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& DEVELOPMENT CORPORATION

Anti-inflammatory Activity of Tea Tree Oil

**A report for the Rural Industries Research
and Development Corporation**

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Foreword

The antibacterial properties of tea tree oil have now been well documented, and there are susceptibility data on a wide range of bacteria. However, there has been no scientific basis for claims that tea tree oil has anti-inflammatory properties. It was therefore essential that scientifically valid data showing tea tree oil as an effective anti-inflammatory product be produced both to satisfy requirements of regulatory authorities and to enable further expansion of other international markets. It was reasoned that rigid scientific evidence of tea tree oil as an anti-inflammatory agent would increase its acceptance for treatment of conditions such as acne, eczema and insect bites and perhaps burns and gingivitis, i.e. conditions in which the inflammatory response, as well as the offending organism/agent, require control.

The inflammatory reaction involves a network of mediators that signal a variety of cell types, including lymphocytes, monocytes/macrophages, and neutrophils, to release products important in the killing of micro-organisms but which also cause tissue damage and pathology.

Neutrophils are the first cells attracted into an inflammatory site. They are phagocytic cells that can engulf and subsequently destroy foreign organisms by expression of a respiratory burst. However, they are short-lived cells and their place is soon taken by monocytes and macrophages. Monocytes are the precursors in blood of tissue macrophages. Monocytes and macrophages are a powerhouse of inflammatory mediators that direct an inflammatory response and the associated tissue damage.

We have examined the effect of tea tree oil on the inflammatory responses of both monocytes and neutrophils *in vitro*. Only the inflammatory activity of monocytes was regulated by the water-soluble components of tea tree oil. We reasoned that tea tree oil may enable neutrophils to be fully active in an acute inflammatory response and thereby eliminate foreign antigens. However, tea tree oil suppresses monocyte production of inflammatory mediators and superoxide and thereby may prevent tissue damage that may be seen in more chronic inflammatory states.

This publication details these experiments and our studies to identify the components of tea tree oil responsible for its ability to modulate the inflammatory activity of human monocytes. The experiments with human neutrophils are detailed in Chapter 3.

This project was funded from industry revenue which is matched by funds provided by the Federal Government.

This report, a new addition to RIRDC's diverse range of over 600 research publications, forms part of our Tea Tree Oil R&D program.

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Abbreviations

| | |
|------------------|---|
| FCS | fetal calf serum |
| fmlp | N-formyl methionyl-leucyl-phenylalanine |
| IL-1 | interleukin-1 |
| LPS | lipopolysaccharide |
| ODRS | oxygen derived reactive species |
| PGE ₂ | prostaglandin E ₂ |
| PMA | phorbol myristate acetate |
| TNF α | tumour necrosis factor α |
| TTO | tea tree oil |

Contents

| | |
|--|------------|
| FOREWORD | III |
| ACKNOWLEDGEMENTS | IV |
| ABBREVIATIONS..... | IV |
| EXECUTIVE SUMMARY | VI |
| CHAPTER 1: INTRODUCTION..... | 1 |
| CHAPTER 2: TERPINEN-4-OL, THE MAIN COMPONENT OF THE ESSENTIAL OIL OF MELALEUCA ALTERNIFOLIA (TTO), SUPPRESSES INFLAMMATORY MEDIATOR PRODUCTION BY Activated HUMAN MONOCYTES..... | 3 |
| 2.1 MATERIALS AND METHODS | 3 |
| 2.2 RESULTS | 5 |
| 2.3 DISCUSSION | 11 |
| CHAPTER 3: THE WATER-SOLUBLE COMPONENTS OF THE ESSENTIAL OIL OF MELALEUCA ALTERNIFOLIA (TTO) SUPPRESS THE PRODUCTION OF SUPEROXIDE BY HUMAN MONOCYTES, BUT NOT NEUTROPHILS, ACTIVATED <i>IN VITRO</i> | 13 |
| 3.1 MATERIALS AND METHODS | 13 |
| 3.2 RESULTS | 15 |
| 3.3 DISCUSSION | 22 |
| CHAPTER 4: REFERENCES..... | 25 |

Executive Summary

Tea tree oil (TTO) is the essential oil steam distilled from the Australian native plant, *Melaleuca alternifolia*. It is a complex mixture of approximately 100 terpenes and hydrocarbons, the main component being terpinen-4-ol which comprises at least 30% of the oil. Besides anecdotal evidence for the anti-inflammatory properties of TTO, components of the oil have been demonstrated to show anti-inflammatory activity in experimental inflammation in animals. For example, in a carageenan-induced hind paw oedema model in rats, terpinen-4-ol had anti-inflammatory activity when applied topically in mg amounts [1]. In the same model, α -terpineol (a minor component of TTO comprising approximately 3% of the oil) was anti-inflammatory when administered subcutaneously as a 7.5% mixture with linalool [2]. However, the mechanisms of the anti-inflammatory effects of TTO remain undefined.

In our first study of the anti-inflammatory activity of TTO *in vitro*, human peripheral blood monocytes were used as a model for tissue macrophages. Upon activation with molecules such as lipopolysaccharide (LPS), these cells produce many mediators including the central mediators of inflammation, tumour necrosis factor- α (TNF α) and interleukin-1 β (IL-1 β). Other important monocyte/macrophage-derived mediators of inflammation include IL-8, IL-10 and prostaglandin E₂ (PGE₂). Together with other products of activated macrophages, these molecules can damage tissue or, in turn, activate other cells to produce pro-inflammatory mediators. It was hypothesised that if anti-inflammatory, TTO would reduce the production *in vitro* of TNF α , IL-1 β , IL-8, and PGE₂ by LPS-activated monocytes.

TTO emulsified by sonication in a glass tube into culture medium containing 10% fetal calf serum (FCS) was toxic for monocytes at a concentration of 0.016% v/v. However, the water soluble components of TTO at concentrations equivalent to 0.125% significantly suppressed LPS-induced production of TNF α , IL-1 β and IL-10 (by approximately 50%) and PGE₂ (by approximately 30%) after 40 h. Gas chromatography/ mass spectrometry identified terpinen-4-ol (42%), α -terpineol (3%) and 1,8-cineole (2%, respectively, of TTO) as the water soluble components of TTO. When these components were examined individually, only terpinen-4-ol suppressed the production after 40 h of TNF α , IL-1 β , IL-8, IL-10 and PGE₂ by LPS-activated monocytes. We concluded that the water-soluble components of TTO can suppress pro-inflammatory mediator production by activated human monocytes.

Oxygen derived reactive species (ODRS) such as superoxide, hydrogen peroxide, singlet oxygen and hydroxyl radical, as well as hypochlorous acid and various chloramines [3], are formed by activated macrophages and neutrophils. ODRS play an important role in immunological host defence, providing anti-microbial, anti-viral and anti-tumour activity, as well as being involved in apoptosis and cell survival [3, 4,]. However, increased levels of ODRS (such as those generated during chronic and acute inflammatory diseases) are cytotoxic and may cause tissue damage through lipid peroxidation, oxidation of amino acid side chains, protein cross-linking and fragmentation, and DNA damage [5-7].

In our second study of the anti-inflammatory properties of TTO, we examined the effects of TTO on the production of ODRS (superoxide) in monocytes and neutrophils stimulated *in vitro*. In the absence of toxicity, the water-soluble fraction of TTO had no significant effect on agonist-stimulated superoxide production by neutrophils, but significantly and dose-dependently suppressed agonist-stimulated superoxide production by monocytes. When the water-soluble components were examined individually, terpinen-4-ol significantly suppressed N-formyl-methionyl-leucyl-phenylalanine (fMLP)- and LPS- but not phorbol myristate

acetate (PMA)-stimulated superoxide production; α -terpineol significantly suppressed fMLP-, LPS- and PMA-stimulated superoxide production; 1,8-cineole was without effect. From this study we concluded that TTO components suppress the production of superoxide by monocytes, but not neutrophils, suggesting the potential for selective regulation of cell types by these components during inflammation.

The physiological relevance of these studies is high as it implies TTO has potential as an anti-inflammatory agent. The results suggest TTO contains water-soluble components, specifically terpinen-4-ol and α -terpineol, that may selectively regulate cell function during inflammation, in particular monocyte activity, and following topical application may control inflammatory responses to foreign antigens in the skin. TTO may enable neutrophils to be fully active in an acute inflammatory response and eliminate foreign antigens, while suppressing monocyte inflammatory mediator and superoxide production and thereby preventing oxidative tissue damage that may be seen in more chronic inflammatory states.

The potential of TTO as a topical anti-inflammatory agent requires confirmation through documentation of a reduction of inflammatory cells and mediators in skin after application of TTO.

Chapter 1: Introduction

TTO is the essential oil steam distilled from the Australian native plant, *Melaleuca alternifolia*. TTO contains over 100 components, the majority being monoterpene and sesquiterpene hydrocarbons and their alcohols. The antibacterial properties of TTO have now been well documented, and there are susceptibility data on a wide range of bacteria [8-16]. There is also considerable information identifying the components of TTO active against bacteria and yeasts [13]. Until recently there have only been anecdotal claims about TTO's anti-inflammatory activity. The inflammatory reaction involves a network of mediators which signal a variety of cell types, including lymphocytes, macrophages and neutrophils, to release products important in the killing of micro-organisms but which also cause tissue damage and pathology.

Several studies have investigated the anti-inflammatory properties of compounds also found in TTO in a rat carrageenan-induced hind paw oedema model [1,2,17,18]. These studies investigated the immunomodulatory effects of oils from *Bupleurum gibraltarium*, *Bupleurum frutescens*, *Zingiber cassumunar* and *Salvia sclarea*, respectively. Collectively, these studies concluded that α -pinene [17,18], α -terpinene [1], terpinen-4-ol [1], α -terpineol [2] and linalool [2] may have direct or indirect anti-inflammatory activity. The implications of these studies for the activity of TTO are uncertain since the composition of the oils and the concentrations used were generally in excess of those found in TTO. However, they go some way to identifying essential components of TTO which possess anti-inflammatory activity. Whether there are synergistic or antagonistic interactions of several potential immunomodulators when they are in the same chemical mixture is not known. Some of the components of TTO previously shown to have anti-bacterial properties, e.g. terpinen-4-ol and α -terpineol, are also anti-inflammatory *in vivo* [13].

The basis of our studies was to investigate if tea tree oil could modulate the production of inflammatory mediators by blood-derived monocytes and neutrophils upon activation *in vitro*. The cells chosen for study, and their products that were investigated, are those well characterised as responsible for the development and maintenance of inflammation and for tissue damage associated with chronic inflammation.

Upon activation of monocytes and macrophages with molecules such as lipopolysaccharide (LPS), many mediators including the central mediators of inflammation, tumour necrosis factor- α (TNF α) and interleukin-1 β (IL-1 β), are produced. Other important monocyte/macrophage-derived mediators of inflammation include IL-8, IL-10 and prostaglandin E₂ (PGE₂). Together with other products of activated macrophages, these molecules can damage tissue or, in turn, activate other cells to produce pro-inflammatory mediators. Oxygen derived reactive species (ODRS) such as superoxide, hydrogen peroxide, singlet oxygen and hydroxyl radical, as well as hypochlorous acid and various chloramines [3], are formed when macrophages and neutrophils are stimulated and components of the NADPH oxidase are assembled on the plasma membrane of the phagocytic cell. Activation of the oxidase promotes the reduction of oxygen in the extracellular milieu. These ODRS play an important role in immunological host defence, providing anti-microbial, anti-viral and anti-tumour activity, as well as being involved in apoptosis and cell survival [3,4]. There is evidence that they also have a role in the action and secretion of some cytokines, growth factors and hormones, and in the regulation of intracellular signalling pathways [3,4].

However, increased levels of ODRS (such as those generated during chronic and acute inflammatory diseases) are cytotoxic and may cause tissue damage through lipid peroxidation, oxidation of amino acid side chains, protein cross-linking and fragmentation, and DNA damage [5-7].

The batch of TTO used in all experiments (97/03) was examined by Wollongbar Agricultural Institute, Wollongbar, Australia, according to the international standard for TTO [19]. The levels of the 15 major components of TTO were as follows: terpinen-4-ol (41.6%), γ -terpinene (21.5%), α -terpinene (10.0%), terpinolene (3.5%), α -terpineol (3.1%), α -pinene (2.4%), 1,8-cineole (2.0%), p-cymene (1.8%), aromadendrene (1.1%), δ -cadinene (1.0%), limonene (0.9%), ledene (0.9%), globulol (0.5%), sabinene (0.4%) and viridiflorol (0.2%)(Table 1.1). For individual study, terpinen-4-ol and α -terpineol were obtained from Fluka, Buchs, Switzerland and 1,8-cineole from Sigma Chemical Co., St Louis, MO.

Table 1.1. Components of tea tree oil separating into aqueous and oil phases under different conditions*

| Component | Concentration in tea tree oil | Glass + Sonication | | Plastic | | |
|-------------------------|-------------------------------|--------------------|-------|-----------|-------|-----------|
| | | 10% FCS | | 0% FCS | | 10% FCS |
| | | Aqueous % | Oil % | Aqueous % | Oil % | Aqueous % |
| 1. α -pinene | 2.4 | 0.2 | 2.8 | - | 2.8 | - |
| 2. sabinene | 0.4 | - | 0.9 | - | 0.9 | - |
| 3. α -terpinene | 10.0 | 0.8 | 9.1 | - | 9.3 | - |
| 4. limonene | 0.9 | - | 1.1 | - | 1.0 | - |
| 5. p-cymene | 1.8 | 0.7 | 6.1 | - | 6.8 | 0.8 |
| 6. 1,8-cineole | 2.0 | 2.8 | 1.7 | 3.3 | 1.8 | 2.5 |
| 7. γ -terpinene | 21.5 | 2.3 | 23.0 | - | 21.0 | 1.3 |
| 8. terpinolene | 3.5 | - | 3.8 | - | 3.5 | - |
| 9. terpinen-4-ol | 41.6 | 80.4 | 34.3 | 83.8 | 37.0 | 80.8 |
| 10. α -terpineol | 3.1 | 6.3 | 2.7 | 6.5 | 2.9 | 6.3 |
| 11. aromadendrene | 1.1 | - | 1.4 | - | 1.3 | - |
| 12. ledene | 0.9 | - | 1.2 | - | 1.1 | - |
| 13. δ -cadinene | 1.0 | - | 1.2 | - | 1.1 | - |
| 14. globulol | 0.5 | - | 0.5 | - | 0.5 | - |
| 15. viridiflorol | 0.2 | - | 0.4 | - | 0.4 | - |

* for details of methodology see Chapter 2, Section 2.1

Chapter 2: Terpinen-4-ol, the main component of the essential oil of *Melaleuca alternifolia* (TTO), suppresses inflammatory mediator production by activated human monocytes

2.1 MATERIALS AND METHODS

Monocyte isolation and culture

Human monocytes were isolated from peripheral blood as published [16,17] to >93% purity by countercurrent centrifugal elutriation and cultured in RPMI-1640 medium (Cytosystems, Castle Hill, Australia) supplemented with 13.3 mM NaHCO₃, 2 mM glutamine, 50 mM β -mercaptoethanol, 100 U/ml penicillin, 100 g/ml streptomycin, and 2 nM 3-(*N*-morpholino)propanesulphonic acid with an osmolality of 290 mmol/kg H₂O ('complete RPMI'). During isolation and subsequent culture of all cells, extreme care was taken to limit LPS contamination of buffers and culture fluids [20,21]. For measurement of regulation of cytokine production, freshly isolated monocytes were cultured at 10⁶ cells/ml with TTO or its water-soluble components and, unless otherwise indicated, 1% heat inactivated fetal calf serum (FCS). LPS from *Escherichia coli* 0111:B4 (Sigma) was added to give a final concentration of 500 ng/ml. Triplicate cultures for each test variable were incubated at 37°C in 5% CO₂. After 20 or 40 h, cultures were centrifuged and the supernatants harvested for measurement of TNF α , IL-1 β , IL-8, IL-10 and PGE₂. For cultures of adherent cells, polystyrene 24-well tissue culture plates (Falcon, Becton Dickinson, Lincoln Park, NJ) were used.

Measurement of toxicity of TTO and its water soluble components

Metabolically active cells were enumerated using the CellTiter 96 AQueous Non-Radioactive Cell Proliferation Assay (Promega Corporation, Madison, WI). Monocytes (2 x 10⁵/200 μ l) were cultured under adherent conditions for 17 or 37 h with TTO or its water-soluble components, with or without LPS. The MTS/PMS substrate (20 μ l) was added and the amount of formazan product measured spectrophotometrically at 490 nm hourly for the next 4 hours.

ELISAs for TNF α , IL-1 β , IL-8 and IL-10

Culture supernatants were stored at -20°C until used. TNF α , IL-8 and IL-10 were measured by sandwich ELISA using mAbs to human TNF α , IL-8 and IL-10 purchased as antibody pairs from PharMingen, San Diego, CA. The antibody pair for measuring IL-1 β was purchased from Endogen, Woburn, MA. One antibody from each pair was purchased as a biotinylated antibody. The assays were sensitive to levels of >40 pg/ml.

Radioimmunoassay for PGE₂

The levels of PGE₂ in the culture supernatants were determined by radioimmunoassay using competitive adsorption to dextran-coated charcoal (³H-PGE₂, Amersham, Bucks, UK; PGE₂ antiserum, Sigma). The assay was sensitive to levels of >30 pg/ml.

Solubility of TTO in water

A sample of TTO (500 mg) was rapidly stirred in water (50 ml) for 20 h at 20°C. The layers were separated and the aqueous layer filtered through filter paper, then extracted with ether (3 x 25 ml). The ether extracts were dried (Na₂SO₄), and the solvent carefully removed by distillation at 50°C in a closed system. The residue weighed 80 mg giving a solubility of 1.6 g/l in water. The organic phase and the oil adhering to the filter paper were extracted into ether, dried (Na₂SO₄) and evaporated as above to give 400 mg oil. Gas chromatographic analysis as above showed the composition to be 84% terpinen-4-ol, 7% α-terpineol and 3% 1,8-cineole.

For separation of TTO preparations in culture medium into water and oil soluble components, very similar procedures were adopted. Immediately before separation, samples of 250 ml were vigorously shaken before separation of the aqueous and oil layers in a separating funnel. The aqueous fraction was filtered through paper, extracted with ether (2 x 25 ml), dried with sodium sulphate and the ether removed by distillation. The oily fraction, and the oil adhering to the filter paper, was extracted in a similar manner. The residues from both fractions were analysed by gas chromatography/mass spectrometry (GC/MS)(Table 1.1).

Expression of results

Cytokine measurements were performed on samples from triplicate cultures and results were normalised to the LPS-induced level in the absence of TTO at 100%. The mean values from each set of replicates were used to determine the mean + SEM for *n* donors. For comparison of responses by cell populations from a number of different donors, the Student's paired *t* test was used. A value of *P* < 0.05 was considered significant.

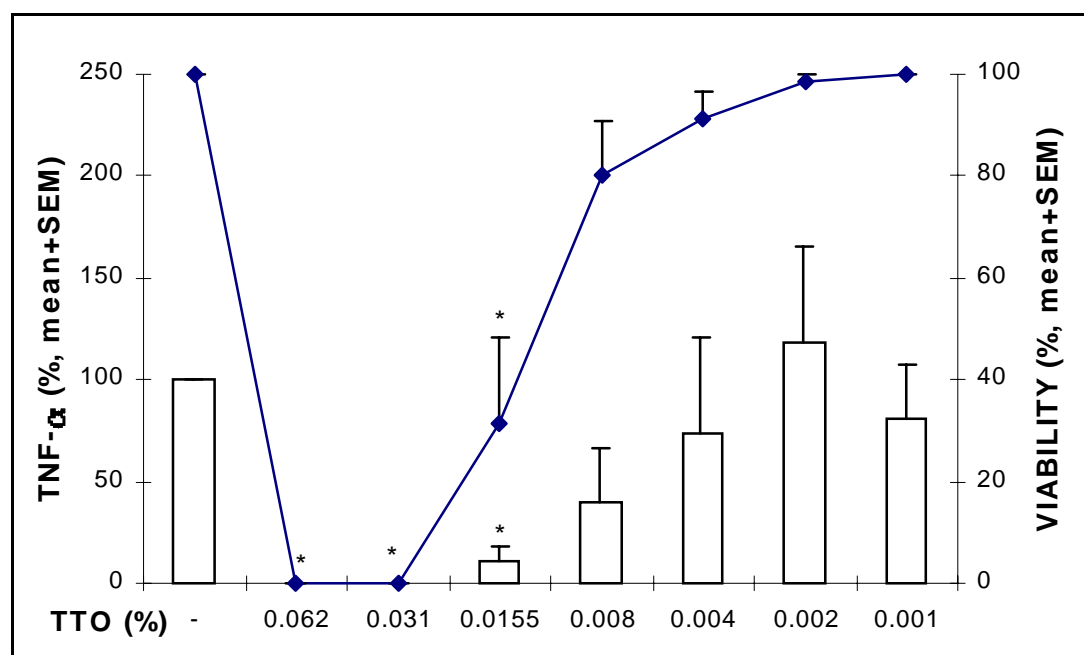
2.2 RESULTS

Regulation of monocyte mediator production by TTO emulsions prepared in glass tubes

To assess the regulatory properties of TTO on inflammatory mediator production by monocytes, it was necessary to prepare emulsions of TTO in culture medium. If TTO was dissolved in propylene glycol or ethanol, the TTO separated from its diluent before a dilution of 1 in 100 required for monocyte culture could be made. Instead, dilutions of TTO were performed in glass tubes with medium containing 10% FCS, followed by sonication for 20 seconds immediately before use. Emulsions of TTO were toxic to adherent monocytes to some extent at concentrations greater than, or equal to 0.004% (v/v). For monocytes from 4 donors, TTO at 0.004% caused a mean toxicity of 9 ± 5 % (not significant), at 0.008% a mean toxicity of 20 ± 11 % (not significant) and at 0.016%, a mean toxicity of 69 ± 17 % ($P = 0.03$). No viable cells were detected after incubation of higher concentrations of TTO with adherent monocytes for 20 h. As shown in Figure 2.1, the suppressive properties of TTO on LPS-induced TNF α production paralleled the toxic properties of TTO. Similar results were detected for regulation of LPS-induced IL-1 β , IL-8, IL-10 and PGE $_2$ (data not shown).

Figure 2.1. The effect of TTO emulsified into medium containing 10% FCS by sonication in glass tubes on TNF α production by monocytes in culture for 20 h

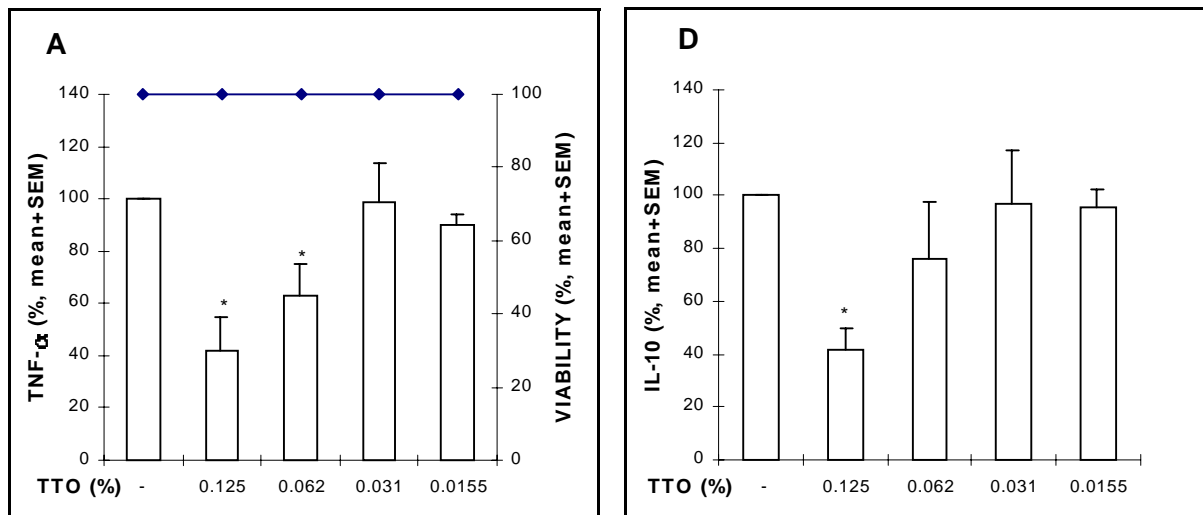
Monocytes from 4 donors were incubated in triplicate for 20 h with LPS (500 ng/ml) and decreasing amounts of tea tree oil. The LPS-induced level was normalised to 100%; the mean result from each experiment was used to calculate the mean + SEM. Regulation of LPS-induced TNF α levels is shown by histograms (left axis). The mean percentage + SEM of viable cells in the cultures is shown by the line (right axis). An asterisk indicates a significant reduction in TNF α production or monocyte viability.

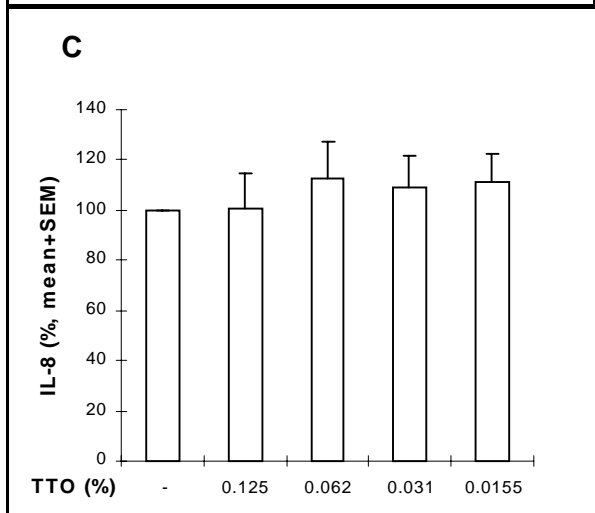
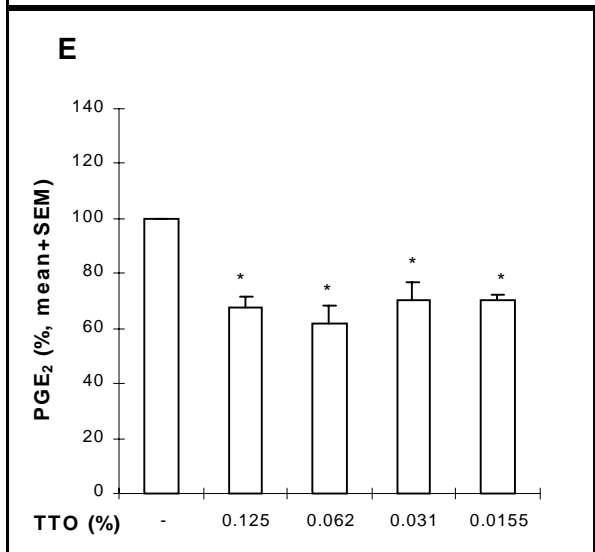
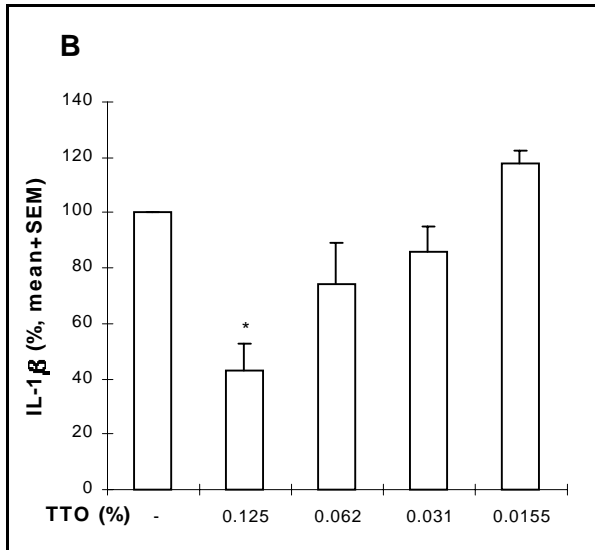


Removal of components of TTO toxic to monocytes by dilution in plastic tubes

We next examined the effect of TTO emulsions prepared in polystyrene plastic, rather than glass tubes. It was noted that the plastic adsorbed a considerable proportion of the oil; we hypothesised that this approach would allow a separation of the water soluble components into the culture medium. Initially, 10% FCS was included in the diluting medium; however, concentrations of TTO greater than, or equal to 0.06% destroyed all monocytes over a 20 h incubation period. Removal of serum from the culture medium used to serially dilute the TTO was then evaluated. TTO mixtures were vortexed for 1 minute immediately before further dilution. Although the diluting medium was serum-free, the monocytes were cultured in complete RPMI supplemented with 1% FCS for 20 and 40 h. Under these conditions, the monocyte toxic components in TTO did not partition into the culture medium; no toxicity was detected in the cultures of monocytes harvested after 40 h (Figure 2.2).

Figure 2.2. The effect of TTO prepared in serum-free medium in polystyrene plastic tubes on monocytes in culture for 40 h. Monocytes from 5 donors were incubated in triplicate under conditions allowing monocyte adherence for 40 h with LPS (500 ng/ml) and decreasing amounts of tea tree oil. The LPS-induced level was normalised to 100%; the mean result from each experiment was used to calculate the mean + SEM. Regulation of LPS-induced A. TNF α , B. IL-1 β , C. IL-8, D. IL-10 and E. PGE $_2$ levels is shown by histograms. For A, the mean percentage + SEM (if sufficiently large) of viable cells in the cultures is shown by the line (right axis). An asterisk indicates a significant reduction in mediator production.





Regulation of monocyte mediator production by TTO solutions prepared in plastic tubes

The TTO solutions had no effect on inflammatory mediator production by monocytes incubated in the absence of LPS. Addition of LPS stimulated mediator production to levels shown in Table 2.1; in Figure 2.2, the mediator production induced by LPS has been normalised to 100%. The dose-dependent suppressive effects of TTO are shown in Figure 2.2. As shown in Table 2.2, the suppressive properties of TTO generally increased with longer incubation times.

Table 2.1. Induction of cytokines and PGE₂ by LPS-activated adherent human peripheral blood monocytes

| | Mediator production | |
|------------------|---------------------|--------------|
| | (mean ± SEM, ng/ml) | |
| | 20 h (n=10) | 40 h (n=5) |
| TNF- α | 6.2 ± 1.8 | 6.8 ± 1.6 |
| IL-1 β | 8.2 ± 1.8 | 8.1 ± 2.3 |
| IL-8 | 250.8 ± 28.2 | 448.5 ± 58.0 |
| IL-10 | 0.3 ± 0.1 | 0.6 ± 0.2 |
| PGE ₂ | 6.1 ± 0.8 | 6.7 ± 1.9 |

Table 2.2. Suppressive effect of the water-soluble components of tea tree oil on LPS-induced cytokine and PGE₂ production by adherent human peripheral blood monocytes

| | Water-soluble components of tea tree oil | | Terpinen-4-ol | |
|------------------|--|------------|---------------|------------|
| | 20 h (n=10) | 40 h (n=5) | 0 h (n=6) | 40 h (n=3) |
| TNF- α | 23% ↓ | 58% ↓ | 58% ↓ | 43% ↓ |
| IL-1 β | 27% ↓ | 57% ↓ | 30% ↓ | 38% ↓ |
| IL-8 | 19% ↓ | - | 31% ↓ | 20% ↓ |
| IL-10 | - | 58% ↓ | - | 36% ↓ |
| PGE ₂ | - | 32% ↓ | 25% ↓ | 32% ↓ |

- = no significant decrease
P < 0.05 for all changes shown

Components of TTO responsible for the anti-inflammatory activity of TTO

The data presented in Figure 2.1 indicated that TTO contained components that were toxic to monocytes in culture. When these were absent, other components of TTO suppressed the production of inflammatory mediators by stimulated monocytes. The components of TTO that partitioned into the culture medium when diluted in glass or polystyrene plastic tubes, in the absence or presence of 10% FCS, were determined. Table 1.1 shows that under the initial conditions of dilution in glass tubes with medium containing 10% FCS, the aqueous phase contained detectable levels of α -pinene, α -terpinene, p-cymene, 1,8-cineole, γ -terpinene, terpinen-4-ol and α -terpineol. With dilution in polystyrene plastic tubes with medium containing 10% FCS, the aqueous phase (which still contained significant toxic activity for monocytes) contained p-cymene, 1,8-cineole, γ -terpinene, terpinen-4-ol and α -terpineol. Using plastic tubes and serum-free diluting medium, three components of TTO were detected in the aqueous phase, namely 1,8-cineole, terpinen-4-ol and α -terpineol, with terpinen-4-ol representing 84% of the recovered material. The identity and relative proportion of these components were identical to those in the aqueous phase when 500 mg TTO was rapidly mixed with 50 ml water in a glass vessel.

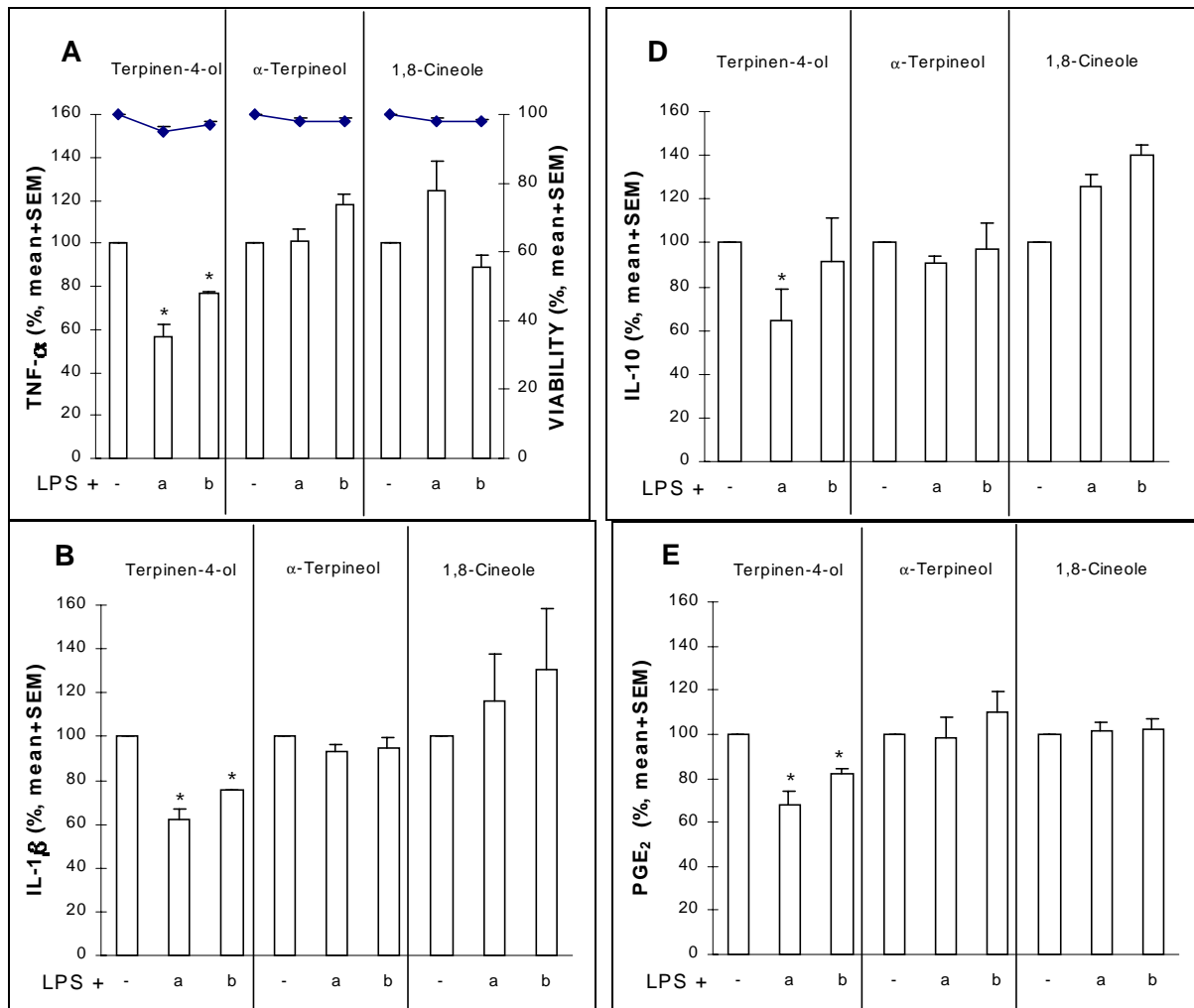
Effects of terpinen-4-ol, α -terpineol and 1,8-cineole on monocyte mediator production

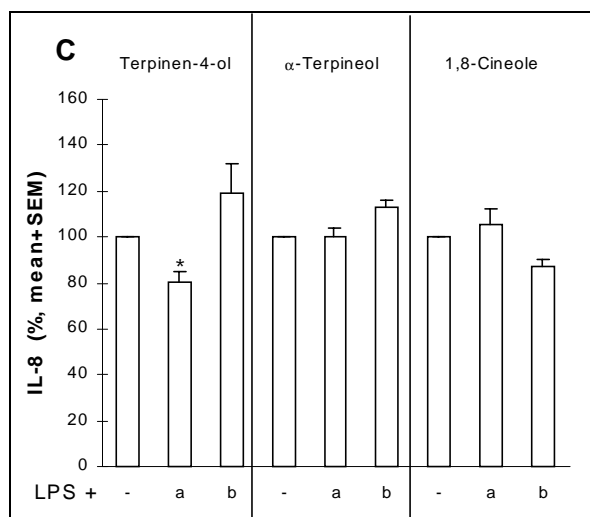
The data in Figure 2.2 and Table 2.1 suggested that water-soluble components of TTO can suppress inflammatory mediator production by monocytes *in vitro*. The GC/MS analyses suggested that terpinen-4-ol, α -terpineol and 1,8-cineole were the components of TTO that were partially soluble in tissue culture medium. It was therefore hypothesised that these three components were not toxic and could suppress inflammatory mediator production by monocytes activated with LPS *in vitro*.

The amounts of terpinen-4-ol, α -terpineol and 1,8-cineole investigated were calculated according to their concentration in a 0.125% solution of TTO. A concentration equal to their level in a solution of 0.062% TTO was also investigated. To maximise the amount of terpinen-4-ol, α -terpineol and 1,8-cineole that remained in the aqueous phase, dilutions were performed in glass tubes. As for the cultures of Figure 2.1 and Table 2.2, dilutions were performed in the absence of serum and monocytes were cultured in medium containing 1% FCS.

When monocytes were incubated with 0.052 and 0.026% terpinen-4-ol, 0.004 and 0.002% α -terpineol and 0.0025 and 0.0013% 1,8-cineole (concentrations equivalent to those in 0.125 and 0.062% TTO, respectively), no toxicity for monocytes was detected (Figure 2.3). After incubation for 20 h, 0.052% terpinen-4-ol significantly suppressed LPS-induced TNF α , IL-1 β , IL-8 and PGE₂ production (Table 2.2). The suppression of LPS-induced IL-10 was not significant. After incubation for 40 h, the lower concentration of terpinen-4-ol significantly suppressed LPS-induced IL-1 β and PGE₂, as well as TNF α (Figure 2.3). After incubation for 20 or 40 h, the other water soluble components of TTO, namely α -terpineol and 1,8-cineole, were without effect (Figure 2.3).

Figure 2.3. The effect of the water-soluble components of TTO prepared in serum-free medium in glass tubes on monocytes in culture for 40 h. Monocytes from 3 donors were incubated in triplicate under conditions allowing monocyte adherence for 40 h with LPS (500 ng/ml) and concentrations of terpinen-4-ol, α -terpineol and 1,8-cineole equivalent to those found in 0.125% (shown as a) and 0.062% (shown as b) tea tree oil. The LPS-induced level was normalised to 100%; the mean result from each experiment was used to calculate the mean + SEM. Regulation of LPS-induced A. TNF α , B. IL-1 β , C. IL-8, D. IL-10 and E. PGE $_2$ levels is shown by histograms. For A, the mean percentage + SEM (if sufficiently large) of viable cells in the cultures is shown by the line (right axis). An asterisk indicates a significant reduction in mediator production.





2.3 DISCUSSION

The potential for TTO to have anti-inflammatory activity (as anecdotally reported) was investigated *in vitro* using activated human monocytes. However, when TTO was emulsified in tissue culture medium containing 10% FCS in glass tubes and incubated at a dilution of 0.016% with adherent monocytes for 20 h, approximately 30% of the cells retained viability. TTO components toxic for human cells in culture have been reported previously. In a Swedish study [22], TTO at concentrations of approximately 0.03% (300 µg/ml) and 0.05% (500 µg/ml) caused 50% killing of gingival epithelial cells and fibroblasts, respectively, after 24 h incubation. In another Australian study [23], the toxicity of TTO and its main water-soluble components (terpinen-4-ol, α-terpineol and 1,8-cineole) on five human cell lines was examined after 4 and 24 h incubation. Susceptibility of the cell lines to the toxicity of TTO varied with 50% killing after 4 h at a concentration of 0.28% for HeLa (epithelioid), K562 (chronic myelogenous leukaemia) and Hep G2 (hepatocellular carcinoma) cells. A concentration of 0.06% TTO over 4 h caused 50% killing of CTVR-1 (B cell-derived from bone marrow of a patient with acute myeloid leukaemia) and Molt-4 (lymphoblastic leukaemia) cell lines. After 24 h, the IC₅₀ for HeLa cells was 0.27%, for K562 cells 0.03%, CTVR-1 cells 0.03%, Molt-4 cells 0.03% and Hep G2 cells 0.002% [23]. Thus, monocytes cultured *in vitro* have a susceptibility to the toxic effects of TTO similar to that of K562, CTVR-1 and Molt-4 cells. When TTO was diluted to a concentration which was not toxic or minimally toxic to monocytes (0.008% or lower), there were some suggestions of reduced production of inflammatory mediators. However, it was difficult to rule out the effects of lowered LPS-stimulated production of mediators by dying cells. In subsequent experiments, partitioning of TTO components into aqueous and oil phases was encouraged with the oil components adhering to the polystyrene plastic tubes. Inclusion of 10% serum provided sufficient emulsifying activity such that TTO was still cytotoxic to monocytes. A similar effect was seen if lower concentrations of serum were used in the presence of 0.001% Tween 20 (data not shown). Only when TTO was diluted with serum-free medium in polystyrene plastic tubes were the components toxic for monocytes not dissolved. Analysis of the components of TTO remaining in the culture medium suggested that either terpinen-4-ol (84% of compounds identified), α-terpineol (7% of compounds identified) or 1,8-cineole (3% of compounds identified) were regulatory for inflammatory mediator production by LPS-activated monocytes *in vitro*. Further studies suggested that terpinen-4-ol was responsible in

large part for the regulatory effects of TTO. The effects of terpinen-4-ol after 40 h closely paralleled the effects of TTO as a whole, suggesting that if there were any other water-soluble anti-inflammatory components in TTO, they were minor.

The plastic-non-adherent, water soluble components of a concentration of TTO of 0.125% were examined in the experiments of Figure 2.2 and Table 2.2. As it is recommended that a 100% solution of TTO is applied to skin, this concentration represents approximately one thousandth of that used and is a level less than the water solubility of TTO (1.6 g/l). The ability of TTO components to reach the epidermal and dermal tissues and enter the systemic circulation has not been measured. However, TTO contains several components known to enhance skin penetration of other compounds, e.g. 1,8-cineole [24], limonene [25], terpinen-4-ol [26] and α -terpineol [26]. The exact concentrations of the water soluble components of TTO in the culture medium were not determined for each experiment but were assumed to be similar to those of Table 3 (84% terpinen-4-ol, 7% α -terpineol and 3% 1,8-cineole). To test the activity of terpinen-4-ol, α -terpineol and 1,8-cineole in isolation, calculations were made according to their concentration in whole TTO and therefore their approximate level in TTO solutions of 0.125%. Thus, in experiments in which the independent activity of the components was analysed, concentrations higher than in the aqueous phase of TTO were examined. It should be noted that even at these higher concentrations, no toxicity was observed. 1,8-Cineole, the main component of eucalyptus oil (85%) was not regulatory in our study. We are uncertain why in a previous study it significantly suppressed LPS-induced TNF α production *in vitro* by monocytes at a concentration of 0.00001% [27] and following administration to asthmatic patients caused reduced leukotriene B₄ and PGE₂ production by monocytes *ex vivo* [28]. Our study identified terpinen-4-ol, the major component of TTO (> 30%), as the component with the ability to suppress the production of TNF α , IL-1 β , IL-8, IL-10 and PGE₂ by LPS-activated monocytes.

It was apparent from our study that all mediators released by activated monocytes are not similarly regulated by the water-soluble components of TTO. In particular, little regulation of LPS-induced IL-8 production was detected at 20 or 40 h. TNF α and IL-1 β production was regulated at 20 h by the water-soluble components in TTO and this regulation was enhanced after 40 h incubation. In contrast, regulation of LPS-induced IL-10 and PGE₂ production was not detected until the later time point. This difference reflects, in part, the production of mediators with autocrine regulatory activity. The greater regulation of TNF α is significant as it is widely recognised that control of TNF α production/activity can limit further production of other inflammatory cytokines [29]. TNF α is regarded as central to the development and maintenance of chronic inflammation; this was evidenced by the ability of anti-TNF α antibodies to reduce inflammatory diseases such as rheumatoid arthritis [30].

Our study suggests that TTO may potentially control inflammatory responses to foreign antigens in the skin. With application of TTO to skin, toxicity would be limited and the anti-inflammatory water-soluble components may penetrate into the vascularised dermis and regulate inflammatory processes. This study supports the selection of superior trees for propagation after identification of productive clones which produce TTO high in terpinen-4-ol [31]. Alternatively, an aqueous extract of TTO which produces a fraction very high in terpinen-4-ol, may be used as an anti-inflammatory agent. As we are uncertain of the concentrations of terpinen-4-ol that may penetrate beyond the stratum corneum, the potential of TTO as a topical anti-inflammatory agent will only be confirmed by documentation of a reduction of inflammatory cells and mediators in skin after application of TTO.

Chapter 3: The water-soluble components of the essential oil of *Melaleuca alternifolia* (TTO) suppress the production of superoxide by human monocytes, but not neutrophils, activated *in vitro*

3.1 MATERIALS AND METHODS

Neutrophil isolation

Neutrophils were prepared from blood of healthy volunteers by a rapid single-step method [32]. Briefly, freshly collected whole heparinized blood was layered onto hypaque-ficoll medium of density 1.114 g/ml and centrifuged at 400 x g for 30 minutes. With centrifugation, the leukocytes resolved into 2 bands allowing distinction between mononuclear cells and neutrophils. The neutrophils were harvested from the lower band and washed in medium 199 to yield a final purity of > 96% and viability of > 99% as determined by exclusion of trypan blue dye (0.1%) and used immediately for assessment of superoxide production.

Monocyte isolation and culture

Human monocytes were isolated as per Chapter 2. Freshly isolated monocytes (10^6 cells/ml) were cultured overnight (16 h) in complete RPMI supplemented with 5% heat inactivated (56°C for 30 min) fetal calf serum (FCS) at 37°C in 5% CO₂ in 40ml Teflon pots (Savillex, Minnetonka, MN) prior to assessment of superoxide production.

Chemiluminescence and superoxide production

Superoxide production was measured as chemiluminescence (CL) resulting from the oxidation of the fluorescent probe lucigenin (bis-*N*-methylacridinium nitrate). This assay provided a direct and specific measure of superoxide production and could be inhibited totally by superoxide dismutase [33].

Neutrophils (10^6 in 100 μ l of Hanks balanced salt solution (HBSS) supplemented with 1% heat inactivated autologous serum (AS) or FCS, pH 7.3) or non-adherent monocytes (10^6 in 100 μ l of HBSS supplemented with 1% heat inactivated FCS, pH 7.3) were transferred into luminometer tubes and pre-incubated for 30 min at 37°C in 5% CO₂ with TTO, its water-soluble components or HBSS before addition of agonist (5×10^{-5} M *N*-formyl-methionyl-leucyl-phenylalanine (fMLP), 500 ng/ml lipopolysaccharide from *Escherichia coli* K-235 (LPS) or 10^{-6} M phorbol 12-myristate 13-acetate (PMA)) and HBSS for a total volume of 500 μ l. Lucigenin (500 μ l, 127.5 μ g/ml) was then added. fMLP, LPS, PMA and lucigenin were obtained from Sigma. The resulting CL was monitored in a water-jacketed (37°C) luminometer chamber (Model 1250, LKB, Wallac, Turku, Finland) and recorded in millivolts

(mV) as the peak initial rate of superoxide production. For fMLP this usually occurred within 1 to 2 minutes of agonist addition producing a response of intermediate size, for LPS within 60 minutes producing the weakest response and for PMA within 15 to 20 minutes producing the strongest response.

Measurement of toxicity of TTO and its water-soluble components

The viability of neutrophils or monocytes incubated with TTO or its water-soluble components was determined by exclusion of trypan blue dye (0.1%) after recording the CL response. Total assay time ranged from 30 minutes for measurement of fMLP-stimulated superoxide production to 1.5 hours for measurement of LPS-stimulated superoxide production.

Measurement of cellular uptake of water-soluble TTO components by neutrophils and monocytes

Neutrophils or monocytes (10^7 in 900 μ l HBSS supplemented with 1% heat inactivated FCS) were transferred into luminometer tubes and incubated for 1 h at 37°C in 5% CO₂ with 100 μ l of the water-soluble fraction of TTO (0.125% v/v). HBSS supplemented with 1% heat inactivated FCS was incubated with or without TTO under identical conditions. The cultures were centrifuged and the supernatants harvested. A 1 μ l aliquot of each supernatant was injected into a Varian Saturn 4D instrument with a J & W DB5MS capillary column (5% phenylmethylpolysiloxane 30 m x 0.25 mm id), oven 80°C isothermal, injector 200°C. All peaks were unambiguously characterised by a mass-spectral library matching service, and the levels of terpinen-4-ol, α -terpineol and 1,8-cineole present in the supernatant were integrated and represented as area (%).

Expression of results and statistical analysis

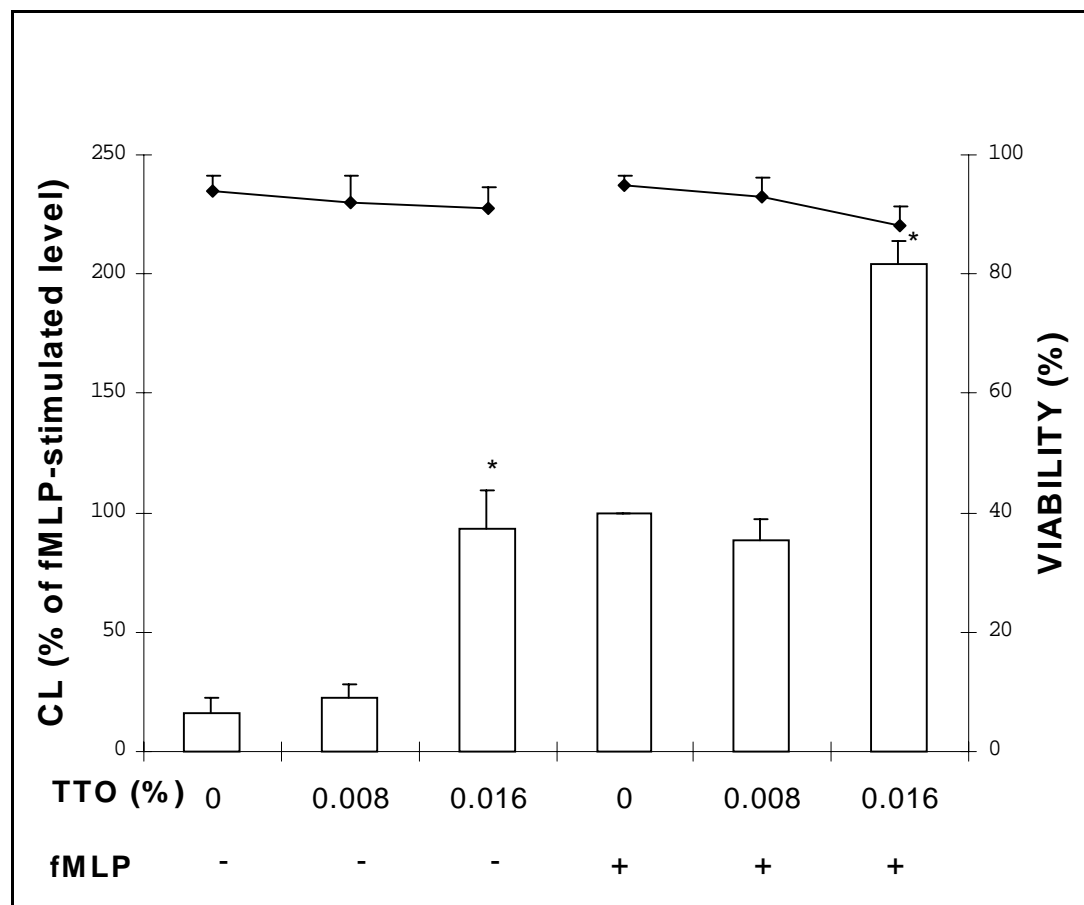
Measurement of superoxide production was performed on duplicate or triplicate samples and results were normalised to the agonist-stimulated level in the absence of TTO at 100%. The mean values from each set of replicates were used to determine the mean + SEM for *n* donors. For responses by cell populations from a number of different donors, a multiple comparison procedure employing a one-way analysis of variance and Fisher's test was used to determine the statistical significance of differences between experimental and control groups. Probabilities less than 0.05 were considered significant.

3.2 RESULTS

Effect of TTO on superoxide production by neutrophils

TTO emulsified by sonication in HBSS containing serum was toxic for neutrophils at concentrations above 0.016% v/v. This concentration of TTO significantly induced the basal and fMLP-stimulated CL response but was without significant effect at half this concentration of TTO (Figure 3.1). For LPS- and PMA-stimulated CL responses, no significant effect of TTO at these concentrations (0.016% and 0.008%) was detected (data not shown).

Figure 3.1. The effect of TTO emulsified into buffer containing 10% FCS by sonication in glass tubes on superoxide production by neutrophils. Neutrophils from 3 donors were incubated for 30 min with increasing amounts of TTO prior to stimulation with fMLP (5×10^{-7} M). Regulation of lucigenin-dependent superoxide production is shown by histograms (left axis; CL); the fMLP-stimulated superoxide level was normalised to 100% and the mean result from each experiment used to calculate the mean + SEM. The mean percentage + SEM of viable neutrophils is shown by the line (right axis). An asterisk indicates a significant enhancement in superoxide production compared with the absence of TTO.

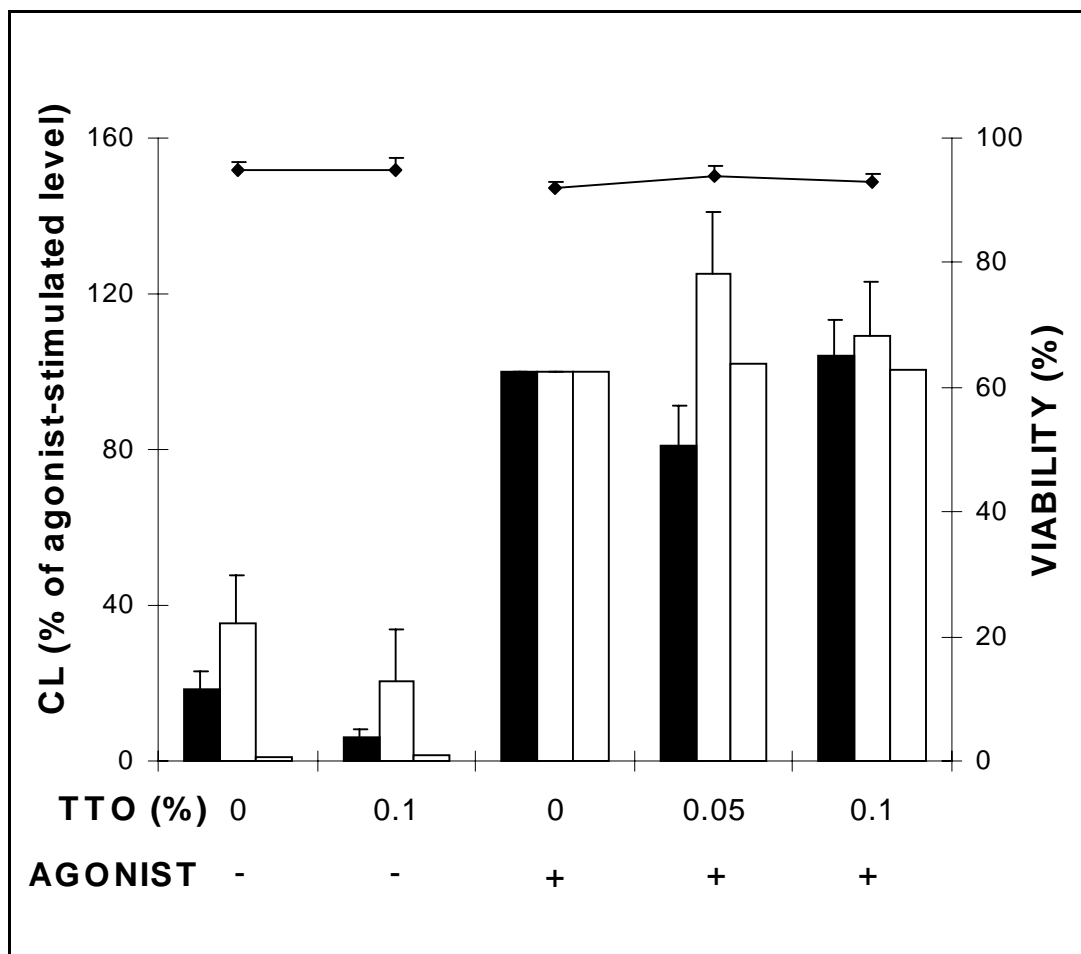


Effect of the water-soluble TTO fraction on superoxide production by neutrophils

The effect of the water-soluble TTO fraction at concentrations of 0.1% v/v and 0.05% v/v on superoxide production by neutrophils in 1% AS in the absence and presence of agonist (fMLP, LPS and PMA) was investigated. There was no significant effect of the water-soluble TTO components on responses measured (Figure 3.2). At a concentration of TTO of 0.25% v/v, any decrease was associated with significant toxicity (data not shown). Addition of FCS instead of AS did not change the inability of the water-soluble TTO fraction to suppress fMLP-stimulated superoxide production by neutrophils (data not shown).

Figure 3.2. The effect of TTO prepared in serum-free buffer in polystyrene plastic tubes on superoxide production by neutrophils.

Neutrophils were incubated for 30 min with increasing amounts of TTO prior to stimulation with fMLP (5×10^{-7} M, n=8), LPS (100 ng/ml, n=3) or PMA (10^{-8} M, n=1). Regulation of lucigenin-dependent superoxide production is shown by histograms (left axis, CL; fMLP, solid bars; LPS, open bars; PMA, hatched bars); the agonist-stimulated superoxide level was normalised to 100% and the mean result from each experiment used to calculate the mean + SEM. The mean percentage + SEM of viable neutrophils is shown by the solid line (reference: right axis).

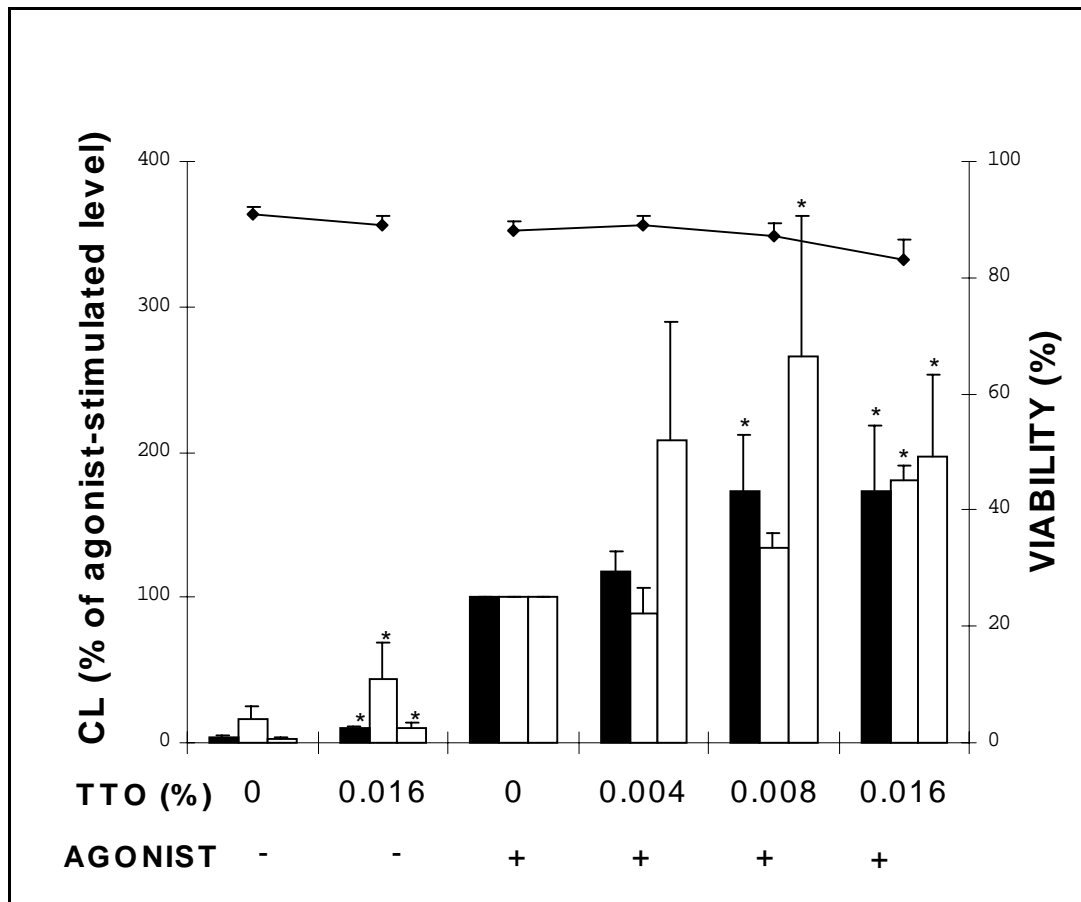


Effect of TTO on superoxide production by monocytes

As for neutrophils, TTO at concentrations greater than 0.016% was toxic for monocytes. However, at non-toxic levels tested (less than, or equal to 0.016%), it significantly induced superoxide production by unstimulated monocytes. Pooled results from 3 donors indicated that TTO significantly induced fMLP-stimulated superoxide production at a concentration greater than, or equal to 0.008%; significantly induced LPS-stimulated superoxide production at a concentration of 0.016%; and significantly induced PMA-stimulated superoxide production at a concentration greater than, or equal to 0.008% (Figure 3.3).

Figure 3.3. The effect of TTO emulsified into buffer containing 10% FCS by sonication in glass tubes on superoxide production by monocytes.

Monocytes were incubated for 30 min with increasing amounts of TTO prior to stimulation with fMLP (5×10^{-7} M, n=3), LPS (100 ng/ml, n=3) or PMA (10^{-8} M, n=3). Regulation of lucigenin-dependent superoxide production is shown by histograms (left axis, CL; fMLP, solid bars; LPS, open bars; PMA, hatched bars). The mean percentage + SEM of viable monocytes is shown by the line (right axis). An asterisk indicates a significant enhancement in superoxide production.

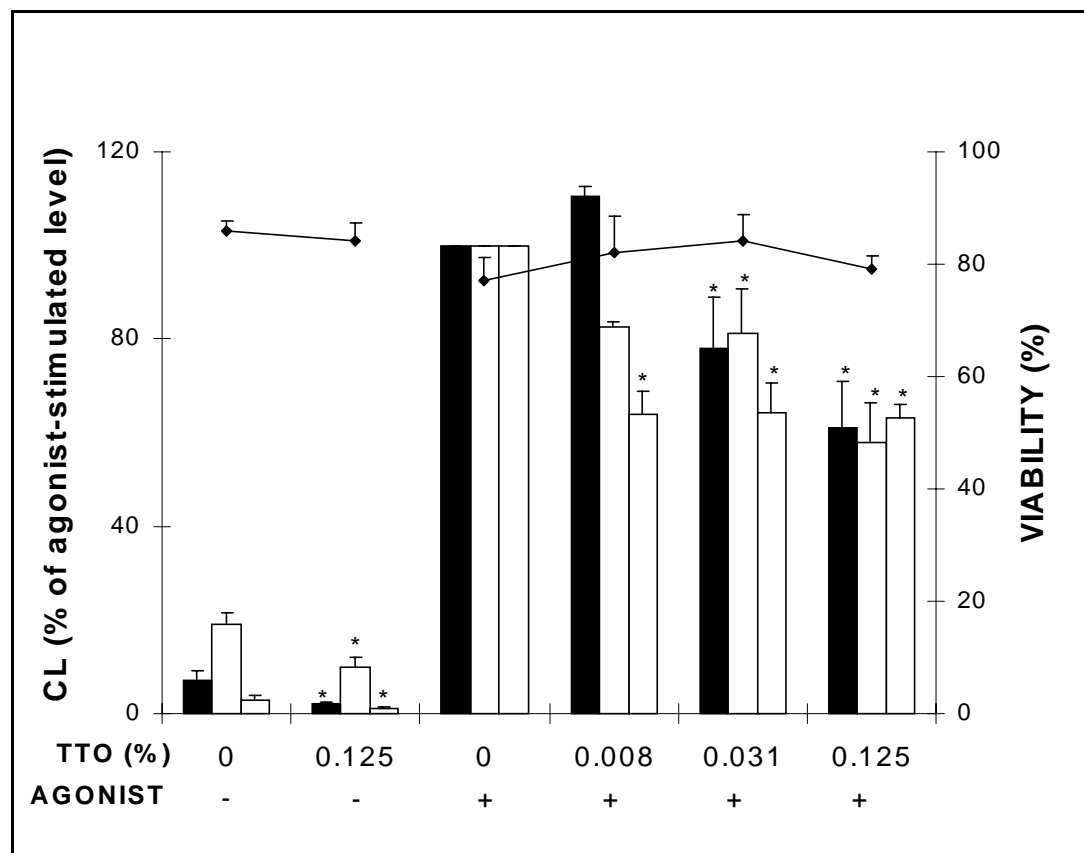


Effect of the water-soluble TTO fraction on superoxide production by monocytes

The effect of the water-soluble TTO fraction at concentrations ranging from 0.125% v/v to 0.008% v/v on monocyte superoxide production was investigated. Monocyte viability assessed by trypan blue exclusion was unaffected by the water-soluble TTO fraction at any of the concentrations investigated (Figure 4). In the absence of agonist (fMLP, LPS or PMA), superoxide production was significantly suppressed at a concentration of TTO of 0.125%. Significant and dose-dependent suppression of superoxide production was seen at a concentration greater than, or equal to 0.031% by fMLP- and LPS-stimulated monocytes from 5 and 4 donors respectively. PMA-stimulated superoxide production was suppressed at a concentration greater than, or equal to 0.008% from 4 donors (Figure 3.4). This suppressive effect was very potent and was removed, for cells from one donor, at a dilution of the water-soluble fraction of TTO of $8 \times 10^{-7}\%$ (data not shown).

Figure 3.4. The effect of TTO prepared in serum-free buffer in polystyrene plastic tubes on superoxide production by monocytes.

Monocytes were incubated for 30 min with increasing concentrations of TTO prior to stimulation with fMLP (5×10^{-7} M, n=5), LPS (100 ng/ml, n=4) or PMA (10^{-8} M, n=4). Regulation of lucigenin-dependent superoxide production is shown by histograms (left axis, CL; fMLP, solid bars; LPS, open bars; PMA, hatched bars). The mean percentage + SEM of viable monocytes is shown by the line (right axis). An asterisk indicates a significant reduction in superoxide production.



Effects of terpinen-4-ol, α -terpineol and 1,8-cineole on superoxide production by monocytes

The water-soluble components of TTO, terpinen-4-ol, α -terpineol and 1,8-cineole (42, 3 and 2% of TTO respectively), were initially examined at concentrations equivalent to those found in 0.125% v/v, 0.031% v/v and 0.008% v/v TTO. Dilutions were performed in glass tubes and serum-free buffer to maximise the amount of terpinen-4-ol, α -terpineol and 1,8-cineole that remained in the aqueous phase. No effect was seen on superoxide production by unstimulated monocytes for any component, and viability remained unaffected.

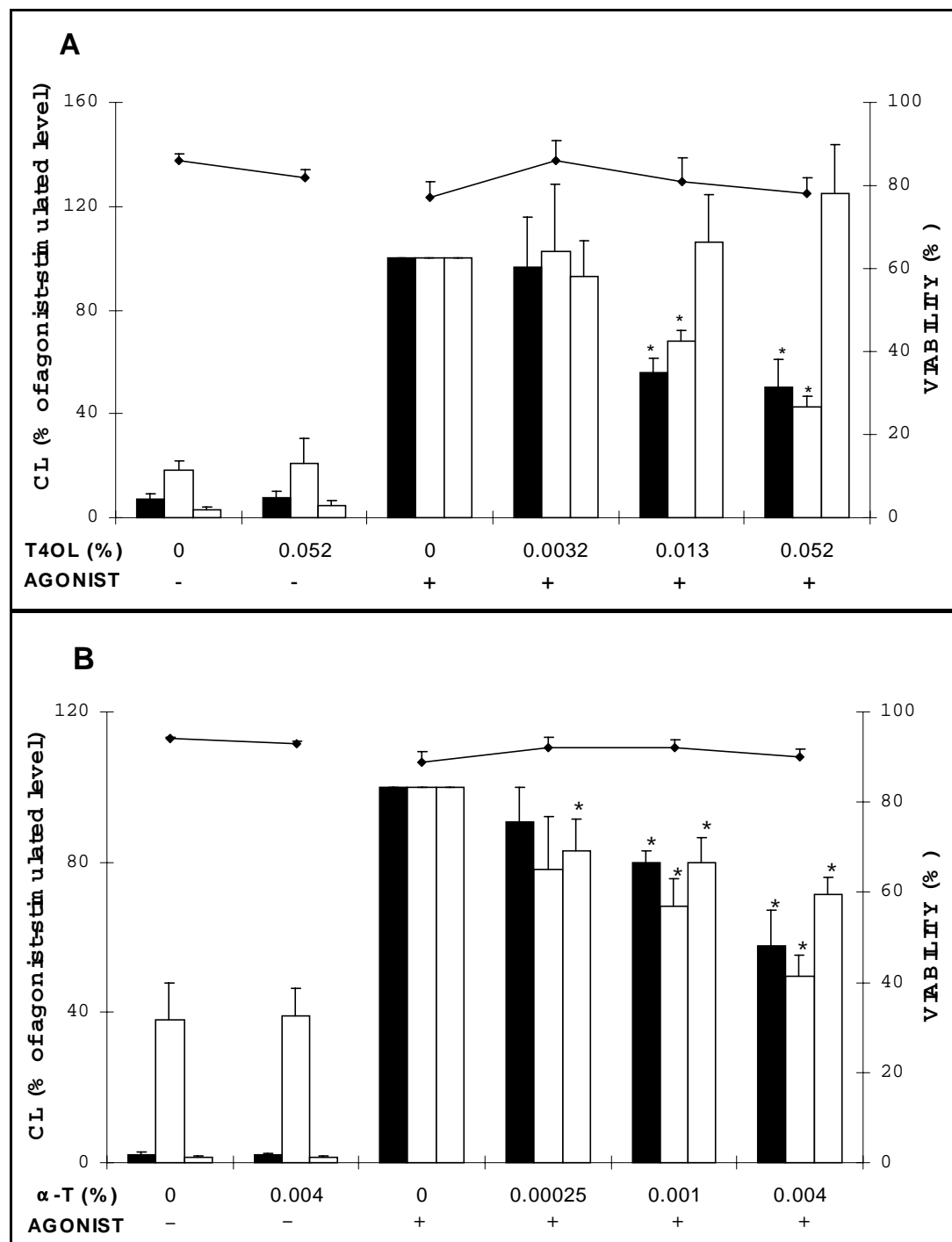
Terpinen-4-ol at a concentration greater than, or equal to 0.013% (equivalent to 0.031% TTO) significantly and dose-dependently suppressed superoxide production by fMLP-stimulated (n=5) and LPS-stimulated (n=4) monocytes. No effect was seen on PMA-stimulated superoxide production by monocytes from 4 donors (Figure 3.5A).

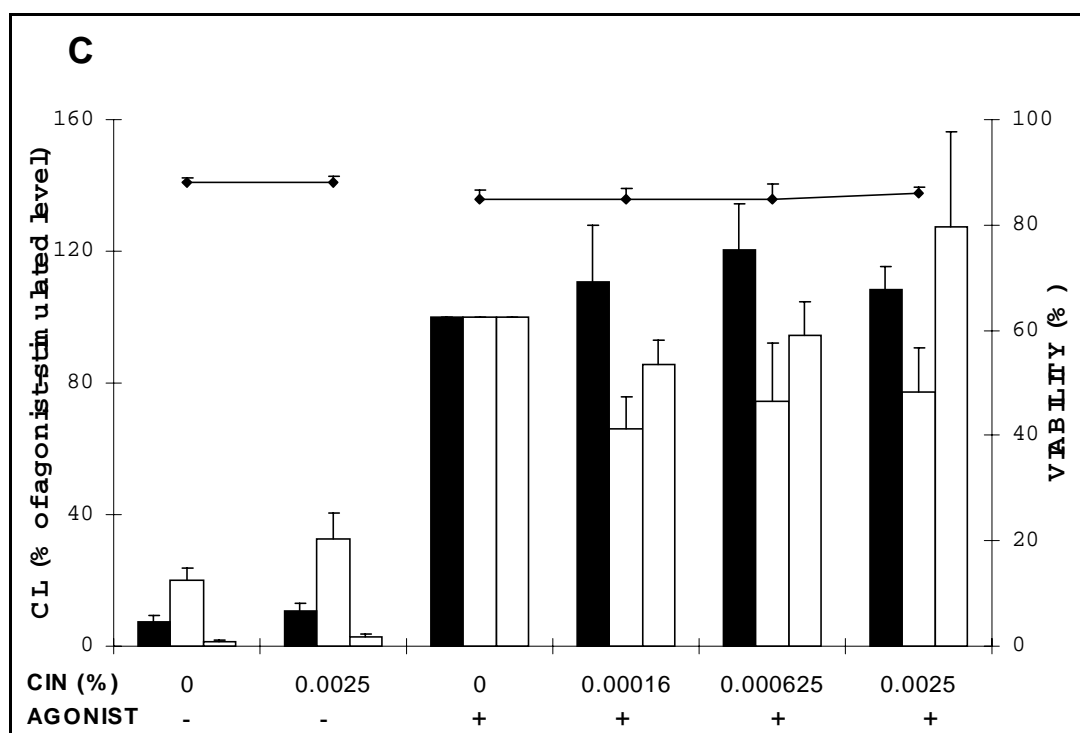
α -Terpineol significantly and dose-dependently suppressed fMLP- and LPS-stimulated superoxide production by monocytes from 3 donors at a concentration greater than, or equal to 0.001% (equivalent to 0.031% TTO) and PMA-stimulated superoxide production by monocytes from 5 donors at a concentration greater than, or equal to 0.00025% (equivalent to 0.008% TTO) (Figure 3.5B). Although for the aggregated data the suppression of superoxide production by monocytes stimulated with PMA appeared to be dose-responsive, 2 distinct patterns were observed. Inhibition was experienced by all donors. However only some responded in a concentration dependent fashion, while the remainder failed to dose respond in a manner similar to that seen with the water-soluble TTO fraction.

There was no significant effect of 1,8-cineole on superoxide production by monocytes stimulated with fMLP, LPS or PMA (Figure 3.5C).

Figure 3.5. The effects of terpinen-4-ol (A), α -terpineol (B) and 1,8-cineole (C) prepared in serum-free buffer in glass tubes on superoxide production by monocytes.

Monocytes were incubated for 30 min with increasing amounts of each component prior to stimulation with fMLP (5×10^{-7} M), LPS (100 ng/ml) or PMA (10^{-8} M). Regulation of lucigenin-dependent superoxide production is shown by histograms (left axis, CL; fMLP, solid bars; LPS, open bars; PMA, hatched bars). The mean percentage + SEM of viable monocytes is shown by the line (right axis). An asterisk indicates a significant reduction in superoxide production.





Uptake of terpinen-4-ol, α -terpineol and 1,8-cineole by neutrophils and monocytes

GC-MS analysis showed no significant differential uptake of any one component by monocytes compared with neutrophils following incubation for 1 hour at 37°C (Table 3.1).

Table 3.1. Water-soluble components of TTO remaining in 1 ml medium after incubation with cells (10^7) and TTO (0.125% v/v, prepared in plastic tubes) for 1 hour

| Incubation with | Terpinen-4-ol (mean \pm SD, %)* | α -Terpineol (mean \pm SD, %) | 1,8-Cineole (mean \pm SD, %) |
|-----------------|--------------------------------------|---|-----------------------------------|
| No cells | 91.1 \pm 2.0 | 6.8 \pm 1.8 | 2.1 \pm 0.4 |
| Monocytes | 91.1 \pm 0.6 | 6.8 \pm 0.4 | 2.1 \pm 0.3 |
| Neutrophils | 90.2 \pm 0.5 | 7.7 \pm 0.6 | 2.1 \pm 0.2 |

* = triplicate cultures

3.3 DISCUSSION

Separation of TTO into its hydrophobic and water-soluble components was encouraged by dilution of TTO in polystyrene plastic tubes with serum-free buffer. Using this method, the hydrophobic TTO components adsorbed to the plastic wall of the tube, while the water-soluble TTO components remained in the aqueous phase. This was confirmed by analysis based on GC-MS where the water-soluble TTO fraction was shown to contain terpinen-4-ol (84%), α -terpineol (7%) and 1,8-cineole (3%) (Table 1.1).

When examined on monocytes at concentrations equivalent to those found in TTO, terpinen-4-ol suppressed superoxide production by monocytes stimulated with fMLP and LPS but not with PMA (data summarized in Table 3.2). Since fMLP and LPS bind to surface receptors and act intracellularly on protein kinase C (PKC), terpinen-4-ol probably acts on signals upstream from PKC, but not at the surface receptor level because the water-soluble components of TTO did not modulate CD14 levels on monocytes during the 30 min incubation prior to LPS addition (flow cytometry data not shown).

As shown in Chapter 2, terpinen-4-ol also suppressed TNF α , IL-1 β , IL-8, IL-10 and PGE₂ production by human peripheral blood monocytes stimulated *in vitro* with LPS. Thus, it is evident that this component of TTO affects the production of two major products of mononuclear phagocytes, pro-inflammatory cytokines and ODRS. These mediators have been established as important contributors to the inflammatory reaction and the tissue damage which occurs in chronic inflammation.

Interestingly, α -terpineol could suppress fMLP-, LPS- and PMA-stimulated superoxide production (data summarized in Table 3.2) but not LPS-stimulated inflammatory mediator production by monocytes (Chapter 2). This difference was further confirmed on cells from each of 2 donors whereby α -terpineol suppressed LPS-induced superoxide production but was without effect on LPS-induced TNF α production (data not shown). This suggested α -terpineol was a potent inhibitor of superoxide production, as it comprises only 3% (compared to 42% for terpinen-4-ol) of TTO but was able to produce a strong effect. Furthermore, α -terpineol regulated NADPH oxidase activity and subsequent production of ODRS by targeting PKC or downstream molecules. The ability of α -terpineol to inhibit LPS-induced superoxide production but not cytokine production suggests that these two monocyte functions are independently controlled and that the mechanism by which terpinen-4-ol inhibits monocyte function is different to that mediated by α -terpineol.

In contrast to the effects on monocytes, the water-soluble TTO components had no significant effect on agonist-stimulated superoxide production by neutrophils. This response could not be explained by differential cellular uptake of terpinen-4-ol, α -terpineol or 1,8-cineole by neutrophils and monocytes. The influence of the water-soluble TTO components on neutrophil and monocyte adherence to fibronectin-coated plates, another function shared by these two cell types, was examined. However, no effect of the water-soluble components of TTO was seen on either cell type (data not shown).

Several studies have suggested an anti-inflammatory activity of TTO and its components [1, 2]. However, TTO is also associated with some cytotoxicity to mammalian cells *in vitro* [22,23]. It was reported that TTO at a concentration of 0.05% v/v caused greater than 85% suppression of superoxide production by neutrophils stimulated *in vitro* [34]. However in our

studies, 0.05% TTO was very toxic, and it was necessary to dilute TTO to 0.016% v/v to avoid toxicity to neutrophils and monocytes during short term exposure. This concentration increased fMLP-stimulated superoxide production but as we have shown previously, was significantly toxic for monocytes after 20 hours (Chapter 2). This suggested TTO contains components that can activate NADPH oxidase and therefore production of ODRS in phagocytic leukocytes, and enhance subsequent responses to other agonists, but may be responsible for the toxicity evident during longer exposure. However, it is likely that neutrophils and monocytes would not be exposed to all TTO components *in vivo*. The *stratum corneum* may act as a selective barrier allowing penetration of the water-soluble components into the vascularised dermis but differentially retaining the hydrophobic components. The selective effect on monocyte but not neutrophil superoxide production implies TTO components do not scavenge oxygen radicals.

In our study 1,8-cineole was inactive, demonstrating no significant effect on cell viability or agonist-stimulated superoxide production at the concentrations tested (data summarized in Table 3.2). Despite contrary reports [27,28], 1,8-cineole was also unable to regulate LPS-stimulated inflammatory mediator production by monocytes (Chapter 2). However, another study has demonstrated that 1,8-cineole possesses local irritant properties, inducing oedema following subplantar injection into the hind paw of rats and causing rat peritoneal mast cell degranulation and release of histamine and serotonin *in vitro* [35]. 1,8-Cineole and other components of TTO including limonene, terpinen-4-ol and α -terpineol [24-26], demonstrate percutaneous penetration enhancing properties. As such, they may increase penetration of potential anti-inflammatory components such as terpinen-4-ol and α -terpineol, beyond the *stratum corneum* into the vascularised dermis resulting in levels that would be biologically significant following topical application. Nevertheless, studies measuring the actual concentration of TTO components penetrating into the skin and their effect on inflammatory cells and mediators *in vivo* are required. Typical protocols for usage of TTO include application of a preparation of up to 100% to skin. In this study we used the highest concentration of TTO that was not toxic to mammalian cells *in vitro*. It represented approximately one thousandth of the recommended dosage and is a level less than the water-solubility of TTO (1.6 g/l).

The physiological relevance of this study is high as it implies TTO has potential as an anti-inflammatory agent. The results suggest TTO contains water-soluble components, specifically terpinen-4-ol and α -terpineol, that may selectively regulate cell function during inflammation, in particular monocyte activity, and following topical application may control inflammatory responses to foreign antigens in the skin. TTO may enable neutrophils to be fully active in an acute inflammatory response and eliminate foreign antigens, while suppressing monocyte superoxide production and thereby preventing oxidative tissue damage that may be seen in more chronic inflammatory states.

The anti-microbial activity of TTO is already well established [8-16]. If its potential anti-inflammatory properties (including prevention of oxidative tissue damage and inhibition of inflammatory mediator production) can be further elucidated, acceptability of TTO for treatment of acne, eczema, burns and periodontal disease will be increased.

Table 3.2. Effect of the water-soluble components of TTO on superoxide production by agonist-stimulated monocytes

| | Water-soluble TTO fraction (0.125% v/v) | | Terpinen-4-ol (0.052% v/v) | | α -Terpineol (0.004% v/v) | | 1,8-Cineole (0.0025% v/v) | |
|--------|--|-----|-------------------------------|-----|-------------------------------------|-----|------------------------------|-----|
| fMLP + | 39% ↓ | (5) | 50% ↓ | (5) | 42% ↓ | (3) | - | (5) |
| LPS + | 42% ↓ | (4) | 58% ↓ | (4) | 50% ↓ | (3) | - | (4) |
| PMA + | 37% ↓ | (4) | - | (4) | 29% ↓ | (5) | - | (4) |

- = no significant effect ($P > 0.05$)

(n) = number of donors

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