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Anti-listerial activity of ethanolic extracts of medicinal plants, *Eremophila alternifolia* and *Eremophila duttonii* in food homogenates and milk

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Abstract

Listeria monocytogenes is a foodborne pathogen responsible for the disease listeriosis. Ethanolic extracts from two native Australian traditional medicinal plants, Eremophila alternifolia and Eremophila duttonii, have been found to inhibit the growth of L. monocytogenes. These plants were investigated for their ability to control the growth of L. monocytogenes in full cream milk and skim milk and in diluted homogenates of salami, pâté and brie cheese. Time-kill experiments indicated that the extracts were able to inhibit the growth of L. monocytogenes in food at 4°C and 37°C. However, components in the food appeared to inhibit the anti-listerial activity of the extracts, necessitating higher concentrations to control microbial growth relative to those used in laboratory media. Preliminary investigations suggested that the active components responsible for the antimicrobial activity of each extract are most likely to be terpenes or sterols. Our study suggests that natural products derived from medicinal plants have the potential to be used as food preservatives.

Key words: antibacterial activity, medicinal plants, phytochemicals
1. Introduction

*Listeria monocytogenes* is a Gram positive bacterium responsible for the severe food-borne illness, listeriosis. Most reports associate listeriosis with the consumption of contaminated ready-to-eat foods such as dairy products, processed or cured meat and poultry, salads, seafood and uncooked eggs (Garcia, Canamero, Lucas, Omar, Pulido & Galvez, 2004). Although most healthy humans are not significantly affected by low doses of the bacteria, the pathogen can be more potent for people with weak immune systems or during pregnancy (Charpentier & Courvalin, 1997; Datta, 2003). Among severe infections, listeriosis has been associated with a mortality rate as high as 30-40% (Datta, 2003).

A range of synthetic antimicrobial agents has been used to inhibit the growth of *L. monocytogenes* in foods, although concerns about the safety of these chemicals have increased consumer demand for naturally processed food. Hence, there has been recent interest in testing natural products, including plant-derived compounds, for anti-listerial properties as these may be used as natural preservatives in foods (Nair, Vasudevan & Venkitanarayanan, 2005). The essential oils of clove, bay, cinnamon, thyme and pimento have all been found to inhibit the growth of *L. monocytogenes* in food, at concentrations less than 1% (Hao, Brackett & Doyle, 1998; Smith-Palmer, Stewart & Fyfe, 2001; Vrinda Menon & Garg, 2001). We have previously found that the ethanolic extracts from the leaves of *Eremophila alternifolia* and *Eremophila duttonii* inhibited the growth of *L. monocytogenes* in standard laboratory media (Shah, Cross & Palombo, 2004). The Aboriginal people of Australia have traditionally used these plants as sources of medicine. *E. alternifolia* was used for general sickness, pain and respiratory infections, and *E. duttonii* was used to treat respiratory tract infections, sore throats, skin cuts, painful ears and inflamed eyes (Semple, Reynolds, O’Leary & Flower, 1998; Palombo & Semple, 2001). The
effectiveness of these plants as potential food preservatives, in particular their activity against *L. monocytogenes*, was explored in the current study.

2. Materials and Methods

2.1. Preparation of the plant extracts

Plant extracts from *Eremophila alternifolia* R. Br. (Myoporaceae) (leaves) and *Eremophila duttonii* F. Muell. (Myoporaceae) (leaves) were supplied in concentrated form by Dr Susan Semple, University of South Australia, Adelaide. The extraction method has been described previously (Semple *et al.*, 1998). Freeze-dried plant material was milled to a powder and 30-50 g placed in a glass column plugged with cotton wool and then extracted into two volumes of ethanol (250-300 ml) at room temperature for 24 h. These two portions were then combined and concentrated in a rotary evaporator at 40°C. The concentrated extract (drug extract ratio of 6-10:1) was centrifuged at 10 000 x g for 20 min at 20°C, and the supernatant filtered through a series of sterile, ethanol resistant filters (AP25 and AP15 pre-filters, and GVHP 0.22 μm filter, Millipore, Bedford, MA, USA). The sterilised extract was stored in pre-sterilised glass vials at –70°C. The concentrations of the *E. alternifolia* and *E. duttonii* extracts were determined as 160 mg/ml and 154 mg/ml, respectively.

2.2. Preparation of food samples

Ten grams of salami, pâté or brie cheese were added to 90 mL of one-quarter-strength buffered peptone water (pH 7.2) in blender bags and homogenised in a Stomacher (Lab-blender 400, FSE, Australia) until smooth. Full cream and skim milk were made from powders and
reconstituted with distilled water according to the manufacturer’s instructions. All prepared food was autoclaved at 121°C for 15 mins prior to use to eliminate contamination from organisms that may already be present in the food. The pH of the food samples ranged between 6 and 7.

2.3. Bacterial media

Brain Heart Infusion Agar (BHIA), Brain Heart Infusion Broth (BHIB), Mueller-Hinton Agar (MHA), and Mueller-Hinton Broth (MHB) were supplied by Oxoid Pty Ltd (Basingstoke, UK). All media was prepared in deionised water and autoclaved at 121°C for 15 minutes prior to use.

2.4. Bacterial cultures

Listeria monocytogenes (ACM 98) was obtained from the culture collection at Swinburne University of Technology. Bacteria were sub-cultured on BHIA at 37°C prior to being grown in BHIB overnight. All overnight (ON) cultures were standardised by matching to the McFarland 0.5 turbidity standard using sterile saline to produce approximately 1.5 x 10^8 colony forming units (cfu) per ml.

2.5. Plate-hole diffusion assay

Two hundred microlitres of a standardised ON culture was inoculated into 15 ml of MHA (at 45°C) and mixed gently. The inoculated agar was poured into a sterile Petri dish and allowed to set at room temperature. Wells were cut into the set agar using a sterile cork-borer (6 mm diameter) and 20 µl of the test substance (extract diluted 1:200 in water; final concentrations of
0.8 mg/ml and 0.77 mg/ml for the *E. alternifolia* and *E. duttonii* extracts, respectively) was dispensed into each well. Plates were left at room temperature for 30 mins to allow the liquid to diffuse into the agar before overnight incubation at 37°C. All assays were carried out in triplicate. A clear zone of inhibited microbial growth surrounded substances exhibiting antimicrobial properties and zones with a diameter greater than 6 mm were considered positive.

2.6. Determination of minimum bactericidal concentration (MBC)

The test substance was dispensed into an appropriate media (MHB, reconstituted milk or food homogenates) and serial, two-fold dilutions were made in the same media to produce dilutions ranging from 1:10 to 1:64000. Ten microlitres of standardised ON culture was added to each dilution. Following overnight incubation at 37°C, 50 µl from each dilution was spread onto BHIA plates and incubated overnight at 37°C. The procedure was performed in MHB, diluted homogenates of salami, pâté and brie cheese, reconstituted full cream milk and reconstituted skim milk. The minimum bactericidal concentration (MBC) was defined as the lowest concentration of plant extract that completely prevented microbial growth, determined by visible inspection of the BHIA plates. MBC assays were carried out in duplicate.

2.7. Time-kill assay

The MBC of test substance in the appropriate media (MHB, reconstituted milk or food homogenates) was inoculated with 10 µl of standardised ON culture, mixed by vortexing and incubated at 4°C or 37°C. A 100 µl sample was collected every hour for 4 hours and diluted in 900 µl of sterile saline. Serial 10-fold dilutions were carried out in sterile saline (0.85% w/v
NaCl), a viable count was performed after overnight incubation at 37°C and the number of cfu/ml was recorded. Untreated control samples (i.e. without extracts) were incubated under the same conditions. Time-kill assays were carried out in duplicate.

2.8. Thin layer chromatography (TLC) and bioautography

Five microlitres of neat plant extract samples were applied 2.5 cm from the base of aluminium-backed silica plates (Sigma-Aldrich) cut to size (5 x 10 cm). After drying, the plates were developed at room temperature with a mixture of toluene:ethanol (96:4). After the solvent was allowed to evaporate, the plates were placed into sterile square Petri dishes (10 x 10 cm). Two hundred microlitres of BHIB bacterial culture were added to 15 ml of molten MHA, mixed well, poured over the TLC plate and allowed to set. The plate was incubated at 37°C overnight, sprayed with 2 mg/ml of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide), incubated at 37°C for a further 30 minutes and examined for zones of bacterial growth inhibition (clear zones within a purple background).

2.9. TLC spray reagents

A freshly prepared solution of 1% (v/v) anisaldehyde and 2% (v/v) sulphuric acid, for detection of terpenes and sterols, was sprayed onto developed TLC plates. The plates were heated at 100°C for 20 minutes (Krebs Heusser & Wimmer, 1969).
3. Results and discussion

A plate-hole diffusion assay was used to determine the anti-listerial activity of the plant extracts. Both extracts inhibited the growth of *L. monocytogenes*, producing clear zones of growth inhibition. *E. alternifolia* produced an average zone of inhibition (ZOI) of 8.8 mm while *E. duttonii* produced an average ZOI of 9.6 mm, from triplicate assays.

MBC assays were performed in each of the prepared foods (diluted salami, pâté or brie cheese homogenates) or milks to determine the lowest concentration of extract that produced bactericidal effect. Both extracts displayed similar MBCs in salami, pâté and full cream milk, while a higher concentration of *E. duttonii* extract was required to prevent growth in skim milk and brie cheese. Table 1 shows the results of the MBC assays.

The inoculated foods required substantially higher concentrations of both extracts to produce a bactericidal effect when compared to the inoculated MHB. This suggests that components in the food interacted with the extract to alter its antimicrobial efficacy. A recent study indicated that the presence of organic matter interfered with the antibacterial efficacy of these plant extracts when tested in laboratory media (Tomlinson & Palombo, 2005). It would be of interest to investigate the relationship between the efficacy of these extracts as food preservatives and interference by ingredients in different foods.

Time-kill assays were used to determine the lethal exposure times for each MBC less than 1:10 at 4°C and 37°C. When viable cells remained after the 4 hour incubation period, the data was graphed and a line of best fit was constructed to enable an estimation of the time required to reduce the viable cell count to zero. Table 2 summarises the lethal exposure times for *L. monocytogenes* at the corresponding MBCs. All food samples supported the growth of *L.*
monocytogenes at 4°C and 37°C (except for salami at 4°C where the inoculum remained constant after 14 days). Doubling times were in the order of days at 4°C and hours at 37°C in all foods.

The most promising results in food were E. duttonii and E. alternifolia in salami at 37°C (both MBCs 1:40, lethal exposure time 1 hour) and E. duttonii in pâté at 4°C (MBC 1:40, lethal exposure time 1 hour). An interesting observation was the noticeable difference between kill times in full cream milk and skim milk. It is possible that the additional fat in full cream milk provides protection for the bacterial cells. It should be noted that the inoculation level used in these experiments was much higher than that expected to contaminate food. Accordingly, the lethal exposure time is expected to be much shorter in ‘real life’ situations.

In this study, the whole plant extracts were added to the food and this imparted a characteristic green colour and grassy aroma that would certainly be disagreeable to consumers. Identification of the active phytochemicals in the extracts could lead to purified compounds being used for their anti-listerial activity, and thus minimise the negative aesthetics produced by the whole extract.

Preliminary investigations used TLC to separate the extracts, with a mixture of toluene:ethanol (96:4) as the developing solvent. Developed TLC plates were overlayed with inoculated agar to locate the components contributing to the extract’s antimicrobial activity. E. alternifolia presented two antimicrobial components, while seven bands from the E. duttonii extract showed antimicrobial activity in addition to the origin. A freshly prepared anisaldehyde-sulphuric acid spray reagent suggested that the active components from the separated plant extracts are terpenes or sterols, although the active components are likely to be terpenes as these are readily extracted with ethanol and are known to display antibacterial properties (Cowan, 1999). In fact, the active component of the E. duttonii extract showing anti-staphylococcal activity has been identified as a terpene (Shah et al., 2004). Interestingly, the extracts showed
different MBCs in brie cheese, skim milk and MHB. This may be indicative of different phytochemical components producing the antibacterial activity in each extract, supporting the bioautography findings that different active components are present in the extracts.

In summary, this study has shown that while whole plant extracts can be used to control the growth of *L. monocytogenes* in a variety of foods, the isolated bioactive components from the extracts may have greater potential as food preservatives. A limitation of the current study is that only one strain of *L. monocytogenes* was tested so further testing against additional strains is warranted. In addition, further analysis is required to assess the efficacy of extracts in foods and their toxicity to humans since medicinal plant extracts do not have ‘generally regarded as safe’ (GRAS) status.

**Acknowledgements**

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References


Table 1

The minimum bactericidal concentration (MBC) of plant extracts against *L. monocytogenes* in Mueller-Hinton broth (MHB), milk and food homogenates

<table>
<thead>
<tr>
<th>Extract</th>
<th>MHB</th>
<th>Salami</th>
<th>Pâté</th>
<th>Brie cheese</th>
<th>Full cream milk</th>
<th>Skim milk</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. alternifolia</em></td>
<td>1:320</td>
<td>1:40</td>
<td>1:10</td>
<td>1:20</td>
<td>1:10</td>
<td>1:20</td>
</tr>
<tr>
<td><em>E. duttonii</em></td>
<td>1:1600</td>
<td>1:40</td>
<td>1:10</td>
<td>&gt;1:10</td>
<td>1:10</td>
<td>1:80</td>
</tr>
</tbody>
</table>
Table 2

Comparison of the lethal exposure times for *L. monocytogenes* at the minimum bactericidal concentration (MBC) for plant extracts in different growth media

<table>
<thead>
<tr>
<th>Plant extract</th>
<th>Growth medium</th>
<th>MBC</th>
<th>Time required to eliminate all microbial growth (hours)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>4°C</td>
<td>37°C</td>
</tr>
<tr>
<td><em>E. alternifolia</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E. alternifolia</td>
<td>MHB&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1:320</td>
<td>21 3</td>
</tr>
<tr>
<td>Salami</td>
<td>1:40</td>
<td>26 1</td>
<td></td>
</tr>
<tr>
<td>Pâté</td>
<td>1:10</td>
<td>9 1</td>
<td></td>
</tr>
<tr>
<td>Brie cheese</td>
<td>1:20</td>
<td>22 1</td>
<td></td>
</tr>
<tr>
<td>Full cream milk</td>
<td>1:10</td>
<td>ND&lt;sup&gt;b&lt;/sup&gt; 3</td>
<td></td>
</tr>
<tr>
<td>Skim milk</td>
<td>1:20</td>
<td>23 1</td>
<td></td>
</tr>
<tr>
<td><em>E. duttonii</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E. duttonii</td>
<td>MHB</td>
<td>1:1600</td>
<td>12 1</td>
</tr>
<tr>
<td>Salami</td>
<td>1:40</td>
<td>8 1</td>
<td></td>
</tr>
<tr>
<td>Pâté</td>
<td>1:10</td>
<td>1 1</td>
<td></td>
</tr>
<tr>
<td>Full cream milk</td>
<td>1:10</td>
<td>ND&lt;sup&gt;a&lt;/sup&gt; 3</td>
<td></td>
</tr>
<tr>
<td>Skim milk</td>
<td>1:80</td>
<td>22 16</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Mueller-Hinton Broth.

<sup>b</sup> Not determined. The number of viable cells observed during these time-kill assays was above a level that could be accurately counted. Visible inspection suggested that there was no obvious decline in cell numbers after 4 hours and that the rate of decline in cell viability would have been accordingly gradual.