 2.1 Showing percentage of expected volume collected into glass vials over 90min to show accuracy of CMA 400 syringe pump.

Micro tube	Weight before (g)	Weight after (g)	Difference	%150µl
A1	0.3868	0.5286	0.141	94
A2	0.3956	0.5383	0.143	95
A3	0.3807	0.5274	0.147	98
A4	0.3809	0.5249	0.144	96
B1	0.3804	0.530	0.1496	99
B2	0.3978	0.5449	0.147	98
B3	0.3881	0.5374	0.149	99
B4	0.3822	0.5342	0.152	101

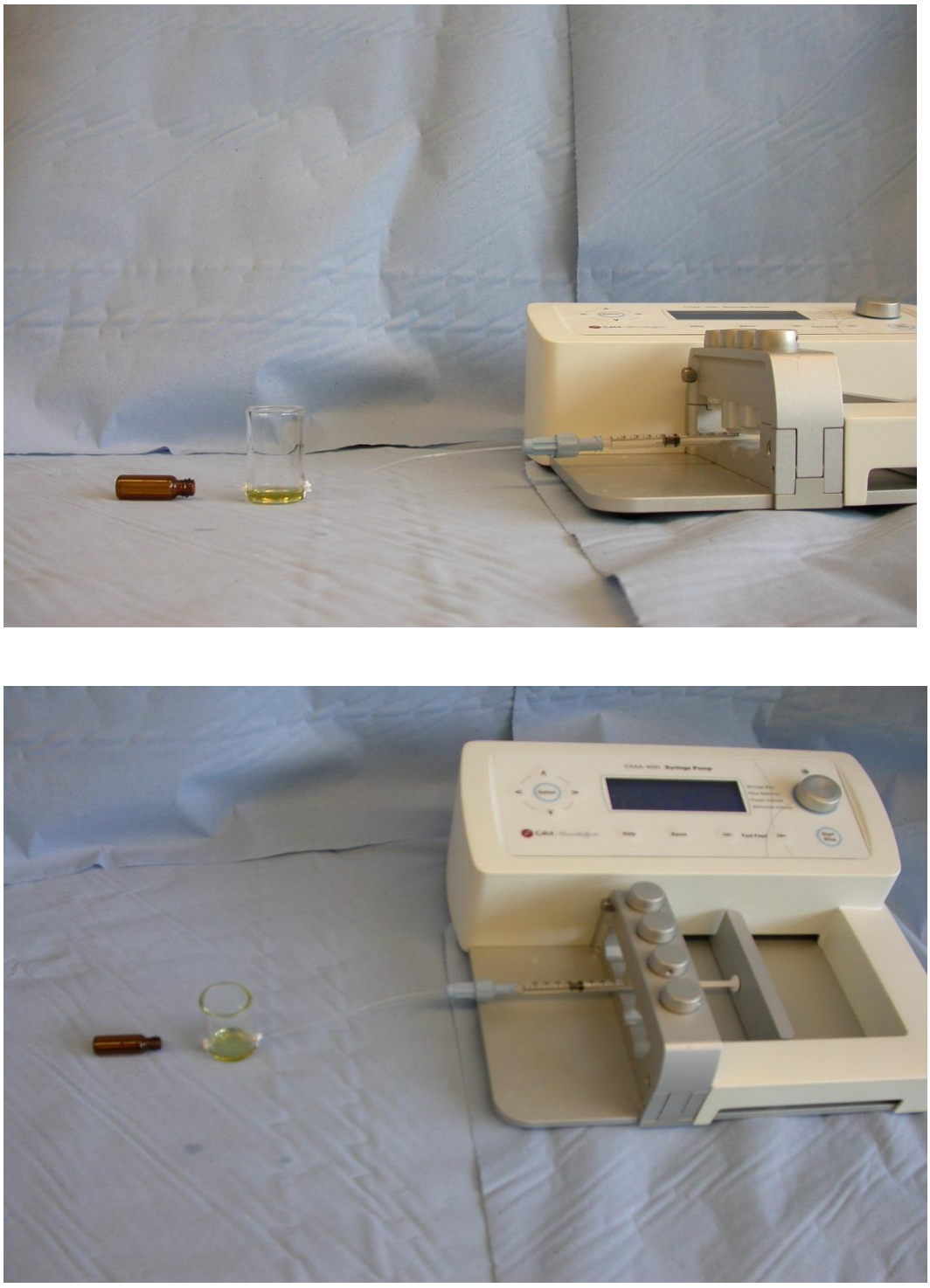


Figure 2.3 Assembly of in vitro microdialysis experiments
The picture shows microdialysis pump, syringe containing PBS as perfusate, glass bath containing 2ml TTO with 2kDa microdialysis membrane inserted and amber glass vial for collection of dialysate.

2.3 *In vivo* microdialysis

The following protocol for *in vivo* microdialysis studies was informed by *in vitro* microdialysis and analysis investigations.

2.3.1 Inclusion/Exclusion Criteria

Following ethical approval from Southampton and South West Hampshire Ethics Committees B (06/Q1704/2), 10 healthy volunteers, mean age 41 years (±20 SEM), were recruited via advertisement. Inclusion criteria is summarised in *table 2.2*.

Table 2.2 A summary of inclusion criteria for healthy participants recruited to the in vivo microdialysis study

Aged between 18-65 years
Male or Female
Non-smoker
Absent from active skin disease
Not pregnant
Not taking medication that may affect the cardiovascular system

Participants acted as their own controls.
Microdialysis membranes were constructed and sterilised as described (*section 2.2.2*).

All investigations were undertaken in a temperature controlled room (22°C± 2°C) within a clinical research facility. All participants arrived at 09.30, the study undertaken at the same time each day to limit variations caused by circadian rhythm (Krielgaard, 2002). Participants were advised to not drink caffeinated drinks, ingest anti-inflammatory medication or participate in vigorous exercise for 12 hours prior to and throughout the experiment due to the effects on dermal vasculature (Krielgaard, 2002).

EMLA local anaesthetic cream (Astra Zeneca) was applied to two areas separated by 5cm, on the non-dominant volar forearm 2cmX1cm and occluded using opsite occlusive dressing (Smith and Nephew) to achieve topical anaesthesia. Application time was 90minutes. During this time participants were free to leave the research facility.

On return to the research facility the EMLA cream was removed by gentle washing with water and the microdialysis membranes were inserted.

2.3.2 Insertion of microdialysis membranes

The microdialysis membranes were checked for patency and integrity by perfusing with PBS using an aseptic technique, any that exhibited leaks or did not flow freely were discarded.

All microdialysis membranes were inserted in the non-dominant arm. The area to be dialysed was measured and two small dots 2cm apart were made with a marker pen, this ensured accurate membrane implanted length. A 23 gauge hypodermic needle (Plastipak, UK) was then inserted as superficially as possible and the microdialysis membrane fed into the point of the needle, exiting via the hub. The needle was then removed, leaving the membrane in position (*figure 2.4*). Entry and exit sites were occluded using an occlusive dressing (Opsite, Smith and Nephew) and the membrane was then secured using surgical tape (Micropore, 3M). This procedure was repeated a further three times, leaving two membranes positioned, 2mm apart, securely in one of the anaesthetised site and two in the other site. The membranes were then covered with a light dressing (Melolin) that had been moistened with sterile water and bandaged.

A period of two hours was allowed between insertion of membranes and commencing microdialysis investigations, to ensure that any insertion trauma caused had resolved and that the topical local anaesthetic cream effects had diminished (Groth and Serup, 1998; Anderson et al., 1994).
On return to the research facility the participant was acclimatised to the environmentally controlled room for 15min. The bandage and micropore dressings were removed and the membranes connected to the microinfusion pump.

2.3.3 Equipment assembly

In vivo microdialysis equipment was assembled as for *in vitro* investigations (*section 2.2.5)*. However the tubing connected to the epidural catheter connector was attached to the inserted membrane as shown in *figure 2.4*.

The perfusate fluid containing 5% Hydroxypropyl Beta Cyclodextrin (HPBCD) (Cargill, North America) in PBS pH 7.4 (Tayside Pharmaceuticals, Dundee) was perfused at a rate of 5µl/min for 5min controlled by a Microdialysis Syringe Pump (CMA 400, Sweden).

A Pasteur pipette was used to apply 0.5ml of TTO (Fragrant Earth, Glastonbury a hill top chamber (25mm, Kraton) and positioned centrally over one pair of membranes and occluded using Opsite occlusive dressing (Smith and Nephew). A hill top chamber was applied over the remaining two membranes and occluded as before with no oil applied to act as control.

2.4 Separation of components of TTO from the dialysate

Many methods of analysis require samples to be free from sodium and other large molecules, as they can obstruct the column and hinder analysis; this is known as a 'clean' sample (Ku et al., 2000). Microdialysate from these investigations includes sodium, therefore a method of separation was required that would not affect the TTO components. A number of existing separation methods exist including solid-phase extraction, reverse-phase and ion-exchange solid-phase extraction and liquid-liquid extraction.

2.4.1 Solid-phase extraction method

The solid-phase extraction method has been used to successfully prepare samples of Chinese medicinal herbs prior to analysis (Ku et al., 2000). This method involves the application of the sample to a column. The column is treated with methanol and water and the sample is washed. The eluate is then collected under reduced pressure to dry and then the residue re-suspended in 2ml of methanol, this is then filtered prior to final analysis, leaving the undesired impurities attached to the column. This method ensures samples are clean prior to analysis and is a straight forward process; however, the amount of sample required is large (2-3ml). Furthermore, there are a number of steps included in the process including not only drying and re-suspension but also filtering of the sample which may reduce the amount of components present in the sample (Ku et al., 2000; Brondi et al., 2004). This method has many adaptations including reverse phase column extraction which is used to extract components based on the polarity of the components; the column is coated in hydrocarbon chains which attracts components of low-mid polarity, thus separating them from the remaining sample. A further condition used is the

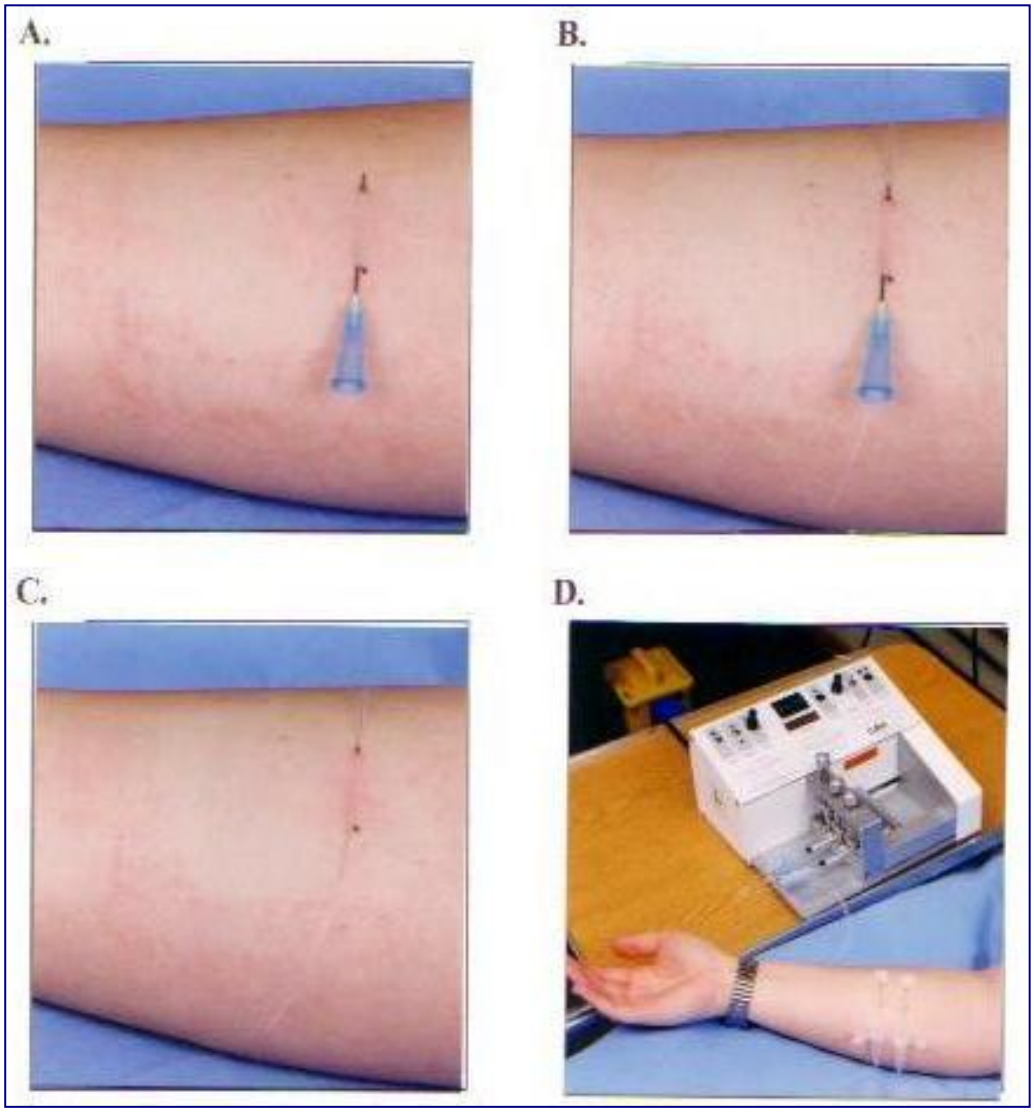


Fig. 2.4 In vivo microdialysis membrane insertion

A = Following removal of EMLA a blue (23 gauge) needle is inserted very superficially for a distance of 2cm.

B = The microdialysis membrane is inserted through the positioned needle.

C = The guide needle is removed, leaving the membrane in position.

D = Following a period of 2 hours to allow for insertion trauma to reduce, the membranes are connected to the pump and the experiment can commence.

ion-exchange solid-phase extraction method: This extracts components from a sample based on their positively charged ions (Ku et al., 2000). Whilst there are benefits to these methods, including the ability to isolate components dependent on their properties, this could be of detriment if the desired components are a mixture of carbons and terpenes, and of mixed polarity. Furthermore these methods require relatively large sample volumes as described before.

2.4.2 Liquid-Liquid extraction

The liquid-liquid extraction method involves the addition of a liquid solvent phase to the sample. The sample is mixed and then allowed to settle. The components of interest will be collected in the solvent phase whilst the impurities remain in the original liquid sample. The solvent phase is removed and retained for analysis (Muller et al., 1998). Whilst this method is beneficial for samples containing volatile substances which may easily be lost in more convoluted processes, care must be taken to ensure the volatile solvent does not evaporate thus diminishing components present within the sample. Furthermore, the process may require repetition to ensure all impurities are removed prior to analysis, this may lead to further reduction in components of interest. However, this method does not require a large sample volume or expensive equipment.

Clearly there are a number of aspects to consider when choosing a method of separation including the properties of the components of interest and volume of the sample. The choice of separation method is limited for these investigations due to the small volume of the dialysate sample (90-150µl). Additionally, TTO components are a mixture of polarity and volatility thus further restricting choice. The solid phase extraction method requires large sample volumes and includes a number of steps whereby sample may be lost; furthermore the process may exclude substance of interest due to polarity or volatility. In contrast, despite limitations of the liquid-liquid extraction method including the potential loss of sample due to the volatility of the solvent phase compared to solid-phase extraction, there is less opportunity for substance of interest to be lost. The process is simple and inexpensive, requiring only a small sample volume.

In summary the liquid-liquid extraction method has been chosen to separate sodium and other large molecules from the dialysate sample. To ensure all traces of sodium are removed from the sample prior to analysis a double liquid-liquid separation will be undertaken whereby the process is repeated.

2.4.3 Double liquid-liquid extraction of microdialysate

Equal amounts of dichloromethane (DCM) and sample were mixed vigorously together in a glass test tube using a vortex (Stuart-equipment) for 1min and then left to allow solutions to separate for 1min. The DCM solvent layer separated to the top due to the specific gravity of the solvent being less that the dialysate solution, this layer was removed carefully using a glass Pasteur pipette (Fisher Scientific, UK) ensuring no part of the dialysate sample was included, and placed in another test tube. The sample (not solvent) was discarded. An equal amount of solvent was then added to the remaining solvent (containing components) and the process was repeated. The solvent layer at the top was placed into an amber glass vial to await analysis whilst the remaining layer containing sodium was discarded.

2.5 Tape stripping

Dermal microdialysis can successfully determine the presence of exogenous and endogenous substances at the dermal epidermal junction (Benfeldt et al., 2007). However, to clearly assess the pharmacokinetics of TTO the investigation of all layers of the skin including the SC would be of benefit. Tape stripping as discussed in *section 2.1.1* is a method which can be utilised to examine substances within the SC and will therefore be used in this study.

2.5.1 SC Depth

The depth of the SC varies between individuals and there have been many attempts to ensure that the amount removed is consistent between volunteers. This includes the measurement of trans-epidermal water loss (TEWL) where increasing amounts of water is lost from the body as the skins barrier is disrupted, as it is during tape stripping (Jacobi, 2005). Chao and Nylander-French (2004) measure TEWL after every 3 tape strips and stopped when TEWL measured $40gm^{-2}h^{-1}$ as it was estimated that 60% of the SC had been removed at this point. Other studies vary in the amount of tape strips used during experiments. As few as 10 to 21 strips have been used in previous

investigations (Cal and Krzyzniak, 2006; Parfitt et al., 2011; Incecayir et al., 2011).

Jacobi et al., (2005) states that the complete removal of the SC is normally necessary to correlate the removed amounts of topically applied substances to their position within the SC but also suggests that topically applied substances are likely to be stored in the uppermost layers of the SC and can be removed with a small number of strips, discussing that it is not always necessary to remove the whole of the SC. This is supported by Caussin et al., (2009) demonstrating that the first strips remove more that the average amount of SC per tape strip.

In an *in vivo* study involving 15 subjects Jacobi et al., (2005) investigates the variability of SC depth between subjects. By measuring in each subject the amount of corneocytes removed with each strip they found that there was a less than 20% deviation concerning the relative amount of SC removed by a consistent number of tape strips. From this they were able to form an equation (*equation 3*) that they suggest can estimate the relative thickness of the SC with the number of removed tape strips.

$$y = 107 - 111e^{-n/21} \quad \textbf{(3)}$$

y = relative thickness of the SC

n = number of tape strips removed

This equation has enabled the estimation of the % of SC removed from the number of tape strips used as in *table 2.3*. From the literature it can be seen that there is variability in the amount of SC removed in studies. This is partly due to the purpose of the study, whether it is investigating bioequivalence or bioavailability of a substance of interest.

Table 2.3. Showing the amount of SC removed by tape strips in % ±SEM

Number of Tape Strips	% SC removed
20	66±12%
30	84±11%
50	95±3%

(Jacobi et al., 2005)

2.5.2 Investigation to determine SC depth

To correlate the number of tape strips used with the amount of SC removed, 2D ultrasound scans (Dermascan C version 3, Cortex Technology, Denmark) were sequentially taken from an area of the volar forearm in two volunteers as follows.

An area 2cmX1cm was marked by 4 dots with a marker pen on the non-dominant volar forearm. Tape strips (Scotch tape) 2cmX1cm were prepared. One ultrasound scan was taken of the area at baseline. Ten tape strips were sequentially applied directly over the area with gentle finger pressure and removed. Another 2D ultrasound scan was taken and this process was repeated after 20, 30 and 50 tape strips. The area of SC removed was then measured using the inbuilt software.

2.5.3 Results

The results are displayed in *table 2.4* and *figure 2.5*. The scans are displayed in *figure 2.6*. The mean depth of SC following 0 strips is 0.2165 ±0.0005 (mm±SEM). The mean depth of SC following the removal of 10 strips is 0.1545 ±0.0125. This represents a 29% reduction of SC following 10 tape strips representing a statistically significant difference in SC depth. Overall the reduction of SC depth between 0 strips and 50 is 37%. There are no other statistical differences observed.

Table 2.4 Mean SC depth (mm) ±SEM following tape stripping measured using a dermascan. (n=2).

	0 strips	10 strips	20 strips	30 strips	40 strips	50 strips
Mean	0.2165	0.1545	0.159	0.162	0.13	0.1365
SEM	0.0005	0.0125	0.008	0.005	0.012	0.0055

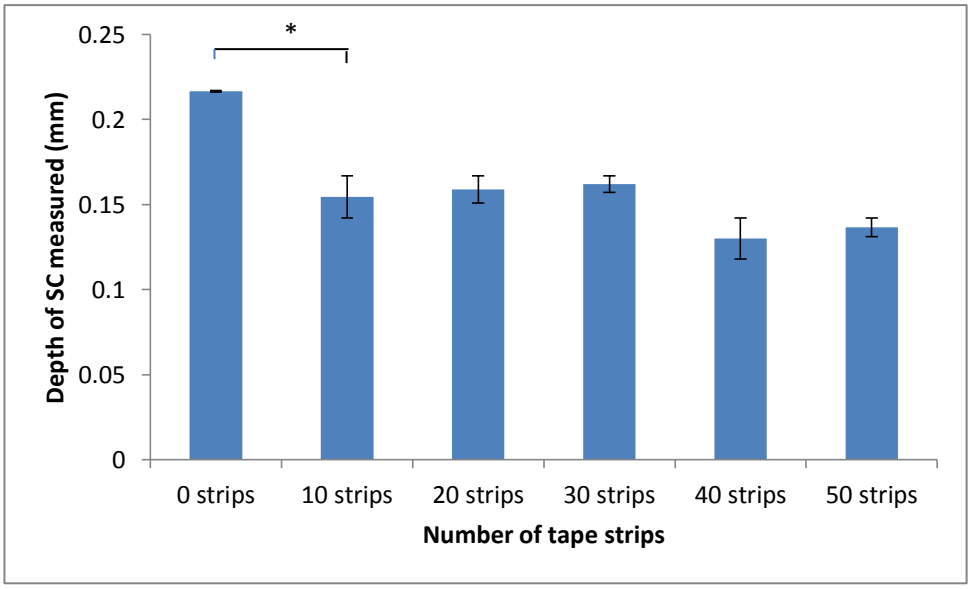


Figure 2.5 Mean SC depth (mm) ±SEM following tape stripping
There is a significant statistical decrease in SC depth observed following the removal of 10 tape strips compared to 0, assessed by the independent samples t-test (p=0.038). There are no other statistical differences observed. (n=2). Statistical significance is displayed by *.

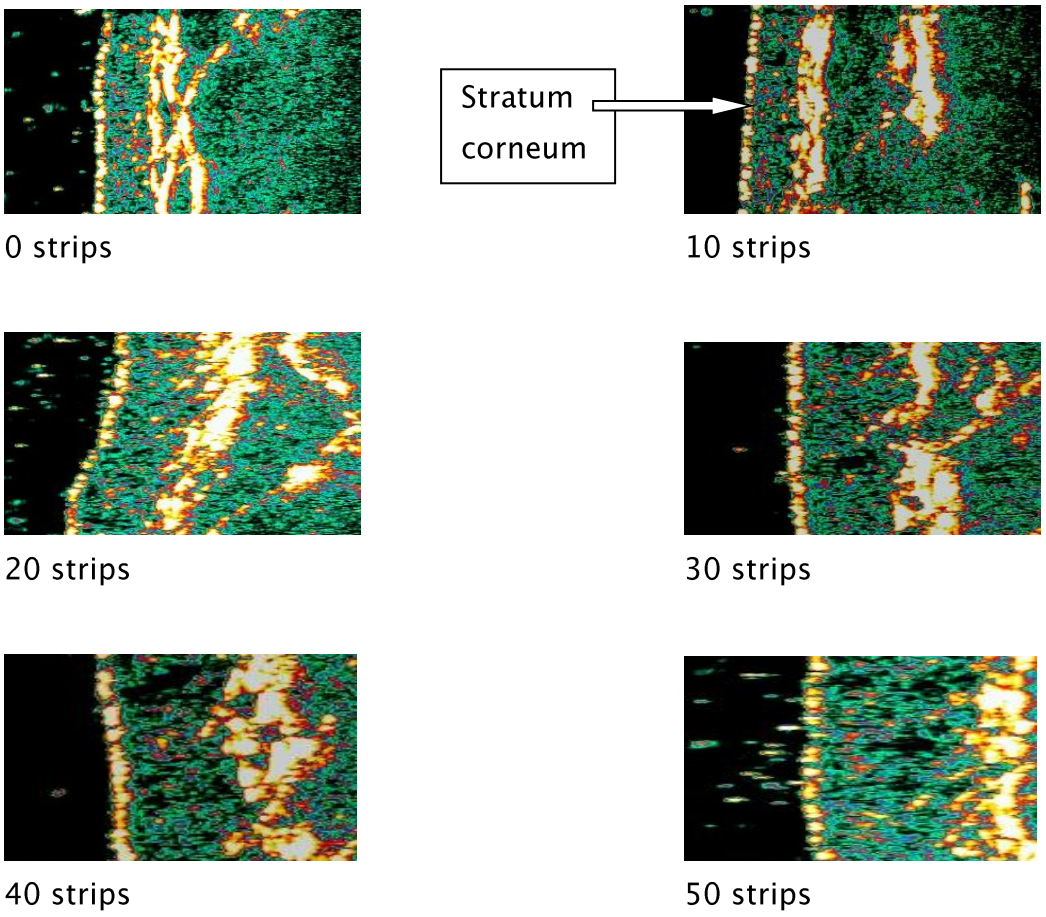


Figure 2.6 Dermal scans displaying the epidermis following SC removal with tape strips.
The numbers of tape strips used are stated under each scan, the SC is indicated by the arrow. The initial depth of SC (mean±SEM) is 0.216mm±0.0005, the final depth following 50 tape strips is 0.1365mm±0.0055. There was a statistical difference between the depth of SC following 0 strips and following 10 strips (p=0.038) assessed by the t-test. There are no other statistical differences between the measurements. (n=2).

2.5.4 Discussion

The results following the tape stripping scans observe less SC removal than previously reported within the literature (Jacobi et al., 2005). This could be due to reasons including the variability between subjects as highlighted within the literature and the differences in pressure exerted onto the tape by the researcher prior to removal from the skin. Only one researcher collected data during this investigation therefore reducing this variable. However, a statistically significant reduction of SC is achieved between 0 and 10 strips. This supports studies within the literature suggesting the majority of SC is

removed during the first tape strips (Caussin et al., 2009). For the present study it is of interest to observe if there are any differences in components recovered at different depths of SC which can be observed following SC removal by 30 strips. The removal of SC with 50 strips does not represent a statistically significant difference in the reduction of SC compared to 30 strips. Furthermore, in house experiments observed that after the removal of 30 tape strips the skin became increasingly sore for the subject. The purpose of this study is to identify components of TTO present within the SC, not to quantify them in this instance, therefore upon the evidence presented here it was concluded that 30 strips would be sufficient.

2.5.5 *In vivo* Tape Stripping

Ethical approval for this study was obtained from Southampton and South West Hampshire Ethics Committee B, 06/Q1704/2. The study was undertaken as before (*section 2.5.2*) in a temperature controlled room within a research facility. Inclusion and exclusion criteria were as before (*table 2.2)*

On arrival at the research facility 0.5ml of TTO was applied to an area on the volar forearm and occluded with a hill top chamber and opsite occlusive dressing. An area 5cm above or below (alternating between participants) was covered with a hill top chamber and occluded with opsite occlusive dressing to act as control.

After 90min the hill top chamber was removed from the treated area and the skin wiped twice with a cotton swab. The hill top chamber was removed from the control site. Sequential tape strips 2X2cm were applied and removed as before (*section 2.5.2*). The first tape strip was discarded and the subsequent tape strips are grouped in the following order: 2-5, 6-9, 10-20, 21-30, each group of strips were placed into individual test tubes containing 5ml DCM and the test tube occluded to avoid evaporation. This process was repeated on the control site.

2.5.6 Separation of TTO components from adhesive tape

The test tube was agitated vigorously on a vibration plate (Stuart-Equipment) at room temperature for 5min and then vortexed vigorously for 1min. The uppermost layer was removed using a Pasteur pipette and placed in an amber glass vial to await analysis.

2.6 Methods of analysis

2.6.1 High Performance Liquid Chromatography and Gas Chromatography Mass Spectrometry

The properties of components within a sample require consideration when identifying a method of analysis. TTO contains components of varying volatility. Furthermore the sample volume produced during microdialysis limits choice. However there are two methods that have been used successfully to analyse essential oils and microdialysate samples, these include High Performance-Liquid Chromatography (HP-LC) and Gas Chromatography-Mass Spectrometry (GC-MS) (Incecayir et al., 2011; Koh et al., 2002; Cox et al., 2001; Nielson et al., 2006). HPLC and GC-MS are similar in that a small volume of sample is injected into the analyser, however during GC-MS analysis the sample proceeds to a chamber containing a polysiloxane coated column the 'stationary phase' within a vacuum containing Helium the 'mobile phase'. The components of the sample adhere to the column, the temperature is increased in preprogramed increments causing the components, depending on their volatility, to disassociate from the column. The components continue to the analyser and a chromatograph is produced depending on the speed of dissociation from the column. In contrast the HPLC column is chosen according to the nature of the sample to be analysed. The column (stationary phase) contains a sorbent exhibiting various particle size and chemical nature. The sample is injected into the column chamber and is incorporated into the mobile phase which is a mixture of solvents and water that flows through the column at high pressure. The components of the sample attach to the column and, depending on interactions between the stationary and mobile phase, dissociate from the column at differing times and proceed to the analyser. If the HPLC or GC is coupled with a mass spectrometer the components continue to the ionising chamber where they and are ionised. The ions proceed through the mass spectrometer containing four poles with opposing charges which switch rapidly causing the particles to proceed in a spiralling motion to the mass spectrometer. The speed of passage through the mass spectrometer will determine the mass spectrum produced. The chromatograph and mass spectrum will give information regarding molecular size and volatility which combined can allow for identification of individual components of a sample (Zhang et al., 2010; Incecayir et al., 2011; Koh et al., 2002; Reed 2003).

Both methods require only a small volume size and in addition can handle a large number of samples. However, GC-MS requires derivatization to create a volatile compound, (non-volatile compounds are not derivatized and will not be detected, therefore limiting its applicability to non-volatile samples). In contrast HPLC does not require derivatization of the sample prior to analysis and can analyse non-volatile samples. This method is often used to characterize compounds and to provide structural information (Zhang et al., 2010).

2.6.2 Summary

In summary, TTO contains volatile components; furthermore the dialysate produced will be of small volume. GC-MS is a validated method often used to analyse essential oils including tea tree (Cox et al., 2001; Nielson et al., 2006). GC-MS requires only 1µl per sample and more significantly can accurately identify components and their abundance through peak identification through the chromatograph and mass spectra (Zhang et al., 2010). Therefore GC-MS was chosen as the method of analysis for dialysate and tape stripping samples. Following preparation of the samples by double liquid-liquid extraction 1µl of sample was placed into a glass vial and the sealed with a cap. This was positioned on the GC-MS with a blank sample, containing DCM only, included to follow each analysis of sample.

2.6.3 GC-MS Settings

Make and setting of GC-MS:
Column: Zebron ZB5-MS 30m X 0.25mm X 0.25 micron (Phenomenex).
Instrument; Thermo Trace GC-MS system with an Electron Ionisation source.

Temperature program:
Initial Temperature: 40°C held for 4 minutes increasing to 320°C at 10°C per minute
and then held at 320°C for 6 minutes.
Injector temperature: 220°C.
Helium carrier gas: a constant flow of 1ml/min.

The resulting mass spectra and chromatographs were analyzed using the on-line library attached to the mass spectrometer.

2.7 Conclusion

Chapter 2 has discussed and justified the methods chosen to identify components of tea tree oil absorbed to the dermal-epidermal junction following topical application. However, the validated methods require adaptation in order to allow for optimal recovery and identification of TTO components. The adaptation and subsequent validation of these methods to this series of studies is described in *Chapter 3*.

Chapter 3
Adaptation and validation of methods

3 Introduction

Chapter 2 presented discussion on the established methods to measure the transdermal absorption of topically applied substances. However, the complex nature of TTO (Hammer et al., 2004) requires adaptations to the chosen methods included in *Chapter 2* in order to thus ensure all components within the skin can be recovered and identified. *Chapter 3* presents adaptations to dermal microdialysis and GC-MS and subsequent validation of these methods.

3.1 Dermal Microdialysis

The successful recovery of substances following topical application using dermal microdialysis is dependent upon the manipulation of a number of variables identified in *table 3.1*. As TTO is a mixture of lipids and hydrophilic hydrocarbons and terpenes (Hammer et al., 2004), particular consideration is required regarding the transport of lipids not only across the membrane but also incorporation into the perfusate fluid, a challenge already documented by others (Benfeldt et al., 1999; Krielgaard, 2002; Sun and Stenken, 2003). *In vitro* microdialysis investigations are recommended to establish membrane efficiency and optimal conditions for recovery *in vivo* (Benfeldt et al., 1999; Holmgaard et al., 2010). Therefore a series of *in vitro* investigations were undertaken to identify optimal conditions to recover absorbed TTO components following topical application using dermal microdialysis.

Table 3.1 Factors influencing the relative and absolute recovery of a substance using dermal microdialysis

Membrane depth	(Schnetz and Fartasch, 2001)
Property of the membrane	(Schnetz and Fartasch, 2001)
Surface area of membrane exposed	(Groth, 1996)
Perfusate flow rate	(Kreilgaard, 2002)
Perfusate type	(Groth, 1996)
Physicochemical properties of the compound of interest	(Benfeldt et al., 1999)
Protein bound components	(Schnetz and Fartasch, 2001)
Temperature	(Kreilgaard, 2002)

3.1.1 Microdialysis membrane material

The hollow single fibre dialysis membranes used in the construction of dermal microdialysis membranes (*section 2.2.2)* are available commercially and are manufactured in a variety of materials and pore sizes (Schnetz and Fartasch, 2001).The correct choice of microdialysis membrane allows for selection and exclusion of particles of interest, for example a small cut-off, 2kDa, will allow the passage of low molecular weight substances, such as terpenes across the membrane. Furthermore, larger proteins such as albumin will be excluded, thus reducing the amount of preparation of the sample required prior to analysis (Groth, 1996), however, this will exclude protein bound molecules of interest. In comparison, larger molecules such as cytokines can be recovered when using microdialysis membranes with a 3000kDa cut-off, but are more prone to 'blocking' in use and will require preparation prior to some methods of analysis. The molecular weight of components of TTO range from 136-154MW (Fragrant Earth, Glastonbury), therefore, for the purposes of these investigations a membrane cut-off of 2kDa was chosen as it will selectively allow the smaller components of TTO passage into the dialysate whilst excluding larger proteins.

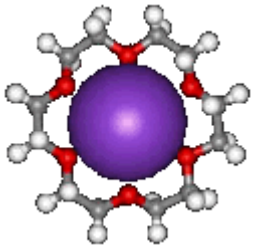
Dialysis membranes with a 2kDa cut-off are commercially available in the cellulose material cuprophan and haemophan. Cuprophan is an unmodified cellulosic dialysis membrane which has no charge. In comparison, haemophan is a modified cellulose membrane which is positively charged (membrana). The effect of both membrane materials on recovery of TTO components will be investigated.

3.1.2 Perfusate fluid

Previous studies demonstrate the difficulty of recovering lipids into perfusate fluid (Anderson et al., 1994; Muller et al., 1998; Kreilgaard, 2001). The membrane is continuously perfused with a physiologically compatible fluid to that which the membrane is placed (Holmgraad et al., 2010). Perfusate fluids previously utilized *in vivo* include Ringer's solution, Phosphate buffered Saline and 5% Glucose, all of which have a low solubility to lipids (Schentz and Fartasch, 2001; Kreilgaard, 2001). However, in recent years, attempts have been made to overcome this limitation. Perfusate has been modified using albumin and cyclodextrins with some success (Sun and Stenken, 2003;

Waleczek et al., 2003; Ao and Stenken, 2003; Fletcher and Stenken, 2008). Albumin has a molecular mass of 67kDa and would therefore be too large for use in these investigations as the membrane has a 2kDa cut off. Cyclodextrins are cyclic-oligosacharides which have the capability to form inclusion complexes with various organic molecules by capture of the guest molecule into a hydrophobic central cavity, its hydrophilic outer body allows its solubility in water as demonstrated *in figure 3.1* (Khramov and Stenken, 1999). Its cavity is a suitable size for common pharmaceutical drugs and molecular weights (200-800g/mol) (Waleczek et al., 2003).

A sub-group of cyclodextrin, which has been used in microdialysis investigations, is hydroxypropyl-β-cyclodextrin (HPβCD) (Fletcher and Stenken, 2008; Ao and Stenken, 2003). HPβ-CD has a molecular mass of approximately 1396 daltons (Sigmaaldritch.com) and would therefore be suitable for these investigations.



(AgricultureSource.com 2013)

Figure 3.1 Hydroxypropyl-β-cyclodextrin: demonstrating the encapsulation of a lipid substance as a guest molecule.

The lipid molecule is encapsulated into the lipophilic centre of HPβCD, the hydrophilic outer layer allows solubility within the perfusate and transport across the hydrophilic membrane. HPβCD has been successfully included in the perfusate of previous microdialysis experiments to ensure the transport of lipophilic moloecules across the hydrophilic microdialysis membrane (Ao and Stenken, 2003; Fletcher and Stenken, 2008).

In addition, HPβ-CD has a solubility in water of >500mg/ml at room temperature compared to the less soluble beta cylodextrin (β-CD), 18mg/ml (Cargill, North America). Fletcher and Stenken, (2008) demonstrated 0.5% HPβ-CD was as effective at recovery of the antibody, methionine-enkephalin, as 1% β-CD. HPβ-CD forms stable complexes with a wide range of drugs and has an

extensive collection of safety data with no adverse reactions (membrana). Therefore, for the present study, the effect of HPβ-CD on the recovery of lipid components of TTO will be explored.

3.1.3 Perfusate flow rate

The recovery of a substance of interest is dependent upon passive diffusion across the membrane (Benfeldt et al., 2007). The effect of perfusate flow rate on recovery of substance of interest is well documented (Kreilgaard, 2002; Incecayir et al., 2011). Whilst many studies demonstrate a slow flow rate of between 2-3µl/min provides optimal conditions for recovery (Incecayir et al., 2011; Benfeldt et al., 1999), others provide evidence that a flow rate of 5µl/min is sufficient for recovery (Kreilgaard, 2001). However, identification of recovered substance requires sufficient dialysate to be collected in order to be prepared and analysed. The length of time required to collect sufficient dialysate has implications regarding the acceptability of the method to the participant and also the physiological changes that occur due to the implantation of a foreign object (the membrane) in the skin (Groth and Serup, 1998). There is a need for balance between sufficient recovery and length of time required for collection of dialysate. The effect of perfusate flow rate on recovery of TTO components is not known, therefore a comparison of flow rates 3 and 5µl/min will be made.

3.1.4 Temperature

Temperature has been demonstrated to have an effect on recovery rate in microdialysis (Kreilgaard, 2001; Ao and Stenken, 2003), as it has on the solubility of HPβCD (Cargill, North America), TTO (Hammer et al., 2004) and blood flow *in vivo* (Schentz and Fartasch, 2001). Therefore all *in vitro* and *in vivo* experiments will be undertaken at room temperature in a temperature controlled laboratory.

In summary, recovery of a substance of interest using dermal microdialysis is dependent on a number of variables including perfusate type, rate, membrane material and temperature. *In vitro* studies are recommended prior to *in vivo* investigations to establish optimal conditions for successful recovery (Benfeldt et al., 1999). Therefore *in vitro* studies will be undertaken comparing the variables identified in *table 3.2*.

Table 3.2: Variables to be compared during *in vitro* microdialysis investigations

Membrane type	Haemophan	And	Cuprophan
Perfusion fluid	Phosphate Buffered Saline (PBS)	And	5% Hydroxypropyl Beta Cyclodextrin (HPBCD) in PBS (determined by in house study)
Flow rate	5µl/min	and	3µl/min

3.2 *In vitro* investigations to determine optimal conditions for the recovery of TTO components during *in vivo* microdialysis

All experiments were undertaken in a temperature controlled laboratory (21°C±2°C). Each experiment was replicated in triplicate. Equipment was assembled as in *section 2.3.3.*

Study 1: Dialysis efficiency for TTO using haemophan membranes, phosphate buffered saline with a perfusate fluid flow rate of 5µl/min.
Haemophan membranes were attached to the microdialysis pump as described in *section 2.2.3*, 1ml of phosphate buffered saline pH 7.4 (Tayside) was used as perfusate fluid.

The microdialysis membrane was initially perfused at a rate of 10µL/min and the membranes purged for 5min to check for membrane viability.
The bath was filled with 2ml of TTO and bath temperature taken. The bath was covered with parafilm to prevent evaporation.
The perfusate rate was then reduced to 5µl/min.

Samples were collected into weighed labelled glass amber vials at 30min intervals over 90min.
Samples were weighed to check efficiency of the pump.
Samples were then stored on ice until the end of the study and then stored at -20°C to await analysis.

Study 1 was replicated to incorporate each variable for comparison (*table 3.2)*.

3.2.1 Separation and Analysis

Samples were separated as in *section 2.4.3* with a double liquid-liquid separation using DCM and analysed using GC-MS, settings as in *section 2.6.3*. Samples were quantified using internal standards.

3.3 Results

Analysis with GC-MS demonstrated that many components of TTO were able to be recovered using *in vitro* microdialysis under certain conditions. However, consistently the components T-4-ol, 1,8 cineole, γ-terpinen and α-pinene were present under all various conditions in the *in vitro* studies. Therefore it was decided that a comparison of amounts recovered of these components would confirm optimal conditions for *in vivo* microdialysis recovery. The results are presented here.

Recovered amounts are expressed as the mean ± SEM, unless otherwise stated.

3.3.1 T-4-ol

The total mean amount of T-4-ol recovered using haemophan membrane with PBS as perfusate at a rate of 3μL/min was 173.06ng ±20.5 *(figure 3.2A)*. In comparison the mean total amount of T-4-ol recovered using cuprophan membrane at a rate of 3μL/min with PBS as perfusate was 470ng±190, thus demonstrating a 63% increase in recovery when using cuprophan membranes as opposed to haemophan with PBS perfusate at a rate of 3μL/min.

The total mean amount of T-4-ol recovered using haemophan membranes at a rate of 3μL/min and using HPβ-CD as perfusate was 511.04ng±54.6 compared to using haemophan membranes at a rate of 3μL/min and using PBS as perfusate which was 173.06g ±20.5. This demonstrates an increase of 66% in

recovery of amount of T-4-ol when using HPβ-CD as perfusate as opposed to PBS using haemophan membranes at a rate of 3μL/min.

The total mean amount of T-4-ol recovered using cuprophan membranes at a rate of 3μL/min using PBS as perfusate was 470.08ng±190. This is in comparison to using cuprophan membranes at a rate of 5μL/min and using PBS as perfusate, the mean total amount recovered was 319.35ng±71.41. This demonstrates a 32% increase in recovery of amount when using a flow rate of 3μL/min as opposed to 5μL/min.

The mean total amount of T-4-ol recovered using cuprophan membranes at a rate of 3μL/min with HPβ-CD as perfusate was 802.6ng±195.12. This is compared to using cuprophan membranes at a rate of 3μL/min with PBS as perfusate where recovery was 470.087ng±190. This demonstrates an increase of 41% in recovery of amount of T-4-ol when using HPβ-CD as perfusate in comparison to PBS when using cuprophan membranes at a rate of 3μL/min.

In summary, the largest amount of T-4-ol recovered was when using cuprophan membranes with 5% HPβ-CD in PBS as perfusate at a rate of 3μL/min. The mean total amount recovered was 802.6ng±195.12.

3.3.2 1,8 Cineole.

The mean total amount of 1,8 Cineole recovered using haemophan membrane at a rate of 3μL/min with PBS as perfusate was 3.48ng±1.11 (*figure 3.2B*) . In comparison the mean total amount of 1,8 Cineole recovered using cuprophan membrane at a rate of 3μL/min with PBS as perfusate which was 8.99±4,36, thus demonstrating a 61% increase in recovery of amount of component when using cuprophan membranes in comparison to haemophan with PBS perfusate at a rate of 3μL/min.

The mean total amount of 1,8 cineole recovered using haemophan membranes at a rate of 3μL/min and using HPβ-CD as perfusate was 20.69±2.03 compared to using haemophan membranes at a rate of 3μL/min and using PBS as perfusate which was 3.48±1.11, thus demonstrating an increase of 83% in recovery of amount of 1,8 cineole when using HPβ-CD as perfusate in comparison to PBS using haemophan membranes at a rate of 3μL/min.

The mean total amount of 1,8 cineole recovered using cuprophan membranes at a rate of 3μL/min using PBS as perfusate was 8.99ng±4.36. In contrast, cuprophan membranes at a rate of 5μL/min and using PBS as perfusate, allowed for the mean recovery of 5.2ng±1.78. This demonstrates a 42% increase in recovery of amount of 1,8 cineole when using a flow rate of 3μL/min in 5μL/min.

The mean total amount of 1,8 cineole recovered using cuprophan membranes at a rate of 3μL/min with HPBCD as perfusate is 29.33ng±9.33, in comparison to using cuprophan membranes at a rate of 3μL/min with PBS as perfusate where recovery was 8.99ng±4.36. This shows an increase of 69% in recovery of amount of 1,8 cineole when using HPβCD as perfusate in comparison to PBS when using cuprophan membranes at a rate of 3μL/min.

In summary the greatest amount of 1,8 cineole recovered was demonstrated when using cuprophan membranes with 5% HPβCD in PBS as perfusate at a rate of 3μL/min. The mean total amount recovered was 29.33ng±9.33.

3.3.3 Gamma-Terpinen. (γ-terpinen)

γ-terpinen was only able to be recovered when using HPβ-CD as perfusate so comparison of recovered amount of γ-terpinen is only able to be made between membrane material and flow rate.

The mean total amount of γ-terpinen recovered using haemophan membranes at a rate of 2μL/min with HPβCD as perfusate was 23.95ng±9.73 (*figure 3.3*), in contrast to cuprophan membranes at a rate of 2μL/min with HPβCD as perfusate where the recovered amount was 25ng±15.2, thus demonstrating an increase in recovery of 4% when using cuprophan as opposed to haemophan membranes.

The mean total amount of γ-terpinen recovered using cuprophan membranes at a rate of 5μL/min was 13ng±7.5. The mean amount of the γ-terpinen recovered using cuprophan membranes at a rate of 2μL/min with HPβ-CD as perfusate was 25ng±15.2. This demonstrates an increase of 48% in amount of γ-terpinen recovered when the perfusate rate is 3μL/min as opposed to 5μL/min when using cuprophan membranes with HPβ-CD as perfusate.

In summary, the largest amount of γ-terpinen recovered was demonstrated when using cuprophan membranes with HPβ-CD as perfusate at a rate of 3μL/min. The mean total amount recovered was 25ng±9.73.

3.3.4 α-pinene

α-pinene was unable to be recovered in quantifiable amounts.

3.3.5 Actual amount recovered

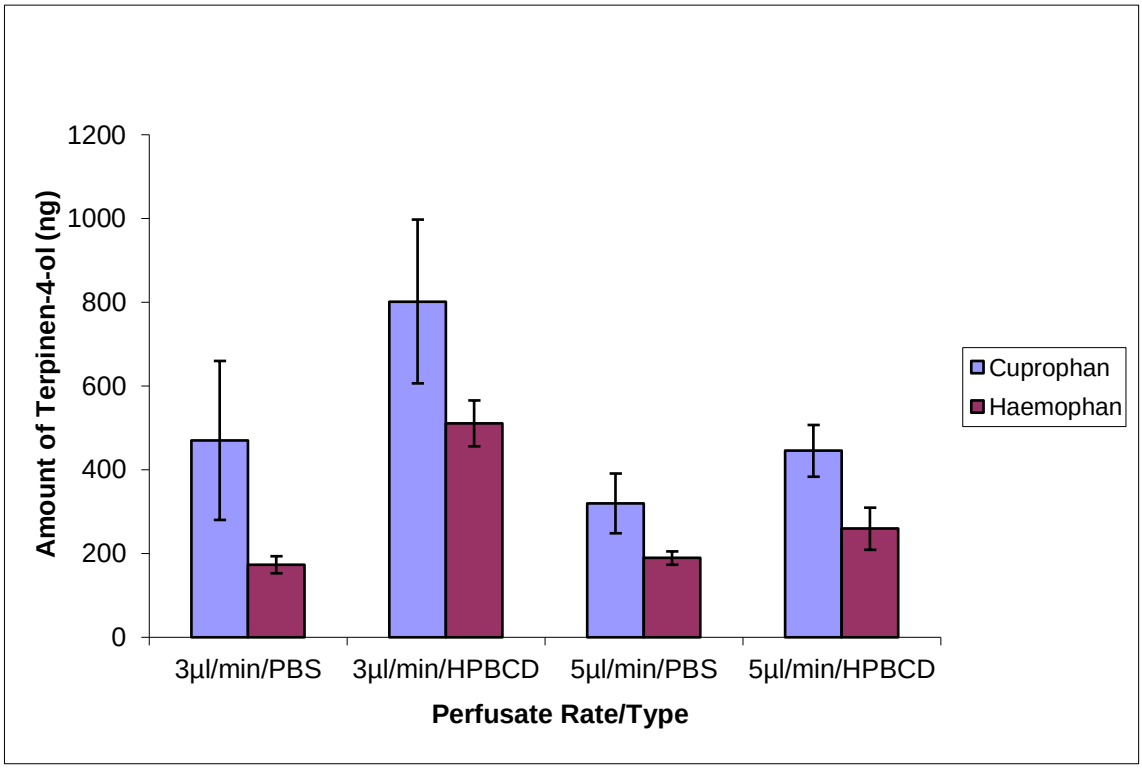
Analysis of the stock TTO used in these studies by GC-MS showed the amounts present for each component to be:

T-4-ol = 4978ng/μL
1,8 Cineole = 229ng/μL
γ-terpinen = 1155ng/μL

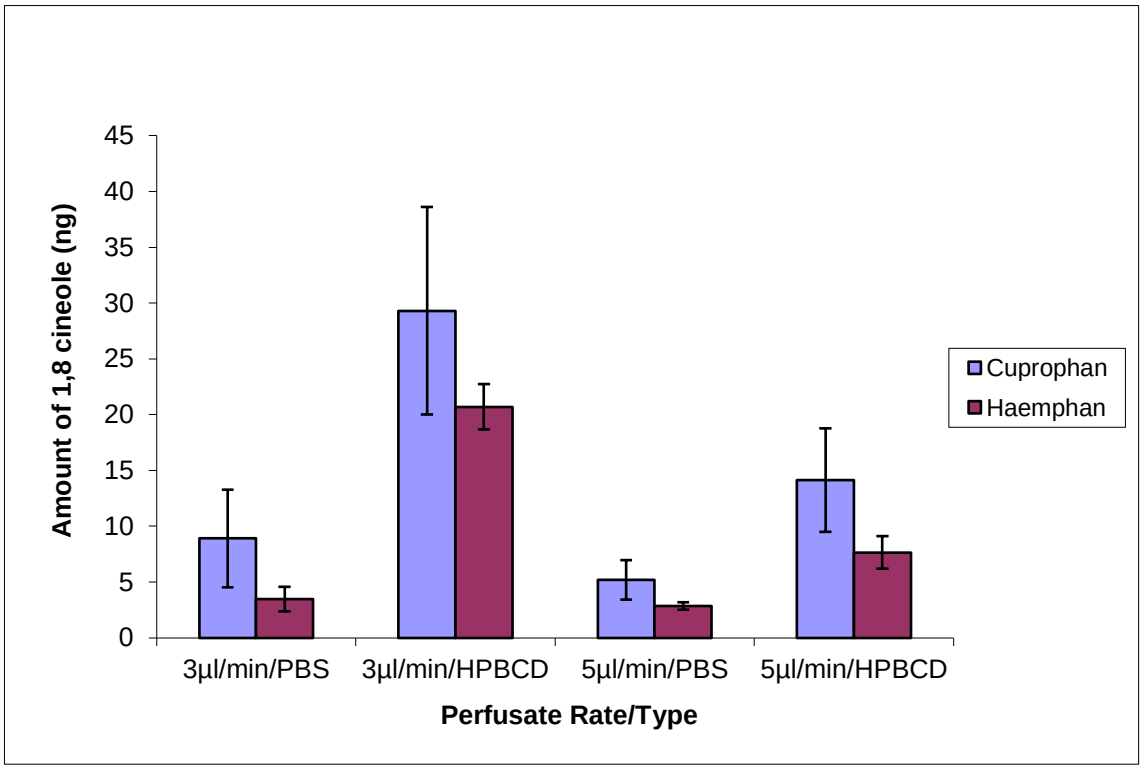
As not all components of the oil have been quantified, comparison of these measured amounts with ISO recommendations for TTO (Hammer 2006) is difficult. However when comparing the three components to each other, the percentages are in accordance with the ISO recommendations. T-4-ol quantified as the largest amount of those measured, γ-terpinen, 23% of the amount of T-4-ol and 1,8 cineole 5% of TTO.

3.4 Conclusion

These investigations demonstrate that TTO components can be successfully recovered using *in vitro* microdialysis. Recovery of TTO components has been shown to be enhanced under the following conditions: Cuprophan membrane with a 2kDa cut off, 5% HPβ-CD at a rate of 3μl/min. However, there are limitations when using microdialysis to recover TTO components, including that α-pinene could not be recovered in quantifiable amounts.



A



B

Figure 3.2 Total amount of T-4-ol (A) and 1,8 Cineole (B) recovered using dermal microdialysis under various conditions. It is consistently observed that the largest recovery is when using HPβCD as perfusate at a rate of 3µl/min with cuprophan membranes. Error bars represent the SEM. n=5.

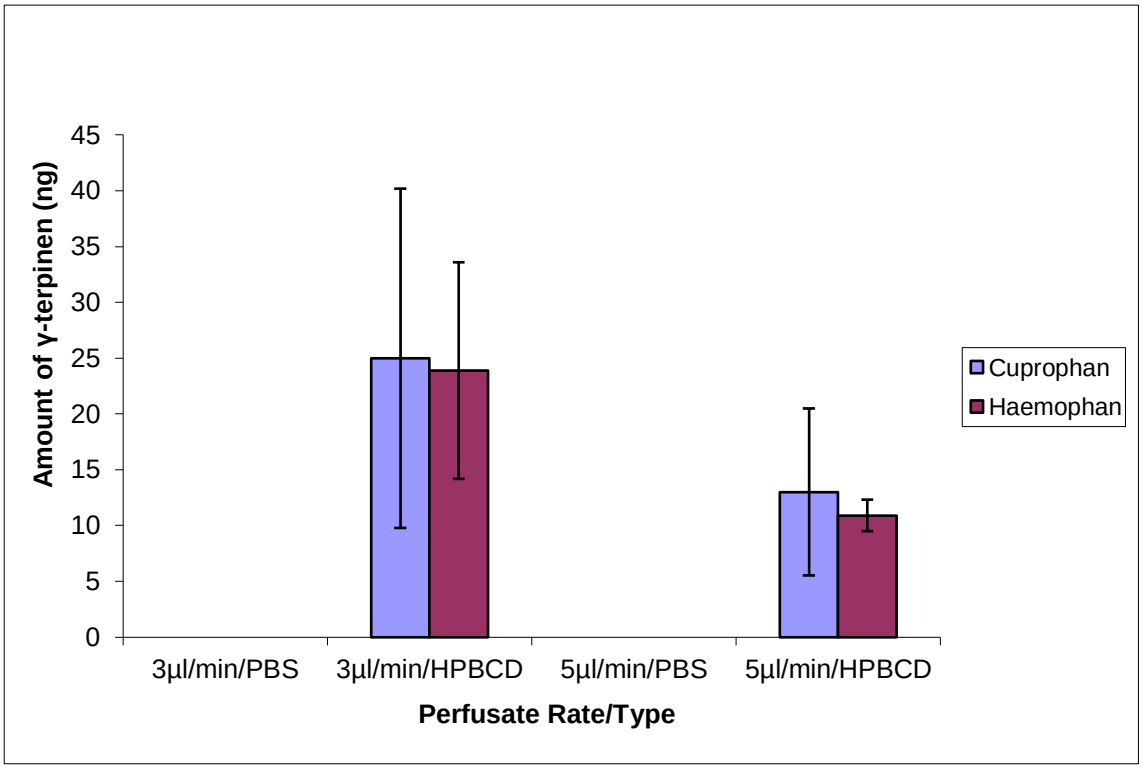


Fig. 3.3 Total mean amount recovered of γ-terpinen (ng) *in vitro* under different experimental conditions Error bars represent the SEM. n=5. γ-terpinen was unable to be recovered with PBS as perfusate. Optimal conditions for the recovery of Υ-terpinen *in vitro* are observed as; perfusate rate; 3µl/min, perfusate type; 5% HPβCD in PBS, membrane material; cuprophan.

3.5 Analysis of dialysate

3.5.1 Liquid-Liquid Separation

A double liquid-liquid separation technique as described in *section 2.4.3* was chosen to prepare dialysate samples for GC-MS analysis. A limitation of this method is the potential loss of substance of interest due to evaporation of the solvent and the level of solubility of the substance of interest within the solvent (Muller et al., 1998). Therefore a series of investigations were undertaken to determine a solvent which would conserve the most components of TTO during GC-MS.

Three solvents immiscible with water, therefore potentially able to extract organic components into the solvent phase were chosen:

- Chloroform ($CHCL_3$),
- Diethylether ($C_4H_{10}O$)
- Dichloromethane (CH_2CL_2).

All studies were undertaken in a fume hood in accordance with Good Laboratory Practice (GLP) due to the toxic fumes emitted by the solvents.

Previous in house microdialysis efficiency studies allowed for the estimation that *in vivo* dialysate will contain 5% of components of TTO. This was therefore mimicked in the controls.

3.5.2 Controls

i. 1ml Chloroform + 0.05ml TTO
ii. 1ml Diethylether + 0.05ml TTO
iii. 1ml Dichloromethane + 0.05ml TTO

Each control was analysed by GC-MS to determine the effect of each solvent on the TTO components. This was then compared with samples 1, 2 and 3 below following analysis:

In vivo dialysate will contain 5% (w/v) HPβ-CD in PBS. A solution was produced that mimicked the anticipated components of the expected *in vivo* dialysate;

3.5.3 Dialysate:

4ml PBS + 0.2g HPβ-CD (5%) +200µL of TTO (5%)

A liquid-liquid extraction was undertaken with each solvent.

1. 1ml dialysate solution + 1ml Chloroform.
2. 1ml dialysate solution + 1ml Diethylether.
3. 1ml dialysate solution + 1ml Dichloromethane.

The resulting sample was then compared with samples i, ii and iii.

3.6 Results

The initial chromatographs produced were inconclusive; the bases of the peaks were wide and inconsistent. A possible explanation was that the butyl rubber seals of the lids of the amber glass vials reacted with the solvent impacting the results. New PTFE/Silicone inert seals were sourced and the process repeated. Clearer chromatographs were obtained as there was no solvent reaction.
It was demonstrated that a number of identifiable TTO components were able to be conserved with all solvents used, as shown in *table 3.3*.

Table 3.3 Number of identifiable peaks shown on a chromatograph following extraction by different solvents

Solvent	Number of identifiable components
Chloroform	12
Dichloromethane	8
Diethylether	4

Diethylether was disregarded due to the low number of identifiable peaks and that due to the volatility of the solvent an accurate chromatograph was unable to be achieved.

Separations using chloroform and dichloromethane (DCM) produced similar good quality chromatographs, providing peaks with small bases. The chloroform chromatograph preserved the greatest number of components (*table 3.2*), however, following a risk assessment and GLP it was decided that although separations were always performed in a fume hood, DCM would be used as it is much safer than chloroform.

The identifiable peaks on the chromatograph using DCM included T-4-ol, 1,8 cineole, α-pinene and γ-terpinen.

3.7 Gas Chromatography-Mass Spectrometry (GC-MS)

GC-MS as described in *section 2.6* has the capacity to identify and quantify volatile components from a small volume of sample. The chromatograph produced following analysis includes peaks at specific retention times, this combined with information regarding its mass spectrum leads to the identification of the substance. In addition, the peak allows for quantification of the substance. However, to do this it is essential to:

- Identify components of neat TTO following analysis with GC-MS
- Identify components of TTO within a dialysate sample following GC-MS
- Quantify the amount of components of TTO present in the sample
    - Create an internal standard for each component of TTO identified
    - Create a standard curve for each component of TTO identified

The investigations undertaken to confirm the identity and quantification of TTO in dialysate are presented here:

3.7.1 Identify components of neat TTO following analysis with GC-MS

A sample of neat TTO was injected into the GC-MS, set as in *section 2.6.3*. However, the resulting chromatograph was unable to be deciphered due to the sample being too concentrated. Therefore a dilution of 1/200 (v/v) (TTO in DCM) was injected into the analyser as before (*figure 3.4*). Each peak on the chromatograph represents an individual component of TTO. TTO components were easily identified with the aid of the mass spectra and the online library attached to the GC-MS, demonstrating GC-MS to be an appropriate method to analyse this oil.

3.7.2 Quantification of TTO components using an internal standard

The peaks produced on a chromatograph following analysis of a substance can be used to quantify the component of interest. To define an internal standard the area under the peak (AUP) was calculated and compared with a peak of a known amount of the component.

The above is repeated with various known dilutions of the component (e.g. 100ng/ml, 50ng/ml) thus allowing a standard curve to be produced from which calculations of the weight of a component in an unknown sample can be made (*figure 3.5).*

There are a number of methods to calculate an internal standard. These include radiolabelling a known weight of the component, allowing the peak to be easily identified against a non-radiolabelled component (Unice et al., 2012). The AUP can be calculated and a standard curve created. Whilst this method enables accurate quantification of the component, it is expensive and furthermore, carries health and safety risks due to radioactivity. An alternative is to purchase individual components of interest of a known weight and create a serial dilution, for example 100ng/ml, 50ng/ml, 25ng/ml, etc. The dilutions are injected into the GC-MS for analysis thus producing chromatographs from which standard curves can be created (*figure 3.5*). This method accurately quantifies individual components and not only carries less health and safety risks but is also less expensive than the former. Therefore standard curves for these investigations were created using this method.

3.7.3 Identification of key components to be used as internal standards

TTO consists of over one hundred components (Hammer et al., 2004), however to quantify all of these would be time consuming and not necessary for this study. *In vitro* microdialysis studies presented in this chapter (*secton 3.2*) identified the components T-4-ol, 1,8 cineole, α-pinene and γ-terpinen to be consistently present within the dialysate. These components are a mixture of hydrophilic and lipophilic components and therefore represent similar physicochemical properties to the remaining TTO components. Accordingly these components were chosen to be used as internal standards for these investigations.

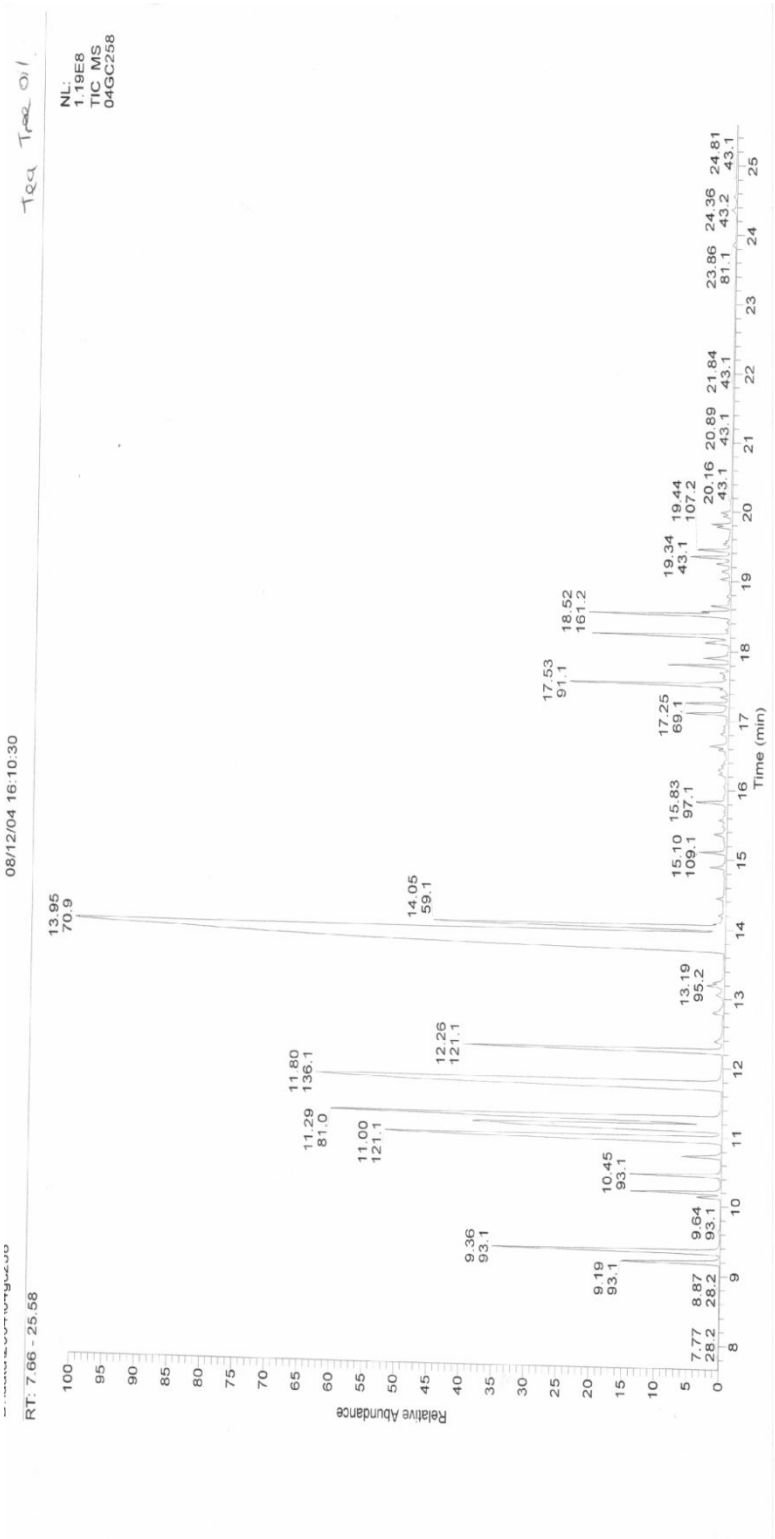


Figure 3.4 Chromatograph produced following GC-MS of a 1/200 dilution of neat TTO in DCM.

Each peak represents a component of the oil. The area under the peak is measured to enable quantification of the component. In this chromatograph T-4-ol is represented at 13.95min. This is the highest peak and so represents the most abundant component of TTO.

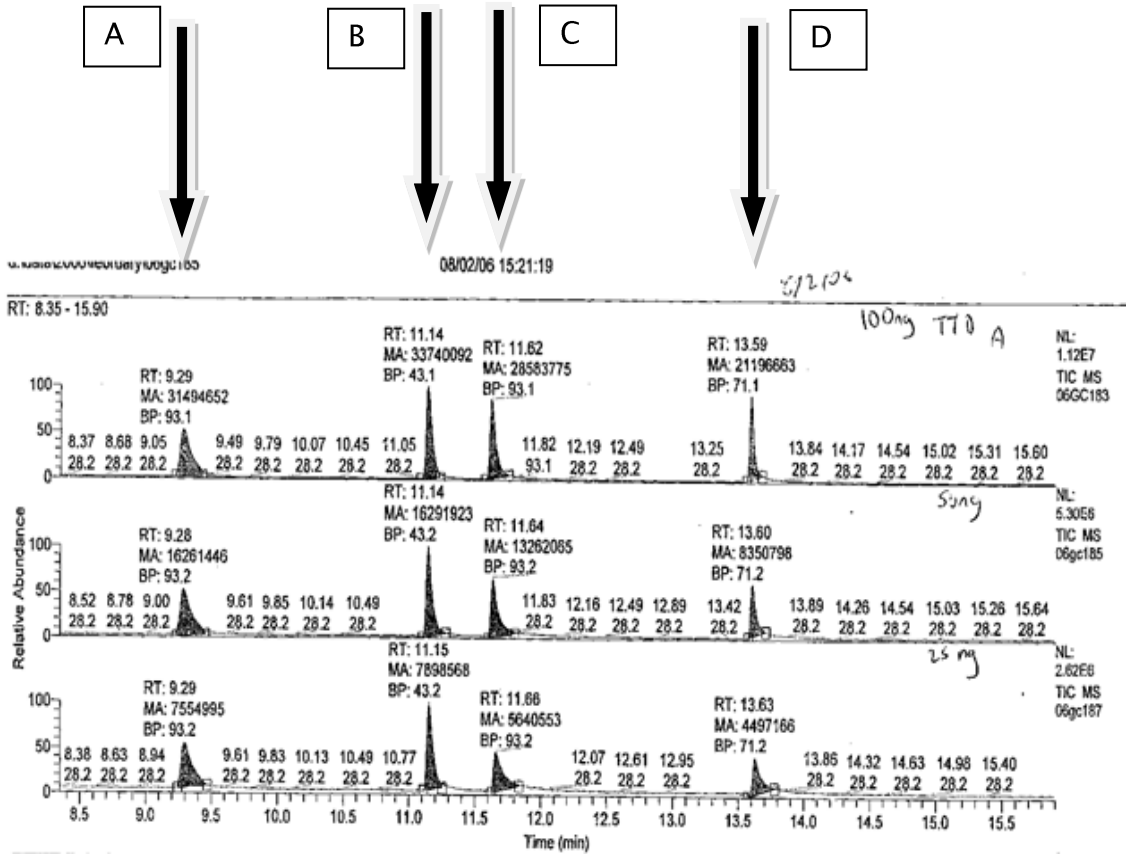


Figure 3.5. Chromatograph following GC-MS of equal amount solution of 4 internal standards.

A = α-pinene

B= 1,8 Cineole

C= γ-terpinen

D= T-4-ol

Each row represents (from the top) 100ng/ml, 50ng/ml and 25ng/ml of component in each sample. The peaks show the retention time of the component on the GC-MS column (RT) and the AUP displayed as MA above each peak. The information gathered from the chromatographs following analysis of the serial dilutions allows for the creation of a standard curve from which the amount of an unknown component included in a dialysate sample can be calculated.

3.7.4 Creating a standard curve for components of TTO analysed using GC-MS

The creation of a standard curve requires a solution of known amounts of the components chosen as internal standards. The chosen internal standards for these investigations are T-4-ol, 1,8 cineole, γ-terpinen and α-pinene (*section 3.7.3).*

3.7.5 Creating a solution of equal weights of internal standard

Each component of TTO exhibits a different weight, for example, 1ml of 1,8 Cineole contains 0.9210g of the component 1ml of T-4-ol contains 0.929g of the component (Fragrant Earth, Glastonbury). Therefore to create an accurate serial dilution a stock solution of equal weights of all four internal standards is required. A calculation was made to determine the amount of fluid, in each case, that would equate to 1g of each component. The calculation was made thus (*equation* 4):

$$\frac{1}{y} = x \qquad (4)$$

y = amount of component in 1ml (as stated by manufacturer)
x = amount of solution in ml containing 1g of component

Therefore, in the case of 1,8 cineole:
1ml of solution contains 0.9210g of 1,8 cineole as shown in *equation 5*.

$$\frac{1}{0.9210} = 1.086\text{ml} \qquad (5)$$

Therefore 1.086ml of 1,8 cineole contains 1g of the component.

This equation was repeated for each of the three remaining internal standards. Next, 1g of each internal standard was mixed together to create an equal amount solution. This was then applied to the GC-MS. However, the solution was too concentrated for this method, the chromatograph was hard to decipher and thus a dilution was made with DCM to create a stock solution containing 100ng each of the components.

Furthermore, a serial dilution was made using DCM, the seven dilutions ranged from 100ng – 1.625ng. The dilutions were subsequently analysed in duplicate by GC-MS.

Figure 3.5 displays the chromatographs produced following analysis of the diluted internal standards. The AUP for each peak was calculated using the online program attached to the GC-MS. The standard curve was calculated by comparing each component the AUP for each dilution and plotting a graph using SPSS (v19, IBM) (*figure 3.6*). A linear regression line was imposed upon the graph and the calculation of an unknown amount of component in the dialysate was calculated (*equation 6*). The AUP of the unknown component is included in the calculation and according to where the AUP lies on the regression line equates to the amount of component in the dialysate. The equation below is that created following analysis of T-4-ol at various known dilutions:

$$\boldsymbol{y = 248914 \times -1000000} \qquad \textbf{(6)}$$

y = the amount of T-4-ol in the sample in ng
x = the AUP of the unknown amount of T-4-ol

To calculate the unknown amount of T-4-ol in dialyste the equation (7) requires rearrangement:

$$\boldsymbol{\frac{\times -1000000}{248914} = y} \qquad \textbf{(7)}$$

Therefore, a sample of dialysate containing a peak at a retention time known to be that of T-4-ol the AUP calculated is 147496717 (*figure 3.7*). This is included in the equation (8):

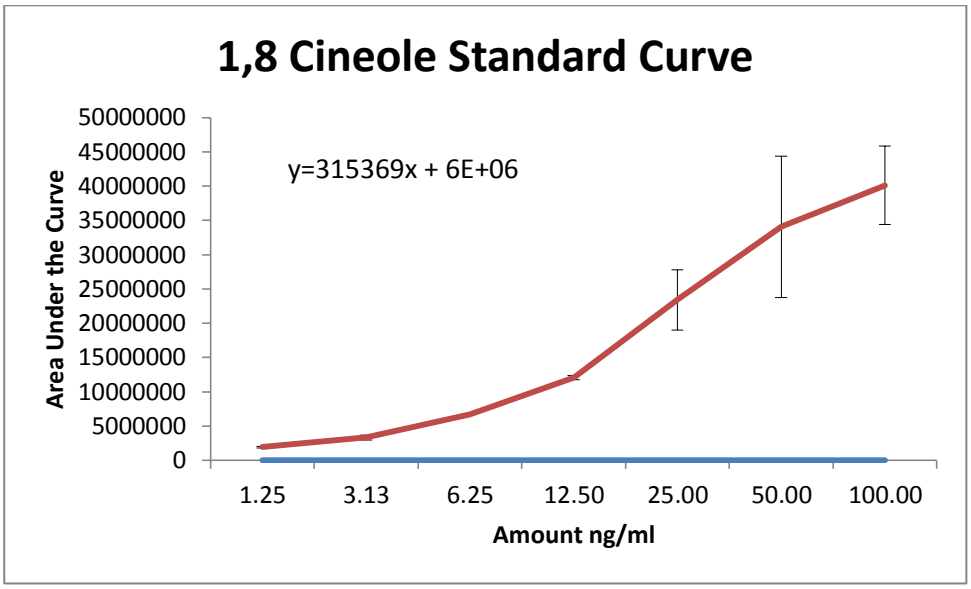
$$\boldsymbol{\frac{147496717 - 1000000}{248914} = 596.6} \qquad \textbf{(8)}$$

Therefore the amount of T-4-ol present in the dialysate would be 596.6ng.

3.8 Conclusion

In summary the methods chosen for identification and quantification of TTO components following topical application in *Chapter 2* are already established and validated. However, limitations of dermal microdialysis have meant adaptations to allow for the recovery of TTO components is required. In recent years attempts have been made to overcome the limitations of this method, including manipulation of perufsate fluid, flow rate and membrane material. This chapter has discussed particular adaptations to enhance the recovery of TTO components resulting in the identification of optimal conditions for *in vivo* dermal microdialysis for the recovery of TTO components. These studies have informed the protocol for *in vivo* microdialysis within this thesis. Furthermore, for the accurate identification and quantification of components recovered in dialysate following analysis using GC-MS, subsequent examination was required. Four TTO components were chosen as internal standards and standard curves were subsequently developed thus allowing for the quantification of TTO components recovered during *in vivo* microdialysis studies presented in *Chapter 4*.

A



B

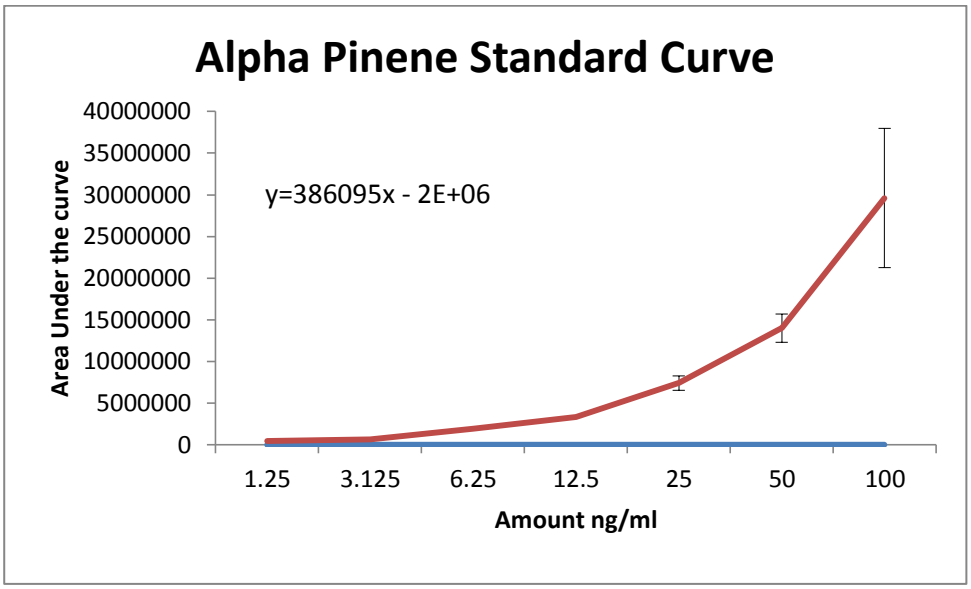


Figure 3.6 Two examples of Standard Curves produced using the computer program SPSS following analysis by GC-MS of the internal standards, 1,8 cineole (A) and α-pinene (B) at varying weights.

Each point corresponds with a measured area under the curve from the peak on the chromatograph (figure 3.6). An equation is produced from the curve which can then be used to calculate the unknown amount of the component of TTO in a dialysate sample. The calculations are shown on the respective graphs (2E+06 is equal to 2000000) (n=4).

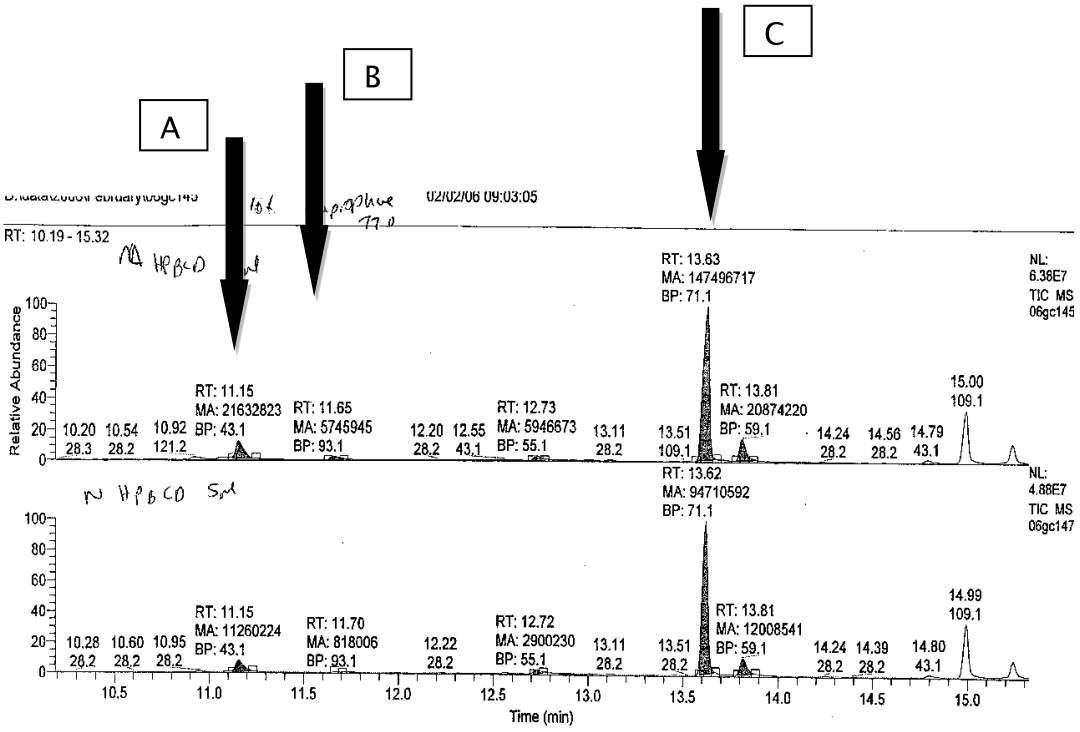


Figure. 3.7 Chromatograph of microdialysate sample from *in vitro* experiment following analysis by GC-MS containing an unknown amount of TTO component.

The labelled arrows identify each component thus:

A = 1,8 Cineole

B = γ- terpinen

C- T-4-ol

The chromatograph shows the area under the peak in arbituary units, labelled as MA, which can be calculated to demonstrate the amount of component in the oil.

Chapter 4
The transdermal absorption of tea tree oil

4 Introduction

The benefits of TTO as an anti-bacterial, anti-fungal and anti-inflammatory agent have, until recently, been based on anecdotal evidence. However, empirical research is emerging that supports this oil as a beneficial product *in vivo* and *in vitro* (Caldefie-Chezet et al., 2004; Dryden et al., 2004; Hart et al., 2000). Within the UK TTO is predominantly applied topically, the benefit of this includes that often the target organ for TTO is the skin. Furthermore, the direct application of the oil to the skin reduces the amount of oil present systemically, therefore minimising side effects (Satchell et al., 2002a). However, a primary function of the skin is to act as a barrier to exogenous agents (Thody and Friedmann, 1986). The SC provides the majority of the skin's barrier, the penetration of which is essential for TTO to exert its effect (Hammer et al., 2006). To date there is limited evidence to demonstrate the transdermal penetration of TTO components following topical application, the majority being *in vitro* investigations and exploring only one or two components (Cal and Krzyzaniak, 2006; Reichling et al., 2006; Cross et al., 2008). The physiological effects of the skin, for instance, tortuosity and blood flow, on penetration of topically applied substance is difficult to replicate *in vitro*, therefore to accurately measure the transdermal penetration of TTO *in vivo* investigation is required (Reichling et al., 2006).

The measurement of the absorption of substance through the skin *in vivo* has long been a challenge (Cross et al., 2008, Reichling et al., 2006). Established methods including skin blisters, skin biopsies and confocal microscopy have limitations, as discussed in *Chapter 2 (section 2.1*). Dermal microdialysis, despite limitations, has been demonstrated to be able to recover components of TTO *in vitro* (*Chapter 3*). More significantly this is a minimally invasive technique which is often used successfully *in vivo* to identify the presence of exogenous and endogenous agents within the epidermis and dermis (Morgan et al., 2003, Sjogren et al., 2012).

Following *in vitro* investigations discussed in *Chapter 3* a protocol for *in vivo* dermal microdialysis for the recovery of TTO components at the dermal/epidermal junction was devised, the results of which are presented in this chapter. Furthermore the presence of TTO components present within the

SC will also be investigated utilizing the tape stripping method as discussed in *Chapter 2 (section 2.5*).

4.1 The Skin Barrier and Percutaneous absorption of substances

The SC forms the main barrier of the skin (Bronaugh and Maibach 1990). Despite providing an excellent barrier, the skin is permeable to some substances, particularly lipophilic, but is also permeable to some hydrophilic substances (Williams and Barry, 2004). There is much debate in the literature as to the formation of the skin barrier and how substances penetrate the skin (Kitson and Thewalt, 2000; Kasting et al 2002).

It is considered that there are four routes of percutaneous penetration, intra-cellular (through the corneocytes), inter cellular (through the lipid bilayers), through the sweat glands and through the hair follicles and sebaceous glands (Kitson and Thewalt, 2000). However, it is generally accepted that the intercellular route is the major pathway for most drugs (Hadgraft and Guy, 2004).

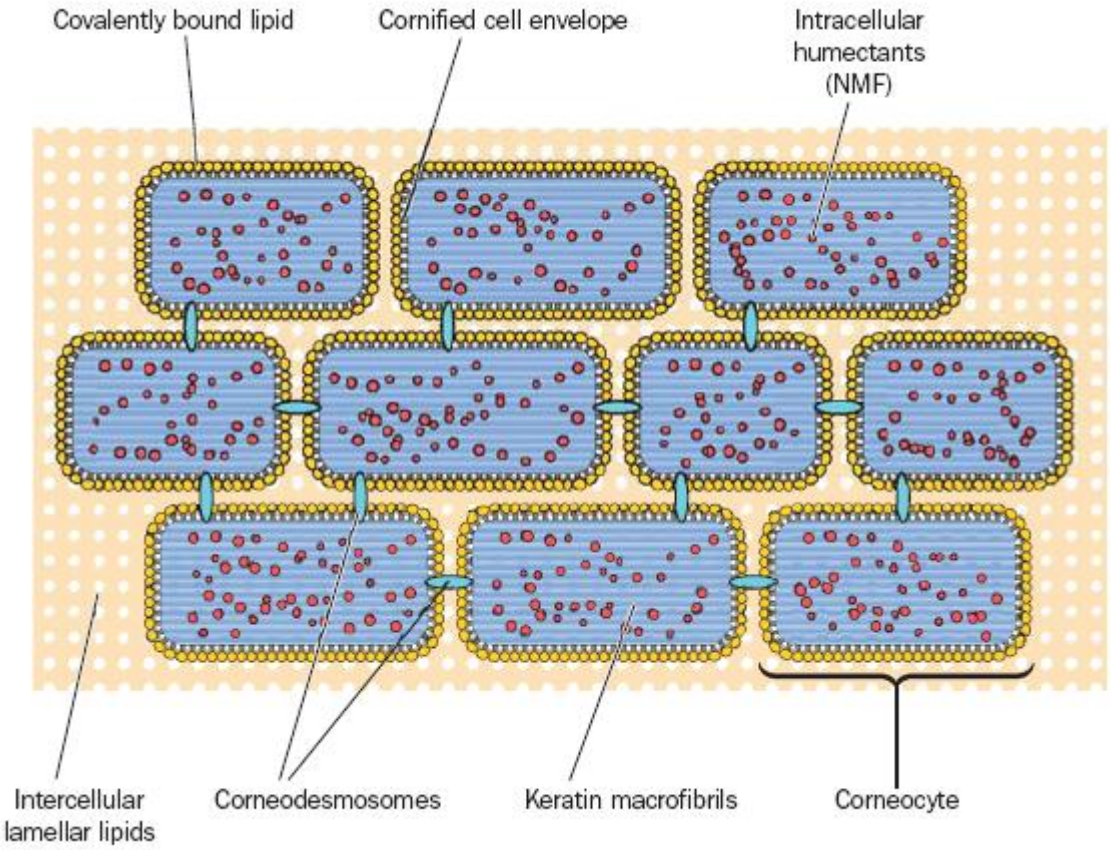
The SC consists of corneocytes surrounded by a lipid bilayer containing ceramides, cholesterol and free fatty acids (Bouwstra et al., 2000). Elias (1983) suggests a bricks and mortar model, the corneocytes being the bricks and an intracellular water-lipid mixture is the mortar (*figure 4.1*). The lipid formation and arrangement has long been debated and theories refined. Forsalind (1994) modified Singer and Nicholson's fluid mosaic model (1972) by suggesting a domain mosaic model for the structure of the intracellular space. He suggests the lipids segregate in such a way that the long chain lipids appear in crystalline or 'gel' domains separated by 'grain borders' of short chain lipids in the liquid crystalline state. Further modifications of this theory are continually discussed in the literature (Kitson and Thewalt, 2000; Bouwstra et al., 2000).The lipids form a bilayer with lipid tails and polar heads (Williams and Barry, 2004).

4.1.1 Permeation of substances through the skin

There are four principles of cutaneous drug delivery:

- Diffusion of substance in delivery vehicle to the skin vehicle interface
- Release of the solute from the topical vehicle
- Absorption into the skin
- Diffusion of substance into systemic circulation. (Simonsen et al., 2002).

There are many factors that can affect percutaneous absorption including the molecular size, water/lipid partition coefficient, ionisation of the test compound, formulation solubility and polarity and volatility and concentration. Distribution in SC, temperature and circulatory effects, condition and hydration of the skin, application dose and duration of exposure of test compound can all effect percutaneous absorption.



(Elias, 1983)

Figure 4.1 'Bricks and Mortar' Model of the SC.

Elias (1983) compares the corneocytes within the SC as being the bricks and an intercellular water-lipid mixture as the mortar, when describing possible routes of percutaneous penetration.

Although there are a number of percutaneous absorption rate limiting factors it is thought that the overall rate determining barrier to percutaneous absorption of hydrophilic and lipophillic substances is the SC (Simonsen et al., 2002). This is further visualised in *figure 4.2.*

Skin Layers	Loss Processes
Vehicle	Surface Loss
_____Partition_____	
SC	Irreversible binding and Metabolism
_____Partition_____	
Viable epidermis	Metabolism
Dermis	Drug removal into circulation

(Bronagh and Maibach 1989)

Figure 4.2 Factors limiting percutaneous absorption of substances

4.1.2 Diffusion of Molecules across the SC

Delivery of a substance through the skin depends on the diffusion and partitioning of the molecule across the SC and viable epidermis. Fick's First Law of diffusion can be adapted to describe permeablility of a compound through the skin and can describe the steady state flux of the compound per unit area (*equations 9 and 10*).

J=KD(Capp – Crec)/h **(9)**

J=Steady State Flux
K=Partition of permeant between skin and applied formulation
D=Diffusion coefficient
h= intracellular channels of diffusional path length
Capp= applied concentration of permeant
Crec= concentration of permeant in receptor phase.

This can be simplified to;

$$J=KpCapp \quad \textbf{(10)}$$

Kp= (KD/h) = permeability coefficient.
(Hadgraft 2004).

This, however, is a very simplistic model and does not take into consideration the complex nature of the skin. Compounds from a drug formulation rarely reach steady state in the skin due to, for example, tortuosity within the skin and protein binding of the drug (Hadgraft and Guy, 2004).

4.1.3 Theories of permeation of compounds into the skin

It is often presumed that lipids easily penetrate the skin and that the skin is virtually impenetrable to hydrophilic substances (Kitson and Thewalt, 2000). Lipids are thought to either penetrate rapidly through the epidermis or accumulate in the upper layers of the SC like a reservoir, and can remain here for days (Bronagh and Maibach 1989). Although hydrophillic substances are not as penetrable, they may be under certain conditions. Well hydrated skin can aid permeation. Water is typically 15-20% of dry tissue. In cases of excessive humidity, water level in the SC can reach equilibrium with that of the underlying epidermal cells, 25-30% is 'bound' structurally in the SC the rest being 'free'. It is thought hydrophilic substances can penetrate through the 'free' water (Williams and Barry, 2004). Occlusion of a test compound by a hill top chamber can increase hydration by preventing trans-epidermal water loss and could therefore increase permeation of hydrophilic substances (Bronagh and Maibach 1989). Williams and Barry (2004) highlight that the mechanism of action by which water increases transdermal delivery is unclear. It is suggested that swelling of the SC could cause disruption of domains possibly by swelling the polar head group region of the bilayers.

Barry (1991) suggests a lipid-protein partitioning theory where substances act by altering skin lipids and proteins and can affect their partitioning behaviour, allowing hydrophilic substances for example to penetrate through the SC. Kitson and Thewalt, (2000) suggests the epidermal barrier is a 'porous medium' and that there is a percolating pathway across the SC. They suggest

that there are interconnected regions of fluid intracellular lipid membranes and permeable associated inter-bilayer space.

Bos et al., (2000) suggests that permeation of a substance through the skin is determined by its molecular weight, and describes a '500 Dalton rule'. Through observation it was noticed that the majority of allergens and drugs known to penetrate the skin are all less than 500 Dalton. Further experimental work appears to uphold this theory that a substance must be below 500 Dalton to penetrate the skin.

It is suggested that a substance with a log partition coefficient of between 1-3 will penetrate the SC most easily (Hadgraft and Guy, 2004). Log P is the logarithm of the octanol water partition coefficient, it shows the hydrophillicity and lipophilicity of a compound. The greater the log P value the more lipophilic the substance. A log P of 1-3 would represent a hydrophilic compound; this, therefore, is surprising as it is thought that a more lipophilic substance would transport more easily through the skin (Cross et al., 2008).

4.1.4 Penetration enhancers

Much has been written on the effect of penetration enhancers (Aquil et al., 2007; Williams and Barry, 2004; Magnusson et al., 1997). A penetration enhancer can be a chemical substance or a mechanical process that decreases the barrier function of the skin. Chemical enhancers act by interacting with the layers of the SC either by interaction with the lipid organisation or intracellular proteins. Physical enhancers, for example, ionotophoresis, use a moderate voltage charge across the skin in order to enhance penetration of ionised substances (Bronagh and Maibach 1989). There are many different penetration enhancers that can be used including alcohol, water and terpenes (Williams and Barry, 1992). The enhancers appear to act on different pathways through the skin. The focus here will be on terpenes because this is a major component of tea tree oil (Hammer et al., 2006). In a study to enhance the penetration of the hydrophilic drug 5-fluorouracil through excised human skin, terpenes were used (Williams and Barry, 1992). It was seen that hydrocarbon terpenes were less potent than alcohol or ketone containing terpenes. The greatest enhancement was seen using 1,8 cineole and terpinen-4-ol (terpenes found in tea tree oil) but when using the same terpenes to enhance the passage of the

lipophilic estradiol the enhancers were only moderately active. The study suggests that the non-polar hydrocarbon terpenes are better at providing enhancement for lipophilic groups and the polar terpenes provide better enhancement for hydrophilic compounds. It is suggested that terpenes act by disrupting the lipid structure of the SC thereby increasing the diffusion coefficient of the polar drug in the membrane. Williams and Barry (1992) suggests that terpenes do not increase the partitioning of the drug into the SC but that they work by increasing diffusivity via SC disruption.

A small angle x-ray diffraction study carried out later by the same author supports this earlier finding by showing that 1,8 cineole disrupts the bilayer lipids, allowing penetration of the hydrophilic substances. The same study suggests that long chain sesquiterpenes reinforce the bilayers by orientating alongside the SC lipids, thus enhancing the penetration of lipophillic substances (Williams and Barry, 2004).

A penetration enhancer should have no pharmacological effect and should only aid penetration of a desired substance (Williams and Barry, 2004). The literature suggests that the terpenes terpinen-4-ol and 1,8 cineole have anti-bacterial and anti-inflammatory effects (Hammer et al., 2002; Koh et al., 2002). This highlights the need for further investigation into the effects of these terpenes and their suitability as penetration enhancers in pharmacological and cosmetic products (Williams and Barry, 2004).

The mechanism by which hydrophilic substances penetrate the skin are not fully understood (Kitson and Thewalt, 2000). There are presumptions that the lipids of tea tree oil will penetrate straight through the barrier of the skin and into the viable epidermis and dermis (Calcabrini et al., 2004), however, noted earlier, this may not be the case with the lipids partitioning into the lipid bi-layers of the SC and remaining there for days (Bronagh and Maibach 1989).

4.2 Dermal microdialysis to identify and quantify the presence of TTO components at the dermal-epidermal junction

4.2.1 Aim

To identify and quantify TTO components present at the dermal-epidermal junction following topical application of neat TTO.

4.2.2 Objectives

- To identify components of TTO present at the dermal-epidermal junction *in vivo* using dermal microdialysis.
- To quantify components of TTO present at the dermal-epidermal junction.

4.2.3 Subjects

Ten healthy subjects male and female (mean age 41years ± 20) were recruited following ethical approval from Southampton and South West Hampshire Ethics Committees B (06/Q1704/2). All were aged between 16-65 years, male and female, absent of active skin disease, not taking medication that may affect the cardiovascular system, not taking non-steroidal anti-inflammatory drugs (NSAIDS), not pregnant and non-smokers.

4.2.4 Study Protocol

The study was an open labelled, placebo controlled design which required each subject to attend the clinical research facility on one occasion.

All participants arrived for their study visit at 09.30hrs. The study was undertaken in a temperature controlled environment (22°C ± 2°C). Participants were asked to refrain from drinking caffeinated drinks and taking part in vigorous exercise for 12 hours prior to the study and throughout the duration of the study.

On arrival written informed consent was given by the participant. Two areas of local anaesthetic cream (EMLA) were applied and occluded as described in *section 2.3.1*. The volunteer was free to leave the facility for 90min. On return

four cuprophan microdialysis membranes with a molecular cut off of 2kDa were inserted as described in *section 2.3.2*, two in each anaesthetised area. The membranes were secured and the area lightly bandaged and the participant was allowed to leave the research facility once again for a period of two hours in order to allow for the effects of the local anaesthetic cream to diminish and any insertion trauma to subside (Petersen et al., 1994).

On return to the facility the participant acclimatised to the environment by lying supine on a bed, head slightly raised with a pillow for 15min. The membranes were then attached to the microinfusion pump (CMA 400, Sweden), which had been previously assembled as described previously (*2.3.3*), the 1ml syringe containing 1ml PBS + 5% HPβCD. The pump was started at a rate of 5µl/min for 10min to ensure that the membranes were patent.

0.5ml TTO was then applied to a hill top chamber (25mm, Kraton) and positioned centrally over one pair of the membranes and occluded using opsite dressing (Smith and Nephew). A hill top chamber was applied over the remaining pair of membranes and occluded as before; this acted as control.

The rate of perfusion was then reduced to 3µl/min, dialysate was collected from each membrane into an amber glass vial (Fisher, UK) over a period of 90min. The dialysate was stored on ice for a maximum of 30min before separation and analysis or storage at -20°C.

The membranes were disconnected from the microinfusion pump, the hill top chambers removed and the area lightly washed prior to the 2D ultrasound scan to assess membrane depth.

4.2.5 Confirmation of microdialysis membrane depth

Depth of microdialysis membrane has been proven to have an effect on recovery rate in microdialysate, a greater depth leads to a lower recovery rate (Andersson et al., 1996). Following completion of the dialysate collection, the membrane was disconnected from the microinfusion pump, the hill top chamber removed and the area gently cleaned with water. A 2D ultra sound scan was then made to measure the depth of the position of the membranes (Dermascan C version 3, Cortex Technology, Denmark). *Figure 4.3* presents a

scan image taken following the *in vivo* microdialysis investigation. The scan permitted the measurement of the depth of the membrane using inbuilt software.

The mean membrane depths for all inserted membranes were measured as; 0.69mm (SEM 0.043mm) (n=36). This is consistent with the depth of the dermal-epidermal junction, 0.06-0.8mm (Tortora, 2009). Finally the membranes are removed and the area cleaned and dressed if necessary. The participant was then free to leave the facility.

4.2.6 Results

Results are expressed as descriptive statistics, stating minimum, maximum, mean and standard error of the mean (SEM).

Two components were recovered using dermal microdialysis at the dermal-epidermal junction, these were T-4-ol and 1,8 cineole. *Table 4.1* presents the total amount of each component recovered in each volunteer (including mean ± SEM).

The membranes were positioned allowing duplicate samples to be taken from the same site.

The total mean amount of T-4-ol recovered using *in vivo* microdialysis was 115.64ng±28.1, this represents 2.3% of T-4-ol present in neat TTO (4978ng/ml).The total mean amount of 1,8 Cineole recovered using *in vivo* microdialysis was 15.05ng±2.6 *(figure 4.4*). This represents 6% of 1,8 cineole present in neat TTO (229ng/ml).

Due to membrane failure because of blockage, T-4-ol was unable to be recovered in two samples. 1,8 cineole was unable to be analysed in four samples due to membrane blockage (Volunteer 2, membrane B and Volunteer 5, membrane A) and because recovered levels of 1,8 cineole being below the lower limit of detection by GC-MS (Volunteer 10 membranes A and B).

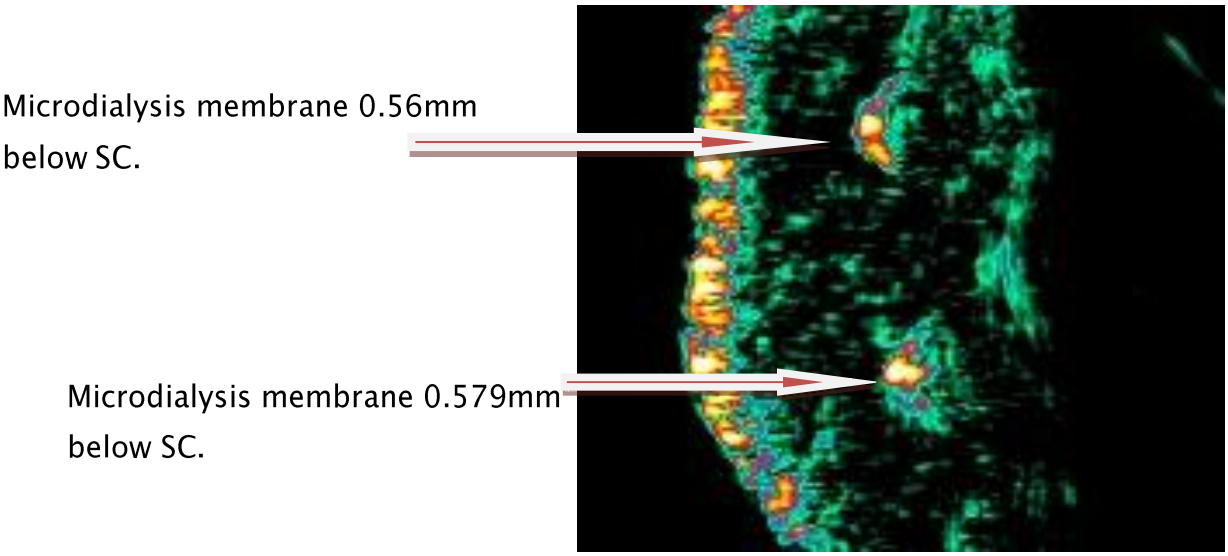


Figure 4.3 Ultrasound image of two implanted microdialysis membranes. Showing a transverse ultrasound image taken at the midpoint of the membranes implanted in the forearm of a participant, confirming the position of the inserted membranes to be at the dermal-epidermal junction. The mean membrane depth (±SEM) was calculated to be 0.69mm (±0.043) which equates with the dermal-epidermal junction. (n=36).

4.2.7 Limitations

The limitations of dermal microdialysis include that it is difficult to assess the true amount of component available in the extracellular fluid as *in vitro* studies, whilst necessary to ensure optimal conditions for recovery *in vivo*, cannot account for tortuosity, blood flow, protein binding etc. However, validation of this method in *Chapter 3* has reduced the number of variables that can impact *in vivo* microdialysis investigations.

Table 4.1 The amount of T-4-ol and 1,8 Cineole recovered (ng) using in vivo microdialysis. Also shown are minimum, maximum, ±SEM

Volunteer	T-4-ol	1,8 Cineole
1A	14.9	1.23
1B	189.9	10.4
2A	51.5	2.1
2B	Blocked	Blocked
3A	20.1	0.9
3B	73	6.6
4A	121.6	6.2
4B	54.7	1.9
5A	Blocked	Blocked
5B	10.8	21.4
6A	17.4	20.4
6B	11.6	19.9
7A	209	23.6
7B	120.8	21.8
8A	365.4	28.2
8B	190.7	30.1
9A	243	21.8
9B	362	24.2
10A	2.4	not detected
10B	22.7	not detected
Min	**2.4**	**0.9**
Max	**365.4**	**30.1**
Mean	**115.64**	**15.05**
SEM	**28.1**	**2.6**

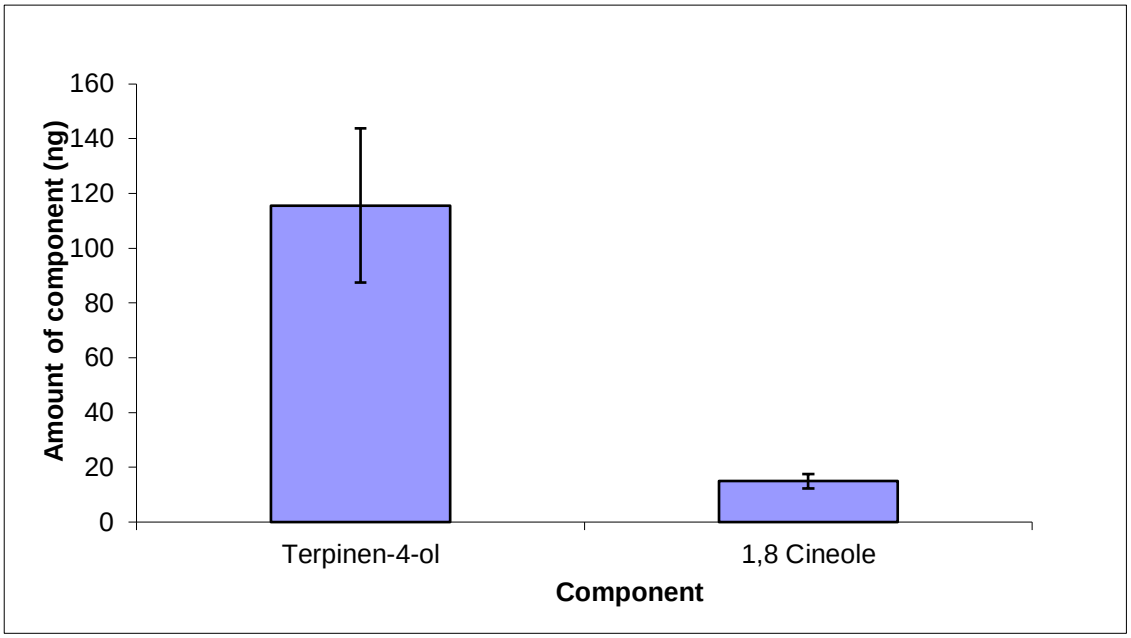


Fig. 4.4 Total amount of T-4-ol and 1,8 cineole (ng) recovered using dermal microdialysis.

Following the topical application of 100% TTO in vivo in humans it was observed that over a 90min period the total mean ±SEM amount of T-4-ol recovered over this period was 115.64ng±28.1 and for 1,8 cineole 15.05ng±2.6. This represents 2% and 6% respectively of the total amount of each component in neat TTO. Error bars represent SEM. (n=10).

4.3 *In vivo* investigation to identify components of TTO present within the SC using the tape stripping method

4.3.1 Aim

- Are TTO components present within the SC following topical application of 100% TTO?

4.3.2 Objectives

- To identify components of TTO present within the SC using the tape stripping method.

4.3.3 Subjects

This *in vivo* study had been ethically approved by Southampton and South West Hampshire Ethics Committee B, 06/Q1704/2. The study was undertaken in the same conditions as the dermal microdialysis investigations (*section 2.5.2*), in a temperature controlled room within a research facility.

4.3.4 Protocol

On arrival at the research facility the volunteer gave informed consent to participate in the study. Following this 0.5ml of TTO was applied to an area on the volar forearm and occluded with a hill top chamber and Opsite™ occlusive dressing. An area 5cm above or below (alternating between participants) was covered with a hill top chamber and occluded with Opsite™ occlusive dressing to act as control.

After 90min the hill top chamber was removed from the treated area and the skin wiped twice with a cotton swab. The hill top chamber was removed from the control site. Sequential tape strips 2X2cm were applied and removed (*section 2.5.2*). The first tape strip was discarded and the subsequent tape strips are grouped in the following order: 2-5, 6-9, 10-20, 21-30 and placed into individual test tubes containing 5ml DCM and the test tube occluded to avoid evaporation. This process was repeated on the control site.
Separation of the oil components was achieved as described in *section 2.5.6*, followed by GC-MS as described in *section 2.6.3*.

4.3.5 Results

There were nine components of TTO able to be identified in the chromatographs produced from the tape strips taken from the TTO exposed area (table 4.2). There were no components of TTO identified following GC-MS analysis of the tape strips taken from the control site. A sample of the chromatographs produced can be seen in *figures 4.5 and 4.6)*.

Table 4.2 Components identified following analysis of samples from tape strips after topical application of TTO. The components are named, the number of times the component was identified (counted as once per subject, even if present in a number of chromatographs from the same subject), if the component is aqueous or lipid, the Log P value and the percentage of component present in neat TTO are shown. (n=7).

Component	Number of times identified in this study	Aqueous or lipid component (Hammer et al., 2006)	Log P (Cal and Krzyaniak, 2006)	Percentage in neat TTO as defined by ISO4730. (Hammer et al., 2006)
T-4-ol	6	Aqueous	2.91	>30
Terpinolene	5	Lipid	3.12	1.5-5
Cadinene	4	Lipid	4.4	Traces -8
α-terpineol	3	Aqueous	2.6	1.5-8
Y-terpinen	3	Lipid	4.5	10-28
α-pinene	2	Lipid	4.8	1-6
1,8 cineole	1	Aqueous	2.94	<15
Aromadendrene	1	Lipid	4.4	Traces-7%
Ledene	1	Lipid	-	0.9

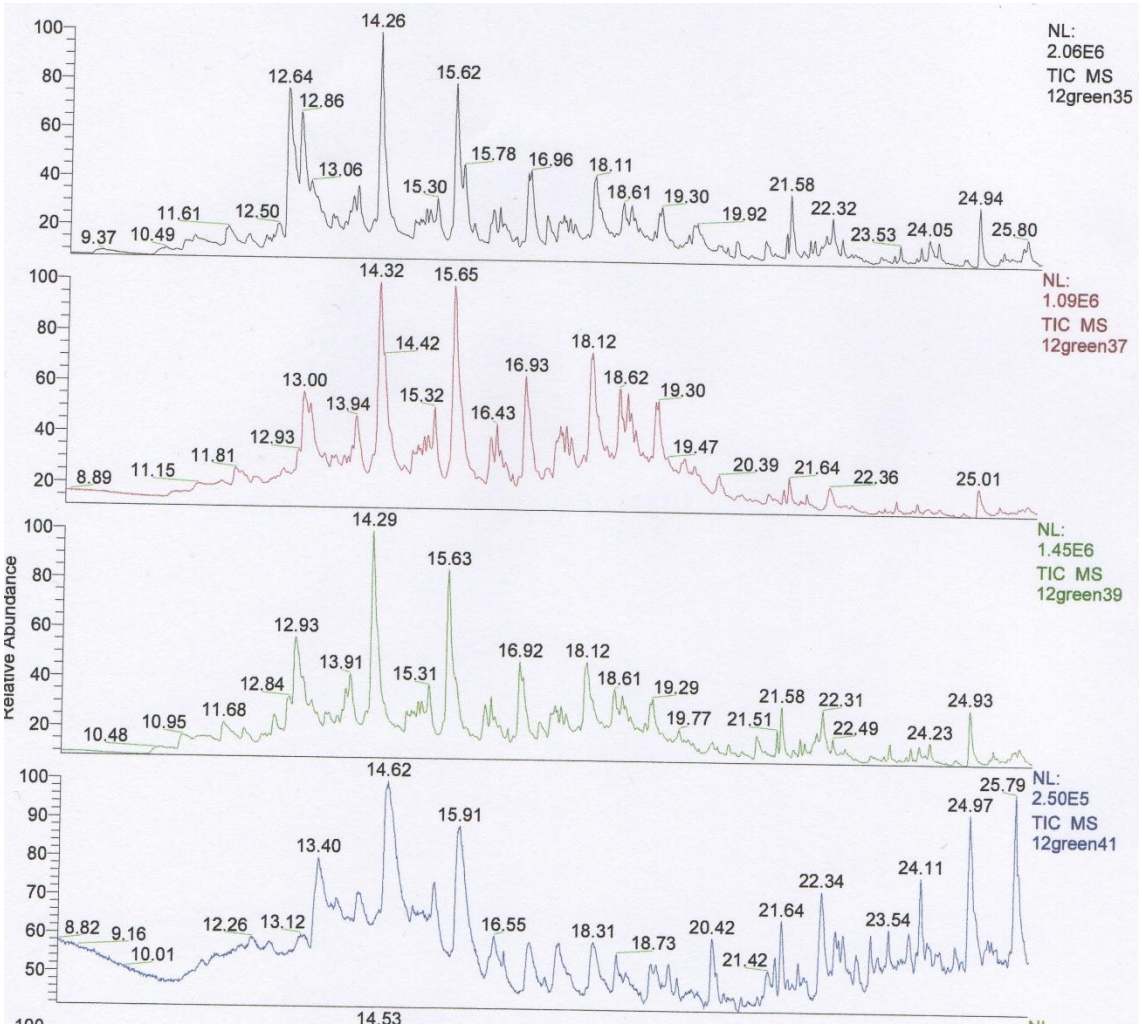


Figure 4.5 Chromatograph produced following tape stripping of an area of skin exposed to neat TTO for 90min. The scans demonstrate strips from subsequently deeper layers of the SC. It can be identified that T-4-ol and terpinoene are easily identified (RT 13.06min and 14.26min respectively, as shown in the top chromatograph).

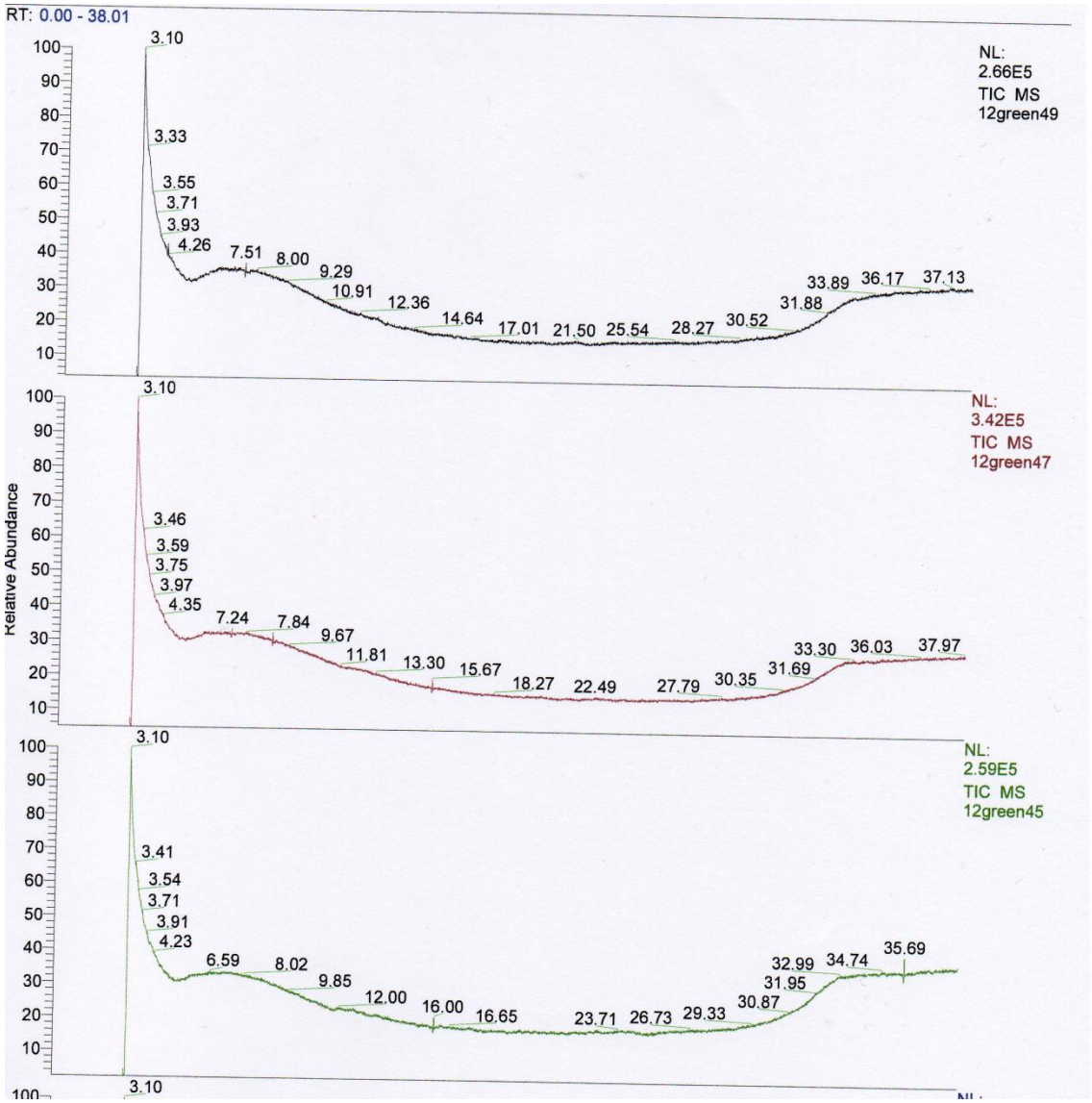


Figure 4.6 Chromatographs produced following GC-MS analysis of tape strips from an area not exposed to TTO (control). The chromatographs show strips taken from subsequently deeper levels of the SC. There are no components identifiable in the control strips.

4.3.6 Summary of key findings

- *In vivo* dermal microdialysis allowed for the recovery of T-4-ol and 1,8 cineole
- The mean total amount ±SEM recovered at the dermal-epidermal junction over a 90min period was: T-4-ol = 115.64ng/ml±28.1, 1,8 cineole = 15.05ng/ml±2.6.
- The amounts recovered represent 2.3 % of T-4-ol in neat TTO and 6% 1,8 cineole of that in neat TTO.
- *In vivo* dermal tape stripping allowed for the identification of 9 lipid and hydrophilic components within the SC including T-4-ol, α-pinene and terpinolene.

4.4 Discussion

The benefit of the topical application of a medicinal product includes not only the direct application to the target organ but also reductions of potential side effects as large amounts of the product are unlikely to reach the systemic circulation. Increasing empirical evidence supports the probable anti-bacterial, anti-fungal and anti-inflammatory effect of TTO (Mondello et al., 2006; Khalil et al., 2004; Dryden et al., 2004). However, the potential of TTO as a medicinal product is dependent upon its penetration through the skin (Hammer et al., 2006; Cross et al., 2008).

There have been limited investigations into the transdermal penetration of TTO, the majority of which are *in vitro* or examine one or two components of the oil only (Cross et al., 2008; Neilson et al., 2006; Cal and Krzyzaniak, 2006). *In vitro* studies fail to replicate the physiological conditions of the skin including blood flow and tortuosity, furthermore the study of one or two components of the oil will not provide an accurate representation of the pharmacodynamics of the oil; this is particularly important as it is not known as yet if TTO components work independently or in synergy (Cox et al., 2001).

The investigations presented in this chapter demonstrate the successful utilisation of *in vivo* dermal microdialysis and dermal tape stripping to identify and quantify components of TTO at the dermal-epidermal junction and within the SC following topical application of neat TTO.

Adaptations to the dermal microdialysis method as discussed in *Chapter 3* allowed for enhancement of recovery of components of TTO. The conditions incorporate a slow rate of perfusion, the inclusion of 5% HPβ-CD in PBS as the perfusate with 2kDa cut off cuprophan membranes.

Reduction of perfusate flow rate to aid recovery of the components by microdialysis is supported in the literature (Cano-Cebrian et al,. 2005; Groth, 1995). Slowing the flow rate allows for greater diffusion of molecules across the membrane and is likely to reflect more closely the actual amount of component present in the area surrounding the membrane.

Limited evidence in the literature demonstrates that recovery of lipids across the hydrophilic membrane can be increased by the inclusion of HPβ-CD in the perfusate (Ao and Stenken, 2003), however its use *in vivo* microdialysis is still infrequent.

Furthermore it is known that the physicochemical properties of the microdialysis membrane can influence recovery. Tao et al., (1992) suggests cuprophan membranes exhibit maximum relative recovery of a water soluble drug (5-hydroxytryptamine) quickly (20min) compared to membranes of different materials (polyacrylonitrile/sodium methallysulfonate copolymer) which required 2 hours to recover the same amount of drug *in vitro* and *in vivo.* This would suggest cuprophan as an appropriate choice to use in experiments with a relatively short collection time such as this (collection time 90min). Haemophan membranes are a relatively new membrane type, a modification of the cellulose membrane, it has a positive charge, compared to no charge exhibited by the cuprophan membrane (membrana).

TTO contains non-polar hydrocarbons and polar terpenes. Non-polar molecules have no charge, polar molecules have no overall electrical charge but small charges at different points; for example, oxygen has a small negative charge and hydrogen has a small positive charge and if bonded in a molecule, for example T-4-ol, which contains an OH group, will exhibit these small charges at certain points. *In vitro* study results presented in *Chapter 3* demonstrated that cuprophan membrane increased recovery of T-4-ol by 63%, 1,8 cineole and γ-terpinen by 61% and 4% respectively when compared to

haemophan membranes. There is a significant difference between recovery of the polar T-4-ol and 1,8 cineole and the non-polar γ-terpinen, thus demonstrating that even though the overall charge of the polar terpenes is small, it may interact with the positively charged haemophan membranes and therefore reduce recovery of the component. The increase in recovery of the non-polar γ-terpinen is much less when using cuprophan in comparison to haemophan membranes thus demonstrating the charge has little effect on recovery of the non-polar molecule.

The absolute amount of T-4-ol (MW 154) recovered *in vitro* represents 16%, for 1,8 cineole (MW 136) 14% and for γ-terpinen (MW 136) 2% of neat TTO. The recovered amount for all three components is lower than that previously reported for similar sized molecules. Previous studies have demonstrated histamine (MW 184) to have an *in vitro* recovery rate of around 50% (Voegeli, 2001). The low recovery rates seen in these studies may be due to the components diffusing in and out of the dialysate across the membrane as the pore size is 2kDa and the components MW are smaller than that of histamine, or due to a difference in release of the component from the vehicle (Bronagh and Maibach 1989).

The *in vivo* study presented in this chapter demonstrated that consistently T-4-ol and 1,8 cineole were able to be recovered using dermal microdialysis. No other components were present when the samples were analysed using GC-MS. These findings are similar to *in vitro* findings presented in the literature (Reichling et al., 2006; Cross et al., 2008; Neilson et al., 2006). However, the results are difficult to compare due to the variety of methods used and also limited availability of data in the literature.

The total mean amount of T-4-ol recovered *in vivo* was 115.64ng±28, the total mean amount for 1,8 cineole was 15.05ng±2.6. *In vitro* microdialysis studies allow for the estimation of the exact amount of a component present in the skin by calculating the recovery rate. The recovery rate is normally assessed following perfusion of various concentrations of substance of interest through the membrane and collected in dialysate as discussed in *section 2.2.1*. However, TTO reacts with the plastic syringe which holds the perfusate, causing the lipids to adhere to the plastic and stopping perfusion, therefore

concluding that this method to measure recovery rate is not appropriate for TTO. Despite this, absolute amounts recovered demonstrate that at least 2% of T-4-ol which is present in neat TTO can be quantified at the dermal-epidermal junction and 6% of 1,8 cineole. This would be an underestimation of what is actually present within the exogenous and endogenous fluid as it does not take into account tortuosity and protein binding of TTO components. Nevertheless at these concentrations and below, clinical efficacy has been demonstrated *in vitro* against fungus, bacteria and inflammation (Hammer et al., 1998; Jose et al., 2002; Caldefie-Chezet et al., 2004; Hammer et al., 2012). However, the toxicity of these concentrations to the cells would require further examination.

The occlusion of the skin is known to enhance penetration of molecules through the skin by reducing trans-epidermal water loss and therefore increasing hydration of the skin (Bronagh and Maibach 1989). Indeed, it has been demonstrated that 1,8 cineole can only be recovered under occlusive conditions (Cross et al., 2008, Nielson et al., 2006). Therefore, it is likely that the dermal penetration of T-4-ol and 1,8 cineole during these investigations was enhanced by occlusion.

The amounts recovered are variable (*Table 4.1*). One possible explanation for this could be variability in the depth of the microdialysis membrane. The depth of the microdialysis membrane is known to influence the recovery of substances from the skin (Muller et al., 1998). However the probe depths determined by ultrasound in this study were shown to be consistent, with a small degree of variability (mean probe depth 0.69mm±0.043 SEM) (*section 4.1.5*). γ-terpinene, a lipid component recovered during *in vitro* microdialysis, was not recovered in the *in vivo* dialysate.

There are a number of theories that may explain the dermal penetration of T-4-ol and 1,8 cineole, including penetration enhancement, Log P value, molecular weight and Fick's First Law of Diffusion. Components of TTO, particularly T-4-ol and 1,8 cineole are often used as penetration enhancers (Williams and Barry, 1992) (section *4.1.5*). It is therefore unsurprising that these components are present at the dermal-epidermal junction. T-4-ol and 1,8 cineole are an alcohol and cyclic ether respectively and are known to

penetrate the SC more readily that hydrocarbon terpenes. Williams and Barry (2002) suggest that these terpenes act by disrupting the lipid structure of the SC, and has demonstrated, 1,8 cineole to disrupt the bilayer lipids, allowing penetration of hydrophilic products.

In addition, the lipophilicity of a compound can be identified using the logarithm of the octanol water partition coefficient (Log P). This describes the ratio of concentrations of partitioning un-ionized components between two aqueous phases with differing pH levels. A higher log P value represents a more lipophilic component. The log P of T-4-ol is 2.91 and for 1,8 cineole 2.94, the log P of γ-terpinene is 4.35 (Cal and Krzyzaniak, 2006). It is known that a log P of between1-3 is the optimum for penetration through the skin (Hadgraft and Guy, 2004). Cal and Krzyzaniak (2006) suggests lipids may partition into the lipid layers of the SC. This is in contrast with the view of Cross et al., (2008) who suggests a lipophilic substance would transport more easily through the skin than a hydrophilic substance.

Furthermore, The penetration of T-4-ol and 1,8 cineole supports the theory of the '500 Dalton rule' suggested by Bos et al., (2000). This theory suggests that only substances below 500 Daltons can penetrate the skin. T-4-ol has a MW of 154 whilst 1,8 Cineole has a MW of 136 and according to this theory would penetrate the skin easily due to their size alone. Moreover T-4-ol and 1,8 cineoles are two of the most abundant components in TTO, >30% and ~15% respectively (Hammer et al., 2006) thus supporting Ficks First Law of Diffusion, the diffusion of components through the skin is concentration dependent (Groth, 1996).

Clearly there are differing views regarding the penetration of substances through the skin, whilst the results of the *in vivo* studies presented here can be explained by the Log P value of the penetrated components, occlusion of the TTO and that the components are said to be penetration enhancers. Nevertheless, according to the '500 Dalton rule' all components under 500 Daltons should penetrate the epidermis readily, yet, the largest component of TTO has a MW of 154, thus suggesting all components of TTO should penetrate the skin including γ-terpinene (MW 136). This has not been supported by the *in vivo* dermal microdialysis studies presented here.

Furthermore the suggestion that lipophilic substances should penetrate the epidermis more rapidly than hydrophilic is again not supported. In addition, whilst the dermal penetration of T-4-ol and 1,8 cineole supports Fick's First Law of Diffusion, γ-terpinene represents 10-28 % of TTO and is therefore present in a comparatively large amount but is not present in dialysate.

It is likely that the penetration of the components to the dermal-epidermal junction following topical application is due to a combination of theories including Log P value, occlusion and that the components are penetration enhancers.

Many authors suggest that T-4-ol and 1,8 cineole are the active components of TTO (Brand et al., 2002a; Hammer et al., 2006). However, the synergistic role of TTO components has yet to be confirmed. Nevertheless, the presence of T-4-ol and 1,8 cineole at the dermal-epidermal junction supports the potential of TTO to exert its positive effects on the cells within the vicinity, for example, keratinocytes, neutrophils and mast cells (Hart et al., 2000; Caldefie-Chezet et al., 2004).

The results following tape stripping identify some inter-subject variability. This may be due to the varying SC structure between subjects (Jacobi et al., 2005). The aqueous component T-4-ol is the most abundant identified within the SC. As the most abundant in neat TTO it would be expected to be and has been identified throughout the layers of the SC in previous studies (Cal, 2008). As discussed there are various theories regarding the penetration of lipids and aqueous substances throughout the literature (Bos et al., 2000; Hammer et al., 2006). However the study presented in this chapter demonstrates the presence of both lipid and aqueous components of TTO throughout the SC. It is interesting that many of the frequently identified components in the samples following tape stripping are the least abundant in neat TTO (*table 4.2*). The log P values of the majority of the lipids identified are >4.4 and this would support the theory regarding easier penetration of substances with lower log P values discussed above. Despite T-4-ol being identified as present throughout the SC, 1,8 cineole is identified in only one of the samples. The identification of this component at the dermal-epidermal junction and the relative lack of evidence of its presence in the SC suggest that it does pass very quickly

through the lipid layers of the SC. However, a previous study suggests 1,8 cineole disrupts the lipid bilayers of the SC (Williams and Barry, 2004). Further investigation is required to ascertain if 1,8 cineole is present at sufficient concentrations within the SC to cause such disruption. Evidence of the presence of additional components of TTO within the SC demonstrate further the potential of the oils efficacy against inflammation and bacteria. A previous study has identified α-terpineol as an anti-inflammatory agent. In a rat model following inducement of a wheal and flare by Substance P injection, α-terpineol was demonstrated to reduce the flare area significantly, thus suggesting it reduces vasodilation (Khalil et al., 2004). Furthermore, α-terpineol has also been demonstrated to reduce superoxide production in activated monocytes (Hart et al., 2000). As the present study identifies the presence of α-terpineol only within the SC and not at the dermal-epidermal junction this would suggest that despite *in vitro* evidence of the anti-inflammatory effect of this component of TTO, *in vivo* evidence suggests it would not exert a similar effect as it would not penetrate past the SC.

The synergistic nature of TTO still requires clarification. Nevertheless, evidence of the presence of these components within the SC may support its use in particular dermatological conditions that occur in the superficial layers of the epidermis. Further work is needed in order to quantify these components.

In summary, the studies within this chapter have demonstrated that the hydrophilic components T-4-ol and 1,8 cineole are present at the dermal-epidermal level at clinically efficacious concentrations. However, more work is required to assess the toxicity of the concentration on local cells. The presence of lipid and hydrophilic components of TTO have been identified within the SC including the potentially effective α-terpineol. Further work is required to determine the concentration of these components in order to assess the effectiveness of topical application of TTO.

There is much evidence to support the anti-bacterial and anti-fungal properties of TTO *in vitro*. Despite this, it is only in recent years that the potential anti-inflammatory effects have been investigated. As the presence of TTO components within the SC and at the dermal-epidermal junction has been confirmed, the study of its effect on local cells is more relevant. *Chapters 5*

and *6* will discuss and undertake methods to assess the modulatory effect of TTO on anti-inflammatory cytokines produced by keratinocytes in response to an inflammatory challenge.

Chapter 5
Cell culture methods

5 Introduction

Currently there are a limited number of investigations that demonstrate the ability of TTO and its components to modulate the production of pro-inflammatory cytokines (Caldefie-Chezet et al., 2004; Hart et al., 2000; Abe et al., 2003).

In vitro experiments using a cell line allow for the investigation of the behaviour of cells following incubation under various conditions, therefore permitting replication and validation of experiments. The investigation of the modulatory effect of TTO and T-4-ol on pro-inflammatory cytokines was investigated using an immortalised keratinocyte cell line (HaCaT) and further described in *chapter 6*. A potential mode of action is also examined in *chapter 7*, methods are describe in this chapter.

5.1 HaCaT cells

Primary cell lines, particularly keratinocytes, grown directly from a sample of human tissue, are difficult to maintain and have only a finite life span leading to difficulties in replicating and validating experiments (Deyrieux and Wilson, 2007). Therefore many cell lines have been immortalised (cells from a source which would not normally proliferate indefinitely but due to mutation is able to continually undergo division) (Olaru and Jensen, 2010). Cell lines originating from animal tissue or a malignancy can be developed to allow a number of passages without altering phenotype (the cell's observable traits) and these cell lines allow for accurate, reproducible conditions in which to undertake investigations and provide a suitable *in vitro* model (Deyrieux and Wilson, 2007; Olaru and Jensen, 2010). Two such immortalised cell lines are HEL-30 (Fusenig et al., 1983) and the HaCaT cell line (Boukamp et al., 1988). HEL-30 is a mouse-derived keratinocyte cell line which has since been superseded by the HaCaT cell, a human derived cell line, demonstrated to be immortal (>140 passages) and non-tumorigenic. The DNA fingerprint pattern has been shown to be unaffected by long term cultivations which enables the HaCaT cell to be a robust cellular model that is able to replicate the traits of keratinocytes (Boukamp et al 1988).

The HaCaT cell line is now widely employed as a keratinocyte model and has been demonstrated as being easy to propagate, having a near-normal phenotype and allows the study of innate immune mechanisms (Olaru and Jensen, 2010; Deyrieux and Wilson, 2007). Therefore the HaCaT cell line was chosen for subsequent investigations.

5.1.1 Maintenance of HaCaT cells

HaCaT cell lines were a kind gift from Dr Chris Pickard (Faculty of Medicine, University of Southampton).

All cell culture work was undertaken in a temperature controlled laboratory (21 ±2°C), within a laminar air flow hood under sterile conditions to reduce the possibility of infection of the cells. Cells had been previously stored in liquid nitrogen and were rapidly defrosted and diluted in 30ml of Dubleccos' Modified Eagles Medium (DMEM) containing 10% foetal calf serum (FCS), 1% Streptomycin, 1% Penicillin and 1% L-glutamine (Sigma, UK) in a T75 flask (NUNC). The cells were incubated at 37°C with 5% CO_2. Cells were passaged every 3-4 days or at 80% confluence, as viewed at 10X magnification using an inverted microscope (*figure 5.1*) (Zbytek et al., 2003; Cheng et al., 2008).

5.1.2 Cell Passage method

When cells were at 80% confluence (= approximately 10^6 cells per ml) the medium was removed using a sterile pipette in a sterile fume hood and discarded. Cells were gently rinsed twice using 10ml of phosphate buffered saline (PBS) (Sigma, UK). Next 3ml EDTA trypsin (Sigma, UK) was added to the flask which was then incubated for 5-10min at 37°C with 5% CO_2. The cells were periodically vigorously shaken and checked under an inverted microscope set at X10 magnification to observe cells lifting from the bottom of the flask. When all cells had lifted, 5ml of medium was immediately added to the flask to avoid over-trypisinisation. The contents were poured into a 50ml sterile falcon tube (Fisher-Scientific). PBS was added until the solution was at 50ml. The cells were centrifuged at 1500rpm for 5min. The supernatant was gently removed using a sterile pipette leaving the pellet of cells. The cells were re-suspended in 1ml of medium, 9ml of medium was subsequently added to the falcon tube and 1ml of the this solution was added to a sterile T75 flask which was supplemented by a further 30ml of medium. The cells were viewed through the

microscope to ensure they were present as before and replaced in the incubator.

5.2 Inducing an inflammatory response in HaCaT cells

Keratinocyte and HaCaT cells are known to innately produce pro-inflammatory mediators including TNFα, IL1β and IL6 but require stimulation to produce mediators such as IFN-Ɣ (Lewis et al., 2006; Cho et al., 2006). Up-regulation of these pro-inflammatory mediators will allow for the action of TTO to be demonstrated more clearly (Brand et al., 2001). Therefore a substance to stimulate the cells to produce pro-inflammatory cytokines as in an inflammatory response was required.

5.2.1 Inflammatory stimuli

Pro-inflammatory responses in HaCaT cells can be induced by ultra violet-B light (UVB), Compound 48/80, TNFα and toll-like receptor (TLR) specific ligands such as Lipopolysaccharide (LPS). The agents induce inflammatory reactions caused by differing pathways including histamine release, cytokine recruitment and cell apoptosis (Brand et al., 2002a; Cho et al., 2006; Lewis et al., 2006).

LPS is a large molecule found on the outer membrane of gram-negative bacteria. It is an endotoxin and produces a strong immune response after recognition by TLR4 on the surface of the cell (Kollisch et al., 2005).

LPS is frequently used as a pro-inflammatory stimulus during *in vitro* experiments on a variety of cells including monocytes, neutrophils, keratinocytes and HaCaT cells at concentrations ranging from 50ng/ml-1µg/ml and has been demonstrated to produce a strong immunological response at 100ng/ml (Cheng et al., 2008; Kollisch et al., 2005; Abe et al., 2003).

LPS was therefore chosen as the pro-inflammatory stimulus for the *in vitro* cell culture studies presented in this thesis.

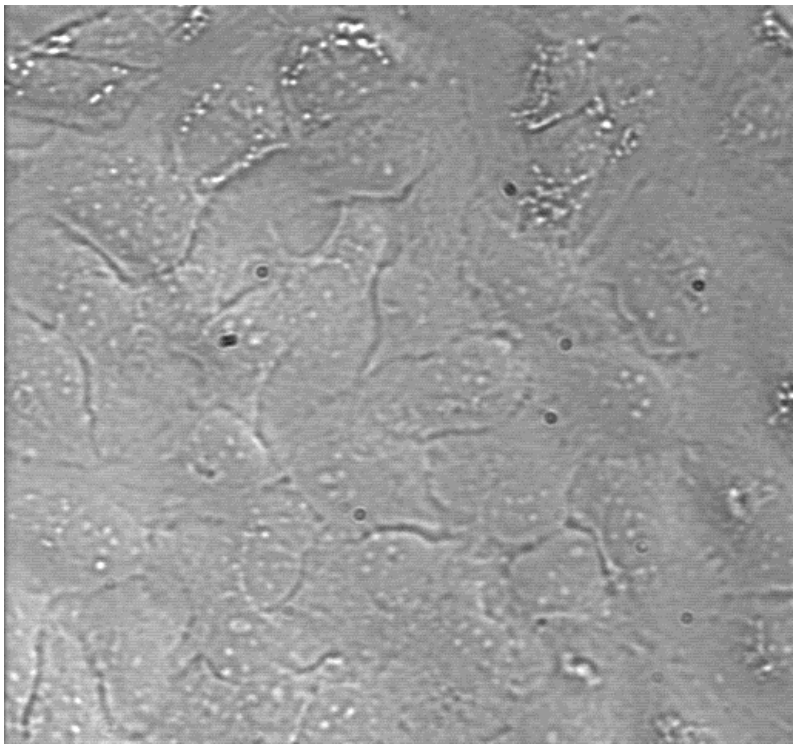


Figure 5.1 Image of HaCaT cells at using a X20 objective lens. Cells are approximately 30µM diameter. The cells have adhered to the T75 flask base and have undergone replication. Prior to passage the cells were required to be 80% confluent (adhered to 80% of the flask base). If confluence was less it would reduce the amount of cells available for investigation and may therefore skew results. If confluence is greater than this level, cells can begin to grow on top of each other and will mutate and thus will not exhibit a response in the way normal HaCaT/keratinocyte cells would.

5.3 Cytotoxicity investigations

The assessment of cell viability following incubation with a substance of interest is necessary prior to on-going investigation. This ensures the concentration of the substance of interest to be tested is not such that will immediately kill viable cells and therefore prevent the investigation of potential benefits of the substance. Cell viability assays allow for the identification of the concentration of the substance of interest that is toxic to the cell line.

5.3.1 Methods to measure cell viability in HaCaT cells

Cell viability can be measured by a number of methods, including the MTT assay, 3-(4,5-dimethhylthiazole-2-yl)-2,5-diphenyltetrazolium bormide, Trypan blue exclusion assay, crystal violet staining and CyQUANT® cell proliferation kit which are described below (Mickuviene et al., 2004). Each method estimates cell viability in differing ways. Mickuviene (et al., 2004) suggests that as the methods are so varied the criteria to determine cell viability should be chosen to fit the needs of each particular study.

5.3.2 MTT Assay

The MTT assay assesses the metabolic reduction of a tetrazolium dye, therefore measuring the cell's ability to maintain and provide energy for metabolic function and growth. A small volume of the MTT solution is added to the cell culture and incubated for approximately 2 hours, this is aspirated and 2-propanol added. Finally the optical density is recorded using a spectrophotometer. The results are expressed as % compared to control, the optical density of which represents 100% viability (Sui et al., 2007; Thangiam et al., 2009).

5.3.3 Trypan Blue Exclusion Assay

The trypan blue exclusion assay assesses plasma membrane integrity. If the cell membrane is compromised the blue dye will be taken up by the cell, thus when observed under a microscope the blue cells indicate dead cells and non-blue cells indicate viable cells. A small volume of 0.4% Trypan blue is added to the cell sample and mixed; immediately the cells can be counted using a haemocytometer. The results can be expressed by % viability compared to control (Brand et al., 2001) or by % toxicity to cells (Rossiter et al., 2010).

5.3.4 Crystal Violet

The addition of the protein dye crystal violet tests the loss of monolayer adherence. Adherent cells become non-adherent when non-viable; the crystal violet assay would show a decrease in the number of stained cells as cell viability decreases. A small percentage of crystal violet is added to the cell sample and incubated for approximately 30min. After this time the cell monolayers are rinsed with tap water. The remaining cell-attached dye is dissolved in acetic acid and ethanol and the optical density is read using a spectrophotometer. Results are expressed as a percentage of the optical density of control (test-compound free culture) which is set at 100% (Mickuviene et al., 2004).

5.3.5 CyQUANT® cell proliferation assay (Invitrogen, UK)

The use of this manufactured assay allows for the measure of total DNA. Following test incubation, the cells are lysed using the manufacturer's solution and incubated with a fluorescent dye. Following incubation the plate is read using a fluorescence microplate reader. The mean fluorescence of the control cells (exposed to test-compound free culture) represented 100% viability; subsequent results are expressed as a % of these controls (Mickuviene et al., 2004).

As adherent cells, the HaCaT cell line requires consideration when identifying a reliable method with which to assess cell viability. Furthermore, suggested modes of action of TTO also require deliberation. Evidence within the literature examining TTO's effect on bacteria suggests that a potential mode of action is that TTO acts on the cell wall causing cell permeability. In addition, TTO components may act in the same way as a membrane active disinfectant, causing leakage of potassium and inhibiting respiration (Hammer et al., 2003a; Cox et al., 1998; Gustafson et al., 1998). Mickuviene's (et al., 2004) study examining the reliability of specific cell viability assays on a range of cells identified the most sensitive test was the assay related to the site of the direct damage.

Whilst the MTT assay has been utilized in the investigation of cell viability following incubation with TTO (Caldefie-Chezet et al., 2004; Hart et al., 2000), these experiments are not with HaCaT cells, using alternatively monocytes and

PBMCs. Crystal violet was identified by Mickuviene (et al., 2004) as the most sensitive assay to assess cell viability across a large range of cell types, however, like the MTT and CyQUANT® assay the results are expressed as % of optical density and do not identify specific cell numbers. Again the consideration of the potential mode of action would suggest that CyQUANT®, MTT and Crystal violet assays may not be sensitive enough to estimate HaCaT cell viability following incubation with TTO and its components (Mickuviene et al., 2004). In contrast, the trypan blue exclusion assay assesses plasma membrane integrity, is simple to use and provides reliable cell number estimation. Despite limitations to this method, including the possibility of cell viability overestimation due to the detachment of the dead cells from the substratum and therefore potential escape from the subsequent cell count, this method can be adapted to ensure that as many cells as possible are included.

In summary, the measurement of HaCaT cell viability following incubation with TTO can be made using a variety of methods including the MTT assay, trypan blue exclusion assay, crystal violet and CyQUANT® cell proliferation assay. Evidence within the literature suggests that to identify the most specific method to measure cell viability, the potential mode of action of the substance of interest requires consideration (Mickuviene et al., 2004). TTO is suggested to exert its effect by compromise of the cell membrane (Hammer et al., 2003a; Cox et al., 1998; Gustafson et al., 1998). The trypan blue method specifically assesses plasma membrane integrity and has been utilized in the assessment of HaCaT cell viability (Brand et al., 2001). Despite adaptations being required to ensure all cells, adherent and non-adherent are included, the trypan blue method was chosen for subsequent measure of HaCaT cell viability within this thesis.

5.4 Investigations into cell viability of HaCaT cells

5.4.1 Aim

- To determine the cytotoxicity of TTO, T-4-ol and LPS to HaCaT cells prior to cytokine modulation assays.

5.4.2 Objectives

- Is TTO at concentrations present at the dermal-epidermal junction toxic to HaCaT cells?
- Is T-4-ol at concentrations present at the dermal-epidermal junction toxic to HaCaT cells?
- Is LPS toxic to HaCaT cells at 100ng/ml?

5.4.3 TTO and T-4-ol concentrations

The concentration of T-4-ol at the dermal epidermal junction following topical application, as demonstrated in *Chapter 4*, is 115.64ng representing 2.23% of T-4-ol in neat TTO. Cell viability following incubation of T-4-ol and TTO at <5% and LPS at <100ng/ml were investigated.

5.4.4 Incubation of cells

HaCaT cells were plated into 3 X 24 well flat bottomed plate containing no additives (NUNC, UK). 1200µl of a 1 in 20 concentration (approx. 12^5 cells per well) were then incubated at 37°C with 5% CO^2 for 24 hours. The HaCaT cells were then challenged as follows:

LPS: 100ng, 50ng, 25ng, 12.5ng, 6ng, 3ng, 1ng and 0ng per ml (medium only, control) in duplicate.

Tea Tree oil: 5%, 2.5%, 1.25%, 0.6%, 0.3%, 0.1%, 0.05% and 0.01% and 0% (medium only, control) in duplicate.

Terpinen-4-ol: 5%, 2.5%, 1.25%, 0.6%, 0.3%, 0.1%, 0.05% and 0.01% and 0% (medium only, control) in duplicate.

The concentrations were decided upon following previous in-house experiments (data not shown) indicating approximately 5% of a substance may reach the dermal-epidermal junction following topical application of 100% of the substance of interest. The literature has revealed that much lower concentrations have been used when establishing anti-bacterial, anti-fungal and anti-inflammatory efficacy and therefore it was decided to analyse for the effect of these concentrations in keratinocytes.

Following application of each challenge the plate was returned to the incubator for 24 hours prior to investigations to determine cell viability following the various challenges.

5.4.5 Trypan Blue Exclusion Assay Method

The addition of trypsin and the combination of the cell supernatant to the cell medium, described below ensured that all cells, viable and non-viable, were captured.

Following incubation under the various conditions for 24 hours, 1% of medium was removed from each well (12µl) and replaced by 12µl of 0.4% Trypan Blue solution (Fisher-Scientific) then incubated at 37°C in 5% CO^2 for 1min.
The supernatant was removed and placed in a labelled amber glass vial.
120µl of Trypsin was added to each well and the plate incubated for 4-5min.The wells were periodically viewed under the microscope to avoid over-typsinisation. Once all cells had lifted, 120µl of DMEM was added to each well to dilute the trypsin and the supernatant was placed into individually labelled micro vials. The vials were then centrifuged at 2000rpm for 5min. The medium was carefully removed leaving only the cell pellet which was re-suspended in the corresponding amber vial of supernatant.

Immediately 1µl of supernatant (including re-suspended cells) was transferred to a dry, clean haemocytometer ensuring the chamber fills fully using capillary action (*figure 5.3*). Using a 10X objective the gridlines on the haemocytometer chamber were focused (*figure 5.4*). The total number of cells was counted in 4 large squares of the haemocytometer grid. A minimum of 100 cells in total is required (Reed, 2003). The non-viable cells (b
lue) were counted. Cell viability was calculated in % (*equation 11*).

$$\% \text{ viability} = 100 - ((Total\ number\ of\ non-viable\ cells)/(total\ number\ of\ cells) \times 100 \quad \mathbf{(11)}$$

5.4.6 Statistical Analysis of Results

Results were repeated in at least triplicate and are expressed as mean % viability ±SEM. To determine statistical difference between challenge and control, a two-way between samples analysis of variance (ANOVA) was employed.

5.5 Results

5.5.1 TTO

Mean cell viability results following incubation with TTO are displayed in *table 5.1* and *figure 5.4*.

A two-way between-groups analysis of variance (ANOVA) was conducted to explore the impact of concentrations of TTO on HaCaT cell viability. TTO concentrations included 5%, 1.25%, 0.6%, 0.3, 0.07 and control (no oil). The interaction between % of TTO and cell viability was statistically significant, F(5, 24) = 5.88, p=0.002, the effect size was large (partial eta squared = 0.620). Post-hoc comparisons using the Tukey honesty significant difference test (HSD) indicated that the mean percentage for cell viability following incubation with 5% TTO (M = 73.8 SEM 6.099) was significantly different to that of control (no oil) (M=89 SEM 6.68).

5.5.2 T-4-ol

Mean cell viability following incubation with T-4-ol is displayed in *table 5.1* and *figure 5.4*.

As above, a two-way between groups ANOVA was conducted to explore the impact of concentration of T-4-ol on HaCaT cell viability. T-4-ol concentration ranged from 5%-0 (control). The interaction between % of T-4-ol and cell viability was statistically significant, F(9, 45) = 32.5, p=2.035E-14, the effect size was large (partial eta squared = 0.893). Post-hoc comparisons using Tukey HSD indicated the mean percentage for cell viability following incubation with 5% (M=90 SEM 1.326), 2.5% (M=82.6 SEM 1.027), 1.25% (M=82.5 SEM 1.148), 0.6% (M=85.75 SEM 1.148), 0.3% (M=87.8 SEM 1.027) and 0.12% (M=92.2 SEM 1.027 were all significantly different to that of control (no oil) (M=98.04 SEM 1.027).

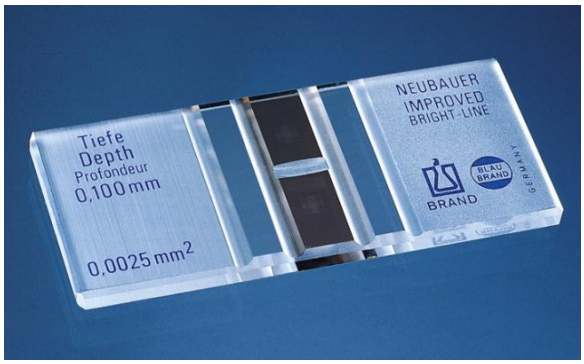


Figure 5.2 A haemocytometer used to count the number of cells in a sample.
1µl of supernatant was added to the channels in the centre. The haemocytometer is then viewed under an inverted microscope x10 magnification. The cells can be clearly viewed positioned with the grid (shown in figure 5.4).

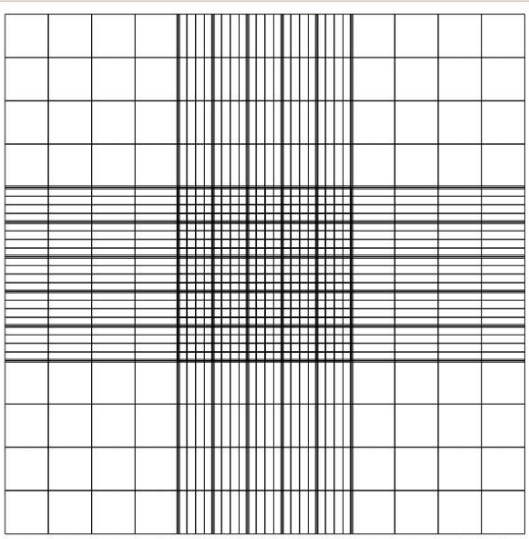


Figure 5.3 A diagrammatic representation of the grid from a haemocytometer viewed through a microscope to aid the counting of cells.
The cells within four large squares were counted, viable cells were not blue, non-viable cells were blue. Cell viability is calculated by dividing the number of viable cells with the number of non-viable and x100.

5.5.3 Lipopolysaccharide

Mean cell viability following incubation with LPS is displayed in *table 5.2* and *figure 5.5.*

As above, a two-way between groups ANOVA was conducted to explore the impact of concentration of LPS on HaCaT cell viability. LPS concentration ranged from 100ng/ml – 0 (control). The interaction between amount of LPS and cell viability was statistically significant, f(9,31)=6.7, p=0.000162, the effect size was large (partial eta squared = 0.742). Post-hoc comparisons using Tukey HSD indicated the mean percentage for cell viability following incubation with 100ng/ml LPS (M=61 SEM 10.15) was statistically reduced compared to that of control (no oil)(M=77.6 SEM 6.8).

5.5.4 Summary

It was interesting to observe that solubility was achieved following the addition of 5% TTO with cell culture medium, however, solubility was not achieved following the addition of 2.5% TTO. It is possible that components within the whole TTO aid solubility of the lipids at a particular concentration (>5%), which is not seen at 2.5%. However, solubility was achieved following 1.25% TTO with cell.

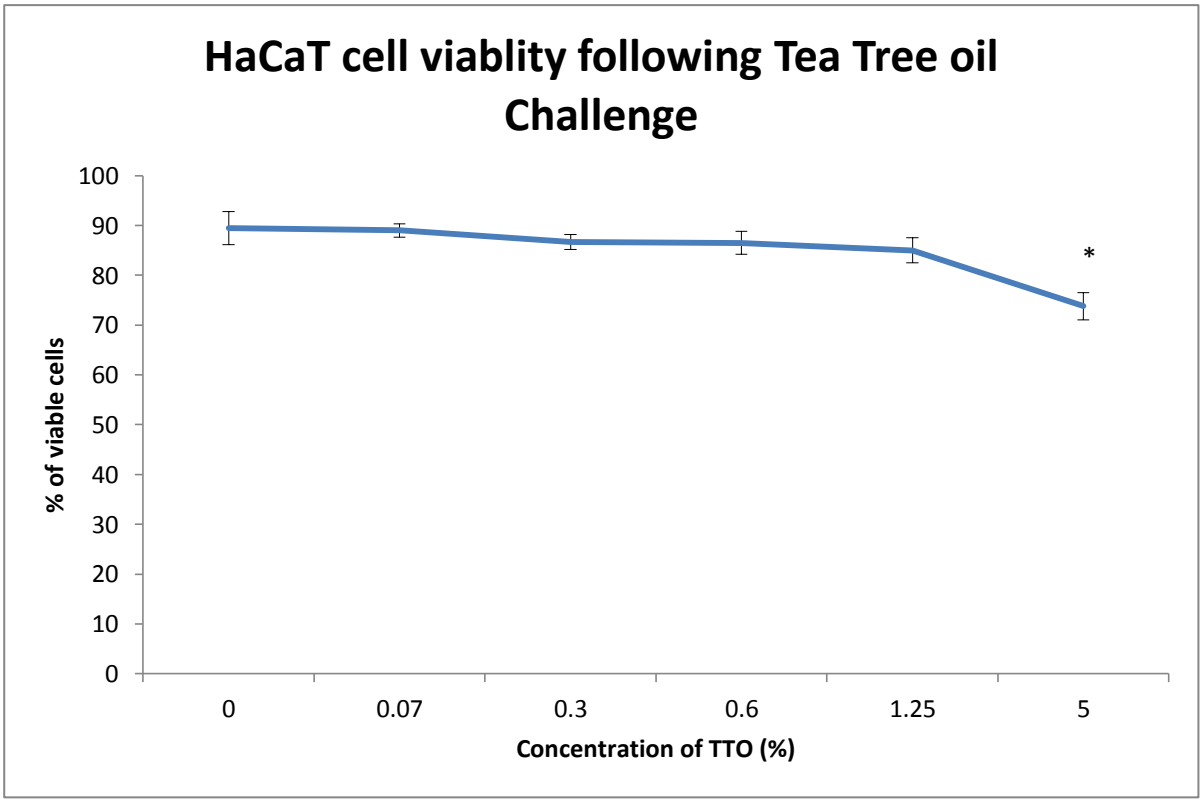
HaCaT cell viability was significantly decreased as identified by an independent t-test following incubation with 5% TTO compared to control. Incubation with of HaCaT cells with T-4-ol led to a significant decrease in cell viability at concentrations greater than 0.15%. LPS significantly decreased cell viability at 100ng/ml. This supports findings in the literature (Cheng et al., 2008; Kollisch et al., 2005; Abe et al., 2003). In Summary, TTO and T-4-ol at 1.25 and 5% will be investigated. LPS at 100ng/ml will be utilized to induce an inflammatory reaction.

Table 5.1 Mean cell viability% ±SEM following incubation with TTO and T-4-ol at various concentrations and control

Incubation concentration %	TTO mean cell viability (%) ±SEM (n=)	T-4-ol mean cell viability (%) ±SEM (n=)
0	98.6 ±0.40 (n=5)	89.5 ±3.3 (n=4)
0.01	97.8 ±0.80 (n=5)	
0.03	96.0 ±0.70 (n=5)	
0.06	96.0 ±0.00 (n=4)	89.0 ±1.3 (n=4)
0.15	92.2 ±0.60 (n=5)	
0.3	87.8 ±1.46 (n=5)	86.7 ±1.5 (n=3)
0.6	85.7 ±0.75 (n=4)	86.5 ±2.3 (n=3)
1.25	82.5 ±0.96 (n=4)	85.0 ±2.5 (n=5)
2.5	82.6 ±1.96 (n=5)	
5	90.0 ±1.50 (n=4)	73.8 ±2.7 (n=5)

Table 5.2 Mean cell viability% ±SEM following incubation with lipopolysaccharide (LPS) at various concentrations and control

Incubation concentration LPS ng/ml	Mean Cell Viability % ±SEM (n=)
0	86 ±3.33 (n=3)
0.3	85 ±0.93 (n=3)
0.7	82 ±1.45 (n=3)
1.5	77 ±0.80 (n=3)
3	75 ±3.38 (n=3)
6	72 ±3.06 (n=4)
12	71 ±1.73 (n=3)
25	69 ±4.84 (n=3)
50	65 ±1.45 (n=3)
100	62 ±5.86 (n=3)



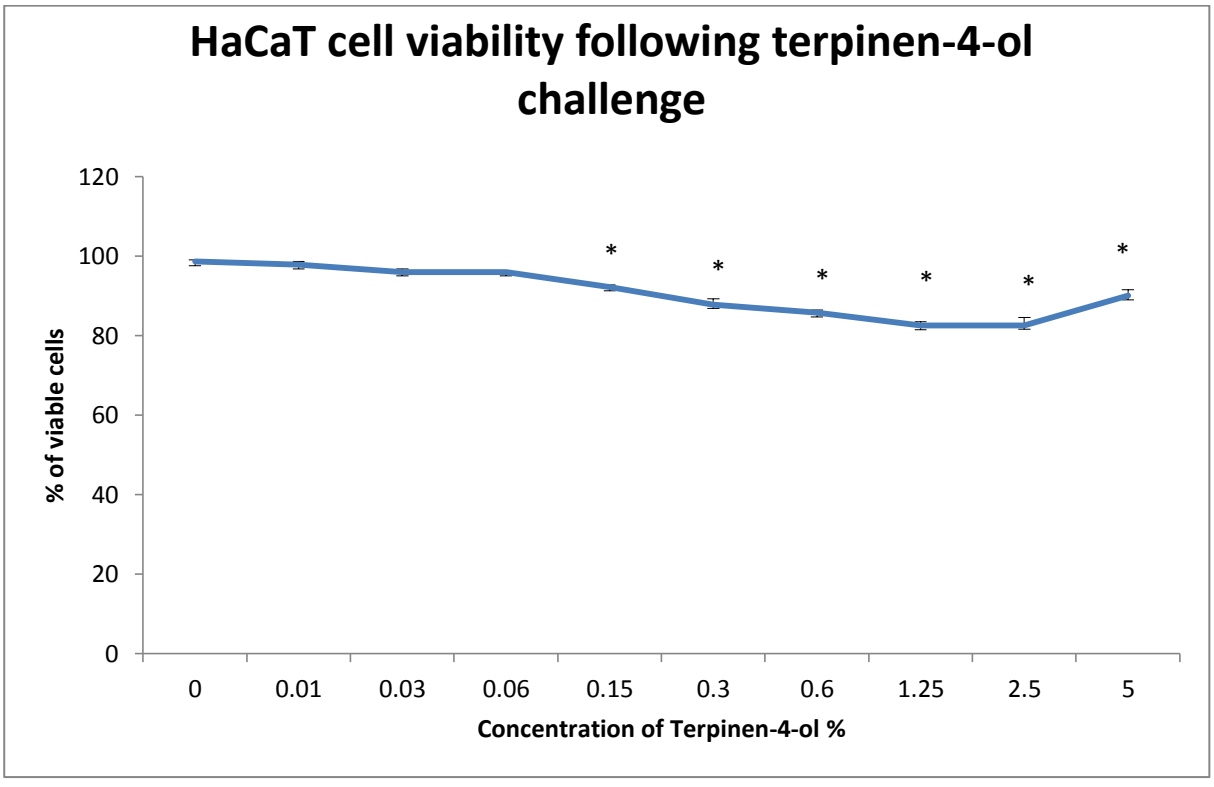


Figure 5.4 Graph to show cell viability of HaCaT cells following incubation with T-4-ol at various concentrations.

*Error bars display SEM. Statistical differences were identified by a t-test. Significant difference to control is identified with *, (n=4-5).*

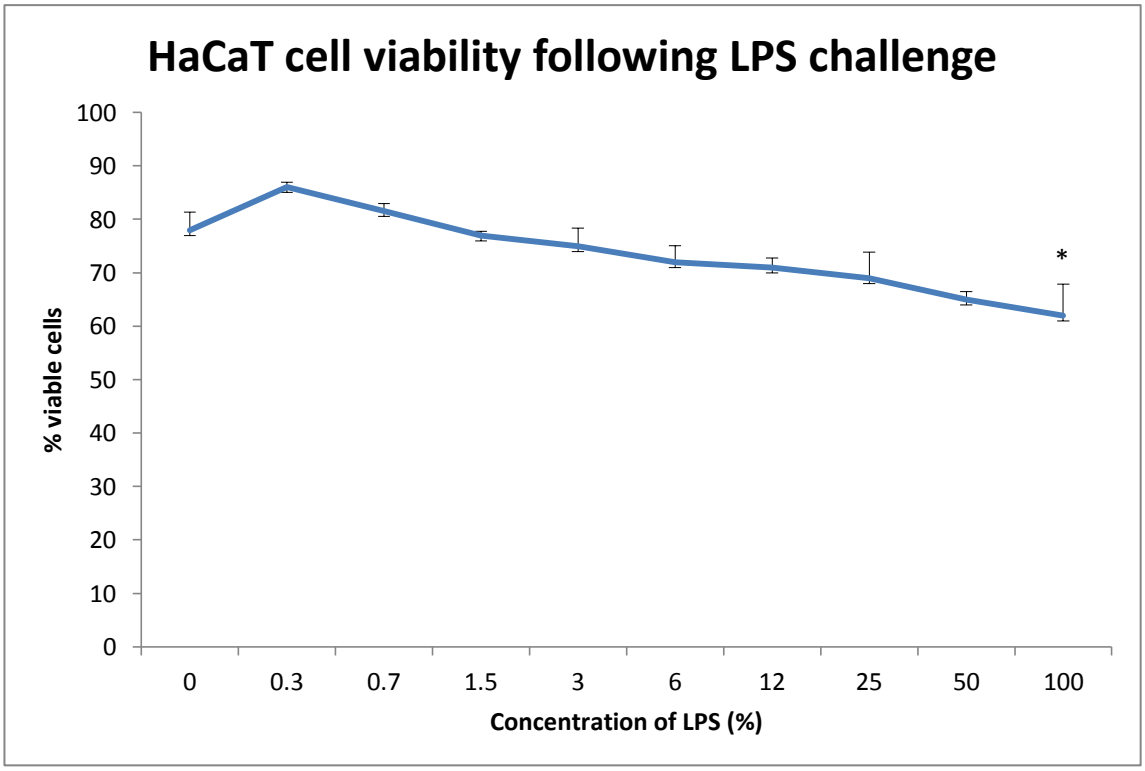


Figure 5.5 Cell viability of HaCaT cells following incubation with lipopolysaccharide at various concentrations.
*Error bars show SEM. Statistical significance is determined by a t-test. Significant difference to control is identified with *, (n=3-4). It was demonstrated that a concentration of LPS 100ng/ml significantly decreases cell viability in HaCaT cells compared to control.*

5.5.5 Conclusion

Following cytotoxicity investigations in conjunction with findings in the literature, it was concluded that a concentration of 1.25 and 5% T-4-ol and TTO would be utilised in *in vitro* experiments. This is estimated to be the amount present at the dermal epidermal-junction. Furthermore, activity of TTO and its components following topical application *in vivo* is suggested to be increased at these concentrations and above.

5.6 Methods to measure cytokine production

The measurement of cytokines in cell supernatant can be achieved in a number of ways including the use of Enzyme linked immunoassays (ELISA), multiplex bead array immunoassays with the inclusion of flow cytometry analysis scan (FACS), and multi-array electrochemiluminescense detection method (Yoon et al., 2010; Angst et al., 2006).

5.6.1 Enzyme Linked Immunoassays

Enzyme Linked Immunoassays (ELISA) are frequently utilised in the measurement of cytokines in cell supernatant (Bercherel et al., 1997; Hart et al., 2000; Yoon et al., 2010). A cell sample is added to a plate with capture molecules embedded in the base of each well, cytokines of interest are captured by the embedded capture antibody. A solution containing a labelled antibody is then added encompassing the cytokine of interest like a sandwich. The plate is then analysed using a plate reader which identifies the labelled antigens enabling the identification and quantification of the cytokine. Whilst this is a reliable method and safer than conventional radioimmunoassays (RIA), using an enzymatic label rather than a radioactive one, ELISAs can be expensive, particularly when analysing for more than one cytokine and at low levels of detection.

5.6.2 Multiplex Bead Array Immunoassay

Multiplex bead array immunoassays are also popular allowing for the detection of many cytokines at one time. Angst et al., (2006) used this method to analyse microdialysate using a 17 plex panel which measured for pro and anti-inflammatory cytokines. This method can be combined with FACS analysis. Much like the ELISA, a cell sample is added to a plate and a solution is added with encapsulated labelled beads which attach to the cytokines. Following

incubation, the sample is injected into the FACs machine which allows for identification and quantification of cytokines. This method includes many benefits, particularly when the content of a sample is unknown. However, this method is expensive and is not necessary for the purposes of the present study where the cytokines of interest have already been identified.

5.6.3 Multi-Array Electrochemiluminescence Detection

More recently a multi-array electrochemiluminescence detection method has been developed (Meso Scale Discovery). This method allows for the rapid analysis of biomarkers at low levels of detection, for example, TNFα at a concentration as low as 0.49 pg/ml, using small amounts of sample (25µl) (MSD, UK). As it is not known the levels of cytokine released from the HaCaT cells and to what level, if any, they will be modulated by TTO, a method to measure low levels of biomarker is necessary. It is also possible to design a plate with the capacity to analyse for the four pro-inflammatory cytokines of interest (IL1β, IL6, IFNγ and TNFα).

In summary, there are a number of methods which can be utilised to identify and quantify cytokine presence in a cell sample. Whilst the ELISA and Multiplex Bead Array produce accurate results and can be highly sensitive, the limitations of the ELISA method include that it can only detect one or two cytokines at a time. Whilst the number of cytokines detectable using the Bead Array is not a limitation, it is unnecessary for the purposes of this study. Despite being a relatively new development, the multi-array electrochemiluminescence detection method allows for the identification and quantification of the four cytokines of interest for this study, in addition, it has a low limit of detection for the mediators (*table 5.4)* and is an accurate, relatively quick method of analysis. Therefore the multi-array electrochemiluminescence detection method was chosen for analysis of cell supernatant in this study.

5.7 MSD multi-array electrochemiluminescence detection

For a 4 multiplex assay each well contains 4 spots (*figure 5.6*). Each spot contains capture antibodies for the specific mediator of interest (IL1β, IL6, TNFα and IFNγ). Capture and detection human antibodies were sourced from mouse monoclonal and goat polyclonal species. The sample was mixed with a solution containing labelled antibodies (anti-IL1β, anti-IL6, anti-TNFα and

anti-IFNγ) attached to an electrochemiluminescent compound which was then added to the plate. Analytes will bind to capture antibodies and be immobilized and the labelled antibodies are then recruited to complete the sandwich. A read buffer was added and the plate was then loaded onto a MSD Sector instrument for analysis. The plate was electrochemically stimulated causing the luminescent label to emit light at varying intensities according to the amount of antibody attached to mediator. This provides a quantitative method to measure the mediators included in the sample.

Figure 5.6 Diagram of the base of an individual well in a MSD 4-plex assay kit, each spot contains capture antibodies for each cytokine.
The cell supernatant is added to the well. The cytokine of interest is captured by the antibody. The labelled antibody is subsequently added prior to being read by the MSD plate reader.

The commercially available Human ProInflammatory 1 4-plex assay ultra-sensitive kit (Meso Scale Discovery, UK) was utilized to analyze HaCaT cell supernatant following various challenges with LPS, TTO and T-4-ol.
The manufacturer claims the kit to have a lower limit of detection (LLOD) as displayed in *table 5.3*.

Table 5.3 Lower limit of detection (LLOD) for cytokines to be assayed using the MSD immunoassay, as stated by the manufacturer (MSD, UK)

	IFNγ	IL1β	IL6	TNFα
LLOD (pg/ml)	0.40	0.50	0.22	0.49

The maximum limit of detection for this kit is 2500pg/ml for all mediators. These lower limits are favorably comparable to other commercially available kits and as it is not known the amount of mediator that will be produced during these studies, a low limit of detection was desirable.

5.7.1 Cell preparation

HaCat cells were cultured as previously described (*section 5.1.1*). Following passage, 200µl of cell supernatant was transferred to each well of a 96 well plate and incubated at 37ºC and 5% CO^2 for 48 hours. The cells were approximately 80% confluent in each well at this time.
The cells were incubated in the conditions shown in figure *5.7* (in duplicate).

5.8 MSD Pro inflammatory cytokine assay

Following the 24 hour incubation time the cell supernatant was assayed immediately.
All procedures were undertaken within a lamellar air flow hood in a temperature controlled laboratory (21 ± 1ºC).

All reagents were brought to room temperature prior to use.

Calibrator and control solutions were prepared. The calibrator was diluted 100-fold by adding 10µl of Calibrator Blend to 990µl of Diluent 2 (provided). This is now stock solution. An 8 point standard curve was prepared by diluting the stock solution in 4-fold serial dilution steps with Diluent 2. This was achieved by adding 50µl of stock solution to 150µl of Diluent 2, creating a solution containing 2500pg/ml of calibrator blend (Standard-01). The four fold dilution is repeated to create Standard02 (containing 625pg/ml) and this continued a further five times until Standard-07 is created containing 0.61pg/ml of calibrator blend. Diluent 2 was standard 8 and acted as control. The assay required 25µl of standard per well and all standards were duplicated.

As tissue culture supernatant was being used, dilution was not required prior to analysis and as the medium contained serum there was no need to add a carrier protein to the sample.

5.8.1 Preparation of solutions

The detection antibody solution is provided at 50X stock solution, a working concentration is 1X, 60µl of stock detection antibody blend was diluted in 2.94ml of Diluent 3.

The read buffer was provided at 4X, a working concentration of 2X was required, and therefore 10ml of 4 X read buffer T was mixed with 10ml of deionized water.

5.8.2 Standard Curves

A sample of standard curves created can be viewed in *figures 5.8*.

5.8.3 Protocol

25µl of Diluent 2 was added to each well. The plate was sealed with an adhesive plate seal (Fisher Scientific, UK) and incubated for 30min at room temperature and vigorously shaken on a plate shaker.

25µl of sample or standard was added to each well, the plate sealed once again and incubated for 2 hours at room temperature on the plate shaker. The shaking of the plate accelerates capture of the mediator to the antibody attached to the well.

The plate was washed 3 times with phosphate buffered saline plus 0.05% Tween-20 (PBS-T).

25µl of the 1X detection antibody solution was added to each well. The plate was sealed and shaken as before for a further 2 hours at room temperature.
After this time the plate was washed 3 times with PBS-T and 150µl of 2X read buffer T was added to each well.

The plate was read by the plate analyzer (Sector Imager, MSD, UK). The inbuilt software provided the measured concentration of cytokine in each well (pg/ml), the mean the mean luminescence intensity signal and % confidence variable.

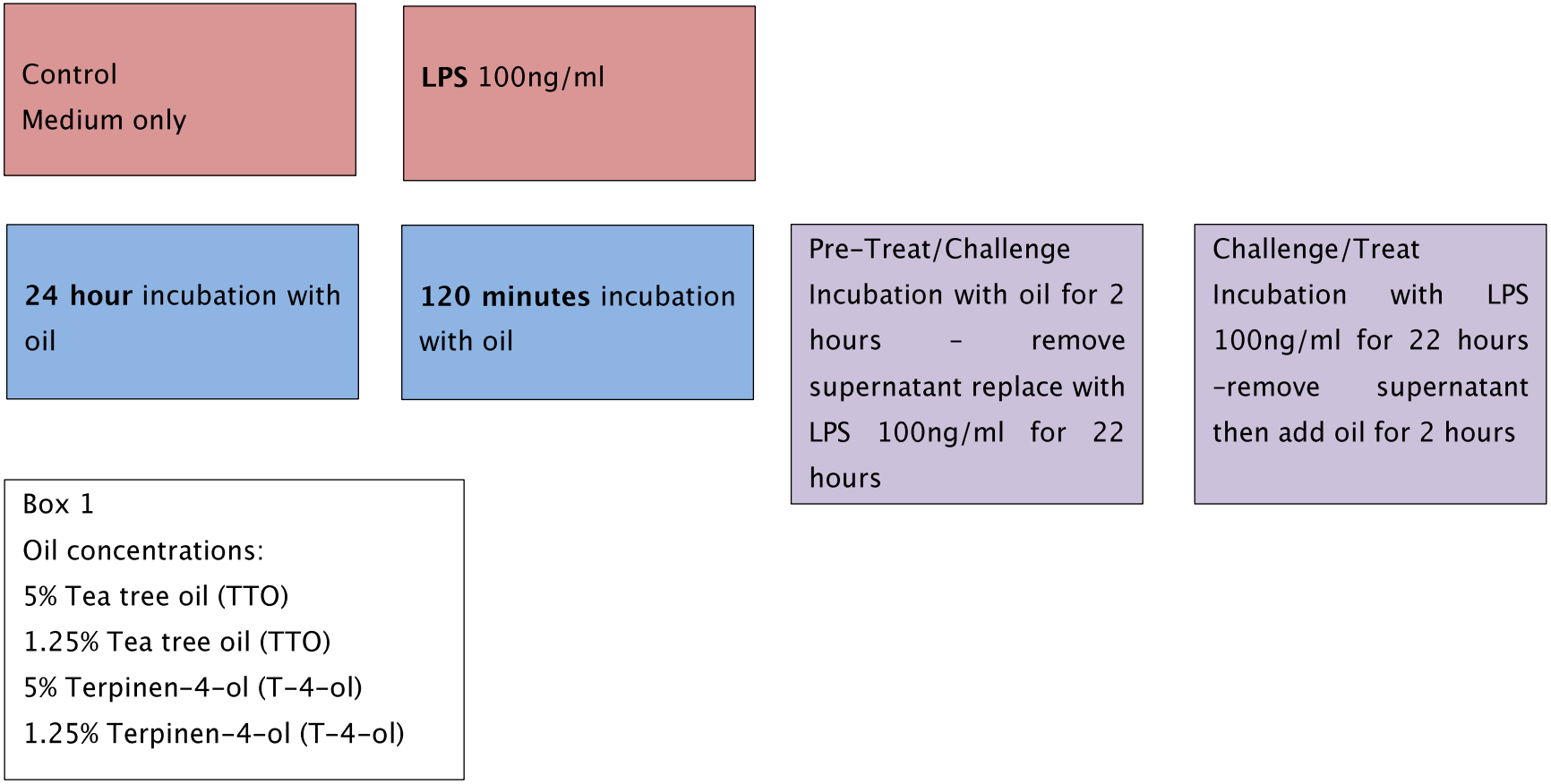
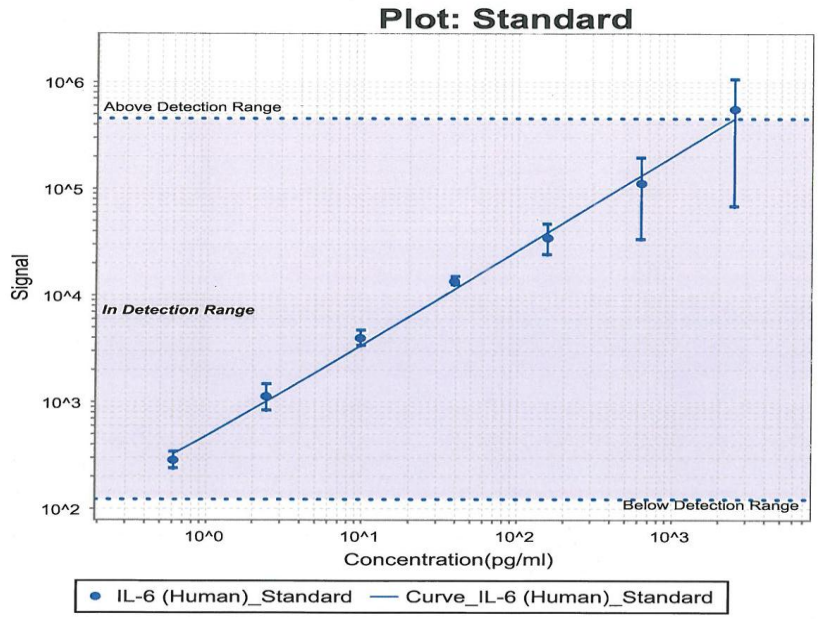
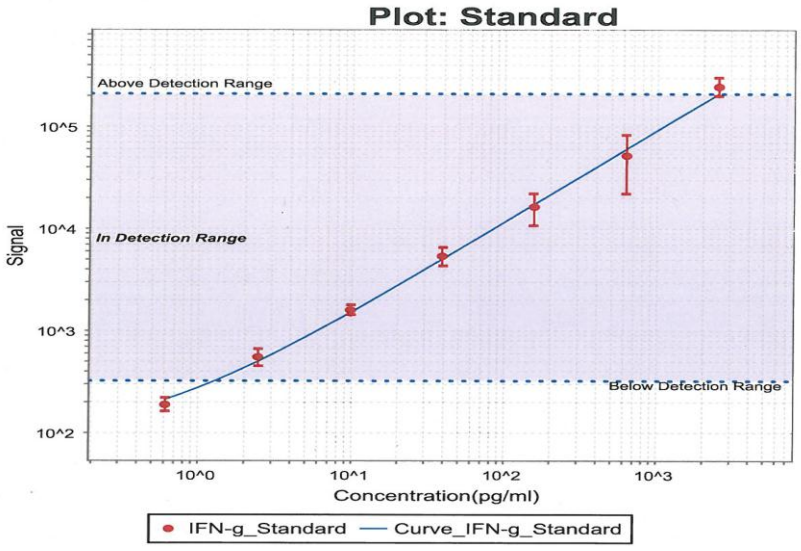


Figure 5.7 Boxes display incubation conditions for HaCaT cells. Oil types and concentrations used are identified in 'box 1'. For each challenge each well contain 1200µl of supernatant



. A



B

Figure 5.8 Standard curve created following addition of standard solutions of A – IL6 and B IFNγ provided with the MSD kit used to analyse 'unknown' samples. The concentrations range from below lower limit of detection to above upper limit of detection. The signal strength produced in an 'unknown' sample is plotted against the slope in the graph above to quantify the concentration of IL6 in the sample. Error bars dispay SEM.

5.9 Methods to measure NFKB presence in a cell sample

NFkB becomes activated following phosphorylation of the IkB and NFkB complex, leading to free NFkB in the cytoplasm which will relocate to the nucleus where it can act as a target gene (Bari et al., 2011). The amount of free NFkB in the cytoplasm is an indication of inflammatory activity, as is NFkB/DNA binding and free and bound IKK and IKB. Due to the location of NFkB the cell requires lysis prior to measurement. Analytical techniques used to quantify NFkB in cell lysate include Western Blot, electrophoretic mobility shift assay (EMSA) and multi-array electrochemiluminescence (Kim et al., 2004; Cheng et al., 2008).

5.9.1 Western Blot

The Western Blot method produces a protein immunoblot which can be used to estimate the amount of protein (NFkB) present in a cell sample. The cells are lysed and protease and phosphate inhibitors are added to prevent digestion of the sample by its own proteins. Detection antibodies are added (e.g. anti-IKK, anti-IkB). Gel electrophoresis allows for the denatured proteins to separate by length of polypeptide or 3-D structure of the protein; smaller proteins migrate faster through the mesh of the gel (usually constructed of acrylamide) and can be separated according to size. The information from the gel is then transferred to a 2-D membrane (consisting of e.g. nitrocellulose). This can be achieved in one of two ways; the membrane can be placed on top of the gel with filter papers placed on top, the stack is placed in a buffer solution allowing protein to move up the membrane by capillary action. Alternatively an electric current is used to pull proteins from the gel into the nitrocellulose membrane, the benefits of the latter include that the protein maintains the organisation they had within the gel (Kim et al., 2004; Kondaiah et al., 2009).

5.9.2 Electro Mobility Shift Assay (EMSA)

EMSA provides evidence of the binding of NFkB with DNA. Following lysis of the cell, the nuclear protein is incubated with various buffers and labelled or activated proteins corresponding to the binding site of interest (NFkB) and annealed by heating. The DNA protein complexes are resolved on a gel by electrophoresis providing bands corresponding to the protein of interest. The bands on the gel transfer to a nylon membrane (Cheng et al., 2008; Kim et al., 2004).

5.9.3 Multi-array electrochemiluminescence

The multi-array electrochemiluminescence method to analyse for NFkB identifies the amount of free NFkB in the cytoplasm. The cell is lysed and then added to a plate (provided by the manufacturer). Each well contains an electrode coated with an antibody for a specific protein target. A solution containing an antibody conjugated with an electrochemiluminescent label is added and the plate incubated. The plate is then read by the Sector Imager (as before *section 5*.9). A voltage is applied to the plate electrodes causing the labels bound to the electrode surface to emit light which can be used to quantify the amount of phosphorylated NFkB present in the sample.

As with all analytical procedures presented here for the identification of NFkB activity in cell lysate, the limitations of these methods include that accurate quantification of the amount of the protein of interest cannot be achieved. The multi array assay provides an indication of ratio of NFkB available for comparison within the same sample. It is a relatively simple method to measure presence of free NFkB providing accurate results and was therefore chosen as the method of choice within this thesis.

5.10 Protocol for the MSD 96-well MULTI-ARRAY Phosphate-NFKB (Ser468) Assay Kit

The MSD multi-array phosphate NFkB (Ser468) assay kit allows for the measurement of phosphorylated NFkB in a sample. The process is as described in *section 5.9*. Phosphorylated NFkB is immobilised by the capture antibody attached to the base of each well, the conjugated detection antibody is then bound to the analyte forming a 'sandwich' which is then read by the MSD Sector Imager. The intensity of light emitted correlates with the amount of NFkB present in the sample. Whilst this cannot be accurately quantified, a comparison can be made of NFkB present following incubation in various conditions.

5.10.1 Preparation of reagents

Tris Wash Buffer

Stock Tris Wash Buffer (provided) was diluted 10X to 1X thus:

35ml of Tris Wash Buffer (10X) in 315ml deionised water.

Blocking Solution

600mg Blocker A (dry powder) was mixed with 20ml 1X Tris Wash Buffer.

Antibody Dilution Buffer

1ml Blocking Solution was combined with 2ml 1X Tris Wash Buffer and set aside on ice.

Complete Lysis Buffer

To 10ml Complete Lysis Buffer (provided), 100µl Protease Inhibitor Solution, 100µl Phosphatase Inhibitor Solution I and 100µl Phosphatase Inhibitor Solution II were added. The complete lysis buffer was stored on ice.

Detection Antibody Solution

2.94ml of antibody dilution buffer was added to 60µl 50X SULFO-TAG Anti-Total NFKB Antibody.

Read Buffer

5ml Read Buffer T (provided) was combined with 15ml deionized water.

5.10.2 Preparation of Cells

Following treatment of cells as described in *table 5.7*, the medium was gently aspirated from each well of the 96 well plate. 50µl of 1X complete lysis buffer was added to each well and incubated for 30min.

5.10.3 Assay Protocol

150µl of blocking solution was added to each well of the MSD plate. The plate was sealed with an adhesive seal and incubated for 1hour with vigorous shaking at room temperature.

The plate was washed three times with 300µl/well of Tris Wash Buffer. 25µl of sample was added to each well, the plate was sealed with an adhesive seal (as before) and incubated for 1 hour with vigorous shaking at room temperature.

The plate was washed 3 times with 300µl/well of Tris Wash Buffer. Next 25µl of detection antibody solution was added to each well of the MSD plate and the plate immediately read using the Sector Imager. Care was taken to ensure no bubbles were introduced during the addition of read buffer as this would interfere with imaging.

5.11 Conclusion

Evidence of the anti-inflammatory activity of TTO and T-4-ol has been demonstrated using monocytes and PBMCs; however, there has been no

confirmation in the literature of contact between TTO components and these cells (Hart et al., 2000; Caldefie-Chezet et al., 2004). The presence of T-4-ol and 1,8 cineole at the dermal epidermal junction, has been confirmed in *Chapter 4* of this thesis, demonstrating contact of the TTO components with keratinocytes. However, there is little evidence of the effect of TTO components on keratinocyte cells which may support its topical use.

This chapter has discussed methods to measure the potential pro-inflammatory action of TTO and T-4-ol on keratinocyte cells *in vitro*, and methods to assess a possible mechanism of action. Cell identification and maintenance have been discussed whilst cytotoxicity studies presented in this chapter identify the concentrations that will be used of substances to be tested. A multi-array electrochemiluminescence method to identify and quantify cytokine modulation in cell supernatant and NFkB have also been described and will be subsequently utilised to identify potential anti-inflammatory action of TTO in *chapters 6* and *7*.

Chapter 6
The modulation of pro-inflammatory mediator production from HaCaT cells by tea tree oil

6 Introduction

Despite anecdotal evidence to support the anti-inflammatory effect of TTO, thus far there has been limited empirical data to support this (Brand et al., 2001; Caldefie-Chezet et al., 2004; Hart et al., 2000). Past investigations explore the potential anti-inflammatory effects of TTO on monocytes and neutrophils (Caldefie-Chezet et al., 2004; Hart et al., 2000). *Chapter 4* of this thesis demonstrates the presence of T-4-ol and 1,8 cineole at the dermal-epidermal junction, therefore confirming potential interaction of the components with cells of the epidermis. T-4-ol and 1,8 cineole have been demonstrated to exert anti-bacterial and anti-fungal effects however there is discussion in the literature regarding the oil working in synergy or individual components exerting effects alone (Cox et al., 2001, Brand et al., 2001). The following chapter explores the potential anti-inflammatory effects of T-4-ol and whole TTO on cells within the epidermis. Despite evidence of potential benefits of 1,8 cineole, there is suggestion of this component leading to dermatitis and therefore leading to an inflammatory response. For this reason 1,8 cineole was not investigated as an individual component.

6.1 The Inflammatory Response

Inflammation is a protective attempt by the body to initiate healing following invasion by an infectious agent, antigen challenge or physical, chemical or traumatic damage. This results in an initial local acute vascular response (vasodilation, increased capillary permeability, hyperaemia, erythema and oedema), followed by cells of the immune system including neutrophils presenting in the area of injury causing leakage of erythrocytes into the local tissue with fibrinogen which will reduce haemorrhage, pus is also formed by the accumulation of dead cells. Macrophages and lymphocytes follow in the next few days clearing cellular and tissue debris and the tissue can then be remodelled (Lydyard et al., 2004).

6.1.1 Immunological components of the skin

Clearly the epidermis and dermis are vulnerable to infection and injury as the skin provides the barrier between the outside environment and the body, however, it is well equipped to defend against insult due to the presence of

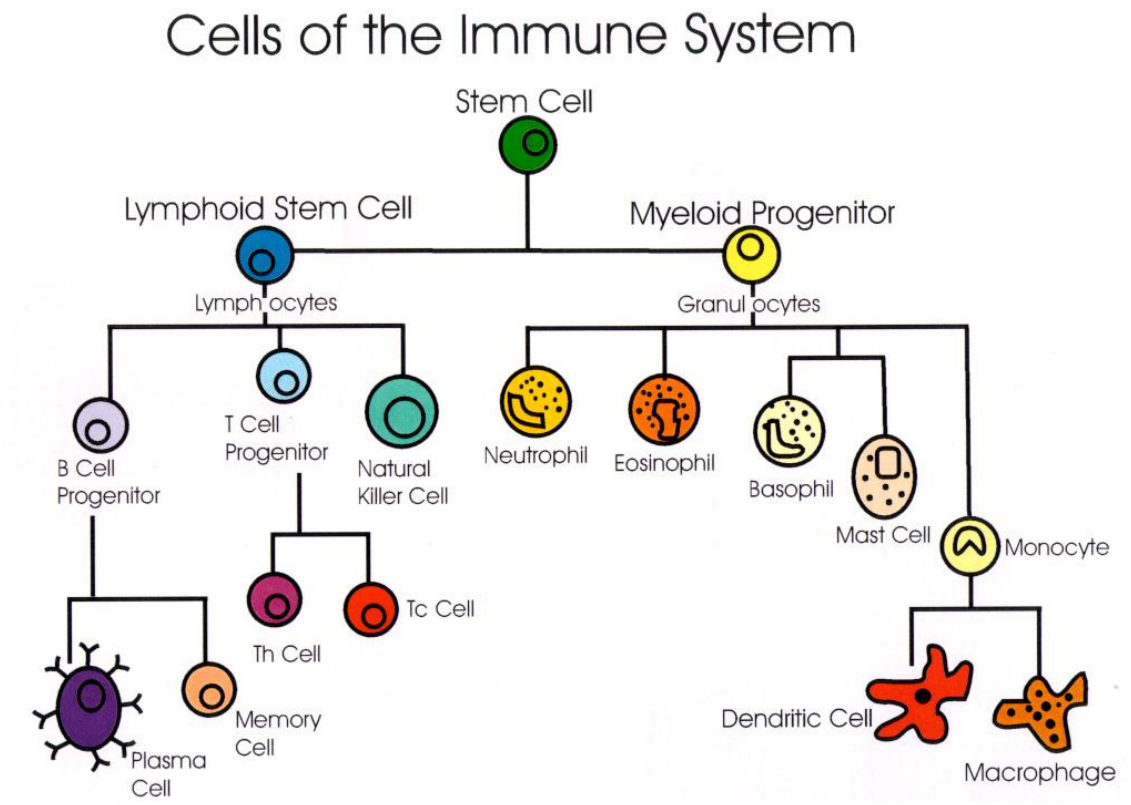


Figure 6.1 Cells of the immune system displaying differentiation of cells with a specific immunological role.
Cells originate in the bone marrow as a stem cell with the ability to develop into any of the above cells. Depending on communication with other cells and encounters with, for example, an antigen, leads the stem cell to develop into one of the cells above exhibiting various functions.

the cells identified in *figure 6.1.*

In vivo dermal microdialysis studies presented in *Chapter 4* provide evidence of T-4-ol and 1,8 cineole at the dermal-epidermal junction, therefore establishing direct contact of these TTO components with keratinocytes. Keratinocytes have the potential to produce cytokines in response to insult or infection, the modulation of which will indicate a pro or anti-inflammatory response which can be measured.

6.1.2 Cytokines produced by keratinocytes

Cytokines are small cell-signalling protein molecules with the potential to control a number of factors including cellular growth and differentiation and can regulate host responses to infection, immune response, inflammation and trauma (Corwin 2000). Depending on type, cytokines elicit a variety of functions which can be classed as pro or anti-inflammatory. The pro-inflammatory cytokines Interleukin 1 (IL1) and tumour necrosis factor (TNF) can lead to a fever, whilst it has been demonstrated that increased temperature during an infectious illness can be of benefit (Lydyard et al., 2004), over expression can lead to tissue destruction, chronic inflammation and, in some cases, shock and death (Dinarello, 2000; Corwin 2000). Therefore modulation of such cytokines will reduce the likelihood of overexpression.

Keratinocytes innately produce the pro-inflammatory cytokines IL1β, TNFα and IL6 and on activation keratinocytes produce interferon gamma (IFNγ), the properties of which are discussed below:

6.1.3 Interleukin 1β (IL1β)

IL1β induces a number of pro-inflammatory actions including the recruitment of macrophages and neutrophils to an area of infection or injury.

IL1β stimulates B cells to maturity causing antibody production and activates natural killer cells which lyse foreign cells and stimulates vascular endothelial cells to produce substances such as nitric oxide and prostaglandins. Wound, trauma and burn pain may be caused by the production of these substances or as a direct result of the cytokine on pain receptors (Junger and Sorkin, 2000).

This cytokine helps promote directional T-lymphocyte migration along a chemotactic concentration gradient to the epidermis, leading to T-cells adhering to keratinocytes. IL1β synthesises its own future production and also that of IL6. Systemic manifestations of IL1β include induction of fever, increased heart rate, decreased appetite and fatigue (Corwin, 2000).

6.1.4 Interleukin 6 (IL6)

In addition to being induced by IL1β, IL6 is also produced by TNFα and in response to bacterial endotoxin and viral infection.

IL6 has been demonstrated to induce fever and produces acute phase proteins such as c-reactive protein (CRP) and several complement proteins such as fibrinogen and fibrin (Lydyard et al., 2004). Acute phase proteins are thought to assist white blood cells when containing a pathogen and neutralizing its toxin (Corwin 2000).

Despite IL6 being described as a pro-inflammatory cytokine, possibly due to its production during an inflammatory response by IL1β and TNFα, IL6 has an inhibitory effect on the production of IL1β and TNFα, acting in a negative feedback manner and so exhibits anti-inflammatory behaviour.

6.1.5 Tumour Necrosis Factor-α (TNFα)

TNFα is released by activated macrophages in response to bacterial endotoxin and increased concentrations of IL1 and IL2. This cytokine activates vascular endothelium and stimulates cell surface expression of major histocompatibility antigens that aid leucocytes when identifying foreign antigens. It increases vascular permeability and induces mobilization of metabolites. TNFα is cytotoxic to some tumours causing lysis and destruction of cells. Systemically it causes decreased appetite, fever and shock (Lydyard et al., 2004). TNFα induces expression of IL1 and IL2 as well as stimulating the production of IL6 (Crowen 2000).

6.1.6 Interferon-γ (IFNγ)

IFNγ is produced by activated NK cells, stimulated by maturing B cells which are induced by IL1β. IFNγ is capable of inhibiting viral replication and targeting uninfected host cells. This molecule increases expression of all major histocompatibility molecules which is essential when identifying virally infected cells (Crowen 2000). IFNγ induces Th1 (pro-inflammatory) and inhibits Th2 (anti-inflammatory) responses (Lydyard et al., 2004).

The modulation of IL1β, TNFα, IL6 and IFNγ released by keratinocytes will be investigated in response to application of TTO and T-4-ol.

6.1.7 Summary

In, summary, inflammation is necessary to protect the body from foreign insult, injury and infection. However, acute inflammation due to over expression of pro-inflammatory cytokines leads to chronic inflammation causing tissue destruction and the inability to resolve the process. *In vivo* dermal microdialysis has demonstrated that the TTO components T-4-ol and 1, 8 cineole are able to penetrate to the dermal-epidermal junction following topical application of the oil (*Chapter 4*) and is therefore in direct contact with keratinocytes. Keratinocytes innately produce the pro-inflammatory cytokines IL1β, TNFα, IL6 and upon activation IFNγ, the reduction of which may lead to the early resolution of an inflammatory response. Therefore the potential anti-inflammatory properties of TTO were investigated by examining the modulation of IL1β, TNFα, IL6 and INFγ produced by keratinocytes in response to the application of TTO and T-4-ol.

6.2 The modulation of inflammatory cytokines by TTO and T-4-ol

6.2.1 Aim

To identify the modulatory effects of TTO and T-4-ol on IL1β, TNFα, IL6 and INFγ produced by keratinocytes following an inflammatory challenge

6.2.2 Objectives

- To establish the modulatory effect of TTO and T-4-ol on cytokines produced by keratinocytes at various time points following an inflammatory challenge.

- To establish the effect of pre-treatment of keratinocyte cells with TTO and T-4-ol on the modulation of cytokines produced.

6.2.3 Protocol

All manipulation of cell culture was undertaken in a lamellar flow cabinet within a temperature controlled tissue culture laboratory (21°C±1°C).

HaCaT cells were maintained as described in *Chapter 5 (section 5.1.1*). Following passage the cells 200µl of cell supernatant (approximately 10^6 cells) were seeded in a 96 well plate, flat bottomed with no additives (NUNC) and incubated at 37°C, 5% CO^2 for 48 hours. The cells were then incubated under the conditions shown previously *(figure 5.5*).

An MSD immunoassay was used to determine the amount of cytokine produced under each condition. The MSD immunoassay was conducted as manufacturer's instructions described in detail in *Chapter 5 (section 5.9*).

Following analysis by the plate analyser (Sector Imager, MSD, UK), the inbuilt software provided the measured concentration of cytokine in each well (pg/ml). Standard curves are presented in *Chapter 5 (figure 5.8*).

6.3 Data Analysis

Following analysis utilising the plate analyser (Sector Imager, MSD, UK) the statistical software package SPSS (IBM v19) was used to analyse for differences in cytokine production between the conditions observed.

The results were split into two groups:
Group A; Incubation time of oil (in later analysis this is compared with control). The results will identify not only if T-4-ol or TTO at 1.25% and 5% modulates cytokine production in un-stimulated HaCaT cells but also if duration of incubation has an effect.

Group B; Pre or post challenge treatment of cells with oil (in later analysis this is compared to cells incubated with LPS). The results will identify if pre-treatment of HaCaT cells with TTO or T-4-ol at 1.25 or 5% concentration has the ability to modulate cytokine production in HaCaT cells and if this differs to treatment after challenge.

Results are expressed as mean (pg/ml) ±SEM. Statistical analysis was undertaken using a between samples two way analysis of variance (ANOVA) and subsequent independent t-test when required. Levene's test for homogeneity of variances was used to ensure the assumption of homogeneity of variance was not violated. If it was found that this assumption was violated,

the data provided by the SPSS programme for 'equal variances not assumed' was used (Pallant ed.,2007). Statistical significance is achieved if p is equal or less than 0.05. Effect size was calculated using eta squared as described below (*equation 12*):

$$\textbf{Eta squared} = \frac{t^2}{t^2+(n1+n2-2)} \qquad (12)$$

*n*1 = number of subjects in group 1
*n*2 = number of subjects in group 2

Cohen's guidelines were used in order to interpret the value:
0.01 = small effect
0.06 = moderate effect
0.14 = large effect (Pallant ed., 2007)

6.4 Results

Results are presented in table 6.1 displaying the mean cytokine production (pg/ml) under each condition.
Statistical analysis is presented in table 6.2. It is evident following the initial analysis that there were no significant statistical differences in the production of cytokine following incubation under the various conditions. However cytokine production was observed to be up-regulated, although not significantly, under certain conditions as displayed in figures 6.2 – 6.9.

6.4.1 Pooled Data

Subsequent data analysis was undertaken on pooled data. Four groups were created: Group 1; All oil types and concentrations (5% TTO, 1.25% TTO, 5% T-4-ol and 1.25% T-4-ol). Group 2; all exposure times (120min and 24 hours). Group 3; Pre-treat and challenge-treat and group 4; HaCaT cells stimulated with LPS alone.
Group 1 data was compared to group 2 data. Results are displayed in *figures 6.10, 6.12, 6.14* and *6.16,* whilst groups 3 and 4 were compared. Results are displayed in figures 6.11, 6.13, 6.15 and 6.17.

Table 6.1 Cytokine production (pg/ml ±SEM) from HaCaT cells in response to incubation under varying conditions.

	IL1β		IL6		TNFα		IFNΥ		
	Mean	±SEM	Mean	±SEM	Mean	±SEM	Mean	±SEM	n=
C	0.30	0.05	6.35	5.07	1.20	0.88	4.48	2.53	4
LPS	0.10	0.02	9.06	8.40	0.96	0.69	2.83	2.10	4
24 5 TTO	2.49	1.50	3.50	0.93	1.07	0.82	0.59	0.14	4
24 1.25 TTO	0.10	0.01	12.07	6.48	0.99	0.54	1.94	1.26	4
24 5 T-4-ol	1.61	0.97	5.50	0.76	0.38	0.16	3.08	2.90	4
24 1.25 T-4-ol	0.14	0.03	4.80	0.99	1.26	1.07	1.54	0/93	4
120min 5 TTO	0.58	0.06	8.18	3.62	0.23	0.06	0.73	0.00	4
120min 1.25 TTO	0.39	0.18	21.45	10.55	0.22	0.02	0.65	0.07	4
120 5 T-4-ol	0.20	0.02	4.83	3.23	0.21	0.06	1.10	0.35	4
120 1.25 T-4-ol	0.25	0.09	6.15	2.12	0.25	0.05	1.28	0.94	4
p/t 5 TTO	0.53	0.19	0.60	0.40	0.77	0.67	2.58	1.48	4
p/t 1.25 TTO	1.16	0.63	9.90	6.06	0.75	0.53	2.12	1.88	4
p/t 5 T-4-ol	1.61	1.13	1.80	0.00	0.30	0.21	2.14	1.37	4
p/t 1.25 T-4-ol	0.34	0.17	2.66	1.80	1.13	0.63	1.67	0.63	4
lps 5 TTO	0.93	0.46	3.23	2.04	1.61	1.30	2.32	2.15	4
lps 1.25 TTO	0.40	0.16	7.69	6.15	0.14	0.06	4.71	3.00	4
lps 5 T-4-ol	0.77	0.43	3.02	2.35	0.22	0.14	2.12	1.69	4
lps 1.25T-4-ol	0.36	0.16	0.70	0.20	1.01	0.81	2.01	0.96	4

Table 6.2 Results following data analysis using a between samples two way analysis of variance (ANOVA). The coloured text represent the following cytokines: TNFα IL6 IFNY IL1β. ***There are no statistically significant results (p = <0.05)***

	Concentration	Time	PT/CT
Concentration	F = 0.129 p = 0.941 F = 0.157 p = 0.923 F = 0.257 p = 0.855 F = 0.500 p = 0.688	F = 0.107 p = 0.954 F = 0.571 p = 0644 F = 0.122 p = 0.952 F = 0.328 p = 0.805	F = 0.175 p = 0.912 F = 1.693 p = 0.204 F = 0.230 p = 0.874 F = 0.674 p = 0.577
Time		F = 1.6 p = 0.224 F = 1.011 p = 0.333 F = 0.415 p = 0.530 F = 2.309 p = 0.148	
PT/CT			F = 0.092 p = 0.765 F = 1.307 p = 0.303 F = 0.249 p = 0.874 F = 0.646 p = 0.429

PT= pre-treat-challenge
CT= challenge-treat

An independent-samples t-test was conducted to compare cytokine production (pg/ml) by group 1 compared with group 2. Whilst there were no statistically significant differences in cytokine production between groups 1 and 2 for cytokines observed for it was evident that there was significant increase in the production of IL1b; group 1 (oil) M=1.24, SEM 0.4, and group 2 (control) M= 0.295, SEM 0.046; t (23.57) = -.380, p=0.029 (two-tailed). The magnitude of the difference in the means (mean difference = 0.94534, 95% CI: 0.106-1.7) was large (eta squared = 0.165).

Following this an independent-samples t-test was conducted to compare IL1β production in pg/ml by HaCaT cells following exposure to TTO (pooled results 1.25% and 5%) and no oil (control) as shown in *figure 6.18*. There was a significant difference between IL1b production following exposure to TTO M =

1.895, SEM 0.7) and control M = 0.2952, SEM 0.046; t(11.093) = 2.177, p = 0.05 (two-tailed). The magnitude of the difference in the means (mean difference – 1.53072, 95% CI: – 0.015 – 3.077) was large (eta squared = 0.166).

An independent-samples t-test was conducted to compare IL1b production in pg/ml by HaCaT cells following exposure to T-4-ol (pooled results 1.25% and 5%) and no oil (control) as shown in *figure 6.19*. There was no significant difference in IL1b production following exposure to T-4-ol, M = 0.655, SEM 0.356 and control, M = 0.2952, SEM 0.046; t (15)= 0.640, p = 0.532 (two-tailed). The magnitude of the difference in the means (mean difference – 0.36, 95% CI: -.84 – 1.56) was small (eta squared = 0.02).

6.4.2 Cytokine production from cells stimulated v non-stimulated

Groups 3 and 4 were compared. Whilst there was no significant difference between groups in the regulation of IL1b, IFNg or TNFa, there was a statistically significant increase in the mean production of IL6 by non-stimulated cells compared with stimulated cells (*figure 6.20*).

An independent-samples t-test was conducted to compare the IL6 production between stimulated and non-stimulated cells. There was a significant increase in IL6 production in non-stimulated cells, M = 14.21 SEM 3.555 compared with stimulated cells, M = 5.78 SEM 1.688; t (27.387) = -2.142, p=.041 (two-tailed). The magnitude of the difference in the means (mean difference = -8.428, 95% CI: -16.497 to -0.359) was large (eta squared = 0.123).

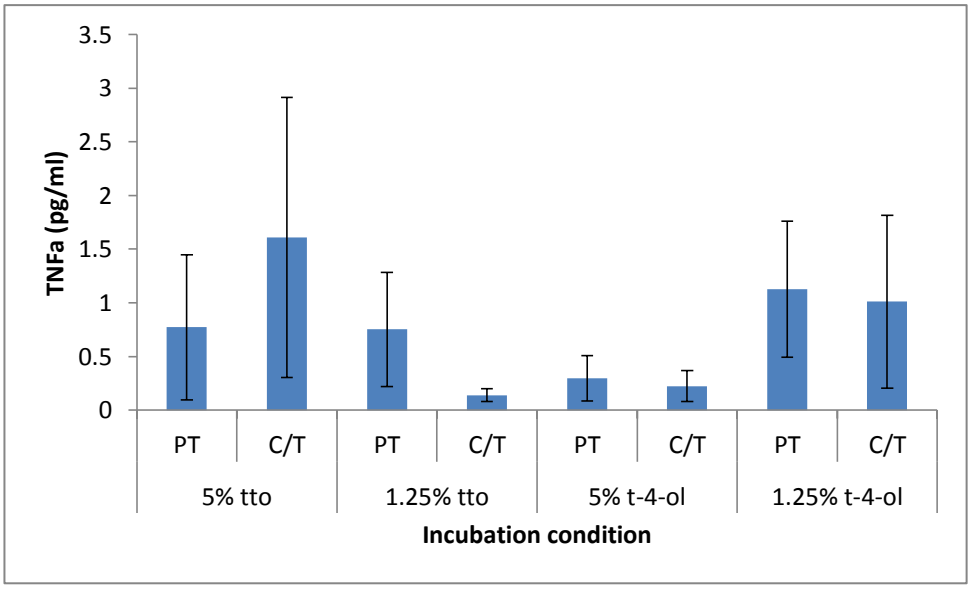


Figure 6.2 Amount of TNFα released following pre-exposure (PT) and then challenge with LPS and challenge then exposure (CT) of HaCaT cells with TTO and T-4-ol at varying.Error bars display SEM. n=4

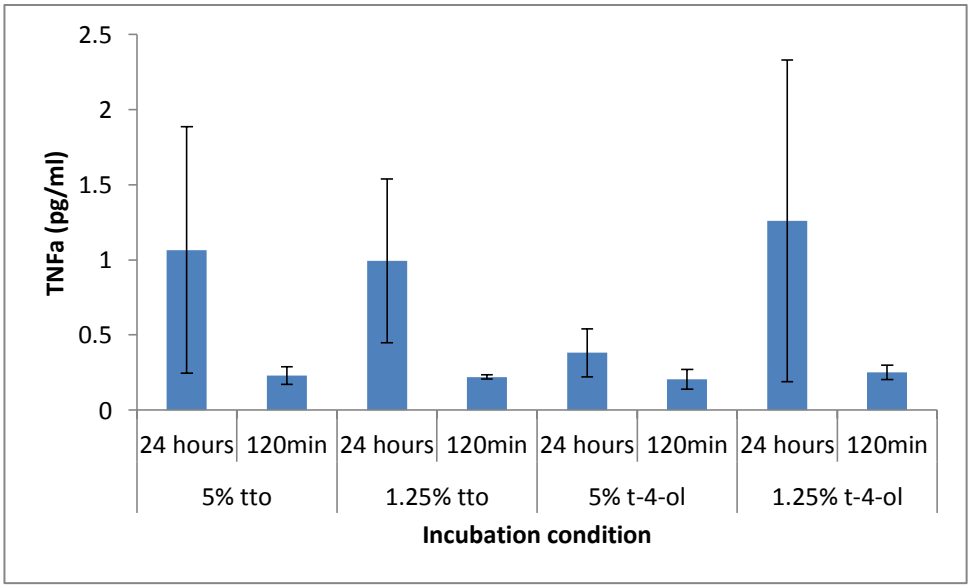


Figure 6.3 Amount of TNFα released following Exposure to 5%, 1.25% TTO (TTO) and T-4-ol (T-4-ol) for 120min and 24 hours. Error bars display SEM. n=4.

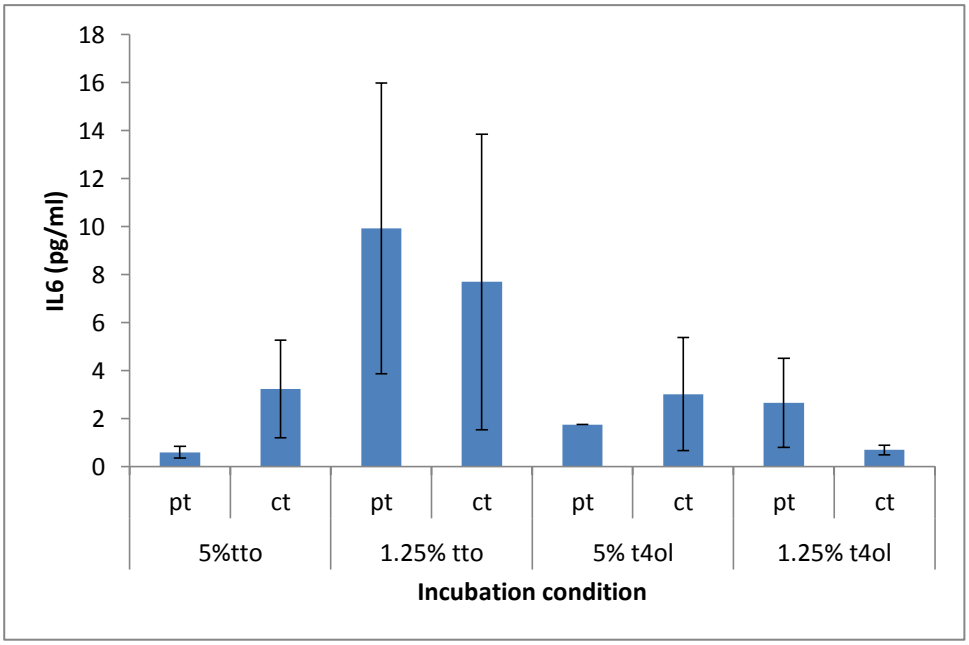


Figure 6.4 Amount of IL6 released following pre-exposure (PT) and then challenge with LPS and challenge then exposure (CT) of HaCaT cells with TTO and T-4-ol. Error bars display SEM. n=4.

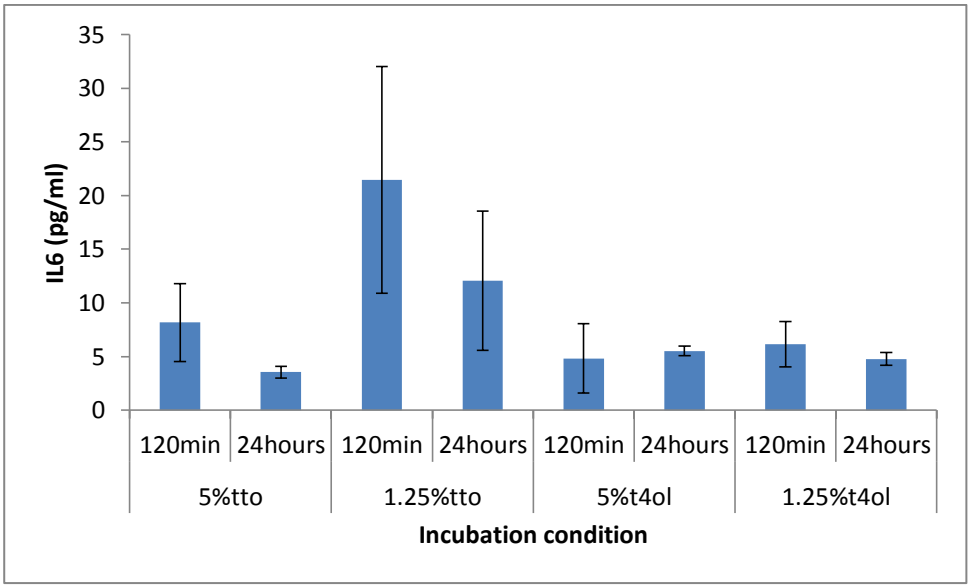


Figure 6.5 Amount of IL6 released following Exposure to 5%, 1.25% TTO (TTO) and T-4-ol (T-4-ol) for 120min and 24 hours. Error bars display SEM. n=4

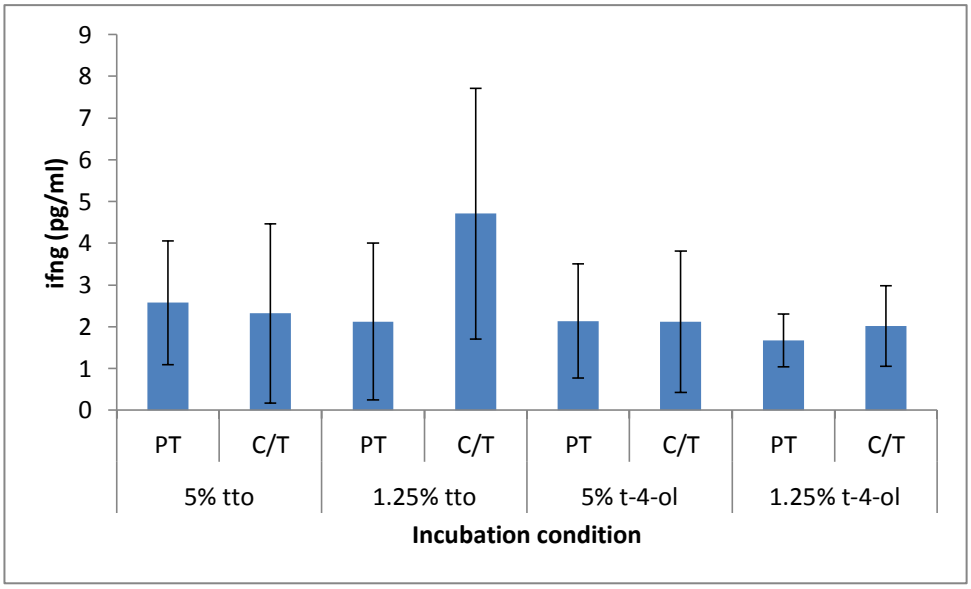


Figure 6.6 Amount of IFN-Y released following pre-exposure (PT) and then challenge with LPS and challenge then exposure (CT) of HaCaT cells with TTO and T-4-ol. Error bars display SEM. n=4.

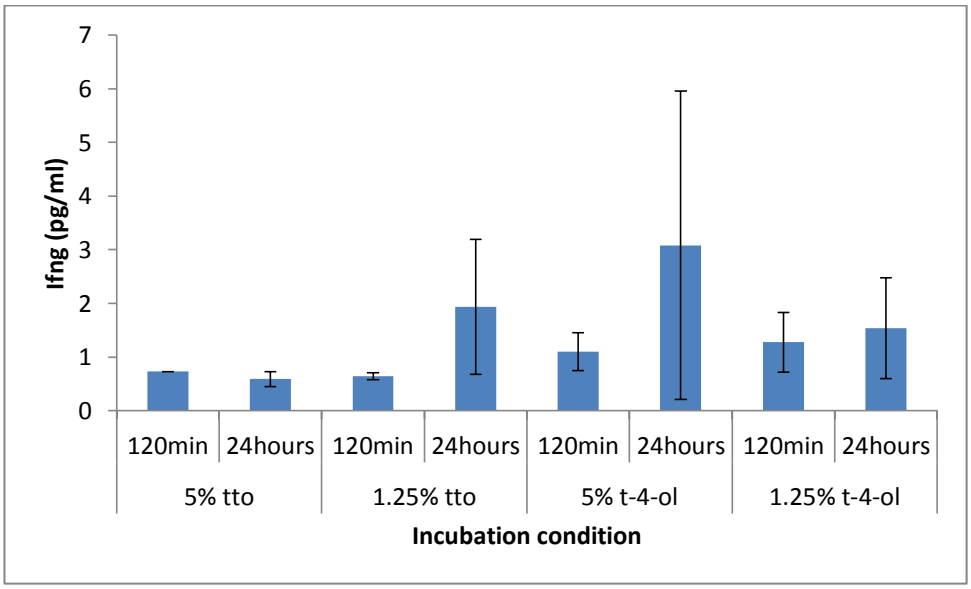


Figure 6.7 Amount of IFNY released following Exposure to 5%, 1.25% TTO (TTO) and T-4-ol (T-4-ol) for 120min and 24 hours. Error bars display SEM. n=4.

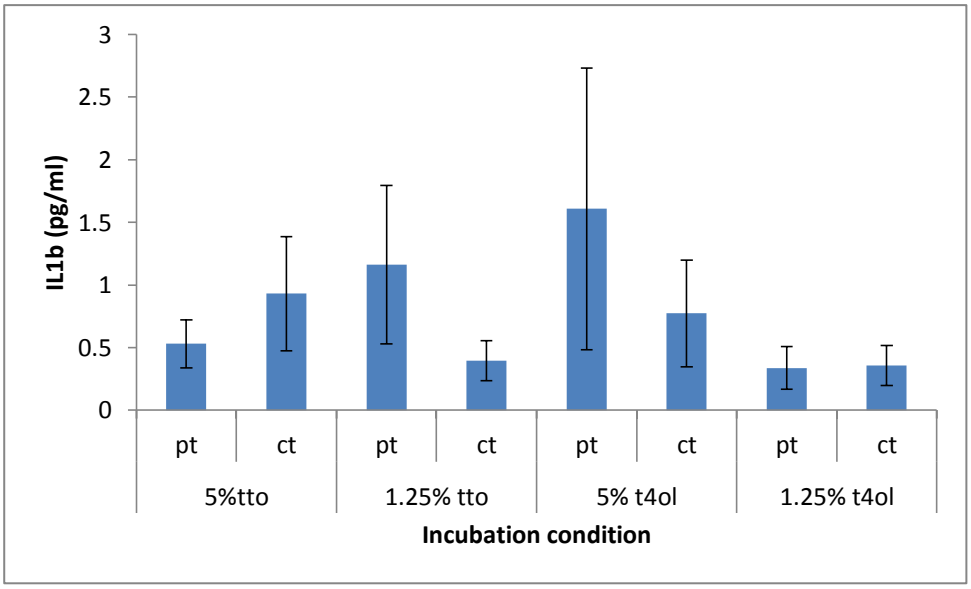


Figure 6.8 Amount of IL1ß released following pre-exposure (PT) and then challenge with LPS and challenge then exposure (CT) of HaCaT cells with TTO and T-4-ol. Error bars display SEM. n=4

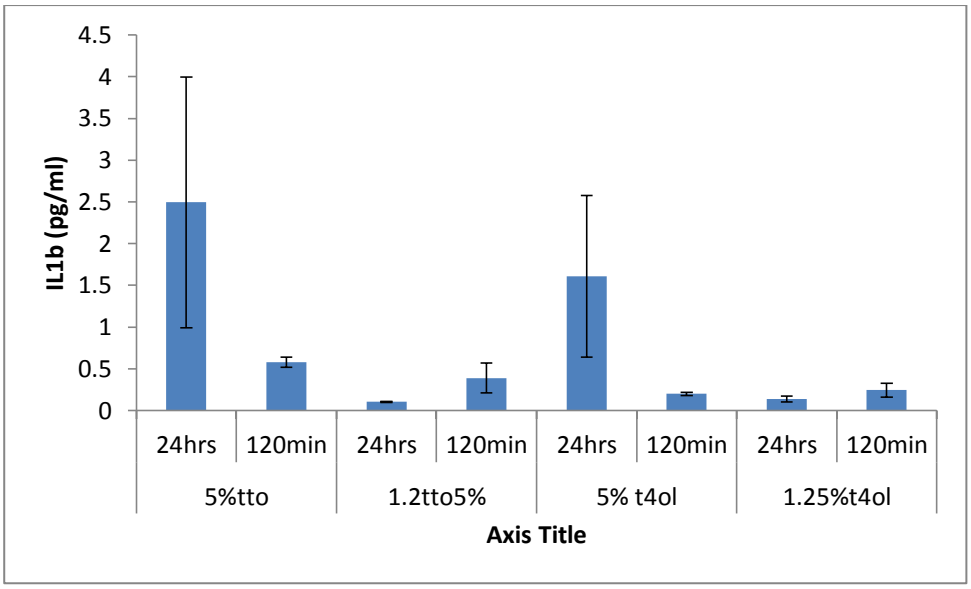


Figure 6.9 Amount of IL1ß released following Exposure to 5%, 1.25% TTO (TTO) and T-4-ol (T-4-ol) for 120min and 24 hours. Error bars display SEM. n=4.

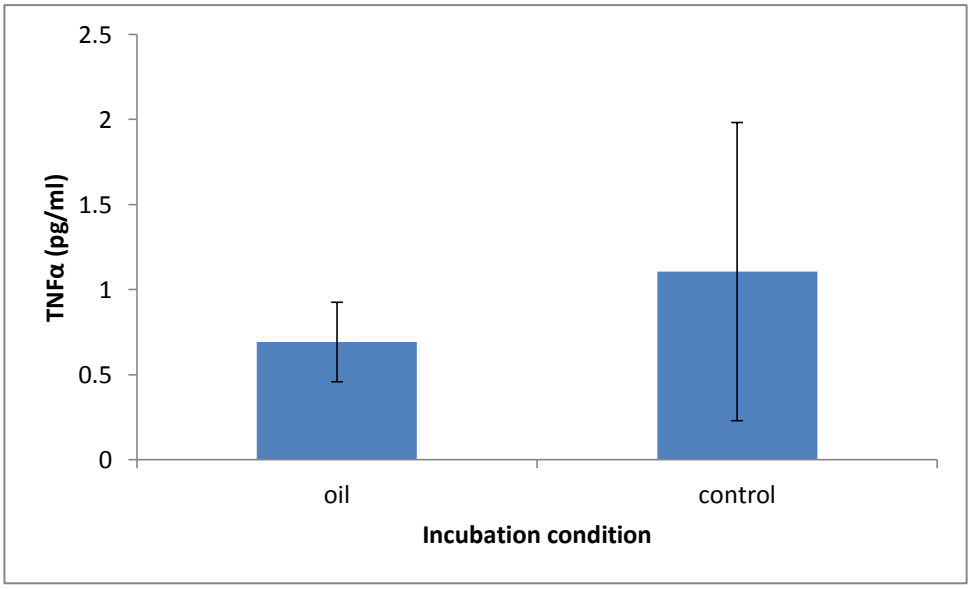


Figure 6.10 Amount of TNFα produced by HaCaT cells following exposure to oil (TTO and T-4-ol) and control. Error bars display SEM. n = 24 (oil) n=4 (control).

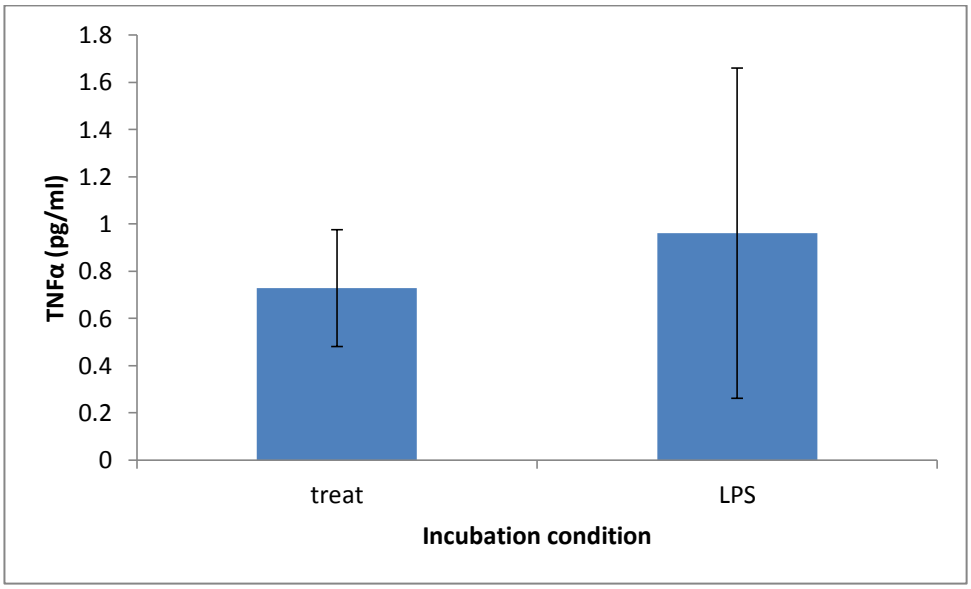


Figure 6.11 Amount of TNFα produced by challenged HaCaT cells following exposure to oil (TTO and T-4-ol) and LPS. Error bars display SEM. n = 24 (treat) n=4 (LPS).

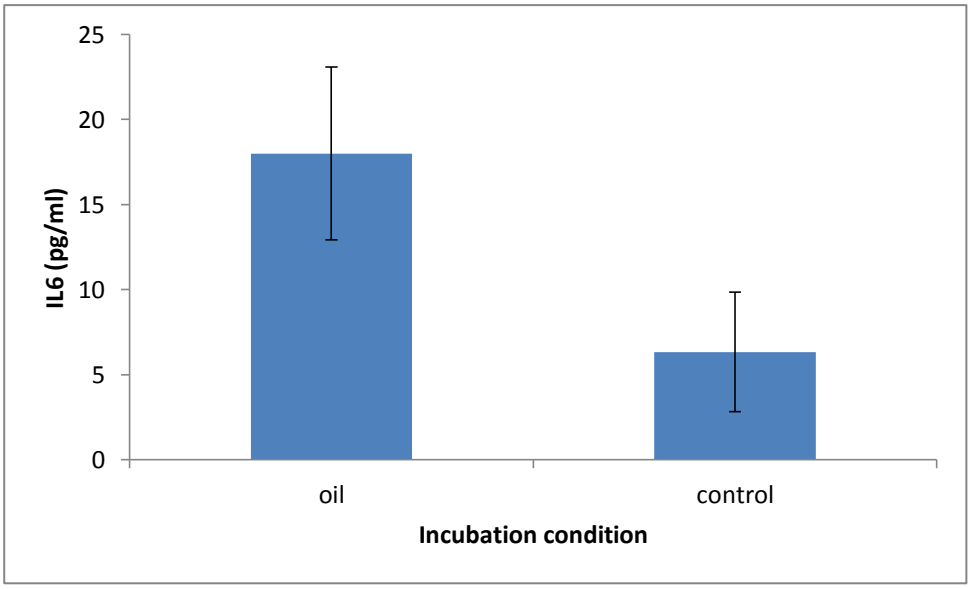


Figure 6.12 Amount of IL6 produced by HaCaT cells following exposure to oil (TTO and T-4-ol) and control. Error bars display SEM. n = 24 (oil) n=4 (control).

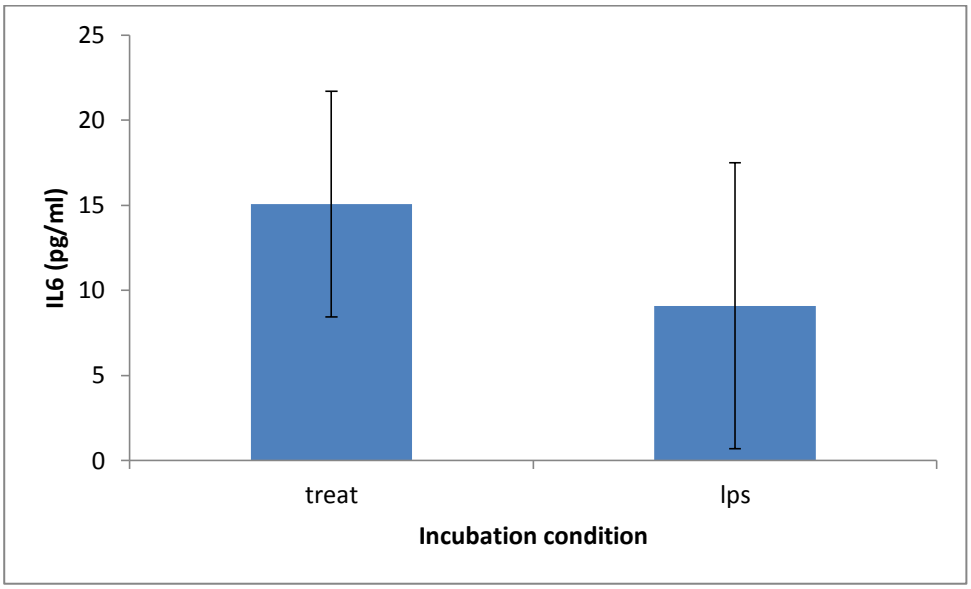


Figure 6.13 Amount of IL6 produced by challenged HaCaT cells following exposure to oil (TTO and T-4-ol) and LPS. Error bars display SEM. n = 24 (treat) n=4 (LPS).

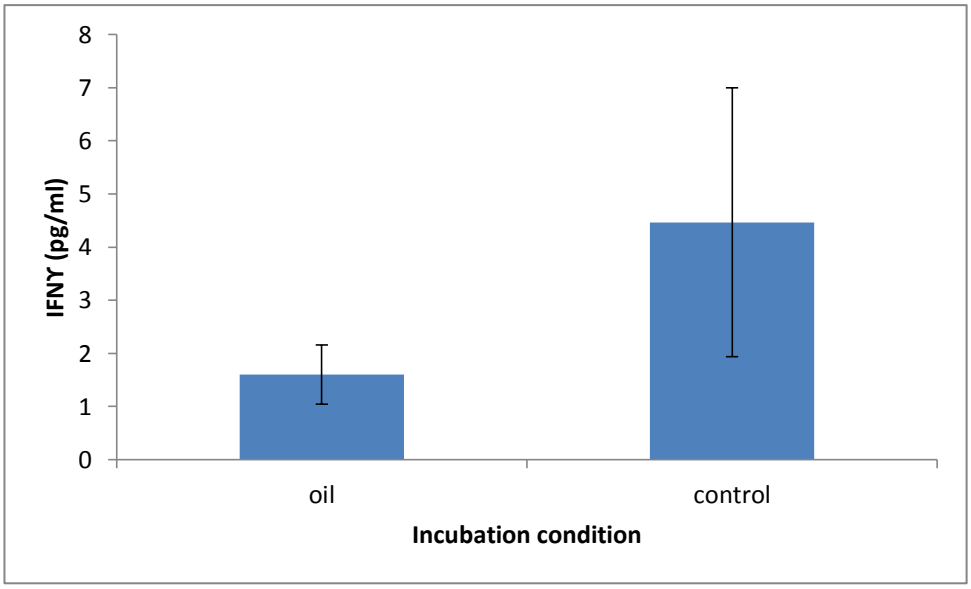


Figure 6.14 Amount of IFNY produced by HaCaT cells following exposure to oil (TTO and T-4-ol) and control. Error bars display SEM. n = 24 (oil) n=4 (control).

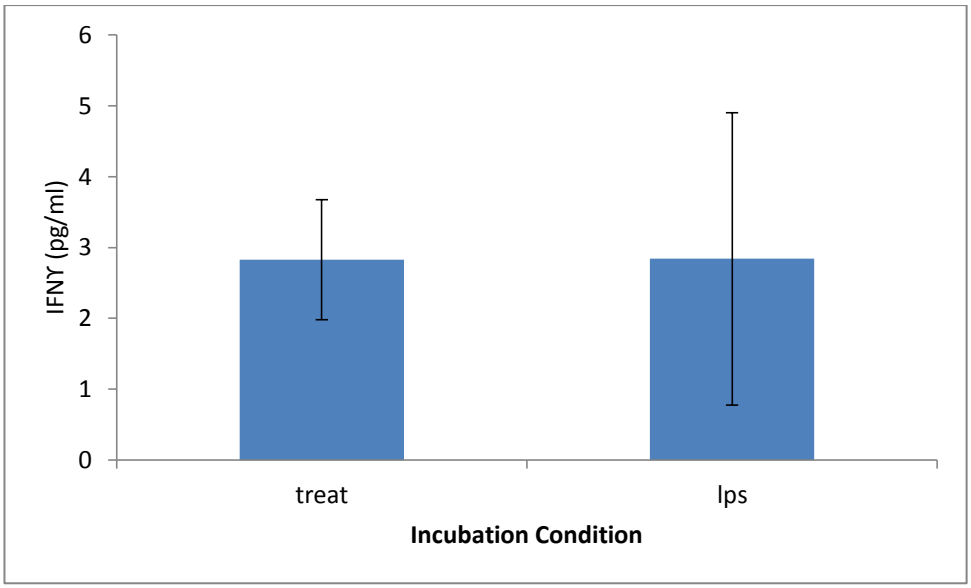


Figure 6.15 Amount of IFNY produced by challenged HaCaT cells following exposure to oil (TTO and T-4-ol) and LPS. Error bars display SEM. n = 24 (treat) n=4 (LPS).

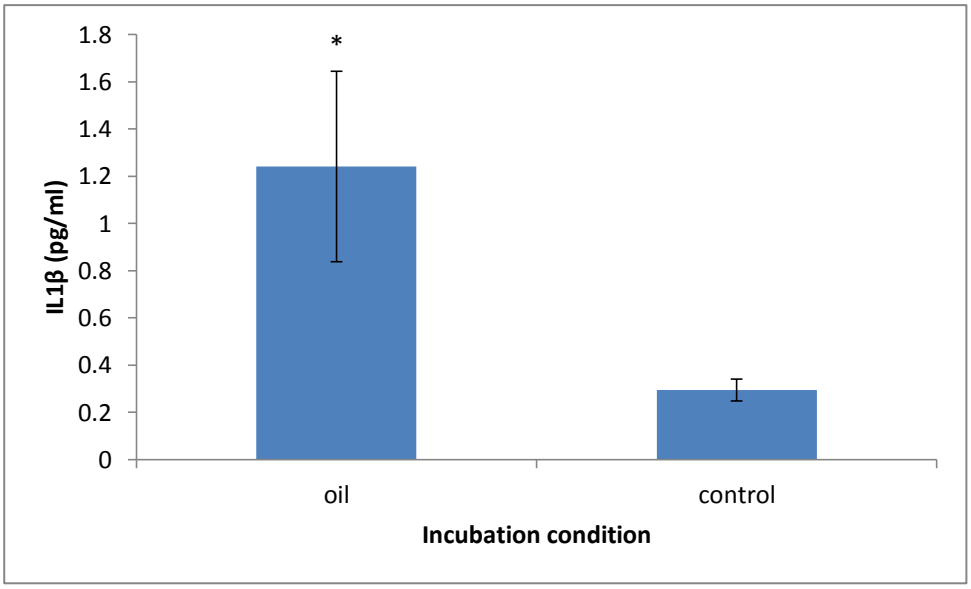


Figure 6.16 Amount of IL1ß produced by HaCaT cells following exposure to oil (TTO and T-4-ol) and control.
*Comparison of the pooled data of groups 1 and 2 identified a 3 fold increase in the production of IL1ß from the cells exposed to oil compared to control (medium only). The difference is statistically significant and is identified with an *. Error bars display SEM. n = 24 (oil) n=4 (control).*

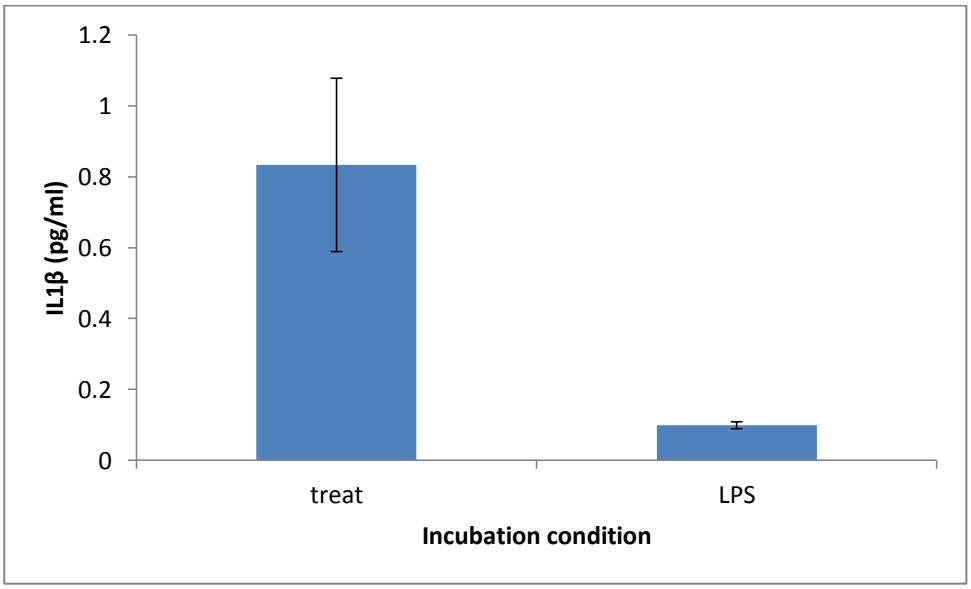


Figure 6.17 Amount of IL1ß produced by challenged HaCaT cells following pre-treatment, challenge/treat compared with incubation with LPS only.
Error bars display SEM. n = 24 (treat) n=4 (LPS) Whilst the difference is not statistically significant, it is clear that HaCaT cells that have been exposed to TTO and T-4-ol exhibit an up-regulation of IL1ß compared with those exposed to LPS alone.

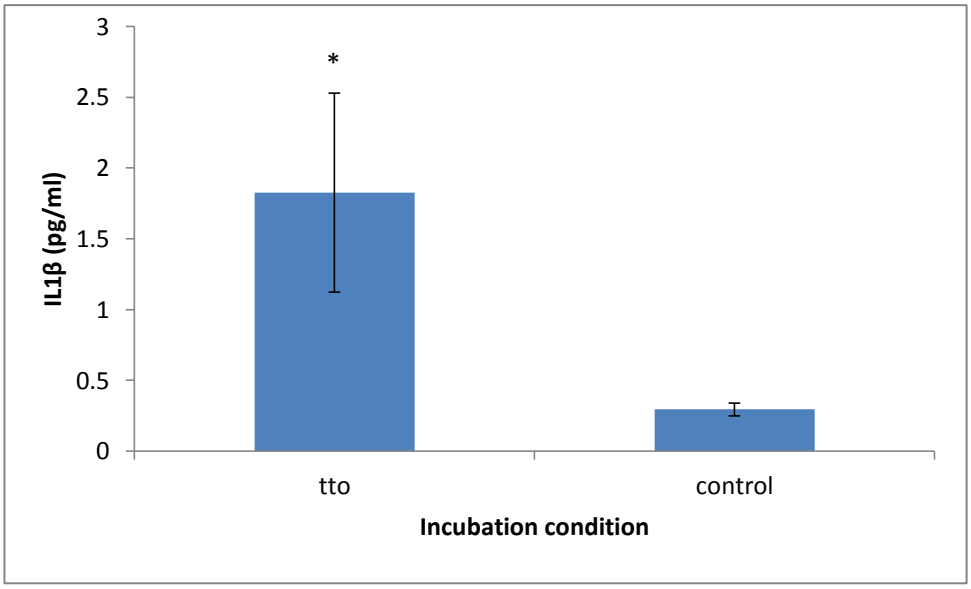


Figure 6.18

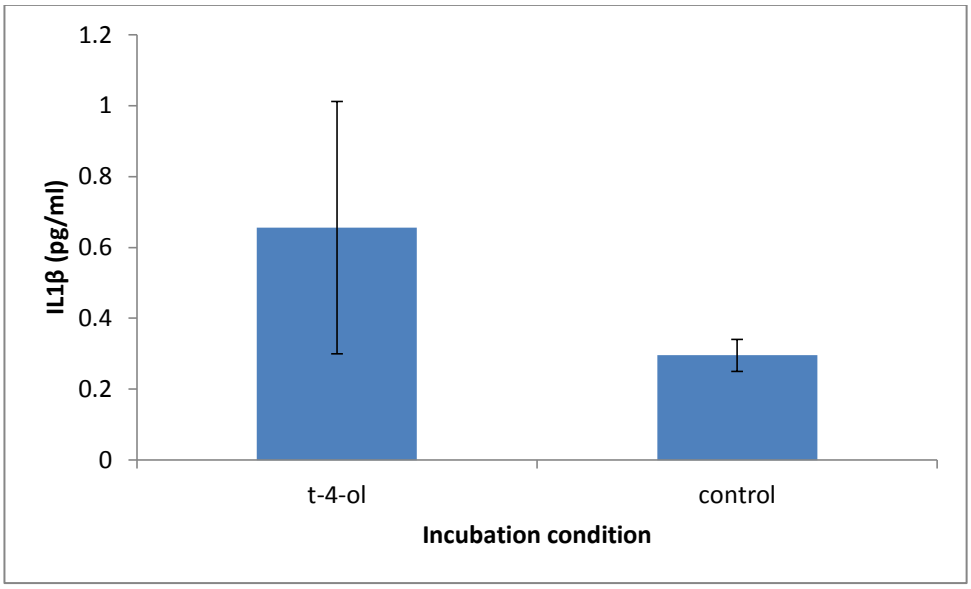


Fig 6.18

Figure 6.18 and 6.19 Amount of IL1ß produced by HaCaT cells following exposure to TTO and T-4-ol.

Further statistical analysis revealed that incubation with TTO (rather than Terpinen-4-ol) led to the statistically significant increase of IL1ß production. Statistically significant difference is demonstrated by *. n=12.

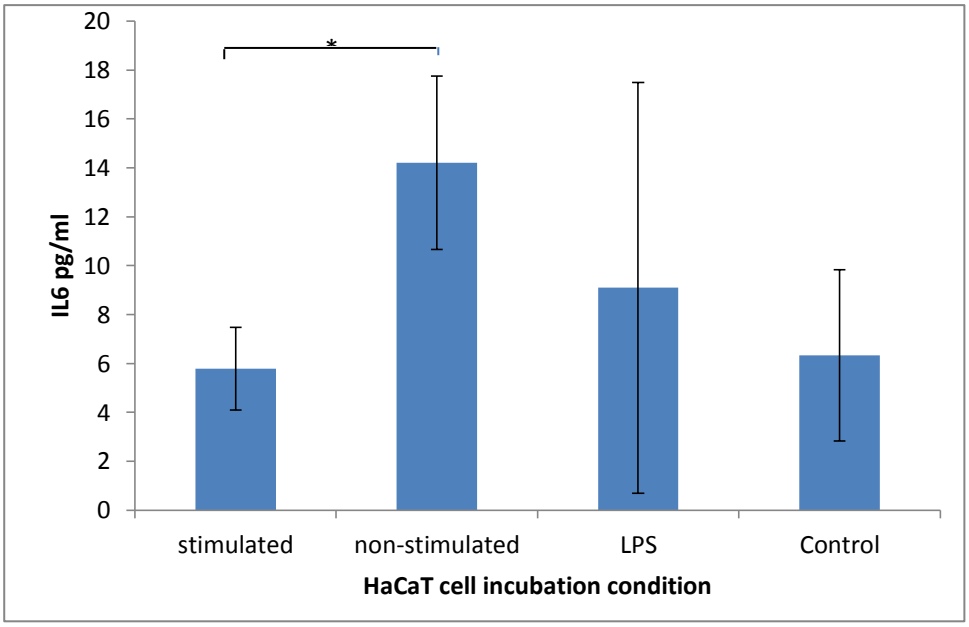


Figure 6.20 IL6 production by HaCaT cells

*Data analysis compared cytokine release from stimulated HaCaT cells (LPS exposed) or non-stimulated (no LPS exposure) incubated with TTO and T-4-ol and HaCaT cells incubated with LPS alone or medium alone (control). IL6 production by HaCaT cells in the control group and LPS were similar. It was observed that non-stimulated HaCaT cells (no LPS) incubated with TTO and T-4-ol significantly up-regulated the production of IL6 compared to cells that were stimulated by LPS and with TTO or T-4-ol. The mean amount of IL6 produced by the stimulated cells incubated with the TTO and T-4-ol is less than the mean production under all other conditions, although this is not significant. Error bars display SEM n=24 stimulated, n=20 non-stimulated. Statistical difference was assessed by t-test (p=0.041) and indicated on the graph by *.*

6.4.3 Summary of key findings

- Incubation of HaCaT cells with 1.25% or 5% TTO or T-4-ol for 120min or 24 hours exerts no statistical difference in the modulation of TNFα, IL6, IFNY or IL1β.

- Pre-treatment of HaCaT cells with 1.25% or 5% TTO or T-4-ol followed by challenge with LPS exerts no statistical difference in the modulation of TNFα, IL6, IFNY or IL1β, compared to cells challenged with LPS and then incubated with 1.25 or 5% TTO or T-4-ol.

- Pooled data from HaCaT cells incubated with both concentrations of TTO oil at all time points demonstrated a statistical increase in IL1β production compared to control (medium only). This was not demonstrated when HaCaT cells were incubated with T-4-ol.

- Pooled data from non-stimulated HaCaT cells incubated with all concentrations of the oil and its component demonstrated a statistical increase in IL6 production by as much as 3-fold compared to pooled data of stimulated HaCaT cells incubated with all concentrations of the oil and its component (pre and post challenge).

6.4.4 Limitations

The limitations of the *in vitro* investigations undertaken within this chapter include the low level of cytokine induced by LPS at 100ng/ml, alternative stimuli should be sought for future experiments to more clearly observe the effect of TTO and T-4-ol on stimulated cells.

The observation of cytokine regulation at various time points in order to map the progression of inflammation would be beneficial in understanding the potential mechanism of action of TTO.

It would also be of interest to observe altered regulatory effects of TTO and T-4-ol in psoriatic skin compared with non-psoriatic skin.

6.5 Discussion

TTO has been demonstrated to exhibit an anti-inflammatory effect on activated monocytes by reducing the pro-inflammatory mediators TNFα, IL1β, IL10 and PGE_2 following incubation for 20 hours, this outcome was replicated when activated monocytes were incubated with T-4-ol for 40 hours. However, this component was also able to down regulate the production of IL8 (Hart et al., 2000). Further studies have identified the ability of TTO to reduce LPS induced superoxide, and other ROS production by activated monocytes and have highlighted in particular the water soluble components T-4-ol and α-terpineol as being particularly potent. Thus, the authors suggest, supporting the use of TTO as an anti-inflammatory agent (Caldiefie-Chezet et al., 2004; Brand et al., 2001).

Whilst monocytes and neutrophils are essential components of the immune system and are recruited and migrate to the site of inflammation, topically applied TTO will be in direct contact with keratinocytes, an innate immune cell which has thus far not been examined in relation to the potential anti-inflammatory effect of TTO. Therefore, the modulation of the pro-inflammatory cytokines TNFα, IL1β, IL6 and IFNγ from LPS stimulated HaCaT cells (keratinocytes) following incubation with 1.25% and 5% TTO and T-4-ol was examined.

6.5.1 Cytokine production following stimulus with LPS

Previous studies have demonstrated the successful up-regulation of pro-inflammatory cytokines from keratinocyte and HaCaT cells following various stimuli including TNFα, histamine and LPS (Cho et al., 2007; Chen et al., 2013; Sjogren and Anderson, 2009; Wilmer et al., 1994; Giustizieri et al., 2004). The production of cytokine by HaCaT cells within the studies in this chapter is lower than that within the literature. A previous investigation demonstrated the production of IL1β following incubation with LPS 100ng/ml to be approximately 50pg/ml (Cheng et al., 2008), in comparison with the study in this chapter, 0.099pg/ml and for control approximately 2pg/ml in comparison with 0.295 pg/ml. It was demonstrated that following incubation with 100ng/ml LPS, TNFα production was approximately 4pg/ml and control, approximately 0.5pg/ml (Cheng et al., 2008), within this study TNFα production was shown to be 0.96pg/ml and 1.2pg/ml respectively (n=4). This

may be due to the incubation time. Cheng (2008) measured cytokine production following 48 hours, whereas this study analysed for cytokines after 24 hours. Cytotoxicity investigations (*section 5.4*), identify a significant decrease in cell viability following incubation of HaCaT cells with 100ng/ml LPS for 24 hours, suggesting that an increase in LPS amount or time of incubation would lead to an increase in cell death and therefore not truly represent conditions as a result of incubation with TTO or its components. Pro inflammatory cytokine production following stimulation with LPS has been measured in other cells including melanocytes, monocytes and neutrophils, producing larger amounts of pro-inflammatory mediators (Tam et al., 2011; Hart et al., 2000; Brand et al., 2001). However, it is known that monocytes and neutrophils are among the primary source of these cytokines and can therefore be predicted to secrete larger amounts (Hart et al., 2000; Brand et al., 2001). As a result of this direct comparison of actual amount of cytokine production between these studies should be undertaken with caution.

6.5.2 The modulatory effect of TTO of pro-inflammatory cytokines on LPS stimulated cells.

The studies within this chapter demonstrate no significant modulatory effect of TNFα IL1β, IL6 and IFNγ following incubation with LPS stimulated HaCaT cells. Furthermore, no significant difference was observed following pre-treatment of the cells with TTO or T-4-ol prior to LPS stimulation or when the oil was applied post challenge. The application of TTO to a murine ear 30min prior to intradermal injection of histamine significantly increased the mean wheal and flare area, however application of TTO 20min post intradermal histamine injection significantly decreased mean wheal and flare area, T-4-ol was demonstrated to be the active component in this instance (Brand et al., 2002a). Similar findings were observed after pre-treatment of a murine model 30min prior to exposure to UVB, oedema was significantly increased, however, application of TTO 24 hours post exposure to UVB exhibited no effect (Brand et al., 2001). Direct comparison of these studies is difficult due to the different methods used. However, it appears that pre-treatment of the skin with TTO prior to insult enhances the inflammatory effect. Oedema and wheal and flare are regulated by varying mechanisms including the regulation of pro-inflammatory cytokines. Therefore it was hypothesised that up-regulation of pro-inflammatory cytokines following pre-treatment of HaCaT cells prior to

LPS stimulation compared to treating cells post challenge would potentially describe a mechanism of action. The results of this *in vitro* study suggests that increased oedema and wheal and flare area following pre-treatment of TTO and T-4-ol is not caused by up-regulation of pro-inflammatory cytokines.

It would also be of interest to investigate the effect of different challenges to the HaCaT cells, for example the effect of fMLP, PMA and UVB has been observed as producing strong inflammatory effects in monocytes and on murine models (Brand et al., 2001).

6.5.3 The modulatory effect of TTO of pro-inflammatory cytokines on non-stimulated cells at different time points.

As discussed, the pre-treatment of murine skin with TTO or T-4-ol prior to exposure to histamine or UVB leads to an increase in oedema and wheal and flare area (Brand et al., 2001). It was considered that the amount of time HaCaT cells are in contact with TTO and T-4-ol may have an effect on pro-inflammatory cytokines as in studies TTO was applied at different times (e.g. 30min prior to insult or 24hr post insult), however the outcome measures were analysed at regular times post insult (e.g. 2, 4, 7 and 24 hours post insult) (Brand et al., 2001). Therefore pro-inflammatory cytokines were analysed for at 120min and 24 hours post application of TTO and T-4-ol to non-stimulated HaCaT cells. It was hypothesised that if pro-inflammatory TTO and T-4-ol will up-regulate production of pro-inflammatory cytokine at one or both of these time points. Furthermore, this will provide information regarding the speed of action.

There were no significant differences observed of production of TNFα IL1β, IL6 and IFNγ following comparison of TTO and T-4-ol at 1.25% and 5% to each other. This suggests that the production of pro-inflammatory cytokines following application of TTO and T-4-ol at 120min and 24 hours at the different concentrations is no different. The results for all conditions were pooled and compared with cytokine production from control (medium only). TNFα, IL6 and IFNγ exhibited no significant difference; however the production of IL1β was significantly increased in the pooled group compared to control. Further statistical analysis revealed TTO (pooled from all concentrations and

time points) produced significantly more IL1β compared with control. T-4-ol did not produce a significant increase of IL1β compared to control.

Incubation of LPS activated monocytes with TTO and T-4-ol has been previously demonstrated to reduce the amount of IL1β production following 20 and 40 hours respectively (Hart et al., 2000).

IL1β exhibits paracrine and endocrine effects: increasing the production of tissue factor (this triggers the blood clotting cascade, stimulates the synthesis and secretion of a variety of the interleukins and helps to activate T cells) thus initiating an adaptive immune response. Endocrine effects include the reduction of blood pressure and the induction of fever (Lydyard et al., 2004).

There is much debate in the literature regarding potential irritant properties of components of TTO, particularly 1,8 cineole and α-terpinene (Housen et al., 2004). Whilst some authors dispute the irritancy of these components, instead suggesting that irritancy is caused by the oxidisation of components in TTO due to the length of storage time of the oil (Hammer et al., 2006; Carson et al., 2001), there is evidence that TTO induces a contact dermatitis reaction in some individuals (Housen et al., 2004) and has been observed during in house *in vivo* experiments (data not shown). The up-regulation of IL1β following application of TTO to non-stimulated cells, which is not seen in those incubated with T-4-ol suggests a component other than T-4-ol is responsible for the up-regulation of this cytokine. Whilst IL1β is effective in activating monocytes which is important in the early stages of inflammation (Lydyard et al., 2004), the increase in relation to only the application of TTO suggests TTO has the ability to cause an inflammatory reaction when applied to normal skin. Ilβ has the ability to activate t-cells. A study investigating the effect of irritants on various cell lines demonstrated IL1β and IL8 to be up-regulated in large amounts following direct contact with an irritant (rather than the sensitizer) (Yoshikawa et al., 2010). If the up-regulation of IL1β in HaCaT cells is indicative of contact irritant reaction this would suggest that a component of TTO other than T-4-ol is an irritant to keratinocytes. A study from as long ago as 1997 following *in vivo* patch testing with various components of TTO identified that individuals reacted mostly to sesquiterpenoid fractions of TTO but not pure monoterpenes (Southwell 1997). This would exclude 1,8 cineole and α-

terpinene as potential irritants and implicate aromadendrene and σ-cadinene (Carson et al., 2006). Further investigation is required to identify the specific component.

It would be of interest to observe the effect of TTO and T-4-ol on pro-inflammatory cytokines following a timescale with a longer incubation period, modulation of IL1β and TNFα has been observed from as little as 15min post insult; in other investigations regulation was not seen until 40hours post insult (Thongradard et al., 2010; Hart et al., 2000). It is possible that TTO and T-4-ol do regulate pro-inflammatory cytokines in non-stimulated cells but at different time points.

6.5.4 The modulatory effect of TTO of pro-inflammatory cytokines on stimulated versus non-stimulated HaCaT cells

Whilst an anti-inflammatory effect has been demonstrated by TTO and T-4-ol on activated monocytes, it has been demonstrated to have no effect on non-activated monocytes (Hart et al., 2000). It has also been shown that TTO and T-4-ol can increase the production of ROS and decrease anti-inflammatory cytokine production (IL2 and IL4) in non-activated monocytes, compared to a reduction in ROS production and an increase in IL2 and IL4 production in activated monocytes (Caldefie-Chezet et al., 2004). It has been demonstrated that a higher concentration of TTO is required to reduce superoxide production in non-stimulated cells compared to stimulated (Brand et al., 2001). Furthermore TTO and T-4-ol have been demonstrated to reduce oedema, erythema and flare following application of a hapten challenge (TNCB and Nickel) of previously sensitized subjects however exhibits no effect in non-sensitized subjects (Pearce et al., 2004; Brand et al., 2001). In addition, whilst TTO exhibited an anti-inflammatory effect in activated monocytes, there was no effect on cell adhesion in activated neutrophils (an indicator of inflammation) or superoxide production (Brand et al., 2001; Abe et al., 2003). These authors dispute the previous findings by Pippin (1994), who identified a strong suppression of superoxide production in activated neutrophils by TTO.

The increase in IL1β production following application of TTO to non-stimulated HaCaT cells, discussed previously, suggests this might be linked to the findings in the literature. It was considered if there was a statistical difference

in the regulation of TNFα IL1β, IL6 and IFNγ of HaCaT cells that had been stimulated and non-stimulated by LPS this would lead to clinical implications for the use of TTO *in vivo*.

Comparison of pooled data from non-stimulated HaCaT cells incubated with TTO and T-4-ol and both concentrations was compared to the pooled data from stimulated HaCaT cells. No statistically significant difference was observed in the regulation of TNFα, IL1β or IFNγ. However there was a statistically significant increase in the amount of IL6 released by non-stimulated cells compared to that of stimulated cells by approximately 3-fold. A slight decrease in IL6 production is observed in stimulated cells compared with control (medium only), although this is not significant. A larger increase of IL6 production was observed between non-stimulated cells and control but again this was not significant.

IL6 has pro-inflammatory and anti-inflammatory functions. It stimulates acute phase protein synthesis as well as production of neutrophils in the bone marrow, supports the maturation of B cells and is antagonistic towards regulatory T-cells. However IL6 also inhibits the production of IL1 and TNFα (Lydyard ed 2004).

It is possible that IL6 inhibited the production of IL1β and TNFα in these circumstances, however, TNFα has the ability to up-regulate IL1β, IL6 and itself, yet throughout the *in vitro* investigations in this chapter there was no significant increase in TNFα which it is speculated would have been evident due to the amount of IL6 present.

The anti-inflammatory effect of TTO when applied to challenged skin or cells appears to be enhanced when the challenge and inflammatory reaction is stronger.

A previous study observes TTO to have an enhanced anti-inflammatory effect on a sub-group of nickel sensitive individuals who had displayed prolonged erythema thought to be associated with a prolonged initiation phase. Following challenge with nickel, TTO was able to regulate the vascular response and

reduce erythema more evidently than those not in the sub-group (Pearce et al., 2005).

Previous investigation has shown IL6 to be significantly up-regulated in primed cells that were then exposed to LPS in a murine model. In this study murine skin was burnt and then LPS applied, comparison of groups who were only burnt, were only exposed to LPS and who were burnt and exposed to LPS were made. Whilst IL6 was up-regulated in all groups, the largest increase was observed in the burnt and LPS challenged skin, there was no difference in the up-regulation of TNFα and no effect on IFNγ (Pallua and Von Heimburg, 2003). Anecdotal evidence suggests TTO is beneficial in the treatment of burns (Hammer et al., 2004). Pallua and Von Heimburg's study (2003) indicates IL6 to be the most up-regulated pro-inflammatory cytokine when murine skin is burnt (primed) and then exposed to bacterial infection (LPS). Pearce's investigation (2005) suggests TTO is more effective in the most inflamed skin. This implies that TTO would be successful in reducing inflammation when applied to burns with acquired secondary infection as it will reduce IL6 production but have no effect on TNFα or IFNγ regulation, which are not shown to be up-regulated under these conditions.

IL6 has been demonstrated to aid healing in barrier perturbed skin. A study investigating the healing of skin disrupted by tape stripping in wild-type and IL6 deficient mice showed delayed healing in the deficient mice (up to 24 hours post injury) compared to wild-type (15min-3hours post injury). The delay was reversed by topical application of IL6 (Wang et al., 2004). This investigation further studied the presence of IL6 in epidermal skin, immunohistochemistry identified little IL6 and the receptor IL6r in un-perturbed epidermis, however significant increase of staining for IL6 and IL6r was observed in epidermis following tape stripping. Furthermore, deficiency of IL6 was shown to have a more pronounced reduction in barrier repair than a deficiency in TNFα and IL1β. In relation to the current investigation discussed within this chapter, the increase of IL6 in non-stimulated cells suggests TTO may disrupt the epidermal barrier, thus up-regulating IL6 in order to repair the area. TTO has been demonstrated to alter skin barrier function in previous studies (Magnusson et al., 1997). Further investigation is required to identify the time course of regulation of IL6 in stimulated and non-stimulated cells.

IL6 is antagonistic to regulatory T-cells which suppress the immune response of other cells, therefore preventing excessive reactions (Lydyard et al., 2004). The up-regulation of IL6 in non-stimulated cells could predispose the area to chronic inflammation by supressing the resolution of the inflammation by anti-inflammatory mediators. IL6 has been implicated in the pathogenesis of a variety of diseases including chronic inflammatory proliferative disease such as psoriasis (Kishimoto 2010). This is supported by a previous study where cytokines were recovered by microdialysis from lesional and non-lesional skin in patients with psoriasis and were compared to control (patients without psoriasis); results demonstrate an increase in pro-inflammatory mediators including IL6 in both lesional and non-lesional skin compared with control. It is suggested that even if the skin appears 'normal' in a patient with psoriasis the cytokine profile is different to that of patients without this skin condition (Sjogren et al., 2004). As demonstrated by Sjogren's and Wang's studies (Sjogren et al., 2004; Wang et al., 2004), IL6 is linked with increased cell proliferation, as evident in psoriasis. As discussed, lesional and non-lesional skin exhibits an increase in IL6 compared with 'normal' skin. TTO has been shown to exert a more potent anti-inflammatory action when applied to stimulated cells which suggests TTO may be more effective in patients with psoriasis than those who do not.

It is suggested that the toll like receptor TLR4 found on the surface of monocytes, neutrophils and keratinocytes is involved in the modulation of IL1β and TNFα (Chen et al., 2013). TLR4 gene expression was significantly increased at 12 and 24 hours post injury and returned to baseline at day 10. TLR4 was observed in all layers of keratinocytes at 6 hours, 1 and 3 days post wound at an area slightly distal to the wound edge rather than immediately next to the wound. TLR4 significantly decreased as the wound healed and returned to baseline levels at day 7-10 post wound. In a study the wound healing of wild type mice was compared with that of TLR4 deficient mice. Keratinocytes at the wound edge of TLR4 deficient mice displayed notably reduced levels of epidermal growth factor (EGF), which can substantially affect proliferation and migration of keratinocytes. These mice had larger wounds and delayed re-epithelisation compared with wild-type mice. Upon analysis it was found that IL6 and IL1β decreased in the first 6 hours post wound and then significantly

increased for a sustained amount of time in TLR4 deficient mice; in comparison, wild-type mice displayed high levels of TLR4 at the wound edge which initially increased IL6 and IL1β (at 6 hours) but then decreased rapidly. It is suggested that in TLR4 deficient mice other toll like receptors, specifically TL2 compensate. IL6 is important to wound repair, however, excessive inflammation can promote scar tissue (Chen et al., 2013).

TLR4 is recruited following injury, i.e., LPS stimulation. TLR4 deficient mice mimic what may happen in a non-stimulated cell (no TLR4 recruitment). The results within this chapter suggest that TLR4 may have an impact upon IL6 and IL1β in HaCaT cells following incubation with TTO and T-4-ol. However, the increase of IL6 could be anti-inflammatory as, in previous investigations, TTO and T-4-ol exerted an anti-inflammatory effect on stimulated monocytes but not neutrophils, it is suggested that the suppression of superoxide production prevents the oxidative tissue damage seen in chronic inflammatory skin whilst allowing the neutrophils to be fully active (Brand et al., 2001).

6.5.5 Potential Mode of Action

The various effects of TTO exhibited on monocytes, neutrophils and keratinocytes discussed here suggest TTO acts upon many pathways (Pallua and Von Heimburg, 2003; Abe et al., 2003; Brand et al., 2001). The mode of action of TTO in the modulation of pro-inflammatory cytokines in activated monocytes is suggested to be regulated by the transcription factor nulear factor kappa B (NFκB). This transcription factor has also been implicated in the up-regulation of IL6, however alternative intracellular signalling pathways including Janus Kinase (JAK)/signal transducer and MAPK have also been suggested (Wei et al., 2013; Rein-Smith et al., 2013). A potential mode of action for the effect of TTO and T-4-ol on the regulation of pro-inflammatory cytokines will be investigated in *Chapter 7.*

6.5.6 In summary

The *in vitro* studies discussed here suggest that TTO and T-4-ol have no effect on TNFα and IFNγ regulation in HaCaT cells. This is contrary to previous investigations demonstrating a reduction in TNFα in activated monocytes. However, a significant increase was observed in IL1β production from non-stimulated HaCaT cells incubated with TTO for up to 24 hours compared with

control; this increase was not observed when incubated with T-4-ol, indicating the possibility of contact hypersensitivity reaction observed in some people following exposure to TTO is not due to T-4-ol. Furthermore a significant increase in IL6 production was observed following incubation with TTO and T-4-ol with non-stimulated cells in comparison with IL6 production in stimulated cells. Previous studies observe a strong anti-inflammatory effect in subjects exhibiting the greatest inflammatory reaction, including the treatment of burns with a secondary infection; this may be due to a number of reasons including the recruitment of TLR4 and suggests that application of TTO to non-inflamed skin should be undertaken with caution.

The investigations within this chapter suggest T-4-ol does not irritate non-inflamed skin in contrast to TTO disrupting the skin barrier thus creating an inflammatory response. TTO and not T-4-ol alone may have a greater anti-inflammatory action on previously challenged skin.

Chapter 7
Modulation of NFκB in HaCaT cells by tea tree oil and terpinen-4-ol

7 Introduction

Chapter 6 confirms the modulatory effect of TTO and T-4-ol on IL6 and IL1β produced by keratinocyte cells (HaCaT) under certain circumstances. As discussed in *Chapter 6* (*section 6.5*) it is thought TTO and T-4-ol act on different pathways to exert their anti-inflammatory/pro-inflammatory effect although as yet, exact mechanisms of action have not been confirmed. Important mediators of cellular response to extracellular signals include protein kinase C, mitogen-activated protein kinase (MAPKs), Janus kinase (JAK), phophatidylinoasitol-3-kinase and nuclear factor kappa B (NFκB) contained within the cytoplasm (Kim et al., 2006). MAPKs and NFκB are thought to play a particular role in the regulation of pro-inflammatory molecules on cellular responses, especially TNFα, IL1β and IL6 (Blackwell et al., 1997). LPS binding to keratinocyte toll like receptor 4 (TLR4) has been demonstrated to lead to NFκB translocation and the secretion of inflammatory cytokines (Collart 1990). Within the literature it is suggested that identification of specific and efficacious inhibitors of NFκB activation may prove beneficial for the treatment of cytokine mediated inflammation (Blackwell et al., 1997). As TTO and T-4-ol have a modulatory effect on inflammatory cytokine production in HaCaT cells, a possible mechanism of action could be the inhibition of NFκB activation. The following chapter will investigate the potential of TTO and T-4-ol to modulate NFκB activation.

7.1 Mechanism of action

Cytokines released as a result of an inflammatory response bind to cell receptors on the cell surface; this is known as the cytokine-receptor complex. The cytokine-receptor complex activates tyrosine kinase (an intracellular protein kinase) which leads to the phosphorylation of a series of intracellular kinases, leading to a transcription protein traveling into the nucleus and binding to a DNA regulatory site. This process then leads to the synthesis of specific proteins that mediate the cytokine's response. One such transcription factor, NFκB, is described in detail here.

7.1.1 Nuclear Factor κ B (NFκB)

The transcription factor NFkB is thought to be one of the most important regulators of pro-inflammatory gene expression (Schulze-Osthoff et al., 1997).

The NFkB system has been shown to play a pivotal role in epidermal development and differentiation, stress and immune responses, apoptosis, chronic inflammatory diseases and cancer. Along with mitogen activated protein kinases (MAPKs) and p38 it plays an important role in the regulation of pro-inflammatory molecules on cellular response, especially TNFα, IL1β and IL6. Activation of this transcription factor increases expression of the adhesion molecules VCAM-1 and ICAM1. Inhibition decreases leucocyte adhesion and transcription (Kim et al., 2004). It is found in cells throughout the body including keratinocytes where it can be found in the cytoplasm of the proliferative basal layer cells and shifts to the nucleus in non-proliferating supra-basal cells (Bari et al., 2011).

NFkB consists of sub units including NFkB1 (p50/p105), NFkB2 (p52/p100), p65 (RelA) and C-Rel. They have various functions: lack of p50 or Rel B has been shown to lead to immunodeficiency in mice where the B cells show abnormal mitogen responses. Rel B has a role in the development and differentiation of dendritic cells. p50 and p65 heterodymers are involved in the activation of inflammatory genes by TNFα and IL1β in human monocytes. These differences allow cells to respond to the external environment at the correct time and with the appropriate genes (Tak et al., 2001).

7.1.2 The NFkB pathway

There are two signalling pathways leading to the activation of NFkB, the canonical pathway and the non-canonical pathway (*fig 7.1*). The non-canonical pathway is responsible for the activation of p100/RelB complexes and occurs in the development of lymphoid organs that will generate B and T lymphocytes. There are only a small number of stimuli known to activate NFκ B via this pathway and they are not relevant to the purpose of this thesis and so will not be discussed further. The canonical pathway however is activated by the binding of ligand to a cell surface receptor leading to the phosphorylation and degradation of IKβ as shown in *figure 7.1* (Hayden and Ghosh 2004).

7.1.3 Toll like Receptors

The immunological barrier created by keratinocytes to protect the body is enhanced by the presence of toll like receptors on the surface of the cells. Recognising a wide range of microbial ligands, including LPS and bacterial

lipoproteins, these receptors bind with the ligands which in turn activate a variety of mechanisms within the cell including the NFkB pathway (Kollisch et al., 2005).

There are a number of toll like receptors which can be expressed innately on keratinocyte cells including TLR2, TLR3, TLR4, TLR5 and TLR10. All have differing specificities, TLR2 for example, has been found to mediate NFkB dependent gene activation of inducible nitric oxide synthetase, cyclooxegenase-2 (COX-2) and IL8 by staphylococcus aureus. TLR4 is a specific receptor for LPS (Kollisch et al., 2005).

7.1.4 IKK complex

At its resting inactive state NFkB resides in the cytoplasm bound to the inhibitor kappa B (IκB). Following the binding of a ligand to a receptor, activation and recruitment of the IKK (IKβ kinase) complex occurs. IKK has three sub units including kinases IKKα and IKKβ (also known as IKK1 and IKK2) and IKKγ. When IKKα and/or IKKβ and two molecules of NEMO join together, this leads to phosphorylation of IκB and the activation of NFκB (Karin et al., 1999). It is thought IKKα is involved in keratinocyte differentiation and formation of the epidermis and IKKβ is involved in the primary pathway by which pro-inflammatory stimuli induce NFκB activation (Tak et al., 2001; Yang et al., 2011).

Activated NFκB then enters the nucleus and binds to specific DNA sequences and regulates the expression of its target genes (Yang et al., 2011).

The NFκB pathway is normally auto-regulating. Once NFκB is activated the IκB gene is expressed and terminates transcriptional activity unless a persistent activation signal is present (Hayden and Ghosh 2004). NFκB is responsible for much of the expression of the inflammatory response in keratinocytes.

TTO and T-4-ol have been demonstrated to modulate the production of cytokines including IL6, TNFα, IFNϒ and IL1β following incubation with HaCaT cells (*Chapter 6*). The identification of a mechanism of action will enhance the knowledge available regarding the use of TTO, leading to more targeted use. A

potential mechanism of action to control production of these cytokines is via the NFκB pathway.

7.1.5 Summary

In summary the production of pro-inflammatory cytokines within the skin is controlled by a number of mechanisms. A major transcription factor thought to be one of the most responsible for controlling the inflammatory response in keratinocytes is the NFκB pathway. Following activation due to infection or inflammation NFκB leads to the cell expressing its target genes, leading to the release of specific cytokines resulting in an inflammatory response. The potential of TTO or T-4-ol to down-regulate NFκB would identify it as a potent anti-inflammatory agent.

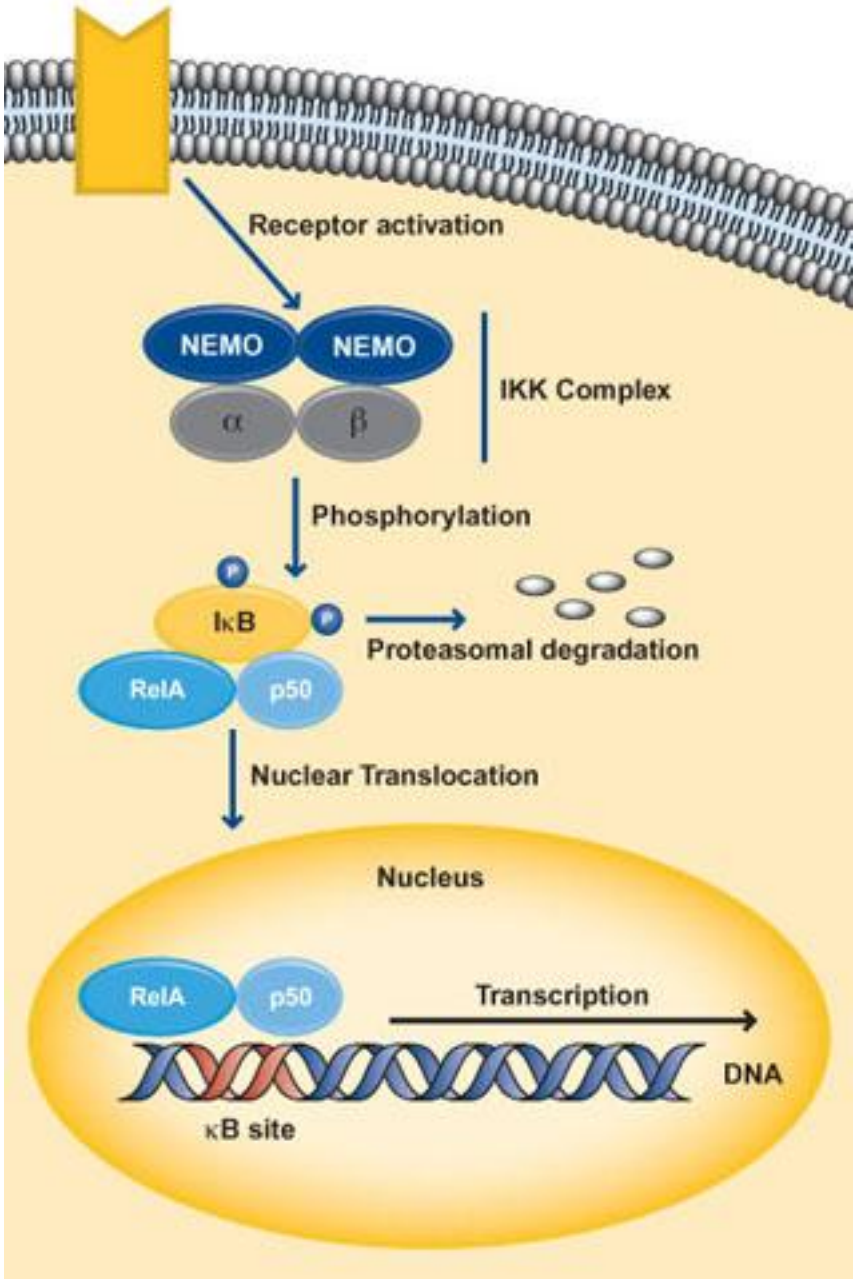


Figure 7.1 Diagrammatic representation of the canonical pathway (Hayden and Ghosh 2004).

This pathway is activated by the binding of ligand to a cell surface receptor, leading to the phosphorylation and degradation of IKβ allowing NFκB to freely enter the cell nucleus and activate the release of cytokines.

7.2 The effect of TTO and T-4-ol on NFκB production in HaCaT cells

7.2.1 Aim

- To establish the effect of TTO and T-4-ol on the production of NFκB in HaCaT cells

7.2.2 Objectives

- Does incubation of HaCaT cells with TTO modulate NFκB production?
- Does incubation of HaCaT cells with T-4-ol modulate NFκB production?

7.2.3 Protocol

In order to provide a direct comparison between modulation of cytokine production and NFKB regulation HaCaT cells were incubated under the same conditions as for the investigation described in *Chapter 6.*

All manipulation of cell culture was undertaken in a lamellar flow cabinet within a temperature controlled tissue culture laboratory (21±1°C).

HaCaT cells were maintained as described in *chapter 5 (section 5.1.1*). Following passage, 200µl of cell supernatant were seeded in each well of a 96 well plate, flat bottomed with no additives (NUNC) and incubated at 37°C, 5% CO_2 for 48 hours. The cells were then incubated in the conditions described in *section 5*.

An MSD immunoassay was used to determine the amount of NFκB production under each condition. The assay was conducted as the manufacturer's instructions described in detail in *chapter 5 (section 5.11*).

The plate analyser (Sector Imager, MSD, UK) provided the mean signal produced by each well, the greater the signal, the larger the amount of NFKB present. Although an actual amount cannot be obtained from this method of analysis it allowed for comparison between incubation conditions within this experiment.

7.2.4 Results

Results are expressed as mean signal strength ±SEM. The results are presented in *table 7.1* and *figure 7.2*.

HaCaT cells under control conditions (medium only) produced a signal strength of 39.75 (±10.647).Whilst HaCaT cells incubated with LPS produced a mean signal strength of 23.25 (±9.338).

The strongest signal strength within this investigation was 41.3 (±7.4), following incubation for 24 hours with 1.25% TTO. This was closely followed equally by HaCaT cells that had been incubated for 24 hours with 100ng/ml of LPS and HaCaT cells pre-treated with 1.25% T-4-ol producing a signal strength of 36.25 (±10.9). The lowest signal strength was 21.71 (± 5.14) following incubation for 24 hours with 1.25% T-4-ol.

A two way between samples ANOVA was undertaken to establish statistically significant difference between 5% and 1.25% TTO and 5% and 1.25% T-4-ol and between pre-treatment and challenge treated with all oils. There were no significant differences between all groups.

The data was pooled as in *section 6.4.1.* An independent samples t-test was used to identify statistical difference between production of NFkB production by cells incubated with TTO and T-4-ol compared with control; there was no significant difference between these two groups (*figure 7.3*). The oil groups were separated into group 1; TTO and group 2; T-4-ol and each compared with control. Once again, no significant difference was demonstrated The data was pooled of the NFkB signal strength that was exhibited following exposure to all oils and LPS and compared with LPS alone (*figure 7.4*). This did not reach statistical significance.

Table 7.1 Displaying signal strength produced following MSD assay for NFκB after incubation of HaCaT cells in various conditions n=8

NFKB	
	Mean signal strength ±SEM
C	39.75 ±10.647
LPS	23.25 ±9.338
24 5 TTO	26.86 ±9.625
24 1.25 TTO	41.13 ±7.484
24 5 T-4-ol	27.50 ±6.525
24 1.25 T-4-ol	21.71 ±5.140
p/t 5 TTO	33.50 ±10.759
p/t 1.25 TTO	30.25 ±5.821
p/t 5 T-4-ol	23.88 ±6.266
p/t 1.25 T-4-ol	36.25 ±10.921
lps 1.25 TTO	31.88 ±7.596
lps 5 TTO	24.75 ±5.706
lps 1.25T-4-ol	27.25 ±5.741
lps 5 T-4-ol	25.63 ±7.817

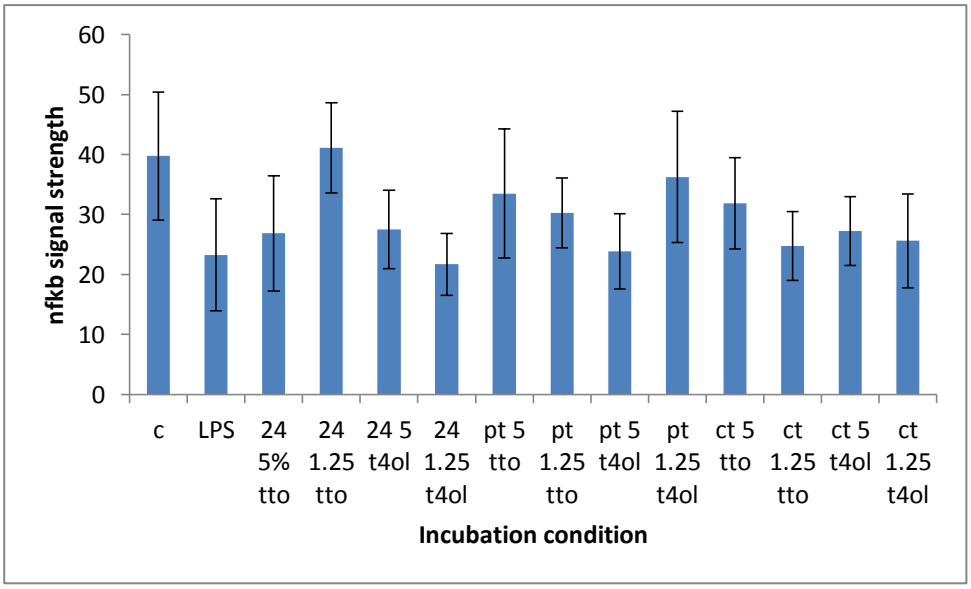


Figure 7.2 The mean signal strength detected following NFkB assay of HaCaT cells incubated under differing conditions.
Included are error bars displaying SEM n=8. There were no statistical differences as assessed by a two way ANOVA. The data is shown for control, LPS 100ng/ml, 24hours of 1.25% and 5% TTO and T-4-ol, pre-treat and expose to LPS and expose to LPS and then treat with TTO and T-4-ol and both concentrations.

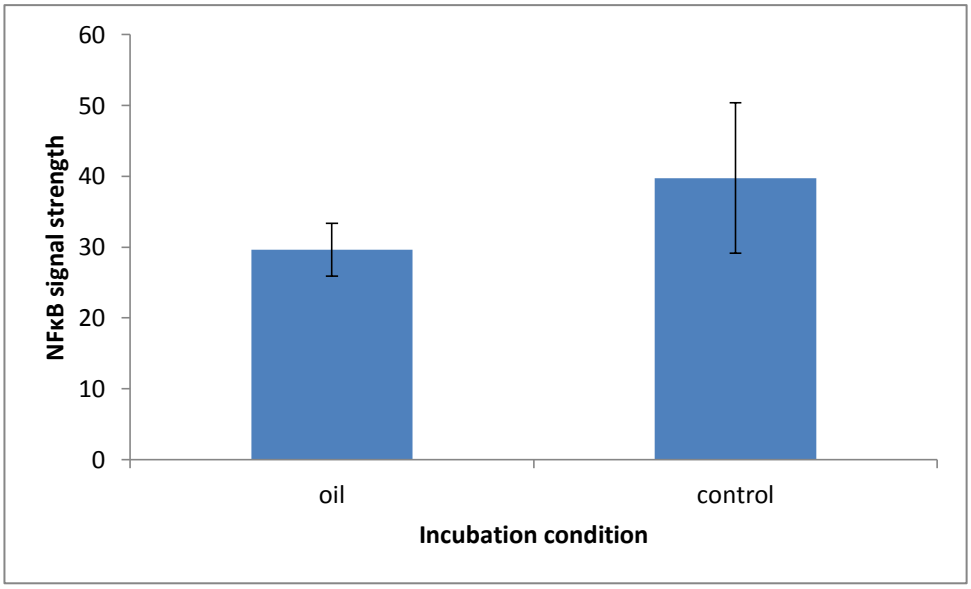


Figure 7.3 The mean signal strength of NFκB following pooling of data from HaCaT cells incubated with TTO and T-4-ol and compared with control. Error bars display SEM n=30 (oil), n=8 (control). There were no statistical differences following analysis using the t-test.

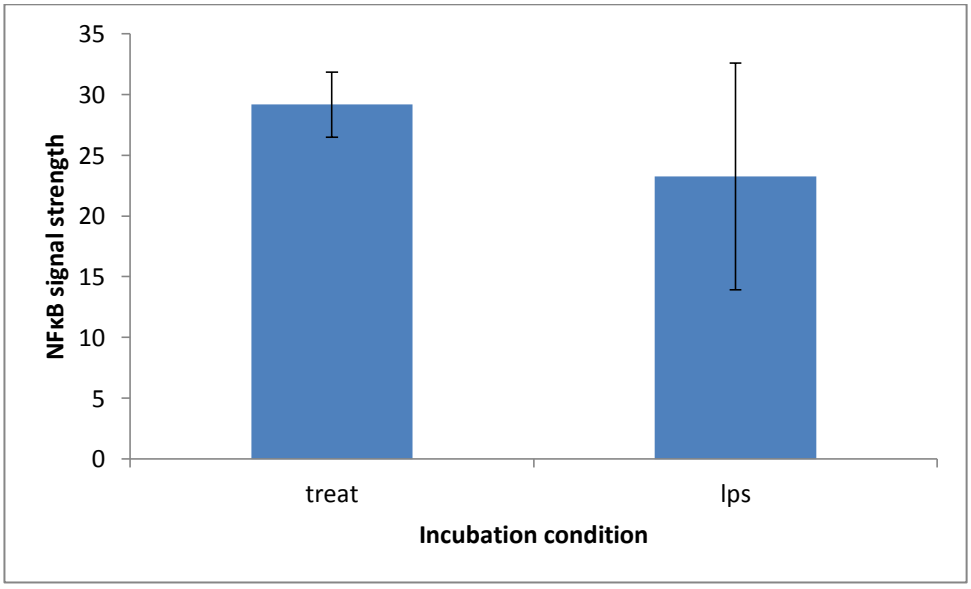


Figure 7.4 The mean signal strength of NFκB following pooling of data from HaCaT cells incubated with TTO and T-4-ol with LPS and compared with HaCaT cells incubated with LPS. Error bars display SEM n=64 (treat), n=8 (control). There were no statistical differences as analysed by the t-test.

7.3 Discussion

The identification of a specific mode of action of TTO during an inflammatory response is difficult due to a number of reasons. These include the number of individual components within the oil (Hammer et al., 2004), the limited existing evidence of the mode of action of TTO during an inflammatory response (Brand et al., 2002a; Khalil et al., 2004) and that the function of transcriptional pathways and signalling processes are still being identified within the literature (Enzler et al., 2009; Verstrepen et al., 2008),

Previous studies have identified the regulatory action of TTO and T-4-ol on pro-inflammatory mediators in activated monocytes but lack evidence to identify a potential mode of action (Hart et al., 2000). A study examining the superoxide production by activated and non-activated monocytes and neutrophils attempted to identify a mechanism of action by challenge of cells with different stimuli; phorbol 12-myristate 13-acetate (PMA), (employed to activate the signal transduction protein kinase C. [PKC]); N-formyl-L-mehionyl-L-leucyl-phenylaline (fMLP), (which plays a large part in chemo attractants) and lipopolysaccharide (LPS). Results are presented in *Chapter 1 (table 1.2*), demonstrating that whilst TTO reduced superoxide production in all cells under all conditions, T-4-ol exhibited no effect following PMA stimulation. However a reduction of superoxide production was observed at very low concentrations of T-4-ol in cells stimulated with fMLP and LPS. α-terpineol reduced superoxide production in cells challenged by all three stimulants. Whilst 1,8 cineole elicited no effect on superoxide production following stimulation by any of the challenges. There is suggestion that the modulatory effects of pro-inflammatory mediators in hapten sensitised subjects is due to multiple pathways (Pearce et al., 2005; Brand et al., 2001).

The production of oedema following UVB, histamine challenge and application of TTO was investigated and in a neuropeptide deficient murine model (unable to produce substance P) it was deduced that TTO induced oedema in much the same way as histamine, T-4-ol reduced oedema and was therefore identified as the active component in this instance. 1,8 cineole and α-terpineol exhibited no effect. This is suggested to identify that T-4-ol has a post-terminal effect (Brand et al., 2002a). This is further supported by a study investigating the action of T-4-ol in regulation of the wheal and flare area in a murine model

and was identified as exerting a post terminal effect whilst the effect of 1,8 cineole and α-terpineol exert a pre-terminal effect (Khalil et al., 2004). Furthermore, it is suggested that the vascular response in nickel sensitive subjects exposed to nickel may be regulated by TTO's effect on antigen presenting cells or the antigen presenting process (Pearce et al., 2005).

7.3.1 Do TTO and T-4-ol initiate the NFκB pathway during an inflammatory reaction?

The transcription factor NFkB is thought to be one of the most important regulators of pro-inflammatory gene expression (Schulze-Osthoff et al., 1997). As TTO and T-4-ol has been demonstrated to modulate IL6 and IL1β production in HaCaT cells (*section 6.4)* it was hypothesised that the mechanism of action could be driven by the initiation of the NFκB pathway.

Limitations of the experiment undertaken within this chapter include that the assay does not allow for quantification of actual amount of NFκB, however comparison of mean signal strength will indicate trends regarding NFκB activity.

Comparison of mean NFκB signal strength under all conditions identified no significant differences between groups. Furthermore the trend of free NFκB in no way mirrored the trend of IL6 and IL1β regulation in HaCaT cells described in *Chapter 6*. Therefore suggesting TTO and T-4-ol do not exert their effects by initiation of the NFκB pathway in HaCaT cells. Furthermore the investigations in *Chapter 6* suggest the anti-inflammatory mechanism of action of TTO and T-4-ol on monocytes is different to that of keratinocytes (Brand et al., 2001).

Following incubation of 100ng/ml LPS with HaCaT cells within *Chapter 6*, it was observed that there was very little up-regulation of pro-inflammatory cytokine identified. LPS elicits a pro-inflammatory response by initiating the NFκB pathway (Cheng et al., 2008). However up-regulation of IL6 and IL1β was observed in non-stimulated HaCaT cells only (*section 6.4*). This, in addition to the evidence provided by the investigation in this chapter, suggests TTO and T-4-ol do not initiate the NFκB pathway. Furthermore, this is supported by

evidence of trans-signalling pathways within the literature. There is evidence that the NFκB pathway elicits IL1β, IL6, IFNγ and TNFα, (Kim et al., 2006). However, IFNγ and TNFα have been suggested to be the most effective cytokine in eliciting chemokine synthesis in keratinocytes and are elicited by NFκB/Signal Transducers and Activators of Transcription-1 (STAT-1).

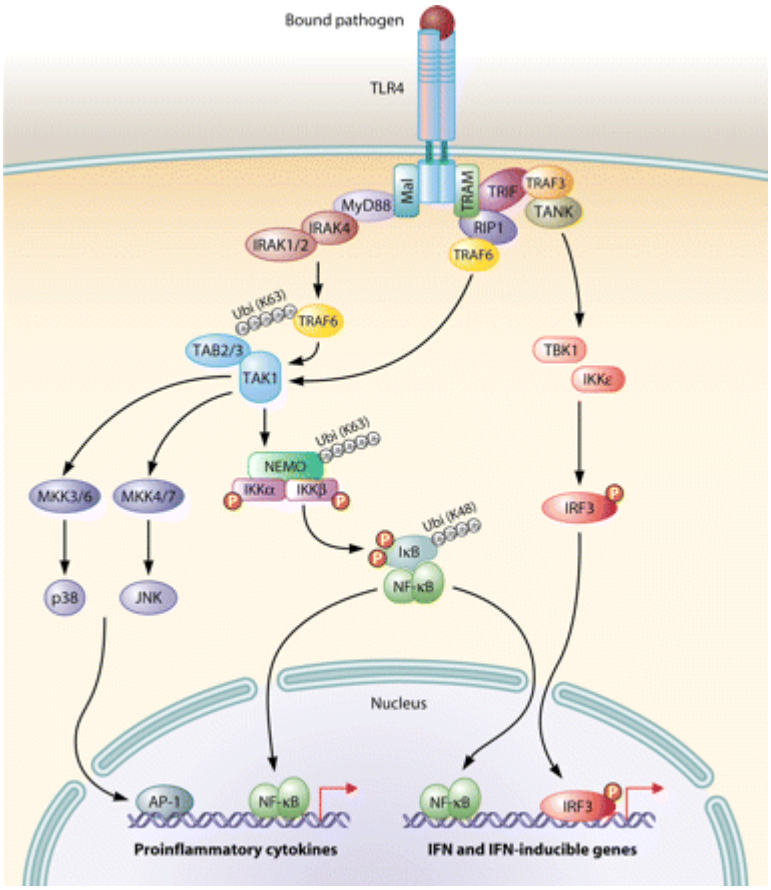
In contrast, IL6 is up-regulated following perturbation of the epidermis (following tape stripping). IL6 deficient mice exhibited delayed barrier repair compared to non-deficient mice which was reversed following the application of IL6. Furthermore, deficiency of IL6 was shown to have a more pronounced reduction in barrier repair than a deficiency in either TNFα or IL1β (Wang et al., 2004). A study considering the effect of a burn and bacterial insult (LPS) on murine skin identified IL6 to be significantly up-regulated when a primed (burn) area of murine skin was then exposed to LPS, compared to burnt skin alone, LPS alone or control (Pallua et al., 2003). This study further identified that IFNγ and TNFα were not up-regulated or altered under the same experimental conditions.

A study attempting to identify a mode of action for up-regulation of IL6 and IL1β in early wound healing considered the involvement of the toll-like receptor, TLR4, (Chen et al., 2013). A murine model was again utilised. Following wound induction, TLR4 was found to be localised in the keratinocytes in the wound edge rather than cells in the dermis in wild-type mice. TLR4 deficient and wild-type mice were wounded. It was observed that TLR4 deficient mice had larger wounds and delayed re-epithelisation compared with wild type mice. IL1β and IL6 were decreased during the first 6 hours post wound in the TLR4 deficient mice and then significantly increased and sustained up-regulation of IL1β and IL6 after this time. In contrast, initially IL6 and IL1β were increased during the first 6 hours post wounding and then decreased rapidly to basal levels after this time. It is suggested that in TLR4 deficient mice, other TLRs compensate, (for example TLR2) and that this inhibits wound healing as although IL6 is important for wound closure, excessive cellular inflammation can be detrimental to wound repair. TLR4 is involved in the initiation of the NFκB pathway, particularly in the induction of TNFα and INFγ following challenge with LPS (a bacterial insult) (Chen et al., 2013). However, it is suggested that the up-regulation of IL6 and IL1β is due to

TLR4 initiating the phosphorylation of c-Jun N-terminal kinases (JNKs) and p38 but not NFκB *(figure 7.5)* (Chen et al., 2013). Furthermore, this study identified a slight increase in inflammatory cells in wound area in TLR4 deficient mice which although subtle supports the notion that excessive inflammation is detrimental to wound repair. The study concludes that skin wound repair in adults is complex and there is a fine line between 'good' and 'bad' inflammation as it pertains to repair and that TLR4 may subtly harness inflammation so that wounds heal functionally with resistance to infection and appropriate restoration of structure (Chen et al., 2013).

The investigations in *Chapter 6* identify the up-regulation of IL1β following application of TTO (not T-4-ol) in HaCaT cells compared with control (medium only) and that IL6 is up-regulated in HaCaT cells that had not been stimulated but were incubated with TTO compared to those that had been stimulated and been incubated with TTO. Previous studies on monocytes and *in vivo* demonstrate the anti-inflammatory effects of TTO when applied to stimulated cells/skin and that TTO at times exhibits no effect or a pro-inflammatory effect in non-stimulated cells (Hart et al., 2000; Brand et al., 2001; Caldiefie-Chezet et al., 2004). This suggests a potential link in the TTO activated transcription pathways involved in the up-regulation of pro-inflammatory mediators following application to non-stimulated cells.

It has been suggested that the epidermis is a shield of sequested IL1 surrounding a host which is waiting to be released upon injury (Murphy et al., 2000). A murine model was used to investigate a potential mode of action, IL1R1 knockout mice (IL1 receptor present in cytoplasm) and wild-type mice. When exposed to radiation there was a significant up-regulation of IL1β mRNA in the wild-type mice that was less pronounced in the knockout mice. It is also suggested that, in the skin, the IL1 pathway is independent of ligand binding. Other pathways that can be activated by the IL1-IL1R complex include MAPK, JNK and p38 (Murphy et al., 2000). It was evident in the investigations in *Chapter 6* that TTO alone induced an up-regulation of IL1B following incubation with non-stimulated cells compared to control; T-4-ol elicited no such effect. It is proposed that a component of TTO (unidentified) is an irritant to keratinocytes, in the absence of TLR4 on the cell surface; the irritant



(Chen et al., 2013)

Figure 7.5 A potential mechanism of action for the up-regulation of IL1β following incubation of TTO with non-stimulated cells. The diagram presents ligand binding with TLR4 and potential transcriptional factors including NFκB, JNK and p38.

activates the IL1R-IL1 complex thus initiating the JNK and p38 pathways, releasing IL1β. T-4-ol may then act upon the JNK and p38 pathways thus inhibiting IL1β release. In monocytes it has been suggested that TTO acts on signals upstream from protein kinase C (PKC), an enzyme causing phosphorylation but not at the surface receptor level as it was found that CD14 levels were unable to be modulated by TTO (Brand et al., 2001). This would support the theory proposed in this chapter as it is suggested that IL1R-IL1 is only independent of ligand binding in keratinocytes (Murphy et al., 2000). In this model, the lack of TLR4 in the non-activated cells would suggest this is not a mechanism of action for IL1β release thus activation of the IL1R-IL1 complex within the cytoplasm could be a potential mechanism of action within these circumstances.

7.3.2 A potential mechanism of action for the up-regulation of IL6 following incubation of non-stimulated HaCaT cells with TTO compared to stimulated cells incubated with TTO.

IL6 was significantly increased when TTO was incubated with non-stimulated HaCaT cells when compared stimulated cells incubated with TTO. It was observed that T-4-ol did not down regulate the production of IL6 under these circumstances.

A previous study on a murine model suggested the reason for a significant increase in oedema following the application of TTO 30min prior to exposure to UVB compared to application of TTO 24 hours post exposure to UVB (no effect on oedema) was due to the effect of the oil on lipids within the epidermis allowing easier penetration of the UVB rays to the dermis (Brand et al., 2001). A study considering the up-regulation of IL6 following UVB exposure of keratinocyte cells observed that UVB induced an increase in steady state levels of IL6mRNA; this was found to be a pre-requisite for keratinocyte IL6 production after UVB irradiation (de Vos et al., 1994). It is possible that the application of TTO in Brand's study induced an increase in IL6mRNA which significantly up-regulated IL6 production following exposure to UVB and therefore led to oedema.

Furthermore, a study using immunohistochemistry has demonstrated little presence of the IL6 receptor IL6R in fibroblasts and lymphocytes but an increased presence in nucleated keratinocytes; IL6 was significantly increased following tape stripping (Wang et al., 2004). Over expression of IL6 is seen in skin disease such as psoriasis, demonstrating the negative effect of a disrupted immune response (Wang et al., 2004; Sjogren et al., 2004). Over expression of IL6 can lead to chronic inflammation. Excessive cellular inflammation can be detrimental to wound repair (Chen et al., 2013). However, IL6 is necessary for cell proliferation in wound repair, as already discussed (Wang et al., 2004). It is suggested that an unidentified component of TTO (other than T-4-ol) induces barrier disruption similar to that of tape stripping. It is possible that a component such as α-terpineol, (as this component has been previously identified as able to reduce the production of superoxide production by three different stimuli including PMA, which T-4-ol was unable

to (Brand et al., 2002a), may induce the expression of IL6 in order to repair the perturbed skin barrier. Brand's study suggests α-terpineol may exert its effects downstream of PKC (Brand et al., 2001). This study observed that α-terpineol was unable to modulate pro-inflammatory cytokine production, however its effect on IL6 was not examined. A study examining IL6 signalling pathway identified that it may occur via the signal-transducing membrane protein gp130 which initiates intracellular signalling via the Janus Kinase (JAK)/signal transducer and activator of MAPK and phosphatidylinositol-3-kinase pathway (Wei et al., 2013). A study investigating the effect of topical application of IL6 on barrier repair in wild-type and IL6 deficient mice identified reduced epidermal barrier repair in the IL6 deficient mice compared to wild-type, furthermore, the presence of STAT-3 (transcription factor) was markedly reduced in the deficient mice (Rein-Smith et al., 2013). An, as yet unidentified component of TTO may induce the up-regulation of IL6 following incubation with non-stimulated HaCaT cells via the JAK/STAT-3 pathway in order to repair the perturbed skin barrier.

It is noteable that increase in IL6 is not observed when TTO is incubated with LPS activated HaCaT cells, despite little up-regulation of pro-inflammatory cytokines induced by LPS alone. Pre-treatment with TTO and then challenge with LPS does not elicit an up-regulation of pro-inflammatory cytokine as discussed when considering the increase in oedema following pre-treatment with TTO prior to UVB exposure. This suggests it is due to a different pathway compared to that situation. However, as observed in the literature, TTO exerts a stronger anti-inflammatory effect on particularly strong inflammatory responses (Brand et al., 2001), therefore the presence of LPS stimulation and potential barrier perturbation by TTO may enhance the anti-inflammatory effect of TTO components (not T-4-ol) thus inhibiting the JAK/STAT-3 pathway or JNK and p38 pathways preventing the induction of IL6.

Alternatively, following disruption of the skin barrier by application of TTO or LPS, TLR4 is increased, as identified in previous studies (Chen et al., 2013), thus 'priming' the skin for further insult. When the subsequent challenge is applied TLR4 is activated. As previously discussed, TLR4 deficient mice exhibit an increase in inflammatory cells and heal more slowly than wild-type mice; furthermore, the study identifies skin that heals extremely well, including

foetal skin exhibits little or no inflammation (Chen et al., 2013). Thus it is concluded that TLR4 is up-regulated during an initial injury, a component of TTO (not T-4-ol) then acts on the JNK and p38 pathways thus down regulating IL6 production.

7.3.3 Summary

In conclusion, TTO increases the production of IL1β in non-stimulated HaCaT cells compared to cells incubated in medium alone (control). The up-regulation of IL1β is not observed following incubation with T-4-ol. It has been demonstrated that this is not due to the activation of the NFκB pathway. An alternative mechanism of action may be that an unidentified component of TTO acts upon the ligand independent IL1R-IL complex thus initiating the JNK and p38 pathways and up regulating IL1β. Subsequently T-4-ol may inhibit the JNK and p38 pathways thus down regulating IL1β production.

IL6 is up-regulated in non-stimulated HaCaT cells; this is not observed when TTO is incubated with LPS stimulated HaCaT cells. This is not due to the action of TTO on the NFκB pathway. An alternative mechanism of action of TTO may be that the barrier is perturbed by application of TTO or LPS increasing TLR4 expression on the cell surface, the cell is thus 'primed' for further insult. Following the subsequent application of TTO or LPS a component of TTO (not T-4-ol) may inhibit the JNK and p38 pathways thus down regulating IL6 production.

Chapter 8
General discussion

8 Aims and Objectives of Thesis

This thesis set out to investigate the transdermal penetration of TTO *in vivo* and the potential of TTO to modulate pro-inflammatory cytokines in HaCaT cells *in vitro*. The aim was to:

- Develop a protocol to identify and quantify the transdermal penetration of TTO at the dermal-epidermal junction *in vivo*.
- Investigate the presence of TTO components of TTO components in the SC *in vivo*.
- Assess the modulatory effects of TTO and its components on pro-inflammatory cytokines produced by stimulated and non-stimulated keratinocyte cells *in vitro*.
- Identify a potential mode of action for any modulatory effects exhibited by TTO in stimulated and non-stimulated keratinocytes cells *in vitro*.

8.1 Introduction

The development of synthetic drugs has revolutionised medical care, however these drugs and treatments can be expensive and not always successful in the treatment of all conditions, therefore alternatives are often sought (Barnes 2003).

TTO is commonly used by the general public and is utilised as an 'alternative medicine' for the treatment of conditions including athlete's foot, dandruff and acne (Hammer et al., 2004). Anecdotal reports support its use, however, there is little robust scientific evidence to support its safety and efficacy (House of Lords, 2000). A scientific report commissioned by Parliament into Complementary and Alternative medicine (House of Lords, 2000), highlights the need for scientific research into CAM to ensure the treatment is efficacious above and beyond the placebo effect, safe, cost effective and that there is some evidence of the mechanism of action (House of Lords, 2000).

TTO is the oil distilled from the leaves of the native Australian plant; *Melaleuca Alternifolia*. There is a growing body of evidence indicating that TTO may be beneficial in the treatment of some anti-fungal and anti-bacterial conditions and that the oil can potentially be used as an anti-inflammatory agent.

However, the methods used in many of the studies to identify these benefits and potential mechanisms of action are not consistent leading to confusion in regard to the effect of TTO and potential mechanisms of action.

TTO is often applied topically, yet despite evidence of its action on peripheral blood mononuclear cells there is little evidence of the transdermal absorption of components of TTO *in vivo*. There are frequent calls within the literature for this (Brand et al., 2001; Hammer et al., 2006).

Previous studies have attempted to identify components of TTO that penetrate the epidermis, yet due to the recovery and analytical method chosen, fail to provide a definitive answer. The identification and quantification of TTO components at the dermal-epidermal junction following topical application *in vivo* will strengthen existing evidence for its potentially beneficial use.

Furthermore, limited studies suggest the potential of TTO and its individual components as an anti-inflammatory agent. The majority of these studies, whilst identifying the potential, are undertaken in murine models or *in vitro* on isolated PBMCs. The most abundant cell in the skin is the keratinocyte, cells which possesses innate immunological functions. As TTO is often topically applied, the effect of the oil upon keratinocyte cells will add to the growing body of knowledge. In addition, the identification of a potential mode of action will direct its safe and efficacious use in a clinical environment.

8.2 Summary of findings and subsequent implications

8.2.1 The development of a protocol to identify and quantify the transdermal penetration of TTO at the dermal-epidermal junction

Consideration of methods used to investigate the transdermal penetration of substances following topical application resulted in the identification of dermal microdialysis as the most appropriate method for the experiments within this thesis. *In vitro* investigations allowed for the adaptation of the method to ensure the optimal recovery of transdermally penetrated lipid and hydrophilic TTO components. Furthermore, the method of analysis Gas Chromatography-Mass Spectrometry was chosen and adapted to ensure accurate identification and quantification of TTO components recovered. Initially *in vitro* studies were

undertaken to compare perfusate flow rate, membrane material and perfusate type.

GC-MS analysis of microdialysate identified a number of lipid and hydrophilic components. Consistently the TTO components T-4-ol, 1,8 cineole and γ-terpinen were present within the microdialysate and therefore chosen as components on which to base internal standards. This allowed for the quantification of components within dialysate following analysis by GC-MS. Following *in vitro* microdialysis studies, it was identified that a perfusate flow rate of 3µl/min using PBS with the addition of 5% hydroxypropyl-β-cyclodextrin and a membrane constructed with a cuprophan fibre allowed for the optimal recovery of lipid and hydrophilic components of TTO (*Chapter 3*). Under these conditions the actual amount of components recovered (mean ±SEM) are as follows; T-4-ol 470ng±190, 1,8 cineole 8.9ng±4.36 and Ɣ-terpinen 25ng ±15.2. These results represent 16%, 14% and 2% respectively of the whole oil. The protocol developed was then used for *in vivo* studies.

The *in vivo* microdialysis studies undertaken to identify transdermally absorbed TTO components following topical application of 100% TTO revealed that T-4-ol and 1,8 cineole alone were present at the dermal-epidermal junction. The actual amount recovered represented as mean ±SEM is; T-4-ol 115.64ng±2.6 and 1,8 cineole 15.05ng±2.6 (*Chapter 4*). This represents 2% and 6% respectively of the amount of each component in neat TTO.

8.2.2 The presence of TTO components within the SC

In vivo dermal tape stripping investigations identified the presence of nine lipid and hydrophilic components within the SC. The most frequently identified was T-4-ol and among the least, 1,8 cineole. The potentially active α-terpineol was also identified to be present within the SC.

8.2.3 Implications of the penetration of TTO and its individual components through the epidermis

The investigations within this thesis are the first to identify and quantify *in vivo* the absorption of TTO components following topical application of neat TTO. Despite limitations of the dermal microdialysis technique, the adaptations made including the choice of membrane material and the addition of HPβCD

ensures that optimal conditions were identified in order to recover transdermally penetrated lipid and hydrophilic components. Previous *in vitro* studies identify the effect of TTO and its individual components on monocytes and PBMCs (Hart et al., 2000), however the amount of TTO component tested was based on prediction of the quantity of component absorbed. The confirmation of exact amounts now supports the previous *in vitro* investigations thus strengthening evidence for the use of TTO as a medicinal product.

Evidence of the presence of T-4-ol and 1,8 cineole at the dermal epidermal junction at a concentration shown *in vitro* to be clinically effective against various fungal and bacterial infections and sufficient to reduce inflammation (Mondello et al., 2006; Soukoulis and Hirsch, 2004; Dryden et al., 2004) supports its use *in vivo*. However, the presence of α-terpineol within the SC only would suggest that it will not come into contact with cells and vessels that it has been shown to have a positive effect upon *in vitro* (Hart et al., 2000; Khalil et al., 2004). There is still a lack of evidence regarding the synergistic nature of TTO components.

There are many theories regarding the penetration of substances through the skin as discussed in *Chapter 4*. The evidence presented within this thesis demonstrates hydrophilic components exhibiting a low log P penetrate the SC quickly. However lipids with higher log P values appear to remain within the SC. Despite this evidence, T-4-ol was present throughout the SC and within the dermal-epidermal junction. This may be expected as it is the most abundant component of neat TTO.

The presence of TTO components within the SC suggests a potential to protect the epidermis and the host against invading pathogens. Further investigation is to confirm the concentration of components and specific actions.

8.2.4 The modulatory effects of TTO and T-4-ol on pro-inflammatory cytokines produced by keratinocyte cells *in vitro*

The potential anti-inflammatory effects of TTO were investigated *in vitro*. The modulation of the pro-inflammatory cytokines IL1β, TNFα, IFNY and IL6 by TTO and T-4-ol was observed in the immortalised keratinocyte HaCaT cell line. The

inflammatory response stimuli lipopolysaccharide (LPS) was chosen. As T-4-ol is present at the dermal-epidermal junction and is identified as exerting potentially beneficial effects in PBMCs, the effect of this component on HaCaT cells was observed in addition to that of the whole TTO. Cytotoxicity experiments revealed HaCaT cell viability to be significantly reduced when incubated for 24 hours with 5% TTO compared with control (medium only). Cell viability was significantly reduced at concentrations of between 0.15-5% T-4-ol compared to control. Finally, cell viability was observed as significantly reduced following incubation for 24 hours with 100ng/ml LPS (*Chapter 5)*. Therefore concentrations of components to be used in subsequent investigation were identified as 1.25% and 5% TTO and T-4-ol and 100ng/ml LPS.

The MSD multi-array electrochimiluminescence detection 4-multi-plex assay was chosen to identify the modulation of TTO and T-4-ol of the pro inflammatory mediators IL1β, TNFα, IFNY and IL6 in HaCaT cells in various conditions including those stimulated with LPS and those that had not (*Chapter 6*).

The modulation of the mediators identified above was observed under the following incubation conditions: TTO and T-4-ol at 1.25 and 5% for 120min and 24 hours. Pre-treatment of cells with TTO or T-4-ol at both concentrations for 120min then challenged with LPS for 22.5 hours or cells were challenged with LPS for 22.5 hours followed by incubation with TTO or T-4-ol at all concentrations.

There were no statistically significant differences in mediators produced following incubation with TTO and T-4-ol at both concentrations for 120min or 24 hours. In addition there were no statistically significant differences observed in mediators produced when cells had been pre-treated then challenged or challenged then treated with the oil and individual component at both concentrations. However, a statistically significant increase in the production of IL1β was demonstrated when all data was pooled for 1.25% and 5% TTO incubation at 120min and 24 hours compared to control (medium only). This was not observed following pooling of the data for T-4-ol. In addition a statistically significant increase in IL6 was observed after data

collected following incubation with TTO (including T-4-ol) with non-stimulated cells compared with pooled data of incubation of the oil with stimulated cells. These results suggest IL1β is attenuated by T-4-ol but released in response to the application of an, as yet, unidentified component of TTO. Furthermore T-4-ol has no effect on the attenuation of IL6 in non-stimulated cells, however following application of TTO to stimulated cells it is observed that the oil does not increase IL6 production.

8.2.5 Implications of the modulatory effects of TTO and T-4-ol on keratinocytes *in vitro*

Previous *in vitro* studies identify the modulatory effects of TTO and its components on inflammatory mediators in monocytes and PBMCs (Hart et al., 2000; Caldefie-Chezet et al., 2004). The keratinocyte is an innate immune cell and is the most abundant within the epidermis. Keratinocyte cells possess unique properties not evident in other cells (Murphy et al., 2000). Topically applied TTO will be in direct contact with keratinocytes and yet there is little evidence of the effect of TTO and its components on these cells. The evidence of regulation of pro-inflammatory cytokines by TTO and T-4-ol on keratinocytes presented in this thesis adds insight into the action of TTO and T-4-ol, particularly as the action, at times, appears detrimental to healing (Brand et al., 2001).

Cytotoxicity tests observed 100ng/ml of LPS to significantly reduce cell viability in HaCaT cells, this corroborates with studies within the literature (Cheng et al., 2008; Kollisch et al., 2005; Abe et al., 2003). However, despite challenging the cells with LPS 100ng/ml, an up-regulation of IL1β, TNFα, IFNϒ and IL6 was not achieved. Nevertheless the effect of application of TTO and T-4-ol on non-activated cells and application of TTO and T-4-ol on non-activated compared with activated was able to be observed and conclusions drawn.

The modulation of pro-inflammatory mediators in activated monocytes has been observed. Down regulation of pro inflammatory cytokines including IL1β, TNFα, IL10 and PGE_2 was observed following incubation of TTO with LPS activated monocytes for 20 hours. This effect was seen following incubation of T-4-ol with activated monocytes for 40 hours, in addition IL8 production was

inhibited (Hart et al., 2000). Furthermore, incubation of TTO and T-4-ol with non-activated cells exhibited no effect. Incubation of 1,8 cineole and α-terpineol elicited no effect when incubated with activated and non-activated monocytes. Furthermore, the water soluble fraction of TTO decreased superoxide production in PMA, fMLP and LPS stimulated monocytes and in at a higher concentration reduced superoxide production in non-stimulated cells. However, T-4-ol elicited no effect on monocytes stimulated with PMA, however demonstrated significant reduction of superoxide production in cells stimulated with fMLP and LPS, there was no effect observed in non-stimulated cells. In contrast α-terpineol significantly reduced superoxide production in monocytes stimulated with PMA, fMLP and LPS, but had no effect on non-activated cells. It was observed that 1,8 cineole elicited no regulation of superoxide production in cells stimulated and non-stimulated cells (Brand et al., 2001). More significantly an increase in ROS production was observed following incubation of TTO with non-activated monocytes and a decrease of ROS production in monocytes activated with phyoemagglutin A. Furthermore the production of the anti-inflammatory cytokines IL2 and IL4 were significantly increased following incubation of TTO with activated monocytes, these cytokines were down regulated following incubation with non-activated monocytes (Caldefie-Chezet et al., 2004). The studies in this thesis identify a potential pro-inflammatory effect of TTO when incubated with non-activated HaCaT cells compared to control. A significant increase of IL1β was observed when TTO was incubated with non-activated HaCaT cells for 24hours compared to control. However, incubation with T-4-ol elicited no such effect. This suggests a component of TTO other than T-4-ol elicits a pro-inflammatory reaction by up-regulating IL1β production. Irritation of the skin has been documented within the literature and is often attributed to 1,8 cineole (Cal, 2008). However, this is disputed by some authors suggesting that incorrect storage of TTO leads to oxidation of TTO components, which can lead to irritation (Carson and Riley, 2002; Hammer et al., 2006). Nevertheless, during the *in vivo* studies undertaken in *Chapter 4* an increase in erythema (suggesting inflammation) was observed in some participants but not in others following application of TTO for 120min, the TTO had been stored correctly. This suggests, in some circumstances, an as yet unidentified component of TTO (not T-4-ol) acts as an irritant to keratinocytes. There are two potential explanations for the effect of T-4-ol, either, it is not an irritant to

keratinocytes and therefore does not up-regulated IL1β production or T-4-ol reduces the production of IL1β, therefore acting in an anti-inflammatory manner.

The differing effect of TTO on sensitised and non-sensitised subjects following application of the sensitizer has been observed. Erythema and flare were significantly decreased following application of TTO to nickel challenge in sensitised subjects *in vivo*, in contrast there was no effect on erythema and flare following application of TTO to nickel challenge in non-sensitised subjects (Pearce et al., 2005). Furthermore, a significant reduction in oedema was observed following TTO application to TNCB challenge in sensitised mice compared to no effect of TTO application on oedema following TNCB challenge in non-sensitised mice (Brand et al., 2002). Investigations within this thesis observe a significant increase in IL6 production following incubation of TTO (including data T-4-ol) with non-activated HaCaT cells, in contrast, IL6 was not up-regulated when TTO was incubated with LPS activated HaCaT cells. IL6 is both a pro and anti-inflammatory cytokine and is essential for cell proliferation which is necessary for wound healing. However, over expression can be detrimental to wound repair (Wang et al., 2004). In addition elevated levels of IL6 are observed in non-lesional and lesional skin in psoriatic patients and is thought to be essential for the on-going expression of psoriasis (Sjogren et al., 2004). Within this thesis the up-regulation of IL6 in non-stimulated cells in response to incubation with TTO may be an attempt by the HaCaT cells to induce cell proliferation, thus repairing a potentially damaged skin barrier. However, a TTO component (not T-4-ol) may inhibit production of IL6 in LPS stimulated cells, thus regulating the inflammatory process, a known function of IL6 (Lydyard et al., 2004). TTO has been observed as exhibiting a more pronounced anti-inflammatory effect when applied to a strongly inflamed area compared to an area exhibiting less inflammation (Pearce et al., 2005; Brand et al., 2001). Further investigation into the mechanism of action for the responses indicated may identify the transcriptional pathways acted upon and direct the appropriate use of TTO.

8.2.6 The effect of TTO and T-4-ol on the NFκB pathway

The effect of TTO and T-4-ol on the NFκB pathway was investigated in order to identify a potential mode of action (*Chapter 7*). It was demonstrated that there

were no statistically significant differences between conditions. Furthermore the trend of release of NFκB in no way mirrored the trend of release of inflammatory cytokines observed in *Chapter 6*. This implies TTO and T-4-ol has no effect on the NFκB pathway in keratinocyte cells and therefore alternative mode of action must be sought. The findings in *Chapter 6* suggest TTO and its components may act on multiple pathways in order to potentiate its effect.

8.2.7 A potential mode of action of TTO in keratinocytes in the modulation of pro-inflammatory cytokines

There was no observed effect of TTO on TNFα or IFNY production following incubation with TTO or T-4-ol thus suggesting TTO acts on specific cellular transcriptional pathways that do not involve the regulation of these cytokines.

The transcription factor nuclear factor kappa B (NFκB) is thought to play a particular role in the regulation of pro-inflammatory molecules on cellular responses, especially TNFα, IL1β and IL6 (Schulze-Osthoff et al., 1997). LPS binding to keratinocyte toll like receptor 4 (TLR4) has been demonstrated to lead to NFκB translocation and the secretion of inflammatory cytokines (Collart et al., 1990). Therefore the potential initiation and inhibition of the NFκB process by TTO and T-4-ol was considered. It was identified that no effect was elicited under any condition studied of TTO and T-4-ol on NFκB in HaCaT cells. Furthermore, the trend of NFκB production in no way mirrored the trend observed in the release of cytokines in chapter 6. TNFα and IFNY are predominantly regulated by the NFκB and STAT-1 pathway. As TNFα and IFNY were not up-regulated it is suggested that this supports the theory that TTO and T-4-ol do not initiate the NFκB pathways. Therefore alternative pathways were considered for future investigation.

Whilst modulatory effects of TTO on inflammatory mediators have not been clearly defined attempts have been made to suggest modes of action. The action of T-4-ol and TTO has been investigated in murine models, a de-nervated murine model was used, and it was suggested that T-4-ol has a post-terminal effect whilst 1,8 Cineole and α-terpineol exert a pre-terminal effect (Brand et al., 2002b; Khalil et al., 2004).

Furthermore following *in vitro* studies on monocytes it was suggested that TTO acts on signals upstream from protein kinase C (PKC) but not at the surface receptor level due to lack of evidence of the modulatory effect of TTO on CD14 levels following incubation with TTO (Brand et al., 2001). A previous study has highlighted the differences in cell signalling pathways in keratinocytes compared to monocytes and has identified that IL1R-IL1 are independent of ligand binding and can subsequently initiate the JNK andp38 pathways which lead to release of cytokines including IL1β. Therefore it is suggested that TTO application to non-inflamed skin perturbs the skin barrier leading to initiation of the IL1R-IL1 complex and subsequent initiation of the JNK and p38 pathways resulting in the up-regulation of IL1β in an attempt by the skin to protect its host. This response is not observed following application of T-4-ol, therefore it is suggested that T-4-ol does not exert an irritant effect and hence does not up-regulated IL1β, alternatively T-4-ol may inhibit the JNK and p38 pathways down regulating the cytokine.

Studies have identified the up-regulation of IL6 as essential to normal wound repair, however over expression can be detrimental to healing (Chen et al., 2013). Furthermore the role of TLR4 has been identified as integral to wound repair. TLR4 deficient mice experienced up-regulation of IL6 and delayed wound healing compared to wild-type mice. TLR4 has been observed as being up-regulated following injury, (Chen et al., 2013). In addition TTO has been observed as exerting a greater anti-inflammatory effect on stronger inflammatory responses compared to a reduced inflammatory response (Pearce et al., 2005; Brand et al., 2001). It is suggested that application of TTO or LPS disrupts the skin barrier leading to an increase in TLR4 and IL6 production following initiation of the JNK and p38 pathway. TLR4 is then activated by the second insult, however a component of TTO (not T-4-ol) inhibits the JNK and p38 pathways thus down regulating IL6 production and successful resolution of the inflammatory response.

8.3 Limitations of the methods used within this thesis

8.3.1 Dermal microdialysis

Following adaptation, the method dermal microdialysis was successful in the recovery of components of TTO at the dermal epidermal junction following topical application. However, limitations of this method whilst investigating TTO include that the estimation of dialysis efficiency was not possible due to the lipophilic nature of TTO. As dialysis efficiency was not established within these studies an estimation of the actual amount of component present at the dermal epidermal junction *in vivo* was not possible. Therefore quantification of components present within the dialysate was measured.

Furthermore despite the use of HPβCD in order to recover lipophilic components, dermal microdialysis utilising a 2kDa membrane cannot account for any protein bound components present at the dermal epidermal junction. Therefore suggesting the possibility of other components present at the dermal epidermal junction but not recovered using dermal microdialysis.
There are a number of variables which may lead to the loss of a substance of interest whilst using this method including the preparation of dialysate prior to analysis with GC-MS. It is essential to remove all sodium from the dialysate prior to addition to the analyser. Investigations within chapter 3 demonstrate that loss of components from the dialysate was limited due to the correct choice of solvent.

8.3.2 Tape stripping

Components of TTO were able to be identified within the SC following topical application using this method. However, limitations include the variation of pressure exerted by the researcher whilst applying the tape to the skin, thus leading to a variation of depth of SC being removed. Furthermore it is essential to remove all residual test solution prior to removal of the SC with the tape strips in order to reduce the risk of contamination of the sample.

8.3.3 MSD multi-array electrochemiluminescence detection using HaCaT cells

Whilst HaCaT cells are frequently used within current literature as a model of keratinocytes, there is evidence that they may not act in a similar way in regards to inflammation (Muller et al., 2003). This may have an impact on the

results obtained within this thesis. Muller suggests NFκB is over expressed in HaCaT cells and that HaCaT cells are unable to express TLR4. However, the results within chapters 6 and 7 suggest that TTO acts upon pathways other than NFkB and furthermore is not reliant upon the presence of TLR4. Therefore the results within this thesis may not have been affected by the use of the HaCaT cell but further investigation is required to identify the effect of TTO on primary keratinocytes to identify the effect of the oil upon TLR4 induced inflammation.

A limitation of the MSD assay includes that the quantification of NFkB is not possible therefore leading to the observation of trends in NFkB phosphorylation only. Despite providing some indication of the effect of TTO on NFkB accurate quantification would not be possible using this method.

8.4 Clinical implications

The investigations within this thesis have enhanced current knowledge of TTO. The identification and quantification of T-4-ol at the dermal-epidermal junction supports the use of TTO as an anti-inflammatory agent as it is proven that this component will be in contact with monocytes. Furthermore, confirmation of the presence of lipids within the SC supports its use for topical application in the treatment of epidermal conditions including fungal and bacterial infection.

The potential of TTO as an anti-inflammatory agent in keratinocytes is not as clearly defined. It is suggested that application of TTO to non-inflamed skin can lead to barrier disruption and up-regulation of IL1β and should therefore be used with caution. However, the barrier perturbation may favourably influence the resolution of an established inflammatory response in epidermal wounds. A component (other than T-4-ol) may act upon the JNK and p38 pathway thus down regulating IL6, leading successful healing of the wound.

There is suggestion that the aqueous extract of TTO with a fraction high in T-4-ol would be beneficial as an anti-inflammatory agent (Hart et al., 2000). However, the demonstration of various effects and potential mechanisms of action of TTO components suggests the probability of a synergistic action of

components of TTO. This may include not only clinical benefits but also enhance the safe use of the oil.

8.4.1 Clinical benefits of TTO.

Scientific evidence suggests TTO is beneficial when used under the conditions presented in *table 8.1* shaded in blue, at the concentrations stated. TTO should not be applied under the conditions shaded in pink. TTO should be applied directly to the affected area; application to healthy skin should be avoided.

Table 8.1 Clinical benefits of TTO and how it should be applied.

Fungal Infection	**Treatment**	**Symptom relief/cure**
Athletes foot (*tinea pedis*)	10% TTO cream w/w	Symptom improvement (Tong et al 2007)
	25% TTO cream w/w	31% mycological improvement
	50% TTO cream w/w	64% mycological improvement (Satchell et al 2002)
Dandruff (*p.ovale)*	5% TTO shampoo v/v for 4 weeks	40% symptom improvement compared with control (Satchell et al 2001)
Bacterial infection	**Treatment**	**Symptom relief/cure**
MRSA	10% TTO cream w/w 5% TTO gel v/v	Clears MRSA from skin lesions and superficial skin sites (but not nasal sites) when compared with chlorohexidine body wash (Dryden et al 2004)
Acne	5% TTO gel v/v	Reduced the severity and number of lesions compared to placebo but required a longer time period than benzyl peroxide (5%) however incurred less side effects (Basset et al 1995)
Inflammation	**Treatment**	**Symptom relief/cure**
Insect bite/sting (histamine)	100% TTO 10 and 20min after bite/sting	Reduced wheal and flare (Brand et al 2002a)
Contact hypersensitivity reaction (TNCB)	100% TTO If nickel sensitive	Reduced oedema and erythema
	NOT if non-nickel sensitive	No effect
	5% TTO ointment w/w	Reduced oedema
	5% TTO gel v/v	No effect (Pearce 2002)
UVB exposure	100% TTO pre-treat 24 hours after UVB exposure	Increased oedema No effect (Brand et al 2002b)

8.5 Future work

The results of the effects of both TTO and some of its components on cultures of HaCaT cells showed relatively little up-regulation of selected inflammatory markers. This questions the effectiveness of the immortalized cell line and indicates the importance of extending the *in vitro* experiments using primary keratinocytes. Although a reliable source of such cells will need to be acquired this offers the potential of examining whether the effects change with age of cell source. In addition, what is important is to examine the temporal profile of these effects. Thus future work would investigate the effect of TTO on pro-inflammatory cytokine modulation in keratinocytes over time (e.g. 10min, 20min, 30min, 1hr, 2hr, 4hr ….. 24hr, 36hr, 48hr). Furthermore the potential synergistic effect of components of TTO warrants investigation. Therefore, in addition to the above time line the interaction between individual components of the oil will be assessed.

It was evident from the studies undertaken that TTO exerts no effect on the NFκB pathway. Whilst the investigations should be repeated with primary keratinocytes as discussed, future studies to identify the effect of TTO on the JNK and p38 pathways will confirm or disprove the theories suggested in this thesis.

In vitro cell culture models provide an indication of the effect of TTO on keratinocyte cells, in order to confirm the effect *in vivo*, dermal microdialysis studies in humans with 'healthy' skin and areas of skin disease will identify cytokine modulation *in vivo* following application of 100% TTO and its individual components.

List of Materials

0.100mm Neubauer Haemocytometer *(Fisher Scientific, UK)*

0.4% Trypan Blue *(Sigma, UK)*
1% L-glutamine (*Sigma, UK)*
1% Penicillin (*Sigma, UK)*
1% streptomycin (*Sigma, UK)*
12 well flat bottomed plate (NUNC, Sigma, UK)
5% w/w EMLA cream (Astra Zenica, Luton, UK)
96 well flat bottomed plate *(NUNC, Sigma, UK)*
Alpha Pinene (Acros Organics, UK)
Amber glass vials *(2ml, Fischer, UK)*
Chloroform (*Sigma, UK)*
Cineole (Acros Organics, UK)
Cuprophan capillary membrane 2kDa cut off, type RC55 8/200 (*Membrana)*
Haemophan capillary membrane 2kDa cut off *(Membrana)*
Dichloromethane *(Sigma, UK)*
Diethylether *(Sigma, UK)*
Dublecco's modified eagles medium (*Sigma, UK)*
EDTA Trypsin *(Sigma, UK)*
Ethylene oxide sterilisation (In Health Decontamination Services, Cardiff,UK)
Foetal calf serum (*Sigma, UK)*
Gamma-terpinen (Acros Organics, UK)
Gilson Pipettes (Fisher Scientific, UK)
Hill top chambers 25mm (*Kiraton, USA*)
Hydroxypropyl Beta-Cyclodextrin (Cargill Food and Pharmacy Specialities, North America)
Loctite 4061 instant adhesive medical line (*BSL, Southampton, UK*}
Melaleuca Alternifolia – organic EO 621 (*Fragrant Earth, Glastonbury, UK)*
Melolin wound dressing (*Smith and Nephew, UK)*
Microdialysis syringe Pump CMA400 (*Sweden)*
MSD 4-multi-plex multiarray electrochemilluiminescence assay *(Meso scale discovery, UK)*
MSD multi-array phosphate NFκB (Ser 468) assay kit *(Meso scale discovery, UK)*

Needles 23g 0.6X25mm (*BD Plastipak, UK*)

Opsite adhesive dressing 6X7cm (*Smith and Nephew, UK*)

Pasteur pipettes (Fisher Scientific, UK)

Phosphate Buffered Saline injection pH7.4 (Tayside Pharmaceuticals, Ninewells Hospital, Dundee)

Pipette pump and pipettes (30ml, 50ml) *(Fisher Scientific, UK)*

Portex epidural catheter 16G, ref.100/382/116 (*Portex Ltd, Kent, UK)*

Portex tubing, fine bore polyethene tubing ID 0.28mm OD 0.58mm ref. 800/100/100 and ID 0.61mm OD 0.96mm ref. 800/100/200 (*Portex Ltd., Kent, UK*)

Silicone (Bathroom) (*B&Q, UK*)

Silicone Remover (B&Q, UK)

Stainless Steel Wire, cut and straightened, diameter 0.1mm (*Goodfellow, Cambridge, UK;*

Syringe 1ml luer lock*(BD Plastipak, UK)*

T75 Flask (NUNC, Sigma, UK)

Terpinen-4-ol (Acros Organics, UK)

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