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Screening of the antimicrobial properties of four Australian native Eucalyptus species

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Screening of the antimicrobial properties of four Australian native *Eucalyptus* species

Screening des propriétés antimicrobiennes de quatre espèces d'*Eucalyptus* d'origine australienne

Objectives

This study was undertaken to screen the *in vitro* antimicrobial properties of four Ausralian native *Eucalyptus* species, i.e., *E. dives*, *E. gunnii*, *E. olida* and *E. staigeriana* against five food-related micro-organisms, i.e., *E. faecalis*, *S. aureus*, *E. coli*, *P. aeruginosa* and *C. albicans*. The influences of two different extraction methods as well as the influence of a surfactant as plant extract dissolver in the water-based culture media used in agar disc diffusion tests and the role of growth location of two different E. olida specimens were investigated on the antimicrobial activity. Plant extracts profile and major compounds were analysed by GC/MS.

Results

Essential oils of the four *Eucalyptus* species displayed a variable degree of antimicrobial activity against the panel of micro-organisms tested, *E. staigeriana* oil showing a greater potential. Hexane extracts displayed no or low antimicrobial properties against the microbial strains tested with the exception of *S. aureus*. *P. aeruginosa* proved to be the most resistent micro-organism tested while *S. aureus* was the most sensitive one. The effect of a surfactant showed to decrease the antimicrobial activity of plant extracts or to not have any influence at all. GC/MS analyses showed that *E. staigeriana* and *E. dives* essential oils contain mostly volatile compounds while essential oils of both *E. olida* specimens contain heavier compounds.

Key words

Antimicrobial properties, disc diffusion method, *Eucalyptus* species, essential oils, hexane extracts, influence of a surfactant, chemical composition, GC/MS

Objectifs

Ce travail a été entrepris dans l'optique de tester les activités antimicrobiennes de quatre espèces d'*Eucalyptus* d'origine australiennes, *E. dives*, *E. gunnii*, *E. olida* et *E. staigeriana* contre cinq micro-organismes d'importance alimentaire, *E. faecalis*, *S. aureus*, *E. coli*, *P. aeruginosa* et *C. albicans*. L'influence de deux méthodes d'extraction et l'influence d'un solvant comme stabilisateur d'extraits de plante dans les milieux de culture aqueux ainsi que le rôle de la provenance de deux différents spécimens de *E. olida* ont été étudiés sur les activités antimicrobiennes. Le profile des extraits de plante ainsi que les composés majeurs ont été analysés par GC/MS.

Résultats

Les huiles essentielles des quatre espèces d'*Eucalyptus* ont montré un degré variable d'activités antimicrobiennes contre le panel de micro-organismes testé, l'huile essentielle de *E staigeriana* montrant le meilleur potentiel. Les extraits d'hexane n'ont montré qu'une faible voir aucune activité antimicrobienne, excepté contre *S. aureus*. *P. aeruginosa* s'est montré comme le micro-organisme le plus résistant et *S. aureus* comme le plus faible. L'effet du solvant a montré une diminution de l'activité des extraits de plante ou bien n'a aucune influence. Les analyses GC/MS ont montré des huiles essentielles riches en composés très volatils pour E. dives ainsi que *E. staigeriana* et des composés plus lourds pour les deux spécimens de *E. olida*.

Mots-clés

Propriétés antimicrobiennes, méthode par diffusion sur disque, *Eucalyptus*, huiles essentielles, extraits d'hexane, influence d'un solvant, GC/MS

Screening der antimikrobiologischen Eigenschaften von vier einheimischen australischen *Eukalyptus* Arten

Ziele

Diese Studie wurde durchgeführt um die antimikrobiologischen Eigenschaften von vier einheimischen australischen *Eukalyptus* Arten zu untersuchen. Es wurden die Eigenschaften von *E. dives*, *E. gunnii*, *E. olida* und *E. staigeriana* gegenüber fünf in der Lebensmitteltechnologie wichtigen Mikroorganismen, das heisst, *E. faecalis*, *S. aureus*, *E. coli*, *P. aeruginosa* und *C. albicans* getestet. Der Einfluss zweier unterschiedlicher Extraktionsmethoden und der Herkunft zweier unterschiedlicher *E olida* Spezies sowie der Einfluss eines Lösungsmittels, welches die Diffusion im Kulturmilieu erleichtert, auf die antimikrobiologischen Eigenschaften wurde untersucht. Das Profil der Pflanzenextrakte sowie die Hauptkomponenten wurden mittels GC/MS analysiert.

Resultate

Die ätherischen Öle der vier *Eukalyptus* Arten zeigten unterschiedliche antimikrobiologische Aktivität gegenüber den Versuchsmikroorganismen. Das ätherische Öl von *E. staigeriana* wies das grösste Potential auf. Die Hexan-Extrakte zeigten nur schwache bis keine antimikrobiologischen Eigenschaften ausser gegenüber *S. aureus*. *P. aeruginosa* stellte sich als resistentester Mikroorganismus heraus und *S. aureus* als der schwächste. Der Einsatz des Lösungsmittels hatte entweder keinen Einfluss oder führte zu einer Abnahme der antimikrobiologischen Aktivität der Pflanzenextrakte. GC/MS Analysen zeigten, dass die ätherischen Öle von *E. staigeriana* and *E. dives* hauptsächlich aus flüchtigen Verbindungen bestanden während die ätherischen Öle der beiden *E. olida* Arten schwerere Verbindungen enthielten.

Schlüsselwörter

Antimikrobiologische Eigenshaften, Disk Diffusion Methode, *Eukalyptus*, ätherische Öle, Hexan-Extrakte, Lösungsmitteleinfluss, GC/MS

Table of contents

- *summary* -

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Abbreviations

ATCC	American Type Culture Collection
bp	boiling point
c.	circa
C. albicans	*Candida albicans*
CFU	colony forming unit
GC/MS	gas chromatography / mass spectrometry
GC/ITD	gas chromatography / ion trap detector
E. coli	*Escherichia coli*
E. dives	*Eucalyptus dives*
E. faecalis	*Enterococcus faecalis*
E. gunnii	*Eucalyptus gunnii*
E. olida^{NSW}	*Eucalyptus olida* from Northern New South Wales
E. olida^{VIC}	*Eucalyptus olida* from Southern Victoria
E. staigeriana	*Eucalyptus staigeriana*
EO	*essential oil*
HE	*hexane extracts*
ITD	Ion Trap Detector
MHA	Mueller-Hinton agar
MHB	Mueller-Hinton broth
MRSA	methicillin-resistant *Staphylococcus aureus*
MIC	minimum inhibitory concentration
mp	melting point
NA	Nutrient Agar
NaCl	Natrium Chloride
NCCLS	National Committee for Clinical Laboratory Standards
NSW	New South Wales
P. aeruginosa	*Pseudomonas aeruginosa*
PCA	Plate Count Agar
OD	optical density
RSD	relative standard deviation
RT DB5	Retention time on DB-5 column, in seconds
S. aureus	*Staphylococcus aureus*
SDA	Sabouraud Dextrose agar
SLM	Sabouraud Liquid Medium
TTC	2,3,5-Triphenyl tetrazolium chloride
VIC	Victoria
v/v	volume/volume
w/w	weight/weight
WHO	World Health Organisation

1. Introduction

In spite of modern improvements in hygiene and food production techniques, food safety is an increasingly important public health issue. It has been reported that two million people died from diarrhoeal disease in 2000 worldwide and that the percentage of people suffering from food borne diseases each year in industrialized countries was up to 30% (WHO, 2002).

The food industry uses chemical preservatives to prevent the growth of food borne and spoilage micro-organisms. Currently, there is a strong debate about the safety aspects of chemical preservatives since they are considered to have carcinogenic and teratogenic attributes as well as residual toxicity (Moreira et al., 2005). In recent years, there has been considerable pressure from consumers to reduce or eliminate chemically synthesized additives in their foods (Tuley de Silva, 1996; Smid & Gorris, 1999).

The increasing incidence of food borne diseases, coupled with the demand for natural and socially more acceptable preservatives, means there is a constant striving to develop naturally occurring antimicrobial agents for food preservation.

Because most plants produce antimicrobial secondary metabolites, either as part of their normal program of growth or in response to special conditions, antimicrobial additives such as plant extracts and essential oils are therefore of increasing interest in food preservation.

1.1. Secondary metabolites

Through primary metabolic pathways, plant cells synthesize, degrade, and generally interconvert a vast number of organic compounds to enable them to live, grow, and reproduce. In contrast, there also exists an area of metabolism concerned with compounds which have a much more limited distribution in nature.

Such compounds, called secondary metabolites, are found in only specific organisms, or groups of organisms, and are an expression of the individuality of species. Secondary metabolites are not necessarily produced under all conditions, and in the vast majority of cases, the function of these compounds and their benefit to the organism is not yet known.

Some are undoubtedly produced for easily appreciated reasons, e.g. as toxic materials providing defence against predators, as volatile attractants towards the same or other species, or as colouring agents to attract or warn other species, but it is logical to assume that all do play some vital role for the well-being of the producing organism. It is this area of secondary metabolism that provides most of the pharmacologically active natural products (Dewick, 2004).

1.2. Essential oils

Essential oils are the odorous, volatile products of the secondary metabolism of an aromatic plant, normally formed in special cells or groups of cells. They are commonly concentrated in one particular region such as leaves, stems, bark or fruits, and when they occur in various organs in the same plant, they frequently have different composition profiles (Conner, 1993).

They can be obtained by expression, fermentation or extraction but the method of steam distillation is most commonly used for commercial production of essential oils. An estimated three thousand essential oils are known, of which about three hundred are commercially important, destined chiefly for the flavours and fragrances market (Burt, 2004).

Essential oils have been proven to be inhibitory against a wide range of food spoilage micro-orgainsms, dependent upon their concentration, method of testing and active constituents present (Skočibušić, Bezić & Dunkić, 2006).

1.3. Antimicrobial properties

The antimicrobial activity of essential oils is assigned to a number of small terpenoid and phenolic compounds, which also in pure form have been shown to exhibit antimicrobial activity. These properties of the compounds are in part associated with their lipophilic character, leading to accumulation in membranes and to subsequent membrane-associated events such as energy depletion (Conner, 1993). Phenolic components of essential oils sensitize the phospholipids bilayer of the cell membrane, causing an increase of permeability and leakage of vital intracellular constitutes or impairment of bacterial enzyme systems (Moreira et al., 2005).

Large differences in the antimicrobial activity of essential oils from the same plant have been reported. The reasons for this variation can be due to the different geographical growth locations, the harvesting seasons, the genotype, the climate, the drying procedure, the distilled part of the plant and the extraction type. All of these significant factors influence the chemical composition and the relative concentration of each constituent in the essential oils (McGimpsey & Douglas, 1994; Salgueiro et al., 1997). Solvent extracted oils should exhibit in general greater antimicrobial activity than corresponding steam distilled oils because some of the highly volatile components are lost during steam distillation (Packiyasothy & Kyle, 2002).

The antimicrobial activity of natural extracts and pure compounds can be detected by observing the growth response of various micro-organisms to samples that are placed in contact with them. Antimicrobial test methods are classified into three main groups, i.e. diffusion, dilution and bioautographic methods. A fourth and upcoming test method is the conductimetric assay, detecting microbial growth as a change in the electrical conductivity or impedance of the growth medium (Cos et al., 2006).

The principles and practice of these test methods are explained in the literature. The NCCLS method for antimicrobial susceptibility testing, which is principally aimed at the testing of antibiotics, has been modified for testing essential oils. It is adapted from "Antibiotic susceptibility testing by a standardized single disk method" originally described by Bauer et al. (1966). The Oxoid method for antimicrobial susceptibility testing is the disc diffusion method which although developed in 1947 remains as the most widely used test (The Oxoid Manual, 1995).

1.4. Eucalyptus

Australia is one of the few countries in the world that is associated in the minds of many people with a single group of plants. These plants are the eucalypts that dominate most of the Australian landscape. *Eucalyptus* is a large genus of the Myrtaceae family that includes about 900 species and subspecies, some of which are known but as yet unpublished (Brooker & Kleinig, 2004).

The Aborigines, believed to have settled in Australia over 60,000 years ago, developed a sophisticated empirical understanding of indigenous plants, such as *Eucalyptus*. They traditionally used its leaves to heal wounds and fungal infections. While much of this knowledge has vanished with its keepers, there is currently a high level of interest in native herbal traditions (Chevallier, 2001).

Leaf extracts of *Eucalyptus* have been approved as food additives, and the extracts are also currently used in cosmetic formulations. Recently, attention has been focused on the medicinal properties of these extracts. Research data has demonstrated that the extracts exhibited various biological effects, such as antibacterial, antihyperglycemic and antioxidant activities (Takahashi et al., 2004).

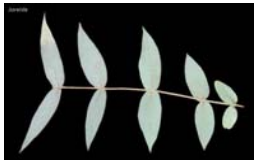
1.5. Objectives

This study was undertaken to screen *in-vitro* antimicrobial properties of four Australian native *Eucalyptus* species, i.e., *E. dives*, *E. gunnii*, *E. olida* and *E. staigeriana* (cf. *Table 1* on page 4), against five food-related micro-organisms, i.e., *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Enterococcus faecalis* and *Candida albicans*. Antimicrobial activity tests were carried out using the agar disc diffusion method.

Specifically, the objectives of this study were as follows:

- to determine the influence of two methods of extraction on the antimicrobial activity of extracts,
- to investigate the role of a surfactant as plant extract dissolver or stabiliser in the water-based culture media used in agar disc diffusion tests,
- to elucidate the role of location in the antimicrobial properties of *E. olida* species and
- to analyse the profiles and identify the major compounds in the extracts by GC/MS.

Table 1 : Description of the four studied *Eucalyptus* species (Brooker & Kleinig, 1999; Brooker & Kleinig, 2004; Brooker et al., 2006)

Botanical name **Common name**	***Tree picture and description***		***Juvenile leaves picture and description***		***Distribution***	
Eucalyptus dives **Broad-leaved peppermint**		Small to medium-sized tree. Bark rough to the large branches, finely fibrous, peppermint-type, grey to grey-brown; smooth bark grey.		Juvenile leaves opposite for many pairs, rarely connate, sessile, ovate to cordate to broadly falcate, 6-14 cm long, 2-7 cm wide, blue-green to blue-grey or glaucous.		Northern Tablelands of New South Wales through the Southern Tablelands to eastern Victoria, from south of the Glen Morrison and Niangala area, often on poor shallow soils.
Eucalyptus gunnii **Cider gum**		Small to medium-sized woodland tree. Bark smooth throughout or with some imperfectly decorticated rough bark; smooth bark mottled white, yellow, pink, brown, green or grey, branchlets often conspicuously glaucous.		Juvenile leaves opposite and sessile for at least 40 nodes, broadly orbicular to cordate, 1.3-4.5 cm long, 1.7-4 cm wide, margin entire or crenulated, grey-green or glaucous.		Endemic to Tasmania, usually occupying poorly drained sites in cold upland areas.
Eucalyptus olida **Strawberry gum**		Small to medium-sized forest tree. Bark rough, thick, flaky and fibrous to branches 5-8 cm diameter, peppermint-type, orange-brown or grey.		Juvenile leaves subsessile to shortly petiolate and opposite for *c.* 5 nodes, then alternate, petiolate, ovate, 4.5-10 cm long, 2.5-5.5 cm wide, base rounded or tapering to petiole, dull, green to blue-green.		Endemic on the eastern Northern Tablelands of New South Wales, from Timbarra Plateau to Gibraltar Range, on granite-derived soils.
Eucalyptus staigeriana **Lemon-scented ironbark**		Small to medium-sized tree. Ironbark to small branches, very hard, dark grey or black. Branchlets yellow, often glaucous.		Juvenile leaves opposite for many nodes before turning alternate, petiolate, ovate, 3.8-7 cm long, 2-4 cm wide, grey-green to glaucous.		Endemic to North Queensland, restricted to hilly country in the Maytown – Palmer River – Maitland Downs area of Cape York Peninsula, on granite or sandstone hills.

2. Materials and methods

2.1. Plant material

Fresh leaves on stems of *E. dives*, *E. gunnii* and a specimen of *E. olida* were provided by Tarnuk Bush Food & Flower, Korumburra VIC, Australia. These specimens were hand harvested late October 2006.

E. staigeriana and another specimen of *E. olida* were provided by Australian Rainforest Products Pty Ltd, Blue Knob NSW, Australia. Fresh leaves on stems were hand harvested early November 2006.

2.2. Plant extracts

2.2.1. Essential oils

A portion (100 g) of *E. dives*, *E. gunnii* and *E. staigeriana* fresh leaves was cut into 5 mm strips and submitted to steam distillation for 3 h using an indirect steam distillation apparatus (cf. *Appendix 1*). The essential oil floating on the top of water was collected into a separatory funnel and then into a scintillation vial before being dried overnight in a desiccator and stored at -18°C.

Because essential oils of both *E. olida* specimens were heavier than water, 100 g of fresh cut leaves with anti-bumping granules and 600 ml of distilled water were submitted to steam distillation for 3 h using a Clevenger-type apparatus (cf. *Appendix 2*). The white opaque crystalline solution obtained was collected into a scintillation vial and freeze dried (Christ, Freeze Dryer Alpha 2-4 LD*plus*) under 0,011 mbar for 15 h before being stored at -18°C.

2.2.2. Hexane extracts

Ten grams (10g) of each *Eucalyptus* species were cut into 5 mm strips and put in a thimble filter (Bonnet Equipment, N8425x80MM) before being submitted to 10 cycles of Soxhlet extraction with 100 ml of n-hexane (Mallinckrodt Chemicals). The hexane was then evaporated under 320 mbar at 40°C using a rotary evaporator (Büchi B-480, R-114). Plant extracts were freeze dried (Christ, Freeze Dryer Alpha 2-4 LD*plus*) under 0,011 mbar for 24 h, and then resolubilized in 3 ml n-hexane before being filtered with 0,45 µm membrane filters (Pall Life Sciences, PN4614) and stored into a scintillation vial at -18°C.

2.3. GC/MS analysis

GC/MS analyses were performed using a Varian 3800 gas chromatograph directly coupled to a Varian Ion Trap (ITD) mass spectrometer, model Saturn 2000. The column used was a J & W DB-5 fused silica capillary column (0.25 mm i.d. x 30 m, 0.25 µm coating thickness, AGI122-5032, Biolab). Chromatograms, identification and quantification were obtained using the Saturn GC/MS workstation (v5.2, Varian).

The GC/ITD was operated under the conditions suggested by Adams (1995, 2001). Briefly: injector temperature – 220°C; transfer line – 250°C; oven temperature programmed – 60°C to 240°C at 3°C/min; carrier gas – He at 0.8 ml/min at 220°C; manual injection using 10 µl syringes (Hamilton 701N 10 µl, Alltech Associates) – 0.2 µl, split 1:20. Acquisition parameters: full scan; scan range 41 – 300 amu; scan time 1.0 sec; threshold 1 count; AGC mode: on; microscans: 5; filament delay: 120 sec. Temperatures transfer line – 250°C; manifold – 60°C.

A 10% (v/v) hexane (Mallinckrodt Chemicals) solution as well as a 1% (v/v) hexane solution of each essential oil was injected in order to allow a better identification of the different compounds. This was necessary to increase the sensitivity for minor compounds, especially for the profiles of both *E. olida* specimens which were similar in their major compound. The chromatograms obtained with the 10% (v/v) hexane solution allowed the identification of some minor compounds while those obtained with the 1% (v/v) hexane solution allowed a more accurate identification of the major compounds, saturated at 10%. Hexane extracts were injected without further dilution. Each hexane extract and diluted essential oil was run three times on the same day.

Column head pressure was adjusted to 9.4 psi with *Lavandula angustifolia* essential oil, used as an internal standard at the Environmental & Analytical Laboratories, Charles Sturt University, Wagga Wagga, to set a flow rate which matches with Adams (1995) retention times. Reference compounds were camphor (RT DB5-0734), borneol (RT DB5-0789) and terpin-4-ol (RT DB5-0820).

The quantification of the identified compounds was carried out with the following parameters: measurement type – area; calibration type – external standard; unretained peak time – 2.0 min (remove solvent peak); ion ratio type – absolute. Integration parameters: peak width – 2.0 sec; slope sensitivity – 5 SN; tangent – 10%; peak size reject – 2000 counts (differentiation with background noise). The three runs of each hexane extract and 10% (v/v) hexane solution of essential oil were quantified.

2.4. Antimicrobial activity

2.4.1. Microbial strains

Essential oil and hexane extract of each Eucalyptus species were tested against a panel of food-related micro-organisms, including Gram-positive bacteria *Staphylococcus aureus* ATCC 25923, *Enterococcus faecalis* ATCC 19433, Gram-negative bacteria *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853 and a yeast *Candida albicans* ATCC 10231. The reference cultures provided by the Department of Biomedical Sciences, Charles Sturt University, Wagga Wagga NSW 2678, were stored at 4°C on Nutrient Agar (Oxoid, CM0003) for bacteria and on Sabouraud Dextrose Agar (Oxoid, CM0041) for the yeast. Cultures for experiments were prepared by transferring a loop of cells from the reference cultures to a test tube containing 10 ml of Mueller Hinton Broth (Oxoid, CM0405) for bacteria and Sabouraud Liquid Medium (Oxoid, CM0147) for the yeast respectively. They were then incubated overnight (16 h) without agitation at 37°C for bacteria and 30°C for the yeast. A control of purity was applied to reference cultures. Each colony was plated on Mueller Hinton Agar (Oxoid, CM0337) for bacteria and SDA (Oxoid, CM0041) for the yeast.

2.4.2. Standard growth curves

In order to use an appropriate inoculum concentration in the agar disc diffusion tests, standard growth curves were determined. According to the literature, an inoculum concentration of about 10^5 CFU/ml is adequate for most bacterial species while for yeasts and fungi between 10^3 and 10^4 CFU/ml is sufficient in dilution methods (Cos et al., 2006). In disc diffusion tests, an inoculum concentration of 10^5-10^6 CFU/ml is recommended by The Oxoid Manual (1995). A low inoculum size (e.g. 10^2 CFU/ml) will create many false-positives while a too high inoculum size (e.g. 10^7 CFU/ml) will hamper endpoint reading and increase the chances for false-negatives (Cos et al., 2006).

The overnight culture of each micro-organism listed above was diluted to 1/100 in 10 ml MHB for bacteria and SLM for the yeast. This dilution was taken as the initial suspension. Test tubes containing 10 ml of the initial suspension were incubated at 37°C for bacteria and 30°C for the yeast. Optical density was measured (UV/VIS Spectrometer, UNICAM 8625) at 420 nm as suggested by Mann & Markham (1997) on initial suspensions as well as on suspensions collected from the incubator every hour for *E. coli*, *S. aureus* and *P. aeruginosa* and every two hours for *C. albicans*. The corresponding microbial growth (CFU/ml) was determined by plating 100 µl of a serial dilution of each suspension on Plate Count Agar (Oxoid, CM0003) for bacteria and on SDA for the yeast. Each dilution was plated in duplicate. Agar plates were incubated for 24h at 37°C for bacteria and 48h at 48°C for the yeast.

2.4.3. Agar disc diffusion method

Antimicrobial activity of plant extracts obtained by steam distillation and Soxhlet extraction with hexane was tested using the agar disc diffusion method (The Oxoid Manual, 1995; NCCLS, 2000a, 2002).

MHA (Oxoid, CM0337) and SDA (Oxoid, CM0041) sterilized in autoclave (High-Pressure Steam Sterilizer ES-315, Tomi) and cooled to 45-50°C were poured into 90 mm Petri dishes (Techno-Plas). According to Elgayyar, Draughon, Golden & Mount (2001) and Mourrey & Canillac (2002), 0.01% (w/w) of TTC (Research Organics, 3086T) were added to MHA after sterilisation to help differentiating bacterial colonies and clarifying the zone of inhibition. Agar plates were stored at 2-8°C before being used.

Fresh bacterial and yeast inoculates were prepared from overnight cultures in order to apply microbial inoculates in the logarithmic growth phase. The overnight culture of each micro-organism was diluted to 1/100 in 10 ml MHB for bacteria or 10 ml SLM for the yeast, and incubated for 4 to 6 h without agitation at 37°C for bacteria and 30°C for the yeast. Optical density was measured at 420 nm and appropriate dilutions were carried out following the standard growth curve data to reach the adequate inoculum concentration of 10^5-10^6 CFU/ml suggested by the Oxoid Manual (1995). This was controlled by plating 100 µl of a serial dilution of the fresh inoculates on Plate Count Agar (Oxoid, CM0003) for bacteria and on SDA for the yeast. Each dilution was plated in duplicate. Agar plates were incubated for 24h at 37°C for bacteria and 48h at 48°C for the yeast.

One hundred microlitres (100 µl) of the 10^5-10^6 CFU/ml fresh inoculate were spread over the surface of MHA for bacteria and SDA for the yeast. Agar plates were incubated at 30°C for approximately 15 min until the microbial overlay had dried. Six susceptibility test discs (Oxoid, CT0998B, Ø = 6 mm) were placed onto the agar plates using a six cartridges disc dispenser (Oxoid, ST6090). Only one or two discs were used when plant extracts showed great inhibition zones. Discs were then individually impregnated with 10 µl of hexane extracts, essential oils and standard antibiotics. Because of its high content in methyl cinnamate (mp: 34-37°C; Sigma-Aldrich, 2007), *E. olida* essential oils needed to be resolubilized by heating before application.

In order to test the influence of using a surfactant as plant extracts dissolver or stabiliser in water-based culture media, discs were also impregnated with 10 µl of hexane extracts and essential oils diluted in 10% (v/v) Tween 80 (2510-500ML, Labchem).

Standard antibiotics as chloramphenicol (30 µg/disc, Oxoid CT0013B) served as a positive control for *E. coli*, *E. faecalis* and *S. aureus* while gentamicin (30 µg/disc, Oxoid CT0072B) served as a positive control for *P. aeruginosa* and nystatin (Nystatin Dihydrate, Sigma-Aldrich) - 10 µl of a 3 mg/ml prepared aqueous solution – served as a positive control for the yeast. Hexane served as a negative control for hexane extracts while Milli-Q water (Milli-Q gradient A10, Quantum Ex Ultrapure Organex Cartridge, Millipore) served as a negative control for essential oils. A 10% (v/v) hexane solution of Tween 80 served as a negative control for hexane extracts and essential oils diluted in Tween 80. Inoculated plates were incubated without any test discs as a control to detect any plant extracts vapour effect.

Agar plates were sealed with laboratory film (Parafilm PM-996, Pechiney Plastic Packaging) and kept at 2-8°C for 2 h in order to lower the detection limit (Rios, Recio & Villar, 1988). They were then incubated for 24h at 37°C for *E. coli*, *P. aeruginosa* and *S. aureus* and 48h at 30°C for *C. albicans*. The diameters of the inhibition zones were measured in millimetres with a digital calliper (Absolute Digimatic 500-196, Mitutoyo). Three agar disc diffusion tests were carried out on each micro-organism. Each test was performed in duplicate.

2.5. Statistical analysis

Values dispersion was analysed with relative standard deviation for the quantification of major compounds identified in plant extracts by GC/MS and with standard deviation for inhibition zones measurements in antimicrobial activity tests.

Student's two-tailed *t*Test allowed comparing means of two populations in order to determine their relation. Populations were deemed to be significantly different when the calculated *p*-value was smaller than the significance level $\alpha = 0.05$.

3. Results

3.1. Plant extracts

3.1.1. Steam distillation yields

The extraction yields obtained by steam distillation for the different *Eucalyptus* species are listed in *Table 2*. The yields are calculated from the fresh weight because essential oils were extracted from fresh plant material.

Table 2 : Steam distillation yield in percentage (w/w) for each *Eucalypus* species

Eucalyptus species	Weight of fresh cut leaves [g]	Weight of collected essential oil [g]	Yield [%]
E. dives	98.91	2.94	2.97
E. gunnii	111.01	0.07	< 0.10
E. olida^{NSW}	100.02	1.12	1.12
E. olida^{VIC}	99.13	3.10	3.12
E. staigeriana	100.11	2.13	2.13

A limited amount of extractable essential oil was available for *E. gunnii* while *E. olida*^{VIC} showed greatest exctractable at around 3%. This was about three times the amount available from *E. olida*^{NSW}. *E. dives* and *E. staigeriana* provided around 3% and 2% extractable content respectively.

3.1.2. Solvent extraction yields

The extraction yields obtained by solvent extraction with hexane for the different *Eucalyptus* species are listed in *Table 3*. The yields are calculated from the fresh weight because hexane extracts were obtained from fresh plant material.

Table 3 : Hexane extraction yield in percentage (w/w) for each *Eucalyptus* species

Eucalyptus species	Weight of fresh cut leaves [g]	Weight of collected essential oil [g]	Yield [%]
E. dives	11.63	0.11	0.96
E. gunnii	10.52	0.15	1.47
E. olida^{NSW}	11.12	0.27	2.46
E. olida^{VIC}	11.34	0.07	0.59
E. staigeriana	11.20	0.06	0.53

A small amount extractable hexane extract was available for *E. staigeriana* and *E. olida*^{VIC} while *E. olida*^{NSW} showed greatest extractable at around 2.5%. *E. dives* and *E. gunnii* provided around 1% and 1.5% extractable content respectively.

3.2. GC/MS analysis

3.2.1. Essential oils

The chemical composition of *E. dives*, both *E. olida* specimens and *E. staigeriana* essential oils was identified and quantified by GC/MS analysis. Dispersion or variability measures were indicated with relative standard deviation. Because *E. gunnii* steam distillation caused problems and that only a limited amount of plant material was available, it was insufficient to produce proper essential oil. Therefore, GC/MS analysis of *E. gunnii* essential oil was not carried out.

3.2.1.1. E. dives

The compounds identified and quantified in *E. dives* essential oil are listed in *Table 4*. Their name with the corresponding retention time, the content average and the relative standard deviation are indicated. The chromatogram is available in *Appendix 3*.

Table 4 : Identification and quantification of the compounds detected in *E. dives* essential oil.

No.	Retention time [min]	Compound	Content average [%][a]	RSD [%]
1	*4.76*	*tricyclene*	*0.9*	*22.3*
2	*4.88*	*α-thujene*	*3.1*	*24.9*
3	*5.06*	*α-pinene*	*0.4*	*32.2*
4	*6.04*	*sabinene*	*0.1*	*31.5*
5	*6.53*	*myrcene*	*1.1*	*44.0*
6	*6.88*	*para-mentha-1(7),8-diene*	*3.3*	*64.7*
7	***6.98***	***α-phellandrene***	***17.4***	***4.5***
8	*7.32*	*α-terpinene*	*1.2*	*2.6*
9	***7.56***	***para-cymene***	***8.5***	***6.9***
10	*7.72*	*β-phellandrene*	*2.8*	*15.5*
11	*7.79*	*1,8-cineole (eucalyptol)*	*0.7*	*81.5*
12	*8.35*	*(E)-β-ocimene*	*0.3*	*8.6*
13	*8.76*	*γ-terpinene*	*0.8*	*8.9*
14	*9.84*	*terpinolene*	*2.4*	*10.0*
15	*10.21*	*linalool*	*0.9*	*2.4*
16	*11.06*	*cis-para-menth-2-en-1-ol*	*1.3*	*2.9*
17	*11.79*	*trans-para-menth-2-en-1-ol*	*0.9*	*4.3*
18	***13.41***	***terpin-4-ol***	***4.7***	***3.5***
19	*13.94*	*α-terpineol*	*1.0*	*6.9*
20	*14.67*	*trans-piperitol*	*0.5*	*21.5*
21	***16.90***	***piperitone***	***40.5***	***7.3***
22	*20.87*	*α-terpinyl acetate*	*0.3*	*11.6*
23	*22.22*	*(E)-methyl cinnamate*	*2.0*	*12.5*
24	*27.18*	*bicyclogermacrene*	*0.8*	*21.4*
		Total:	***95.9***	

[a][%] figures are their relative proportions as percent of total ion current (TIC).
The dominant compounds are indicated in bold.

GC/MS analysis resulted in the identification of 24 compounds representing 95.9% of the *E. dives* essential oil. The dominant compounds were piperitone with 40.5%, α-phellandrene with 17.4%, para-cymene with 8.5% and finally terpin-4-ol with 4.7%.

3.2.1.2. *E. olida*^{NSW}

The compounds identified and quantified in the essential oil of *E. olida*^{NSW} are listed in *Table 5*. Their name with the corresponding retention time, the content average and the relative standard deviation are indicated. The chromatogram is available in *Appendix 4*.

Table 5 : Identification and quantification of the compounds detected in the essential oil of *E. olida*^{NSW}.

No.	Retention time [min]	Compound	Content average [%][a]	RSD [%]
1	*5.61*	*benzaldehyde*	*<0.1*	*44.4*
2	*8.35*	*(E)-β-ocimene*	*<0.1*	*39.6*
3	*10.24*	*linalool*	*<0.1*	*26.8*
4	*12.39*	*isoborneol*	*<0.1*	*32.8*
5	*13.96*	*α-terpineol*	*<0.1**	*62.9**
6	*15.62*	*citronellol*	*0.1*	*16.8*
7	*16.12*	*neral*	*<0.1*	*31.9*
8	*16.75*	*geraniol*	*0.1*	*24.0*
9	*17.05*	*methyl citronellate*	*<0.1*	*61.0*
10	*17.42*	*geranial*	*<0.1*	*23.8*
11	*17.60*	*nopol*	*<0.1*	*36.8*
12	*18.90*	*(Z)-methyl cinnamate*	*0.1*	*17.5*
13	*19.82*	*methyl geranate*	*<0.1*	*43.9*
14	***22.59***	***(E)-methyl cinnamate***	***99.4***	***0.5***
15	*23.97*	*(E)-caryophyllene*	*<0.1**	*41.3**
16	*25.72*	*seychellene*	*<0.1**	*55.0**
17	*27.18*	*viridiflorene*	*<0.1**	*36.7**
18	*28.33*	*δ-cadinene*	*<0.1**	*41.1**
19	*30.46*	*spathulenol*	*<0.1**	*47.1**
		Total:	***99.8***	

[a][%] figures are their relative proportions as percent of total ion current (TIC).
*: Average and relative standard deviation calculated from two values instead of three.
The dominant compounds are indicated in bold.

The essential oil GC/MS analysis of *E. olida*^{NSW} allowed the identification of 19 compounds. Total average content of compounds quantified was 99.8%. With an average content of 99.4%, (E)-methyl cinnamate was determined as the dominant compound.

3.2.1.3. *E. olida*^{VIC}

The compounds identified and quantified in the essential oil of *E. olida*^{VIC} are listed in *Table 6*. Their name with the corresponding retention time, the content average and the relative standard deviation are indicated. The chromatogram is available in *Appendix 5*.

Table 6 : Identification and quantification of the compounds detected in the essential oil of *E. olida*^{VIC}.

No.	Retention time [min]	Compound	Content average [%][a]	RSD [%]
1	*5.61*	*benzaldehyde*	*<0.1**	*58.2**
2	*8.00*	*(Z)-β-ocimene*	*<0.1**	*90.0**
3	*8.35*	*(E)-β-ocimene*	*<0.1*	*42.1*
4	*10.23*	*linalool*	*<0.1**	*104.5**
5	*16.61*	*piperitone*	*<0.1**	*28.3**
6	*16.83*	*linalool acetate*	*<0.1**	*110.0**
7	*17.58*	*nopol*	*<0.1**	*28.3**
8	*18.86*	*(Z)-methyl cinnamate*	*0.1*	*12.3*
9	***22.29***	***(E)-methyl cinnamate***	***98.8***	***1.0***
10	*25.71*	*(E)-ethyl cinnamate*	*0.1*	*13.2*
11	*27.23*	*bicyclogermacrene*	*<0.1*	*10.2*
12	*30.46*	*spathulenol*	*<0.1*	*25.6*
13	*30.69*	*globulol*	*<0.1*	*10.4*
14	*31.03*	*viridiflorol*	*<0.1*	*31.5*
15	*31.13*	*khusimone*	*<0.1*	*24.2*
16	*31.46*	*guaiol*	*<0.1*	*10.6*
17	*32.26*	*10-epi-γ-eudesmol*	*<0.1*	*17.3*
18	*32.61*	*γ-eudesmol*	*<0.1*	*19.3*
19	*33.29*	*α-eudesmol*	*<0.1*	*24.1*
20	*33.43*	*7-epi-α-eudesmol*	*0.1*	*4.0*
		Total:	***99.4***	

[a][%] figures are their relative proportions as percent of total ion current (TIC).
*: Average and relative standard deviation calculated from two values instead of three.
The dominant compounds are indicated in bold.

The essential oil GC/MS analysis of *E. olida*^{VIC} allowed the identification of 20 compounds representing 99.4% of the oil. With an average content of 98.8%, (E)-methyl cinnamate was determined as the dominant compound.

3.2.1.4. E. staigeriana

The compounds identified and quantified in *E. staigeriana* essential oil are listed in the following *Table 7*. Their name with the corresponding retention time, the content average and the relative standard deviation are indicated. The chromatogram is available in *Appendix 6*.

Table 7 : Identification and quantification of the compounds detected in *E. staigeriana* essential oil.

No.	Retention time [min]	Compound	Content average [%][a]	RSD [%]
1	*4.88*	*α-thujene*	*1.8*	*11.2*
2	*5.06*	*α-pinene*	*0.5*	*9.4*
3	*6.06*	*sabinene*	*0.7*	*6.3*
4	*6.52*	*myrcene*	*1.5*	*4.9*
5	***6.97***	***α-phellandrene***	***8.8***	***2.4***
6	*7.34*	*α-terpinene*	*0.9*	*1.5*
7	*7.57*	*para-cymene*	*1.1*	*15.8*
8	***7.83***	***1,8-cineole (eucalyptol)***	***34.8***	***3.0***
9	*8.38*	*(E)-β-ocimene*	*0.5*	*3.4*
10	*8.77*	*γ-terpinene*	*1.3*	*2.6*
11	*9.85*	*terpinolene*	*1.5*	*2.8*
12	*10.25*	*linalool*	*0.9*	*0.8*
13	*11.10*	*cis-para-menth-2-en-1-ol*	*0.5*	*0.8*
14	*11.81*	*trans-para-menth-2-en-1-ol*	*0.3*	*2.1*
15	*12.05*	*(E)-tagetone*	*0.2*	*2.9*
16	*12.86*	*lavandulol*	*0.6*	*7.6*
17	*12.92*	*para-mentha-1,5-dien-8-ol*	*0.2*	*4.3*
18	*13.41*	*terpin-4-ol*	*2.3*	*1.6*
19	*13.63*	*thuj-3-en-10-al*	*1.0*	*3.2*
20	*13.95*	*α-terpineol*	*0.7*	*1.6*
21	*14.69*	*trans-piperitol*	*0.3*	*1.0*
22	*15.61*	*nerol*	*1.4*	*2.3*
23	***16.20***	***neral***	***10.8***	***3.4***
24	*16.80*	*geraniol*	*3.2*	*3.0*
25	***17.54***	***geranial***	***10.8***	***4.0***
26	*19.84*	*methyl geranate*	*5.2*	*3.8*
27	*21.61*	*neryl acetate*	*2.1*	*4.4*
28	*22.24*	*(E)-methyl cinnamate*	*0.6*	*2.7*
29	*22.46*	*geranyl acetate*	*3.1*	*5.7*
		Total:	***97.7***	

[a][%] figures are their relative proportions as percent of total ion current (TIC).
The dominant compounds are indicated in bold.

Finally, GC/MS analysis of *E. staigeriana* resulted in the identification of 29 compounds representing 97.7% of the oil. The dominant compounds were 1,8-cineole with 34.8%, both neral and geranial with 10.8% and α-phellandrene with 8.8%.

3.2.2. Hexane extracts

Hexane extracts were analysed but the identification and the quantification of compounds were not undertaken due to insufficient time. The chromatograms are available in *Appendix 7*, *8*, *9*, *10* and *11*.

3.3. Antimicrobial activity

3.3.1. Standard growth curves

Optical density measurements at 420 nm and colony plate counts collected for each micro-organism during the logarithmic growth phase allowed establishing a relationship shown in *Fig 1*. Raw data are available in *Appendix 12*.

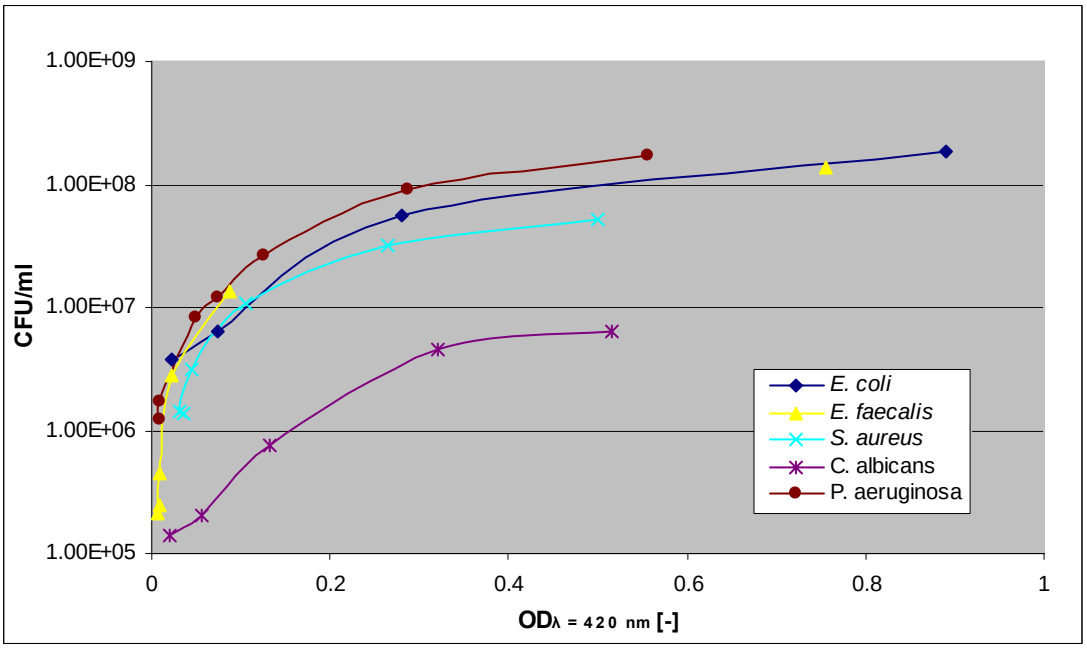


Fig 1 : Relationship between number of CFU/ml and optical density measured at 420 nm for *E. coli*, *E. faecalis*, *S. aureus*, *C. albicans* and *P. aeruginos*a in the logarithmic growth phase.

Curves obtained from the relationship shown in *Fig. 1* indicated clearly the logarithmic growth phase. Initial suspension values for all micro-organisms fitted exactly in the range of 10^5-10^6 CFU/ml suggested by the Oxoid Manual (1995) for the agar disc diffusion tests. This relationship was therefore used to standardize the inoculum concentration.

3.3.2. Agar disc diffusion tests

The *in-vitro* antimicrobial activity of the four *Eucalyptus* species plant extracts against the panel of food-related micro-organisms selected was qualitatively and quantitatively assessed by the presence or absence of inhibition zone and zone diameters. Antimicrobial activities are compared with standard antibiotics such as chloramphenicol, gentamicin and nystatin used as positive controls. Values dispersion or variability was indicated with standard deviation.

The influence of a surfactant as plant extracts dissolver or stabiliser in water-based culture media was graphically represented by comparing the inhibition zone diameters of pure plant extracts with those of plant extracts diluted in 10% (v/v) Tween 80. Dispersion or variability measures were indicated with standard deviation. Populations deemed to be significantly different ($p < 0.05$) were indicated with asterisk.

3.3.2.1. Essential oils

Results from the agar disc diffusion tests for essential oils are summarized in *Table 8*. For the same reason as mentioned in the GC/MS analysis part, antimicrobial activity of *E. gunnii* essential oil was unable to be tested. Raw data are available in *Appendix 13* to *17*.

Table 8 : Antimicrobial activity of investigated essential oils and standard antibiotics against a micro-organisms inoculum concentration of about 10^5-10^6 CFU/ml.

Micro-organisms	Inoculum conc. [a] [CFU/ml]	Inhibition zone diameter [b] [mm]						
		Essential oils				***Antibiotics***		
		E. dives	*E. olida*^{NSW}	*E. olida*^{VIC}	*E. staigeriana*	CH	GE	NY
Gram$^+$								
E. faecalis	3.88E+06	11.1±0.4	11.6±1.1	9.1±0.2	20.3±1.2	22.5±0.7	-	-
S. aureus	2.75E+06	52.3±5.6	25.4±0.5	25.0±2.7	>90	21.9±1.1	-	-
Gram$^-$								
E. coli	6.43E+05	14.8±0.4	10.8±0.7	10.4±0.6	15.8±1.1	21.9±0.9	-	-
P. aeruginosa	3.05E+06	9.1±0.3	nd	nd	7.7±0.4	-	22.8±0.5	-
Yeast								
C. albicans	3.72E+06	15.4±0.8	15.6±1.0	12.6±1.2	26.7±0.7	-	-	11.0±1.0

[a] : results are presented as mean (N=3)
[b] : results are presented as mean ± standard deviation (N=6). The diameter of the discs (Ø = 6 mm) is included.
CH: chloramphenicol 30 µg/disc; GE: gentamicin 30 µg/disc; NY: nystatin 30 µg/disc
- : not tested; nd : not detected; >90 : inhibition zone diameter exceed Petri dishes diameter

According to *Table 8*, the outcomes obtained from the disc diffusion assays indicated that the essential oils from the investigated *Eucalyptus* species displayed a variable degree of antimicrobial activity on the different tested strains. Gram-positive *S. aureus* and yeast *C. albicans* were the most sensitive ones while Gram-negative *P. aeruginosa* was the most resistant.

The influence of using a surfactant as essential oils dissolver or stabiliser in water-based culture media in agar disc diffusion tests is illustrated for each micro-organisms in *Fig. 2* to *6* below.

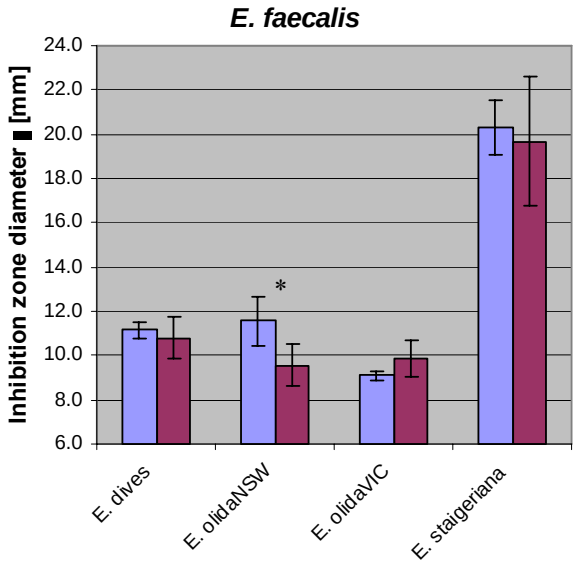


Fig 2 : Comparison of inhibition zone diameters measured for pure EOs and EOs diluted in 10% (v/v) Tween 80 on *E. faecalis*.
[1] : Diameter of the discs (Ø = 6 mm) not included
□ : EOs / ■ : EOs diluted in 10% (v/v) Tween 80
* : $p < 0.05$

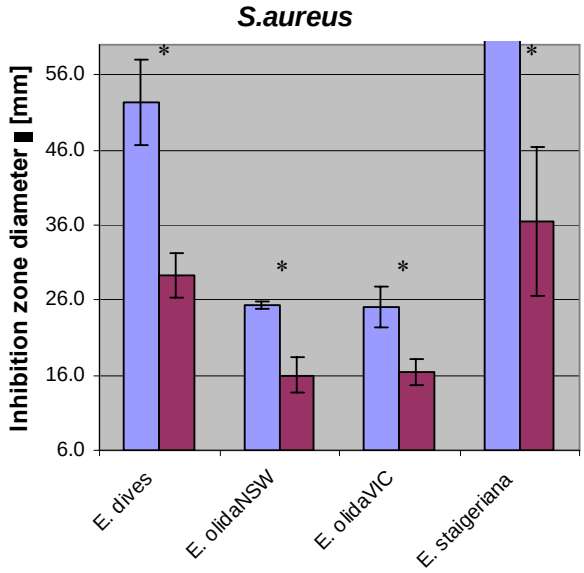


Fig 3 : Comparison of inhibition zone diameters measured for pure EOs and EOs diluted in 10% (v/v) Tween 80 on *S. aureus*.
[1] : Diameter of the discs (Ø = 6 mm) not included
□ : EOs / ■ : EOs diluted in 10% (v/v) Tween 80
* : $p < 0.05$

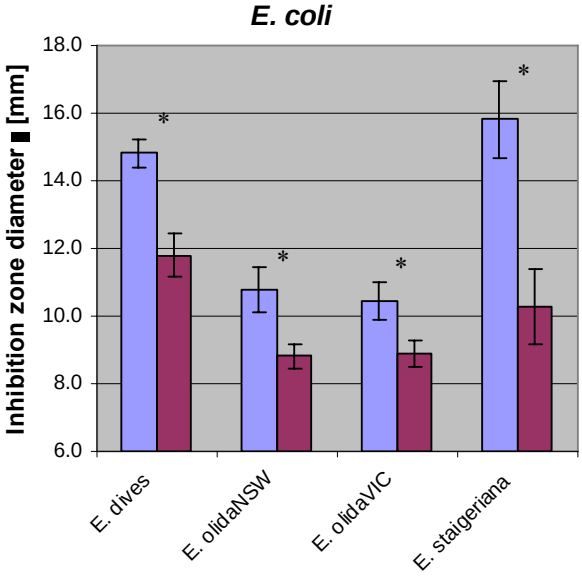


Fig 4 : Comparison of inhibition zone diameters measured for pure EOs and EOs diluted in 10% (v/v) Tween 80 on *E. coli*.
[1] : Diameter of the discs (Ø = 6 mm) not included
▮: EOs / ▮: EOs diluted in 10% (v/v) Tween 80
* : $p < 0.05$

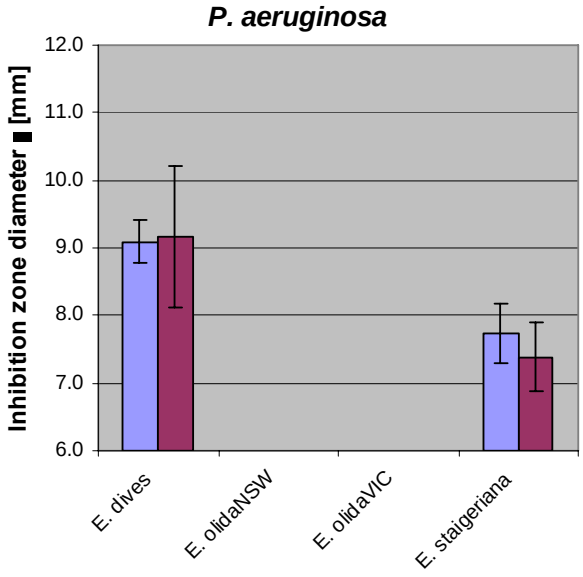


Fig 5 : Comparison of inhibition zone diameters measured for pure EOs and EOs diluted in 10% (v/v) Tween 80 on *P. aeruginosa*.
[1] : Diameter of the discs (Ø = 6 mm) not included
▮: EOs / ▮: EOs diluted in 10% (v/v) Tween 80
* : $p < 0.05$

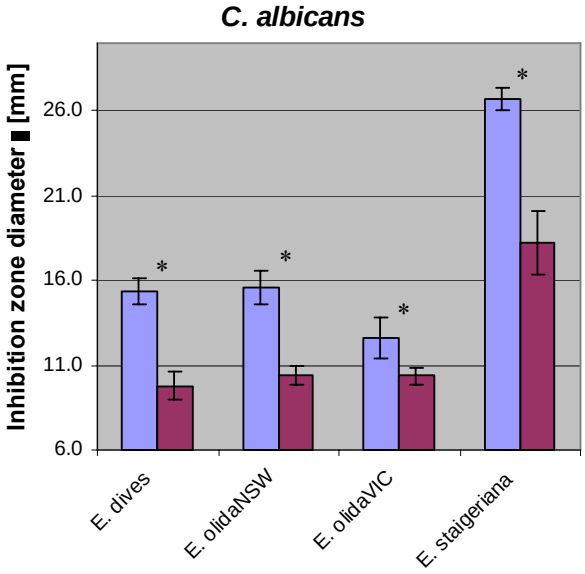


Fig 6 : Comparison of inhibition zone diameters measured for pure EOs and EOs diluted in 10% (v/v) Tween 80 on *C. albicans*.
[1] : Diameter of the discs (Ø = 6 mm) not included
▮: EOs / ▮: EOs diluted in 10% (v/v) Tween 80
* : $p < 0.05$

Regarding *Fig. 2* to *6*, the effect of the surfactant showed to decrease the antimicrobial activity of the essential oils or to have no influence at all. When the inhibition zones of pure essentials oil and essential oils diluted in 10% Tween were deemed to be significantly different ($p < 0.05$), a decrease of the antimicrobial activity was observed. Because *E. staigeriana* essential oil inhibition zone exceeded the Petri dishes diameter against Gram-positive *S. aureus*, its range was left opened.

3.3.2.2. Hexane extracts

Results from the agar disc diffusion tests for hexane extracts are listed in *Table 9*. Raw data are available in *Appendix 13* to *17*.

Table 9 : Antimicrobial activity of investigated hexane extracts and standard antibiotics against a micro-organisms inoculum concentration of about 10^5-10^6 CFU/ml.

Micro-organisms	Inoculum conc. [a] [CFU/ml]	Inhibition zone diameter [b] [mm]							
		Hexane extracts					***Antibiotics***		
		E. dives	*E. gunnii*	*E. olida*^{NSW}	*E. olida*^{VIC}	*E. staigeriana*	CH	GE	NY
Gram$^+$									
E. faecalis	3.88E+06	nd	nd	9.8±0.3	nd	nd	22.5±0.7	-	-
S. aureus	2.75E+06	11.3±2.0	10.3±1.6	19.1±3.7	9.2±2.2	12.4±1.7	21.9±1.1	-	-
Gram$^-$									
E. coli	6.43E+05	nd	nd	9.3±0.5	nd	nd	21.9±0.9	-	-
P. aeruginosa	3.05E+06	nd	nd	nd	nd	nd	-	22.8±0.5	-
Yeast									
C. albicans	3.72E+06	nd	nd	8.7±0.7	nd	nd	-	-	11.0±1.0

[a] : results are presented as mean (N=3)
[b] : results are presented as mean ± standard deviation (N=6). The diameter of the discs (Ø = 6 mm) is included.
CH: chloramphenicol 30 µg/disc; GE: gentamicin 30 µg/disc; NY: nystatin 30 µg/disc
- : not tested; nd : not detected; >90 : inhibition zone diameter exceed Petri dishes diameter

Data given in *Table 9* indicated that hexane extracts showed low antimicrobial activity. Gram-positive *S. aureus* was the only tested strain being sensitive to all *Eucalyptus* species hexane extracts. Gram-negative *P. aeruginosa* did not exhibit any inhibition zones.

The role played by a surfactant as hexane extracts dissolver or stabiliser in water-based culture media in agar disc diffusion tests is illustrated for each micro-organism in *Fig. 7* to *10*. *P. aeruginosa* did not allow any investigation following what has been determined above.

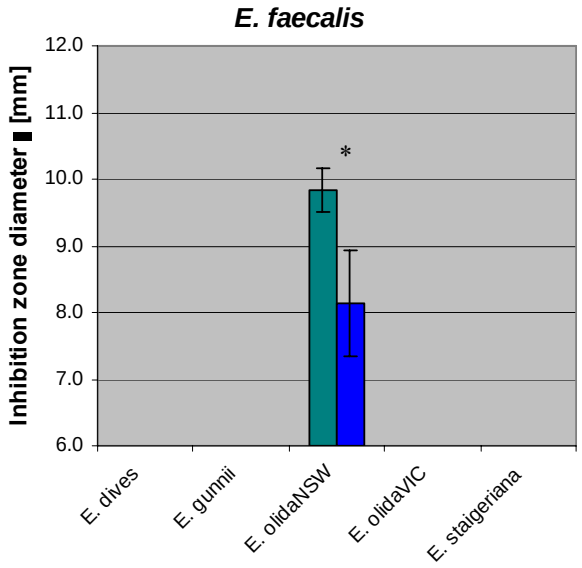


Fig 7 : Comparison of inhibition zone diameters measured for pure HEs and HEs diluted in 10% (v/v) Tween 80 on *E. faecalis*.
[1] : Diameter of the discs (Ø = 6 mm) not included
■ : HE / ■ : HE diluted in 10% (v/v) Tween 80
* : $p < 0.05$

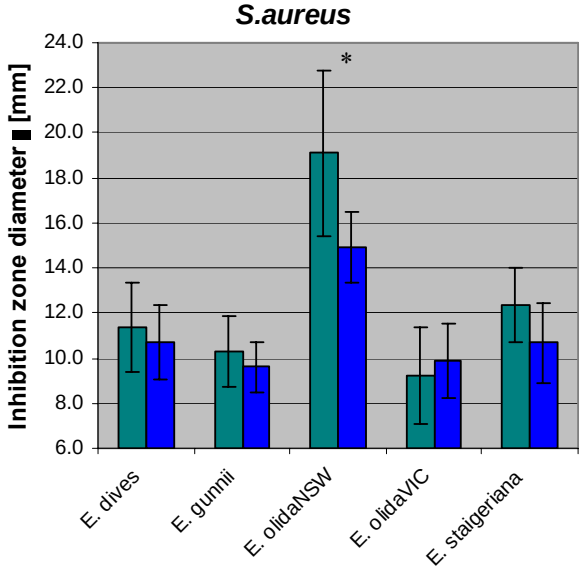


Fig 8 : Comparison of inhibition zone diameters measured for pure HEs and HEs diluted in 10% (v/v) Tween 80 on *S. aureus*.
[1] : Diameter of the discs (Ø = 6 mm) not included
■ : HEs / ■ : HEs diluted in 10% (v/v) Tween 80
* : $p < 0.05$

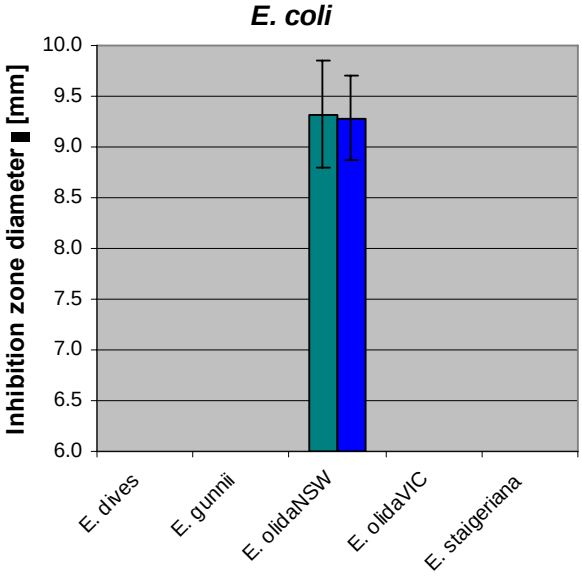


Fig 9 : Comparison of inhibition zone diameters measured for pure HEs and HEs diluted in 10% (v/v) Tween 80 on *E. coli*.
[1] : Diameter of the discs (Ø = 6 mm) not included
■ : HE / ■ : HE diluted in 10% (v/v) Tween 80
* : $p < 0.05$

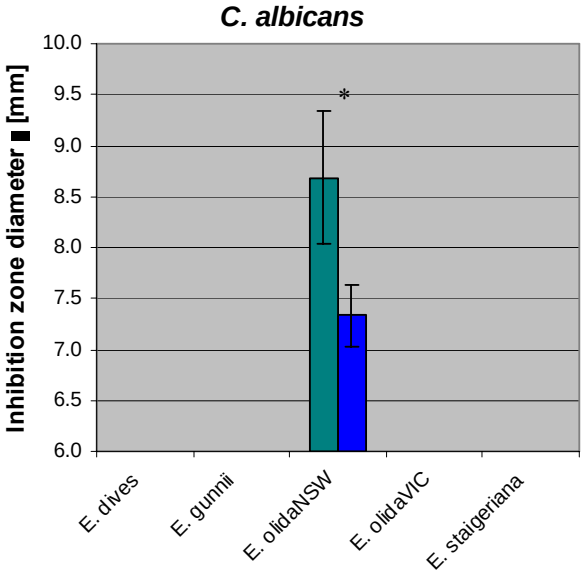


Fig 10 : Comparison of inhibition zone diameters measured for pure HEs and HEs diluted in 10% (v/v) Tween 80 on *C. albicans*.
[1] : Diameter of the discs (Ø = 6 mm) not included
■ : HE / ■ : HE diluted in 10% (v/v) Tween 80
* : $p < 0.05$

Although hexane extracts allowed less comparison between antimicrobial activity of pure hexane extracts and hexane extracts diluted in 10% Tween 80 (cf. *Fig 7* to *10*), the same observations as for the essential oils were noticed. The effect of the surfactant showed to decrease the antimicrobial activity of the hexane extracts or to have no influence at all. When the inhibition zones of pure hexane extracts and hexane extracts diluted in 10% Tween were deemed to be significantly different ($p < 0.05$), a decrease of the antimicrobial activity was observed.

4. Discussion

4.1. Plant extracts

4.1.1. Steam distillation

E. dives and *E. staigeriana* indirect steam distillation has worked well while *E. gunnii* was less successful. At the end of the 3 h steam distillation, *E. gunnii* essential oil amounted to only a few droplets floating on the top of water. Regarding the extraction yield which is less than 0.1% (w/w), *E. gunnii* seemed to have a limited extractable essential oil or a very soluble essential oil. It has been suggested that the essential oil of *E. gunnii* is rich in oxygenated terpenes and that total essential oil content is rather low. It is possible that the essential oil is in fact completely solubilized in the steam distilled water (pers.con. Dr. Matthias Wüst, 01.12.06). In this case, it would have been possible to extract the solubilized oil by addition of NaCl to the steam distilled water before extracting with an organic solvent like diethyl ether, pooling the organic phase and finally, removing the solvent. Unfortunately only a limited amount of plant material was available - an amount that was insufficient to test this hypothesis.

Extraction of *E. olida* essential oils was first attempted using indirect steam distillation but was found to form within the condenser. Moreover, the few essential oil which was collected crystallized and sunk to the bottom of the separatory funnel. It has been concluded that the essential oil of *E. olida* is heavier than water and crystallized in the presence of a small amount of water, due to the high concentration of the amphipathic methyl-cinnamate (mp: 34-37°C; Sigma-Aldrich, 2007). Indeed, Curtis et al. (In Smale et al., 2000) reported that *E. olida* interpopulational studies on essential oil showed (E)-methyl cinnamate levels of 94-99% in fresh leaves. *E. olida* essential oils have therefore been extracted on a Clevenger-type apparatus, which is adapted for collecting essential oils heavier than water.

4.1.2. Solvent extraction

Soxhlet extraction with hexane was carried out to obtain another profile of chemical compounds. While steam distillation had mostly extracted hydrophilic compounds, Soxhlet extraction with hexane removed mostly lipophilic compounds. Moreover, solvent extractions generally allow extracting at lower temperatures (hexane bp: 69°C; Sigma-Aldrich, 2007) than steam distillation, which is recommended to not destroy any thermolabile constituent. Cos et al. (2006) reported that appropriate measures must be taken to guarantee that potential active constituents are not lost, altered or destroyed during the preparation of the extracts. According to this, fresh plant material was therefore preferred to dry plant material for both extraction methods. Leaves were also cut in small strips with a pair of scissors instead of being milled. More particularly, hexane was removed from the extracts under pressure at 40°C and hexane extracts were finally freeze-dried. Hexane extractions were operated without any complication.

4.1.3. Distillation and extraction yields

Curtis et al., (Smale et al., 2000) reported that *E. olida* interpopulational studies on essential oils showed a 2-6% range in oil yield in fresh leaves. The extraction yield of 3.12% obtained for *E. olida*^{VIC} fits into this range while the yield of 1.12% obtained for *E. olida*^{NSW} is lower than expected. As reported by McGimpsey & Douglas (1994) and Salgueiro et al. (1997), the climate, the genotype, the harvesting regime, the watering and the geographical growth location play a role in the total essential oil content. *E. dives* and *E. staigeriana* provided 2.97% and 2.13% extractable content. *E. gunnii* oil extraction was already discussed above (cf. *4.1.1. Steam distillation*).

Hexane extraction yields were in general lower than those obtained for steam distillation. A very small amount of extractable hexane extract was available for *E. staigeriana* and *E. olida*^{VIC}, namely 0.53% and 0.59% respectively, while *E. olida*^{NSW} showed a greatest extractable content of 2.46%. Without taking the yield of *E. gunnii* essential oil into consideration, *E. olida*^{NSW} showed the lowest steam distillation yield but the highest solvent extraction yield. *E. dives* and *E. gunnii* provided an extractable content of 0.96% and 1.47% respectively.

4.2. GC/MS analysis

4.2.1. Essential oils

GC/MS analyses showed that *E. staigeriana* and *E. dives* essential oils contain mostly volatile compounds, the majority of these appearing in the first 20 minutes. On the contrary, essential oils of both *E. olida* specimens showed a content of heavier compounds, most of those coming out after 18 min. (E)-methyl cinnamate, appearing after 22.59 min and 22.29 min, represented 98.8% and 99.4% of the essential oil total content of both *E. olida* specimens. This fits with Curtis et al. (In Smale et al., 2000) which reported that essential oils of *E. olida* specimens showed (E)-methyl cinnamate levels of 94-99% in fresh leaves and suggest that the essential oils extraction was complete.

The identification of major compounds of the four *Eucalyptus* species essential oils showed expected similarities between each species since they are of the same genus. (E)-β-ocimene, linalool and (E)-methyl cinnamate are three compounds they all have in common. α-terpineol is present in *E. dives*, *E. olida*^{NSW} and *E. staigeriana* essential oils. Piperitone and bicyclogermacrene are two compounds which are found in *E. olida*^{VIC} and *E. dives* essential oils. Neral, geraniol, geranial and methyl geranate are four compounds included in *E. olida*^{NSW} and *E. staigeriana* essential oils. *E. dives* and *E. staigeriana* essential oils have a very similar profile as they count 18 compounds in common.

Both *E. olida* specimens have 7 compounds in common, which is less than *E. dives* and *E. staigeriana* have together, although they are from the same species. According to Smale et al. (2000), the chemical composition of both *E. olida* specimen essential oils showed an interspecies variability which seems to depend on the genetic characteristics of the plant and on the conditions under which they have grown.

Literature review revealed a limited amount of research in that area, except for *E. dives*. Small variation were shown between the chemical composition of different *E. dives* essential oils. Oussalah et al. (2006) reported that piperitone with 42.9% and α-phellandrene with 30.0% were found to be the dominent compounds of an *E. dives* essential oil extracted from australian leaves. Delaquis et al. (2002) reported that the heavy end of an *E. dives* essential oil from South Africa was rich in piperitone while light end distillate contained elevated levels of α-phellandrene, α-thujene and α-terpinolene combined with several minor volatiles.

4.2.2. Hexane extracts

Identification and quantification of the compounds in hexane extracts were not undertaken due to insufficient time. With essential oils showing stronger antimicrobial activity, their composition analyses were favoured.

An initial review of the different hexane extracts chromatogram compared to those from essential oils showed some notable peak similarities for both *E.olida* specimens. Both solvent extraction and steam distillation extracted expected methyl-cinnamate since it is an amphipathic compound.

4.2.3. Statistical analysis

The indication of the relative standard deviation helped evaluate accuracy and precision of the manual injections. If relative standard deviation values are rather low, consistency in injection volume and operation is indicated. If relative standard deviation values are rather high, important values differences between the three runs are shown. However, as the equation $RSD = \frac{STDEV}{AVERAGE} * 100$ shows, the content average may have a great incidence on the relative standard deviation calculation following if it is a very small or a very large value.

In the case of both *E. olida* specimens essential oil, the average content of (E)-methyl cinnamate is so dominant in the essential oil composition that other compounds content averages are automatically very low. Considering the equation above, a low denominator gives big relative standard deviation. Moreover, peak areas of some compounds were sometimes so small that the software was not enough sensitive to detect it or distinguish it from the background noise. Relative standard deviations were therefore calculated from two values instead of three, which makes the result varied as well.

Regarding *E. dives* essential oil analyses, most of relative standard deviations are low, especially for the dominant compounds, which indicate that manual injections were well operated. Those of para-mentha-1(7),8-diene, 1,8-cineole and myrcene are slightly higher. This is due to bigger value differences between the three runs. Most of relative standard deviation values are rather high for both *E. olida* essential oils, except for the dominent compound (E)-methyl cinnamate. In this case, very low content averages of minor compounds influenced the relative standard deviation calculation. Relative standard deviations of *E. staigeriana* essential oil analyses are low, which indicate that manual injections were well operated.

4.3. Antimicrobial activity

4.3.1. Standard growth curves

According to The Oxoid Manual (1995), one of the very critical factors for accuracy and precision in disc diffusion tests is the inoculum preparation. It is therefore important to use a technique which will always yield a uniform suspension of the correct number of organisms, 10^5-10^6 CFU/ml. The relationship established for each micro-organism between optical density and microbial growth (CFU/ml) during the logarithmic growth phase allowed the adequate inoculum concentration to be reached. A routine procedure was operated so that inoculates dilution and application were standardized for each test. Colony plate counts controls carried out for each agar disc diffusion test showed that the technique was accurate and precise.

4.3.2. Agar disc diffusion tests

4.3.2.1. Essential oils

Data obtained from the disc diffusion assays indicated that the investigated essential oils displayed a variable degree of antimicrobial activity. Gram-positive *S. aureus* was the most sensitive tested microbial strain, especially to *E. staigeriana* essential oil which cleared all the Petri dishes area. With inhibition zones of around 52 mm, *E. dives* essential oil showed a great antimicrobial activity against *S. aureus*. According to the literature (Cos et al., 2006; Burt, 2004; Dupont et al., 2006), Gram-positive bacteria were found to be in general more sensitive than Gram-negative bacteria.

Both *E. olida* specimens essential oil did not show any antimicrobial activity against Gram-negative *P. aeruginosa* while only weak inhibition zones (7-9 mm) were measured for *E. dives* and *E. staigeriana* oils. *P. aeruginosa* is known to have a high level of intrinsic resistance against many antimicrobials and antibiotics due to a very restrictive outer membrane barrier, highly resistant even to synthetic drugs (Skočibušić et al., 2006).

The four *Eucalyptus* species essential oils showed high antimicrobial activity against the yeast *C. albicans* as they were all more effective than 30 µg of nystatin, the antibiotic standard. The highest antimicrobial activity was observed for *E. staigeriana* oil with inhibition zones measured around 27 mm. Although *E. staigeriana* oil showed a slightly lower antimicrobial activity than *E. dives* oil against *P. aeruginosa*, it is the essential oil which showed the highest antimicrobial activity potential. Indeed, it showed to be more effective than 30 µg of chloramphenicol against *S. aureus* and 30 µg of nystatin against *C. albicans*. Antimicrobial activity of both *E. olida* specimens essential oil was not significantly different confirming the GC/MS results which showed essential oils profiles with respect to the major compound, (E)-methyl cinnamate.

4.3.2.2. Hexane extracts

Inhibition zones observed for hexane extracts demonstrated varying levels of antimicrobial activity as well. Again, Gram-positive bacteria were found to be more sensitive than Gram-negative. *S. aureus* was the only strain to be sensitive to all the five *Eucalyptus* species hexane extracts. With inhibition zones of around 19 mm (which were the best by far because other hexane extracts turned around 10-12 mm), *E. olida*^{NSW} hexane extract showed the highest antimicrobial activity against *S. aureus*. This excepted, only *E. olida*^{NSW} hexane extract exhibited weak inhibition zones against *E. faecalis*, *E. coli* and *C. albicans*. Considering that *E. olida*^{NSW} appeared to have the best extraction yield (2.46%) from all, it is therefore not surprising that *E. olida*^{NSW} hexane extract was more effective. The more compounds extracted the better the chance is to exhibit antimicrobial activity.

4.3.2.3. Influence of the surfactant

Literature review reported that Tween 80 increased the antimicrobial properties of plant extracts because of its intrinsic antimicrobial properties as disinfectant (Burt, 2004). In this study, the effect of the surfactant showed to decrease the antimicrobial activity of both essential oils and hexane extracts or to not have any influence at all. When inhibition zones of pure plant extracts and plant extracts diluted in 10% Tween were deemed to be significantly different ($p < 0.05$), a decrease of the antimicrobial activity was observed. Tween 80 increased twice the antimicrobial activity of plant extracts, *E. olida*^{VIC} essential oil against *E. faecalis* and *E. olida*^{VIC} hexane extracts against *S. aureus*, but there was not any significantly difference ($p > 0.05$)

5. Conclusion and perspective

The four *Eucalyptus* species essential oils displayed a variable degree of antimicrobial activity while hexane extracts showed low antimicrobial activity. Gram-positive bacteria were found to be in general more sensitive than Gram-negative bacteria. *S. aureus* was the most sensitive strain to both essential oils and hexane extracts while *P. aeruginosa* was the most resistant.

The role of growth location in the antimicrobial properties of *E. olida* species appeared to affect the amount of extractable essential oils and hexane extracts. The effect of the surfactant showed to decrease the antimicrobial activity of the plant extracts or to not have any influence at all. The effect of the surfactant on the antimicrobial properties seemed to be micro-organisms dependent more than plant extracts dependent.

As perspectives, the minimum inhibitory concentration (MIC) of the plant extracts which have exhibited antimicrobial properties could be determined by the broth microdilution method. Identification and quantification of the compounds in hexane extracts could be carried out from the obtained chromatograms. The dominant compounds identified by GC/MS in essential oils showing antimicrobial properties with the screening could be ordered in pure form and submitted to agar disc diffusion and broth microdilution test methods to determine their intrinsic antimicrobial properties and their influence in the antimicrobial activity that the plant extract has shown.

As next step, *in vivo* antimicrobial tests could be undertaken with the essential oils showing antimicrobial properties in order to determine whether they have potential use as natural agent for food preservation. The Charles Sturt University cheese factory manufactures some cheddar with some dried cut pieces of native bush foods like alpine pepper, bush tomato (*Solanum centrale*), forest berry, lemon myrtle (*Backhousia citrodora*) and native mint (*Prostanthera incisa*). It could be possible to introduce essential oils showing antimicrobial activity with an appropriate inhibitory concentration in the cheese instead of dried cut pieces.

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7. Appendix

Appendix 1 : pattern of the indirect steam distillation apparatus
Appendix 2 : pattern of the Clevenger-type apparatus
Appendix 3 : *E. dives* essential oil GC/MS chromatogram and raw data
Appendix 4 : *E. olida*^{NSW} essential oil GC/MS chromatogram and raw data
Appendix 5 : *E. olida*^{VIC} essential oil GC/MS chromatogram and raw data
Appendix 6 : *E. staigeriana* essential oil GC/MS chromatogram and raw data
Appendix 7 : *E. dives* hexane extract GC/MS chromatogram
Appendix 8 : *E. gunnii* hexane extract GC/MS chromatogram
Appendix 9 : *E. olida*^{NSW} hexane extract GC/MS chromatogram
Appendix 10 : *E. olida*^{VIC} hexane extract GC/MS chromatogram
Appendix 11 : *E. staigeriana* hexane extract GC/MS chromatogram
Appendix 12 : raw data of the standard growth curves determination
Appendix 13 : Table of *E. coli* agar disc diffusion tests collected data
Appendix 14 : Table of *P. aeruginosa* agar disc diffusion tests collected data
Appendix 15 : Table of *E.faecalis* agar disc diffusion tests collected data
Appendix 16 : Table of *S. aureus* agar disc diffusion tests collected data
Appendix 17 : Table of *C. albicans* agar disc diffusion tests collected data