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Full Length Research Paper

Response of *Flavobacterium* Species Isolates Associated with Fish to Cinnamaldehyde, Vanillin, and Extracts from *Kigelia africana* Fruits

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Phytochemicals are being explored as therapeutic alternatives in aquaculture since they have de-stressing, growth-promoting, appetite-increasing, immune-stimulating, and antimicrobial properties. The susceptibility of 28 *Flavobacterium johnsoniae*-like isolates and nine selected *Flavobacterium* spp. isolates to three phytochemicals, viz.: cinnamaldehyde (10 - 250 µg/ml), vanillin (5 - 500 µg/ml) and four crude *Kigelia africana* extracts (4 – 10 mg/ml ethyl acetate, dichloromethane, methanol and hexane), were assessed using disk diffusion assays and compared to standard antimicrobial agents, ampicillin and tetracycline using activity indices. Cinnamaldehyde (250 µg/ml) was more effective than 250 µg/ml vanillin, which was ineffective even at higher concentrations. *K. africana* extract (4 mg/ml) antibacterial efficacy decreased in the following order: Ethyl acetate, methanol, dichloromethane and hexane. The 10 mg/ml methanolic *K. africana* extract was most effective, with 100% of isolates displaying susceptibility, irrespective of the isolation source. Methanolic extract (10 mg/ml) activity indices ≥ 1 were obtained for 67.9 and 71.4% of isolates, respectively, relative to AMP10 and TE30. Cinnamaldehyde and the *K. africana* methanol extract are promising candidates to be tested for their efficacy in the treatment of *Flavobacterium*-associated fish infections. These phytochemicals might be environmentally-friendly, cost-effective alternatives to antimicrobial agent use in aquaculture, with a lesser potential of resistance development.

Key words: Aquaculture, *Flavobacterium,* phytotherapy.

INTRODUCTION

Aquaculture is one of the fastest growing industries with ~80 million tons of farmed fish and shellfish being produced annually. Over-crowding and increased stress levels in aquaculture systems predispose fish to develop flavobacterial infections, leading to significant economic losses (Flemming et al., 2007; Rattanchaikunsopon and Phumkhachorn, 2009; Schrader, 2008). A number of *Flavobacterium* spp. (Gram-negative, oxidase-positive, yellow-pigmented, and non-fermenting rods with gliding motility) are pathogenic or regarded as opportunistic pathogens in a wide variety of organisms, including plants and fish. *Flavobacterium columnare, F. psychrophilum, F. branchiophilum, F. aquatile and F. johnsoniae* have been associated with fish disease and have also been detected in surrounding water in the presence of disease outbreaks. *Flavobacterium johnsoniae*-like isolates appear to be significant in the Southern African context as aquaculture pathogens (Flemming et al., 2007; Basson et al., 2008), being isolated predominantly from diseased cultured trout. Prevention of flavobacteria epizootics is

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hampered by the ubiquitous presence of *Flavobacterium* spp. in soils and waters and the identification of multidrug resistant strains (DeClerq *et al*., 2013), which makes control of flavobacterial disease outbreaks a significant challenge. *F. johnsoniae* isolates contain chromosomally located β-lactamase genes (Naas et al., 2003) and chloramphenicol-inducible resistance-nodulation-division family multidrug efflux pump system, FmeABC1 (Clark et al., 2009).

Thus the appropriate choice of effective antimicrobial agents for treatment of *Flavobacterium* spp. is challenging due to their decreased susceptibility to many antimicrobial agents (Clark et al., 2009). Although these infections may be resolved using synthetic antimicrobial agents, the dangers of multi-drug resistance, environmental pollution, residues in fish and the need for organic aquaculture has stimulated the search for bioactive materials from plants that have potent antimicrobial/anti-virulence activity (Chakraborty and Hancz, 2011).

Phytochemicals are plant-derived compounds, which based on their chemical structure, can principally be categorized into alkaloids, flavonoids, pigments, phenolics, terpenoids, steroids and essential oils. Phytochemicals have multiple effects, with the antibacterial active components of plants being able to lyse the cell wall, block protein synthesis and DNA synthesis (Chakraborty and Hancz, 2011), while the anti-virulence components inhibit enzyme secretions and interfere with the signaling mechanisms of the quorum sensing pathway (Chenia, 2013; Packiavathy et al., 2012). Cinnamaldehyde or 3-phenyl-2-propenal occurs naturally in the bark and leaves of cinnamon trees of the genus *Cinnamomum*. This potent aromatic compound, with GRAS status, demonstrates a broad spectrum of antimicrobial activity (Nuryastuti et al., 2009). Cinnamaldehyde acts by inhibiting the proton motive force, respiratory chain, electron transfer and substrate oxidation, resulting in uncoupling of oxidative phosphorylation, inhibition of active transport, loss of pool metabolites, and disruption of synthesis of DNA, RNA, proteins, lipids, and polysaccharides. The resulting extensive leakage from bacterial cells or the exit of critical molecules and ions leads to cell death (Nuryastuti et al., 2009).

Vanillin (4-hydroxy-3-methoxybenzaldehyde) is a major component of natural vanilla, (a widely used flavoring material with GRAS status), obtained from the bean of the tropical orchid *Vanilla planifolia* (Kappachery et al., 2010). The mode of action of the phenylpropene phenolic aldehyde, vanillin, is not well understood, but it appears to be bacteriostatic. The inhibitory action of vanillin on *E. coli, Lactobacillus plantarum* and *Listeria innocua* cells was due to its ability to negatively affect cell membrane integrity, which resulted in a loss of the ion gradient, pH homeostasis and an inhibition of respiration (Fitzgerald et al., 2004). *Kigelia africana* (Lam.) Benth., commonly known as the sausage tree, of the *Bignoniaceae* family is found in South, Central and West Africa. This plant is used against dysentery, venereal diseases and as a topical application on wounds and abscesses in many African countries. The antimicrobial properties of *K. africana* leaves, fruits and stem-bark against Gram-negative and Gram-positive bacteria (Grace et al., 2002; Eldeen and Van Staden, 2007; Jeyachandran and Mahesh, 2007; Shai *et al*., 2008; Saini et al., 2009) and the anti-quorum sensing potential of *K. africana* fruit extracts (Chenia, 2013) have been investigated, giving credence to the use of this plant in traditional medicine.

Plant-based antimicrobials provide a vast untapped source of natural antimicrobial agents with potential therapeutic use in fish culture practice because they may be used for the effective treatment of infectious fish diseases; enhancing fish health and food safety and quality while conserving the aquatic environment (Chakraborty and Hancz, 2011). Thus, screening and proper evaluation of phytochemicals in medicinal plants that may be both sustainable and environmentally-acceptable, could offer possible alternatives to chemotherapeutic agents, reducing the side-effects of applying antimicrobial agents and the cost of therapy. Therefore, this study investigated the antimicrobial activity and efficacy of the phytochemicals cinnamaldehyde, vanillin and four crude *K. africana* fruit extracts on selected *Flavobacterium* spp. type strains, as well as 28 *F. johnsoniae*-like isolates from diverse South African aquaculture systems.

MATERIALS AND METHODS

Maintenance of bacterial isolates

Twenty-eight *F. johnsoniae*-like isolates previously isolated from moribund or healthy koi carp, longfin eel, trout and aquaculture tank biofilms (Flemming et al., 2007) were screened for susceptibility (Table 1). An additional nine *Flavobacterium* spp. strains from culture collections were also screened, viz., *F. aquatile* ATCC 11947^T, *F. columnare* C#2, *F. flevense* ATCC 27944^T, *F. johnsoniae* NCIMB 11054 (a), *F. johnsoniae* NCIMB 11054 (b), *F. johnsoniae* ATCC 17061^T (UW101), *F. pectinovorum* ATCC 19366^T, *F. psychrophilum* ATCC 49510, *F. psychrophilum* FPC830 (Table 1). All *Flavobacterium* spp. isolates were maintained on enriched Anacker and Ordal's (EAO) agar plates and stored at 4°C and for long-term storage in EAO broth containing 20% glycerol at -70°C (Flemming et al., 2007).

Preparation of crude fruit extracts

K. africana fruits were collected around the Westville Campus of University of KwaZulu-Natal. A voucher specimen of the *K. africana* plant (voucher specimen Chenia 1) is archived in the Ward Herbarium, University of KwaZulu-Natal, Westville campus (International herbarium acronym UDW). The material was chopped, oven-dried at 60°C, milled and stored in polythene bags at 4°C. Crude extracts were prepared by sequential exhaustive extraction with ethyl acetate, dichloromethane, methanol and hexane by maceration and continuous shaking on an orbital shaker at room temperature for 48 h (Kiplimo et al., 2011). Solvent extracts were concentrated using a vacuum rotary evaporator, dried, dissolved in dimethylsulfoxide (DMSO) to a final concentration of 100 mg/ml and stored at 4°C.

Table 1. Zones of inhibition (mm) obtained with cinnamldehyde, vanillin and four crude *Kigelia africana* extracts as well as standard antimicrobial agents, ampicillin and tetracycline, against fish-associated *F. johnsoniae*-like isolates.

Isolates	Source	C 125*	C 250*	V250*	V 500*	EX 1*	EX 2*	EX 3*	EX 3 (10 mg/ml)	EX 4*	AMP10*	TE30*
F. aquatile ATCC 11947^T		0	8	8	8	18	18	13	23	9	0	30
F. columnare C#2		12	14	10	17	22	22	12	30	11	46	42
F. flevense ATCC 27944^T		0	0	9	9	18	16	12	26	10	8	25
F. johnsoniae NCIMB 110546(a)		8	11	9	17	15	14	12	22	13	0	16
F. johnsoniae NCIMB 110546(b)		18	20	9	9	17	14	12	26	34	24	34
F. johnsoniae ATCC 17061^I		0	0	13	18	15	12	14	25	0	13	28
F. pectinovorum ATCC 19366^T		0	12	0	0	13	10	15	22	0	0	20
F. psychrophilum ATCC 49510		0	0	0	0	13.5	11	11	20	0	6	10.5
F. psychrophilum FPC830		0	0	8	8	11	9	13	17	0	0	10
YO12	Trout	0	0	8	9	12.5	11	10	23	9	12	30
YO15	Trout	0	38	0	15.5	12.5	13	12	30	11	14	24.5
YO19	Trout	0	0	0	0	27	18	11	34	0	20	40.5
YO20	Koi carp	0	0	8	8	18	12	11	22	9	34	18
YO21	Trout	0	0	8	8	11	14	10	25.5	0	13	19
YO26	Trout	0	0	0	0	11.5	11.5	12	22.5	11	14.5	25
YO34	Trout	0	12	0	0	18.5	11	11	29.5	0	16	29.5
YO35	Trout	0	0	0	0	29	38	10	44	0	11.5	31
YO38	Trout	0	11	0	0	10.5	11	11	24.5	10	12.5	19.5
YO45	Trout	0	10	8	8	13	12	10	28	11	13	21
YO49	Trout	0	0	0	0	11	9	12	27	0	13.5	20
YO50	Trout	0	0	0	0	11	9	12	20	10	0	10
YO51	Trout	0	33	12.5	22	15	12.5	15	28	13	0	26
YO52	Koi carp	0	0	0	0	11.5	10	11	23.5	0	24.5	14
YO53	Koi carp	0	0	0	0	11	9	0	29	10	15.5	21
YO54	Koi carp	0	0	0	0	23	26	14	29	0	19.5	23.5
YO55	Koi carp	0	0	0	0	21.5	10	13	28.5	9	16.5	22.5
YO56	Koi carp	0	16	0	0	19	13	8	38	10	19	19
YO57	Longfin eel	14.5	22.5	11	12	10	0	15	23	11	0	17.5
YO59	Longfin eel	0	0	0	0	0	0	15	18.5	0	0	10
YO60	Longfin eel	0	0	0	0	11.5	8.5	13	23	0	0	14
YO61	Longfin eel	0	0	0	0	13.5	9.5	15	24.5	13	0	21.5
YO62	Longfin eel	0	17	0	0	17.5	12	13	26	12	19	31
YO63	Longfin eel	0	0	9	9	11.5	7.5	14	22.5	9	18	24
YO64	Longfin eel	0	0	9	9	16	13.5	10	28	9	17	31

Table 1. Contd.

YO65	**Biofilm**	**30**	**37.5**	**0**	**15.5**	**11**	**13**	**12**	**21**	**13**	**13**	**28**
YO66	Biofilm	0	0	9	9	14	16	14	26	11	15.5	16
YO67	Biofilm	0	16.5	9	9	11.5	10.5	13	23.5	0	0	26
E. coli ATCC 25922		0	10	0	0	8	8	0	10	9	22	28

*C 125, cinnamaldehyde 125 µg/ml; C 250, cinnamaldehyde 250 µg/ml; V 250, vanillin 250 µg/ml; V 500, vanillin 500 µg/ml; EX 1, 4 mg/ml *K. africana* ethyl acetate extract; EX 2, 4 mg/ml *K. africana* dichloromethane extract; EX 3, 4 mg/ml and 10 mg/ml *K. africana* methanol extract; EX 4, 4 mg/ml *K. africana* hexane extract; AMP10, 10 µg/ml ampicillin; TE30: 30 µg/ml tetracycline.

Antimicrobial susceptibility testing

Antimicrobial susceptibility to cinnamaldehyde, vanillin and four crude *K. africana* fruit extracts were determined using the disc diffusion method. Blank discs (MAST, UK) were impregnated with 10 µl DMSO; 10, 25, 125 and 250 µg/ml of cinnamaldehyde (Sigma); 5, 25, 250, 400 and 500 µg/ml of vanillin (Sigma) and crude *K. africana* ethyl acetate (EX 1, 4 mg/ml), dichloromethane (EX 2, 4 mg/ml) methanol (EX 3, 4 mg/ml and 10 mg/ml), hexane (EX 4, 4 mg/ml) extracts and allowed to dry.

Bacterial isolates were grown overnight on EAO agar plates at 26°C and the turbidity of cell suspensions were adjusted equivalent to that of a 0.5 McFarland standard. These were used to inoculate dilute (1/10) Mueller-Hinton (MH) agar plates by streaking swabs over the entire agar surface followed by the application of the respective phytochemical extract discs (CLSI, 2007). Plates were then incubated for 21 h at 26°C. Testing was done in duplicate and ampicillin (AMP10; 10 µg/ml) and tetracycline (TE30; 30 µg/ml) discs were used as standard antimicro-bial agent controls. Zone diameters were determined and averaged. The following zone diameter criteria were used to assign susceptibility or resistance to phytochemicals tested: Susceptible (S) ≥ 15 mm, Intermediate (I) = 11 – 14 mm, and Resistant (R) ≤ 10 mm (Chenia, 2013). Criteria for assigning susceptibility or resistance to AMP10 was as follows: (S) ≥ 17 mm, (I) = 14 to 16 mm, (R) ≤ 13 mm, while those for TE30 were: (S) ≥ 19 mm, (I) 15 to 18 mm, (R) ≤ 14 mm (CLSI, 2007).

Activity indices of each extract were calculated by comparing zones of inhibition obtained with each of the extracts with those obtained with the standard antimicrobial agents, ampicillin and tetracycline. The following equation was used: Relative activity index (RAI) = Inhibition diameter (mm) with test extract/Inhibition diameter (mm) with standard antimicrobial agent (Jeyachandran and Mahesh, 2007).

Statistical analysis

The variations between experiments were estimated by standard deviations, and statistical significance of changes was estimated by one way repeated measures analysis of variance, with $p \leq 0.05\%$ being regarded as statistically significant (SigmaStat V3.5, Systat Software, Inc, CA, USA).

RESULTS

Ampicillin zone diameters ranged from 0 to 46 mm (Table 1) for selected *Flavobacterium* spp. Isolates, with 66.67% (6/9) of isolates demonstrating resistance (Table 2). Zone diameters of 0 to 34 mm were obtained with *F. johnsoniae*-like isolates (Table 1), with 46.43% (13/28) displaying resistance (Table 3). With tetracycline, zone diameters of selected *Flavobacterium* spp. isolates ranged from 10 to 42 mm (Table 1), of which 66.67% (6/9) were susceptible (Table 2). *F. johnsoniae*-like isolates produced tetracycline zones ranging from 10 to 40.5 mm (Table 1), with 75% (21/28) displaying susceptibility (Table 3).Concentration-dependent differences were observed in the antimicrobial effects of cinnamaldehyde and vanillin against the nine selected *Flavobacterium* spp. isolates and the 28 *F. johnsoniae*-like isolates (Tables 2 and 3). All isolates were resistant to 10 and 25 µg/ml of cinnamaldehyde (Tables 2 and 3); with no zones of inhibition being observed for selected *Flavobacterium* spp. isolates as well as *F. johnsoniae*-like isolates. Although zones of inhibition were observed with 125 and 250 µg/ml of cinnamaldehyde (Table 1);

> 50% of the selected *Flavobacterium* spp. isolates as well as *F. johnsoniae*-like isolates demonstrated resistance (Tables 2 and 3).

Resistance was also observed to 5 and 25 µg/ml of vanillin (Tables 2 and 3), with no zones of inhibition being observed for both selected *Flavobacterium* spp. isolates as well as *F. johnsoniae*-like isolates. Although zone diameters ranged from 0 to 13 mm for *Flavobacterium* spp. isolates with 250 µg/ml of vanillin, only the *F johnsoniae* ATCC 17061^{T} isolate displayed intermediate resistance. With 250 µg/ml of vanillin, 92.86% (26/28) of. the *F. johnsoniae*-like isolates demonstrated resistance (Table 3), with zone diameters ranging from 0 to 12.5 mm. Similarly for 400 and 500 µg/ml of vanillin, 66.67% (6/9) of selected *Flavobacterium* spp. isolates (Table 2) and 85.71% (24/28) of *F. johnsoniae*-like isolates (Table 3) demonstrated resistance, respectively.

At 250 µg/ml, cinnamaldehyde was more effective as an antimicrobial agent than 250 µg/ml vanillin

Table 2. Susceptibility analysis of nine selected *Flavobacterium* spp. isolates to phytochemical extracts and standard antimicrobial agents.

Phytochemical	% Susceptibility/resistance phenotype (number of isolates)		
	Susceptible	Intermediate	Resistant
DMSO	0	0	100 (9/9)
Cinnamaldehyde (µg/ml)			
10	0	0	100 (9/9)
25	0	0	100 (9/9)
125	11.11 (1/9)	11.11 (1/9)	77.78 (7/9)
250	11.11 (1/9)	33.33 (3/9)	55.56 (5/9)
Kigelia africana			
4 mg/ml EX 1*	66.67 (6/9)	33.33 (3/9)	0
4 mg/ ml EX 2^	33.33 (3/9)	44.44 (4/9)	22.22 (2/9)
4 mg/ml EX 3*	11.11 (1/9)	88.89 (8/9)	0
10 mg/ml EX 3*	100 (9/9)	0	0
4 mg/ml EX 4*	11.11 (1/9)	22.22 (2/9)	66.67 (6/9)
Vanillin (µg/ml)			
5	0	0	100 (9/9)
25	0	0	100 (9/9)
250	0	11.11 (1/9)	88.89 (8/9)
400	33.33 (3/9)	0	66.67 (6/9)
500	33.33 (3/9)	0	66.67 (6/9)
Ampicillin (AMP10)	22.22 (2/9)	11.11 (1/9)	66.67 (6/9)
Tetracycline (TE30)	66.67 (6/9)	11.11 (1/9)	22.22 (2/9)

*EX 1, *K. africana* ethyl acetate extract; EX 2, *K. africana* dichloromethane extract; EX 3, *K. africana* methanol extract; EX 4, *K. africana* hexane extract.

or even the higher 400 to 500 µg/ml concentrations.

Selected *Flavobacterium* spp. isolates had zone diameters ranging from 11 to 22 mm (Table 1) with *K. africana* ethyl acetate EX 1 and 66.67% (6/9) of these isolates demonstrated susceptibility (Table 2). Ethyl acetate EX 1 zone diameters for *F. johnsoniae*-like isolates ranged from 0 to 29 mm (Table 1), with 35.71% (10/28) of isolates demonstrating susceptibility (Table 3). Zone diameters for selected *Flavobacterium* spp. isolates ranged from 9 to 22 mm with the dichloromethane EX 2 (Table 1), with 22.22% (2/9) of isolates displaying susceptibility (Table 2). For *F. johnsoniae*-like isolates, zones of inhibition with dichloromethane EX 2 ranged from 0 to 38 mm (Table 1), with 14.29% (4/28) of isolates demonstrating susceptibility (Table 3). With *K. africana* methanol extract EX 3 (4 mg/ml), selected *Flavobacterium* spp. isolates had zone diameters ranging from 11 to 15 mm (Table 1), with 88.89% (8/9) of isolates being intermediately susceptible (Table 2). For *F. johnsoniae*-like isolates, zone diameters ranged from 0 to 15 mm with methanol EX 3 (Table 1), with 14.29% (4/28) of isolates demonstrating susceptibility (Table 3). Selected *Flavobacterium* spp. isolates had zone diameters ranging from 0 to 34 mm (Table 1) with the *K. africana* hexane EX 4, with 66.67% (6/9) of isolates demonstrating resistance (Table 2). Hexane EX 4 zone diameters for *F. johnsoniae*-like isolates ranged from 0 to 13 mm (Table 1), with 67.86% (19/28) of isolates demonstrating resistance (Table 3). At 4 mg/ml, the *K. africana* ethyl acetate extract (EX 1) was most effective in its antimicrobial activity, followed by the methanol, dichloromethane and hexane extracts (Tables 2 to 3). *K. africana* methanol EX 3 at a concentration of 10 mg/ml was most effective, with all tested isolates demonstrating susceptibility (Tables 2 and 3), with zone diameters ranging from 17 to 44 mm (Table 1). Isolates from all sources (fish host or biofilm; Table 4) displayed increased susceptibility on exposure to 250 µg/ml cinnamaldehyde. Although decreased resistance was observed for trout and biofilm isolates, eel and koi isolates did not display increased susceptibility on exposure to increasing vanillin concentrations. Analysis of percent resistance based on source of isolation (Table 4) suggested that ethyl acetate EX 1 was more effective than ampicillin for inhibition of *F. johnsoniae*-like isolates (Table

Table 3. Susceptibility analysis of 28 *F. johnsoniae*-like isolates to phytochemical extracts and standard antimicrobial agents.

Phytochemical	% Susceptibility/resistance phenotype (number of isolates)		
	Susceptible	Intermediate	Resistant
DMSO	0	0	100 (28/28)
Cinnamaldehyde (µg/ml)			
10	0	0	100 (28/28)
25	0	0	100 (28/28)
125	3.57 (1/28)	3.57 (1/28)	92.86 (26/28)
250	25 (7/28)	7.14 (2/28)	67.86 (19/28)
Kigelia africana			
4 mg/ml EX 1*	35.71 (10/28)	57.14 (16/28)	7.14 (2/28)
4 mg/ ml EX 2*	14.29 (4/28)	50 (14/28)	35.71 (10/28)
4 mg/ml EX 3*	14.29 (4/28)	60.71 (17/28)	25 (7/28)
10 mg/ml EX 3*	100 (28/28)	0	0
4 mg/ml EX 4*	0	32.14 (9/28)	67.86 (19/28)
Vanillin (µg/ml)			
5	0	0	100 (28/28)
25	0	0	100 (28/28)
250	0	7.14 (2/28)	92.86 (26/28)
400	7.14 (2/28)	7.14 (2/28)	85.71 (24/28)
500	10.71 (3/28)	3.57 (1/28)	85.71 (24/28)
Ampicillin (AMP10)	28.57 (8/28)	25 (7/28)	46.43 (13/28)
Tetracycline (TE30)	75 (21/28)	10.71 (3/28)	14.29 (4/28)

*EX 1, *K. africana* ethyl acetate extract; EX 2, *K. africana* dichloromethane extract; EX 3, *K. africana* methanol extract; EX 4, *K. africana* hexane extract.

3). The hexane EX 4 was the least effective with no isolates, irrespective of source, demonstrating complete susceptibility (Tables 2 to 4).

Compared to 4 mg/ml methanol EX 3, 10 mg/ml of methanol extract EX 3 was more effective than ampicillin and tetracycline, against *F. johnsoniae*-like isolates, irrespective of isolation source.

Based on zones of inhibition obtained with phyto-chemicals and standard antimicrobial agents, the RAIs were determined (Table 5). An extract was considered effective against an isolate if the RAI was ≥ 1 (Table 6). Ampicillin was regarded as a poor standard for comparison since 46.43% (13/28) of the study isolates exhibited resistance (Table 3). The percentage of selected *Flavobacterium* spp. and *F. johnsoniae*-like isolates demonstrating RAIs ≥ 1 is indicated in Table 6.

Relative to ampicillin, 125 and 250 µg/ml cinnamal-dehyde RAIs of selected *Flavobacterium* spp. isolates ranged from 0 to 0.750 and 0 to 0.833 (Table 5), while those of *F. johnsoniae*-like isolates ranged from 0 to 2.308 and 0 to 2.885 (Table 5), respectively. Relative to tetracycline, 125 and 250 µg/ml cinnamaldehyde RAIs ranged from 0 to 1.071 and 0 to 1.551 for *F. johnsoniae*-like isolates (Table 5). With 250 and 500 µg/ml vanillin, RAIs of selected *Flavobacterium* spp. isolates ranged from 0 to 1.125 and 0 to 1.385 (Table 5), while those of *F. johnsoniae*-like isolates ranged from 0 to 0.667 and 0 to 1.192, relative to ampicillin. Relative to tetracycline, 250 and 500 µg/ml vanillin RAIs ranged from 0 to 0.563 and 0 to 1.063 for selected *Flavobacterium* spp. isolates and from 0 to 0.563 and 0 to 0.846 for *F. johnsoniae*-like isolates (Table 5).

RAIs ranging from 0 to 2.250, relative to ampicillin, were obtained with ethyl acetate EX 1 for selected *Flavobacterium* spp. isolates, while RAIs with tetracycline ranged from 0 to 1.286 (Table 5). For dichloromethane EX 2 relative to ampicillin, RAIs for selected *Flavobacterium* spp. Isolates ranged from 0 to 2, while those relative to tetracycline ranged from 0 to 1.048 (Table 5). With 4 mg/ml methanol EX 3, RAIs for selected *Flavobacterium* spp. isolates ranged from 0 to 1.883 (Table 5), while EX 3-tetracycline RAIs were between 0.286 to 1.300 (Table 5). 10 mg/ml methanol EX 3-ampicillin RAIs for selected *Flavobacterium* spp. isolates

Table 4. Percentages of resistance of *F. johnsoniae*-like isolates to cinnamaldehyde, vanillin, four *Kigelia africana* fruit extracts, ampicillin and tetracycline, are represented based on isolation source.

Source	C 125*	C 250*	V 250*	V 500*	EX 1*	EX 2*	EX 3*	EX 3 (10 mg/ml)*	EX 4*	AMP10*	TE30*
Trout (n = 12)	100 (12/12)	66.67 (8/12)	91.67 (11/12)	83.33 (10/12)	0	16.67 (2/12)	33.33 (4/12)	0	66.67 (8/12)	58.33 (7/12)	8.33 (1/12)
Eel (n = 7)	85.71 (6/7)	71.43 (5/7)	85.71 (6/7)	85.71 (6/7)	28.6 (2/7)	71.43 (5/7)	14.29 (1/7)	0	57.14 (4/7)	57.14 (4/7)	28.57 (2/7)
Koi (n = 6)	100 (6/6)	83.33 (5/6)	100 (6/6)	100 (6/6)	0	50 (3/6)	33.33 (2/6)	0	100 (6/6)	0	16.67 (1/6)
Biofilm (n = 3)	66.67 (2/3)	33.33 (1/3)	100 (3/3)	66.67 (2/3)	0	0	0	0	33.33 (1/3)	66.67 (2/3)	0

*C 125, Cinnamaldehyde 125 µg/ml; C 250, Cinnamaldehyde 250 µg/ml; V 250, vanillin 250 µg/ml; V 500, vanillin 500 µg/ml; EX 1, 4 mg/ml *K. africana* ethyl acetate extract; EX 2, 4 mg/ml *K. africana* dichloromethane extract; EX 3, 4 and 10 mg/ml *K. africana* methanol extract; EX 4, 4 mg/ml *K. africana* hexane extract; AMP10, 10 µg/ml ampicillin; TE30, 30 µg/ml tetracycline.

Table 5. Activity indices of cinnamaldehyde, vanillin and four crude *K. africana* fruit extracts, relative to the standard antimicrobial agents, ampicillin (AMP10) and tetracycline (TE30), against *Flavobacterium* spp. Isolates.

Isolates	Activity index AMP10							Activity index TE30						
	C 250*	V 500*	EX 1*	EX 2*	EX 3*	EX 3*	EX 4*	C 250*	V 500*	EX 1*	EX 2*	EX 3*	EX 3*	EX 4*
F. aquatile ATCC 11947^{I}	0.000	0.000	0.000	0.000	0	0.000	0.000	0.267	0.267	0.600	0.600	0.433	0.767	0.300
F. columnare C#2	0.304	0.370	0.478	0.478	0.261	0.652	0.239	0.333	0.405	0.524	0.524	0.286	0.714	0.262
F. flevense ATCC 27944^{T}	0.000	1.125	2.250	2.000	1.500	3.250	1.250	0.000	0.360	0.720	0.640	0.480	1.040	0.400
F. johnsoniae NCIMB 110546(a)	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.688	1.063	0.938	0.875	0.750	1.375	0.813
F. johnsoniae NCIMB 110546(b)	0.833	0.375	0.708	0.583	0.500	1.083	1.417	0.588	0.265	0.500	0.412	0.353	0.817	1.000
F. johnsoniae ATCC 17061^{I}	0.000	1.385	1.154	0.923	1.077	1.923	0.000	0.000	0.643	0.536	0.429	0.500	0.893	0.000
F. pectinovorum ATCC 19366^{T}	0.000	0.000	0.000	0.000	1.500	0.000	0.000	0.600	0.000	0.650	0.500	0.750	1.100	0.000
F. psychrophilum ATCC 49510	0.000	0.000	2.250	1..833	1.833	3.333	0.000	0.000	0.000	1.286	1.048	1.048	1.905	0.000
F. psychrophilum FPC830	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.800	1.100	0.900	1.300	1.900	0.000
YO12	0.000	0.750	1.042	0.917	0.833	1.917	0.750	0.000	0.300	0.417	0.367	0.333	0.767	0.300
YO15	2.714	1.107	0.893	0.929	0.857	2.143	0.786	1.551	0.633	0.510	0.531	0.490	1.224	0.449
YO19	0.000	0.000	1.350	0.900	0.550	1.700	0.000	0.000	0.000	0.667	0.444	0.272	0.840	0.000
YO20	0.000	0.235	0.529	0.353	0.324	0.647	0.265	0.000	0.444	1.000	0.667	0.611	1.222	0.500
YO21	0.000	0.615	0.846	1.077	0.769	1.962	0.000	0.000	0.421	0.579	0.737	0.526	1.342	0.000
YO26	0.000	0.000	0.793	0.793	0.828	1.552	0.759	0.000	0.000	0.460	0.460	0.480	0.900	0.440
YO34	0.750	0.000	1.156	0.688	0.688	1.844	0.000	0.407	0.000	0.627	0.373	0.373	1.000	0.000
YO35	0.000	0.000	2.522	3.304	0.870	3.826	0.000	0.000	0.000	0.935	1.226	0.323	1.419	0.000
YO38	0.880	0.000	0.840	0.880	0.880	1.960	0.800	0.564	0.000	0.538	0.564	0.564	1.256	0.513
YO45	0.769	0.615	1.000	0.923	0.769	2.154	0.846	0.476	0.381	0.619	0.571	0.476	1.333	0.524
YO49	0.000	0.000	0.815	0.667	0.889	2.000	0.000	0.000	0.000	0.550	0.450	0.600	1.350	0.000

Table 5. Contd.

YO50	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**1.100**	**0.900**	**1.200**	**2.000**	**1.000**
YO51	0.000	0.000	0.000	0.000	0.000	0.000	0.000	1.269	0.846	0.577	0.481	0.577	1.077	0.500
YO52	0.000	0.000	0.469	0.408	0.449	0.959	0.000	0.000	0.000	0.821	0.714	0.786	1.679	0.000
YO53	0.000	0.000	0.710	0.581	0.000	1.871	0.645	0.000	0.000	0.524	0.429	0.000	1.381	0.476
YO54	0.000	0.000	1.179	1.333	0.718	1.487	0.000	0.000	0.000	0.979	1.106	0.596	1.234	0.000
YO55	0.000	0.000	1.303	0.606	0.788	1.727	0.545	0.000	0.000	0.956	0.444	0.578	1.267	0.400
YO56	0.842	0.000	1.000	0.684	0.421	2.000	0.526	0.842	0.000	1.000	0.684	0.421	2.000	0.526
YO57	0.000	0.000	0.000	0.000	0.000	0.000	0.000	1.286	0.686	0.571	0.000	0.857	1.314	0.629
YO59	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	1.500	1.850	0.000
YO60	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.821	0.607	0.929	1.643	0.000
YO61	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.628	0.442	0.698	1.140	0.605
YO62	0.895	0.000	0.921	0.632	0.684	1.368	0.632	0.548	0.000	0.565	0.387	0.419	0.839	0.387
YO63	0.000	0.500	0.639	0.417	0.778	1.250	0.500	0.000	0.375	0.479	0.313	0.583	0.938	0.375
YO64	0.000	0.529	0.941	0.794	0.588	1.647	0.529	0.000	0.290	0.516	0.435	0.323	0.903	0.290
YO65	2.885	1.192	0.846	1.000	0.923	1.615	1.000	1.339	0.554	0.393	0.464	0.429	0.750	0.464
YO66	0.000	0.581	0.903	1.032	0.903	1.677	0.710	0.000	0.563	0.875	1.000	0.875	1.625	0.688
YO67	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.635	0.346	0.442	0.404	0.500	0.904	0.000

*C 250, Cinnamaldehyde 250 µg/ml; V 500, vanillin 500 µg/ml; EX 1, 4 mg/ml *K. africana* ethyl acetate extract; EX 2:, 4 mg/ml *K. africana* dichloromethane extract; EX 3, 4 and 10 mg/ml *K. africana* methanol extract; EX 4, 4 mg/ml *K. africana* hexane extract; AMP10, 10 µg/ml ampicillin; TE30, 30 µg/ml tetracycline.

ranged from 0 to 3.333, while EX 3-tetracycline RAIs were between 0.714 to 1.905 (Table 5). For the hexane EX 4, ampicillin RAIs ranged from 0 to 1.417, while tetracycline RAIs ranged from 0 to 1 (Table 5).

For *F. johnsoniae*-like isolates, RAIs for ethyl acetate EX 1 relative to ampicillin ranged from 0 to 2.522, while relative to tetracycline, RAIs ranged from 0 to 1.100 (Table 5). For dichloro-methane EX 2, relative to ampicillin, RAIs of 0 to 3.304 were obtained, while those relative to tetracycline were between 0 to 1.226 (Table 5). With 4 mg/ml methanol EX 3, RAIs ranged from 0 to 0.923 (Table 5) relative to ampicillin, while relative to tetracycline, RAIs ranged from 0 to 1.200. Methanol EX 3 (10 mg/ml)-ampicillin RAIs for *F. johnsoniae*-like isolates ranged from 0 to 3.826, while EX 3-tetracycline RAIs were between 0 and 2 (Table 5). For *F. johnsoniae*-like isolates, RAIs for hexane EX 4 relative to ampicillin were between 0 and 1, while relative to tetracycline, RAIs ranged from 0 to 1 (Table 5). The 10 mg/ml *K. africana* methanol extract (EX 3) appeared to be the most effective with RAIs ≥ 1 for 71.43% (20/28) of *F. johnsoniae*-like isolates and for 55.56% (5/9) of selected *Flavobacterium* spp. isolates, when compared to tetracycline (Table 6).

DISCUSSION

Common treatments of *Flavobacterium*-associated infections include bath treatment with chemicals such as copper sulfate and potassium permanganate in response to epizootics or the inclusion of antimicrobial agents such as oxytetracycline and related compounds in fish feed (prophylactic treatment). However, their efficacy is impacted by water quality variables, they are highly phytotoxic, they have a broad-spectrum toxicity, consequently also killing beneficial organisms, and most importantly, the bioaccumulation of these compounds in the environment and fish tissue is problematic (Schrader, 2008). Although a number of antimicrobial agents are used as feed additives, prophylactically or therapeutically, the increasing incidence of drug-resistant pathogens, zoonoses, accumulation of antimicrobial agents in both fish and the environment and horizontal gene transfer of antimicrobial resistance determinants suggest the need for alternative, sustainable,

Table 6. Percent of isolates with activity indices ≥ 1 for cinnamaldehyde, vanillin and *K. africana* extracts relative to ampicillin (AMP10) and tetracycline (TE30).

Compounds	% of isolates with activity indices ≥ 1			
	F. johnsoniae-like		*Flavobacterium* spp.	
	Ampicillin (AMP10)	Tetracycline (TE30)	Ampicillin (AMP10)	Tetracycline(TE30)
125 µg/ml cinnamaldehyde	3.57 (1/28)	3.57 (1/28)	0	0
250 µg/ml cinnamaldehyde	7.41 (2/28)	14.29 (4/28)	0	0
250 µg/ml vanillin	0	0	22.22 (2/9)	0
400 µg/ml vanillin	7.41 (2/28)	0	22.22 (2/9)	0
500 µg/ml vanillin	7.41 (2/28)	0	22.22 (2/9)	11.11 (1/9)
4 mg/ml *K. africana* EX 1*	28.57 (8/28)	10.71 (3/28)	33.33 (3/9)	22.22 (2/9)
4 mg/ml *K. africana* EX 2*	14.29 (4/28)	10.71 (3/28)	22.22 (2/9)	11.11 (1/9)
4 mg/ml *K. africana* EX 3*	0	7.14 (2/28)	33.33 (3/9)	22.22 (2/9)
10 mg/ml *K. africana* EX 3*	67.86 (19/28)	71.43 (20/28)	44.44 (4/9)	55.56 (5/9)
4 mg/ml *K. africana* EX 4*	3.57 (1/28)	3.57 (1/28)	22.22 (2/9)	11.11 (1/9)

*EX 1: 4 mg/ml *K. africana* ethyl acetate extract; EX 2: 4 mg/ml *K. africana* dichloromethane extract; EX 3: 4 and 10 mg/ml *K. africana* methanol extract; EX 4: 4 mg/ml *K. africana* hexane extract; AMP10: 10 µg/ml ampicillin; and TE30: 30 µg/ml tetracycline.

cost-economic and environmentally-friendly therapeutic options (Chakraborty and Hancz, 2011). Phytochemicals are thus being investigated as alternative therapeutic options for the treatment of *Flavobacterium*-associated infections in aquaculture (Rattanchaikunsopon and Phumkhachorn, 2009, 2010; Seong Wei et al., 2009). Castro et al. (2008) observed that *F. columnare* was the microorganism most susceptible to majority of the 46 tested methanolic Brazilian plant extracts, in comparison to *Aeromonas hydrophila* and *Streptococcus agalactiae* isolates.

The majority of isolates in the present study were susceptible to tetracycline while displaying resistance to ampicillin, which was expected because of the presence of chromosomal β-lactamases (Naas et al., 2003). *F. johnsoniae*-like and selected *Flavobacterium* spp. isolates appeared to be resistant to 10 to 125 µg/ml cinnamaldehyde, although an increase in susceptibility was observed with 250 µg/ml cinnamaldehyde. This is in agreement with Chang et al. (2001) who observed that *Cinnamomum osmophloeum* essential oil extract B with 76% cinnamaldehyde had excellent antibacterial activity against diverse Gram-negative and Gram-positive bacteria at 250 to 1000 µg/ml concentrations. Ooi et al. (2006) also observed that cinnamaldehyde inhibited both Gram-negative and Gram-positive bacteria at concentrations from 75 to 600 µg/ml. Cinnamaldehyde appeared to work in a concentration-dependent manner, so concentrations >250 µg/ml could potentially demonstrate increased inhibitory activity against *F. johnsoniae*-like isolates. An added advantage is that cinnamaldehyde is a legally registered flavouring and foodstuff with international food safety organisations (Zhou et al., 2007), making its potential application in aquaculture more acceptable.

At 5 and 25 µg/ml, vanillin did not inhibit study isolates. Exposure to 250 µg/ml of vanillin was not significantly inhibitory in the present study with 92.86% of isolates displaying resistance. Kappachery et al. (2010) and Ponnusamy et al. (2009) also observed that vanillin did not have an antimicrobial effect against *A. hydrophila* at concentrations ranging from 63 to 250 µg/ml but rather inhibited quorum sensing and biofilm development. Fitzgerald et al. (2004) observed that vanillin had a time of exposure, concentration and species-specific dependency to its antimicrobial activity. In this light, exposure to 400 to 500 µg/ml vanillin reduced resistance of isolates in the present study to 85.71%.

Ripe or unripe *K. africana* fruits are often dried and powdered and applied directly or in topical preparations to treat a variety of skin, digestive and genito-urinary tract infections (Grace et al., 2002). A furanone derivative, eleven iridoids, 3b, 19a-dihydroxyurs-12-ene-28oic acid, caffeic acid, chlorgeric acid, and 6-*p*-coumaroyl-sucrose, together with a diverse group of phenylpropanoid and phenylethanoid derivatives and a flavonoid glycoside, have been isolated from the fruit (Saini et al., 2009). At 4 mg/ml, extract antibacterial efficacy decreased in the following order: ethyl acetate > methanol > dichloromethane > hexane. According to Grace et al. (2002), the antibacterial activity of *K. africana* fruits against Gram-positive and Gram-negative bacteria was due to a mixture of three fatty acids in an ethyl acetate fruit extract. In the present study, 4 mg/ml of the ethyl acetate extract (EX 1) moderately inhibited study isolates while the dichloromethane extract (EX 2) proved less effective against study isolates with resistance being displayed by 35.7% of isolates, while the hexane extract (EX 4) was the least effective. While *K. africana* methanol extract (EX 3) demonstrated limited inhibitory activity at 4 mg/ml, exposure to 10 mg/ml was most effective with

100% of isolates displaying susceptibility. This is in keeping with data from Agyare et al. (2013) who obtained MICs of 5.5 and 7.5 µg/ml following challenge of *E. coli* and *Pseudomonas aeruginosa*, respectively, with methanolic *K. africana* leaf and stem bark extracts.

A notable efficacy of the *K. africana* methanolic extract on Gram-negative bacteria has been previously reported (Eldeen and Van Staden, 2007; Jeyachandran and Mahesh, 2007; Shai et al., 2008). This might be a result of methanol being the best solvent for extraction since most phytochemical components are either polar or non-polar and methanol is thus able to solubilise the antibacterial components of medicinal plants (Jeyachandran and Mahesh, 2007). The antibacterial and antifungal activity of methanolic *K. africana* root and fruit extracts might be due to naphthoquinones, kigelinone, iso-pinnatal, dehydro-α-lapachone, and lapachol, and the phenylpropanoids, *p*-coumaric acid and ferulic acid (Saini et al., 2009). The antimicrobial activity of the crude *K. africana* extracts is most likely the result of the synergistic action of the multiple bioactive compounds found within them.

The susceptibility of bacteria to any given antimicrobial agent may be dependent on the bacterial species or strain, extraction process or mode of action (Rattanachaikunsopon and Phumkhachorn, 2010). Strain- and species-specific differences in susceptibility were observed for cinnamaldehyde, vanillin and *K. africana* extracts in this study. *Chryseobacterium* and *Aeromonas* spp. isolates also display differences in their responses to *K. africana* extracts as well as cinnamaldehyde and vanillin (unpublished data).

It appears that 10 mg/ml of the methanolic extract (EX 3) is an alternative to ampicillin and tetracycline as an antimicrobial agent against *F. johnsoniae*-like isolates, while 4 mg/ml of the ethyl acetate EX 1 was more effective than ampicillin. The present study, which is the first examining the efficacy of cinnamaldehyde, vanillin and *K. africana* extracts against *Flavobacterium* spp. isolates indicates that cinnamaldehyde and the *K. africana* ethyl acetate and methanol extracts are promising candidates to be tested for their efficacy in the treatment of *Flavobacterium*-associated fish infections. Dada *et al*. (2010) compared the effect of incorporation of 50 to 200 g *K. africana* fruit meal to one kg fish feed (five isonitrogenous diets) and observed positive effects on fecundity, hatching rate and percentage survival of catfish, *Clarias gariepinus*, following the use of 100 g dried *K. africana* fruit meal/kg feed. Thus, the extract has an important role as fertility enhancer, minimizing the use of synthetic fertility-enhancing drugs, in addition to its antimicrobial effect. The use on catfish indicates the non-toxic nature of the *K. africana* fruit (upto 200 g fruit meal/kg feed) against fish and highlights its potential beneficial application in an aquaculture setting. Further investigations will have to be carried out to ascertain the effects of antimicrobial agent synergy with *K. africana* phytochemical compounds and practical phytotherapy of infected fish with these phytochemicals.

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ABBREVIATIONS

DMSO, Dimethyl sulfoxide; **EAO,** enriched Anacker and Ordal; **TS,** tryptic soy; **EX 1,** *K. africana* ethyl acetate extract; **EX 2,** *K. africana* dichloromethane extract; **EX 3,** *K. africana* methanol extract; **EX 4,** *K. africana* hexane extract; **MH,** Mueller-Hinton; **AMP10,** ampicillin; **TE30,** tetracycline; **S,** susceptible; **I,** intermediate; **R,** resistant; **RAI,** relative activity index; **MICs,** minimum inhibitory concentrations.

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