The Growth Inhibitory Activity of Tasmannia lanceolata (Poir.) A.C. Sm against the Food-poisoning Pathogen Yersinia enterocolitica

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ABSTRACT

Introduction: Yersinia enterocolitica is a major source of food poisoning via the consumption of contaminated meat products, causing acute gastroenteric yersiniosis. Tasmannia lanceolata has been widely documented for its antiseptic properties, repressing the growth of an extensive range of bacteria. Despite this, Tasmannia lanceolata has yet to be been tested for its inhibitory capacity against Y. enterocolitica. Methods: T. lanceolata leaf and berry extracts were prepared by maceration and growth inhibitory activity against a clinical strain of Y. enterocolitica was examined by disc diffusion assays. The MIC values of the extracts were determined to quantify and compare their relative efficacies. Toxicity was determined using an Artemia franciscana nauplii bioassay. Results: T. lanceolata leaf and berry extracts displayed potent growth inhibitory activity in the disc diffusion assay against Y. enterocolitica. The ethyl acetate and chloroform leaf extracts (MICs of 30 and 53 µg/mL respectively) and the hexane berry extract (MIC = 34 µg/mL) were particularly potent growth inhibitors. The methanol and water extracts of both the berry and leaf, as well as the leaf ethyl acetate extract, also had strong growth inhibitory activity against Y. enterocolitica, albeit with a higher MIC values (250-300µg/mL). All other extracts had lower efficacy, although their MIC values also indicated good inhibitory activity (with the exception of the chloroform berry extract). When assessed for toxicity, all T. lanceolata extracts were non-toxic (LC50 values >1000 µg/mL) in the Artemia franciscana bioassay. Conclusion: The non-toxicity of the T. lanceolata berry and leaf extracts, combined with the potent inhibitory bioactivity observed against Y. enterocolitica, demonstrates their potential as therapeutic agents in the prevention and treatment of yersiniosis. Key words: Yersinia enterocolitica, Yersiniosis, Antioxidant, Zoonotic, Tasmannia lanceolata, Tasmanian pepper.

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INTRODUCTION

Tasmannia lanceolata (Poir.) A.C.Sm (Tasmanian pepper) is a shrub indigenous to the woodlands and cool temperate rainforests of Tasmania and the south-eastern coast of the mainland Australia, to the north of Sydney.1 It is a medium-large shrub of approximately 2-5 m in height. The berries leaves and bark of this plant are traditionally used medicinally. Australian Aborigines used T. lanceolata as a therapeutic agent to treat stomach disorders.1,2 T. lanceolata has also been used traditionally for the treatment and cure of colic, venereal diseases, skin disorders and stomach aches.2 European colonists also documented the therapeutic properties of T. lanceolata and the bark was commonly used as a substitute for other herbal remedies and to treat scurvy (due to its high anti-antioxidant content).2,3 Despite its extensive ethnobotanical usage, limited scientific studies into the therapeutic properties of T. lanceolata have been performed. It has been theorised that the plant’s high antioxidant capacity may provide therapeutic benefits. Indeed, investigations of T. lanceolata within our laboratory have highlighted the inhibition of bacterial growth by T. lanceolata berries, leaves and peppercorns against panels of several distinct pathogenic and food spoilage bacteria* and protozoal parasites.7

The genus Yersinia (Y.) includes over than a dozen species of facultative anaerobes.8 Members of the genus are widespread in the environment but are also extensively present within animal populations. These bacteria are clinically important opportunistic pathogens and can cause human illness. The zoonotic aspect of these bacteria also poses a unique set of challenges in preventing infection, as they can persist in non-human hosts for an indefinite period until they encounter new hosts to infect. Diseases caused by Yersinia spp. vary in extremity and mortality, from the bubonic/pneumonic plagues (Yersinia pestis) to the yersiniosis (Yersinia enterocolitica).9,10 Yersiniosis is an acute, bacterial gastroenteric infection and accounts for >100,000 illnesses annually in the United States.11 Characterized by abdominal aching, fever and/or diarrhoea, Y. enterocolitica infections frequently originate through the ingestion of infected food/water. Indeed, almost 90% of all reported cases originate from this mode of infection.12 The prevention of yersiniosis is complicated by the fact that Y. enterocolitica can grow at 4°C. Therefore, refrigeration alone does not afford adequate protection from contracting the illness.13 Although it is seldom life-threatening, the economic drain caused through temporary incapacitation from yersiniosis makes probing for effective treatment strategies particularly important. One tactic involves the utilization of natural resources such as plants that have been documented for their antibacterial capabilities. These relatively untapped reservoirs may impede Y. enterocolitica growth and thus may offer new management options for yersiniosis. Despite the documented ability of T. lanceolata to inhibit the growth of many bacterial species, to the best of our knowledge there have been no studies focusing on T. lanceolata against Y. enterocolitica growth. T. lanceolata berry and leaf extracts were prepared and their antibacterial potential was assessed against this pathogen.
**MATERIALS AND METHODS**

**Plant collection and extraction**

Seedless, semi-dried *T. lanceolata* berries and dried leaf materials were acquired from Go Wild Harvest, Australia and stored at -30°C. Extracts were prepared as previously described. Briefly, plant materials were thawed and ground into a coarse powder. One-gam quantities of the ground plant materials were weighed into tubes in triplicate, followed by the addition of 50 mL of water, methanol, hexane, chloroform or ethyl acetate respectively. All solvents were obtained from Ajax Fine Chemicals, Australia (AR grade). The berry and leaf materials were extracted in each solvent for 24 hr at 4°C with gentle shaking. The extracts were filtered through with filter paper (Whatman No. 54) under vacuum, followed by drying by rotary evaporation in an Eppendorf concentrator 5301. The dried extracts were weighed and resuspended in 10 mL deionised water (containing 1% DMSO).

**Qualitative phytochemical studies**

Phytochemical analyses of the extracts for the presence of alkaloids, flavonoids, phenolic compounds, polyesters, anthraquinones, cardiac glycosides, saponins, tannins and triterpenoids were conducted as previously described. 

**Antioxidant capacity**

The antioxidant capacity of each extract was assessed using the DPPH free radical scavenging method with modifications. Briefly, ascorbic acid references (0-25 µg per well) were prepared and the absorbances recorded at 515 nm. All assays were completed alongside controls on each plate and all tests performed three times in triplicate. The antioxidant capacity (based off of DPPH free radical scavenging ability) was calculated for each extract and expressed as µg ascorbic acid equivalents per gram of the original plant material extracted.

**Clinical *Yersinia enterocolitica* strain**

The clinical isolate strain of *Yersinia enterocolitica* used in this study was supplied by Ms. Jane Giffins of the School of Natural Sciences Griffith University, Australia. Confirmation of bacterial identity was confirmed as previously described. All growth studies were performed using nutrient agar (Oxoid Ltd., Australia) under aerobic conditions. Incubations were at 30°C and the bacterium was subcultured and maintained in nutrient broth at 4°C. Subculture purity was periodically confirmed as previously described.

**Evaluation of antimicrobial activity**

The antimicrobial activity of the *T. lanceolata* extracts were determined using a modified disc diffusion assay. Briefly, 100 µL of *Y. enterocolitica* was grown in 10 mL of fresh nutrient broth until they reached a count of ~10⁶ cells/mL. Aliquots (100 µL) of the bacterial suspensions were spread onto plates and each extract was tested for antibacterial activity using 6 mm sterilised filter paper discs. Discs were infused with 10 µL of *T. ferdinandiana* extracts, allowed to dry and placed onto the inoculated plates. The plates were allowed to stand at 4°C for 2 hr before incubation at 30°C for 24 hr. The diameters of the inhibition zones were measured to the closest whole millimetre. Each assay was performed three times, with internal triplicates (n=9). Mean values (± SEM) are reported in this study. Standard discs of chloramphenicol (2 µg) were prepared and used as positive controls to compare antibacterial activity. Filter discs infused with 10 µL of distilled water were used as a negative control.

**Minimum inhibitory concentration (MIC) determination**

The Minimum Inhibitory Concentrations (MIC) of the extracts was determined as previously described. Briefly, the plant extracts were diluted in deionised water and tested across a range of concentrations. Discs were infused with 10 µL of the extract dilutions, allowed to dry and placed onto inoculated plates. The assay was performed as outlined above and graphs of the zone of inhibition versus concentration were plotted. Linear regression was used to determine MIC values.

**Toxicity screening**

**Reference toxin for toxicity screening**

Potassium dichromate (K₂Cr₂O₇) (AR grade, Chem-Supply, Australia) was prepared using 0.22 µM filter-sterilised, distilled water (4 mg/mL) and serially diluted in synthesised, artificial seawater for use in the *Artemia franciscana* nauplii bioassay.

**Artemia franciscana nauplii toxicity screening**

Toxicity was tested using an adapted *Artemia franciscana* nauplii mortality assay. Briefly, 400 µL of seawater containing ~43 (mean 43.2, n = 155, SD 14.5) *A. franciscana* nauplii were added to wells of a 48 well plate and immediately used in the bioassay. Volumes of 400 µL of reference toxin or the diluted plant extracts were transferred to the wells and incubated at 25 ± 1°C under artificial light (1000 Lux). A 400 µL volume of seawater was used as a negative control and was run in triplicate for each plate. All treatments were performed three times, each with internal triplicates (n=9). The wells were checked at regular intervals and the deceased nauplii were counted. Nauplii death was determined if no appendage movement was observed within a 10 sec interval (or any period thereafter). After 24 hr, all nauplii were sacrificed and counted to determine the total % mortality per well. The LC₅₀ with 95% confidence limits for each treatment was calculated using probit analysis.

**Statistical analysis**

Data is expressed as the mean ± SEM of three independent experiments, each with technical triplicates (n=9).

**RESULTS**

Extraction of 1 g of the *T. lanceolata* plant materials with the solvents yielded dried plant extracts ranging from 9 mg (*T. lanceolata* leaf hexane extract) to 144 mg (methanolic *T. lanceolata* leaf extract; Table 1). Aqueous and methanolic extracts generally gave relatively high yields of dried extracted material compared to the other extracts. The dried extracts were resuspended in 10 mL of deionised water (containing 1% DMSO), resulting in the extract concentrations shown in Table 1.

Qualitative phytochemical studies showed little difference between the aqueous and methanolic extracts. However, there were notable differences between these and the ethyl acetate extracts. High levels of phenolics (both water soluble and insoluble) were extracted in the aqueous and methanolic samples. There were substantially lower levels detected in the corresponding ethyl acetate extracts. Similarly, there was a lower level of flavonoids detected in the ethyl acetate extracts than the corresponding aqueous and methanolic extracts. Triterpenes were detected in both methanolic and ethyl acetate extracts, although they were absent in the aqueous extracts.

To assess the inhibitory activity of the crude *T. lanceolata* plant extracts against *Y. enterocolitica*, 10 µL aliquots of each were screened with a disc diffusion assay. Antibacterial activity against *Y. enterocolitica* was observed in all 10 extracts tested (Figure 1). The methanolic berry extract was the most potent inhibitor of growth, with inhibition zones of 11.3 ± 0.3 mm. Similarly, strong inhibitory zones were observed in ethyl...
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acetate leaf extract (10.0 ± 0.6 mm) as well as the hexane (11.0 ± 0.0 mm) and ethyl acetate (11.0 ± 0.6 mm) berry extracts. The chloramphenicol control had inhibitory zones of 9.0 ± 0.6 mm which is notably less than several of the T. lanceolata extracts.

The antimicrobial efficacy was further quantified by determining the MIC values (Table 2). All the extracts were determined to be potent inhibitors of Y. enterocolitica growth, with MIC <1000 μg/mL for all extracts except the chloroform berry extract. The T. lanceolata leaf extracts were generally more potent Y. enterocolitica growth inhibitors than the corresponding berry extracts. Indeed, a MIC of 30 μg/mL was determined for the ethyl acetate T. lanceolata leaf extract.

All extracts were initially screened at 2000 µg/mL in the Artemia nauplii assay as a measure of toxicity (Figure 2). For comparison, the reference toxin potassium dichromate (1000 µg/mL) was also tested in the bioassay. The potassium dichromate reference toxin was rapid in its onset, promoting nauplii death within the first 3 hr of exposure, with 100% mortality evident following 4-5 hr (unpublished results). Similarly, all the T. lanceolata extracts displayed significant mortality rates following 24 hr exposure (>50%).

DISCUSSION

Despite the initial efficacy of clinically used antibiotics, recent increases in bacterial resistance have made the development of new antibiotic
Table 2: Minimum inhibitory concentration (µg/mL) of the plant extracts and LC₅₀ values (µg/mL) in the Artemia nauplii bioassay.

<table>
<thead>
<tr>
<th>Extract / Control</th>
<th>MIC</th>
<th>LC</th>
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<tbody>
<tr>
<td>TLLW</td>
<td>370</td>
<td>2665</td>
</tr>
<tr>
<td>TLLM</td>
<td>318</td>
<td>2096</td>
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<tr>
<td>TLLC</td>
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<tr>
<td>TLLB</td>
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<tr>
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<tr>
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</tr>
<tr>
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<td>3132</td>
</tr>
<tr>
<td>PD</td>
<td>-</td>
<td>186</td>
</tr>
<tr>
<td>SW</td>
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</tbody>
</table>

Numbers indicate the mean MIC and LC₅₀ values of triplicate determinations. - indicates no inhibition.

Figure 2: The lethality of the T. lanceolata extracts (2000 µg/mL) and the potassium dichromate control (1000 µg/mL) towards Artemia franciscana nauplii after 24 hr exposure. TLLW = aqueous T. lanceolata leaf extract; TLLM = methanolic T. lanceolata leaf extract; TLLC = chloroform T. lanceolata leaf extract; TLLH = ethyl acetate T. lanceolata leaf extract; TLBL = chloroform T. lanceolata berry extract; TLLB = aqueous T. lanceolata berry extract; TLLC = methanolic T. lanceolata berry extract; TLLD = ethyl acetate T. lanceolata berry extract; PD = potassium dichromate control; SW = artificial seawater control. Results are expressed as mean % mortality ± SEM.

therapies a high priority. A parallel decrease in the introduction of new antibiotic therapies in recent years has further compounded this problem. As a result, interest in re-evaluating medicinal plants for new antibiotic chemotherapies has escalated substantially in recent years. Our study was undertaken to investigate the potential of the endemic Australian plant T. lanceolata to inhibit the growth of Y. enterocolitica, thereby blocking yersiniosis and treating it once it is established. T. lanceolata was selected for this study as it has a history of therapeutic use to treat microbial infections, and numerous recent investigations have reported on its antibacterial properties against a broad panel of bacterial pathogens, including several bacteria associated with diarrhea and dysentery. T. lanceolata extracts have also recently been reported to inhibit the proliferation of the gastrointestinal protozoan parasite Giardia duodenalis, indicating its therapeutic potential against both prokaryotic and eukaryotic pathogens. However, despite the relative wealth of information into the therapeutic potential of T. lanceolata, it is yet to be comprehensively studied for antibacterial activity against many bacterial species associated with food poisoning. Here we report growth inhibitory activity for T. lanceolata berry and leaf extracts against the bacterial cause of the gastrointestinal disease yersiniosis; an acute infection acquired through the ingestion of food (particularly pork) contaminated with Y. enterocolitica.

Potent Y. enterocolitica growth inhibitory activity was evident in the T. lanceolata fruit and leaf extracts. Indeed, with the exception of the chloroform berry extract, MIC values of substantially <1000µg/mL were calculated for all extracts. The ethyl acetate and chloroform leaf extracts (MICs of 30 and 53 µg/mL respectively) and the hexane berry extract (MIC = 34 µg/mL) were particularly potent growth inhibitors. The berry and leaf methanolic and water extracts, as well as the ethyl acetate leaf extract, also exhibited strong growth inhibitory activity against Y. enterocolitica; albeit with higher MIC values (250-300µg/mL). Therefore, these extracts show good potential in the prevention and treatment of yersiniosis and are potential targets for future drug discovery. Furthermore, given that T. lanceolata berries and leaves are edible herbs and spices, their addition to foods would not only provide pleasant flavour enhancements, but could also inhibit food spoilage and the chances of contracting food poisoning (including yersiniosis).

Detailed analyses into the phytochemistry of the T. lanceolata fruit and leaf extracts was beyond the scope of this study. However, several notable compounds have been previously been reported in T. lanceolata extracts and essential oils. These include multiple monoterpenoids (e.g. 1, 8-cineole, terpinen-4-ol, α-pinene and β-pinene) and sesquiterpenoids (particularly polygodial), flavonoids (including quercetin and rutin), other phenolics (including coumaric acid and caffeic acid) and hydrocarbons. Many of these compounds have also been isolated from other plant species and have been shown to have potent antimicrobial activity. Therefore, these components may also contribute to the Y. enterocolitica growth inhibitory properties of the extracts tested in this study. Of particular note, relatively high abundances of the sesquiterpenoid polygodial have been reported in T. lanceolata berry and leaf extracts, with higher relative levels detected in the berry extracts. Indeed, polygodial can account for approximately 40% of commercial T. lanceolata essential oil components. Interestingly, several studies have reported the therapeutic properties of this compound, including its antibacterial, antifungal, anti-hyperalgesia, anti-inflammatory, anti-largolic and vasorelaxation activities. Other structurally similar sesquiterpenoids have also been reported in similar T. lanceolata extracts. Of particular interest, salidroside was present in all inhibitory T. lanceolata berry extracts. Salidroside has been linked with antibacterial and anti-parasitic activity in multiple plants. Plants of the genus Warburgia are known to have significant levels of salidroside and several Warburgia spp. have been reported to have strong antimicrobial and antiparasitic activities. T. lanceolata berry and leaf extracts have also been reported to contain an abundance of gallotannin components. Gallotannins have been reported to inhibit the growth of a broad spectrum of microbial species via binding cell surface lipoteichoic acid and proline-rich cell surface proteins, and by inhibiting glucosyltransferase enzymes. The stilbene combretastatin A1 was also putatively identified in all T. lanceolata berry extracts, although the relatively low peak size indicates that it is present in low abundance. Combretastatin A1 are well known for their potent ability to block cancer cell progression and induce apoptosis by binding intracellular tubulin, thereby disrupting microtubule formation. Combretastatin A1 act in a similar fashion to that of colchicine (N-[7S]-1,2,3,10-tetramethoxy-9-oxo-5,6,7,9-tetrahydrobenzo[a]heptlen-7-yl)
acetylomide) by binding the colchicine's binding site in tubulin.\textsuperscript{41} Thus, the \textit{T. lanceolata} combretastatin's may block bacterial replication events.

The findings reported here also indicate that the \textit{T. lanceolata} berry and leaf extracts were nontoxic, with \textit{LC}_{50} values substantially >2000 \mu g/mL. Toxicity was assessed in this study using the test organism \textit{A. francisca}. The lack of toxicity of the \textit{T. lanceolata} extracts in our study indicates that the extracts are safe for medicinal usage. This is hardly surprising as \textit{T. lanceolata} is highly nutritious and has long been used as a spice (berries) and herb (leaves). However, whilst the extracts examined in this report have potential as \textit{Y. enterocolitica} growth inhibitory agents, caution is needed before these compounds can be applied to medicinal purposes. Toxicity towards \textit{A. francisca} has previously been shown to correlate well with toxicity towards human cells for many toxins.\textsuperscript{7} However, further studies are required to determine whether this is also true for the \textit{T. lanceolata} extracts examined in these studies. The results of this study indicate that the \textit{T. lanceolata} extracts warrant further study due to their \textit{Y. enterocolitica} growth inhibitory activity. Purification and identification of the bioactive components is needed to examine the mechanisms of action of these agents.

**CONCLUSION**

The results of this study demonstrate the potential of \textit{T. lanceolata} berry and leaf extracts to inhibit \textit{Y. enterocolitica} growth. The mid-low polarity ethyl acetate, chloroform and hexane \textit{T. lanceolata} extracts were particularly potent growth inhibitors, with MIC values < 100 \mu g/mL quantified. However, before being deemed acceptable for therapeutic uses, further cell line toxicity studies are required to verify the safety of these extracts. Furthermore, studies aimed at the purification and identification of the bioactive components are required to examine the mechanisms of action of these extracts.

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**CONFLICT OF INTEREST**

The authors have no conflicts of interest.

**ABBREVIATIONS**

DMSO: Dimethyl sulfoxide; \textit{LC}_{50}: The concentration required to achieve 50\% mortality; MIC: Minimum inhibitory concentration.

**REFERENCES**


