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ORIGINAL ARTICLE

The *in vitro* antibiofilm activity of selected culinary herbs and medicinal plants against *Listeria monocytogenes*

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Keywords
antiadhesion, antibiofilm, biofilm, *Listeria monocytogenes*, natural products.

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Abstract

Aims: The antibiofilm activity of extracts obtained from selected herbs, spices, beverages and commercially important medicinal plants was investigated on *Listeria monocytogenes*.

Methods and Results: The growth and development of the biofilm was assessed using the crystal violet (CV) assay. The respiratory activity was assessed using the 2, 3-bis [2-methyloxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide (XTT) reduction assay. The majority of extracts tested prevented cell adhesion to the polyvinyl chloride (PVC) surface. Seven of the 15 extracts reduced biofilm adhesion of both the clinical and the type strains by at least 50%. In contrast, inhibition of a preformed biofilm was more difficult to achieve, with only three extracts (*Rosmarinus officinalis, Mentha piperita and Melaleuca alternifolia*) inhibiting the growth of both strains by at least 50%.

Conclusions: Although most extracts were able to reduce initial cell attachment, inhibition of growth in a preformed biofilm was more difficult to achieve.

Significance and Impact of the Study: The ability to reduce biofilm biomass as shown by several plant extracts warrants further investigation to explore the use of natural products in antibiofilm adhesion.

Introduction

Micro-organisms exist as free-floating cells or, more often, in a community of cells attached to a substrate. This sessile form of life is referred to as a biofilm. By definition, a biofilm is a community of cells attached to either a biotic or abiotic surface enclosed in a complex exopolymeric substance (EPS) (Costerton *et al.* 1999; Mah and O'Toole 2001; Hugo and Russell 2004). Biofilms allow micro-organisms to trap nutrients and withstand hostile environmental conditions, a key feature for their survival (Poulsen 1999). Approximately 60% of human infections are reported to be a result of biofilm formation on human mucosa.

Listeria monocytogenes is a Gram-positive bacterium, which is pathogenic to both humans and animals. Infection results in listeriosis, a serious condition caused by eating food contaminated with the bacterium. The disease primarily affects pregnant women, newborns and adults with weakened immune systems posing potential risk to human health (Doyle *et al.* 2007). *Listeria monocytogenes* has become a very difficult pathogen to control because of its unusual mechanisms of survival under adverse environmental conditions (Gandhi and Chikindas 2007). The ability to form a biofilm adds greater resistance to deleterious agents such as antibiotics, sanitizers and disinfectants, thus allowing it to overcome food preservation and other control processes (Gandhi and Chikindas 2007).

Plant-derived compounds have gained widespread interest in the search to identify the alternatives for microbial control (Essawi and Srour 2000). The compounds are widely accepted because of the perception that they are safe and have a long history of use in folk medicine for the prevention and treatment of diseases and infections (Guarrera 2005). Previous research on plants and the active constituents has almost exclusively focused on the effects of these against planktonic bacteria with little emphasis on the biofilm forms that are more resistant

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to antimicrobial agents and therefore more difficult to control. This study was therefore undertaken to investigate for the first time the *in vitro* antibiofilm potential of some commonly used dietary and medicinal plants against the clinically relevant *L. monocytogenes* biofilms.

Materials and methods

Preparation of plant material

Table 1 lists the plants that have been reported to possess a broad spectrum of antimicrobial activity and were therefore selected to investigate the potential in anti-biofilm activity (Cowan 1999). Most of the plant materials were obtained in powder form from Warren Chem Specialties, Herbs-A-Plenty, and spices were obtained from Unilever South Africa.

However, materials obtained in a more crude form were initially air dried at room temperature and ground to a fine powder in a coffee grinder. The garlic bulbs were peeled to expose the flesh before cutting into small pieces and ground in a mortar and pestle.

Preparation of extracts

Ground plant material (approx. 15 g) was weighed in a conical flask, followed by the addition of 150 ml of dichloromethane/methanol (CH_2CI_2/MeOH, 1 : 1). The flask was sealed with foil and incubated at room temperature for 3 h with shaking at intervals. Following incubation, the extracts were vacuum filtered using a Buchner funnel through Whatman (No. 1) filter paper. The residue collected was resuspended in fresh 100 and 50 ml of CH_2CI_2/MeOH for the second and third extractions, respectively. The filtrates obtained from all three filtration processes were pooled and concentrated using a rotavapor (BUCHI Rotavapor R-200, Flawil, Switzerland) at 50°C under vacuum. The concentrate was resuspended in a minimal volume of CH_2CI_2/MeOH and transferred to a glass petri dish. The petri dish was placed in a vacuum oven (Vismara SrI VO65, Zerbo, Italy) at 60°C to dry for at least 24 h. Following drying, the extracts were scraped out, weighed to obtain percentage yields and stored in sealed vials.

Preparation of bacterial cultures

An American Type Culture Collection (ATCC 19111) and a clinical (CI 001) strain of *L. monocytogenes* were used in this study. The glycerol stock cultures were prepared and kept at −80°C prior to use. They were revived by streaking onto sterile tryptone soya agar (TSA) and incubated at 37°C for 18 h. Following incubation on agar, the bacteria were inoculated into sterile tryptone soy broth (TSB) and incubated

Table 1 Plants investigated for potential antibiofilm activity (Ankri and Mirel 1999; Bozin *et al.* 2007; Coetzee *et al.* 2008; Cowan 1999; Schempp *et al.* 1999; van Wyk and Gericke 2000; van Wyk and Wink 2004)

Scientific name	Common name	Uses and relevance
Agathosma betulina	Buchu	It is commonly used as a flavouring agent and its medicinal attributes include the treatment of kidney and urinary tract infections
Allium sativum	Garlic	The bulbs and leaves are used for flavour. Medicinally, it is used to reduce risks associated with high blood pressure. The active constituent, allicin possesses a range of antimicrobial properties
Aloe vera	Aloe	It has applications in cosmetics and is also taken in health drinks. Medicinally, it is used for the treatment of wounds and burns
Aspalathus linearis	Rooibos	It is taken as a herbal tea and possesses good antioxidant properties. The extracts are potential natural antifungal agents
Camellia sinensis	Green tea	It is taken as a herbal tea. Medicinal uses include treatment of skin disorders
Echinacea angustifolia	Echinacea	It is used for the treatment of respiratory tract infection, wounds and snake bites
Glycyrrhiza glabra	Liquorice	It is used as a sweetening agent. Medicinally, it is used for respiratory infections
Hypericum perforatum	St John's wort	It is used for cosmetic preparations and therapeutically for the relief of anxiety and sleeping disorders. The extracts possess a wide range of anti-inflammatory and antimicrobial properties
Leptospermum petersonii	Lemon scented tea tree	Commonly taken as a lemon-flavoured herbal tea. Traditional uses include treatment of coughs and colds
Melaleuca alternifolia	Tea tree	The essential oil is normally used for topical skin care products, toothpaste and in aromatherapy
Mentha piperita	Peppermint	It is commonly used as a flavouring agent. Medicinally, it relieves nausea, vomiting and respiratory infections
Rosmarinus officinalis	Rosemary	It is a flavouring agent, and the oils are ingredients in cosmetic products. The oils have strong anti-infective properties against a range of micro-organisms
Syzygium aromaticum	Clove	It is used for flavour and spicing in households. Medicinally, it treats toothaches and skin sores
Thymus vulgaris	Thyme	It is a common flavouring agent, and it possesses strong antiseptic properties
Vaccinium macrocarpon	Cranberry	It is commonly taken as a health juice. Medicinally, it is used to treat urinary tract infections

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in a shaking incubator at 37°C overnight. The overnight cultures were standardized to yield a concentration of $1{\cdot}0 \times 10^6$ CFU ml^{-1} before biofilm formation. This was achieved by diluting the overnight cultures with TSB to obtain an absorbance ($OD_{590\ nm}$) of 0·02 using a spectrophotometer (Helios α; Thermoelectron Corp., London, UK).

Determination of antibiofilm activity

Inhibition of cell attachment

The CH_2CI_2/MeOH extracts at a concentration of 1 mg ml^{-1} were tested for their potential antiadhesion properties. The concentration of 1 mg ml^{-1} was chosen based on the previous study (Ríos and Recio 2005) which reported that only extracts exhibiting MIC values <1 mg ml^{-1} are noteworthy. Stock solutions of 2 mg ml^{-1} were prepared using water. Acetone was used as a co-solvent for extracts which were difficult to dissolve in water. One hundred microlitres of the extracts was added to the 96-well microtitre plates, and equal volumes of water and ciprofloxacin (0·00125 mg ml^{-1}) (MIC value) were added as negative and positive controls, respectively. The standardized culture (1.0×10^6 CFU ml^{-1}) (100 μl) was then pipetted into the wells to yield a final volume of 200 μl in each well. The cultures were added into the wells in triplicate, and sterile TSB was added as an additional control to ensure that the media remained sterile during the course of the experiment. The plates were sterile sealed with sealing tape and incubated at 37°C for 8 h without shaking to allow cell attachment and biofilm development. Following incubation, the modified crystal violet assay was performed to assess biofilm biomass, and the results expressed as percentage inhibition (Eqn 1).

$$\text{Percentage inhibition} = \frac{OD_{\text{Negative control}} - OD_{\text{Experimental}}}{OD_{\text{Negative control}}} \times 100 \quad (1)$$

Inhibition of biofilm growth and development

A biofilm was allowed to preform for 4 h prior to the addition of plant extracts at a final concentration of 1 mg ml^{-1} in the wells. Biofilm formation was achieved by aliquoting 100 μl of a standardized ($1{\cdot}0 \times 10^6$ CFU ml^{-1}) *L. monocytogenes* culture into a 96-well microtitre plate. The duplicate plates were incubated at 37°C for 4 h to allow cell attachment. Following the 4 h incubation, 100 μl of each plant extract was added to yield a final concentration of 1 mg ml^{-1} in the wells, and equal volumes of water and ciprofloxacin at a concentration of 0·00125 mg ml^{-1} (MIC value) were added as negative and positive controls, respectively. The plates were further incubated for 24 h before the crystal violet and XTT reduction assays were performed concurrently.

Assessment of biofilm biomass (Crystal violet staining assay)

Cell attachment was indirectly assessed using the modified crystal violet (CV) assay (Djordjevic *et al.* 2002). Following incubation, the plates were washed three times with sterile distilled water to remove loosely attached cells. The plates were air-dried and then oven-dried at 60°C for 45 min. Following drying, the wells were stained with 100 μl of 1% crystal violet and incubated at room temperature for 15 min after which the plates were washed three times with sterile distilled water to remove unabsorbed stain. The semi-quantitative assessment of biofilm formation was performed by adding 125 μl of ethanol to destain the wells. One hundred microlitres (100 μl) of the destaining solution was then transferred to a new plate, and the absorbance determined at 590 nm using a microplate reader (Universal microplate reader EL_X 800; Bio-tek instruments Inc., Winooski, USA). The mean absorbance ($OD_{590\ nm}$) of the samples was determined, and percentage inhibition obtained (Eqn 1). Percentage inhibition was plotted against the extract concentrations using Microsoft Excel®.

Assessment of biofilm metabolic activity [2, 3-bis (2-methyloxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide reduction assay]

The metabolic (respiratory) activity of the biofilm was assessed using the modified [2, 3-bis (2-methyloxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide] (XTT) reduction assay. The XTT (cell proliferation kit) was supplied by Roche Diagnostics (Mannheim, Germany) in a pack containing the XTT-labelling reagent and the electron coupling reagent in separate vials. The working solution was prepared prior to use in quantities sufficient for use at that time. The XTT-labelling and electron coupling reagents were thawed prior to use in a 37°C water bath. The working solution was prepared by mixing 5 ml of XTT-labelling reagent with 0·1 ml of electron coupling reagent. Fifty microlitres (50 μl) of the working solution was added to each well following the washing of the plates. The plates were covered and incubated at 37°C for 2 h after which the absorbance was measured at 450 nm. The data obtained were analysed using Microsoft Excel®. The antimicrobial effect of each extract was determined by comparing the results of the controls to those of the extracts.

Results

Inhibition of cell attachment

Generally, the use of single extracts to reduce *L. monocytogenes* attachment to PVC was successful (Fig. 1); however, none of the extracts was able to inhibit cell attachment completely, including the positive control, ciprofloxacin.

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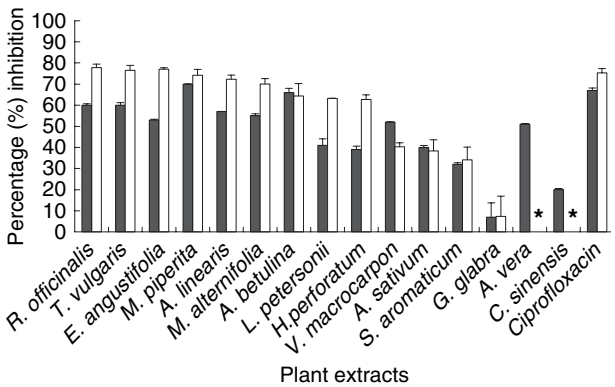

Figure 1 The effect of plant extracts on the attachment of *Listeria monocytogenes* ATCC 19111 (■) and clinical CI 001 (□), expressed as percentage inhibition. *Plant extracts that enhanced growth of biofilm (negative inhibition).

The extracts that showed the best activity comparable to ciprofloxacin (75%) were *Rosmarinus officinalis* (78%), *Echinacea angustifolia* (77%), *Thymus vulgaris* (77%) and *Mentha piperita* (74%). In total, seven of fifteen extracts successfully inhibited cell attachment of both clinical and ATCC isolates by at least 50%. The ATCC 19111 isolate was more resistant than the clinical CI 001 isolate as evidenced by the lower percentage inhibition values.

Inhibition of biofilm growth and development

The extracts that showed at least 50% reduction in cell attachment were used in the preformed biofilm assay. The extracts showed variable effects on the development of a preformed biofilm (Fig. 2). Some extracts exhibited good antibiofilm activity against *L. monocytogenes*, while others enhanced biofilm growth shown as 0% inhibition (Fig. 2). Three of the nine extracts showed good antibiofilm activity with percentage inhibition values of > 50% against both isolates. These extracts were *R. officinalis*, *M. piperita* and *Melaleuca alternifolia*. In total, six extracts inhibited the development of either isolates by at least 50% with the ATCC isolate proving to be more sensitive than the clinical isolate. These results show that inhibition of biofilm growth proved to be more difficult to achieve than cell attachment.

Assessment of metabolic activity of a preformed biofilm

Generally, most extracts (*R. officinalis*, *M. piperita*, *M. alternifolia*, *E. angustifolia* and *T. vulgaris)* reduced the activity of biofilms of both isolates compared to the control biofilm, while an increase in activity was observed with *Aloe vera* and *Vaccinium macrocarpon* (Fig. 3). The results indicate that in addition to reducing biomass, most of the extracts had an effect on the metabolic activity.

Discussion

The success of plant extracts in inhibiting cell attachment as shown in this study is a promising tool for reducing microbial colonization on surfaces and epithelial mucosa which subsequently leads to infections (Bavington and Page 2005). The ease with which the plant extracts inhibited cell attachment is the confirmation of previous reports where it was found that inhibition of cell attachment to a substrate is easier to achieve than inhibiting the growth of an already established biofilm (Cerca *et al.* 2005). The success in inhibiting cell attachment can be explained in a number of ways. First, the cell attachment is the initial stage in biofilm formation following surface

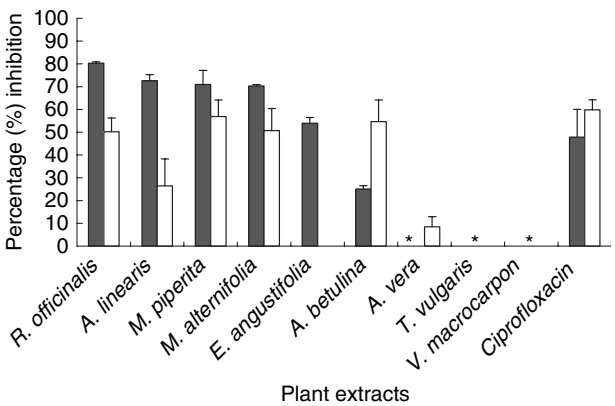

Figure 2 The effect of plant extracts on the growth and development of preformed *Listeria monocytogenes* ATCC 19111 (■) and clinical CI 001 (□) biofilms, expressed as percentage inhibition. *Plant extracts that enhanced growth of biofilm (negative inhibition).

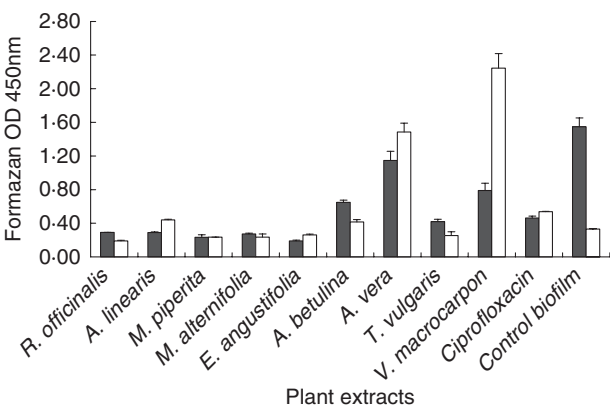

Figure 3 The effect of single extracts on the metabolic activity of preformed *Listeria monocytogenes* ATCC 19111 (■) and clinical CI 001 (□) biofilms.

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conditioning which creates a favourable environment for bacterial attachment (Kumar and Anand 1998). Surface conditioning is achieved by the adsorption of substances that include nutrients, organic and inorganic molecules that are important for the growth of the cells, which in turn promotes cell adhesion. It can therefore be postulated that pretreatment of the surface with plant extracts produced an unfavourable film that promotes detachment, thereby reducing the surface adhesion. Milk constituents and chitosan have been reported to inhibit cell attachment (Cai *et al.* 1994; Sharon and Ofek 2002). Some researchers have also demonstrated the success of coating medical devices with biocides such as silver to reduce microbial adhesion (Klueh *et al.* 2000; Hashimoto 2001). The increased sensitivity observed in the ATCC isolate compared to the clinical isolate confirms previous reports where clinical isolates of *L. monocytogenes* were able to withstand harsh environmental conditions compared to type strains (Vialette *et al.* 2003). The resistance of clinical isolates in both Gram-positive and Gram-negative bacteria to antibiotics is well known (Aiello *et al.* 2003). This has been attributed to prior exposure to antimicrobial agents during therapy or other disinfection processes resulting in secondary/acquired resistance (Kontoyiannis and Lewis 2002).

Inhibition of growth in a preformed biofilm was also successful; however, the extent of inhibition was much less compared to initial attachment. The reduced antibiofilm activity towards a preformed biofilm is evidence that cells in a biofilm are more resistant to antimicrobial agents compared to free-floating cells (Frank and Koffi 1990; Krysinski *et al.* 1992). Several factors have been attributed to the resistance in biofilms; the presence of an EPS (glycocalyx) that surrounds biofilm cells, the negative charge on the EPS that restricts the penetration of molecules by charge attraction thereby imparting resistance to the biofilm (Hugo and Russell 2004). Other mechanisms include degradation or inactivation of the antimicrobial agent and efflux pumps that expel drugs from the cells (Hugo and Russell 2004). The slower growth rate in biofilms compared to planktonic cells as a result of reduced nutrient and oxygen supply has been reported as another factor (Costerton *et al.* 1999; Mah and O'Toole 2001). Many antimicrobial agents have been shown to be effective against microorganisms that are rapidly growing and dividing, with only a few exceptions known. As a consequence, the slow growth rate observed in biofilms makes them less susceptible to antimicrobial agents that are normally effective against metabolically active cells (Lewis 2001).

The enhanced biofilm development observed with some extracts in this study may be because of the presence of certain compounds within the extracts that provided a conditioning film promoting microbial adhesion. Some compounds that include albumin, gelatin, fibrinogen and casein have been reported to promote microbial adhesion (Meadows 1971). The enhancement effect observed in this study correlates with previous reports that some natural compounds promote the growth of micro-organisms (Ofek *et al.* 2003; Sandasi *et al.* 2008). Sandasi *et al.* (2008) showed that some essential oil components promote the growth and development of a preformed *L. monocytogenes* biofilm *in vitro*. Ofek *et al.* (2003) reported that plant lectins may enhance the adsorption of cells onto a surface by acting as receptors of bacterial glycans, thereby promoting cell attachment. The enhanced attachment observed upon exposure to some extracts can therefore be postulated to be a result of the presence of compounds that favour the development of these biofilms.

The results of the effect of extracts on metabolic activity do not show a correlation between the biomass and metabolic activity. Previous work, however, has shown a relationship between the biomass and metabolic activity. Machado *et al.* (2006) reported an inverse relationship between *S. sciuri* biomass and metabolic activity when exposed to quaternary ammonium compounds. However, a different result was observed when the same compound was tested on *Pseudomonas fluorescens*. Cocuaud *et al.* (2005) reported variation on the activity of *Candida albicans* biofilms following the exposure to caspofungin. Some isolates did not respond to the caspofungin, while some showed reduced activity. These reports show that variation in microbial response to antimicrobial agents in a biofilm is common and may only be understood when the mechanisms involved are thoroughly explored.

Conclusions

The results of the study show potential antibiofilm activity for most plant extracts used as they are able to inhibit the initial stage of biofilm formation and subsequent growth. Although most extracts were able to inhibit cell attachment, the inhibition of growth in a preformed biofilm was more difficult to achieve. Isolation and identification of the constituents that exhibit antibiofilm properties might be essential to include these as alternatives in the control of biofilms.

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