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**Rural Industries Research and
Development Corporation**

Antiviral Activity of Tea Tree Oil

In vitro and in vivo

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

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Foreword

While the production of tea tree oil has grown considerably in Australia in the last decade, there has not been a comparable increase in worldwide demand for the oil. Tea tree oil is sold on the strength of its medicinal properties, not all of which are documented scientifically. For many years tea tree oil was grandfathered into markets on the basis of its long history of safe use. In a growing number of markets, the regulatory environment is such that existing data are no longer considered adequate.

Scientific evidence proving the claims made for tea tree oil is now an absolute requirement.

This publication details evidence for the antiviral activity of tea tree oil and explores how this activity may be exploited. It also provides preliminary evidence that tea tree oil products may be efficacious in the treatment of cold sores.

This project was funded from tea tree oil industry revenue (provided by Australian Bodycare Continental A/S) which was matched by funds provided by the Federal Government.

This report, a new addition to RIRDC's diverse range of over 1200 research publications, forms part of our Tea Tree Oil R&D program, which aims to support the continued development of an environmentally sustainable and profitable Australian tea tree oil industry that has established international leadership in marketing, in value-adding, and in product reliability and production.

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Peter O'Brien

Managing Director

Rural Industries Research and Development Corporation

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Abbreviations

DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
g	gravitational force
GM	growth medium
HF 32	human fibroblast, type 32
HSV 1	herpes simplex virus type 1
HSV 2	herpes simplex virus type 2
MEM	modified Eagle's medium
MEM-PR	modified Eagle's medium without phenol red indicator
µl	microlitres
MM	maintenance medium
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide
n	number
NCP	never culture positive
OM	overlay medium
PCR	polymerase chain reaction
pfu/ml	plaque forming units per millilitre
RHL	recurrent herpes labialis (cold sores)
TTO	tea tree oil
v/v	volume/volume

Contents

Foreword	iii
Acknowledgements	iv
Abbreviations	iv
Executive summary	vii
1 Introduction	1
2 Objectives	3
3 In vitro methods	3
3.1 Human fibroblast cell line	3
3.2 Herpes simplex virus (HSV)	4
3.3 Tea tree oil.....	4
3.4 Assays.....	5
3.5 Antiviral activity testing.....	8
4 Results and discussion of in vitro studies	10
4.1 Cytotoxicity testing	10
4.2 Antiviral activity	18
4.3 Discussion	25
5 In vivo application of tea tree oil	28
5.1 Introduction	28
5.2 Methods	30
5.3 Results	33
5.4 Discussion	37
6 Implications	39
7 Recommendations	40
8 References	41

Executive summary

Tea tree oil (TTO) is a popular ingredient in many cosmetic and medicinal products in Australia. Few regulatory dictates apply to ingredients for cosmetic products in Australia except that they must be safe. Since TTO is considered safe in Australia and enjoys enormous popularity with the general population, it has been incorporated into a vast array of cosmetic, non-therapeutic products. In contrast, regulatory requirements for medicinal products dictate that in addition to being safe, therapeutic claims must be supported by evidence. The level of evidence required has increased over the last few decades and still varies depending on the claims being made. In recent years, the TTO industry has endeavoured to continue making therapeutic claims for TTO that were acceptable in the past and in some cases, to make greater claims. In the contemporary national and international regulatory environment, only claims that are supported by scientific data are sustainable.

Substantial progress has been made with regard to the antibacterial and antifungal properties of TTO. Significant characterisation of the spectrum, degree and mechanism of antibacterial and antifungal activity has been conducted and reported in the scientific literature. These data have formed the basis of preliminary clinical studies with several reporting promising results. In contrast, there have been very little data available on the antiviral activity of TTO. The work described here is the first attempt to address this deficiency and shows that TTO has significant antiviral activity against herpes simplex viruses. This antiviral activity appears to be largely confined to a direct effect on the extracellular virus particle, prior to penetration into a host cell. No significant antiviral activity could be detected once virus had entered cells or after pre-exposing cells to TTO prior to infection. Several of the components of TTO were tested and shown to have antiviral activity. Terpinen-4-ol, α -terpineol and 1, 8-cineole appeared slightly more active *in vitro* than whole oil, although the differences may not be clinically significant. ρ -Cymene and α - and γ -terpinene did not exhibit significant antiviral activity in this model, however, but the possibility that these and other components may contribute to the overall activity of the oil should not be disregarded.

Proof of the *in vitro* antiviral activity of TTO opens up the possibility that TTO may be a potentially useful topical treatment for cutaneous viral infections such as cold sores, caused

by HSV. Since there was no data on this subject, a preliminary investigation was undertaken in the form of a pilot study. Twenty patients suffering from an episode of cold sores, also known as recurrent herpes labialis, were recruited and randomised to receive 6% tea tree oil ointment or placebo (no active ingredient) ointment. Patients' cold sores were assessed daily and a swab of the cold sore was also taken daily. The presence or absence of herpes simplex virus in the cold sore was determined by two methods. These were culture (to detect viable virus) and polymerase chain reaction (PCR) to detect viral DNA.

One patient in the TTO group was withdrawn from the study due to an adverse reaction to the ointment. No herpes virus was detected in specimens from the site of this patient's suspected lesion and no cold sore developed. Another patient, randomised to the placebo group, failed to attend any visits after the first one. These two patients were excluded from the analysis. Results from the remaining 18 patients were analysed. For the nine patients that received the TTO ointment, the median number of days taken for the cold sore to heal completely was 9. For the nine patients that received the placebo ointment, the median number of days to healing was 12.5. The median number of days of culture positivity in the TTO group was 3 while in the placebo group it was 4. However, the median number of days on which viral DNA could be detected by the PCR test was 6 in both groups. The median number of days to crust was also the same in both treatment groups and was 4. Of the two methods used to detect herpes virus, the PCR test detected virus for 3-4 days longer than the culture test. However, the PCR test only detects virus in terms of presence/absence. The culture method allows the number of virus particles present to be estimated. Results from this pilot study suggest that the number of virus particles present in cold sores treated with TTO ointment is lower than in those treated with the placebo ointment. All of these outcomes suggest that TTO ointment speeds the healing of cold sores although none of them was statistically significant. The results of this pilot study have been published in the *Journal of Antimicrobial Chemotherapy* (48:450-451).

TTO has promise as a topical antiviral treatment and further work should be conducted to determine whether or not it is an effective treatment for cold sores.

1 Introduction

The antimicrobial activity of tea tree oil (TTO), the essential oil distilled from the Australian plant *Melaleuca alternifolia*, has been clearly established by a number of Australian (Southwell *et al.*, 1993; Carson & Riley, 1994; Carson *et al.*, 1995a, 1995b; Carson *et al.*, 1996; Hammer *et al.*, 1996; Gustafson *et al.*, 1998; Hammer *et al.*, 1999) and international (Ånséhn, 1990; Raman *et al.*, 1995; Harkenthal *et al.*, 2000; Banes-Marshall *et al.*, 2001; Christoph *et al.*, 2001) studies. Most of this work has focussed on documenting the in vitro antibacterial and antifungal properties of the oil in the form of susceptibility data. The results have generated much interest in a variety of therapeutic and disinfectant applications for the oil although little in vivo work has been reported. Apart from extending existing claims about the antimicrobial activity of the oil, these susceptibility data are necessary to justify proceeding to in vivo work. As these data have become available, in vivo work on fungal and bacterial conditions has commenced and some favourable results have been observed (Jandourek *et al.*, 1998; Caelli *et al.*, 2000). In contrast, there has been little in vitro or in vivo exploration of the antiviral properties of TTO despite anecdotal evidence that it is useful in the topical treatment of some viral infections such as recurrent herpes labialis (RHL), also known as cold sores. Preliminary work done at this University (Love, 1996) suggested that the oil may have potentially useful activity against herpes simplex viruses (HSV). In order to be able to explore the in vivo efficacy of TTO in the treatment of HSV infections, additional in vitro data characterising the degree and extent of TTO's antiviral activity were required. Apart from supporting the case to conduct in vivo work, these data also have implications for the type of products developed and the range of suitable indications.

A critically important element of the success of TTO in recent years has been the scientific substantiation of claims regarding its antiseptic and disinfectant capacity. Scientifically valid data demonstrating the ability of TTO to inhibit a wide range of bacteria and fungi have increased its acceptability as a naturally-occurring antimicrobial agent, both nationally and internationally. Demonstrating that TTO has in vitro activity against viruses such as HSV is the first step in validating its use in treating viral infections. Confirmation of the antiviral properties of TTO also has implications for its use in disinfectants and antiseptics.

This study sought to investigate the antiviral activity of TTO *in vitro*, its mode of action and the components involved in this action, and to explore its possible applications *in vivo*. In order to achieve this, it was necessary to evaluate the effect TTO and its components have on the tissue culture test systems used to determine antiviral activity and to modify these to accommodate the oil. HSV was selected as the test virus since there are established techniques for studying this virus *in vitro* and because HSV is a common cause of viral infections in humans. In the most common form, these viruses cause latent infections in the central nervous system resulting in recurrent oro-facial and genital lesions. The two common types of HSV, designated type 1 (HSV 1) and type 2 (HSV 2), have biochemical and serological differences. HSV are obvious potential targets for TTO therapy. HSV 1 is carried in 50-90% of adults, and HSV 2 by 20-60%. Both viruses can cause regular outbreaks on the body surface; HSV 1 usually manifests as cold sores on the lips whilst HSV 2 commonly causes genital lesions. Genital herpes is one of the most common sexually transmitted diseases in the western world. While the exact incidence of attacks is not known, many cases are diagnosed each year in Australia. Cold sores are also extremely common, with 20-40% of the population experiencing recurrent outbreaks (Spruance *et al.*, 1977). Currently there is no treatment which will eliminate these viruses. Drug treatment is largely limited to topical or systemic antiviral drugs. The latter are very effective at suppressing attacks if taken every day but are very expensive. Therapy confined to acute attacks is much less effective and still relatively expensive. There is a clear need for a non-toxic, effective and inexpensive topical agent for herpes infections, particularly for cold sores. None of the current therapies fulfil these criteria. Consequently, the *in vitro* susceptibility of viruses that cause infections which may be treated topically was of interest and HSV was chosen as a model system for examining the antiviral activity of TTO. The potential of TTO as an antibacterial and antifungal skin wash has already been shown to be significant. The addition of viruses to the spectrum of antimicrobial activity will considerably enhance the profile of this emerging product.

2 Objectives

The aim of this project was to develop and validate methods suitable for testing the antiviral activity of TTO *in vitro* and to use these methods to assess its antiviral activity. The second aim of this project was to examine TTO's potential as a topical antiviral therapeutic agent. The outcomes are the development and validation of these methods and the assessment of the suitability of TTO as a topical antiviral agent.

3 In vitro methods

3.1 Human fibroblast cell line

A cell line of human fibroblasts (HF 32) was used for the culturing and susceptibility testing all isolates of HSV. The HF 32 cells were obtained from the Virology section of PathCentre at passage 15 and stored at -120°C over liquid nitrogen in 1 ml aliquots in cell preserving medium (10% DMSO in Hanks balanced salt solution with 15% foetal calf serum) at a concentration of approximately 10^6 cells per ml.

Passaging of tissue culture cells

When required, HF 32 cells were defrosted and immediately diluted into 25 ml of pre-warmed growth medium. The growth medium (GM) was modified Eagle's medium (MEM) supplemented with foetal calf serum (10% v/v), glutamine and antibiotics (vancomycin and streptomycin). Cells and medium were seeded into a tissue culture flask (75 cm²) and incubated at 37°C. The HF 32 cells were propagated by division once confluent monolayers had formed (approximately 3-4 days). The medium was poured from the flask and the monolayer was rinsed with 2 ml Hanks balanced salt solution for 10 seconds. To detach the cells from the plastic flask, the cells were covered with 2 ml working trypsin solution for 2 minutes. The trypsin was removed and the flasks were incubated for 4 minutes at 37°C. Cells were gently washed from the flask surface and suspended in 70 ml GM. This cell suspension was used to seed monolayer cell cultures in a variety of tissue culture vessels. Culture flasks were seeded with 25 ml of cell suspension, the 24-well trays (Falcon, USA) used in most

assays were seeded with 1.5 ml of cell suspension per well and the 96-well trays used in the viability assays received 300 µl of cell suspension per well. The trays were incubated at 35°C in 2.5% CO₂ for 48 - 60 hours, until early confluence was reached. Flasks were incubated for 3 - 4 days at 37°C. Monolayers that had reached confluence were maintained in maintenance medium (MM) made up of MEM supplemented with foetal calf serum (2% v/v), glutamine and antibiotics (vancomycin and streptomycin). Cell cultures were discarded once the cell growth slowed, usually after passage 30.

3.2 Herpes simplex virus (HSV)

HSV isolates

Clinical isolates of HSV 1 and HSV 2 were obtained from the Virology section of the Division of Microbiology and Infectious Diseases at The Western Australian Centre for Pathology and Medical Research (PathCentre). Additional HSV 1 isolates (n=14) were collected from patients recruited into the cold sore pilot study described in section 5 of this report.

Propagation and harvesting of HSV isolates

HF 32 cells were grown to confluence in tissue culture flasks (75 cm² surface area) with GM after which virus was inoculated onto the cell monolayers and the flasks incubated at 37°C until about 75% cytopathic effect was evident on the monolayer (4-6 days). The cells and the growth medium were removed from the flask, sonicated to release intracellular virus and centrifuged at 1500 × g for 20 minutes to sediment cell debris in the pellet. The pellet was discarded and the supernatant containing the virus was ultra-centrifuged at 32000 × g at 4°C for 2 hours to concentrate the virus in a final volume of 5-10 ml of media. Virus concentrate was stored at -120°C over liquid nitrogen in 0.5 ml aliquots until required.

3.3 Tea tree oil

TTO is a complex mixture of over 100 components (Brophy *et al.*, 1989). The TTO used in this study (Australian Plantations batch 97/1) was analysed by gas chromatography-mass spectrometry at the Wollongbar Agricultural Institute, Wollongbar, NSW, Australia and met

the requirements of the international standard for TTO (International Organisation for Standardisation, 1996). The major components are given in Table 1. When dilutions of TTO were prepared, TTO was always measured volume/volume (v/v).

Table 1 Ten major components of tea tree oil, batch number 97/1.

Component	Percentage
terpinen-4-ol	41.5
γ -terpinene	21.2
α -terpinene	10.2
terpinolene	3.5
α -terpineol	2.9
α -pinene	2.5
1,8-cineole	2.1
ρ -cymene	1.5
aromadendrene	1.0
δ -cadinene	1.0

3.4 Assays

Viability assays to determine cytotoxicity

There are a variety of methods for evaluating cytotoxicity of substances to cell lines. Cell counting methods such as trypan blue exclusion by viable cells are considered the standard (Hayashi *et al.*, 1997) although there are some cell lines, including the fibroblasts used in this work, which are difficult to assess with these methods. The method chosen in this study was a tetrazolium based assay. It does not involve the time consuming cell counting step and is well documented as an excellent predictor of cell viability (Mosmann, 1983; Denizot & Lang, 1986). The method is based on the transformation of a compound to a different coloured product by metabolically active cells (Slater *et al.*, 1963). The compound used in this method was 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), a yellow crystalline tetrazolium salt, which is reduced into a blue formazan product by mitochondrial dehydrogenases in metabolically active cells (Slater *et al.*, 1963). The degree of colour change correlates well with the degree of cellular metabolic activity and is taken as an estimation of

cell viability. Modifications to this assay introduced by Denizot and Lang (1986) improved its overall accuracy and effectiveness.

HF 32 cell monolayers were grown to confluence (2-3 days) in 96-well flat-bottomed trays. Dilutions of TTO and the components to be tested were prepared in MM. All oil compounds were tested at a range of concentrations from 0.000001% to 0.2%. The compounds tested were terpinen-4-ol, α -terpineol, 1, 8-cineole, ρ -cymene, α -terpinene and γ -terpinene. The dehydrogenase activity of cells was determined after 1 hour and 48 hours exposure to the test compounds.

A 300 μ l volume of the test compound was placed in each of 4-6 wells containing confluent HF monolayers. The tray was incubated at 37°C in 2.5% CO₂ for the desired testing time (1 hour or 48 hours). For the 1 hour assay, the medium was tipped from the wells at 1 hour and replaced by 300 μ l MM. The trays were incubated for 48 hours in 2.5% CO₂. The metabolic activity of the cells was then estimated. For the 48 hour assay, test compounds were left in contact with the HF 32 monolayer for 48 hours.

MTT (Sigma) was prepared in MEM minus phenol red indicator (MEM-PR) to a concentration of 1 mg/ml. GM was removed from the confluent cells by tipping, flicking and blotting the tray. Each well received 150 μ l of MTT in MEM-PR (1 mg/ml) and cells were incubated at 37°C for 3 hours. Medium was removed from cells by tipping, flicking and blotting the tray. Isopropanol (50 μ l) was placed into each well in order to dissolve the formazan product formed. The trays were incubated at room temperature for 10 minutes.

The tray was shaken well using the automated shaking function and read on a microplate spectrophotometer (SPECTRAmax 250) at wavelengths of 560 nm and 640 nm. The background spectrophotometric reading at 640 nm was subtracted from the reading at 560 nm to give the result for each well. Each well was read three times and the values averaged. Each dilution of each compound was tested in six wells. The results from all six wells were averaged and the assay was repeated at least once.

Eight wells containing untreated cells on each plate were kept as a control. Viability was expressed as the dehydrogenase activity (determined spectrophotometrically) in treated cells compared to that in untreated cells, giving a proportion of viability.

Plaque assays

Plaque reduction assays are considered the standard technique for assessing the *in vitro* antiviral activity of compounds (Swierkosz *et al.*, 1995). Normal replication of a virus on a host monolayer results in the formation of plaques, or areas of damage to the host cell monolayer. Plaque reduction assays are based on the principle that antiviral agents present in the medium will inhibit the formation of viral plaques. The degree to which plaque formation is inhibited is taken as an indication of the antiviral activity of the compound. Varying the times at which the compound is added to the assay can indicate at what stage the compound exerts its antiviral activity.

In this method, confluent monolayers of HF 32 cells in each well of 24-well trays were inoculated with approximately 100 plaque forming units (pfu) of HSV diluted in MM to a final volume of 1 ml. The trays were centrifuged at $2000 \times g$ at 37°C for 1 hour. The excess medium containing virus that had not adsorbed or penetrated the monolayer was tipped from the wells, and replaced with 1.5 ml of overlay medium (OM, MM supplemented with 5% carboxymethylcellulose) containing serial 10-fold dilutions of TTO over the range 10^{-1} - 10^{-5} %. The cells were incubated for 2 days at 35°C in 2.5% CO_2 to allow viral plaques to develop.

The viral plaques were then stained using the immunostaining method described by Pietroboni *et al.* (1989). Briefly, OM was poured from the wells and each well was washed twice with 1 ml phosphate buffered saline. The cells were fixed by immersing them in 2 ml 85% acetone in deionised water and incubating at -15°C for 25-30 minutes. The acetone was removed and the monolayers were air-dried. 300 μl mouse monoclonal antibody to HSV in rabbit serum (PathCentre) was placed into each well. The trays were incubated at 37°C for 1 hour. The antibody was tipped off and the cells washed twice with 1 ml phosphate buffered saline. 300 μl goat-anti-mouse Ig(H+L)- β -galactosidase conjugate solution (made up at 0.1% in phosphate buffered saline) was placed into each well. The trays were incubated at 37°C for 1 hour. The medium was tipped off and the cells washed twice with 1 ml phosphate buffered saline. 300 μl of freshly prepared 6-bromo- α -naphthyl- β -D-galactopyranoside/Fast Garnet substrate was placed into each well. Staining of plaques was achieved by incubating the trays at room temperature for 30 minutes. Excess stain was poured from the wells, which were then

filled with water to improve microscope lighting optics. Plaques stained in this manner were counted at $40\times$ magnification under an inverted microscope. All resultant values were converted to pfu/ml as an average of at least 4 wells, and all assays were repeated at least once.

3.5 Antiviral activity testing

A variety of assays was used to determine the antiviral activity of TTO and its components against HSV.

Activity of tea tree oil against a range of HSV 1 clinical isolates

Clinical isolates of HSV (n=14) were diluted in MM to a concentration of approximately 10^6 pfu/ml. 100 μ l of virus was incubated with TTO diluted in MM to a final concentration of 2% for 30 minutes at room temperature. Samples of 100 μ l were removed and serially diluted 10-fold over the range 10^{-2} – 10^{-6} in MM. Volumes of 1 ml of each dilution were placed into each of 4 wells of a 24-well plate containing a confluent layer of HF 32 cells (2 day growth). The plates were incubated for 48 hours. The titre of viable virus remaining after treatment with TTO was measured by plaque assays. This experiment was run twice for each isolate.

Activity of individual components of tea tree oil against HSV 1

The antiviral activity of the following components against HSV 1 was examined: terpinen-4-ol, α -terpineol, 1,8-cineole, ρ -cymene, α -terpinene and γ -terpinene. Since these components exhibited cytotoxicity in the conventional test system, their activity against extracellular virus particles was evaluated.

100 μ l of HSV 1 (PathCentre clinical isolate) at a concentration of approximately 10^6 pfu/ml, was incubated in a treatment vial with each of six of the major components of TTO at a final concentration of 2% in MM, at room temperature for 30 minutes. Samples of 100 μ l of the treated virus were then serially diluted 10-fold in MM over the range 10^{-2} – 10^{-6} . 1 ml of each dilution was placed in each of 4 wells of a 24-well plate containing confluent HF 32 cells. The titre of viable virus after this treatment was ascertained by plaque assays. The experiment was run twice for each of these six components of TTO.

Time-kill tests

As with the components of TTO, efforts were made to characterise the effect of TTO on extracellular viruses. These experiments were designed to ascertain the length of time taken to achieve a reduction in viral titre at a variety of TTO concentrations.

200µl of the PathCentre laboratory isolate of HSV 1 was incubated in a treatment vial with TTO diluted in MM to final concentrations of 2%, 4% and 6%, respectively. After 30 minutes, 60 minutes and 120 minutes, a 100 µl sample was removed from each treatment vial and serially diluted to concentrations of 10^{-2} – 10^{-6} . A 1 ml volume of each dilution was inoculated onto each of 4 wells of a 24-well tray, grown with a confluent layer of HF 32 cells, and the titre of viable virus was obtained by plaque assay. This experiment was run at least 2-3 times at each dilution of TTO.

Antiviral activity staging assays

Staging assays are designed to ascertain the stage of the viral replicative cycle at which the antiviral compound is most active. The stage of infection at which an agent exerts its effects can provide some clues as to the mechanism of action of the compound. In these experiments TTO was added at different stages of virus infection and the antiviral activity assessed using plaque assays.

TTO was diluted in MM to final concentrations of 1%, 0.1%, 0.01%, 0.001%, 0.0001% and 0.00001%. TTO dilutions were incubated with the HF 32 monolayer and then removed prior to inoculation (stage 1). They were mixed with virus for 30 minutes prior to inoculation onto cells (stage 2). They were added to the HF 32 cells at the same time as virus inoculation (stage 3) and then replaced with MM, or added to the OM after virus had been incubated with cells for 1 hour (stage 4). The effect of the addition of TTO at the various stages was ascertained by the degree to which plaque formation changed.

4 Results and discussion of in vitro studies

4.1 Cytotoxicity testing

TTO and six of its major components were tested for their cytotoxic effects on HF 32 cells at concentrations of between 0.00001% and 0.2% after 1 hour and 48 hour exposures. These results are shown graphically in Figures 1-7. At concentrations of 0.05% and above, TTO significantly reduced the metabolic activity of the HF 32 cells, suggesting that viability was being compromised. At concentrations in excess of 0.1%, cytotoxicity was microscopically visible with cell monolayers detaching from culture vessels. Of the components examined, α -terpineol and α -terpinene showed a spectrum of toxicity very similar to TTO with approximately 50% reductions in the activity of the dehydrogenase enzyme at 0.05% for cells exposed for 48 hours. At concentrations less than 0.1%, terpinen-4-ol did not significantly reduce dehydrogenase activity. 1, 8-cineole, ρ -cymene and γ -terpinene showed similar patterns of toxicity with significant reductions in dehydrogenase activity not apparent until 0.2%. For TTO, terpinen-4-ol and α -terpineol, 48 hour exposure was more cytotoxic than 1 hour. This temporal effect was not seen with 1, 8-cineole or ρ -cymene at the concentrations tested.

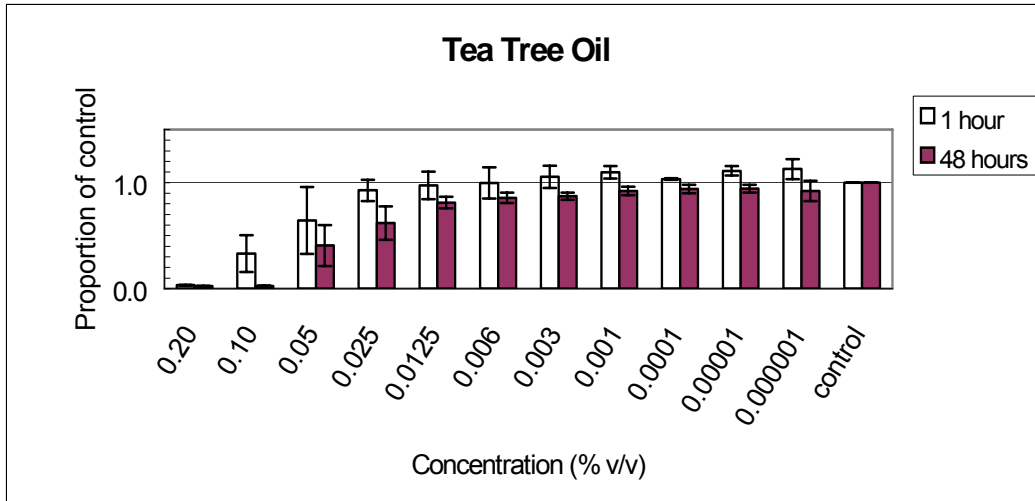


Figure 1. Viability of HF 32 cells after 1 hour and 48 hours exposure to tea tree oil.

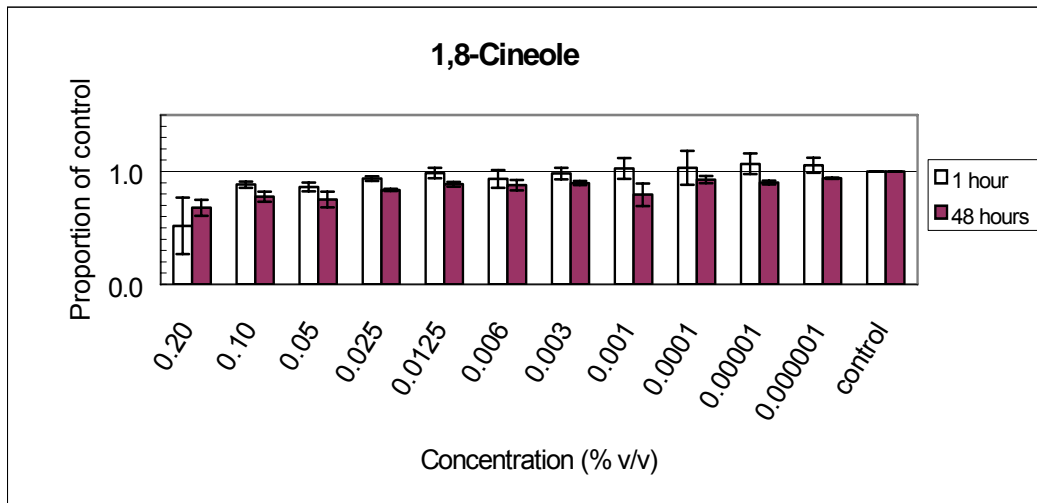


Figure 2. Viability of HF 32 cells after 1 hour and 48 hours exposure to 1, 8-cineole.

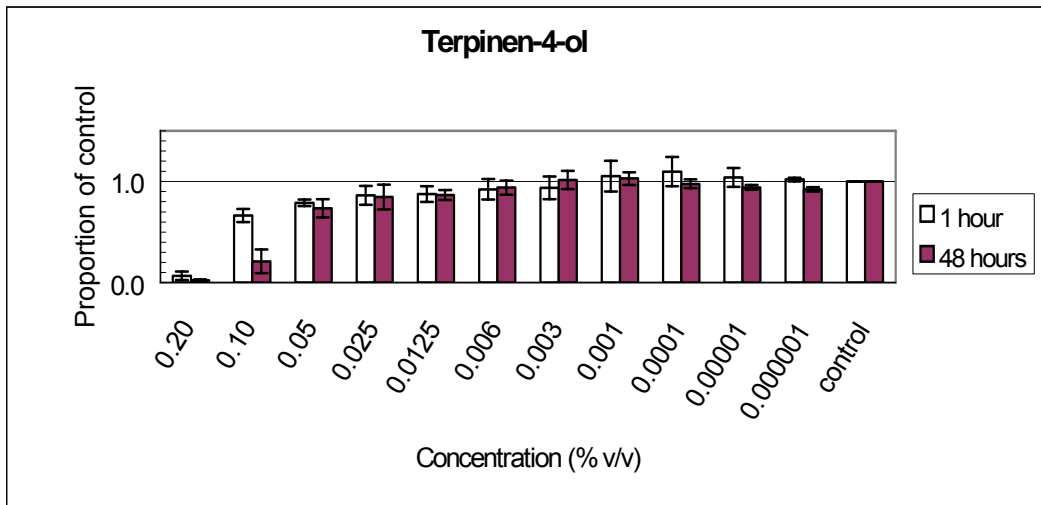


Figure 3. Viability of HF 32 cells after 1 hour and 48 hours exposure to terpinen-4-ol.

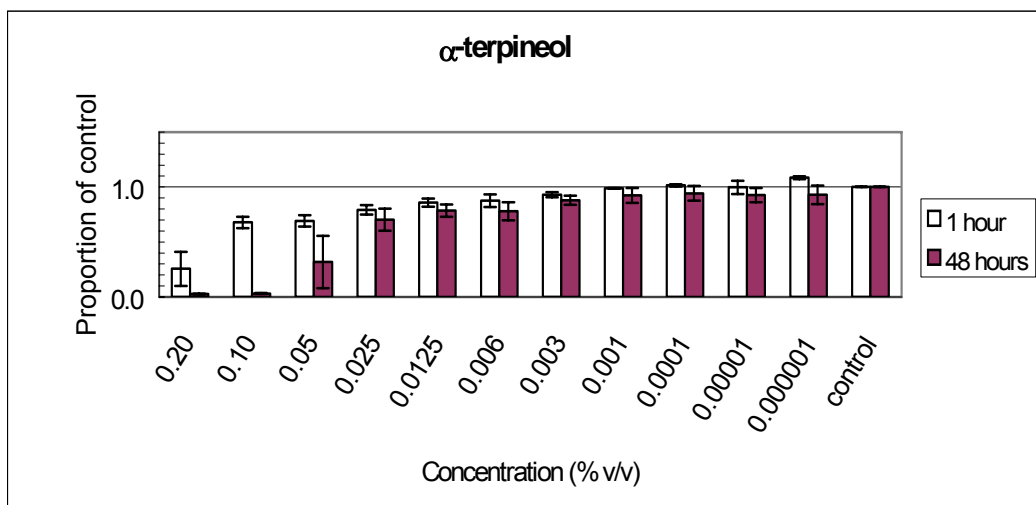


Figure 4. Viability of HF 32 cells after 1 hour and 48 hours exposure to α-terpineol.

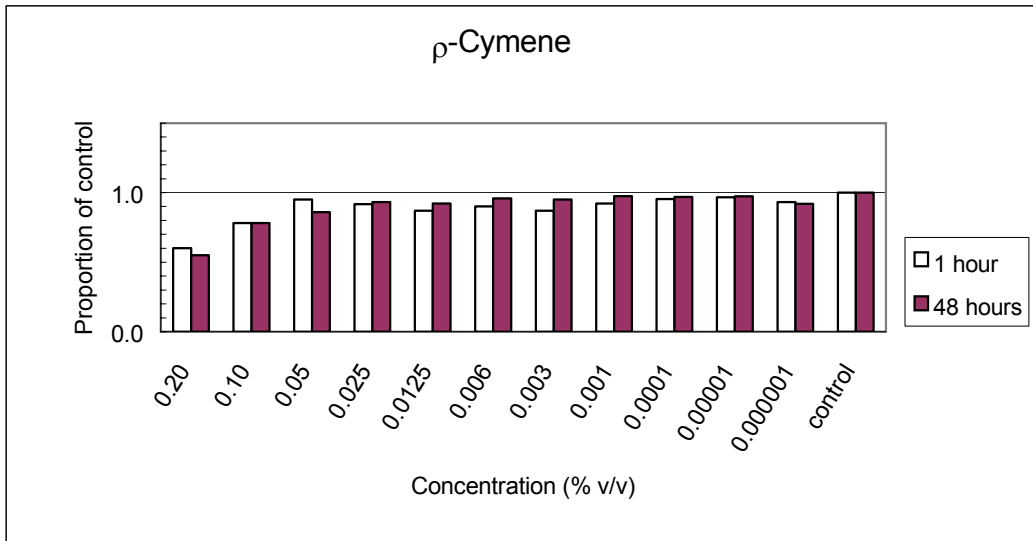


Figure 5. Viability of HF 32 cells after 1 hour and 48 hours exposure to p-cymene.

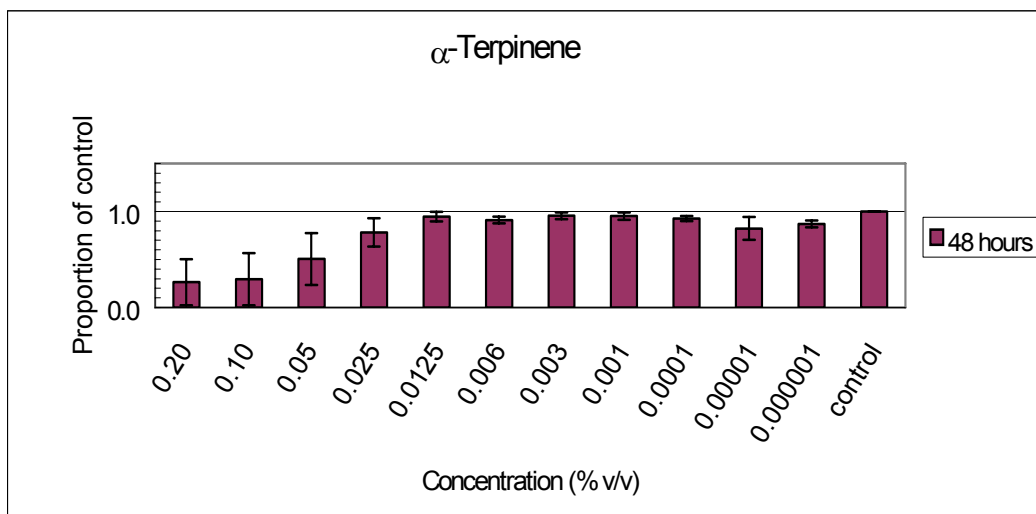


Figure 6. Viability of HF 32 cells after 48 hours exposure to α -terpinene.

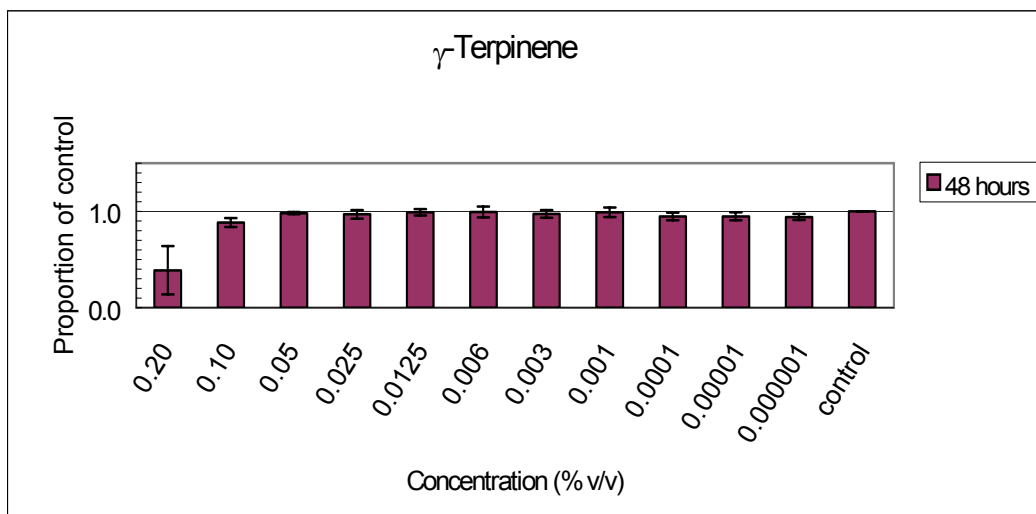


Figure 7. Viability of HF 32 cells after 48 hours exposure to γ -terpinene.

4.2 Antiviral activity

Stage of infection

When the HF 32 monolayer was exposed to TTO for 1 hour and the oil was removed prior to inoculation with HSV 1 (stage 1), there was no significant change in the number of plaques formed (Figure 8). Similarly, when HSV 1 and HF 32 cells were exposed to TTO during the adsorption stage (stage 3) (Figure 9), or when TTO was incorporated into the OM (stage 4) (Figure 11, grey bars), there was no reduction in the number of plaques formed. In contrast, when virus particles were pre-exposed to TTO prior to inoculation onto the cell monolayer (stage 2), there was a significant reduction in the number of plaques formed (Figure 10).

When virus was exposed to 0.1 % TTO for 1 h, there was a 50% reduction in the number of plaques formed. At a concentration of 1%, plaque formation was reduced to less than 0.01% of the control.

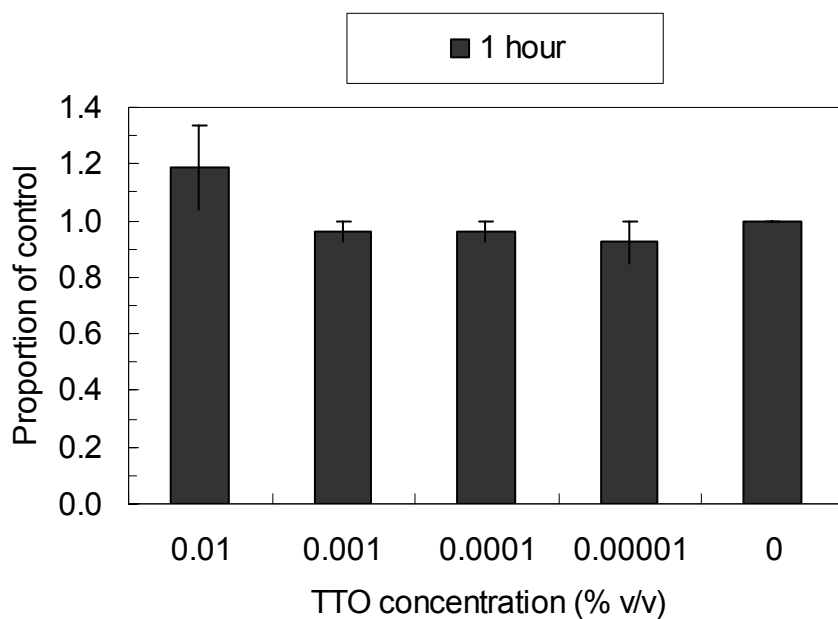


Figure 8. The effect on plaque formation of pre-exposing HF 32 cells to TTO prior to inoculation with HSV 1.

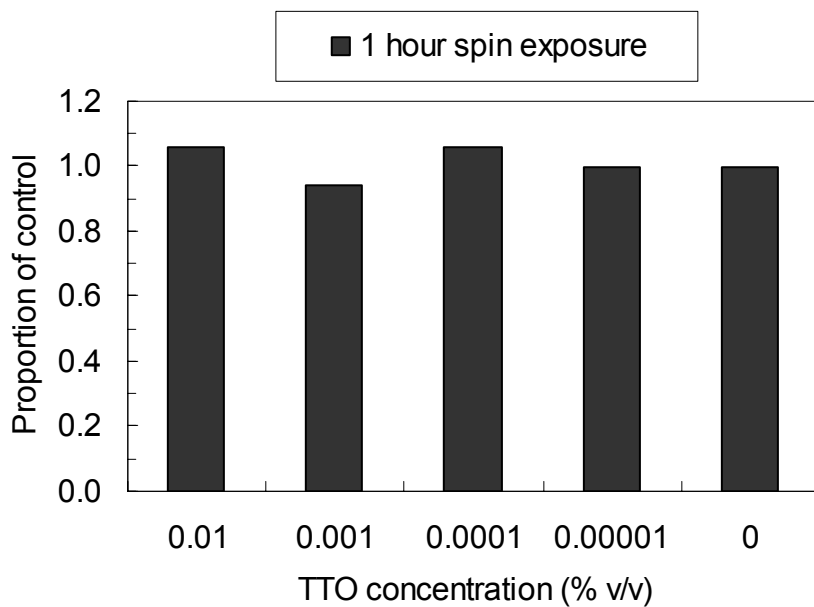


Figure 9. The effect on plaque formation of exposing HF 32 cells and HSV 1 to TTO during the adsorption phase.

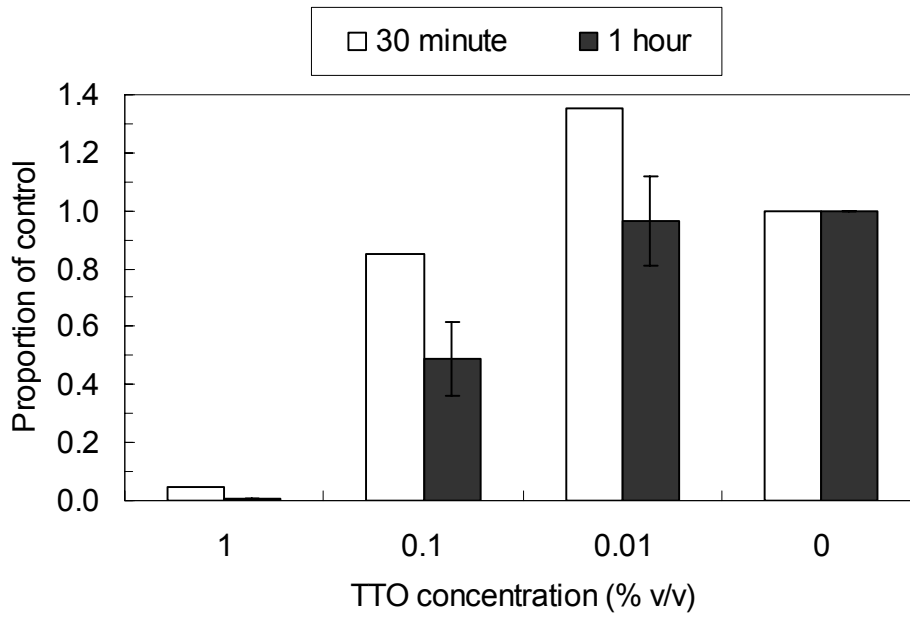


Figure 10. The effect on plaque formation of pre-exposing HSV 1 to TTO for 30 minutes or 1 hour prior to inoculation onto the HF 32 monolayer.

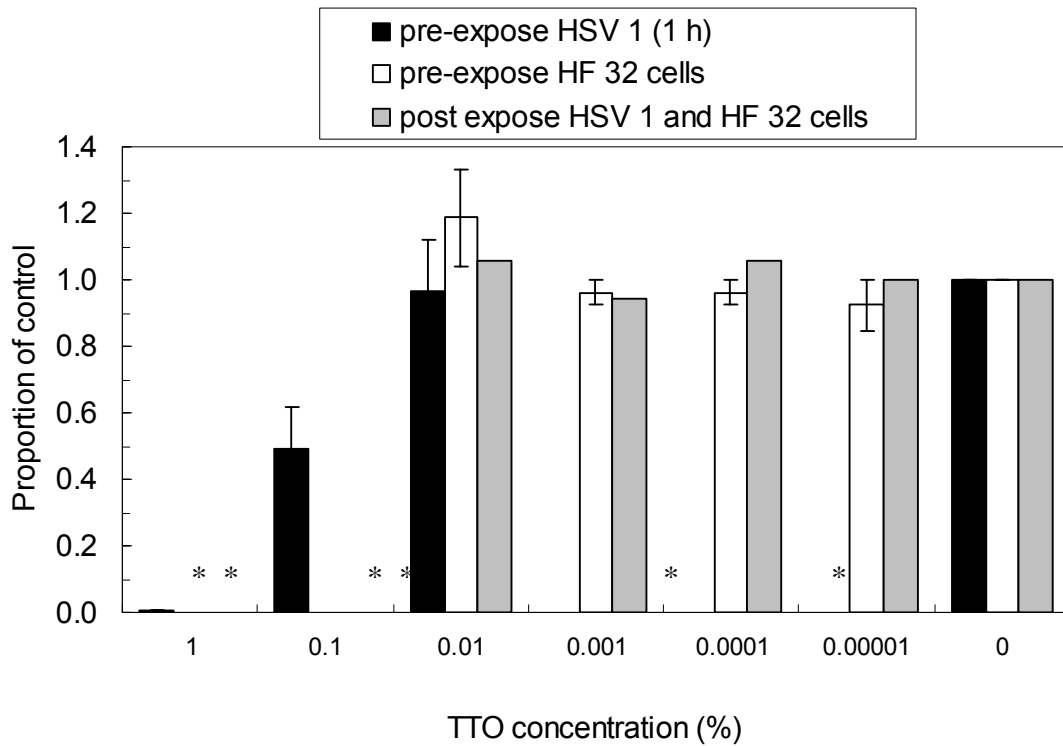


Figure 11. Comparative effects on plaque formation of exposing HSV 1, HF 32 cells or both to TTO at different stages.

* not done

Components of tea tree oil

Figure 12 show the results of experiments to assess the antiviral effect of TTO and six of its major components on HSV 1 virus. The degree of antiviral activity demonstrated by these components after HSV was exposed to 2% of the treatment agent for 30 minutes was: terpinen-4-ol = α -terpineol > 1, 8-cineole > TTO >> ρ -cymene > γ -terpinene > α -terpinene. Terpinen-4-ol and α -terpineol treatment reduced plaque formation to less than 0.0005%, the limit of detection. 1, 8-Cineole also showed a high degree of antiviral activity with virus inactivation exceeding 99.99%. Whole TTO showed a similar level of activity. ρ -Cymene was also active at this concentration with about 95% virus inactivation. The two least active components were γ -terpinene and α -terpinene which inactivated approximately 90% and 55% of the virus, respectively.

Concentration and time-kill tests

Results of the time-kill assays are shown graphically in Figure 13. At higher concentrations of TTO (4% and 6%), most of the antiviral activity occurred in the first 30 minutes of exposure to the oil. At 2%, there was almost 99% viral inactivation in the first 30 minutes, with a further 99% inactivation in the following 30 minutes. Between 60 and 120 minutes, there appeared to be a slower rate of inactivation, although this concentration of virus was nearing the detection limit of the assay which was 100 pfu/ml or approximately 0.0005% of the control.

Activity of tea tree oil against a variety of clinical isolates of HSV 1

The ability of 2% TTO to inactivate 14 clinical isolates of HSV 1 was assessed. The results of these tests are shown graphically in Figure 14. The survival of virus treated with TTO ranged between 0.001% and 0.45% indicating that TTO has activity against a wide variety of clinical isolates of HSV 1.

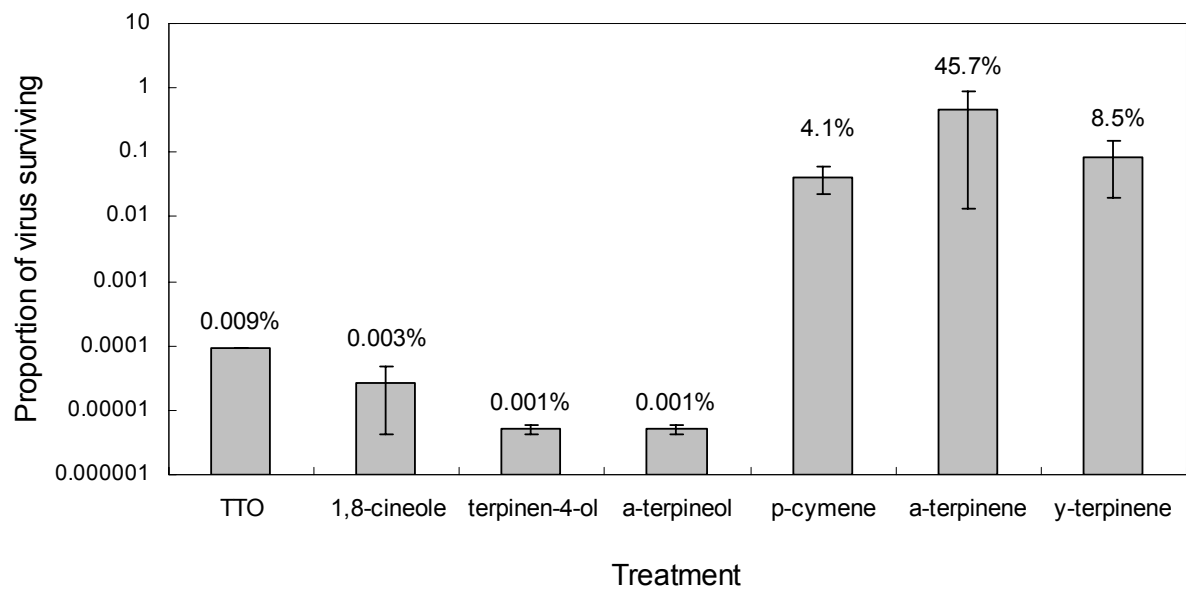


Figure 12. The effect of pre-treatment of HSV 1 with TTO or components (2% v/v) on plaque formation.

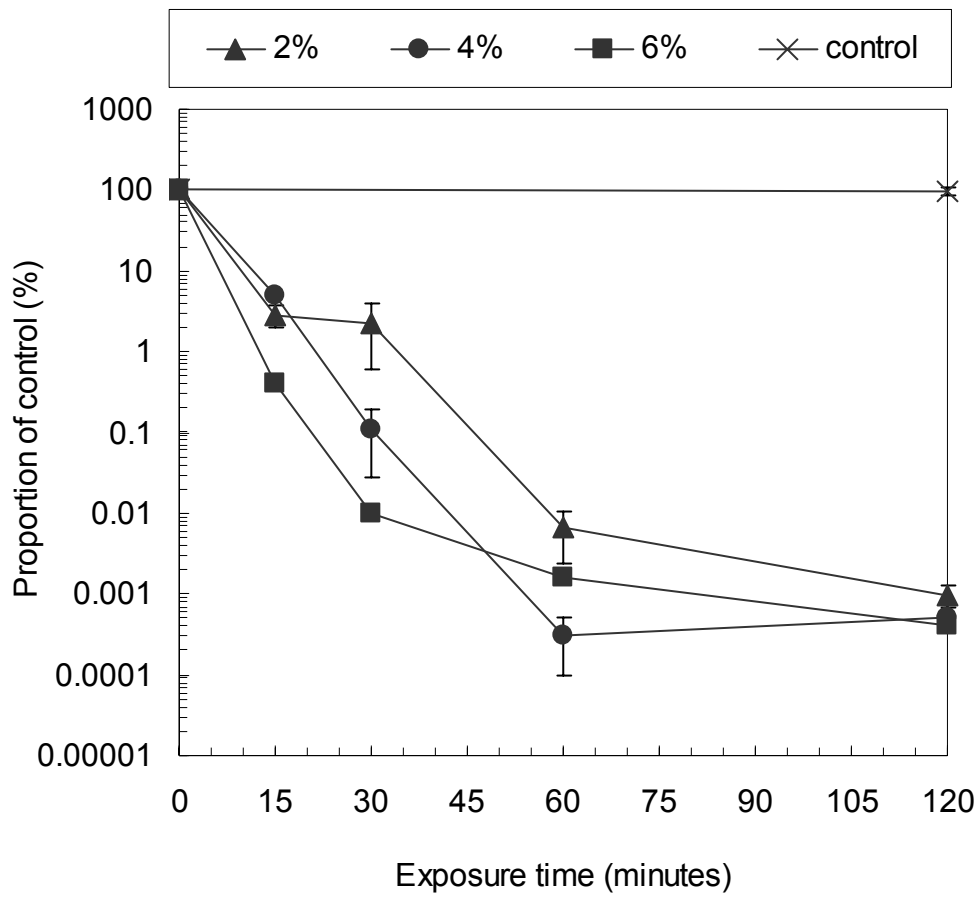


Figure 13. The effect of pre-treatment of HSV 1 with TTO at various concentrations.

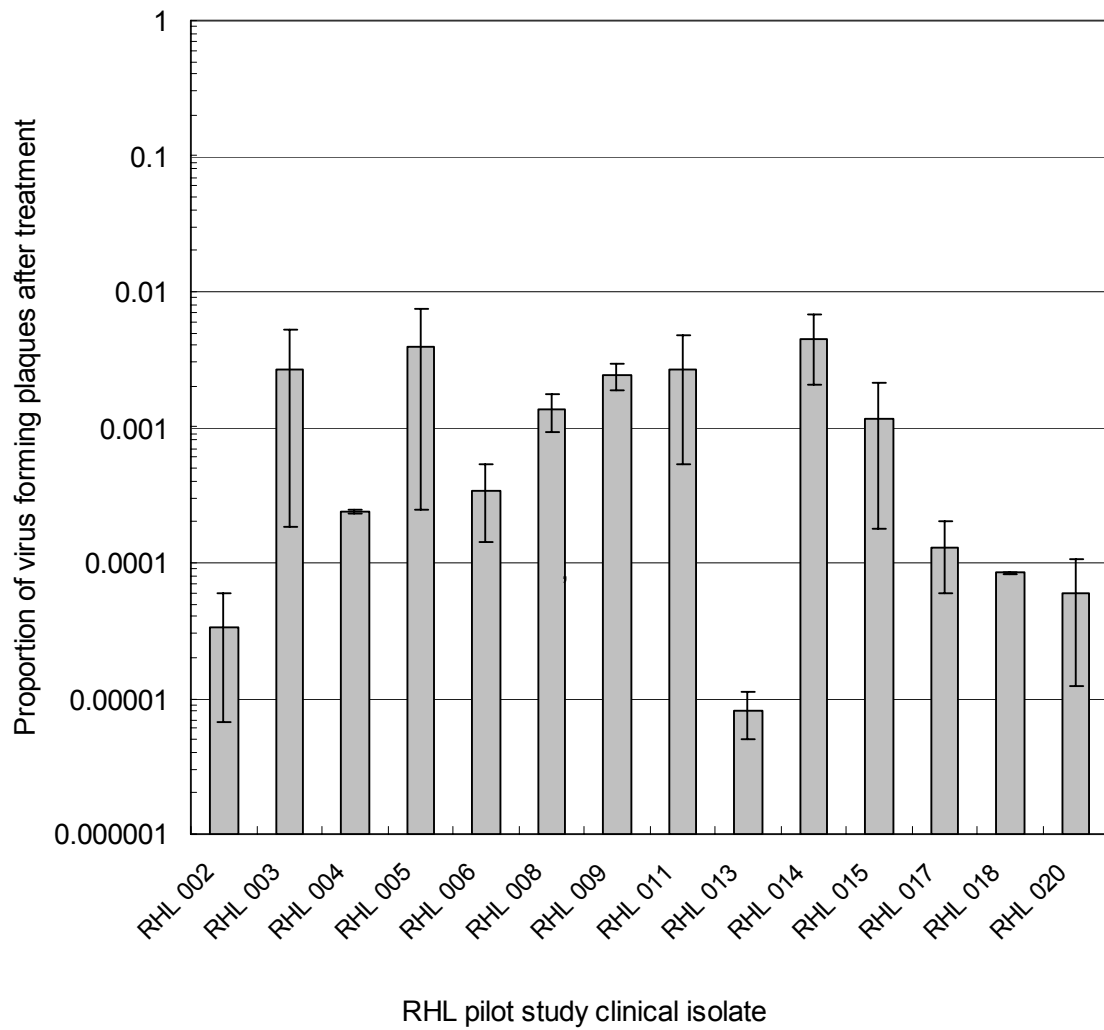


Figure 14. Proportion of HSV 1 able to form plaques after 30 minutes treatment with 2% TTO.

4.3 Discussion

Cytotoxicity of tea tree oil and components

TTO and all of the components tested showed at least some cytotoxicity in the test system. In each case, cytotoxicity was proportional to the test concentration and the time of exposure, with the exception of α - and γ -terpinene that were tested for 48 hours only. TTO was significantly cytotoxic at concentrations of 0.1% and greater, to the extent that cell monolayers were destroyed. Söderberg *et al.* (1996) previously reported that 600 g/ml of TTO (~0.06%) was sufficient to produce a 50% reduction in the growth of fibroblasts after 24 hours exposure. Although the assays measure different outcomes, this is consistent with the approximately 50% reduction in cellular dehydrogenase activity seen with 0.05% TTO for 48 hours. Hayes *et al.* (1997) used a range of human cell lines (but no fibroblasts) and a simplified version of the method used here. They found that concentrations of TTO over the range 0.02 - 2.8 g/l (~0.002 – 0.28%) reduced dehydrogenase activity by 50%. Different cell lines have different susceptibilities to toxic compounds and such a wide range is not unusual. The upper end of this range is the toxicity of TTO for HeLa cells, a cervical epithelial cancer cell line, and is comparable to our results. Of the cell lines examined in their work, these are the ones least different in character to fibroblasts. Hart *et al.* (2000) used a method similar to ours for estimating viability and found that 0.016% TTO effected a 69% reduction in viability. However, the test cells were freshly isolated human monocytes and very different from the fibroblasts used here, making comparison difficult. In addition, their tests were performed in glass vessels, as opposed to plastic, and this is known to influence the solubility of many TTO components (Hart *et al.*, 2000). Finally, other cytotoxicity work done as a prelude to in vitro antiviral testing found that 0.006% TTO reduced the growth of RC-37 cells by 50% (Schnitzler *et al.*, 2001).

When compared to the toxicity of the individual components, our results suggest that TTO is very similar in toxicity to α -terpineol. Of the six components tested, α -terpineol appeared to be the most toxic, followed by α -terpinene, terpinen-4-ol, γ -terpinene and then p -cymene and 1, 8-cineole that were very similar in their toxicity. This corresponds well with other work in which the descending toxicity was reported as α -terpineol > terpinen-4-ol > 1, 8-cineole (Hayes *et al.*, 1997). It is further supported by work which showed that eucalyptus oil was less toxic than TTO by a factor of 5 (Schnitzler *et al.*, 2001). The lesser degree of toxicity

demonstrated by 1, 8-cineole compared to whole oil and the other components is ironic considering the concerted effort made to reduce its level in TTO due to its false reputation as a skin irritant.

In vitro antiviral activity of tea tree oil and components

The results of this work show that TTO has in vitro antiviral activity. HSV 1 isolates collected during the pilot study were all susceptible to TTO indicating that it is active against a range of clinical isolates. Assays in which HSV 1 or the cell monolayer were exposed to TTO at different times, showed that TTO reduced plaque formation most significantly when virus was exposed to TTO prior to inoculation. TTO exposure during the adsorption stage or after penetration did not reduce plaque formation. Likewise, pre-exposure of the HF 32 monolayer was not protective. These results indicate that most of the antiviral action of TTO is exerted prior to attachment of virus to the cell.

Prior to this work, there was limited evidence suggesting that TTO has antiviral activity. Bishop *et al.* (1995) showed that tobacco plants inoculated with tobacco mosaic virus and sprayed with TTO developed less disease than control plants. In addition, anecdotal evidence from the use of TTO to treat cold sores suggested that the oil may have antiviral activity. This work confirms that TTO has significant antiviral activity. More recently, Schnitzler *et al.* (2001) reported that 1 hour room temperature exposure to TTO at 0.0009% and 0.0008% reduced HSV 1 and HSV 2 plaque formation by 50%. These concentrations are approximately 10-fold lower than those found to be effective here.

When the components of TTO were examined, 1, 8-cineole, terpinen-4-ol and α -terpineol exhibited equivalent or greater antiviral activity than whole oil. Since terpinen-4-ol comprises 30-40% of whole oil, it is likely that it is responsible for most of the antiviral activity of TTO. The equivalent activity of α -terpineol is interesting since TTO may contain up to 8% α -terpineol (International Organisation for Standardisation, 1996). However, it usually contains about 2-4% and it is unlikely that this component contributes significantly to antiviral activity overall unless it acts synergistically with other components. Given the complexity of TTO, it is important not to exclude the possibility of synergy between components enhancing the antiviral activity of TTO. Notably, two of the TTO components with considerable antiviral activity, terpinen-4-ol and α -terpineol, are also the components most responsible for

antibacterial and antifungal activity (Carson & Riley, 1995). The upper compositional limit for 1, 8-cineole in TTO is 15% and considerable effort has gone into reducing its level in TTO due to its erroneous reputation as a skin irritant. Although only a minor component of TTO, 1, 8-cineole is the main component of eucalyptus oil, often comprising in excess of 80% of the oil. Our data suggest that eucalyptus oil may have greater antiviral activity than TTO. However, previous work assessing the antiviral activity of both these oils found that eucalyptus oil had approximately 10-fold lower activity than TTO (Schnitzler *et al.*, 2001).

TTO is incorporated into products up to concentrations of approximately 20%. Many products contain <10% TTO and are used in the treatment of skin conditions. Exposure of HSV 1 to 2-6% TTO inactivated significant proportions of the virus with more than 99.99% inactivated after 60 minutes. If TTO is used to treat topical viral infections such as cold sores, contact time between product and virus is unlikely to be this long. However, even after 15 minutes, more than 90% of the virus was inactivated by 2-6% TTO indicating that topically applied TTO may be capable of inactivating significant amounts of HSV during typical use.

5 In vivo application of tea tree oil

5.1 Introduction

Recurrent herpes labialis (RHL), also known as cold sores, affects 20-40% of the population, some of whom develop frequent or extensive attacks (Spruance *et al.*, 1977; Higgins, 1993). Although this is a mild illness, RHL may cause considerable discomfort and cosmetic problems to the sufferer. The lesions are characteristic and usually accurately self diagnosed by patients (Lamey & Biagioni, 1996), making this an ideal indication for self-treatment should effective therapies be available. During the course of a recurrence, a number of clinical stages have been well described by Spruance *et al.* (1977). A prodrome characterized by pain, tingling and burning sensations is experienced by two out of three of those suffering from cold sores. Following this, the site becomes red and raised. A vesicle forms which then ulcerates. A soft crust forms followed by a hard crust. The crust is eventually lost and re-epithelialisation occurs. Complete healing (re-epithelialisation) occurs within 8-10 days. Spruance *et al.* (1977) proposed that these clinical stages may be useful in determining the antiviral efficacy of any treatments. In addition, they also suggest that duration of pain, lesion size and duration of viral shedding are important measures of antiviral effect. Recent studies evaluating antiviral agents propose that the clinical stages which are most indicative of antiviral efficacy are the time to loss of crust, time to total healing (re-epithelialisation), duration of pain and time to cessation of viral shedding (Spruance *et al.*, 1979; 1982; 1984; 1990a; 1990b; Raborn *et al.*, 1987; 1989; Fiddian *et al.*, 1983). Duration of each clinical stage may also be a useful parameter (Van Vloten *et al.*, 1983).

There has been considerable interest in therapy for RHL although current treatments are limited in effectiveness and/or are expensive. Oral therapy with acyclovir, valaciclovir or famciclovir can suppress or shorten the duration of attacks (Balfour, 1999), however, these are prohibitively expensive; none are listed on the Australian Pharmaceutical Benefits Scheme for RHL and the cost of these agents limits their utility. Many trials have been conducted to determine the efficacy of a 5% topical acyclovir cream for the treatment of cold sores, however these studies have produced mixed results. An analysis of these studies by Worrall (1991) concluded that there was insufficient evidence to support the use of acyclovir as a treatment for recurrent cold sores. Despite this, topical therapy with acyclovir cream

remains popular. In some countries acyclovir cream is available over-the-counter while in others it is available by prescription only. Topical therapy with penciclovir is available by prescription for the treatment of cold sores in the USA and there is evidence to support the efficacy of this treatment (Spruance *et al.*, 1997; Boon *et al.*, 2000). Overall, the limited treatment options available mean that the formulation and evaluation of alternative therapies is desirable.

TTO has been used for the past 70 years for its antiseptic activity and is currently available in a range of products including creams, throat lozenges and lip balm. Some evidence of the antiviral activity of TTO has been demonstrated (Bishop, 1995; Love, 1996). There has been little work on its *in vivo* effectiveness as a clinical treatment despite promising anecdotal reports and its current popular use. Our *in vitro* research, and that of others (Schnitzler *et al.*, 2001), indicates susceptibility of HSV 1 and HSV 2 to TTO. Anecdotal reports of symptom relief and accelerated resolution time when TTO is used to treat cold sore lesions have also been made (Cabot, 1989). TTO is inexpensive and poses little threat of inducing resistance to systemic antiviral agents.

A possible concern regarding the use of a topical perioral TTO product is its toxicity. Case reports of contact dermatitis in those patients who are allergic to TTO have been made in the literature (Carson and Riley, 1995) and there are limited data available on the oral toxicity of TTO. The lethal oral dose in the rat was reported as approximately 2 g/kg body weight (Altman, 1990). Cases of central nervous system toxicity and gastrointestinal toxicity following ingestion of TTO have been reported. The volume of TTO ingested in these cases ranges from a teaspoon to half a cup of 100% TTO. A number of oral and perioral products containing TTO such as toothpaste, throat lozenges, cold sore cream and lip balm are currently available over the counter. The TTO contained in these products ranges from 0.4% to 25%. No reports of oral toxicity from the use of these products have been made.

This pilot study aimed to:

- 1) Assess the effects of a 6% TTO ointment on the various clinical stages of RHL previously described.
- 2) Assess the effectiveness of such a product in reducing the duration of virus shedding.

The TTO product was compared to the placebo product (base product without the TTO).

5.2 Methods

Pilot study design

The pilot study was designed as a randomised, placebo-controlled study. TTO has a distinct odour which makes double-blinding difficult. The pilot study was single blinded (investigator-blinded). Although treatment allocation was not discussed openly, participants were probably aware of which treatment they received. They were all advised not to indicate to the investigators which treatment group they were in.

This pilot study used a placebo control instead of a comparative topical acyclovir control due to the inconclusive data regarding the efficacy of acyclovir for the treatment of RHL lesions.

Patients

The sample population was mainly recruited from male and female students and staff at Sir Charles Gairdner Hospital and The University of Western Australia who reported a history of recurrent cold sores. Recruitment was achieved via posters, advertisements in local publications and announcements to students and staff.

Interested patients completed a telephone screening questionnaire to determine their suitability. If selection criteria were met, they were invited to enrol in the pilot study.

Inclusion criteria were as follows:

- males & females aged 18 - 50 years
- history of recurrent cold sores which is defined as two or more occurrences in the past year

The exclusion criteria were:

- receiving long term topical steroid therapy
- immunocompromised patients
- receiving antiviral therapy in the month preceding the pilot study
- known allergy to TTO
- inability to understand the nature of the pilot study and the consent form
- inability to comply with treatment and follow-up visits

- pregnancy or breast feeding

Assessment visits

The first visit took place at PathCentre, Queen Elizabeth II Medical Centre, where patients who had developed a cold sore and were interested in participating in the pilot study were given information about the pilot study to read. A consent form was also given to the participant to read. Following any questions, the form was signed and a copy of it and the information sheet given to them to take home.

Once enrolled in the study, participants completed a questionnaire pertaining to the history and symptoms of their cold sores. They were randomised into one of the two treatment groups. Information and instructions on how to use the treatment were provided.

Patients were instructed to apply the treatment to the lesion 5 times daily at approximately 3-4 hour intervals. They were instructed to return for clinical and virological assessment each day until their lesion was re-epithelialised.

The treatment groups were:

- TTO therapy: 6% TTO ointment
- Placebo therapy: base ointment

Patients were briefed in the recognition of the various clinical stages of cold sores and filled in a daily diary sheet to note the onset of any changes in the clinical stages of the lesion, changes in symptoms experienced, the time of each treatment application and the occurrence of adverse reactions.

Subsequent visits occurred daily (except Sundays) after randomisation/treatment commencement and at least 3 hours after the last treatment application to minimise any carryover effects of the treatment product on the virological analysis.

At each visit, assessment of the clinical stage of the lesion was made by a study investigator (L. Ashton or C. Carson). A swab of the lesion was taken to determine the virological status

of the lesion. The presence of HSV in the lesion was determined using a PCR technique and viral tissue culture. Adverse reactions were recorded.

Sample size estimates

In the absence of any preliminary quantitative data, no sample size calculations were possible. The study was planned as a pilot study to collect preliminary data on the efficacy of TTO for the treatment of cold sores. The sample size consisted of 10 patients per treatment group. Statistical analysis was undertaken after 20 subjects had completed treatment. Information gained from this pilot study will be used to plan a larger study. Those participants who experience frequent recurrences will be invited to enrol in any subsequent trials.

Endpoints and data analysis

The clinical outcomes assessed were those proposed by Spruance *et al.* (1977, 1990):

- a) Days to loss of crust
- b) Days to complete healing (re-epithelialisation)

The virological outcomes assessed were:

- c) Days to cessation of viral shedding as determined by PCR and viral culture

The randomised and comparison treatment groups were compared to assure a balance of relevant characteristics. Differences in clinical and virological outcomes between the two treatment groups were assessed using appropriate statistical analysis. Duration of parameters measured was taken from the time that the patient first noticed their lesion and results were compared using the Mann-Whitney U rank sum test.

Processing of lesion specimens

Swabs were placed in a standard viral transport medium and stored at 4°C until processed. Within 48 hours of collection one aliquot was processed by PCR and the other stored at –75°C for later cell culture. In-house primers directed at the glycoprotein B gene of HSV 1 and HSV 2 were selected (Genbank listings S65444 and U12175, respectively) and used in a nested PCR using 45 cycles per round with an annealing temperature of 60°C. Perkin Elmer TaqGold enzyme was used to provide a 'hot-start'. PCR products were detected in agar gel electrophoresis with ethidium bromide staining. Viral culture was performed by standard

methods using HF 32 cells. Titrations were done in quadruplicate using serial 10-fold dilutions of the original virus transport medium.

5.3 Results

Table 2 shows the characteristics of the treated patients. Ten patients were randomised to each treatment group. There were no significant differences between the groups for age, number of cold sores/year or days to presentation.

One of the 10 patients in the TTO group did not develop clinical RHL, was PCR negative and was withdrawn due to an adverse event. One of the 10 patients from the placebo group was lost to follow-up. All remaining patients (n=18) were PCR positive for HSV 1 and developed clinical RHL. Table 3 shows the outcomes for each of the parameters measured. The median number of days to re-epithelialisation after treatment with TTO was 9 days compared to 12.5 days after placebo. TTO treatment also effected a modest reduction in the median number of days of culture positivity from 4 days to 3 days. However, after both treatments, the median number of days of PCR positivity was 6 days and the median number of days to crust formation was 4 days. HSV was detectable by PCR for 3-4 days longer than by culture in both groups. Viral titres appeared lower in the TTO group than in the placebo group on the third day of the cold sore (5.1×10^4 to 5.8×10^6 pfu/ml, respectively) and on the fourth day of the cold sore (10 to 2.1×10^3 pfu/ml, respectively) (see days 2 and 3 on Figure 15), but these differences did not reach statistical significance. Three TTO patients and one placebo patient were never HSV culture positive but their median number of days to presentation was 2 and 1 days, respectively, and later presentation may have contributed to the failure to detect HSV by culture.

Table 2. Characteristics of patients enrolled in the cold sore pilot study

Parameter	Treatment group	
	Tea tree oil (n = 9)	Placebo (n = 9)
No. females/males	7/2	7/2
mean age (years)	44.4 ± 14	50.1 ± 15
mean days to presentation	1.0 ± 1.1	0.8 ± 1.0
mean no. cold sores/year	4.4 ± 3.1	9.9 ± 7.9
stage at enrolment: erythema	1	0
papule	0	3
vesicle	5	3
ulcer	1	2
crust	2	1

Table 3. Outcomes for patients treated in the pilot study

Parameter	Treatment group (median \pm stand dev.)		Z value [†]
	Tea tree oil	Placebo	
Days to presentation (TTO n [‡] = 9, Placebo n = 9)	1.0 \pm 1.1	1.0 \pm 1.0	-0.378
Days to crust (TTO n = 9, Placebo n = 9)	4.0 \pm 1.4	4.0 \pm 0.8	-0.457
Days to re-epithelialisation (TTO n = 9, Placebo n = 8)	9.0 \pm 3.6	12.5 \pm 2.5	-1.407
Days PCR positive (TTO n = 8, Placebo n = 9)	6.0 \pm 2.0	6.0 \pm 4.9	-0.195
Days culture positive (TTO n = 9, Placebo n = 9)	3.0 \pm 1.3	4.0 \pm 2.1	-0.809
No. of patients never culture positive (NCP) (median time to presentation)	3 (2.0)	1 (2.0)	NA [§]
Days culture positive (excl. NCP patients) (TTO n = 6, Placebo n = 8)	3.5 \pm 1.4	4.0 \pm 2.0	-1.257

[†] Mann Whitney U rank sum test. The value of Z must exceed -1.96 or +1.96 to be statistically significant

[‡] number of patients available for analysis

[§] not applicable

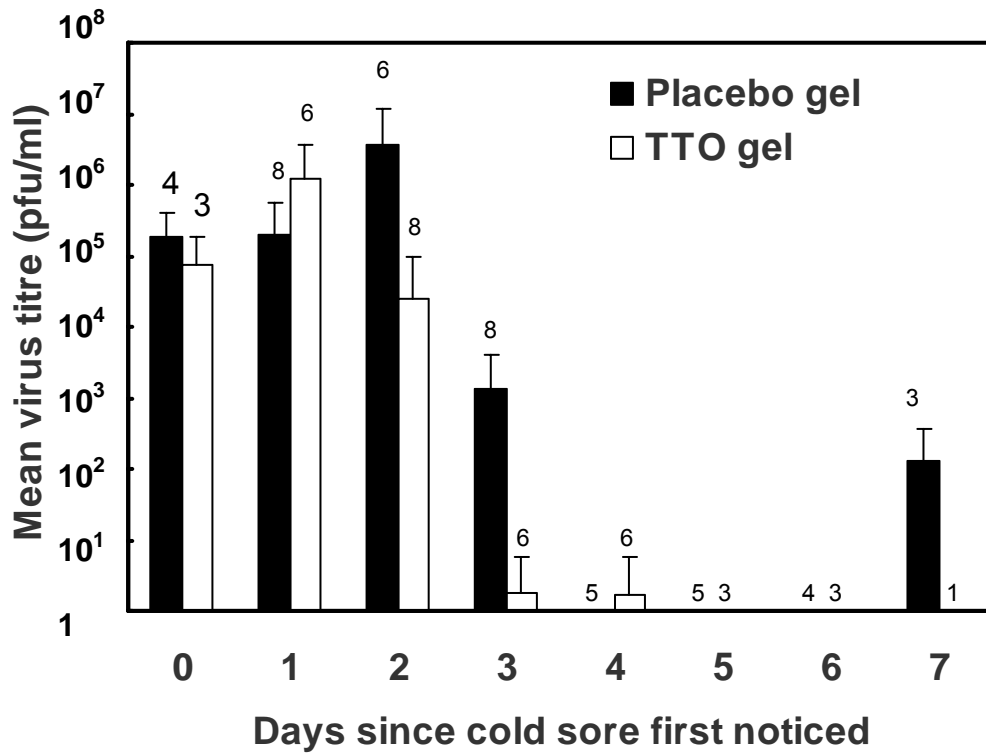


Figure 15. Mean virus titres from daily herpes labialis specimens

*number of patients in this group

5.4 Discussion

This pilot study was designed to provide preliminary data on the use of TTO in the treatment of cold sores. Each outcome favoured the TTO group but none of the differences between groups reached statistical significance, probably due to the small sample size and possibly influenced by the randomisation of patients with lesions beyond the papule stage. In addition, eight of the nine patients in the TTO group commenced treatment at the vesicle stage or beyond compared to six in the placebo group and earlier therapy may be more effective. The reduction in time to re-epithelialisation seen in the TTO group was similar to reductions reported previously for other topical therapies (Spruance *et al.*, 1997; Boon *et al.*, 2000) suggesting that further work in the form of a larger clinical trial is warranted.

Putative benefits in the time to healing seen in this pilot study after treatment with 6% TTO may not be solely attributable to the antiviral activity of TTO. The immune response to the HSV lesion results in inflammation at the site which contributes to overall tissue damage. Since TTO also has anti-inflammatory activity (Hart *et al.*, 2000; Brand *et al.*, 2001), it is possible that this may also reduce or eliminate inflammation and prevent much of the resulting tissue damage. Previous work has shown that when topical corticosteroid gel was used to treat RHL in combination with oral famciclovir, median maximum lesion size was reduced compared to oral famciclovir alone (Spruance & McKeough, 2000). If anti-inflammatory agents do have a role to play in the management of RHL, then the combination of antiviral and anti-inflammatory properties may be another advantage that TTO has over other agents. Similarly, the putative ability of TTO to penetrate intact skin may enhance its antiviral activity by allowing the oil to reach intradermal sites of infection. If TTO proves efficacious in the treatment of cold sores, it may also be useful in the treatment and management of genital herpes since there are many similarities between oral and genital herpes infections.

This pilot study was designed to provide preliminary data about TTO and cold sores and was necessarily simple in design. It is likely that the failure to demonstrate a significant difference between groups was due to a lack of power associated with the small size of the study. A larger clinical trial would provide the opportunity to address some of the methodological weaknesses contained in this pilot study. The first reported trial evaluating the efficacy of 5%

acyclovir did not demonstrate a statistically significant benefit compared to placebo ointment (Spruance *et al.*, 1982). As in this pilot study, ointments were made available and therapy was initiated upon presentation of the patient at the clinic. Consequently, there may have been a delay between the onset of the cold sore and the initiation of treatment. In contrast, the first trials to show significant improvements in healing times did not use clinic-initiated treatment but instead provided healthy prospective patients with ointment and allowed them to initiate treatment as soon as they felt the symptoms of their next cold sore (Fiddian & Ivanyi, 1983; Fiddian *et al.*, 1983; Van Vloten *et al.*, 1983). Since prompt initiation of treatment appears to enhance the effects of 5% acyclovir, it seems reasonable to assume that it may also influence the efficacy of TTO ointment and future clinical trials should allow patient-initiated treatment.

The stage of the lesion when treatment is initiated may also affect the efficacy of the treatment intervention. Trials with acyclovir or penciclovir generally limited enrolment to patients not beyond the papule stage (Spruance *et al.*, 1984; Raborn *et al.*, 1989; Spruance *et al.*, 1997, Boon *et al.*, 2000). In this pilot study there was no maximum time after which patients were ineligible to be randomised and begin treatment. Consequently, some patients enrolled in the study at the ulcer and crust stages limiting the scope for their treatment to be effective. It is possible that earlier treatment would be more effective. Similarly, the time from lesion onset to initial patient presentation may also influence the data collected and any subsequent work should set an upper limit on the time allowed for presentation and first assessment by clinic staff.

TTO may be a potentially useful alternative treatment for cold sores which is relatively inexpensive, acceptable to patients and which does not have the capacity to induce resistance to systemic antiviral agents. A larger study is required to further evaluate TTO as a topical treatment for RHL.

6 Implications

The primary implication of this work is that TTO appears to have promise as a topical treatment for cold sores. The preliminary data reported here require further corroboration in the form of a large, randomised, controlled clinical trial. Since most government pharmaceutical regulatory bodies require results from at least two clinical trials to support a therapeutic claim, it is likely that more than one trial will be necessary.

In addition, the results of this work will be published in peer-reviewed medical and scientific journals, providing data on the antiviral activity of TTO for healthcare professionals and researchers. Publications arising from earlier RIRDC-sponsored work at UWA investigating the activity of TTO against bacteria have had several effects. They have:

1. stimulated international interest in TTO as an alternative antimicrobial agent
2. encouraged other researchers to consider or investigate TTO as an antiviral agent
3. contributed significantly to the body of scientific evidence documenting the antimicrobial activity of TTO
4. been valuable in obtaining regulatory approvals for TTO in a number of countries
5. enhanced the profile of TTO amongst current and potential industry stakeholders

It is anticipated that publications arising from this study will facilitate similar outcomes.

The impact of this work on the TTO industry in Australia may not be immediately obvious in the short-term because these results alone are unlikely to expand markets. However, in the long-term, these results are the first step in attaining a wider market for TTO. It is now up to product developers, manufacturers and marketers to act upon these results so that the industry can benefit commercially from these results. Suitable products must be formulated and evaluated in clinical trials.

7 Recommendations

The results obtained in the study clearly indicate that TTO and several of its components can inactivate herpes virus *in vitro*. Furthermore, the preliminary evidence gained from the pilot study suggests that TTO may be effective in the treatment of cold sores. For these results to be exploited commercially, appropriate products must be clinically evaluated in large, randomised clinical trials.

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