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Published

2021

Journal Title

Pharmacognosy Communications

Version

Version of Record (VoR)

DOI

10.5530/pc.2021.2.24

Pharmacogn. Commn. 2021;11(2):133-137.
A multifaceted peer reviewed journal in the field of Pharmacognosy and Natural Products
www.phcogcommn.org

Original Article

Evaluating the Antimicrobial Potential of *Eucalyptus baileyana* F. Muell. and *Eucalyptus major* (Maiden) Blakely against the Fish Spoilage-causing Bacterium *Shewanella putrefaciens*

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ABSTRACT

Introduction: *Eucalyptus baileyana* (Bailey's stringy bark) and *Eucalyptus major* (Queensland grey gum) have been previously used as antimicrobials against a variety of ailments. This study evaluated the effectiveness of *E. baileyana* and *E. major* as inhibitory agents against *Shewanella putrefaciens*, a bacterium widely associated with fish spoilage. **Methodology:** *E. baileyana* and *E. major* extracts were prepared using the leaves of each plant with methanol or water as the extraction solvent. Growth inhibition and minimal inhibitory concentrations were determined against *S. putrefaciens* through disc diffusion assays. MIC values were subsequently quantified to evaluate the extracts efficacies as antibacterial agents. Finally, the toxicity of each extract was determined using the *Artemia franciscana* nauplii bioassay. **Results:** *E. baileyana* aqueous and methanolic leaf extracts inhibited the growth of *S. putrefaciens* in the disc diffusion assay, with MIC values of 1411 and 1221 µg/mL respectively. Similarly, *E. major* leaf extracts also showed growth inhibition of *S. putrefaciens*, with MIC values of 1686 µg/mL for the aqueous extract, and 1160 µg/mL for the methanolic extract. However, toxicity studies of the extracts revealed that all extracts were toxic and likely unsuitable for human consumption (LC_{50} values 455-1146 µg/mL) as determined by the *Artemia franciscana* bioassay. **Conclusion:** While the *E. baileyana* and *E. major* leaf extracts were effective in preventing microbial growth, given their relatively high levels of toxicity, they would not be suitable for use as a preservative in the prevention of fish spoilage. However, the antibacterial capacity of the extracts indicates that the extracts may show promise as a surface disinfectant, and this should be investigated further.

Key words: Fish spoilage, *Shewanella putrefaciens, Eucalyptus major, Eucalyptus baileyana*, Eucalypts, Medicinal plants.

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DOI: 10.5530/pc.2021.2.24

INTRODUCTION

Food spoilage is the deterioration of food that results in it becoming undesirable or unfit for consumption. This can be chemical spoilage (oxidation, chemical/metal contamination) or biological spoilage (insects, microbial growth), resulting in adverse changes to taste, texture, appearance and/or smell.[1] Microbial spoilage arises through the contamination of foods by acids and other wastes, which are typically biproducts produced as a result of metabolic processes.[2] While most major organisms associated with food contamination are non-pathogenic, these processes often render the food inedible and microbes are estimated to contribute to 25% of all spoilage.[3] As such, food producers employ numerous strategies to mitigate spoilage, including, but not limited to, the addition of preservatives. These are typically chemical compounds that are well-documented as antimicrobial agents. However, these preservatives are often viewed by the general public in a negative light, despite often being well-documented as safe for human ingestion.[3] For industries concerned about consumer perception, natural products (plants containing antimicrobial agents) can be substituted as a more favourable alternative.[4] The suitability of employing natural products to mitigate spoilage caused by microbial growth depends on several factors:

- Antimicrobial capacity: For any intervention to be successful, the plant being used must contain antimicrobials that effectively inhibit microbial growth.
- Toxicity: The plant cannot be poisonous to the humans/animals that will ingest the food product.
- Secondary spoilage: The plant cannot cause secondary spoilage. This can occur either through chemical reactions between the product and the food, or through undesirable tastes/aromas associated with the plant itself.

Undertaking an all-encompassing study on the effectiveness of a plant in preventing food spoilage is a massive endeavour. This paper focuses on one of the prevailing bacteria associated with fish spoilage, *Shewanella putrefaciens*, and its susceptibility to the native Australian plants, *Eucalyptus major* and *Eucalyptus baileyana*, as well toxicity studies to evaluate their suitability for inclusion into food products.

Plants from the genus *Eucalyptus*, including *E. major* and *E. baileyana*, are widely documented for their antiseptic properties. Historically, *E. major* and *E. baileyana* have been used to remedy throat infections, achieved through the crushing of the leaves and subsequent inhalation of released volatiles.[5] Furthermore, preparation of oils/infusions from these leaves produce effective topical antiseptics. These concoctions, when applied to skin diseases or wounds, offer protection against infection.[6] Studies investigating the antibacterial properties of *E. major* and *E. baileyana* extracts have shown strong levels of inhibition against a variety of pathogenic bacteria, including *Bacillus anthracis*, the causative agent of anthrax.[7] *Eucalyptus*-derived essential oils remain incredibly popular today worldwide, serving as a natural alternative to the treatment of many common ailments.

MATERIALS AND METHODS

Plant source and extraction

Eucalyptus baileyana and *Eucalyptus major* leaf materials were collected from Toohey Forest, Brisbane and were identified with reference to a taxonomic key to Toohey Forest plants.[8] The leaf materials were dried thoroughly using a Sunbeam Food Dehydrator and stored at -30°C until used. Prior to use, the leaf materials were thawed and ground into a coarse powder. Individual 1 g quantities were weighed into separate tubes and 50 mL of either methanol or water added, followed by a 24-hr incubation period at 4°C with gentle shaking. Finally, the extracts were filtered using Whatman No. 54 filter paper under vacuum, followed by drying by rotary evaporation in an Eppendorf Concentrator 5301. The resultant dry extract was weighed and redissolved in 10 mL deionised water (containing 1 % DMSO).

Qualitative phytochemical studies

Phytochemical analysis of the extracts for the presence of flavonoids, triterpenoids, cardiac glycosides, polysteroids, saponins, phenolic compounds, anthraquinones, tannins and alkaloids were conducted by previously described assays.[9,10]

Environmental *Shewanella putrefaciens* strain

Shewanella putrefaciens strain CN-32 was obtained from Professor Kenneth Nealson of the University of Southern California (USA). Antibacterial screening was achieved using a modified peptone/yeast extract (PYE) agar as previously described,[11] containing: 1 g/L peptone, 1.5 g/L yeast extract, 7.5 g/L NaCl, 1 g/L ammonium persulfate, 2.4 g/L HEPES buffer (pH 7.5) and 16g/L bacteriological agar. Incubation was at 30°C and the stock culture was subcultured and maintained in PYE media at 4°C. The media nutrient components were supplied by Oxoid Ltd. The GenBank accession number for the complete genome for *S. putrefaciens* CN-32 is CP000681.

Evaluation of antimicrobial activity

Antimicrobial activity was determined using a modified disc diffusion assay as previously described.[6,12] Briefly, 100 µL of *S. putrefaciens* was grown in 10 mL of PYE media until they reached a count of ~10^8 cells/mL. An amount of 100 µL of bacterial suspension was evenly spread onto PYE agar plates. The extracts were assessed for antibacterial activity using 6 mm sterilised filter paper discs. Discs were treated with 10 µL of an extract, allowed to dry before being placed onto inoculated plates. The plates were set at 4°C for 2 h before incubation at 30°C for 24 h and the inhibition zone diameters measured in millimetres. All measurements were rounded to the closest whole millimetre and performed in triplicate. Mean values (± SEM) are reported in this study. Standard discs of chloramphenicol (10 µg) were obtained from Oxoid Ltd. and served as a positive control. Filter discs treated with 10 µL of sterilised deionised water were used as a negative control.

Minimum inhibitory concentration (MIC) determination

The minimum inhibitory concentration (MIC) of the extracts was determined as previously described.[13,14] Briefly, each extract was diluted in deionised water and tested across a range of concentrations. Discs were treated with 10 µL of the test dilutions, allowed to dry and placed onto inoculated plates. The assay was performed as outlined above, and graphs plotting the zones of inhibition versus concentration were visualised. Linear regression was used to determine the MIC values.

Toxicity screening

Reference toxin for toxicity screening

Potassium dichromate ($K_2Cr_2O_7$) (AR grade, Chem-Supply, Australia) was prepared as a 4 mg/mL solution in sterilised deionised water, and serially diluted in artificial seawater for use in the *Artemia franciscana* nauplii bioassay as previously described.[15]

Artemia franciscana nauplii toxicity screening

Toxicity was tested using a modified *Artemia franciscana* nauplii lethality assay as previously described.[15,16] Briefly, 400 µL of seawater containing approximately 43 (mean 43.2, n = 155, SD 14.5) nauplii were added to a 48 well plate and immediately used for bioassaying. Volumes of 400 µL of diluted extracts, or the reference toxin, were transferred to the wells and incubated at 25 ± 1°C under artificial light (1000 Lux). A negative control (400 µL seawater) was run in triplicate for each plate and all treatments were performed in triplicate. The wells were checked at regular intervals and the dead nauplii tabulated. The nauplii were considered dead if no movement of the appendages were detected within 12 sec. After 24 hr all nauplii were sacrificed and counted to determine the total % mortality per well. The LC_{50} with 95% confidence limits for each treatment was determined using ProBit analysis.

Statistical analysis

Data is presented as the mean ± SEM of at least three independent experiments.

RESULTS

Liquid extraction yields and qualitative phytochemical screening

Extraction of 1 g of the *E. baileyana* and *E. major* leaf plant materials with the solvents yielded dried plant extracts ranging from 125 mg (*E. baileyana* aqueous extract) to 280 mg (*E. major* methanolic extract) (Table 1). The methanolic extracts generally gave higher yields of dried extracted material compared with the corresponding aqueous extracts in both eucalypts, although this difference was much less significant with *E. baileyana*. The dried extracts were resuspended in 10 mL of sterile deionised water (containing 1 % DMSO) and resulted in the extract concentrations shown in Table 2.

Antimicrobial activity

To assess the ability of the leaf extracts to inhibit *S. putrefaciens* growth, aliquots (10 µL) of each extract were screened using disc diffusion assaying as previously described.[17,18] Bacterial growth was inhibited by

Table 1: Minimum inhibitory concentration (µg/mL) of the leaf extracts and LC_{50} values (µg/mL) in the *Artemia* nauplii bioassay.

Species	Extract	MIC (µg/mL)
E. baileyana	W	1411
E. baileyana	M	1221
E. major	W	1686
E. major	M	1160
Potassium Dichromate		

W = aqueous; M = methanolic. Numbers indicate the mean MIC and LC_{50} values of triplicate determinations. - indicates no bacterial growth inhibition was evident, or that an LC_{50} value could not be obtained as the mortality did not reach 50% for any dose tested.

Table 2: The mass of dried leaf material, the concentration after resuspension in deionised water, qualitative phytochemical screenings of the plant extracts.

Species	Extract	Mass of Dried Extract (mg)	Concentration of Resuspended Extract (mg/ml)	Total Phenolics	Water Soluble Phenolics	Water Insoluble Phenolics	Cardiac Glycosides	Saponins	Triterpenes	Polysteroids	Alkaloids (Mayer Test)	Alkaloids (Wagner Test)	Flavonoids	Tannins	Free Anthraquinones	Combined Anthraquinones
E. baileyana	W	125	12.5	+++	+++	+	-	+	-	-	-	-	++	+	-	-
	M	143	14.3	+++	+++	+	-	+	-	-	-	-	+	++	-	-
E. major	W	222	22.2	+++	+++	+	-	+	-	-	-	-	+++	+	-	-
	M	280	28	+++	+++	+	-	+	-	-	-	-	++	++	-	-

W = aqueous; M = methanolic. +++ indicates a large response; ++ indicates a moderate response; + indicates a minor response; - indicates no response detected.

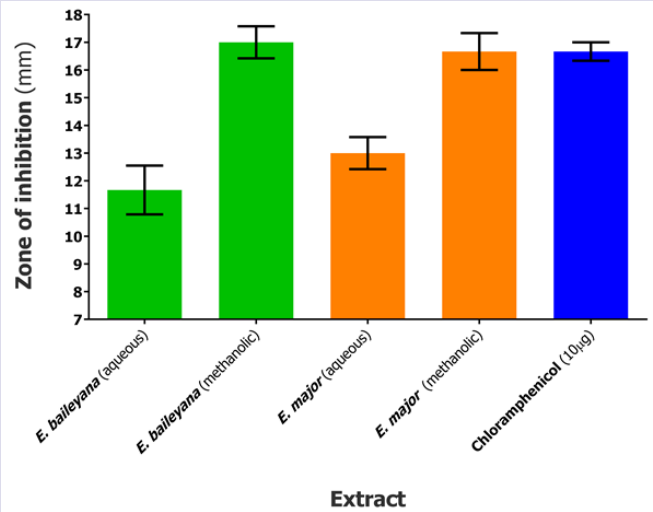

Figure 1: Growth inhibitory activity of plant extracts against *S. putrefaciens* measured as zones of inhibition (mm). Results are expressed as mean zones of inhibition ± SEM.

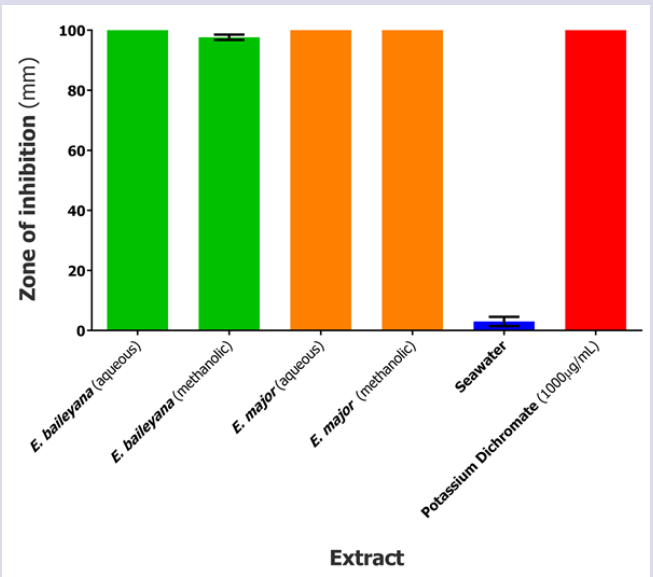

Figure 2: The lethality of the leaf extracts alongside the potassium dichromate (1000 µg/mL) and seawater controls towards *Artemia franciscana* nauplii after 24 hours exposure. Results are expressed as mean % mortality ± SEM.

all the extracts tested (Figure 1). Comparatively, the methanolic leaf extracts were more potent inhibitors of growth (as judged by zone of inhibition) compared to their aqueous counterparts, with inhibition zones of 17 ± 0.6 mm and 16.7 ± 1.0 mm respectively. These results compare favourably with those of the chloramphenicol control, which provided inhibitory zones of 16.7 ± 0.3 mm (10µg).

The antimicrobial efficacy was further quantified by determining the MIC values (Table 2). The methanolic *E. baileyana* and *E. major* methanolic extracts were particularly effective at inhibiting microbial growth, with MIC values against *S. putrefaciens* <1221 µg/mL (*E. baileyana*) and <1160 µg/mL (*E. major*) (~12 µg impregnated in the disc). Similarly, the aqueous extracts also provided strong levels of inhibition, yielding MICs of *putrefaciens* <1411µg/mL (*E. baileyana*) and <1686 µg/mL (*E. major*).

Quantification of toxicity

All extracts were initially screened at 2000 µg/mL in the assay (Figure 2). Potassium dichromate (1000 µg/mL) was used as a reference toxin, and seawater used as a negative control. The potassium dichromate screening showed onset of mortality, inducing nauplii death within the first 3 hr of exposure and 100 % mortality after 4-5 hr (results omitted). All the leaf extracts tested displayed > 95 % mortality rates at 24 h.

To further quantify the effect of toxin concentration on mortality, the leaf extracts were serially diluted in artificial seawater to test across a range of concentrations in the *Artemia* nauplii bioassay. To further quantify the effect of toxin concentration on mortality, the leaf extracts were serially diluted in artificial seawater to test across a range of concentrations in the *Artemia* nauplii bioassay. Table 1 shows the LC_{50} values each extract towards *A. franciscana*. Significant toxicity was noted for both *E. baileyana* extracts as well as the *E. major* leaf methanolic extract with LC_{50} values substantially <1000 µg/mL, indicating that they are toxic. Similarly, the *E. major* leaf aqueous extract returned an LC_{50} of 1146 µg/mL, indicating that it was nontoxic. The relative toxicity of each extract using the LC_{50} values obtained was achieved as previously described.[15,16]

DISCUSSION

Previous studies have highlighted the ability for *E. major* and *E. baileyana* to inhibit pathogenic bacterial species,[5] although this study is the first to investigate their potential in the prevention of food spoilage. From an antimicrobial standpoint, both the aqueous and methanolic *E. major* and *E. baileyana* leaf extracts were effective in preventing *S. putrefaciens* growth. Unfortunately, subsequent toxicity studies revealed that these extracts are unsuitable for human consumption and thus cannot be used as preservatives. However, these results are still promising in that during the preparation for the market, fish are generally gutted and sliced into smaller pieces, so they are ready for purchase by the customer. During this process, surface contamination by bacteria is inevitable and Eucalypt leaf extracts may offer a natural alternative as a cleaning agent for these environments. Still, determining the suitability of *E. major* and *E. baileyana* extracts would require additional testing and was not within the scope of this study.

Studies using extracts from other plants have also reported potent MIC values signifying potent *Shewanella* spp. growth inhibitory activity. One recent study reported an MIC value of 512 µg/mL against a different environmental *S. putrefaciens* isolate by an ethanolic *Zataria multiflora* extract.[19] *Zataria multiflora* is commonly used in the Middle East as both a natural food preservative, and as a medicinal plant. Based on its antiseptic properties, it is considered to have potent growth inhibitory properties against a wide variety of pathogenic and non-pathogenic bacteria.[19] A different study reported moderate growth inhibition (2 mg/mL) of *S. putrefaciens* by aqueous *Terminalia catappa* extracts.[20] That plant is widely regarded for its antibacterial properties and is believed to have potent broad spectrum antibacterial activity.[21] More recently, we reported potent inhibitory activity of *Terminalia ferdinandiana* Exell extracts against four *Shewanella* spp.[4]

Although phytochemical analyses were outside the scope of our investigation, previous literature has highlighted that the members of genus *Eucalyptus* are widely associated with their high terpenoid content.[22,23] Of note, numerous *Eucalyptus* spp. have been reported to contain high levels of 1,8-cineol, a terpenoid well-documented as an antibacterial agent[24,25] and it is likely that this compound contributes to the activity reported herein. This compound has also been reported to be toxic[26,27] and may account for the toxicity reported in our study. Whilst this toxicity would impact on the usefulness of these extracts as natural fish preservatives, that would not preclude its use as topical medicinal agents and further work is required to explore other uses of these extracts.

CONCLUSION

The results of this study demonstrate that despite the noteworthy *S. putrafaciens* inhibitory activity of *E. major* and *E. baileyana* their toxicity would limit their potential as natural fish/seafood preservatives.

ACKNOWLEDGEMENT

The authors are most grateful to Professor Kenneth Nealson of the University of Southern California, USA for the gift of all environmental *Shewanella* spp. strain, used in this study. Financial support for this work was provided by the Environmental Futures Research Institute and the School of Environment and Science, Griffith University, Australia.

CONFLICT OF INTEREST

The authors report no conflicts of interest.

ABBREVIATIONS

DMSO: Dimethyl sulfoxide; **LC_{50}:** The concentration required to achieve 50 % mortality; **MIC:** minimum inhibitory concentration; **ZOI:** zone of inhibition.

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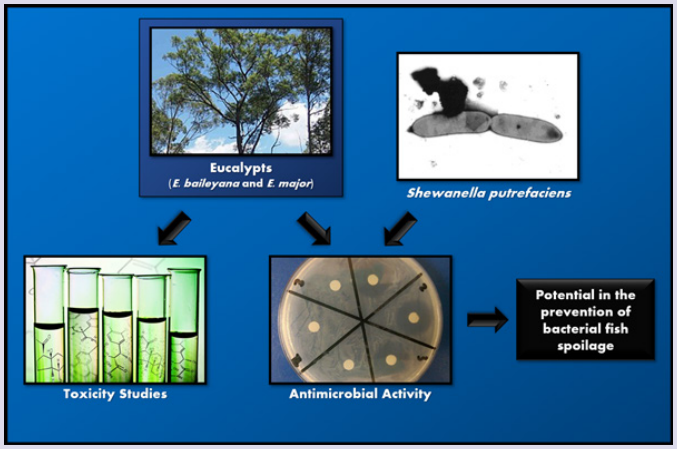
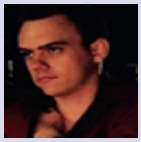
PICTORIAL ABSTRACT



SUMMARY

- *Eucalyptus baileyana* and *Eucalyptus major* extracts were tested for the ability to inhibit the growth of fish spoilage bacteria.
- The extracts were also tested for toxicity in the *Artemia nauplii* bioassay.
- All extracts were good inhibitors of *Shewanella putrefaciens* growth but displayed substantial toxicity.
- The usefulness of the *Eucalyptus* extracts as a fish preservative would be limited by their toxicity.

ABOUT AUTHORS



Dr. Mitchell Henry Wright is a Geomicrobiologist who received his Ph.D. in 2014 for his work investigating the manganese reduction/oxidation characteristics of environmental bacteria. From 2016 to 2018 he undertook a postdoctoral researcher role under the mentorship of Prof. Bradley Tebo, where he explored the bacterial oxidative formation and removal of complexed Mn(III) and the implications of these processes on the global ocean. Upon returning to Australia, Dr. Mitchell H. Wright was recruited by First Choice College and to date, oversees their Department of Research and Development. Additionally, he has returned to his former lab (lead by Dr. Ian Cock) to continue his research into the antimicrobial potential of native plants.

Dr. Anthony Carlson Greene is a senior lecturer and researcher at Griffith University, Brisbane Australia. He obtained his PhD in Microbiology from the University of New South Wales and focuses on extreme environments, bioremediation and geomicrobiology. His specific interests include the microbial ecology of thermophilic, saline and alkaliphilic environments and the mechanisms and industrial potential of extremophilic bacteria contained therein.

Dr. Ian Cock leads a research team in the Environmental Futures Research Institute and the School of Natural Sciences at Griffith University, Australia. His research involves bioactivity and phytochemical studies into a variety of plant species of both Australian and international origin, including *Aloe vera*, South Asian and South American tropical fruits, as well as Australia plants including *Scaevola spinescens, Pittosporum phylliraeoides, Terminalia ferdinandiana* (Kakadu plum), Australian Acacias, Syzygiums, Petalostigmas and *Xanthorrhoea johnsonii* (grass trees). This range of projects has resulted in over 150 publications across a variety of peer reviewed journals.