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ANTIMICROBIAL ACTIVITY OF CINNAMALDEHYDE, VANILLIN AND *KIGELIA AFRICANA* FRUIT EXTRACTS AGAINST FISH-ASSOCIATED *CHRYSEOBACTERIUM* AND *MYROIDES* SPP. ISOLATES

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Running Title: Phytotherapy of *Chryseobacterium* and *Myroides spp*.

Abstract

Background: Members of the family Flavobacteriaceae exhibit intrinsic multi-drug resistance, which hampers their effective eradication. Phytochemicals are being explored as alternatives to the use of antimicrobial agents in aquaculture since they have growth-promoting, immune-stimulating, and antimicrobial properties.
Materials and Methods: The susceptibility of 36 *Chryseobacterium* and seven *Myroides* spp. isolates from salmon, tilapia and trout as well as 19 selected Flavobacteriaceae type strains to cinnamaldehyde, vanillin and four crude *Kigelia africana* extracts (ethyl acetate, dichloromethane, methanol and hexane), was assessed using disc diffusion assays and compared to standard antimicrobial agents, ampicillin and tetracycline using activity indices.
Results: Cinnamaldehyde (≥250 µg/ml) was the most effective (77. 8 – 100% susceptibility) while vanillin was the least effective with inhibitory activity only at 1000 µg/ml. The *K. africana* hexane extract (4 mg/ml) was the most effective, with only 11.3% of isolates displaying resistance, while 94.4% of isolates demonstrated resistance to ampicillin and 38.9% susceptibility to tetracycline. *K. africana* extract inhibitory efficacy decreased in the following order: hexane > ethyl acetate > dichloromethane > methanol. Cinnamaldehyde and *K. africana* EX 4 activity indices ≥ 1 were obtained for 83.3 - 97.2% and 25% of *Chryseobacterium* spp. isolates, respectively, relative to tetracycline.
Conclusions: Cinnamaldehyde and *K. africana* fruit hexane extracts are promising candidates to be tested for their efficacy in the treatment of *Chryseobacterium/Myroides*-associated fish infections. These phytochemicals might serve as environmentally-friendly, cost-effective alternatives to the use of antimicrobial agents in aquaculture farms, with a lesser chance of resistance development.

Keywords: phytotherapy; cinnamaldehyde; vanillin; *Kigelia*; *Chryseobacterium*; *Myroides*

List of Non-standard abbreviations:
Dimethyl sulfoxide (DMSO), Enriched Anacker and Ordal's (EAO), Tryptic soy (TS), *K. africana* ethyl acetate extract (EX 1), *K. africana* dichloromethane extract (EX 2), *K. africana* methanol extract (EX 3), *K. africana* hexane extract (EX 4),
Mueller-Hinton (MH), Ampicillin (AMP10), Tetracycline (TE30), Susceptible (S), Intermediate (I), Resistant (R), Activity index (AI), Minimum inhibitory concentrations (MICs).

Introduction

Intensive aquaculture activities often lead to an increased incidence of infectious diseases resulting in severe economic loss. Antimicrobial agents are used by almost every sector of the aquaculture industry, prophylactically as feed additives to prevent fish disease, in addition to their therapeutic use (Chakraborty and Hancz, 2011). There has been an increase in the frequency of fish clinically presented infections associated with the genus *Chryseobacterium* (yellow-pigmented, non-fermentative Gram-negative bacilli), with an increasing number of *Chryseobacterium* species being considered potentially emerging pathogens in farmed Atlantic salmon, rainbow trout and yellow perch (Illardi et al., 2009; Pridgeon et al., 2012; Zamora et al., 2012). *Chryseobacterium arothri, C. balustinum, C. chaponense, C. joostei, C. oncorhynchi, C. piscicola, C. shigense, C. scophtalmum, C. tructae and C. viscerum* have been isolated from different diseased fish species (Zamora et al., 2012). *Chryseobacterium indologenes*, which is normally associated with human infections, acting as sporadic but severe opportunistic nosocomial pathogen usually in neonates or immuno-compromised patients, has been found to be pathogenic to yellow perch (Pridgeon et al., 2012).

While *Myroides* strains, *Myroides odoratus* and *M. odoratimimus*, have been primarily isolated from clinical sources, they are, however, widely distributed in the aquatic environment with three novel species (*M. pelagicus, M. profundi*, and *M. marinus*) being isolated from seawater. These low-grade opportunistic pathogens have been implicated in urinary tract infection, endocarditis, and ventriculitis and cutaneous infections (surgical wound infections, cellulitis, and necrotizing fasciitis), usually in severely immune-compromised patients (Benedetti et al., 2011). Myroides strains cultured from South Atlantic fish species at a fish processing site have been regarded as potential food spoilage organisms, rather than significant pathogens at aquaculture sites (Jacobs and Chenia, 2009).

The development of multi-drug resistant pathogens, environmental pollution, the accumulation of antimicrobial agent residues in both fish and the environment and the need for organic aquaculture has stimulated the search for phytochemicals that have potent antimicrobial activity to improve fish health and for disease management (Chakraborty and Hancz, 2011). Phytochemicals have multiple effects, with the antibacterial components 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 quorum sensing pathways (Chenia, 2013; Packiavathy et al., 2012). Phytochemicals promote various activities like anti-stress, growth promotion, appetite-stimulation, tonic and immune-stimulation and have aphrodisiac and antimicrobial properties in finfish and shrimp larviculture due to the active principles such as alkaloids, flavanoids, pigments, phenolics, terpenoids, steroids and essential oils (Citarasu, 2010). Phytochemicals provide an untapped source of natural antimicrobial agents for the

effective treatment of infectious fish diseases (broad spectrum of pathogen activity and oral administration); to enhance fish health and food safety and quality, while reducing the cost of therapy and conserving the aquatic environment (Chakraborty and Hancz, 2011).

The choice of effective antimicrobial agents against *Chryseobacterium* and *Myroides* infections is difficult since clinical strains are intrinsically resistant to nearly all penicillins, narrow-spectrum cephalosporins and carbapenems, due to the production of chromosomally encoded IND-type metallo-β-lactamases (Benedetti et al., 2011; Maravic et al., 2013). This suggests the need for alternative, sustainable, cost-economic and environmentally-friendly therapeutic options. This study, therefore, investigated the antimicrobial efficacy of cinnamaldehyde, vanillin and four crude *K. africana* fruit extracts on aquatic *Chryseobacterium* and *Myroides spp* isolates as well as other selected members of the family Flavobacteriaceae.

Materials and Methods

Maintenance of bacterial isolates

Thirty-six *Chryseobacterium* spp. isolates and seven *Myroides odoratus* (Jacobs and Chenia, 2009) isolates previously isolated from moribund or healthy Atlantic salmon (*Salmo salar*), tilapia (*Oreochromis mossambicus*) and trout (*Oncorhynchus mykiss*) were screened in the present study (Table 1). Ten *Chryseobacterium* spp. type strains and nine additional type strains of the family Flavobacteriaceae were also screened. Study isolates were maintained on enriched Anacker and Ordal's (EAO) or tryptic soy (TS) agar plates and stored at 4 °C and for long-term storage in EAO or TS broth containing 20 % glycerol at -70 °C.

Preparation of crude fruit extracts

Kigelia. 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). Material was washed, dried, chopped, oven-dried at 60 °C, milled to yield a finely ground material and stored in polythene bags at 4 °C. Crude extracts were prepared by exhaustive sequential extraction with ethyl acetate, dichloromethane, hexane and methanol by maceration and continuous shaking on an orbital shaker at room temperature for 48 h (Kiplimo et al., 2011). These extraction solvents were chosen to most efficiently extract the diverse phytochemicals contained with the *K. africana* fruit material. Solvent extracts were concentrated using a vacuum rotary evaporator, dried, dissolved in dimethyl sulfoxide (DMSO) to a final concentration of 100 mg/ml and stored at 4 °C.

Antimicrobial susceptibility testing

Antimicrobial susceptibility to cinnamaldehyde, vanillin and four crude *K. africana* fruit extracts was determined using the disc diffusion method. Blank discs (6 mm; MAST, UK) were impregnated with cinnamaldehyde (0.1, 0.25, 0.5, 0.75 and 1 mg/ml), vanillin (0.1, 0.25, 0.5, 0.75 and 1 mg/ml) or crude *K. africana* ethyl acetate (EX 1, 4 mg/ml), dichloromethane (EX 2, 4 mg/ml), methanol (EX 3, 4 and 10 mg/ml) and hexane (EX 4, 4 mg/ml) extracts and allowed to dry. Bacterial isolates were grown overnight on TS agar plates and the turbidity of cell suspensions were adjusted equivalent to that of a 0.5 McFarland standard. These were used to inoculate 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 hr at 30 °C. Testing was done in duplicate and tetracycline (TE30, 30µg/ml) and ampicillin (AMP10, 10 µg/ml) discs (Oxoid, Basingstoke, UK) were used as standard antimicrobial agent controls, while DMSO-impregnated disks were used as negative controls. Ampicillin was used as a negative antimicrobial agent control since *Chryseobacterium* spp. often display intrinsic, chromosomally-mediated resistance to β-lactams (Benedetti et al. 2011; Maravic et al., 2013). 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 – 16 mm, (R) ≤ 13 mm, while those for TE30 were: (S) ≥ 19 mm, (I) 15 – 18 mm, (R) ≤ 14 mm (CLSI, 2007).

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

Results

Zone diameters for cinnamaldehyde (250 and 1000 µg/ml), vanillin (250 and 1000 µg/ml) and four *K. africana* extracts (4 mg/ml) are given in Table 1, along with zone diameters for ampicillin and tetracycline. The percentage of type strains and fish-associated isolates demonstrating susceptibility, intermediate susceptibility and resistance to each of the phytochemicals tested at various concentrations is indicated in Table 2.

High levels of resistance were observed for ampicillin (Table 2), while 51.6% (32/62) of the isolates displayed tetracycline susceptibility (Table 2). Cinnamaldehyde was the most effective at ≥ 250 µg/ml against all four examined groups (Table 2). Overall total susceptibility was observed with ≥ 750 µg/ml cinnamaldehyde exposure (Table 2). Vanillin was the least effective of the phytochemicals tested (Table 2) with inhibitory activity being observed only against *Chryseobacterium* type strains (1000 µg/ml; 10% susceptibility) and fish-associated isolates (1000 µg/ml; 27.8% susceptibility). Of the four *K. africana* extracts tested at 4 mg/ml, the hexane (EX 4; 29% susceptibility) and ethyl acetate (EX 1; 16% susceptibility) extracts were the most effective overall. Overall extract inhibitory efficacy decreased in the following order: hexane > ethyl acetate > dichloromethane > methanol (Table 2). While 4 mg/ml of the methanolic extract (EX 3) was ineffective, at 10 mg/ml susceptibility was observed for 66.1% of overall isolates tested (Table 2).

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Table 1: Zones of inhibition (mm) obtained with cinnamaldehyde, vanillin and *Kigelia africana* extracts as well as standard antimicrobial agents, ampicillin and tetracycline, against fish-associated *Chryseobacterium* and *Myroides* spp. isolates and Flavobacteriaceae type strains

Isolates and Source		C 250	C 1000	V 250	V 1000	EX 1	EX 2	EX 3	EX 3 (10 mg/ml)	EX 4	AMP10	TE30
***Bergeyella zoohelcum* NCTC 11660^T**		26	42	0	14	10	9	10	17	17	30	36
***Elizabethkingia meningoseptica* NCTC 10016^T**		26	34	0	0	10	8	8	13	11	0	8
***Empedobacter brevis* NCTC 11099^T**		19	30	0	8	9	8	7	15	11	16	23
***Flexibacter aurantiacus* ATCC 23107^T**		0	35	0	0	20	25	8	18	12	20	36
***Flexibacter flexilis* ATCC 23079^T**		24	34	0	8	13	13	12	18	14	15	26
***Myroides odoratus* NCTC 11036^T**		10	26	0	8	13	13	0	14	8	26	10
***Sphingobacterium multivorum* NCTC 11343^T**		21	30	0	8	10	8	8	14	12	0	24
***Terrimonas ferruginea* NCTC 11634^T**		30	38	0	14	13	13	8	18	12	0	22
***Weeksella virosa* NCTC 11634^T**		24	44	8	14	17	13	15	18	17	0	24
MY1	Tilapia	17	30	0	8	12	10	8	17	12	0	18
MY2	Tilapia	17	33	0	8	13	10	8	17	12	0	8
MY2B	Tilapia	15	30	0	10	11	16	10	17	15	8	9
MY3	Tilapia	16	30	7	8	10	9	0	13	15	0	13
MY3B	Tilapia	18	34	0	9	16	11	10	18	16	8	8
MY4	Tilapia	22	38	0	10	8	7	8	9	12	13	8
MY5	Trout	12	32	0	9	18	22	9	20	16	27	26
***Chryseobacterium balustinum* NCTC 11212^T**		22	44	0	8	15	15	8	16	14	26	30
***Chryseobacterium defluvii* DSM 14219^T**		17	32	0	8	18	18	8	18	10	11	26
***Chryseobacterium gleum* NCTC 11432^T**		17	30	0	8	14	13	8	15	10	0	20
***Chryseobacterium indologenes* NCTC 10796^T**		24	32	0	8	11	14	8	15	11	0	21
***Chryseobacterium indoltheticum* ATCC 27950^T**		22	31	0	10	10	12	10	17	13	0	18
***Chryseobacterium joostei* LMG 18212^T**		20	36	8	8	11	13	8	17	12	0	18
***Chryseobacterium piscium* LMG 23089^T**		18	34	0	0	14	15	8	18	10	0	15
***Chryseobacterium scophtalmum* LMG 13028^T**		16	30	0	8	15	11	8	14	15	10	20
***Chryseobacterium shigense* DSM 17126^T**		30	42	0	15	12	15	12	17	11	8	20

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***Chryseobacterium vrystaatense* LMG 22846^T**		23	38	0	12	14	12	11	13	15	0	20
CH1	Tilapia	18	36	7	15	10	9	14	14	16	0	14
CH1B	Tilapia	26	42	7	14	12	11	12	16	17	0	12
CH2	Tilapia	20	34	0	10	12	12	14	15	13	0	14
CH2B	Tilapia	22	48	0	14	9	8	10	13	17	0	12
CH3	Tilapia	32	50	0	14	11	11	14	15	14	0	13
CH4	Tilapia	17	36	8	10	11	10	0	14	10	0	14
CH4B	Tilapia	23	38	6	12	13	12	12	18	17	0	15
CH5	Tilapia	22	36	8	8	11	10	11	14	13	0	14
CH6	Tilapia	21	40	0	8	10	9	8	15	17	0	14
CH7	Tilapia	12	26	0	0	12	13	0	17	10	0	21
CH8	Tilapia	22	34	0	10	10	14	10	16	14	0	11
CH9	Tilapia	20	38	0	18	12	13	11	16	16	0	15
CH10	Tilapia	20	31	8	10	10	11	10	16	11	0	14
CH11	Tilapia	20	40	15	24	11	14	10	14	13	0	21
CH12	Trout	20	36	12	20	12	11	10	16	16	0	28
CH13	Trout	24	40	0	8	10	9	10	16	14	30	50
CH14	Tilapia	28	42	0	8	10	11	0	15	14	18	42
CH15	Trout	22	44	0	14	10	10	10	14	14	0	20
CH16	Trout	30	42	8	18	14	18	15	17	21	0	22
CH17	Trout	28	40	0	13	13	10	10	14	12	0	20
CH18	Trout	20	36	8	9	11	13	12	15	14	0	20
CH19	Trout	25	38	0	12	14	14	10	17	13	0	20
CH21	Trout	15	34	0	10	15	11	10	16	12	0	21
CH22	Trout	28	40	8	15	12	12	12	17	14	0	20
CH23	Trout	24	40	0	34	10	9	8	13	13	0	23
CH24	Trout	22	35	0	10	10	11	8	16	11	0	13
CH25	Tilapia	22	34	0	8	13	14	10	18	11	0	14
CH26	Tilapia	20	36	0	12	11	10	12	14	15	0	16
CH27	Tilapia	25	40	0	14	17	12	20	14	18	0	13

CH28	Tilapia	15	36	0	8	12	11	10	13	12	0	15
CH29	Salmon	30	45	0	16	10	10	10	15	13	0	18
CH30	Salmon	32	45	8	20	10	9	12	14	14	0	18
CH31	Trout	16	36	0	8	11	8	11	14	13	0	14
CH32	Trout	30	45	0	18	15	14	12	16	14	0	15
CH33	Trout	26	41	0	13	12	13	10	15	13	0	18
CH34	Trout	30	41	0	8	9	8	0	11	8	0	20
***E. coli* ATCC 25922**		10	18	0	0	8	8	0	10	9	20	27

*C 250: cinnamaldehyde 250 µg/ml; C 1000: cinnamaldehyde 1000 µg/ml; V 250: vanillin 250 µg/ml; V 1000: vanillin 1000 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; and TE30: 30 µg/ml tetracycline.

Based on fish host analyses, trout isolates were more susceptible to tetracycline (75%; 12/16) compared to tilapia isolates, of which 68% (17/25) of isolates displayed resistance (Table 3). Cinnamaldehyde at ≥ 250 µg/ml was effective against all isolates irrespective of fish host (Table 3). Chryseobacteria from salmon, tilapia and trout demonstrated increased susceptibility to vanillin at 1000 µg/ml, however, all *Myroides* spp. isolates were resistant irrespective of fish host and concentration (Table 3). Although no significant differences were observed between tilapia and trout chryseobacteria for *K. africana* extracts 1 - 3, tilapia isolates (42.1%) were more susceptible to the hexane extract (EX 4) compared to trout isolates (13.3%), while 80% of the trout isolates displayed intermediate susceptibility compared to 47.4% of the tilapia isolates (Table 3).

Based on zones of inhibition obtained with phytochemicals and standard antimicrobial agents, ampicillin and tetracycline, the AIs were determined and the AI ranges are represented in Table 4. Differences were observed for each of the four groups tested with respect to the AI ranges obtained with each set of phytochemicals tested (Table 4).

An extract was considered effective against an isolate if the activity index was ≥ 1. Ampicillin was regarded as a poor standard for comparison since 85.48% (53/62) of the isolates tested exhibited ampicillin resistance (Tables 1 and 2). The percentage of Flavobacteriaceae type strains, *Myroides* spp. isolates, *Chryseobacterium* type strains and *Chryseobacterium* spp. isolates demonstrating AIs ≥ 1 is indicated in Table 5. The efficacy of cinnamaldehyde, relative to both ampicillin and tetracycline, is indicated in Table 5. By comparison, majority of the vanillin AIs were ≤ 1 (Table 5), suggesting that this compound has poor antimicrobial activity in comparison to both ampicillin and tetracycline. *K. africana* extracts while not as effective as cinnamaldehyde, demonstrated better AIs compared to vanillin.

Discussion

The choice of an effective drug for the empirical treatment of clinical and aquaculture infections due to *Chryseobacterium* spp. is hampered by the breadth of resistance to extended-spectrum penicillins, first- and second-generation cephalosporins, ceftriaxone, aminoglycosides, aztreonam, chloramphenicol, erythromycin, imipenem, meropenem and ticarcillin-clavulanate (Lin et al., 2010). The treatment of *Myroides* infection is also often difficult, since most strains are resistant to β-lactams, including aztreonam and carbapenems, and exhibit variable susceptibility to aminoglycosides, quinolones, and sulfamethoxazole (Benedetti et al., 2011). Although *Chryseobacterium* and *Myroides* are emerging opportunistic pathogens associated with human and aquaculture infections, there are very few studies investigating alternative therapeutic strategies. Phytochemicals as alternative therapeutic options for the treatment of *Chryseobacterium*-associated infections in aquaculture have only been described by a few research groups and typically have focused on a single *Chryseobacterium* spp. isolate (Adomi and Umukoro, 2010; Laith et al., 2012; Menghani and Sharma, 2011; Rasoarivelo et al., 2011).

Cinnamaldehyde or 3-phenyl-2-propenal, a natural flavoring substance, occurs in the bark and leaves of cinnamon trees of the genus *Cinnamomum*. This potent aromatic compound 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 (Nuryastuti et al., 2009). Type strains and fish-associated isolates demonstrated concentration-dependent susceptibility to cinnamaldehyde at ≥ 250 µg/ml (Tables 1- 2). This is in agreement with Chang et al. (2001) and Ooi et al. (2006) who observed that cinnamaldehyde had excellent antibacterial activity against diverse Gram-negative and Gram-positive bacteria at 250 - 1000 µg/ml and 75 – 600 µg/ml, respectively. Cinnamaldehyde worked effectively against all *Chryseobacterium* and *Myroides* spp. isolates, irrespective of the fish host origin (Table 3). Based on the AIs, cinnamaldehyde could serve as alternative to antimicrobial agents given its efficacy in comparison to ampicillin and tetracycline (Table 5). An added advantage is that cinnamaldehyde is a legally registered flavoring and foodstuff with international food safety organizations (Zhou et al., 2007), making its potential application in aquaculture more acceptable.

Vanillin (4-hydroxy-3-methoxybenzaldehyde) is a major component of natural vanilla, obtained from the bean of the tropical orchid *Vanilla planifolia* (Kappachery et al., 2010). 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). Vanillin, at all concentrations tested, was ineffective against selected Flavobacteriaceae type strains and *Myroides* spp. isolates from both trout and tilapia (Tables 2 – 3). Vanillin did not demonstrate an antimicrobial effect against the fish pathogen *Aeromonas hydrophila* at concentrations ranging from 63 - 250 µg/ml but rather inhibited quorum sensing and biofilm development (Kappachery et al., 2010), thus attenuating its pathogenicity. Fitzgerald et al. (2004) observed that vanillin had a time of exposure, concentration and species-specific dependency to its antimicrobial activity. This could be observed with the fish-associated *Chryseobacterium* spp. isolates which demonstrated increasing susceptibility with an increase in concentration (Table 2) and isolates from trout appeared more susceptible than isolates from tilapia (Table 3).

Kigelia africana (Lam.) Benth., (sausage tree) of the Bignoniaceae family, has a long history as a medicinal plant in South, Central and West Africa. Ripe or unripe *K. africana* fruits are dried and powdered and applied directly or in topical preparations to dermal complaints, ulcers, septic sores, haemorrhoids, rheumatism, as a purgative, to increase lactation in breast-feeding mothers and for digestive and genito-urinary tract infections (Grace et al., 2002; Saini et al., 2009). 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 (Saini et al., 2009) are potentially associated with the medicinal properties attributed to *K. africana* fruit extracts (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.

At 4 mg/ml, extract antibacterial efficacy decreased in the following order: hexane > ethyl acetate > dichloromethane > methanol. Although the hexane extract (EX 4) proved to be the most effective against *Chryseobacterium* and *Myroides* spp. isolates, there are no other similar reports for comparison. Grace et al. (2002) obtained minimum lethal concentrations of 2.5 mg/ml against both Gram-positive and Gram-negative bacteria using ethyl acetate *K. africana* fruit extracts. This antibacterial activity was suggested to be the result of a mixture of three fatty acids (palmitic acid, nonanoic acid and 8-heptadecenoic acid) in the ethyl acetate fruit extract, even though Gram-negative bacteria were less susceptible than Gram-positives. Although Eldeen and van Staden (2007) and Shai et al. (2008) have reported the efficacy of dichloromethane *K. africana* bark and leaf extracts against Gram-positive and Gram-negative bacteria, the dichloromethane extract (EX 2) was less efficacious in the present study.

Table 2: Susceptibility analyses of fish-associated *Chryseobacterium* (n = 36) and *Myroides* (n = 7) spp. isolates, *Chryseobacterium* type (n = 10) and Flavobacteriaceae type (n = 9) strains to cinnamaldehyde, vanillin and *Kigelia africana* extracts as well as standard antimicrobial agents, ampicillin and tetracycline

Phytochemical	Flavobacteriaceae Type Strains			*Myroides*			*Chryseobacterium* Type Strains			*Chryseobacterium* Study Isolates		
	%			%			%			%		
	S	I	R	S	I	R	S	I	R	S	I	R
Cinnamaldehyde												
100 µg/ml	11.11 (1/9)	33.33 (3/9)	55.56 (5/9)	0	14.28 (1/7)	85.71 (6/7)	10 (1/10)	10 (1/10)	80 (8/10)	22.22 (8/36)	27.78 (10/36)	50 (18/36)
250 µg/ml	77.78 (7/9)	0	22.22 (2/9)	85.71 (6/7)	14.28 (1/7)	0	100	0	0	97.22 (35/36)	2.78 (1/36)	0
500 µg/ml	88.89 (8/9)	11.11 (1/9)	0	100	0	0	100	0	0	100	0	0
750 µg/ml	100	0	0	100	0	0	100	0	0	100	0	0
100 µg/ml	100	0	0	100	0	0	100	0	0	100	0	0
Vanillin												
100 µg/ml	0	0	100	0	0	100	0	0	100	0	0	100
250 µg/ml	0	0	100	0	0	100	0	0	100	2.78 (1/36)	2.78 (1/36)	94.44 (34/36)
500 µg/ml	0	0	100	0	0	100	0	0	100	8.33 (3/36)	5.56 (2/36)	86.11 (31/36)
750 µg/ml	0	0	100	0	0	100	0	0	100	11.11 (4/36)	22.22 (8/36)	66.67 (24/36)
1000 µg/ml	0	0	100	0	0	100	10 (1/10)	10 (1/10)	80 (8/10)	27.78 (10/36)	27.78 (10/36)	44.44 (16/36)
Kigelia Africana												
4 mg/ml EX 1	22.22 (2/9)	33.33 (3/9)	44.44 (4/9)	28.57 (2/7)	42.86 (3/7)	28.57 (2/7)	30 (3/10)	60 (6/10)	10 (1/10)	8.33 (3/36)	55.56 (20/36)	36.11 (13/36)
4 mg/ml EX 2	11.11 (1/9)	44.44 (4/9)	44.44 (4/9)	28.57 (2/7)	14.28 (1/7)	57.14 (4/7)	40 (4/10)	60 (6/10)	0	2.77 (1/36)	58.33 (21/36)	38.89 (14/36)
4 mg/ml EX 3	11.11 (1/9)	11.11 (1/9)	77.78 (7/9)	0	0	100	0	20 (2/10)	80 (8/10)	5.56 (2/36)	38.89 (14/36)	55.56 (20/36)
10 mg/ml EX 3	66.67 (6/9)	33.33 (3/9)	0	71.43 (5/7)	14.28 (1/7)	14.28 (1/7)	80 (8/10)	20 (2/10)	0	61.11 (22/36)	38.89 (14/36)	0
4 mg/ml EX 4	22.22 (2/9)	66.67 (6/9)	11.11 (1/9)	57.14 (4/7)	42.86 (3/7)	0	20 (2/10)	50 (5/10)	30 (3/10)	27.78 (10/36)	63.89 (23/36)	8.33 (3/36)

Ampicillin (10 µg/ml)	33.33 (3/9)	22.22 (2/9)	44.44 (4/9)	14.28 (1/7)	0	85.71 (6/7)	10 (1/10)	0	90 (9/10)	5.56 (2/36)	0	94.44 (34/36)
Tetracycline (30 µg/ml)	77.78 (7/9)	0	22.22 (2/9)	14.28 (1/7)	14.28 (1/7)	71.43 (5/7)	70 (7/10)	30 (3/10)	0	38.89 (14/36)	22.22 (8/36)	38.89 (14/36)

[a] S = susceptibility, I = intermediately resistant and R = resistant.
*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.

Table 3: Susceptibility analyses of *Chryseobacterium* and *Myroides* spp. isolates to cinnamaldehyde, vanillin and *Kigelia africana* extracts as well as standard antimicrobial agents, ampicillin and tetracycline, based on fish species source

Genus	Fish species	Phenotype	C 250	C 1000	V 250	V 1000	EX 1	EX 2	EX 3	EX 3 (10 mg/ml)	EX 4	AMP10	TET30
Myroides (n = 7)	**Trout (n = 1)**	**S**	0	100	0	0	100	100	0	100	100	100	100
		I	100	0	0	0	0	0	0	0	0	0	0
		R	0	0	100	100	0	0	100	0	0	0	0
	Tilapia (n = 6)	**S**	100	100	0	0	16.67 (1/6)	16.67 (1/6)	0	66.67 (4/6)	50 (3/6)	0	0
		I	0	0	0	0	50 (3/6)	16.67 (1/6)	0	16.67 (1/6)	50 (3/6)	0	16.67 (1/6)
		R	0	0	100	100	33.33 (2/6)	66.67 (4/6)	100	16.67 (1/6)	0	100	83.33 (5/6)
Chryseobacterium (n = 36)	**Salmon (n = 2)**	**S**	100	100	0	100	0	0	0	50 (1/2)	0	0	0
		I	0	0	0	0	0	0	100	50 (1/2)	100	0	100
		R	0	0	100	0	100	100	0	0	0	100	0
	Trout (n = 15)	**S**	100	100	0	26.67 (4/15)	13.33 (2/15)	6.67 (1/15)	6.67 (1/15)	66.67 (10/15)	13.33 (2/15)	6.67 (1/15)	73.33 (11/15)
		I	0	0	6.67 (1/15)	26.67 (4/15)	53.33 (8/15)	53.33 (8/15)	26.67 (4/15)	33.33 (5/15)	80 (12/15)	0	13.33 (2/15)
		R	0	0	93.33 (14/15)	46.67 (7/15)	33.33 (5/15)	40 (6/15)	66.67 (10/15)	0	6.67 (1/15)	93.33 (14/15)	13.33 (2/15)
	Tilapia (n = 19)	**S**	94.74 (18/19)	100	5.26 (1/19)	5.26 (1/19)	5.26 (1/19)	0	5.26 (1/19)	57.89 (11/19)	42.11 (8/19)	5.26 (1/19)	15.79 (3/19)

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	I	5.26 (1/19)	0	0	36.84 (7/19)	63.16 (12/19)	68.42 (13/19)	42.11 (8/19)	42.11 (8/19)	47.37 (9/19)	0	21.05 (4/19)
	R	0	0	94.74 (18/19)	57.90 (11/19)	31.58 (6/19)	31.58 (6/19)	52.63 (10/19)	0	10.52 (2/19)	94.74 (18/19)	63.16 (12/19)

*C 250: cinnamaldehyde 250 µg/ml; C 1000: cinnamaldehyde 1000 µg/ml; V 250: vanillin 250 µg/ml; V 1000: vanillin 1000 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; and TE30: 30 µg/ml tetracycline.

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Table 4: Cinnamaldehyde, vanillin and four crude *Kigelia africana* activity indices ranges, relative to ampicillin (AMP10) and tetracycline (TE30), *Chryseobacterium* and *Myroides* spp. isolates from fish as well as selected *Chryseobacterium* spp. and Flavobacteriaceae type strains

Phytochemical	**Activity Indices**				
	Flavobacteriaceae type Strains	***Myroides***	***Chryseobacterium* type Strains**	***Chryseobacterium* study Isolates**	***E. coli* ATCC 25922**
			Ampicillin		
Cinnamaldehyde					
100 µg/ml	0.000 – 0.800	0 – 1	0 – 2	0 – 0.556	0
250 µg/ml	0 – 1.6	0 – 2.250	0 – 3.750	0 – 1.556	0.5
500 µg/ml	0 – 2	0 – 3.250	0 – 5	0 – 1.778	0.7
750 µg/ml	0 – 2.133	0 – 4	0 – 5	0 – 2	0.8
100 µg/ml	0 – 2.267	0 – 4.250	0 – 5.250	0 – 2.333	0.9
Vanillin					
100 µg/ml	0	0	0	0	0
250 µg/ml	0	0	0	0	0
500 µg/ml	0 – 0.533	0 – 0.615	0 – 1	0	0
750 µg/ml	0 – 0.533	0 – 8.75	0 – 1.250	0 – 0.444	0
1000 µg/ml	0 – 0.533	0 – 1.250	0 – 1.875	0 – 0.444	0
Kigelia africana					
4 mg/ml EX 1	0 – 1	0 -2	0 – 1.636	0 -0.556	0.4
4 mg/ml EX 2	0 – 1.250	0 – 2	0 – 1.875	0 – 0.611	0.4
4 mg/ml EX 3	0 – 0.8	0 – 1.250	0 – 1.5	0 – 0.333	0
10 mg/ml EX 3	0 – 1.2	0 – 2.250	0 – 2.125	0 – 0.833	0.5
4 mg/ml EX 4	0 – 0.933	0 - 2	0 – 1.5	0 – 0.778	0.450
			Tetracycline		
Cinnamaldehyde					
100 µg/ml	0 – 1.5	0.346 – 1.375	0 – 0.800	0 – 1.538	0
250 µg/ml	0 – 3.250	0.462 – 2.750	0.654 – 1.5	0.480 – 2.462	0.370
500 µg/ml	0.556 – 4	0.885 – 3.750	1.1 - 2	0.600 – 3.231	0.519
750 µg/ml	0.944 – 5	1.077 – 4.5	1.154 – 2	0.760 – 3.500	0.593
100 µg/ml	0.972 – 4.250	1.231 – 4.750	1.231 – 2.267	0.8 - 4	0.667
Vanillin					
100 µg/ml	0	0 – 0.538	0	0	0
250 µg/ml	0 – 0.33	0 – 0.538	0 – 0.444	0 – 0.714	0
500 µg/ml	0 – 0.417	0 – 1	0 – 0.444	0 – 0.889	0
750 µg/ml	0 – 0.800	0 – 1	0 – 0.5	0.160 – 1.067	0
1000 µg/ml	0 – 0.800	0 – 1.250	0 – 0.750	0.160 – 1.478	0
Kigelia africana					
4 mg/ml EX 1	0.278 – 1.3	0.667 - 2	0.5 – 0.933	0.2 – 1.30	0.296
4 mg/ml EX 2	0.250 – 1.3	0.556 – 1.778	0.5 – 1	0.18 – 1.273	0.296
4 mg/ml EX 3	0 – 1	0.346 – 1.250	0.267 – 0.6	0 – 1.538	0
10 mg/ml EX 3	0.472 – 1.625	0.769 – 2.250	0.533 – 1.2	0.320 – 1.455	0.370
4 mg/ml EX 4	0.333 – 1.375	0.615 - 2	0.385 – 0.750	0.280 – 1.417	0.333

*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.

Table 5: Phytochemical activity indices ≥ 1 of cinnamaldehyde, vanillin and four crude *K. africana* fruit extracts in comparison to standard antimicrobial agents, ampicillin (AMP10) and tetracycline (TE30) for *Chryseobacterium* and *Myroides* spp. isolates from fish as well as selected *Chryseobacterium* spp. and Flavobacteriaceae type strains

Phytochemicals	**% of isolates with activity indices ≥ 1**							
	Flavobacteriaceae Type strains (n = 9)		***Myroides* (n = 7)**		***Chryseobacterium* Type Strains (n = 10)**		***Chryseobacterium* Study Isolates (n = 36)**	
	Ampicillin (AMP10)	**Tetracycline (TE30)**	**Ampicillin (AMP10)**	**Tetracycline (TE30)**	**Ampicillin (AMP10)**	**Tetracycline (TE30)**	**Ampicillin (AMP10)**	**Tetracycline (TE30)**
Cinnamaldehyde								
100 µg/ml	0	11.11 (1/9)	28.57 (2/7)	42.86 (3/7)	10 (1/10)	0	0	27.78 (10/36)
250 µg/ml	22.22 (2/9)	44.44 (4/9)	42.86 (3/7)	71.43 (5/7)	30 (3/10)	60 (6/10)	2.78 (1/36)	83.33 (30/36)
500 µg/ml	33.33 (3/9)	77.78 (7/9)	42.86 (3/7)	85.71 (6/7)	40 (4/10)	100 (10/10)	5.56 (2/36)	86.11 (31/36)
750 µg/ml	33.33 (3/9)	88.89 (8/9)	57.14 (4/7)	100	40 (4/10)	100 (10/10)	5.56 (2/36)	94.44 (34/36)
1000 µg/ml	44.44 (4/9)	88.89 (8/9)	57.14 (4/7)	100	40 (4/10)	100 (10/10)	5.56 (2/36)	97.22 (35/36)
Vanillin								
100 µg/ml	0	0	0	0	0	0	0	0
250 µg/ml	0	0	0	0	0	0	0	0
500 µg/ml	0	0	0	14.29 (1/7)	10 (1/10)	0	0	2.78 (1/36)
750 µg/ml	0	0	0	28.57 (2/7)	10 (1/10)	0	0	16.67 (6/36)
1000 µg/ml	0	0	28.57 (2/7)	57.14 (4/7)	20 (2/10)	10 (1/10)	0	27.78 (10/36)
Kigelia Africana								
4 mg/ml *K. africana* EX 1*	11.11 (1/9)	22.22 (2/9)	28.57 (2/7)	57.14 (4/7)	30 (3/10)	0	0	8.33 (3/36)
4 mg/ml *K. africana* EX 2*	11.11 (1/9)	22.22 (2/9)	28.57 (2/7)	42.86 (3/7)	30 (3/10)	10 (1/10)	0	5.56 (2/36)
4 mg/ml *K. africana* EX 3*	0	11.11 (1/9)	28.57 (2/7)	42.86 (3/7)	10 (1/10)	0	0	13.89 (5/36)
10 mg/ml *K. africana* EX 3*	11.11 (1/9)	22.22 (2/9)	28.57 (2/7)	57.14 (4/7)	30 (3/10)	10 (1/10)	0	47.22 (17/36)
4 mg/ml *K. africana* EX 4	0	11.11 (1/9)	28.57 (2/7)	42.86 (3/7)	20 (2/10)	0	0	25 (9/36)

*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.

Jeyachandran and Mahesh (2007) have reported the broad-spectrum efficacy of methanolic *K. africana* bark extracts, while Binutu et al. (1996) observed that the methanolic extracts of *K. africana* fruit were active against Gram-positive bacteria, but displayed no significant activity against Gram-negative pathogens. At 4 mg/ml, the methanolic extract (EX 3) was the least effective of the four crude extracts tested. However, 10 mg/ml of the methanolic fruit extract (EX 3) was extremely effective against the Flavobacteriace type strains and fish-associated *Chryseobacterium* and *Myroides* spp. isolates. This is in keeping with Agyare et al. (2013) who obtained minimum inhibitory concentrations of 5.5 and 7.5 mg/ml following challenge of *E. coli* and *Pseudomonas aeruginosa*, respectively, with *K. africana* methanolic leaf and stem bark extracts.

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-, genus-, and host-specific differences in susceptibility were observed for cinnamaldehyde, vanillin and *K. africana* extracts in this study. This has also been observed for antimicrobial-resistant, aquatic *Aeromonas* spp. isolates (Okolie and Chenia, 2013).

Although fish-associated *Chryseobacterium* and *Myroides* spp. isolates and Flavobacteriaceae type strains displayed resistance to ampicillin (Table 1), due to their chromosomally-encoded β-lactamases (Benedetti et al. 2011; Maravic et al., 2013), cinnamaldehyde and the *K. africana* hexane extract EX 4 displayed inhibitory activity against these resistant bacteria. Activity indices ≥ 1, relative to tetracycline, were obtained with 85.7 - 100% and 86.1- 97.2% of fish-associated *Myroides* and *Chryseobacterium* spp. isolates with 250 – 1000 µg/ml of cinnamaldehyde (Table 5). With *K. africana* hexane extract (EX 4), AIs ≥ 1, relative to tetracycline, were obtained for 42.9% and 25% of fish-associated *Myroides* and *Chryseobacterium* spp. isolates. Activity indices ≥ 1 were also obtained against *Chryseobacterium gleum* with *Tribulus terrestris and Piper cubeba* extracts (Menghani and Sharma, 2011). Cinnamaldehyde and the *K. africana* hexane extract (EX 4) are potential antimicrobial agent alternatives to ampicillin and tetracycline against members of the genera *Chryseobacterium* and *Myroides* (Table 5).

The present study, which is the first examining the efficacy of cinnamaldehyde, vanillin and *K. africana* extracts against *Chryseobacterium* and *Myroides* spp. isolates indicates that cinnamaldehyde and *K. africana* hexane extract are promising candidates to be tested for their efficacy in the treatment of *Chryseobacterium*-associated fish infections. In addition to having GRAS status (Zhou et al., 2007), cinnamaldehyde has been demonstrated to stimulate induce the secretion of the digestive enzymes (amylase production), which results in appetite-stimulation and increased food consumption and efficiencies (Citarasu, 2010). Cinnamaldehyde used at sub-inhibitory levels is not only a potent inhibitor of autoinducer-2-based quorum sensing (QS), but also impacted *in vitro* the production of multiple virulence factors and biofilm formation, and reduced *in vivo* the mortality of *Artemia* shrimp exposed to *Vibrio harveyi* BB120 (Brackman et al., 2008). According to Brackman et al. (2008), cinnamaldehyde and cinnamaldehyde derivatives are potentially useful antipathogenic lead compounds for treatment of vibriosis. Dada et al. (2010) compared the effect of incorporation of 50 – 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 cinnamaldehyde and *K. africana* phytochemical compounds and practical phytotherapy of infected fish with these phytochemicals.

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