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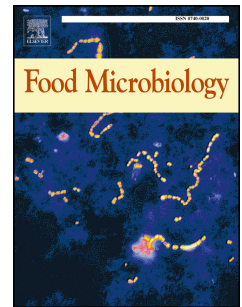
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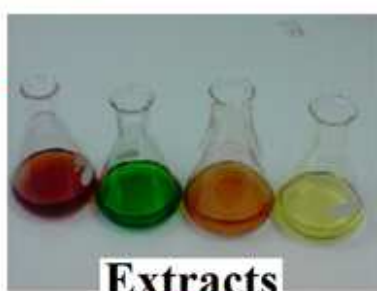
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**Inhibition of
Shewanella spp.
growth**



**Microbial fish
spoilage**



***Terminalia ferdinandiana* Exell: extracts inhibit *Shewanella* spp. growth and prevent fish spoilage**

Running Title: *T. ferdinandiana* extracts inhibit fish spoilage

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***Terminalia ferdinandiana* Exell: extracts inhibit *Shewanella* spp. growth and prevent fish spoilage**

ABSTRACT

Shewanella spp. are major causes of fish spoilage. *Terminalia ferdinandiana* (Kakadu plum) extracts were investigated for their ability to inhibit *Shewanella* spp. growth. Leaf and fruit extracts displayed potent growth inhibitory properties against all *Shewanella* spp. The methanolic leaf extract was a particularly potent inhibitor of *S. putrefaciens* (DD MIC 93; LD MIC 73 $\mu\text{g/mL}$), *S. baltica* (DD MIC 104 $\mu\text{g/mL}$; LD MIC 85 $\mu\text{g/mL}$), *S. frigidimarina* (DD MIC 466 $\mu\text{g/mL}$; LD MIC 391 $\mu\text{g/mL}$) and *S. loihica* (DD MIC 95 $\mu\text{g/mL}$; LD MIC 55 $\mu\text{g/mL}$) growth. The aqueous and ethyl acetate leaf extracts were also potent growth inhibitors, with MIC values generally substantially <1000 $\mu\text{g/mL}$. Treatment of *Acanthopagrus butcheri* Munro fillets with methanolic Kakadu plum extracts significantly inhibited bacterial growth for 15 days at 4°C. All Kakadu plum extracts were nontoxic in the *Artemia franciscana* bioassay. LC-MS analysis identified several compounds which may contribute to the inhibition of *Shewanella* spp. growth.

Keywords: *Terminalia* extracts, Food spoilage, *Shewanella*, Kakadu plum, Combretaceae, natural preservative.

1. Introduction

Microbial spoilage is a major contributing factor to food deterioration, particularly in perishable foods, accounting for an estimated 25% of all food wastage (Ghaly et al., 2010). In many instances these bacteria are non-pathogenic, although they may cause spoilage through malodour formation or change in taste and texture. Methods aimed at inhibiting microbial growth must effectively control initial populations, regrowth of post-processing microbial survivors and contaminant induced populations. Whilst many bacterial species may contribute to the spoilage of fish and other seafood, *Shewanella* spp. are generally acknowledged as a major cause of spoilage as they are psychrotolerant, grow both aerobically and anaerobically, and tolerate a wide pH range (Ng et al., 2015). Thus, they are often relatively unaffected by physical preservative methods which may limit the growth of other bacteria.

The genus *Shewanella* encompasses a large number of facultative anaerobic bacteria that can be psychrophilic, psychrotrophic or mesophilic. Found in both freshwater and marine environments, the genus has been widely investigated due to their ability to utilise a range of electron acceptors, including trimethylamine-N-oxide (TMAO), a common osmolyte in marine fish (Boskou and Debevere, 1998; Macdonald and Khajepour, 2013). Under anoxic conditions, *Shewanella* spp. can utilise TMAO as an alternative to oxygen. Coupled with an appropriate electron donor, TMAO is reduced into trimethylamine (TMA), producing the “fishy aroma” commonly associated with spoilage (Boskou and Debevere, 1998). Although *Shewanella putrefaciens* has long been considered the primary spoilage bacterium of stored marine fish, studies have revealed that *Shewanella baltica* and a number of other *Shewanella* spp. may also contribute to fish spoilage (Vogel et al., 2005).

The psychrophilic and psychrotrophic nature of many *Shewanella* spp., as well as their ability to grow under anoxic conditions, limits the effectiveness of storing fish products on ice or under anaerobic conditions. Some chemical treatments are successful in food

spoilage management. However, customers are often concerned by the potential health risks associated with synthetic chemical compounds (Mahmoud et al., 2006). Investigating natural plant based preparations with known antimicrobial properties potentially offers a safe and effective means of preventing bacterially-driven fish spoilage. Antimicrobial plant extracts with high antioxidant contents are particularly attractive as they may block oxidation of fish macromolecules, as well as inhibiting microbial growth and thus have pluripotent preservative effects. Recent studies have demonstrated the potent bacterial growth inhibitory activity of several high antioxidant fruits and herbs against a wide panel of food spoilage and pathogenic bacteria (Sirdarta et al., 2015; Winnett et al., 2014; Wright et al., 2016).

The genus *Terminalia* encompasses approximately 200-250 species of flowering trees and has an extensive association with usage in traditional medicinal systems (Cock, 2015). The antibacterial activity of this genus has been extensively reported. Extracts prepared from the fruit of the Australian species *Terminalia ferdinandiana* Exell. (Kakadu plum) have potent growth inhibitory activity against an extensive panel of pathogenic bacteria including bacteria associated diarrhoea and dysentery (Cock and Mohanty, 2011) as well as the bacterial triggers of several autoimmune inflammatory diseases (Courtney et al., 2015; Sirdarta et al., 2015a; Sirdarta et al., 2015b). Further studies subsequently reported that Kakadu plum fruit extracts delay spoilage and substantially increase shelf life of commercially harvested *Penaeus monodon* (tiger king prawn) (Sultanbawa et al., 2012). Whilst that study did not differentiate between bacterial species, *Shewanella* spp. are likely to contribute to *P. monodon* spoilage and inhibition of their growth may account for inhibition of spoilage reported in that study. Whilst the antibacterial properties of Kakadu plum leaf extracts have been less extensively reported, recent studies indicate that they inhibit growth of many of the same bacteria (Courtney et al., 2015). Indeed, the leaf extracts are often more potent inhibitors of bacterial growth than are fruit extracts (McManus et al., 2017; Courtney

et al., 2015). Kakadu plum has an extremely high antioxidant capacity (Netzel et al., 2007). Thus, treatment of fish with Kakadu plum fruit and/or leaf extracts may inhibit both oxidative rancidity and microbial spoilage. Despite this, growth inhibitory properties of Kakadu plum extracts against the *Shewanella* spp. are yet to be evaluated. The current study was undertaken to examine the potential of Kakadu plum fruit and leaf extracts as natural preservatives against several *Shewanella* spp. Furthermore, the extracts were tested in a cold stored fish model, which may have numerous bacterial species on their surfaces, some of which (including *Shewanella* spp.) would cause fish spoilage. Thus, the aim of the study was to evaluate the potential of the Kakadu plum extracts to prolong the shelf life and palatability of fish.

2. Materials and methods

2.1. Plant source and extraction

Kakadu plum leaves and fruit pulp were supplied and verified by David Boehme of Wild Harvest, Northern Territory (Australia). The pulp was frozen prior to transport and kept at -10 °C until processed. The pulp and leaves were extensively dehydrated separately in a Sunbeam food dehydrator at ambient temperature (23°C) and the desiccated material was stored at -30 °C. Voucher specimens of the dried pulp (KP2014GD) and leaves (KP2015LA) are stored at the School of Natural Sciences, Griffith University. The dried plant materials were ground into a coarse powder prior to use. A mass of 1g of ground fruit and leaf powders was extensively extracted in 50 mL of either methanol, deionised water, ethyl acetate, chloroform or hexane or for 24 h at 4 °C with gentle shaking. All solvents were supplied by Ajax, Australia (AR grade). The extracts were filtered through Whatman No. 54 filter paper and air dried at room temperature. The aqueous extract was lyophilised by rotary evaporation in an Eppendorf concentrator 5301. The resultant pellets were dissolved in 10 mL deionised

water (containing 0.5 % DMSO) and subsequently passed through a 0.22 µm filter (Sarstedt) and stored at 4 °C until used.

2.2. Qualitative phytochemical studies

Phytochemical screening of the Kakadu plum fruit and leaf extracts to detect the presence of various phytochemical classes was achieved as previously described (Wright et al., 2016; Courtney et al., 2015).

2.3. Antioxidant capacity

The antioxidant capacity of each sample was assessed using a modified DPPH free radical scavenging method (Jamieson et al., 2014) and quantified against an ascorbic acid standard curve. All tests and controls were performed in triplicate and are expressed as µg ascorbic acid equivalents per gram of original plant material extracted.

2.4. Antibacterial screening

2.4.1. Environmental *Shewanella* strains

The *Shewanella putrefaciens* strain 200, *Shewanella baltica* strain OS155, *Shewanella frigidimarina* strain NCIMB 400 and *Shewanella loihica* strain PV-4 used in this study were kindly donated by Professor Kenneth Nealson of the University of Southern California, United States of America. Antibacterial growth and screening conditions were achieved using standard conditions (Wright *et al.* 2016), with the following modifications: *S. putrefaciens* and *S. loihica* cultures were grown in PYE media at 30 °C for 24 h. *S. baltica* and *S. frigidimarina* cultures were incubated at 15 °C for 72 h.

2.4.2. Evaluation of antibacterial activity

Antibacterial activity screening of the Kakadu plum fruit and leaf extracts was assessed in triplicate using a modified disc diffusion assay as previously described (Wright et al., 2016; Courtney et al., 2015). Briefly, 100 μ L of individual bacterial suspensions were spread onto separate nutrient agar plates. A volume of 10 μ L of the Kakadu plum fruit and leaf extracts was infused into 6mm discs of filter paper and placed onto the inoculated plates. Plates inoculated with *S. putrefaciens* or *S. loihica* cultures were incubated at 30 °C for 24 h. *S. baltica* or *S. frigidimarina* cultures were incubated at 15 °C for 72 h and the mean values inhibition zones (\pm SEM) were calculated. Standard discs containing 10 μ g ampicillin discs (Oxoid Ltd., Australia) or sterile distilled water were included on every plate to compare antibacterial activity.

2.5. Minimum inhibitory concentration (MIC) determination

2.5.1. Microplate liquid dilution MIC assay

The MICs of the extracts were evaluated by standard methods (Eloff 1998). Briefly, overnight bacterial cultures were added dropwise to fresh nutrient broth and the turbidity was visually adjusted to produce a McFarlands number 1 standard culture. This was subsequently diluted 1 in 50 with nutrient broth, resulting in the MIC assay inoculum culture. A volume of 100 μ L sterile broth was added to all wells of a 96 well plate. Test extracts or control antibiotics (100 μ L) were then added to the top row of each plate and 1 in 2 serial dilutions were prepared in each column of wells by transferring 100 μ L from the top well to the next well in each column, etc. A growth control (without extract) and a sterile control (without inoculum) were included on each plate. A volume of 100 μ L of bacterial culture inoculum was added to all wells except the sterile control wells. Plates inoculated with *S. putrefaciens* or *S. loihica* cultures were incubated at 30 °C for 24 h. Plates inoculated with *S. baltica* or *S. frigidimarina* cultures were incubated at 15 °C for 72 h. p-Iodonitrotetrazolium violet (INT) was obtained from Sigma, Australia and dissolved in sterile deionised water to prepare a 0.2

mg/mL INT solution. A 40 μ L volume of this solution was added into all wells and the plates were incubated for a further 6 hours at 30 °C. Following incubation, the MIC was visually determined as the lowest dose at which colour development was inhibited.

2.5.2. Disc diffusion MIC assay

The minimum inhibitory concentrations (MIC) of the extracts was also evaluated by disc diffusion assay across a range of concentrations as previously described (Wright et al., 2016).

2.6. Inhibition of bacterial growth on fish fillets by Kakadu plum extracts

2.6.1. Inoculation of southern black sea bream fillets

Freshly filleted southern black sea bream (*Acanthopagrus butcheri* Munro) was obtained from Underwood Seafood Market, Brisbane Australia. All fish fillets were stored fresh at 4°C and were purchased at 10 am on the same day as harvesting. The edges were aseptically excised and discarded from each fillet. The remainder of the fillets were excised aseptically to produce fish fillet cubes with 1cm square ends, each with 2 surfaces (each 1cm³) which had been exposed to atmospheric bacterial contamination. The cubes were separated into 5 groups (n=45):

- (1) immersion in 1M NaCl solution (control),
- (2) immersion 2000 μ g/mL methanolic Kakadu plum fruit extract in 1M NaCl solution,
- (3) immersion 500 μ g/mL methanolic Kakadu plum fruit extract in 1M NaCl solution,
- (4) immersion 2000 μ g/mL methanolic Kakadu plum leaf extract in 1M NaCl solution,
- (5) immersion 500 μ g/mL methanolic Kakadu plum leaf extract in 1M NaCl solution.

All test groups were immersed in the respective treatments for 6 hours. The cubes were subsequently removed from the treatments and allowed to drain aseptically. Three portions

of each group were immediately sampled (day 0). The remainder of the portions for each group were stored separately in closed sterile containers at 4°C. Three further portions were sampled from each group at 5, 10 and 15 days following inoculation for growth time course studies.

2.6.2. Determination of colony forming units (cfu) in southern black sea bream fillets

To examine the effect of Kakadu plum fruit and leaf methanolic extracts on bacterial growth time in the southern black sea bream fillet, individual portions were sampled in triplicate for each treatment at 0, 5, 10 and 15 days following treatment. Each portion was individually homogenised using an overhead immersion blender and filtered through Whatman No. 54 filter paper at 4°C. Following homogenisation, 1 in 10 serial dilutions were prepared from each homogenate in a 1M NaCl solution across the range 10^{-3} – 10^{-7} . For enumeration of viable bacteria number, a volume of 100 µL of each suspension was spread onto individual nutrient agar plates. The plates were incubated at 30 °C for 24 h and the bacterial load (colonies/mL of sample) was enumerated by direct colony counts and expressed as a % \pm SEM of the untreated control colony counts (group 1) for each time point.

2.7. Toxicity screening

2.7.1. *Artemia franciscana* nauplii toxicity screening

Toxicity was assessed using a modified *Artemia franciscana* nauplii lethality assay (Ruebhart et al., 2009). Briefly, 400 µL of *A. franciscana* nauplii were exposed to 400 µL of the reference toxin or the diluted plant extracts in individual wells of a 48 well plate. Following a 24h incubation, the number of dead nauplii were counted and expressed as the total % mortality per well. Each extract was screened over a range of concentrations and the LC₅₀ was calculated.

2.8. HPLC-MS/MS analysis

Chromatographic separations were achieved by LC-MS using conditions previously developed in our laboratory (Wright et al., 2016). Compound lists were generated and screened against a database generated in our laboratory (800 compounds) and the Metlin metabolomics database (24,768 compounds) for identification of compounds in the extracts.

2.9. Statistical analysis

Data is expressed as the mean \pm SEM of three independent experiments, each performed in triplicate (i.e. ≥ 9 replicates). One way ANOVA was used to calculate statistical significance between control and treated groups with a *P* value < 0.01 considered to be statistically significant.

3. Results

3.1. Liquid extraction yields and qualitative phytochemical screening

Kakadu plum fruit and leaf extractions (1 g) yielded dried plant extracts ranging from 18 mg to 483 mg (fruit extracts) and 58 mg to 471 mg (leaf extracts) (Table S1). Aqueous and methanolic extracts provided significantly greater yields of extracted material relative to the chloroform, ethyl acetate and hexane counterparts, which gave low to moderate yields. The dried extracts were resuspended in 10 mL of deionised water (containing 1 % DMSO). The concentrations obtained for each extract are presented in Table S1.

3.2. Antioxidant content

Antioxidant capacity for the plant extracts (Table S1) ranged from 0.4 mg (hexane leaf extract) to a high of 660 mg ascorbic acid equivalence per gram of dried plant material extracted (methanolic fruit extract). The aqueous and methanolic extracts generally had

higher antioxidant capacities than the corresponding chloroform, hexane and ethyl acetate extracts.

3.3. Growth inhibition of *Shewanella* spp.

To determine the ability of the Kakadu plum fruit and leaf extracts to inhibit *Shewanella* spp. growth, 10 μ L of each extract was screened using a disc diffusion assay. *S. putrefaciens* growth was inhibited by the methanolic, aqueous and ethyl acetate Kakadu plum fruit extracts and all of the leaf extracts (Fig. 1a). Only the chloroform and hexane fruit extracts were devoid of *S. putrefaciens* growth inhibitory activity. The methanolic and aqueous leaf extracts were particularly potent inhibitors of *S. putrefaciens*, each with zones of inhibition substantially >11 mm. This compares favourably with the ampicillin control (10 μ g) which had zones of inhibition of 8.3 ± 0.6 mm. As *S. putrefaciens* is a main causative agent for microbial quality detraction (at both mesophilic and psychrophilic conditions), these are particularly noteworthy results. Whilst the methanolic, aqueous and ethyl acetate fruit extracts, as well as the chloroform and hexane leaf extracts, also inhibited *S. putrefaciens* growth, they were generally less potent than the corresponding methanolic extracts. The lower efficacy of the low polarity extracts compared to the higher polarity extracts indicates that the most potent and/or most abundant growth inhibitory compounds are polar.

As seafood is generally stored using low temperature conditions, other psychrotrophic and psychrophilic *Shewanella* spp. have increased importance at lower temperatures (Vogel et al., 2005). Control of *S. baltica* growth, and to a lesser extent *S. frigidimarina* growth, become more important when fish are stored at lower temperatures for extended periods and the contribution of *S. putrefaciens* decreases (Vogel et al., 2005). The growth of *S. baltica* was also susceptible to the Kakadu plum fruit and leaf extracts (Fig. 1b). Consistent with the trend noted for *S. putrefaciens* growth inhibition, *S. baltica* also appeared more susceptible to

the methanolic extracts than to the aqueous extract and the less polar extracts. Furthermore, the leaf extracts were substantially more potent growth inhibitors than were the corresponding fruit extracts. As reported for *S. putrefaciens* growth inhibition, the methanolic and aqueous leaf extracts were particularly potent inhibitors of *S. baltica* growth, with inhibition zones of 14.6 ± 0.3 mm and 12.7 ± 0.6 mm respectively. The inhibition of *S. baltica* growth was particularly noteworthy as the growth of this bacterium was unaffected by ampicillin, indicating that this is an antibiotic resistant strain. The methanolic fruit extract was also a good inhibitor of *S. baltica* growth (inhibition zone = 9.8 ± 0.4 mm). With the exception of the fruit chloroform and hexane extracts which were devoid of inhibitory activity, all other extracts were moderate inhibitors of *S. baltica* growth (as determined by zones of inhibition).

Growth of *S. frigidimarina* was also inhibited by several of the Kakadu plum fruit and leaf extracts (Fig. 1c). As evident for the inhibition of the growth of *S. baltica* and *S. frigidimarina*, the methanolic extracts were generally more potent *S. frigidimarina* growth inhibitors than were the other corresponding solvent extracts. The methanolic leaf extract was particularly potent, with an inhibition zone of 18.6 ± 0.6 mm. Notably, as seen for the other psychrotrophic bacterial species (*S. baltica*), *S. frigidimarina* growth was also resistant to ampicillin exposure. The aqueous leaf extract was also a potent growth inhibitor (inhibition zone = 9.8 ± 0.4 mm). The fruit methanolic, aqueous and ethyl acetate extracts, as well as the leaf ethyl acetate extracts also inhibited *S. frigidimarina* growth, albeit with smaller zones of inhibition indicative of moderate inhibitory activity. All chloroform and hexane extracts were completely devoid of *S. frigidimarina* growth inhibitory activity.

S. loihica and *S. putrefaciens* share similar genotypic and phenotypic characteristics and have similar optimal growth conditions (Gao et al., 2006). Thus, the ability of the Kakadu plum fruit and leaf to inhibit *S. loihica* was also tested (Fig. 1d). In contrast with the

other *Shewanella* spp., *S. loihica* was particularly susceptible to the ampicillin control (zone of inhibition of 19.6 ± 1.3 mm). Whilst substantially smaller zones of inhibition were recorded against most of the Kakadu plum fruit and leaf extracts, several displayed potent *S. loihica* growth inhibition. Indeed, exposure of *S. loihica* to the methanolic fruit and leaf extracts produced >10 mm zones of inhibition. As evident for the growth inhibition of the other *Shewanella* spp., the methanolic Kakadu plum fruit extract was the most potent growth inhibitor (as assessed by the inhibition diameter), with an inhibition zone of 15.3 ± 1.2 mm measured. In contrast, an inhibition zone of 10.8 ± 0.8 mm was measured for the methanolic fruit extract. Whilst the fruit aqueous, ethyl acetate and chloroform extracts, as well as the leaf hexane extracts also inhibited *S. loihica* growth, substantially smaller zones of inhibition were measured.

3.4. Quantification of minimum inhibitory concentration (MIC)

The relative level of antimicrobial activity was further quantified by determining the MIC values (Table 1) for each extract against the *Shewanella* spp. which were shown to be susceptible in the disc diffusion screening assays. A similar trend was noted as seen for the screening assays i.e. the Kakadu plum leaf extracts were substantially better inhibitors of all *Shewanella* spp. growth than were the corresponding fruit extracts. Furthermore, the methanolic extracts were generally the most potent growth inhibitors. The methanolic leaf extract was a particularly potent *S. putrefaciens* growth inhibitor, with disc diffusion (DD) and liquid dilution (LD) MIC values of 93 and 73 $\mu\text{g/mL}$ respectively. This is substantially more potent than the methanolic fruit extract (DD MIC 1160 $\mu\text{g/mL}$; LD MIC 980 $\mu\text{g/mL}$). The Kakadu plum methanolic leaf extract was also a potent inhibitor of *S. baltica* (DD MIC 104 $\mu\text{g/mL}$; LD MIC 85 $\mu\text{g/mL}$), *S. frigidimarina* (DD MIC 466 $\mu\text{g/mL}$; LD MIC 391 $\mu\text{g/mL}$) and *S. loihica* growth (DD MIC 95 $\mu\text{g/mL}$; LD MIC 55 $\mu\text{g/mL}$). The aqueous and ethyl acetate Kakadu plum leaf extracts also had low MIC values against all *Shewanella*

spp., (generally $\leq 800 \mu\text{g/mL}$ against all *Shewanella* spp.), also indicating their potential as fish preservatives. The methanolic, aqueous and ethyl acetate Kakadu plum fruit extracts were also generally good growth inhibitors of *Shewanella* spp. growth, albeit with MIC values often an order of magnitude higher than for the corresponding leaf extracts. In further contrast to the leaf extracts where the methanolic extract had the greatest potency, the ethyl acetate extract was generally the most potent of the fruit extracts.

3.4. Inhibition of bacterial growth on southern black sea bream fillets

Whilst psychrotrophic and psychrophilic *Shewanella* spp. are generally acknowledged as a major cause of cold stored fish spoilage, other bacterial species would also contribute to spoilage. The MIC assay methods used in our study provide important information of the ability of the extracts to inhibit *Shewanella* spp. growth *in vitro*. However, they do not necessarily accurately portray bacterial spoilage in commercial cold stored fish as this may be caused by several bacterial species. Therefore, the Kakadu plum fruit and leaf extracts were tested for the ability to inhibit total bacterial growth in fish fillets under cold storage conditions. As the methanolic extracts were generally the most effective growth inhibitors (Table 1), only these extracts were tested in the fish fillet spoilage study. This study did not discriminate between bacterial species, instead measuring the total viable bacteria as colony forming units (cfu). Furthermore, as cold storage is the most common preservation method for fresh fish, the fish fillets used in this study were also stored at 4°C throughout the study period. The results are expressed as a % cfu of the control fish fillets (no treatment, held at 4°C) to determine the degree of improvement over cold storage alone.

All treatments with the methanolic fruit and leaf Kakadu plum extracts were effective at reducing the number of viable bacteria on the fillets immediately following treatment, indicating that the extracts were bactericidal at 0.5 mg/mL (Fig. 2a). Indeed, the cfu's for all

treated groups were inhibited by approximately 95% compared to the control fillets (Fig. 2b). Whilst the Kakadu plum methanolic leaf extract appeared slightly more effective than the fruit extract at both concentrations tested, the differences were not statistically significant. Interestingly, with the exception of the 0.5 mg/mL methanolic fruit extract, all extracts remained approximately as effective following 10 days cold storage as at the start of the test, each still inhibiting bacterial growth by approximately 95% compared to the control fillets. Although the 0.5 mg/mL methanolic fruit extract was not as effective following 10 days growth, it still inhibited bacterial growth by approximately 35% compared to the control fillets. Following 15 days cold storage, the fruit extract (both concentrations) and the 0.5 mg/mL leaf extract treatment were less effective than earlier in the trial, with bacterial growth increasing to 50-85% of the levels in the control fillets. These values represent a significant reduction in bacterial growth and indicate that all of extracts significantly decrease spoilage for at least 15 days. Notably, the 2 mg/mL methanolic leaf extract treatment was still very effective at inhibiting bacterial growth at 15 days. Indeed, there was still approximately 90% reduction in bacterial growth at day 15 of the trial for this treatment. Thus, treatment with 2mg/mL methanolic Kakadu plum leaf extract substantially decreases bacterial spoilage at 15 days, indicating its potential for increasing the shelf life of cold stored fish.

3.5. Quantification of toxicity

The Kakadu plum fruit and leaf extracts were initially screened in the *Artemia* nauplii assay at 2000 $\mu\text{g/mL}$ (Fig. 3). Additionally, potassium dichromate was also tested in the bioassay as a reference toxin. The reference toxin was rapid in its onset of mortality, promoting nauplii death within the first 3 h of exposure, with 100 % mortality evident within 5 hours (unpublished results). All of the methanolic and aqueous extracts also induced 100% mortality following 24 h exposure. Similarly, the ethyl acetate leaf extract also induced 100

% mortality at 24 h exposure. All other extracts induced <50% mortality and were therefore deemed to be nontoxic.

A 24 h LC₅₀ value was determined for each extract which gave >50% mortality in the screening assay to further quantify toxicity (Table 1). No LC₅₀ values are reported for any chloroform or hexane extracts, nor for the fruit ethyl acetate extract as < 50 % mortality was seen in all tested concentrations. LC₅₀ values substantially >1000 µg/mL were determined for all of the other extracts. As extract with LC₅₀ values >1000 µg/mL towards *Artemia nauplii* have been defined as being nontoxic in this assay (Ruebhart et al., 2009), all of the Kakadu plum fruit and leaf extracts were deemed to be nontoxic.

3.6. HPLC-MS/MS analysis

As the methanolic leaf extract had the greatest antibacterial efficacy (as determined by MIC; Table 1), it was deemed the most promising extract for further phytochemical analysis. Optimised HPLC-MS/MS parameters were developed and used to search for specific compound classes in the methanolic leaf extract and identify the individual components. The resultant total compound chromatograms (TCC) for the positive ion and negative ion chromatograms are presented in Fig. 4a and Fig. 4b respectively. The negative ion chromatogram had significantly higher background absorbance levels than the positive ion chromatogram, due to ionisation of the reference ions in this mode, possibly masking the signal for some peaks of interest. The positive ion chromatogram (Fig. 4a) revealed the presence of numerous peaks, particularly in the early and middle stages of the chromatogram corresponding to the elution of polar compounds. Nearly all of the methanol extract compounds had eluted by 15 minutes (corresponding to approximately 40 % acetonitrile). Indeed, several major peaks eluted in the first 1 minute with 5 % acetonitrile. Similarly, the majority of the peaks detected in the negative ion methanolic Kakadu plum leaf extract TCC

had eluted by 15 min. Several prominent peaks were also evident at elution times up to 30 min (100 % acetonitrile), indicating that lower polarity compounds were also present in this extract.

The metabolomics fingerprinting approach used in this study targeted two specific phytochemical classes. High tannin contents are a defining feature of *Terminalia* spp. and high tannin contents have been reported in Kakadu plum (Cock, 2015). Furthermore, a recent study characterised a number of tannin components in Kakadu plum leaf and correlated them with the inhibition of the growth of several pathogenic bacteria (Courtney et al 2014). In total, 10 tannins were putatively identified in the methanolic Kakadu plum leaf extract by comparison to the Metlin metabolomics, forensic toxicology (Agilent) and phytochemicals (developed in this laboratory) databases. Chebulic acid (2.2% total peak area in + ionisation mode), chebulagic acid (1.7% total peak area in - ionisation mode), corilagen (7.2% total peak area in - ionisation mode), ellagic acid (1.0% total peak area in - ionisation mode) and trimethyl ellagic acid esters (1.7% total peak area in + ionisation mode), exifone (1.9% total peak area in + ionisation mode) and punicalin (2.4% total peak area in - ionisation mode) were present in particularly high relative abundance (as assessed by their relative % peak area). All other tannins were present in lower relative abundances.

Flavonoids are another major class of phytochemicals present in leaves of most plants. Numerous studies have reported potent antibacterial activity for multiple flavonoids (Prasad et al., 2010; Lin et al., 2008). Thus, the metabolomics fingerprinting analysis in our study also screened for the presence of this phytochemical class. Three flavonoids (including 2 flavonol glycosides) were detected in appreciable quantities. Luteolin was present in the greatest relative abundance (2.6% total peak area in - ionisation mode) and in substantially higher levels than the 2 flavonoid glycosides (rutin and quercetin), although rutin was also

present in high relative abundance (1.1% total peak area in + ionisation mode). Whilst still producing a prominent peak, quercetin was present in lower relative abundance (0.5% total peak area in + ionisation mode).

4. Discussion

The main method of preserving fresh fish and other seafood is currently by storage at low temperatures. This is generally an effective method of controlling the growth of many food spoilage bacteria. However, it is inefficient at inhibiting the growth of psychrophilic and psychrotrophic bacteria such as the *Shewanella* spp. and other preservation methods are required. Decreasing the water activity by drying the fish and/or by adding salt, or alteration of the pH of the fish muscle by fermenting fish or directly adding acids (e.g. acetic, citric, lactic) are effective at inhibiting bacterial growth in stored fish. However, these methods also have profound effects on the taste and textural characteristics of the fish. Furthermore, health concerns associated with excess sodium consumption has resulted in a decreased use of salt as a preservative in recent years. Other methods of delaying fish spoilage entail the addition of chemical preservatives. Commonly used chemical food preservatives include butylhydroxyanisole (BHA), butylated hydroxytoluene (BHT), nitrates, nitrites, sulfur dioxide (SO₂) and sulfites (SO₃) (Ray and Bhunia, 2008). Natural antimicrobial alternatives are increasingly being sought to increase the shelf life and safety of processed foods.

Kakadu plum fruit and leaf extracts were selected for screening for the ability to block the growth of fish spoilage bacteria as they have potential to positively influence the shelf life of fish in several ways. A major portion of fresh fish spoilage is the result of oxidative spoilage. The treatment of fish with preparations containing high antioxidant contents decreases lipid oxidation and thus inhibits oxidative rancidity (Pazos et al., 2005). Kakadu plum fruit and leaf extracts have previously been reported to have very high antioxidant

capacities (Courtney et al., 2015; Netzel et al., 2007) and thus have potential in reducing oxidative rancidity. Indeed, Trolox equivalency antioxidant content (TEAC) values of approximately 205 $\mu\text{mol TE/g}$ of extracted fruit were reported for Kakadu plum fruit methanolic extracts (compared to 39 $\mu\text{mol TE/g}$ for blueberries) (Netzel et al., 2007). The same study also reported ascorbic acid levels of 71 $\mu\text{mol/g}$ extracted fruit for Kakadu plum fruit extracts (compared to 0.08 $\mu\text{mol ascorbic acid /g}$ blueberries). Our study also reports high antioxidant capacities for the Kakadu plum fruit and leaf extracts using an alternative measure of antioxidant capacity (DPPH free radical scavenging assay). We measured antioxidant capacities to be as high as 660 mg ascorbic acid equivalence per g of dried extracted plant material (methanolic Kakadu plum fruit extract). This is an exceptionally high antioxidant capacity (equating to 66 % of the original dry fruit) and would be expected to provide a high degree of protection against oxidative fish spoilage. Notably, the ascorbic acid equivalents measured in our study is higher than previously measured in the Netzel et al (2007) study, which reported ascorbic acid levels of 71 $\mu\text{M/g}$ of extracted fruit (which would equate to approximately 12 mg/mL). These differences are likely because our study determined the total antioxidant capacity as ascorbic acid equivalents, whereas the Netzel et al (2007) study recorded the actual ascorbic acid concentrations. The contribution of antioxidant protection to food spoilage has previously been extensively reported (Pazos et al., 2005) and was not a focus of our study.

The other criterion for our selection of Kakadu plum fruit and leaf extracts was their ability to inhibit the growth of other bacteria. Previous studies have reported antibacterial activity for Kakadu plum fruit (Cock and Mohanty, 2011) and leaf extracts (Courtney et al., 2015) against a wide variety of pathogenic and food spoilage bacteria. Our study confirmed the potential of Kakadu plum extracts for delaying bacterial fish spoilage and increasing seafood shelf life. Both fruit and leaf extracts displayed considerable growth inhibitory

activity against all *Shewanella* spp. tested, although the leaf extracts were substantially more potent growth inhibitors. The methanolic Kakadu plum leaf extract was particularly promising, with MIC values of approximately 73, 85, 391 and 55 $\mu\text{g/mL}$ against *S. putrefaciens*, *S. baltica*, *S. frigidimarina* and *S. loihica* respectively. Studies using extracts from other plants have reported comparable or considerably higher MIC values as signifying potent *Shewanella* spp. growth inhibitory activity. One recent study reported an MIC value of 512 $\mu\text{g/mL}$ against a different environmental *S. putrefaciens* isolate by an ethanolic *Zataria multiflora* extract (Motevasel et al., 2011). *Zataria multiflora* is commonly used in the Middle East as both a natural food preservative and as a medicinal plant. It is considered to have potent growth inhibitory properties against a wide variety of pathogenic and non-pathogenic bacteria (Motevasel et al., 2011). Another study reported moderate growth inhibition (2 mg/mL) of *S. putrefaciens* by aqueous *Terminalia catappa* extracts (Chansue and Assawawongkasem, 2008). This plant is widely regarded for its antibacterial properties and is believed to have potent broad spectrum antibacterial activity (Cock, 2015). The significantly greater potency reported in our study emphasises the efficacy of the Kakadu plum extracts. However, it is noteworthy that the *T. catappa* study used a different MIC assay than either of the assays used in our study and this may account for the relatively high MIC value reported therein.

Considerably fewer studies have examined the inhibitory properties of plant extract against other *Shewanella* spp. However, an interesting trend was noted in the literature: Several authors reported antibiotic resistance in multiple *Shewanella* spp. (Poirel et al., 2005). In particular, many strains were highly resistant to β -lactam antibiotics. Similarly, both of the psychrotrophic *Shewanella* spp. examined in our study (*S. baltica*, *S. frigidimarina*) were completely unaffected by relatively high doses of ampicillin. Interestingly, *S. baltica*, *S. frigidimarina* were both highly susceptible to the Kakadu plum extracts, indicating that the

bioactive compounds in these extracts do not have β -lactam structures and/or function via mechanisms different to the conventional β -lactam antibiotics. This has interesting implications beyond the use of Kakadu plum extracts as natural fish preservatives. Indeed, these extracts may have further potential as antibiotics against β -lactam resistant pathogens. Further studies are currently underway in our laboratory, both to screen these extracts against multiple bacterial species/strains, and to determine their antibiotic mechanism(s).

The Kakadu plum extracts were also effective at inhibiting total bacterial growth in a cold store fish model system. We chose to examine the effects of the extracts on total bacterial growth in this study, rather than focussing on the growth of the *Shewanella* spp. in the cold stored fish as this is a more realistic comparison to using the extracts as a commercial fish preservative. Other bacterial species including *Photobacterium* spp. and *Hafnia alvei*, *Morganella psychrotolerans* and *Morganella morganii* are also common causes of spoilage in stored fish. Thus, whilst our in vitro screening studies focussed on *Shewanella* spp. as they are a major cause of fish spoilage, the impact of other bacteria on spoilage should not be neglected. Interestingly, the Kakadu plum extracts also inhibited total bacterial growth in the fish model, indicating that the extracts may also inhibit the growth of other fish spoilage bacteria, including some or all of those listed above. Further studies are required to verify this. Furthermore, whilst the cold store fish test model used in our study does verify the potential of the Kakadu plum as a natural fish preservative, it does not provide information on the efficacy of this treatment for other storage methods. Vacuum packaging and storage under modified atmospheres are frequently used commercially for long-term storage. As these methods reduce oxygen access to the microflora, such storage methods favour the growth of anaerobic bacteria, including *Shewanella* spp. Because the Kakadu plum extracts are effective at inhibiting the growth of bacteria under both aerobic and anaerobic conditions,

they may be particularly beneficial at further increasing the shelf life of fish stored using both of these methods, although further studies are required to verify this.

As the methanolic leaf extract had the most potent *Shewanella* spp. growth inhibitory activity (and total bacterial growth in the cold store fish model), it was deemed the best extract for phytochemical analysis. A total of 145 unique mass signals were identified in the methanolic leaf extract (unpublished data). Of these, 10 compounds were identified as tannins. As well as having a wide diversity of tannin components, the tannins were present as major constituents. The chebulagic acid, corilagen, ellagic acid and punicalin peaks accounted for approximately 1.7%, 7.2%, 1% and 2.4 % of the total peak areas respectively for the negative ionisation mode chromatogram. Chebulic acid, trimethyl ellagic acid and exifone accounted for approximately 2.2%, 1.7% and 1.9% of the total chromatographic peak area in positive ionisation mode. All other tannins were present in lower relative abundances. It is likely that the high tannin contents in the methanolic Kakadu plum leaf extract contributes to the inhibitory activity against the *Shewanella* spp. Gallotannins have been reported to inhibit the growth of a broad spectrum of bacterial species (Buzzini et al., 2008) through a variety of mechanisms including binding cell surface molecules including lipoteichoic acid and proline-rich cell surface proteins (Hogg and Embury, 1982), and by inhibiting glucosyltransferase enzymes (Wu-Yuan et al., 1988). Ellagitannins are also potent inhibitors of bacterial growth, with MIC values as low as 62.5 $\mu\text{g/mL}$ (Buzzini et al., 2008). Ellagitannins function to inhibit bacterial growth via several mechanisms including by disrupting bacterial cell walls and via interaction with cytoplasmic oxidoreductases (Buzzini et al., 2008)

Three flavonoids were also determined to be major components of the methanolic leaf extract. Luteolin was present in the greatest abundance, accounting for approximately 2.6%

of the total peak area for the negative ionisation mode chromatogram. Rutin and quercetin were also present in moderate to high relative abundance, accounting for 1.1% and 0.5% total peak area in positive ionisation mode respectively. Several flavonoids (including the 3 identified in our study) inhibit the growth multiple bacterial species. Quercetin and rutin are potent inhibitors of *Pseudomonas maltophilia* and *Enterobacter cloacae* growth (Waage and Hedin, 1985). Another study tested the inhibitory activity of a panel of 38 flavonoids against methicillin resistant *Staphylococcus aureus* (MRSA) and reported moderate antibacterial activity for several flavonoids including quercetin and luteolin. Rutin was also shown to have a low MIC against multi-resistant β -lactamase producing *Klebsiella pneumoniae* (Özcelik et al. 2008). Thus, luteolin, rutin and quercetin are likely to contribute to the *Shewanella* spp. growth inhibitory activity of the methanolic Kakadu plum leaf extract.

It is likely that other phytochemical classes may also contribute to the *Shewanella* spp. Growth inhibitory properties of these extracts. Alkaloids, anthraquinones, flavonoids, polyphenolics, phytosterols, saponins, stilbenes and terpenes have also been linked with antibacterial activity in different plant species and thus may be responsible (at least in part) for the bacterial growth inhibitory activities reported here. Previous studies have used GC-MS analysis to identify several monoterpenoids in methanolic Kakadu plum fruit and leaf extracts (Wright et al. 2016). That study reported a relative abundance of cis- and trans-linalool oxide, camphor and borneol in the fruit extract. The same study identified linalool oxide and cineole as major components in Kakadu plum leaf extracts. Several other important terpenoids have also been reported in Kakadu plum extracts using different analytical techniques (Sirdarta et al., 2015b). Monoterpenoids were particularly prevalent in those studies, with isomycorene, cineole, cuminol, camphor and isomenthol reported in Kakadu plum fruit extracts. Many of these terpenoids have potent broad spectrum antibacterial activity (Cock, 2015; Cock, 2013) and therefore may contribute to the *Shewanella* spp. growth inhibitory activity.

Whilst none of these terpenoid compounds were detected in our study, it is possible that they may be present and may contribute to the growth inhibitory properties of the Kakadu plum leaf extracts. Our study examined the phytochemical composition of the extracts using HPLC-MS/MS, whereas the previous studies reporting their presence used GC-MS analysis. Generally, HPLC-MS/MS is a good choice for metabolomic profiling studies as it detects a larger amount of compounds of varying polarities than does GC-MS. However, HPLC-MS/MS analysis is limited to studies of the mid-highly polar compounds and is not as useful for studies aimed at highly non-polar compounds. The terpenoids are relatively nonpolar compounds and it is possible that our analysis protocol was unable to detect them. However, this is unlikely as we have used this protocol routinely in our lab and have previously detected low polarity compounds including several of these terpenoids. It is more likely that if present, their levels were below the threshold of detection in our system and that they did not contribute significantly to the potent antibacterial activity reported here.

Whilst, the studies reported here are interesting and highlight the potential of Kakadu plum extracts as food preservatives, further studies are required before their commercial use. In particular, the extracts (as well as fish treated with the extracts) must be evaluated by a raft of organoleptic tests to ensure that any commercial fish products are palatable to the eventual consumers. Furthermore, whilst our findings demonstrate that all Kakadu plum extracts were nontoxic towards *Artemia* nauplii, further toxicity studies in human cell lines are warranted to further ensure that the extracts are safe to use as natural fish preservatives.

5. Conclusions

Kakadu plum fruit and leaf extracts are potent inhibitors of *Shewanella* spp. growth and therefore have potential as natural fish/seafood preservatives. The leaf extracts were particularly effective against all *Shewanella* spp. and thus have potential for both fresh and cold storage fish preservation. However, other bacterial species including *Photobacterium*

spp. and *Enterobacteria* spp. including *Hafnia alvei*, *Morganella psychrotolerans* and *Morganella morganii* may also cause fish spoilage and the effects of the Kakadu plum extracts should also be tested against these bacteria in future studies. Notably, the extracts also inhibited total viable bacterial counts in a cold stored fish model, indicating that they also inhibit the growth of other bacterial species. Thus, the preservative properties of these extracts are more versatile than just as inhibitors of *Shewanella* spp. growth.

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Figure Legends:

Figure 1 Growth inhibitory activity of the Kakadu plum extracts against (a) *S. putrefaciens*, (b) *S. baltica*, (c) *S. frigidimarina* and (d) *S. loihica* environmental isolates measured as zones of inhibition (mm). M = methanolic extract; W = aqueous extract; E = ethyl acetate extract; C = chloroform extract; H = hexane extract; Amp = ampicillin (10 µg). Results are expressed as mean zones of inhibition ± SEM. # indicates fruit extracts which are significantly less potent than the corresponding leaf extracts ($p < 0.01$); * indicates leaf extracts which are significantly more potent than the corresponding fruit extracts ($p < 0.01$).

Figure 2 Inhibition of bacterial growth on southern black sea bream fish fillets by methanolic Kakadu plum fruit and leaf extracts. Total viable bacterial growth was calculated across a 15 day period as (a) individual growth curves for each treatment expressed as \log_{10} CFU/g and (b) % of the untreated bacterial growth for each treatment. Bacterial growth for all treatment groups were measured at 5 day intervals following inoculation. Results are expressed as mean

zones of inhibition \pm SEM of 3 portions in triplicate at each time interval. * indicates results that are significantly different to the untreated control ($p < 0.01$).

Figure 3 The lethality of the Kakadu plum extracts (2000 $\mu\text{g/mL}$) and the potassium dichromate (1000 $\mu\text{g/mL}$) and seawater controls towards *Artemia franciscana* nauplii after 24 hours exposure. M = methanolic extract; W = aqueous extract; E = ethyl acetate extract; C = chloroform extract; H = hexane extract; NC = negative (seawater) control; PC = potassium dichromate control (1000 $\mu\text{g/mL}$). Results are expressed as mean % mortality \pm SEM.

Figure 4 Total compound chromatograms (TCC) of 2 μL injections the methanolic Kakadu plum leaf extract in (a) positive and (b) negative ion RP-HPLC mode. Notable compounds putatively identified are indicated in the chromatograms.

Table S1 The mass of dried extracted material, the concentration after resuspension in deionised water, qualitative phytochemical screenings and antioxidant capacities of the Kakadu plum fruit and leaf extracts.

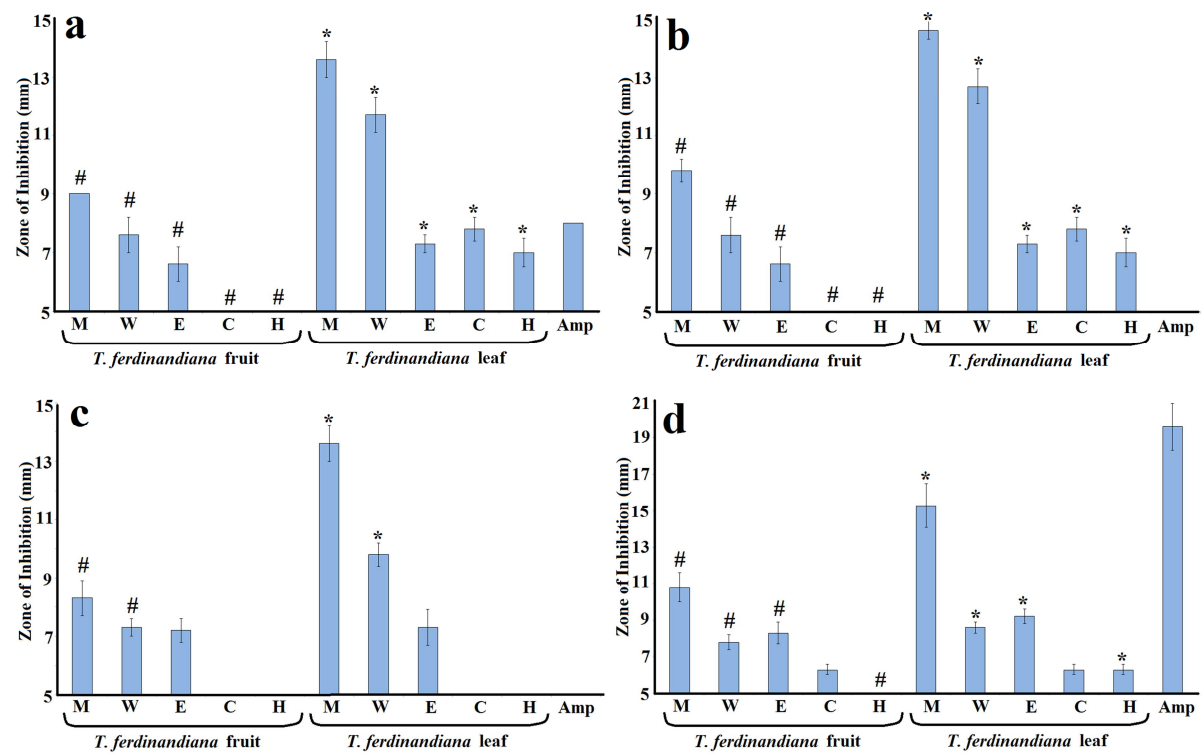
Extract	Mass of Dried Extract (mg)	Concentration of Resuspended Extract (mg/mL)	Antioxidant Capacity (mg Ascorbic Acid Equivalency)	Total Phenolics	Water Soluble Phenolics	Water Insoluble Phenolics	Cardiac Glycosides	Saponins	Triterpenes	Phytosteroids	Alkaloids (Mayer Test)	Alkaloids (Wagner Test)	Flavonoids	Tannins	Free Anthraquinones	Combined Anthraquinones
FM	359	35.9	660	+++	+++	+++	-	++	+	-	+	+	+++	++	-	-
FW	483	48.3	264	+++	+++	+++	-	+	-	-	-	-	+++	++	-	-
FE	30	3	39	++	++	+	-	+	++	-	-	-	++	-	-	-
FC	62	6.2	7	+	-	-	-	-	-	-	-	-	-	-	-	-
FH	18	1.8	1	-	-	-	-	-	-	-	-	-	-	-	-	-
LM	331	33.1	150	+++	+++	+++	+++	++	+	-	+	+	++	+++	+	+
LW	471	47.1	340	+++	+++	+++	++	+++	++	-	-	-	++	+++	+	+
LE	59	5.9	22	+++	+++	+++	-	-	-	-	-	-	++	++	-	-
LC	59	5.9	5	+	-	-	-	-	-	-	-	-	-	-	-	-
LH	58	5.8	0.4	+	-	-	-	-	-	-	-	-	++	+	-	-

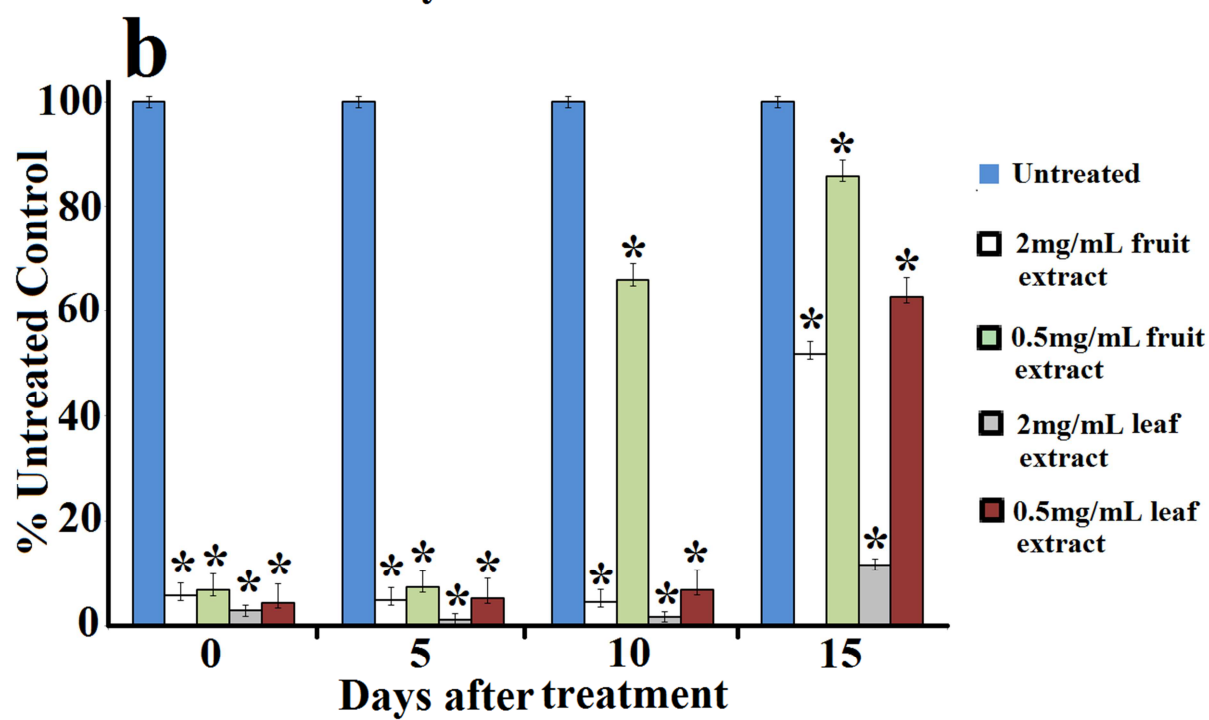
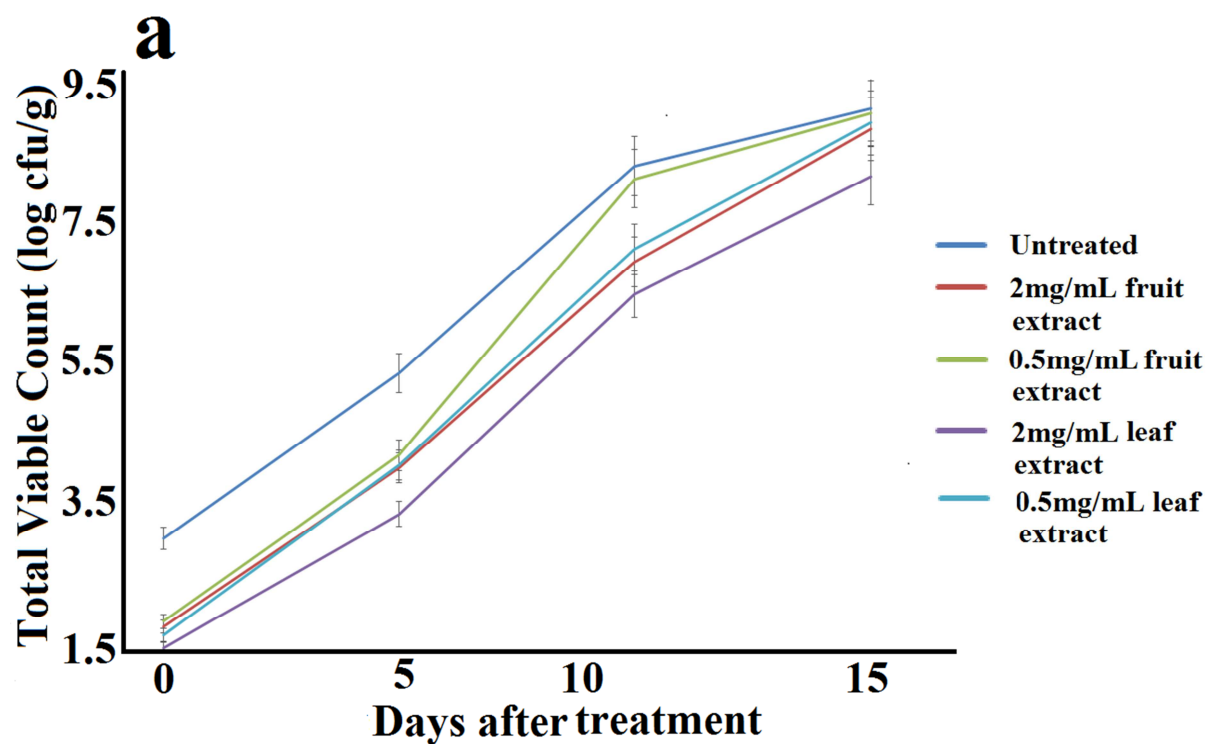
+++ indicates a large response; ++ indicates a moderate response; + indicates a minor response; - indicates no response in the assay. FM = methanolic Kakadu plum fruit extract; FW = aqueous Kakadu plum fruit extract; FE = ethyl acetate Kakadu plum fruit extract; FC = chloroform Kakadu plum fruit extract; FH = hexane Kakadu plum fruit extract; LM = methanolic Kakadu plum leaf extract; LW = aqueous Kakadu plum leaf extract; LE = Kakadu plum ethyl acetate leaf extract; LC = chloroform Kakadu plum leaf extract; LH = hexane Kakadu plum leaf extract. Antioxidant capacity was determined by DPPH reduction and is expressed as mg ascorbic acid equivalence per g plant material extracted.

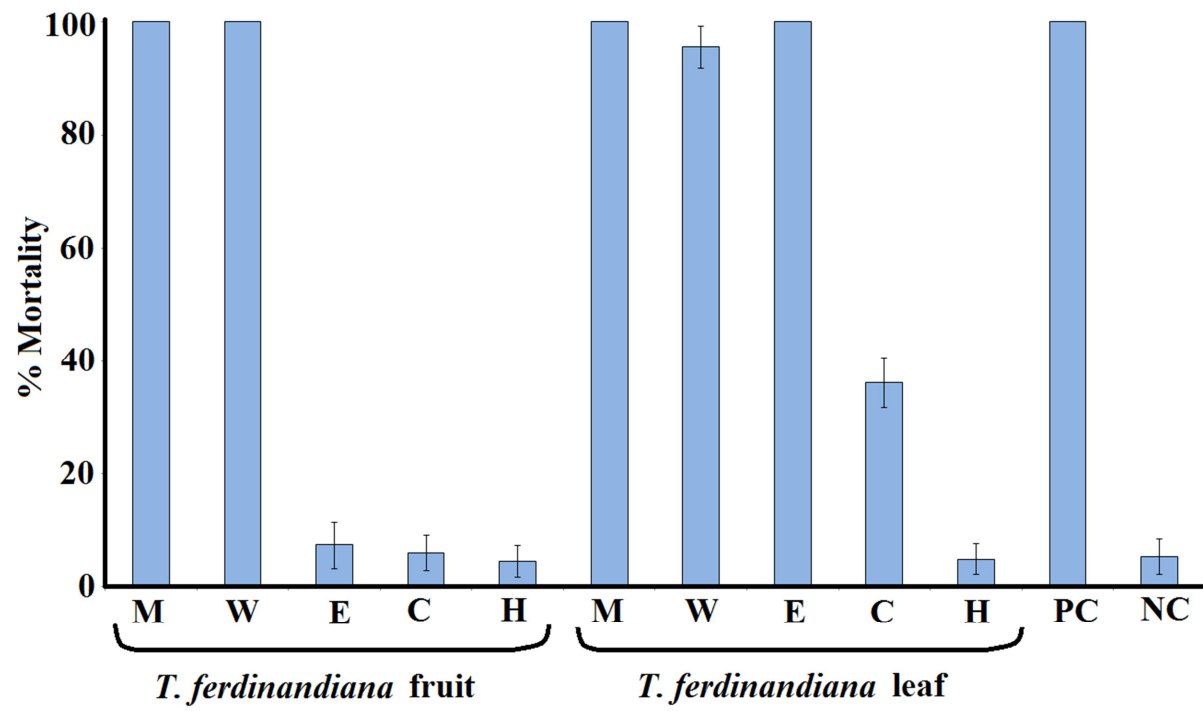
Table 1 Disc diffusion and liquid dilution MICs against *S. putrefaciens*, *S. baltica*, *S. frigidimarina* and *S. loihica* growth ($\mu\text{g/mL}$) and *Artemia* nauplii bioassay LC_{50} values ($\mu\text{g/mL}$) of Kakadu plum fruit and leaf extracts.

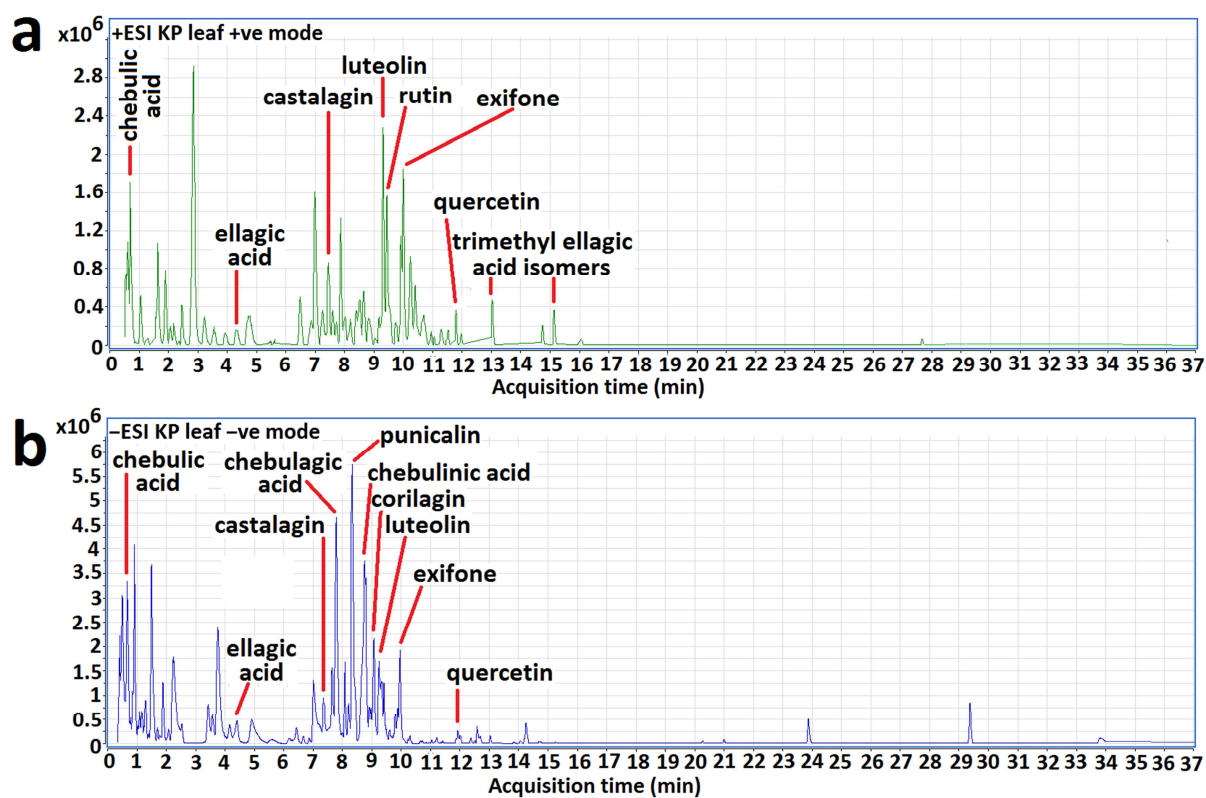
	MIC ($\mu\text{g/mL}$)								<i>Artemia</i> nauplii bioassay
	<i>S.</i> <i>putrefaciens</i>		<i>S. baltica</i>		<i>S.</i> <i>frigidimarina</i>		<i>S. loihica</i>		LC_{50} ($\mu\text{g/mL}$)
	D.D.	L.D.	D.D.	L.D.	D.D.	L.D.	D.D.	L.D.	
F M	1160	986	1077	991	1711	1471	1187	934	2028
F W	1869	1645	2202	2400	2774	2015	2636	1582	1980
F E	1000	860	4326	>5000	1331	1025	932	692	1655
F C	-	-	-	-	-	-	4485	>5000	-
F H	-	-	-	-	-	-	-	-	-
L M	93	73	104	85	466	391	95	55	1283
L W	472	321	625	669	856	753	256	197	1375
L E	311	252	846	770	784	533	469	219	1068
L C	881	960	862	765	-	-	2562	1614	-
L H	841	807	1396	1160	-	-	2829	1924	-

D.D. = disc diffusion; L.D. = liquid dilution; Numbers indicate the mean D.D. MIC, L.D. MIC and LC_{50} values of triplicate determinations. - indicates no inhibition; F = fruit; L = leaf; M = methanolic extract; W = aqueous extract; E = ethyl acetate extract; C = chloroform extract; H = hexane extract.









Highlights:

- *Terminalia ferdinandiana* extracts displayed potent growth inhibitory properties against all *Shewanella* spp. tested.
- The methanolic leaf extract was a particularly potent inhibitor of *S. putrefaciens*
- Treatment of sea bream fillets with *T. ferdinandiana* extract significantly inhibited bacterial growth.
- LC-MS analysis identified 10 tannins and 3 flavonoids which may contribute to *Shewanella* spp. growth inhibition.