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Original Article

Natural Methods for Preventing Fish Spoilage Using Indian *Terminalia* spp. Extracts: Growth Inhibition of *Shewanella* spp.

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ABSTRACT

Introduction: *Shewanella* spp. are a major cause of fish spoilage. *Terminalia* spp. have a long history of medicinal uses, including being used to treat bacterial infections. Despite their well-established antibacterial properties, the Indian *Terminalia* spp. have not been tested for the ability to inhibit the growth of fish spoilage bacteria. **Methods:** Solvent extracts were prepared using Indian *Terminalia* spp. known to inhibit microbial growth. The growth inhibitory activity of the extracts was investigated by disc diffusion assay against four *Shewanella* spp. environmental isolates. Their MIC values were calculated to quantify and compare their relative efficacies. Toxicity was determined using the *Artemia franciscana* nauplii bioassay. **Results:** Extracts prepared from several Indian *Terminalia* spp. displayed potent antibacterial activity in the disc diffusion assay against the environmental *Shewanella* spp. isolates. The methanolic *T. chebula* fruit extract was particularly effective at inhibiting *Shewanella* spp. growth, with MIC values of 198, 329, 162 and 176 µg/mL against *S. putrefaciens*, *S. baltica*, *S. frigidimarina* and *S. loihica* respectively. The *T. chebula* fruit ethyl acetate and *T. catappa* fruit methanolic extracts were similarly potent, with MIC values generally substantially <1000 µg/mL against all *Shewanella* spp. In contrast, the *T. catappa* bark and all *T. arjuna* extracts were only moderate growth inhibitors (MIC values 1000-5000 µg/mL). All other extracts were either inactive or of only low growth inhibitory activity. All the extracts were nontoxic, with all recorded LC_{50} values substantially >1000 µg/mL. **Conclusions:** The potent growth inhibitory activity of the methanolic and ethyl acetate *T. chebula* fruit extracts against all *Shewanella* spp. indicates their potential in the prevention of fish spoilage. Furthermore, the lack of toxicity of these extracts indicates their suitability for use as natural fish preservatives.

Key words: Food Spoilage, Microbial Rancidity, *Shewanella, Terminalia chebula, Terminalia catappa, Terminalia arjuna*, Natural Preservative.

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INTRODUCTION

Food wastage through spoilage is a major global problem, resulting in large amounts of wasted food, as well as a possibility of foodborne illnesses. Spoilage may result in a deterioration of flavour, visual appearance, texture or nutritional value of food, rendering it unpalatable and/or increasing the risks of diseases and food poisoning. From initial harvesting through to handling and processing, food products are exposed to a variety of abiotic (temperature, heat, oxygen) and biotic elements (yeasts, fungi, insects, bacteria).[1] The resultant undesirable odours, tastes or changes in texture produce an unfavourable product which is subsequently discarded. Alternatively, if spoiled food is consumed, food spoilage microbes may cause gastrointestinal distress and a variety of pathogenic diseases.[2]

Microbial spoilage is estimated to account for an estimated 25% of all food wastage.[3] Spoilage can occur either via the introduction of foreign microbes as a result of improper handling/storage techniques, or through the proliferation of pre-existing microbes when conditions are favourable for growth.[4] Several of the common food spoilage microbes may also cause serious food poisoning. Indeed, incidences of food-borne illnesses were estimated at 76 million cases annually in the USA alone in a 1999 study, with at least 5000 deaths annually directly attributed to food poisoning.[5] This is a particular area of concern and there is much effort to develop improved preservation strategies. Methods aimed at inhibiting microbial growth must effectively control initial populations, regrowth of post-processing microbial survivors and contaminant induced populations. This may be achieved by a number of methodologies including alteration of temperature (heating, chilling), pH (fermentation end products), water activity (dehydration) or oxygen availability (canning, shrink wrap, reduced oxygen packaging, high pressures), irradiation or by chemical preservation.[2]

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.[6] 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 may be psychrophilic, psychrotrophic or mesophilic. *Shewanella* spp. occur in both freshwater and marine environments, often as part of fish microbial flora. Under anoxic conditions, *Shewanella* spp. are capable of utilising trimethylamine-N-oxide (TMAO) as an alternative electron acceptor to oxygen. When coupled with an appropriate electron donor, TMAO is reduced into trimethylamine (TMA), producing the 'fishy aroma' commonly associated with spoilage.[7] Although *Shewanella putrefaciens* is generally considered the primary spoilage bacterium of stored marine fish, *Shewanella baltica* and a number of other *Shewanella* species may also contribute to fish spoilage.[8] 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.[9] Investigating natural plant based preparations with known antimicrobial properties 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.[10-12]

The genus *Terminalia* encompasses approximately 200-250 species of flowering trees and has an extensive association with usage in traditional medicinal systems.[13] The antibacterial activity of this genus has been extensively reported. Extracts prepared from the fruit of the Australian species *Terminalia ferdinandiana* (Kakadu plum) have potent growth inhibitory activity against an extensive panel of pathogenic bacteria including bacteria associated diarrhoea and dysentery[14] as well as the bacterial triggers of rheumatoid arthritis (*Proteus mirabilis*)[15] and multiple sclerosis (*Acinitobacter baylyi* and *Pseudomonas aeruginosa*).[10] Leaf extracts from the same species have also been shown to inhibit growth of the same bacteria, as well as a microbial trigger of ankylosing spondylitis (*Klebsiella pneumoniae*).[16] Similarly, African *Terminalia* spp. are potent bacterial growth inhibitors. *Terminalia stenostachya* and *Terminalia spinosa* have strong antibacterial activity against a broad spectrum of medicinally important bacteria including several *Mycobacterium* spp., *Streptococcus faecalis*, *Staphylococcus aureus*, *Vibrio cholera*, *Bacillus anthracis*, *Klebsiella pneumoniae*, *Salmonella typhi*, *Pseudomonas aeruginosa* and *Escherichia coli*.[17] Recent studies have also demonstrated the growth inhibitory activity of *Terminalia sericea* and *Terminalia pruinoides* against pathogenic.[18-20] and food spoilage bacteria.[21]

Many Indian *Terminalia* spp. also have an extensive history of ethnobotanical use. Furthermore, many of the traditional uses of the Indian *Terminalia* spp. are related to microbial infections[13] and numerous recent investigations have reported on their antibacterial properties. Leaf and branch extracts of *Terminalia arjuna*, have antibacterial activity against a wide panel of microbes.[22,23] *Terminalia chebula* has traditional uses in Ayurveda for the treatment of numerous diseases and conditions[13-24] and has potent antibacterial activity.[22] Similarly, *Terminalia alata*, *Terminalia bellirica* and *Terminalia catappa* have broad spectrum antibacterial activity.[23] However, despite the wealth of antibacterial studies for *Terminalia* spp., there is a lack of studies screening *Terminalia* spp. for the ability to inhibit *Shewanella* spp. growth. Our study was undertaken to examine the ability of selected Indian *Terminalia* spp. with extensive usage in Ayurvedic medicine for the ability to inhibit fish spoilage by retarding *Shewanella* spp. growth.

MATERIALS AND METHODS

Plant source and extraction

The *Terminalia chebula*, *Terminalia catappa* and *Terminalia arjuna* plant materials used in this study were a gift from Dr Paran Rayan, Griffith University. Voucher samples of all plant specimens have been stored at the School of Natural Sciences, Griffith University, Brisbane Australia. The plant materials were thoroughly desiccated in a Sunbeam food dehydrator and the dried materials stored at -30 °C until use. Prior to usage, the materials were thawed and ground into a coarse powder. Individual 1 g quantities of the material were weighed into separate tubes and 50 mL of methanol, deionised water, chloroform, hexane or ethyl acetate were added. All solvents were obtained from Ajax and were AR grade. The ground plant materials were individually extracted in each solvent for 24 hours at 4 °C with gentle shaking. The extracts were then filtered through filter paper (Whatman No. 54) under vacuum, followed by drying by rotary evaporation in an Eppendorf concentrator 5301. The resultant extracts were weighed and redissolved in 10 mL deionised water (containing 1 % DMSO).

Qualitative phytochemical studies

Phytochemical analysis of the Indian *Terminalia* spp. extracts for the presence of cardiac glycosides, alkaloids, saponins, tannins, flavonoids, phenolic compounds, phytosterols, flavonoids and triterpenoids was achieved as previously described.[25,26]

Antibacterial screening

Environmental *Shewanella* strains

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 screening was achieved using a modified peptone/yeast extract (PYE) agar 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 as previously described.[27] The *S. putrefaciens* and *S. loihica* cultures were incubated at 30 °C for 24 h. The *S. baltica* and *S. frigidimarina* cultures were incubated at 15 °C for 72 h. All stock cultures were subcultured and maintained in PYE media at 4 °C. The media nutrient components were supplied by Oxoid Ltd., Australia.

Evaluation of antibacterial activity

Antibacterial activity of the *Terminalia* spp. extracts was assessed using a modified disc diffusion assay.[28-30] Briefly, 100 µL of each individual *Shewanella* spp. was grown separately in 20 mL of fresh nutrient broth until an approximate count of 10^8 cells/mL was reached. A volume of 100 µL of each bacterial suspension was spread onto nutrient agar plates and the extracts were tested for antibacterial activity using 5 mm sterilised filter paper discs. Discs were infused with 10 µL of the *Terminalia* spp. extracts, allowed to dry and placed onto the inoculated plates. The plates were left to stand at 4 °C for 2 h before incubation. 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. The diameters of the inhibition zones were measured to the closest whole millimetre. Each assay was completed in at least triplicate. Mean values (± SEM) are reported in this study. Ampicillin discs (10 µg) were obtained from Oxoid Ltd., Australia 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 also evaluated by disc diffusion assay as previously described.[30,31] Briefly, the *Terminalia* spp. 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 then placed onto the inoculated plates. The assay was achieved as outlined above and graphs of the zone of inhibition versus concentration were plotted. Determination of MIC values were achieved using ln-linear regression.

Toxicity screening

Reference toxin for toxicity screening

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

Artemia franciscana nauplii toxicity screening

Toxicity was assessed using a modified *Artemia franciscana* nauplii lethality assay.[32,33] Briefly, 400 µL of seawater containing ~47 (mean 47.4, n = 125, SD 11.7) *A. franciscana* nauplii were added to wells of a 48 well plate and immediately used in the bioassay. A volume of 400 µL of the reference toxin or the diluted plant extracts were transferred to the wells and incubated at 25 ± 1 °C under artificial light (1000 Lux). For each plate, a 400 µL seawater negative control was run in triplicate. The wells were assessed at regular intervals and the number of dead counted. The nauplii were deemed dead if no movement of the appendages was ob-

Table 1: The mass of dried extracted plant material, the concentration after resuspension in deionised water and qualitative phytochemical screenings of the plant extracts.

Plant Species	Plant Part	Extract	Mass of Dried Extract (mg)	Concentration of Resuspended Extract (mg/mL)	Total Phenolics	Water Soluble Phenolics	Water Insoluble Phenolics	Cardiac Glycosides	Saponins	Triterpenes	Phytosterols	Alkaloids (Mayer Test)	Alkaloids (Wagner Test)	Flavonoids	Tannins	Free Anthraquinones	Combined Anthraquinones
T. arjuna	Br	M	140	14	+++	+++	+++	-	+++	-	-	-	-	+++	+++	+	-
		W	144	14.4	+++	+++	+++	-	++	-	-	-	-	+++	++	+	-
		E	22	2.2	+	-	-	-	-	-	-	-	-	+	+	-	-
		C	92	9.2	+	+	-	-	-	-	-	-	-	-	-	-	-
		H	136	13.6	-	-	-	-	-	-	-	-	-	-	-	-	-
T. catappa	F	M	231	23.1	+++	+++	++	+	-	-	-	++	++	++	+++	-	-
		W	144	14.4	+++	+++	++	+	++	-	-	++	++	++	+++	-	-
		E	353	35.3	+	+	-	-	-	-	-	-	-	+	++	-	-
		C	434	43.4	-	-	-	-	-	-	-	-	-	-	-	-	-
		H	447	44.7	-	-	-	-	-	-	-	-	-	-	-	-	-
	L	M	382	38.2	+++	+++	+++	-	++	-	-	+	-	+++	+++	-	-
		W	320	32	+++	+++	+++	-	++	-	-	+	-	+++	+++	-	-
		E	108	10.8	+	+	-	-	-	-	-	-	-	++	++	-	-
		C	275	27.5	-	-	-	-	-	-	-	-	-	-	-	-	-
		H	126	12.6	-	-	-	-	-	-	-	-	-	-	-	-	-
	B	M	183	18.3	+++	+++	+++	-	++	-	-	-	-	+++	+++	-	-
		W	224	22.4	+++	+++	+++	-	++	-	-	-	-	+++	+++	-	-
		E	31	3.1	+	+	+	-	-	-	-	-	-	++	++	-	-
		C	128	12.8	-	-	-	-	-	-	-	-	-	-	-	-	-
		H	147	14.7	-	-	-	-	-	-	-	-	-	-	-	-	-
T. chebula	F	M	634	63.4	+++	+++	+++	-	+++	-	-	-	-	+++	+++	++	-
		W	438	43.8	+++	+++	+++	-	+++	-	-	-	-	+++	+++	++	-
		E	62	6.2	+++	++	+	-	-	-	-	-	-	+++	+++	-	-
		C	93	9.3	-	-	-	-	-	-	-	-	-	-	+	-	-
		H	104	10.4	-	-	-	-	-	-	-	-	-	-	-	++	-
	L	M	473	47.3	+++	+++	+++	-	++	-	-	-	-	+++	+++	-	-
		W	387	38.7	+++	+++	+++	-	+++	-	-	-	-	+++	+++	-	-
		E	52	5.2	++	++	+	-	-	-	-	-	-	++	++	-	-
		C	128	12.8	-	-	-	-	-	-	-	-	-	-	-	-	-
		H	63	6.3	-	-	-	-	-	-	-	-	-	-	-	-	-

+++ indicates a large response; ++ indicates a moderate response; + indicates a minor response; - indicates no response in the assay. Br = branch; F = fruit; L = leaf; B = bark; M = methanolic extract; W = aqueous extract; E = ethyl acetate extract; C = chloroform extract; H = hexane extract, are expressed as mean % mortality ± SEM.

served within 10 seconds. After 24 h, all nauplii were sacrificed and counted to determine the total % mortality per well. The LC_{50} with 95% confidence limits for each treatment was calculated using probit analysis.

Statistical analysis

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

RESULTS

Liquid extraction yields and qualitative phytochemical screening

Extractions of the Indian *Terminalia* spp. plant materials (1 g) with solvents of varying polarity yielded dried plant extracts ranging from 22 mg (*T. arjuna* branch ethyl acetate extract) to 634 mg (*T. chebula* fruit methanolic extract) (Table 1). Higher polar solvents tended to extract higher yields than lower polarity solvents. The majority of the ethyl acetate extracts yielded relatively low masses of extracted material compared to the methanolic and aqueous extracts, which generally gave substantially greater yields. The exception was the *T. catappa* fruit ethyl extract (353 mg) which yielded a substantially higher mass than did the corresponding methanolic (231 mg) and aqueous (144 mg) extracts. Chloroform and hexane generally extracted intermediate masses, with the exception of the *T. catappa* fruit extracts, where chloroform and hexane extractions had the highest yields. This indicates that the majority of compounds in *T. catappa* fruit are of low polarity, whilst the compounds in the other *Terminalia* spp. plant materials were of higher polarity. The dried extracts were resuspended in 10 mL of deionised water (containing 1 % DMSO), resulting in the concentrations presented in Table 1.

Growth inhibition of *Shewanella* spp.

To determine the ability of the Indian *Terminalia* spp. extracts to inhibit *Shewanella* spp. growth, 10 µL of each extract was screened using a disc diffusion assay. *S. putrefaciens* growth was inhibited by 26 of the 30 Indian *Terminalia* spp. extracts screened (87 %) (Figure 1). Only the *T. catappa* leaf, *T. chebula* fruit and leaf hexane extracts, as well as the *T. chebula* leaf chloroform extract, were devoid of *S. putrefaciens* growth inhibitory activity. All other Indian *Terminalia* spp. extracts inhibited *S. putrefaciens* growth, often with zones of inhibition >8 mm. The methanolic extracts were generally the most potent inhibitors of *S. putrefaciens* growth for each *Terminalia* spp. and plant part extracted (as judged by zone of inhibition). Whilst the aqueous, ethyl acetate and chloroform 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. The *T. chebula* methanolic fruit extract was particularly potent, with an inhibition zone of 14.7 ± 0.6 mm determined. This compares favourably with the ampicillin control (10 µg) which had zones of inhibition of 8.3 ± 0.6 mm. As *S. putrefaciens* is a main causative agent for microbial fish spoilage (at both mesophilic and psychrophilic conditions), this is a noteworthy result.

As seafood is generally stored using low temperature conditions, other psychrotrophic and psychrophilic *Shewanella* spp. have increased importance at lower temperatures.[8] 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.[8] The growth of *S. baltica* was also susceptible to the Indian *Terminalia* spp. extracts (Figure 2). 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. As reported for *S. putrefaciens* growth inhibition, the *T. chebula* methanolic fruit extract was a particularly potent inhibitors of *S. baltica* growth, with an inhibition zone of 14.7 ± 0.6 mm measured. Furthermore, 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 *T. catappa* fruit (10.3 ± 0.6 mm) and leaf methanolic (13.0 ± 1.0 mm) extracts were also potent inhibitors of *S. baltica* growth (as determined by zones of inhibition).

Growth of *S. frigidimarina* was also inhibited by several of the Indian *Terminalia* spp. extracts (Figure 3), albeit generally with substantially smaller inhibition zones than determined for *S. baltica* and *S. frigidimarina*. As evident for the inhibition of the growth of the other *Shewanella* spp., the methanolic extracts were generally more potent bacterial growth inhibitors than were the other corresponding solvent extracts.

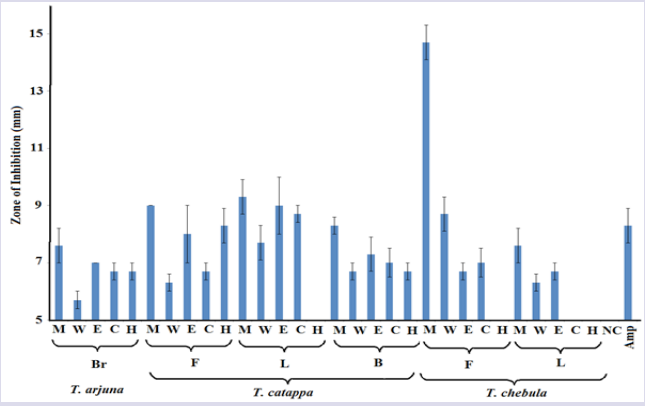

Figure 1: Growth inhibitory activity of the Indian *Terminalia* spp. extracts against the *S. putrefaciens* environmental isolates measured as zones of inhibition (mm). M = methanolic extract; W = aqueous extract; E = ethyl acetate extract; C = chloroform extract; H = hexane extract; Br = branch; F = fruit; L = leaf; B = bark; NC = negative (water) control; Amp = ampicillin (10 µg). Results are expressed as mean zones of inhibition ± SEM.

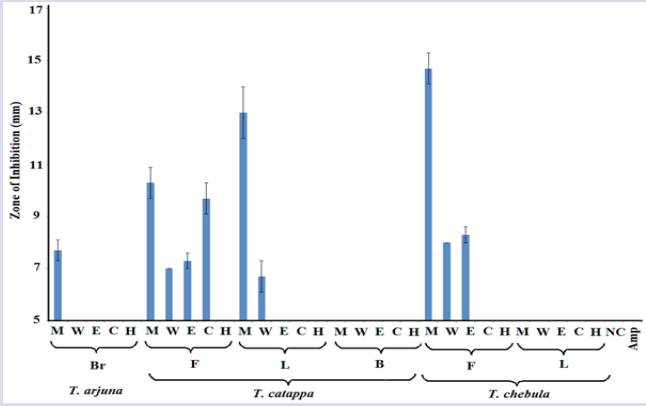

Figure 2: Growth inhibitory activity of the Indian *Terminalia* spp. extracts against the *S. baltica* environmental isolates measured as zones of inhibition (mm). M = methanolic extract; W = aqueous extract; E = ethyl acetate extract; C = chloroform extract; H = hexane extract; Br = branch; F = fruit; L = leaf; B = bark; NC = negative (water) control; Amp = ampicillin (10 µg). Results are expressed as mean zones of inhibition ± SEM.

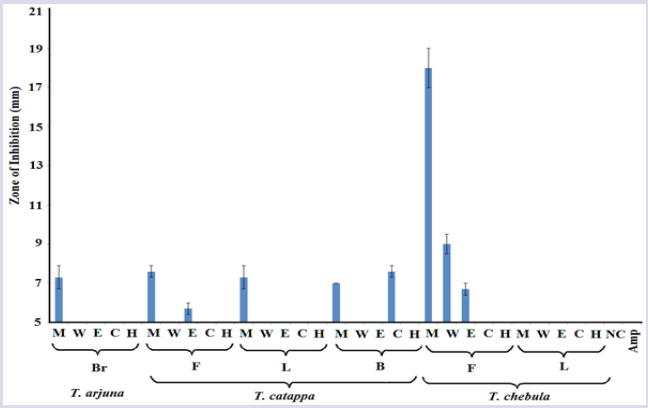

Figure 3: Growth inhibitory activity of the Indian *Terminalia* spp. extracts against the *S. frigidimarina* environmental isolates measured as zones of inhibition (mm). M = methanolic extract; W = aqueous extract; E = ethyl acetate extract; C = chloroform extract; H = hexane extract; Br = branch; F = fruit; L = leaf; B = bark; NC = negative (water) control; Amp = ampicillin (10 µg). Results are expressed as mean zones of inhibition ± SEM.

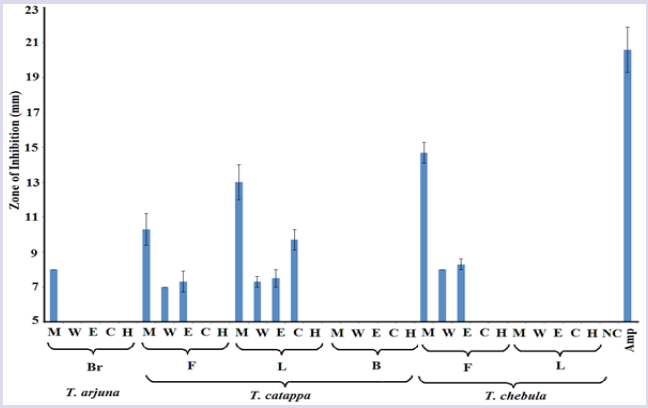

Figure 4: Growth inhibitory activity of the Indian *Terminalia* spp. extracts against the *S. loihica* environmental isolates measured as zones of inhibition (mm). M = methanolic extract; W = aqueous extract; E = ethyl acetate extract; C = chloroform extract; H = hexane extract; Br = branch; F = fruit; L = leaf; B = bark; NC = negative (water) control; Amp = ampicillin (10 µg). Results are expressed as mean zones of inhibition ± SEM.

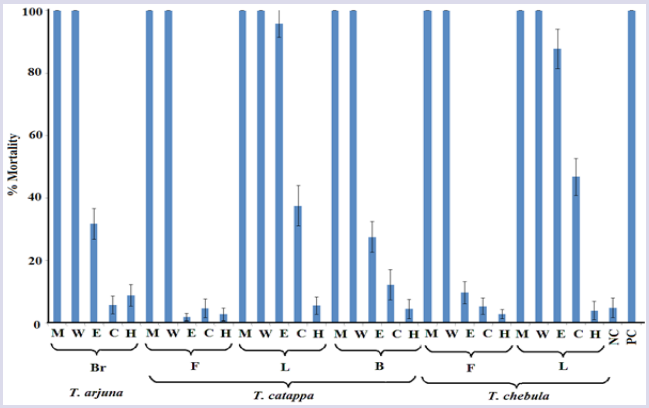

Figure 5: The lethality of the Indian *Terminalia* spp. extracts (2000 µg/mL) and the potassium dichromate (1000 µg/mL) and seawater controls towards *Artemia franciscana* nauplii after 24 h exposure. Br = branch; F = fruit; L = leaf; B = bark; M = methanolic extract; W = aqueous extract; E = ethyl acetate extract; C = chloroform extract; H = hexane extract. NC = negative (seawater) control; PC = potassium dichromate control (1000 µg/Ml.

The methanolic *T. chebula* fruit extract was particularly potent, with an inhibition zone of 18.0 ± 1.0 mm. Notably, as seen for the other psychrotrophic bacterial species (*S. baltica*), *S. frigidimarina* growth was also resistant to ampicillin exposure.

S. loihica and *S. putrefaciens* share similar genotypic and phenotypic characteristics and have similar optimal growth conditions.[34] Thus, the ability of the Indian *Terminalia* spp. to inhibit *S. loihica* was also tested (Figure 4). In contrast with the other *Shewanella* spp., *S. loihica* was particularly susceptible to the ampicillin control (zone of inhibition of 20.6 ± 1.3 mm). Whilst substantially smaller zones of inhibition were recorded against most of the Indian *Terminalia spp.* extracts, several displayed potent *S. loihica* growth inhibition. Indeed, exposure of *S. loihica* to the methanolic *T catappa* fruit and leaf extracts, and the *T. chebula* fruit extracts all produced >10 mm zones of inhibition. As evident for the growth inhibition of the other *Shewanella* spp., the methanolic *T. chebula* fruit extract was the most potent growth inhibitor (as assessed by the inhibition diameter), with a an inhibition zone of 14.7 ± 0.6 mm measured.

Quantification of minimum inhibitory concentration (MIC)

The relative level of *Shewanella* spp. growth inhibitory activity was further evaluated by determining the MIC values (Table 2) 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. The methanolic and ethyl acetate extracts of all plant

Table 2: MICs of the *T. arjuna, T. catappa* and *T. chebula* extracts measured against *S. putrefaciens, S. baltica, S. frigidimarina* and *S. loihica* growth (µg/mL), and *Artemia* nauplii bioassay LC_{50} values (µg/mL).

Plant Species	Plant Part	Extract	MIC (µg/mL)			LC_{50} (µg/mL)	
			S. putrafaciens	*S. baltica*	*S. frigidimarina*	*S. loihica*	*Artemia* nauplii
T. arjuna	Br	M	1833	889	2891	623	1683
		W	2775	-	-	-	2094
		E	520	-	-	-	-
		C	3000	-	-	-	-
		H	4286	-	-	-	-
T. catappa	F	M	283	697	1651	705	1452
		W	4226	4244	-	4486	1873
		E	1886	2698	> 5,000	2808	-
		C	> 5,000	912	-	-	-
		H	1631	-	-	-	-
	L	M	684	679	1695	613	1328
		W	1255	> 5,000	-	> 5,000	1765
		E	255	-	-	2405	-
		C	2376	-	-	1432	-
		H	-	-	-	-	-
	B	M	2270	-	> 5,000	-	1730
		W	> 5,000	-	-	-	2247
		E	320	-	-	-	-
		C	2686	-	2158	-	-
		H	> 5,000	-	-	-	-
T. chebula	F	M	198	329	162	176	1883
		W	1716	2495	2060	2237	2246
		E	344	727	244	874	-
		C	2875	-	-	-	-
		H	-	-	-	-	-
	L	M	3240	-	-	-	1387
		W	> 5,000	-	-	-	1945
		E	428	-	-	-	-
		C	-	-	-	-	-
		H	-	-	-	-	-

Numbers indicate the mean MIC and LC_{50} values of triplicate determinations. - indicates no inhibition. Br = branch extract; F = fruit extract; L = leaf extract; B = bark extract. M = methanolic extract; W = aqueous extract; E = ethyl acetate extract; C = chloroform extract; H = hexane extract.

parts were generally good inhibitors of *Shewanella* spp. growth. The methanolic and ethyl acetate *T. chebula* extracts were particularly potent growth inhibitors of *S. putrefaciens* (MIC's of 198 and 344 µg/mL respectively), *S. baltica* (329 and 727 µg/mL), *S. frigidmarina* (162 and 244 µg/mL) and *S. loihica* (176 and 874 µg/mL). The *T. catappa* leaf and fruit methanolic extracts were also potent *Shewanella* spp. growth inhibitors, albeit with MIC values generally several fold greater than the *T. chebula* methanolic and ethyl acetate extracts. Despite this, as the *T. catappa* extracts MIC's were generally <1000 µg/mL against most *Shewanella* spp., they were therefore also considered potent growth inhibitors. In contrast, MIC values determined for the *T. catappa* bark and all *T. arjuna* extracts were generally 1000-5000 µg/mL and therefore these extracts were considered to be moderate growth inhibitors. All other extracts were either inactive or of only low growth inhibitory activity.

Quantification of toxicity

All extracts were initially screened in the assay at 2000 µg/mL (Figure 5). 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 h (unpublished results). Similarly, all methanolic and aqueous extracts induced 100% mortality at 24 h. The ethyl acetate extracts also induced significant mortality at 24 h, although the % mortality was generally <50 %. Thus these extracts were deemed to be nontoxic. In contrast, the % mortality induced by the chloroform and hexane extracts was not significantly different from the seawater negative control.

To further quantify the effects of toxin concentration on the initiation of mortality, the extracts were serially diluted in artificial seawater to test

across a range of concentrations in the *Artemia* nauplii bioassay. The 24 h LC_{50} values of the Indian *Terminalia* spp. extracts towards *A. franciscana* are displayed in Table 2. No LC_{50} values were determined for the ethyl acetate, chloroform or hexane extracts of any of the Indian *Terminalia* spp. as < 50 % mortality was seen in all tested concentrations. LC_{50} values substantially >1000 µg/mL were determined for all of the other extracts. As extract with LC_{50} values >1000 µg/mL towards *Artemia* nauplii have been defined as being nontoxic in this assay,[35] all of the Indian *Terminalia* spp. extracts were deemed to be nontoxic.

DISCUSSION

Low temperature storage is currently the main method used for preserving fresh fish and other seafood by retarding microbial growth. Whilst this is an effective method of controlling the growth of many bacteria, it is inefficient at inhibiting the growth of psychrophilic and psychrotrophic bacteria such as the *Shewanella* spp. Other preservation methods are required to ensure food safety and to decreases losses. Reducing 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 also 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 butylhydroxyanisol (BHA), butylated hydroxytoluene (BHT), nitrates, nitrites, sulfur dioxide (SO_2) and sulfites (SO_3).[2] However, the safety of many of the chemical preservatives used in food has yet to be determined and in some cases these preservatives have been linked with serious health problems. Studies have indicated that commonly used chemical food preservatives may cause respiratory problems,[36] aggravate attention deficit hyperactivity disorder (ADHD)[37] and cause anaphylactic shock in susceptible individuals.[36] Due to greater consumer awareness and the negative perceptions of artificial preservatives, consumers are increasingly avoiding foods containing preservatives of chemical origin. Natural antimicrobial alternatives are increasingly being sought to increase the shelf life and safety of processed foods.[38]

The Indian *Terminalia* spp. examined in this study 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 (e.g. some plant extracts) decreases lipid oxidation and thus inhibits oxidative rancidity.[39] *T. arjuna, T. catappa* and *T. chebula* have previously been reported high to have very high antioxidant capacities[13] and thus have potential in reducing oxidative rancidity. The contribution of antioxidant protection to food spoilage has been extensively reported elsewhere [39] and was therefore not a focus of our study.

The other criterion for our selection of *T. arjuna, T. catappa* and *T. chebula* as potential fish preservatives was their ability to inhibit the growth of other bacterial species. Previous studies have reported antibacterial activity for extracts of these species against a wide variety of pathogenic and food spoilage bacteria.[40-43] Furthermore, potent growth inhibitory activity has also been reported for Australian.[10-15,16] and African *Terminatia* spp.[18-21] Despite this, there is a lack of previous studies examining the ability of *T. arjuna, T. catappa* and *T. chebula* to inhibit *Shewanella* spp. growth. Our study has confirmed the potential of Indian *Terminalia spp.* extracts for delaying fish spoilage and increasing seafood shelf life. All *Terminalia* spp. displayed considerable growth inhibitory activity against all *Shewanella* spp. tested. The methanolic and ethyl acetate *T. chebula* fruit extracts were particularly promising, with MIC values of approximately 190 and 340 µg/mL (for the methanolic and ethyl acetate extracts respectively) against *S. putrefaciens,* 330 and 730 µg/mL (for the methanolic and ethyl acetate extracts respectively) against *S. baltica*, 160 and 240 µg/mL (for the methanolic and ethyl acetate extracts respectively) against *S. frigidimarina*, and 170 and 870 µg/mL (for the methanolic and ethyl acetate extracts respectively) against *S. loihica*. In addition, several of the other Indian *Terminalia* spp. extracts were also potent inhibitors of *Shewanella* spp. growth, albeit with higher MIC values. This indicates that they also have potential as fish preservatives. For example, the *T. catappa* leaf and fruit methanolic extracts were also potent *Shewanella* spp. growth inhibitors, albeit with MIC values generally several fold greater than the *T. chebula* methanolic and ethyl acetate extracts. The *T. catappa* bark and all *T. arjuna* extracts also displayed moderate growth inhibitory activity and may therefore be useful for retarding fish spoilage.

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 µg/mL against a different environmental *S. putrefaciens* isolate by an ethanolic *Zataria multiflora* extract.[44] *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.[44] A different study reported moderate growth inhibition (2 mg/mL) of *S. putrefaciens* by aqueous *Terminalia catappa* extracts.[45] This plant is widely regarded for its antibacterial properties and is believed to have potent broad spectrum antibacterial activity.[13] Thus, the significantly greater potency reported in our study emphasises the efficacy of the Indian *Terminalia* spp. extracts. However, it is noteworthy that the previous *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 in that study. Whilst the earlier studies examined *S. putrefaciens* growth inhibition, neither screened against the other *Shewanella* spp. tested in our study, so these comparisons are not available.

Considerably fewer studies have examined the inhibitory properties of plant extract against the other *Shewanella* spp. examined in our study. However, an interesting trend was noted in the literature: Several authors reported antibiotic resistance in multiple *Shewanella* spp.[46] 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 Indian *Terminalia* spp. 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 the Indian *Terminalia* spp. 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).

Whilst a detailed investigation of the phytochemistry of the Indian *Terminalia* spp. extracts tested was beyond the scope of our study, the qualitative phytochemical studies highlighted several classes of compounds which may contribute to their bacterial growth inhibitory properties. Flavonoids were particularly prevalent in all extracts. Many studies have reported potent antibacterial activities for a wide variety of flavonoids.[47] Tannins were also generally present in relative abundance in the extracts with the greatest inhibitory activity. Multiple tannins have been

reported to inhibit the growth of a broad spectrum of bacterial species[48] through a variety of intra- and extracellular mechanisms.[48-51] It is likely that other phytochemical classes may also contribute to the growth inhibitory properties of these extracts. Further phytochemical evaluation studies and bioactivity driven isolation of active components is required to evaluate the mechanism of *Shewanella* spp. growth inhibition. The findings reported here demonstrate that all Indian *Terminalia* spp. fruit extracts were nontoxic towards *Artemia* nauplii and are thus safe to use as natural fish preservatives. However, further toxicity studies using human cell lines and subsequent *in vivo* studies are required to confirm the safety of these extracts before they are accepted as natural fish preservative alternatives.

CONCLUSIONS

The results of this study demonstrate that the Indian *Terminalia* spp. extracts are potent inhibitors of *Shewanella* spp. growth and therefore have potential as natural fish/seafood preservatives. The *T. chebula* fruit methanolic extract was particularly effective against all psychrotrophic and mesophilic *Shewanella* spp. and thus have potential for both fresh and cold storage fish preservation.

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CONFLICT OF INTEREST

The authors report no conflicts of interest.

ABBREVIATION USED

DMSO: Dimethyl sulfoxide; LC50: The concentration required to achieve 50 % mortality; MIC: minimum inhibitory concentration.

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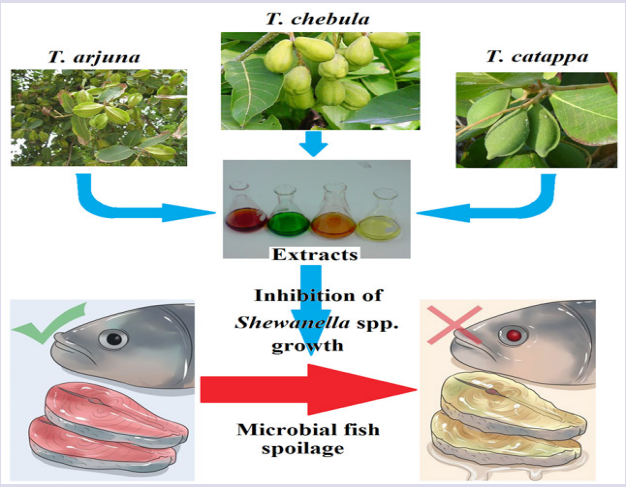
PICTORIAL ABSTRACT



- Extracts of 3 Indian *Terminalia* spp. inhibited *Shewanella* spp. growth.
- *T. chebula* fruit methanolic and ethyl acetate extracts were particularly potent (MIC's substantially <1000 µg/mL against all *Shewanella* spp).
- *T. catappa* fruit methanolic extracts were similarly potent (MIC values substantially <1000 µg/mL against all *Shewanella* spp).
- *T. catappa* bark and all *T. arjuna* extracts were moderate growth inhibitors (MIC values 1000-5000 µg/mL).
- All other extracts were either inactive or of only low growth inhibitory activity.
- All Indian *Terminalia* spp. extracts were non-toxic in the *Artemia* nauplii assay.

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Ms Samantha Webster is an undergraduate student studying for forensics/biomolecular sciences and criminology dual degrees. She has an interest in laboratory based studies and has completed several projects examining medicinal plants and bacterial pathogens in Dr Ian Cock's research group.

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Dr Anthony 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 nearly 200 scientific publications in a variety of peer reviewed journals.