CHARACTERIZATION OF ANTIMICROBIAL RESISTANCE GENES OF *AEROMONAS* SPP. ISOLATED FROM FISH AND INVESTIGATION OF PHYTOCHEMICAL TREATMENT EFFICACY AGAINST RESISTANT ISOLATES

by

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As the candidate's supervisor I have/have not approved this thesis/dissertation for submission.

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ABSTRACT

The dissemination of resistance determinants, associated with mobile genetic elements, by horizontal gene transfer is responsible for the increasing antimicrobial resistance of *Aeromonas* spp. Phytochemicals are thus being explored as alternatives to the use of antimicrobial agents since they have antimicrobial, anti-virulence and immuno-stimulating properties. Aeromonads from fish and aquatic sources were examined for some of their resistance gene array and the antimicrobial and anti-biofilm effects of phytochemicals were assessed as an alternative therapeutic avenue. The presence of β-lactam resistance genes (bla_{TEM} 1 and 2), extended spectrum β-lactam resistance genes (bla_{SHV-1}, $bla_{CTX-M-15}$ and bla_{CTX-II}) and integron-associated genes in multi-drug resistant *Aeromonas* spp. isolates was investigated using polymerase chain reaction (PCR). The antimicrobial effect of three phytochemicals, viz; cinnamaldehyde, vanillin and crude *Kigelia africana* fruit extracts on multi-drug resistant *Aeromonas* spp. isolates was assessed using disk diffusion assays. Anti-biofilm effect of cinnamaldehyde, vanillin, 10% ethanolic *K. africana* extract and crude *K. africana* fruit extracts against *A. bestiarum* isolates was investigated using microtiter plate assays. Amongst test isolates, 17.1% (17/99) and 29.2% (29/99) were positive for bla_{TEM} (1) and bla_{TEM} (2), respectively. None of the test isolates were positive for the extended spectrum β-lactamase SHV gene while three isolates (3.03%; 3/99) were positive for both CTX-M-I5 and CTX-M genes. The *intI* gene was found in 10.1% (10/99) of test isolates, while 23.2% (23/99) had the *intII* gene, and this was correlated to amplification of their variable regions CS 10.1% (10/99) and Hep 19.1% (19/99), respectively. The *qac*, *sulI* and *sulII* genes were found in 64.6% (64/99), 29.2% (29/99), and 17.1% (17/99) of test isolates, respectively. None of the study isolates displayed zones of inhibition with 1 mg/ml cinnamaldehyde, 100µg/ml hexane *K. africana* extract as well as with all concentrations of vanillin. Cinnamaldehyde (all concentrations) and *K. africana* 10 mg/ml methanol extract proved bactericidal for study isolates. Sub-inhibitory concentrations of cinnamaldehyde (50 and 100 µg/ml) were most effective against *A. bestiarum* biofilms in the initial attachment and mature biofilm assays. The bla_{TEM} was the most prevalent of the β-lactamases and extended spectrum β-lactamases genes amongst test isolates. Cinnamaldehyde and *K. africana* fruit extracts appear to be promising and sustainable phytochemicals that may be used as alternatives to antimicrobial agents in aquaculture against *Aeromonas* spp. and *A. bestiarum* biofilms.

LIST OF FIGURES

LIST OF TABLES

TABLE OF CONTENTS

CHAPTER ONE
INTRODUCTION AND LITERATURE REVIEW

1.1 The genus *Aeromonas*

Aeromonas species are Gram-negative bacteria, found in aquatic environments including sewage, polluted and un-polluted water, sea-water and potable water (Fontes *et al*., 2011; Igbinosa *et al*., 2012). They have even been isolated from raw milk, ice-cream, meat, poultry, vegetables, sea foods, sediments and soil (Janda and Abbott, 2010; Xanthopoulos *et al*., 2010 ; Zaky *et al*., 2010). This genus belongs to the family *Aeromonadaceae* along with other genera, viz:, *Oceanimonas*, *Oceanisphaera* and *Tolumonas* (Igbinosa *et al*., 2012). Members of the genus *Aeromonas* are biochemically characterised into three groups, viz:, *Aeromonas hydrophila*, *Aeromonas caviae* and *Aeromonas sobria*, which contain a variety of genomospecies (Janda and Abbott, 2010). The taxonomy of the genus *Aeromonas* has undergone continual change, due to extended description of existing species and the addition of newly described taxa (Igbinosa *et al*., 2013). The current classification of the genus *Aeromonas* is based on DNA-DNA hybridization and 16S ribosomal DNA relatedness (Fontes *et al*., 2011). Currently there are 26 phenospecies names that have been described in the genus *Aeromonas* (Beaz-Hidalgo *et al*., 2013). This list of species comprises of twelve new species added to the 14 that are already known, i.e., *A. tecta, A. aquariorum, A. bivalvium, A. pisicola, A. fluvialis, A. taiwanensis, A. sanarellii, A. rivuli, A. simiae*, *A. molluscorum*, *A. diversa* and *A. dhakensis*, which was recently proposed to designate *Aeromonas* spp. HG13 (Enteric Group 501) (Fontes *et al*., 2011).

Aeromonads are straight, cocco-bacillary to bacillary, non-sporing bacteria with rounded ends measuring 0.3 - 1.0 × 1.0 - 3.5 µm across (Parker and Shaw, 2011). *Aeromonas* species have an optimum pH range of 5.5 - 9 and optimum sodium chloride concentration range of 0 - 4% (Igbinosa *et al*., 2012). Many motile strains produce a single polar flagellum, while peritrichous or lateral flagella may be formed on solid media in some species (Parker and Shaw, 2011). *Aeromonas* species are divided into two broad groups according to their optimum temperatures and motility, the mesophilic, motile species with optimum temperatures of 35 – 37 °C typified by *A. hydrophila* and the psychrophilic non-motile species with optimum temperatures of 22 - 25 °C typified by *A. salmonicida* (Janda and Abbott, 2010). However, there are some species that can grow at temperature range of 40 - 45 °C (Janda and Abbott, 2010). The mesophilic motile species such as *A. hydrophila*, *A. caviae* and *A. veronii* bv *sobria* are

associated with human infections while the psychrophilic non-motile species such as *A. salmonicida* are associated with fish diseases (Janda and Abbott, 2010; Chuang *et al*., 2011). *A. hydrophila* and *A. veronii* have also been implicated in various fish diseases (Janda and Abbott, 2010).

Aeromonas species are facultatively anaerobic, oxidase-positive, catalase-positive, indole-positive, glucose-fermenting bacteria that are resistant to the 0/129 vibriostatic agent and are chemoorganotrophic (Parker and Shaw, 2011; Igbinosa *et al*., 2012). These microorganisms produce different extracellular hydrolytic enzymes such as arylamidases, esterases, elastase, amylases, deoxyribonucleases, chitinases, peptidases and lipases (Igbinosa *et al*., 2012). Members of the genus *Aeromonas* have the ability to utilize urease, pectinase, ornithine decarboxylase, tryptophan and phenylalanine deaminases (Parker and Shaw, 2011). While some members of this genus have the ability to ferment D-mannitol and sucrose, important distinguishing qualities from other genera such as *Plesiomonas* include their inability to grow in the presence of 6.5% sodium chloride, inability to ferment i-inositol, inability to grow on thiosulphate citrate bile salts sucrose agar, variable presence of ornithine decarboxylase activities and the ability to liquefy gelatine (USEPA, 2006).

1.1.1 *Aeromonas* species in aquatic environments

Members of the genus *Aeromonas* are known to be autochthonous to aquatic environments, since they may be isolated from all types of water environments, e.g. rivers, lakes, ponds, seawater, estuaries, chlorinated drinking water and non-chlorinated drinking water, ground water, waste water and sewage (Janda and Abbott, 2010). Calhau *et al*. (2010) detected the presence of *A. media*, *A. molluscorum*, *A. sobria*, *A. veronii*, *A. salmonicida*, *A. hydrophila*, *A. bivalvium*, *A. caviae*, *A. bestiarum*, *A. popoffii*, *A. allosaccharophila*, *A. tecta* and *A. encheleia* in environmental waters. Carvalho *et al*. (2012) observed that *A. media*, *A. veronii*, *A. salmonicida*, *A. hydrophila*, *A. caviae*, *A. bestiarum*, *A. popoffii*, *A. allosaccharophila*, *A. eucrenophila*, *A. tecta* and *A. encheleia* are prevalent in surface waters in Portugal. *Aeromonas allosaccharophila*, *A. aquariorum*, *A. enteropelogenes*, *A. eucrenophila*, *A. hydrophila*, *A. jandaei*, *A. media*, *A. punctata*, *A. sanarellii*, *A. taiwanensis* and *A. veronii* were detected in raw surface water, ground water, chlorinated and ozonated drinking water (Figueira *et al*., 2011). In Brazil, *A. encheleia*, *A. allosaccharophila*, *A. hydrophila* and *A. jandaei* were detected in wells and collective reservoir waters used for human consumption (Razzolini *et al*., 2010). In South Africa, Igbinosa and Okoh

(2013) detected the presence of *A. hydrophila* and *A. caviae* in fresh and waste waters in the Eastern Cape Province.

1.1.2 *Aeromonas* species in aquaculture

Aeromonads are ubiquitous in water environments and as a result form part of the normal micro-flora of fish and other aquatic animals (Shayo *et al*., 2012). *Aeromonas* species are etiological agents of a variety of infections in fish and their diversity in isolated species from aquaculture has been studied (Adeleye *et al*., 2010; Kadlec *et al*., 2011; Nagar *et al*., 2011; Sarria-Guzmàn *et al*., 2013; Ye *et al*., 2013; Vega-Sanchez *et al*., 2014). *A. hydrophila* has been reported to be the species with highest incidence in fish and aquaculture (Adeleye *et al*., 2010; Kadlec *et al*., 2011; Nagar *et al*., 2011; Sarria-Guzmàn *et al*., 2013; Ye *et al*., 2013; Vega-Sanchez *et al*., 2014). This can be correlated with the increased isolation of *A. hydrophila* from clinical samples (Shayo *et al*., 2012; Ye *et al*., 2013; Vega-Sanchez *et al*., 2014). Fish hemorrhagic diseases due to *A. hydrophila* are responsible for huge economic losses in aquaculture every year in China (Ye *et al*., 2013). Sarria-Guzmàn *et al*. (2013) reported the presence of *A. veronii*, *A. hydrophila*, *A. salmonicida*, *A. media*, *A. punctata*, *A. sobria*, *A. caviae* and *A. allosaccharophila* in moribund cultured *Cyprinus carpio*. Multidrug resistant *A. hydrophila* were isolated from commercialized seafood in Lagos, Nigeria (Adeleye *et al*., 2010). Vega-Sanchez *et al*. (2014) observed that *A. hydrophila*, *A. bestiarum* and *A. veronii* biovar biovar *sobria* were implicated in hemorrhagic septicemia and furunculosis of farmed rainbow trout under stress conditions in Mexico. Other species identified were *A. allosaccharophila*, *A. popoffi*, *A. salmonicida*, *A. media* and *A. encheleia*. Nonmotile *A. salmonicida* was implicated in a typical furunculosis among salmonids (*Salmo salar*) and goldfish ulcers in non-salmonids and have been identified with *A. hydrophila* in cultured fish for consumption and ornamental fish in Germany (Godoy *et al*., 2010; Kadlec *et al*., 2011). Dias *et al*. (2012) observed the prevalence of *A. caviae*, *A. hydrophila*, *A. veronii*, *A. jandaei*, *A. media* and *A. aquariorum* in ornamental fish from Portugal. Shayo *et al*. (2012) observed that motile *Aeromonas* species *A. caviae*, *A. hydrophila*, *A. punctata*, *A. ichthiosmia* and *A. veronii* were responsible for motile aeromonad septicemia (MAS) in tilapia subjected to elevated water temperatures, decreased oxygen concentration and increased ammonia and carbon dioxide concentrations. Outbreaks of hemorrhagic diseases in China have been associated with *A. bestiarum*, *A. hydrophila*, *A. salmonicida*, *A. sobria* and *A. piscicola* (Beaz-Hidalgo *et al*., 2010).

Nagar *et al*. (2011) reported the predominance of *A*. *salmonicida* and *A*. *veronii* bv *sobria* among *A*. *hydrophila*, *A*. *caviae*, *A*. *jandaei*, *A*. *trota* and *A*. *eucrenophila* identified in commercialised cultured fresh water and marine water fishes from India. Similarly in Brazil *A*. *allosaccharophila*, *A*. *caviae*, *A*. *veronii* bv *sobria*, *A*. *hydrophila* and *Aeromonas* spp. were detected in reared and commercial *Thunnus* spp., *Oncorynchus* spp., *Centropomus* spp., *Paralichthys* spp., and *Mycteroperca* spp., farmed for human consumption (Da Silva *et al*., 2010). Biological films of cultured adult and juvenile abalone (*Haliotis discus hannoi*) have been shown to harbour *Aeromonas* species among other Gram-negative bacteria (Choresca *et al*., 2010). *Aeromonas* spp. were detected in mucus of farmed adult abalone (*Haliotis discus hannai*) (Choresca *et al*., 2010).

1.1.3 *Aeromonas* species infections in humans

Aeromonads are responsible for gastrointestinal and extraintestinal infections such as septicaemia and wound infections in healthy and immunocompromised humans (Janda and Abbott, 2010). Humans usually get infected with aeromonads via contact or consumption of contaminated water or food (Khajanchi *et al*., 2010). The role of aeromonads in waterborne infections has been established with *A*. *caviae* and *A*. *media* being the most prevalent (Khajanchi *et al*., 2010; Pablos *et al*., 2010). *Aeromonas* species that are associated with human gastroenteritis are *A*. *caviae*, *A*. *hydrophila*, *A*. *media*, *A*. *bestiarum* and *A*. *veronii* (biovars *veronii* and *sobria*) (Pablos *et al*., 2010). Mesophilic species *A*. *hydrophila*, *A*. *caviae* and *A*. *veronii* were reported to be responsible for monobacterial bacteremias in humans (Chuang *et al*., 2011). *Aeromonas trota*, *A*. *sobria* and *A*. *jandaei* have been implicated in gastroenteritis in humans (Igbinosa *et al*., 2012). *Aeromonas hydrophila*, *A*. *caviae* and *A*. *veronii* biovars *veronii* and *sobria*), *A*. *jandaei* and *A*. *schubertii* are associated with peritonitis, meningitis, and various infections of the eye, joint and bone in humans (Janda and Abbott, 2010). *Aeromonas caviae, A. hydrophila,* and *A. veronii* biotype *sobria* have been associated with opportunistic infections of humans such as food-borne gastroenteritis, diarrhoea and wound infections (Naviner *et al*., 2011). These infections are usually treated with antimicrobial agents, in both humans and animals, although increasing frequency of occurrences of antimicrobial resistance have been reported (Janda and Abbott, 2010).

1.2 Bacterial antimicrobial resistance

Antimicrobial resistance is largely the consequence of drug overuse in human medicine and animal agriculture and is of great public health concern (WHO, 2004). The abuse of antimicrobial agents in animal, plant and fish farms has contributed immensely to the emergence of resistant pathogenic and non-pathogenic bacteria (Kűmmerer, 2009). Antimicrobial resistance is facilitated by the presence of antimicrobial resistance genes (ARG), and it is established that these genes usually have environmental origins (Lupo *et al*., 2012; Marti *et al*., 2013). In addition to food and animal production farms, hospital effluents have been identified as a source of these ARGs that eventually get transported and transferred by horizontal gene transfer in aquatic environments (Picào *et al*., 2013). Mobilization of these ARGs by genetic elements and mobile genetic elements (MGE) such as integrons, transposons and plasmids, means that they can arrive at drinking water supplies, food products and eventually humans (Kűmmerer, 2009; Lupo *et al*., 2012; Marti *et al*., 2013).

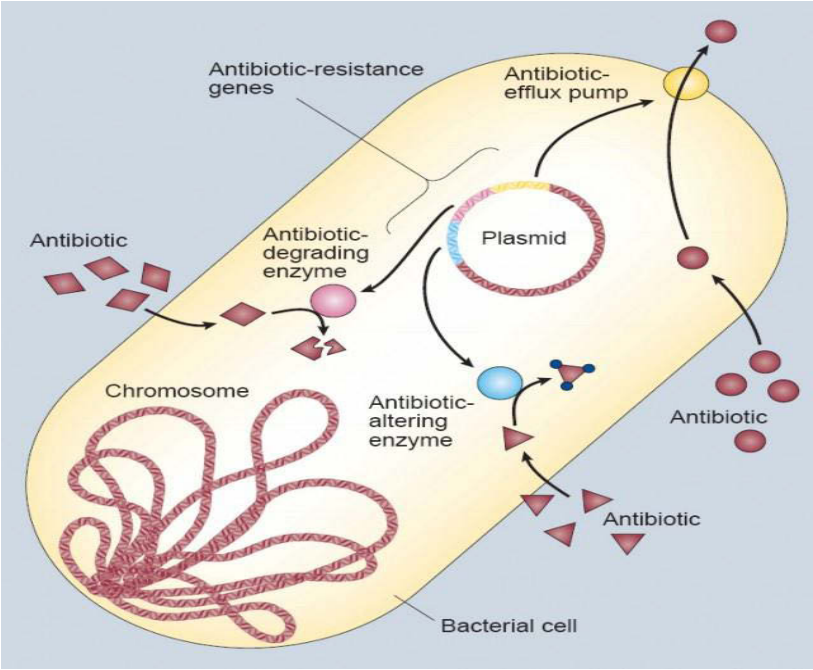

Figure 1.1: Representation of various mechanisms of bacterial resistance (Levy and Marshal, 2004)**.**

There are four main mechanisms of antimicrobial resistance in bacteria, (Fig. 1.1) viz.; the innate hydrolysing activities of enzymes, e.g., beta-lactamases against antimicrobial agents, the activities of efflux pumps that expel antimicrobials from cells, the use of *DE novo* metabolic

pathways which provides altered cell walls that do not possess active binding sites for antimicrobials, and a plethora of mutations that lead limitation of antimicrobials to target sites (Tenover, 2006).

1.2.1 Antimicrobial resistance in *Aeromonas* spp.

World-wide reports of the members of *Aeromonas* spp. suggests that aeromonads are readily developing single or multiple antimicrobial resistance phenotypes (Janda and Abbott, 2010). *Aeromonas* spp. are known to be resistant to β-lactams, tetracyclines, quinolones, and second- and third-generation cephalosporins (Jacobs and Chenia, 2007; Jun *et al*., 2010; Surek *et al*., 2010; Igbinosa and Okoh, 2012).

Matyar *et al*. (2010) observed that aquatic environments are possible reservoirs of antimicrobial resistant *Aeromonas* spp strains. This increases the risk of contamination of surface waters and ground water (Gõni-Urriza, 2000; Huddleston *et al* 2006; Hoa *et al*., 2011), which in-turn highlights the fact that potable water can be contaminated by these waters and will indeed transfer antimicrobial resistance to humans and human pathogens (Xi *et al*., 2009; Pablos *et al*., 2010). Figueira *et al*. (2011) observed that aeromonads were relevant in the dissemination of antimicrobial resistance in water environments and *A. media* and *A. punctata* were important environmental reservoirs of quinolone resistance. Figueira *et al*. (2011) observed antimicrobial resistance in aquatic *A. caviae*, *A. hydrophila*, *A. veronii, A. jandaei* and *A. allosaccharophila* from Portugal to beta-lactams, cephalosporins and nalidixic acids. Similarly in the Eastern Cape Province of South Africa, *Aeromonas* spp. isolated from waste water treatment plants were observed to be multidrug resistant to beta-lactams, tetracycline, trimethoprim, sulphamethoxazole and trimethoprim-sulfamethoxazole (Igbinosa and Okoh, 2012).

Jun *et al*. (2010) observed multi-resistance to amoxicillin/clavulanic acid, ampicillin, chloramphenicol, tetracycline and trimethoprim-sulfamethoxazole in *A. hydrophila* isolated from farmed Korean *Misgurnus anguillicaudautus*. Ornamental koi carp (*Cyprinus carpio koi*) and common carp (*Cyprinus carpio*) from Czech Republic were observed to possess *A. hydrophila*, *A. sobria* and *A. veronii* sub-species *sobria* exhibiting resistance to oxytetracycline, ciprofloxacin, chloramphenicol, spectomycin, oxolinic, streptomycin, florfenicol and trimethoprim (Čižek *et al*., 2010).

In Brazil ampicillin, cephalothin and cefazolin resistance was detected in clinical strains of *A. caviae*, *A. hydrophila*, *A. veronii* bv *sobria* and *Aeromonas* spp. from humans with

diarrhoea (Surek *et al*., 2010). Obi *et al*. (2007) also detected multi-drug resistance in *A. caviae*, *A. hydrophila* and *A. sobria* isolated from clinical samples in the Limpopo Province of South Africa.

1.2.2 Mobile genetic elements associated with antimicrobial resistance in *Aeromonas* spp.

Antimicrobial resistance genes are elements that encode resistance to antimicrobials and may be passed on to members of the same species through vertical transfer or to different species of bacteria through horizontal gene transfer via MGEs. Thus antibiotic resistance genes can be transferred between bacteria in the environment through different MGEs, viz.; plasmids, integrons and transposons (Akinbowale *et al*., 2007). Horizontal gene transfer often involves plasmids, and in *Aeromonas* spp. antimicrobial resistance has been correlated with the possession of plasmids and integrons (Palu *et al*., 2006; Ndi and Barton, 2011).

1.2.2.1 Plasmid-associated antimicrobial resistance in *Aeromonas* spp.

Plasmid-associated resistance genes have been found to encode for multidrug resistance in bacteria including quinolone, aminoglycoside and trimethoprim-sulfamethoxazole resistance (Samaha-Kfoury and Araj, 2003; Poole, 2004). Plasmids with varying molecular weights have been reported in several strains of *Aeromonas* spp. High molecular weight plasmids are responsible for multiple drug resistance in *A. salmonicida* (Sorum *et al*., 2003) and *A. hydrophila* (Majumbar *et al*., 2006) and this resistance could be transferred along with the transfer of plasmid (Sorum *et al*., 2003; Casas *et al*., 2005). In Brazil, human and food aeromonad isolates have been shown to harbour plasmids (Palu *et al*., 2006), while a high prevalence of small (2.3 kb) plasmids has been observed in Malaysian retailed fish (Radu *et al*., 2003). These plasmids offer the greatest source and route of dissemination of antimicrobial resistance genes and their determinants such as integrons. *A. caviae* isolated in Brazil displayed resistance to tetracycline which was associated with presence of a 15 kb plasmid (Palu *et al*., 2006). *A. salmonicida* isolates possessing multi-resistance plasmids have been described globally (Najimi *et al*., 2008). A broad-host range plasmid pRAS1, first isolated from an atypical *A. salmonicida,* encoded phenotypic resistance to sulphonamides, trimethoprim, and tetracycline, which were determined by *sul1, dfr16* and *tetA* genes (L'Abee-Lund and Sorum, 2001; Balassiano *et al*., 2007). McIntosh *et al*. (2008) reported the occurrence of *A. salmonicida* harbouring a large transferable

IncA/C plasmid, and nucleotide sequence analysis revealed that the genes encoding the resistance phenotypes were organized into three separate resistance cassettes. These cassettes were a class I integron containing an *aadA*7 gene encoding for an aminoglycoside-3′-adenyltransferase, a cassette with 99% nucleotide sequence homology to a cassette previously identified in the *Salmonella enterica* IncA/C plasmid pSN254, containing *floR*, *tetA*, *sulII* and *strA/strB* sequences and a third cassette showed 100% nucleotide sequence similarity to a transposon-like element, containing a bla_{CMY-2} β-lactamase in association with *sugE* and *blc* sequences.

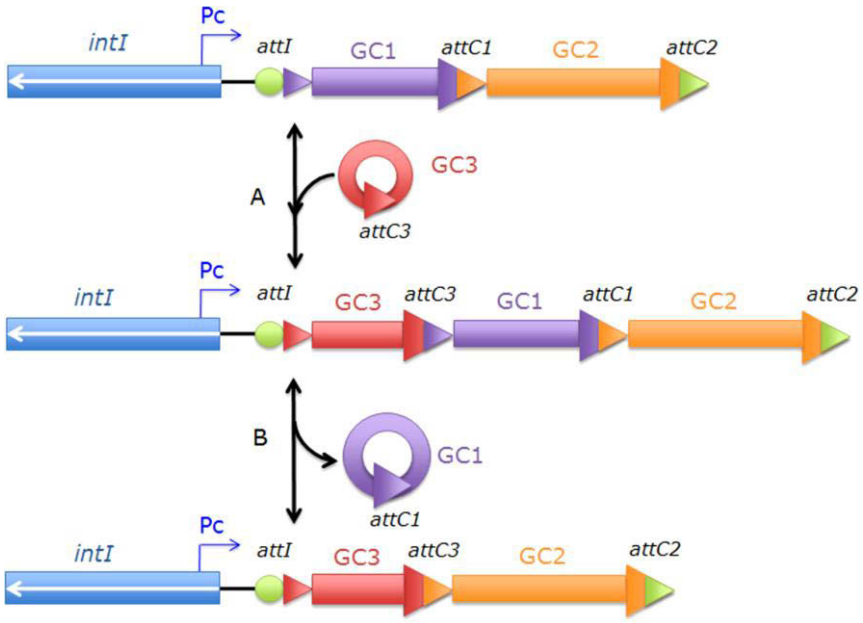

Figure 1.2: General organization of an integron and gene cassette (GC) recombination mechanism. TheIntI1protein catalyzes the insertion **(A)** and excision **(B)** of the GC in the integron, with GC integration occurring at the *attI* recombination site. In example **(A)**, the circularized GC3 is integrated in linear form inside the integron platform *via* a specific recombination mechanism between the *attI* site and the *attC3* site of the GC3. GC excision preferentially occurs between two *attC* sites. In example **(B)**, the GC1 is excised following there combination between the two *attC1* and *attC3* sites. Pc: gene cassette promoter; *attI*: integron recombination site*; attC1*, *attC2*, and a*ttC3*: *attC* GC recombination sites; *intI*: the integrase gene; GC1, GC2, GC3 are the gene cassettes, and arrows indicate the direction of coding sequences (Stalder *et al*., 2012).

1.2.2.2 Integron-associated antimicrobial resistance in *Aeromonas* spp.

Integrons are defined as genetic elements that play a key role in the dissemination of resistance determinants in the environment (Stalder *et al*., 2012). They do this through encoding a site-

specific recombination system that recognises and captures mobile genetic cassettes (Ma *et al*., 2011). The integron structure consists of a functional platform (Fig. 1.2) viz.; 5'conserved segments (CS) and 3'CS, an *intI* gene encoding an integrase protein, a specific recombination site *attI*, and a promoter, Pc (Stalder *et al*., 2012). Gene cassettes are non-replicable mobile elements which consist of a 59-base element known as the *attC* recombination site and an open reading frame (*orf*) but lacking a promoter (Chang *et al*., 2007; Stalder *et al*., 2012). The gene cassettes are usually located between the 5'CS and 3'CS (Fig. 1.2) within the functional platform and they consist of one or more antimicrobial resistance genes which leads to the multi-drug resistance that integrons confer to bacteria (Ma *et al*., 2011; Lupo *et al*., 2013). There are over 130 known gene cassettes and most encode antimicrobial resistance (Stalder *et al*., 2012). Although not mobile, integrons are often associated with MGEs such as plasmids and transposons that disseminate them in the environment and among bacteria (Ma *et al*., 2011; Ndi and Barton, 2011).

There are five different classes of mobile integrons currently from classes 1 to class 5, with the class 1 integrons being more prevalent than other classes (Ma *et al*., 2011; Stalder *et al*., 2012). Class 1 integrons are ubiquitous elements occurring naturally in aquatic and land environments, and are the most widely distributed among Gram-negative bacteria (Chang *et al*., 2007; Stalder *et al*., 2012). They have been associated with the evolution and spread of antimicrobial resistance in clinical Gram-negative bacteria (Chang *et al*., 2007; Lupo *et al*., 2012). The classic structure of class 1 integrons includes the *intI* gene, the *attI* integration site, the common promoter in the 5'CS and the *qacEΔ1* and *sulI* genes in the 3'CS region conferring resistance to quaternary ammonium compounds and sulphonamides, respectively (Chang *et al*., 2007). Clinical isolates containing class 1 integrons are usually similar in gene cassette content and structure, while those found in environmental isolates are usually varied in terms of gene cassettes and structure (Stalder *et al*., 2012). Class 1 integrons are associated with the T*n*402-transposons and it has been established that they originated from environmental bacterial communities (Lupo *et al*., 2012).

Class 2 integrons are less prevalent than the class 1 integrons and also have low incidence in aquatic environments (Stalder *et al*., 2012). Their basic structure is truncated, as the *intII* gene has a stop codon which means that the class 2 integrons lacks the 5'CS and 3'CS regions (Stalder *et al*., 2012). However, their gene cassette area is more stable than the class 1 integrons and usually consists of *dfrΔ1*, *aadA1*, *sat2* and an unknown *orfx*. Their presence may lead to

trimethoprim, spectinomycin, streptomycin and streptothricin resistances in bacteria that carry them. They are associated with Tn7 transposons, which ensure their dissemination (Stalder *et al*., 2012).

Class 3 integrons are suggested to be involved in the dissemination of antibiotic resistance in both clinical and environmental settings and also participating in exchange of antibiotic resistance gene cassettes (GC) between these two ecosystems (Stalder *et al*., 2012). This class of integrons are abundant in aquatic environments, and are usually resistance integrons (Stalder *et al*., 2012). In literature only five classes of integrons have been characterised; the first was detected in a clinical sample, the second was identified in an *Enterobacteriaceae* strain, the third and the fourth were both detected in environmental *Delftia* strains and the fifth was detected in an *Enterobacter cloacae* isolate from a hospital effluent (Stalder *et al*., 2012). In South Africa Jacobs and Chenia (2007) detected the presence of *intI3* gene in an *A*. *veronii* biovar *sobria* isolate from aquaculture systems. Wen *et al*. (2012) also detected the presence of the *intI3* gene in *Flexneri* spp. clinical isolates. Class 3 integrons are characterised as frequently as class 1 integrons, however when identified, it mediates IMP-1 Metallo-Beta-lactamase (Stalder *et al*., 2012).

Class 4 integrons are called super integrons as they contain hundreds of gene cassettes that encode antimicrobial resistance and adaptations (Stalder *et al*., 2012). The presence of class 4 integrons in bacterial chromosomes has been traced back to pre-antibiotics times and in recent years *intISXT* has been characterized among *Vibrionaceae* and *Pseudomonas* spp. (Stalder *et al*., 2012). Class 5 integrons are rarely characterised, and in literature the *intIHS* has only been detected once in a *Vibrio* spp. (Stalder *et al*., 2012)

In *Aeromonas* spp., class 1 integrons are most prevalent (Chang *et al*., 2007; Lee *et al*., 2008; Sarria-Guzmàn *et al*., 2013) although class 2 have been detected in members of this genus from different geographical regions (Jacobs and Chenia, 2007; Carvalho *et al*., 2012; Maravic *et al*., 2013). Chang *et al*. (2007) observed that many *Aeromonas* spp. that were integron-positive were multi-drug resistant to three or more commonly used antimicrobial agents. Sarria-Guzmàn *et al*. (2013) detected the presence of class 1 integrons in *A. caviae*, *A. hydrophila*, *A. veronii, A. media*, *A. salmonicida*, *A. sobria*, *A. punctata* and *A. allosaccharophila* isolated from commercialised fresh fish in Mexico with antimicrobial gene arrays of *aadA*2, *aadA*5, *dhfrA17*, *dhfrA1*, *oxa2*, $bla_{\text{OXA-10}}$ and *catB3* conferring resistance to adenylyl transferase, dihydrofolate reductases, beta-lactamase, oxacillinase and chloramphenicol, respectively. In Portugal,

Carvalho *et al*. (2012) detected the presence of both class 1 and 2 integrons from raw water, both with similar antimicrobial resistance gene arrays, viz.; *aadA1*, *aadA*2, *dhfrA12*, *dhfrA1*, *sat2*, *catB8*. Jacobs and Chenia (2007) observed that *Aeromonas* spp. isolates from tilapia, trout and koi aquaculture systems in South Africa harboured class 1 integrons with gene cassettes *ant(3'')Ia*, *aac(6')Ia*, *dhfr1*, *oxa2a* and/or *pse1*. Lee *et al*. (2008) observed that *dhfrA1* and *aadA*2 were the most common integron-associated genes encountered in clinical isolates of *A. caviae*, *A. hydrophila*, *A. veronii,* and *A. sobria* in Taiwan. The most commonly found genes among class 1 integrons are the *aadA* genes (Chang *et al*., 2007; Sarria-Guzmàn *et al*., 2013), which accounts for the prevalent aminoglycoside resistance observed among *Aeromonas* spp. isolates. In a study of the occurrence and diversity of integrons and β - lactamase genes among ampicillin-resistant isolates from estuarine waters, Henriques *et al*. (2006) detected the presence of bla_{TEM}, bla_{SHV}, bla_{CphA} and $bla_{\text{OXA-B}}$ in *Aeromonas* strains.

1.2.3 Beta-lactam resistance in *Aeromonas* spp.

Bacterial resistance to β-lactams by the expression of β-lactamases is one of the most common mechanisms of resistance in bacteria. Antimicrobial resistance in *Aeromonas* species is usually chromosomally mediated, but β-lactamase production by *Aeromonas* may occasionally be encoded by plasmids or integrons (Aravena-Romàn *et al*., 2011). The β-lactamase genes and the enzymes they encode continuously undergo mutations as a strategy to beat the diversity of β-lactams in use (Samaha-Kfoury and Araj, 2003). These continuous mutations are due to heavy antimicrobial agent use which has led to the emergence of extended spectrum β-lactamase enzymes (Samaha-Kfoury and Araj, 2003; Poole, 2004).

Aeromonas spp. isolates are resistant to many β-lactams as a result of multiple inducible, chromosomally-encoded β-lactamases which are under a single mechanism of coordinate expression (Goni-Urriza *et al*., 2000). These organisms are able to produce three different β–lactamases including group 1 molecular class C cephalosporinase, a group 2d molecular class D penicillinase and a group 3 molecular class B metallo-β–lactamases (Fosse *et al*., 2003; Talavera *et al*., 2006; Libisch *et al*., 2008). Different strains of β–lactamase producing *Aeromonas* spp. have been grouped into five major classes viz.; *A. hydrophila* complex strains expressing class B, C and D β-lactamases, *A. caviae* strains expressing class C and D β-lactamases, *A. veronii* group strains containing class B and D β-lactamases, *A. schubertii* strains harbouring class D β-lactamases, and *A. trota* strains with class C β-lactamases. *Aeromonas* spp. are among the few

microorganisms harbouring different chromosomal β–lactamase genes including *cphA*, *cepH*, and *ampH*, encoding for classes B, C and D β-lactamases (Carminato *et al*., 2007).

1.2.3.1 Penicillin resistance in *Aeromonas* spp.

In aeromonads, resistance to penicillins are encoded for by bla_{TEM} native genes, which express class D penicillinases (Balsalobre *et al*., 2010). In China, Deng *et al*. (2014) observed the presence of bla_{TEM} gene in *A*. *hydrophila* isolated from cultured fresh water fish. The intrinsic occurrence of bla_{TEM} genes in *Aeromonas* spp. from aquatic environments (Carminato *et al*., 2010) points to the possibility of the aquatic environment being the reservoir of these β–lactamase genes (Henriques *et al*., 2006; Hernould *et al*., 2008). The bla_{TEM} family β–lactamases are plasmid-mediated and common among environmental Gram-negative bacteria, particularly *Aeromonas* spp. This Bush group 2d penicillinase of β–lactamases confers resistance to narrow and broad spectrum penicillin resistance penicillin and ampicillin (Janda and Abbott, 2010). The bla_{TEM} genes are contained in ampicillin resistance transposons (*TnA*), and are associated with the prevalent *Tn1*, *Tn2* and *Tn3* transposons (Tristram *et al*., 2005; Tristram *et al*., 2012).

TEM-I β-lactamases have been noted to be the progenitor of TEM-type extended β-lactamases (ESBL) due to diverse point mutations in the $bla_{TEM\text{-}I}$ gene (Tristram *et al*., 2005). Single base mutations are responsible for amino acid substitutions such as glutamate to serine at position 104, arginine to serine or histidine at position 164, glycine to serine at position 238 and glutamate to lysine at position 240 which produces the ESBL phenotype in TEM-type genes (Chroma and Kolar, 2010). These diverse point mutations in the TEM β–lactamases genes has given rise to enzymes with extended activity against first, second and third generation cephalosporins in addition to penicillins.

1.2.3.2 Extended spectrum beta–lactam resistance in *Aeromonas* spp.

The production of extended spectrum β–lactamase (ESBL) genes in *Aeromonas* species has been reported (Marchandin *et al*., 2003; Fosse *et al*., 2004; Picào *et al*., 2008; Lu *et al*., 2010; Girlich *et al*., 2011). ESBLs belong to the class A β–lactamases and confer resistance to penicillins, aztreonam and first, second and third-generation cephalosporins but are susceptible to clavulanate and tazobactams (Chikwendu *et al*., 2011; Chen *et al*., 2012). Environmental isolates of *A*. *media* and *A*. *hydrophila* harbouring ESBLs genes has been described (Picào *et al*., 2008; Lu *et al*., 2010; Girlich *et al*., 2011) and *A*. *allosaccharophila*, a non-clinical important species,

was observed to harbour the PER-6 extended spectrum gene (Girlich *et al*., 2010). Even though production of ESBLs is not an intrinsic feature of aeromonads (Janda and Abbott, 2010), studies suggest that environmental *Aeromonas* spp. could be important reservoirs for ESBL dissemination (Girlich *et al*., 2010; Carminato *et al*., 2010).

In recent years ESBLs derived from the TEM and SHV families are gradually being replaced by the $bla_{\text{CTX-M}}$. The CTX-M enzymes are class A β–lactamases that are able to hydrolyse cefotaxime more than ceftazidime (Chen *et al*., 2012). The genus *Kluyvera* has been identified as the reservoir of these $bla_{\text{CTX-M}}$ genes and is known to contain the progenitor of these ESBLs in their chromosome (Lupo *et al*., 2011). *Kluyvera* spp. are known environmental bacteria, hence the reason why these $bla_{\text{CTX-M}}$ genes are increasingly described from environmental and aquatic isolates (Lupo *et al*., 2011). The $bla_{\text{CTX-M}}$ genes are classified into five groups based on aminoacid homology viz.; $bla_{\text{CTX-M-1}}$ $bla_{\text{CTX-M-2}}$ $bla_{\text{CTX-M-8}}$ $bla_{\text{CTX-M-9}}$ and $bla_{\text{CTX-M-25}}$. The $bla_{\text{CTX-M-}\textit{15}}$ gene is the most disseminated ESBLs among Gram-negative bacteria and have also been detected in fish pathogenic *Aeromonas* spp. (Ye *et al*., 2010; Gomez-Garces *et al*., 2011). Gõmez-garcés detected the presence of $bla_{\textit{CTX-M-15}}$ in a clinical isolate of *A. hydrophila* in Spain and in China clinical isolates of *A. caviae* was observed to be a CTX-M-3 producer (Ye *et al*., 2010). In Croatia Maravic *et al*. (2013) detected the presence of $bla_{\text{CTX-M-}\textit{15}}$ gene in aquatic isolates of *A. caviae* and *A. hydrophila*. In *Aeromonas* spp. these ESBLs are usually acquired through horizontal gene transfer mediated by MGEs (Chen *et al*., 2012). The transferability of these extended spectrum β–lactamases genes via horizontal gene transfer is of public health importance (Ye *et al*., 2010; Gomez-Garces *et al*., 2011). Therefore, the prevalence of these ESBL genes amongst aquatic aeromonads suggests that *Aeromonas* spp. are possible agents of dissemination of β–lactamases and ESBLs (Girlich *et al*., 2011).

1.2.3.3 Metallo-beta–lactam resistance in *Aeromonas* spp.

Under selective pressure of certain antimicrobials, emergence of β–lactamase over-expressing carbapenem resistant *Aeromonas* spp. has been observed (Libisch *et al*., 2008). These carbapenemases are metallo-β–lactamases (MBL), and are located chromosomally in aeromonads (Sànchez-Cespedes *et al*., 2009; Chen *et al*., 2012). They hydrolyze oxyimino-cephalosporins, cephamycins, carbapenems and monobactams to a lesser extent. They are not readily inhibited by clavulanate acids (Sànchez-Cespedes *et al*., 2009). Amongst *Aeromonas* spp., the CphA is the most widely described MBL (Chen *et al*., 2012). The CphA

carbapenemases have a relatively narrow substrate profile (Libisch *et al*., 2008) and contain a zinc ion in its active site (Libisch *et al*., 2008). The prevalence of CphA in *Aeromonas* spp. is species-specific (Chen *et al*., 2012). Aquatic isolates of *A*. *veronii* and *A*. *caviae* were observed to harbour CphA (Rossolini *et al*., 1995). Balsalobre *et al*. (2009) detected the presence of CphA in aquatic environment isolates of *A*. *jandaei* and *A*. *hydrophila*. Ornamental fish isolates of *A*. *aquariorum* have been found to carry these CphA MBLs (Martinez-Murcia *et al*., 2008). Although they carry chromosomal *Aeromonas* MBLs, wild type isolates are categorized as being susceptible to carbapenems (Neuwirth *et al*., 2007; Libisch *et al*., 2008), but on exposure to certain β–lactamases inhibitors will over-express carbapenemases due to selective pressures (Libisch *et al*., 2008; Balsalobre *et al*., 2009; Sànchez-Cespedes *et al*., 2009).

Another MBL that is 98% identical to the CphA β–lactamase in terms of amino acid content is the ImiS β–lactamase (Wu *et al*., 2012). It has been detected in clinical isolates of *A*. *veronii* biovar *sobria.* This has been correlated to imipenem resistance in clinical isolates of *Aeromonas* spp. (Tsai *et al*., 2006) and it also confers resistance to meropenem but susceptibility to other β–lactam (Sànchez-Cespedes *et al*., 2009).

Some MBLs have been found to be associated with integrons. Neuwirth *et al*. (2007) and Libisch *et al*. (2008) observed that $bla_{\text{IMP-19}}$ and $bla_{\text{VIM-4}}$, respectively, were borne-on integrons. In addition to the three groups of β–lactamases that *Aeromonas* spp. possess and confer as resistance to β – lactams, efflux pumps are also involved in the mode of resistance to β – lactam antimicrobials (Henriques *et al*., 2006).

1.3 Bacterial biofilm development

Bacterial biofilms are communities of microorganisms that form and grow attached to surfaces, encased in an exopolysaccharide matrix (Kostakioti *et al*., 2013). Biofilms may comprise of single or multiple species colony, such as dental plaque biofilms (Kreth *et al*., 2008). Bacteria possess the ability to form biofilms on many surfaces (Kostakioti *et al*., 2013). Tthis increases the detrimental effects of these microbial communities in the medical and industrial settings that they occur in (Lopez, 2010). For example in food industries, they contribute to biofouling and normal sanitary procedures are no longer sufficient in eradicating these sessile communities (Simoes, 2010). In medical settings biofilms can form in hospital hot water systems, dental unit water lines and domestic shower heads can act as a source of infection in immune compromised patients (Jacques *et al*., 2010).

Biofilm formation is a bacterial survival response to adverse environmental changes, which consists of five basic stages. The first stage is the initial attachment, here bacteria respond to nutrient composition and utilizes flagella or chemotaxis in order to overcome surface repelling hydrodynamic (Lopez *et al*., 2010; Kostakioti *et al*., 2013). However this stage is reversible owing to sheer surface forces that can slough bacteria off desired niches, various surface properties, nutrient levels, ionic compositions and temperature (Kostakioti *et al*., 2013). Adherence to attached surface follows, usually through secreted adhesins or adhesive appendages such as pilli, curli, fimbriae and antigen 43 (Kostakioti *et al*., 2013). Successive adherence leads to the irreversible attachment of bacteria and sessile growth of cells along with exopolysaccharide (EPS) construction by resident cells can begin. As cells actively multiply and exchange genetic material such as resistance genes, there is up-regulation of biofilm phenotype factors that encourage sessility and stabilize biofilms (Fuente-Nunez *et al*., 2012; Kostakioti *et al*., 2013). The mature biofilm community is characterised by different gradients of nutrients, oxygen and toxic by-products, as a result of active and dead cells within sessile community. Inevitably biofilm dispersal follows as a natural end of sessile community. This dispersal can be passive as a result of environment stress or exogenous dispersal inducing compounds. Dispersal can also be active and initiated by resident cells due to environmental cues viz.; nutrient availability, oxygen fluctuations, increased toxic products and stress induced conditions (Fuente-Nunez *et al*., 2012). Cells within biofilms have been found to be resistant to adverse environmental effects, ultraviolet damage, metal toxicity, anaerobic conditions, acid exposure, salinity, pH gradients, desiccations, bacteriophages and a host of other hostilities (Fuente-Nunez *et al*., 2012).

1.3.1 Role of biofilms in antimicrobial resistance

Antimicrobial resistance in biofilms is suggested to be a combination of various mechanisms (Drenkard, 2003). Bacteria existing in biofilms are 1000-fold more resistant to treatment with antimicrobials than planktonic cells (Fuente-Núńez *et al*., 2013). This is because the resistance mechanisms within a biofilm differ from resistance mechanisms present in planktonic cells (Drenkard, 2003; Simoes *et al*., 2010). Biofilm bacteria express sets of genes that differ from planktonic bacteria, including specific antimicrobial resistance genes that are activated as part of the unique biofilm phenotype (Mah and O'Toole, 2001; Drenkard, 2003; Simoes *et al*., 2010).

The various mechanisms of biofilm resistance (Fig. 1.3) are dependent on the species of bacteria in question and antimicrobial in use (Mah and O'Toole, 2001; Fuente-Nunez *et al*., 2013). There are also general mechanisms that include the structure and composition of the biofilm (Simoes *et al*., 2010; Fuente-Nunez *et al*., 2013). The exopolysaccharide (EPS) matrix of the biofilm consists of polysaccharides, proteins and extracellular DNA (eDNA), and it has been shown to be a protective barrier against exogenous stress, host immune phagocytosis and antimicrobial substance (Kostakioti *et al*., 2013). In addition to the EPS, the heterogeneous population of biofilms with bacteria at different growth states also play a part in the resistance of biofilm bacteria to certain antimicrobials (Fuente-Nunez *et al*., 2013). Exogenous stresses such as starvation, anaerobic conditions, sub-inhibitory concentrations of antimicrobials and heat shock have been suggested to activate adaptive resistances in bacteria within a biofilm (Fuente-Nunez *et al*., 2013).

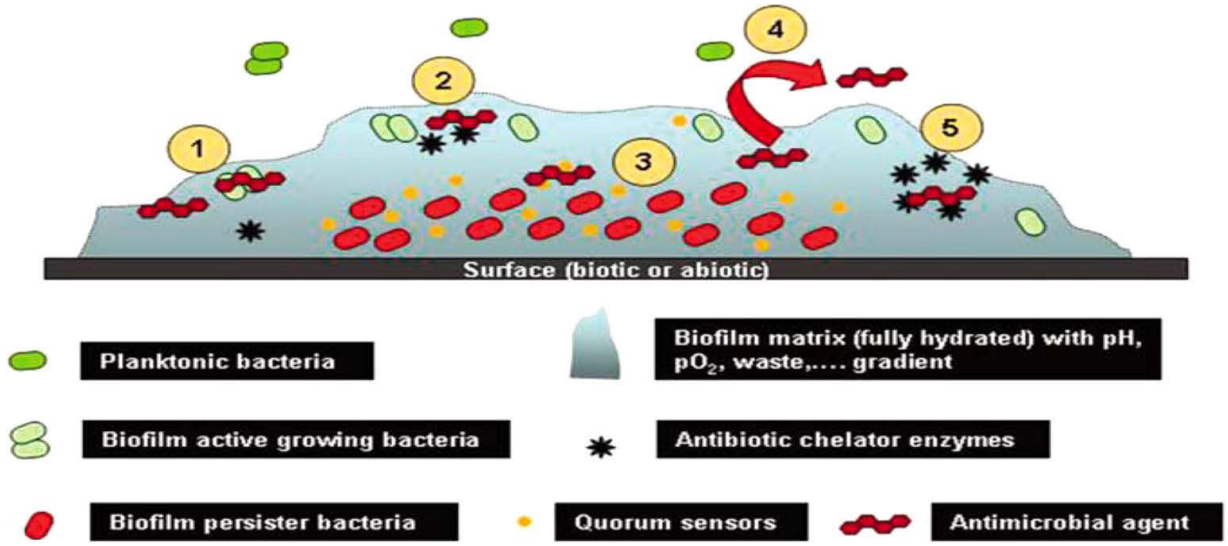

Figure 1.3: Proposed-biofilm associated resistance mechanisms: (1) Antimicrobial agents may fail to penetrate beyond the surface layers of the biofilm. Outer layers of biofilm cells absorb damage. Antimicrobial agents action may be impaired in areas of waste accumulation or altered environment (pH, pCO2, pO2, etc). (2) Antimicrobial agents may be trapped and destroyed by enzymes in the biofilm matrix. (3) Altered growth rate inside the biofilm. Antimicrobial agents may not be active against nongrowing microorganisms (persister cells). (4) Expression of biofilm-specific resistance genes (e.g., efflux pumps). (5) Stress response to hostile environmental conditions (Del Pozo and Patel, 2007).

Other mechanisms include varying physiological gradients of oxygen and nutrients and several biofilm specific phenotypes such as activation of certain efflux pumps, activity of perisplasmic glucans and persister cells (Drenkard, 2003; Lewis, 2005; Fuente-Nunez *et al*.,

2013). Persister cells are non-dividing biofilm bacteria that express toxin-antitoxins that block antimicrobials targets (Lewis, 2005). These cells have been suggested to be bacteria that tolerant to antimicrobials and are there to insure the propagation of cell species (Lopez, 2010).

1.3.2 Biofilm formation by *Aeromonas* spp.

Aeromonas spp. are strongly inclined to form colonies on surfaces known as biofilms (Medino-Martinez *et al*., 2006; Khajanchi *et al*., 2009; Ponnusamy *et al*., 2009; Santos *et al*., 2010). They are able to efficiently colonize various surfaces like contact lenses, glass, stainless steel and micro-titre plates (Merino *et al*., 2001; Lynch *et al*., 2002; Gavin *et al*., 2002; Bechet and Blondeau, 2003; Asha *et al*., 2004). Biofilm-forming *A. hydrophila* was identified on commercialised green-leafy vegetables in Saudi Arabia (Elhariry, 2011). Aeromonads are an important constituent of bacterial biofilms in water distribution systems or food processing for environmental strains, and in the gastrointestinal tract for clinical strains (Scoaris *et al*., 2007). *Aeromonas* spp. are known to possess a polar unsheathed monotrichous flagellum (*fla*) expressed for the swimming ability in liquid environments and inducible lateral flagella (*laf*) expressed for swarming motility on solid media. This phenomenon is associated with expansion of area of colonization, biomass production and biofilm formation (Kirov *et al*., 2002; Gavin *et al*., 2003). Aeromonad efficacy in micro-colony development has been attributed to hyperpiliation of *Aeromonas* spp. strains through the presence of type IV pilli and to the cell surface hydrophobicity, which would promote auto-aggregation of bacterial cells (Bechet and Blondeau, 2003). As biofilm formation is a feature of persistent infections and characterize up to 30% of *Aeromonas* gastroenteric infections, it is important to understand the mechanisms of antimicrobial resistance within aeromonad biofilms with a view to develop alternatives in the treatment of *Aeromonas* infections both in humans and animals.

1.3.3 Control of biofilms

The control of biofilms targets the different stages of biofilm development (Fig 1.4) (Simoes *et al*., 2010; Fuente-Núńez *et al*., 2013). The initial biofilm formation stage known as adhesion phase, has been explored as a strategy to prevent biofilm development. Several compounds such as polysaccharides secreted by mature biofilms, human and animal antimicrobial peptides, glycoside hydrolase, compounds that inhibit pili and curli syntheses and lyse mature biofilms have been explored (Fuente-Núńez *et al*., 2013). The use of substances that are able to cause

biofilm self-destruction is a factor that can be explored in seeking strategies to eradicate biofilm because most biofilm e.g., *Pseudomonas fluoresens* lyse their exopolysaccharides, liberating cells within the biofilms on oxygen and substrate depletion (Drenkard, 2003). Lytic phages that lyse bacteria cells have also been explored in controlling biofilms at microcolony formation and mature stages. Chelating agents such as sodium citrate and the use of silver nanoparticles have been observed to be effective at controlling bacteria at microcolony formation and mature stages as well (Kostakioti *et al*., 2013). The dispersal of mature biofilms by the use of enzymes such as DNAse 1, D-amino acids, polyamine norspermidine, nitric acid and dispersin have all been explored in the search for effective and efficient methods of controlling biofilms (Defoirdt *et al*., 2003; Fuente-Núńez *et al*., 2013). Kaplan *et al*. (2005) observed that *Actinobacillus actinomycetemcomitans* produces a soluble glycoside hydrolase called dispersin B, which degrades polysaccharide intercellular adhesins. Eradication of persister cells phenotype using a synergy of biofilm dispersal compounds and conventional antimicrobials may be effective therapy for biofilm-associated infections (Lewis, 2001). The engineering of universal signaling molecule 3, 5 cyclic diguanylic acid (c-di-GMP). Deactivation of cell-to-cell communication known as quorum sensing has been explored as a means of inhibiting biofilm development (Lynch *et al*., 2002; Fuente-Núńez *et al*., 2013). The uses of quorum-quenching enzymes and inhibitors have been identified as a possible strategy in biofilm dismantling (Dong *et al*., 2005). Phytochemicals have also been established to interfere with the signalling pathways of certain Gram-negative bacteria (Husain *et al*., 2013).

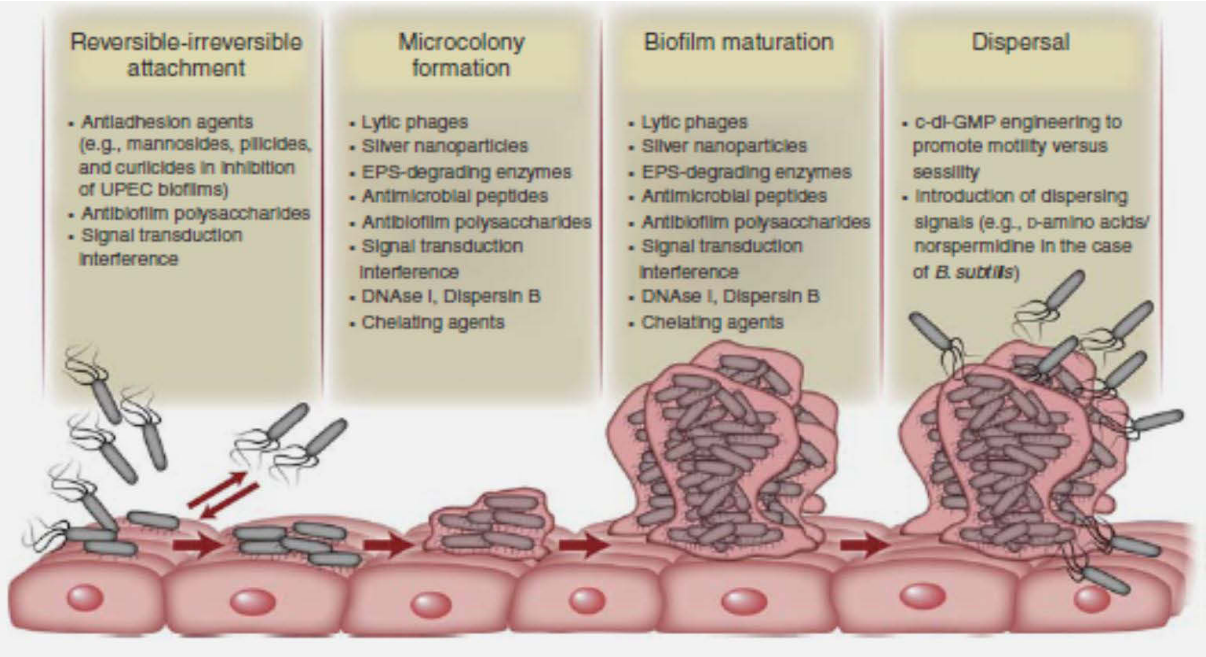

Figure 1.4: Schematic outlining of the stages in biofilm development and listing the strategies aimed at inhibiting and/or disrupting biofilm formation at specific stages (Kostakioti *et al*., 2013).

1.3.3.1 The use of phytochemicals in biofilm control

Phytochemicals are defined as bioactive, non-nutrient plant compounds found in fruits, vegetable, grains and other plant products (Doughari *et al*., 2009). They are also known as constitutive secondary metabolites that enable plants to overcome temporary or continuous threats integral to their environment, while also controlling essential functions of growth and reproduction (Molyneux *et al*., 2007). Medicinal plants have always been used successfully and approximately 80% of the world's population still rely on them as primary health care. The optimal effectiveness of a medicinal plant may not be due to one main bioactive component, but a concerted action of all the secondary metabolites within the plant (Senthilkumar *et al*., 2005). The use of plant antimicrobials has many advantages, including the lack of deleterious side effects compared to synthetic antimicrobials, their vast therapeutic potential. There are thousands of phytochemicals that have been discovered and they are grouped according to function and source. Four different groups of phytochemicals exist, including flavonoids and flavones, quinones, tannins and phenols and phenolic acids (Cowan, 1999). The antibacterial active components of plants may lyse the cell wall, block protein synthesis and DNA synthesis, inhibit enzyme secretions and/or interfere with the signaling mechanisms of the quorum sensing pathway (Chakraborty and Hancz, 2011).

1.3.3.2 Antimicrobial potential of phytochemicals

Phytochemical compounds present in plant extracts have been in use for the treatment of ailments of bacterial and fungal origins (de Britto *et al*., 2011; de Britto *et al*., 2012a, b; Dhayanithi *et al*., 2012). Cinnamaldehyde is a phytochemical belonging to phenol class and has been shown to have antibacterial activity against Gram-negative and Gram-positive bacteria, e.g., *Staphylococcus aureus*, *Clostridium botulinum*, *Escherichia coli* O157:H7, *Salmonella. typhimurium* and *Vibrio* species (Brackman *et al*., 2008; Jia *et al*., 2011). This is attributed to its hydrophobicity, which enables it to enter and disturb the lipid bilayer of the cell membrane and cause increased permeability to protons. Nascimento *et al*. (2000) observed that plant extracts of *P. granatum*, *Syzygyum joabolanum* (jambolan) and *Carophyllus aromatus* (clove) exhibited inhibitory properties against antimicrobial resistant strains of *Pseudomonas aeruginosa* and other resistant bacteria tested. Methanolic extracts of five green vegetables (*Coriandrum sativum*, *Lactuca sativa*, *Menthe pierita*, *Portulaca oleracea* and *Raphanus sativus*) were assessed for their antibacterial activities and all were observed to possess inhibitory properties against clinical strains of *Staphylococcus aureus*, *Streptococcus pyogenes*, *Escherichia coli*, *Bacillus subtilis* and *Pseudomonas aeruginosa* (Bhat and Al-Daihan, 2014).

The antibacterial activity of several phytochemicals has been explored using *Aeromonas* spp. as models (Siri *et al*., 2008; Dhayanithi *et al*., 2012; de Britto *et al*., 2012a, b). Methanolic extracts of leaves, stem, flowers and fruits of *Murraya koenigii* (L) were observed to be antibacterial against *A. hydrophila* (de Britto *et al*., 2012b). Siri *et al*. (2008) demonstrated that *Punica granatum* aqueous extract exhibited antibacterial activity against *A. caviae*. Methanol extracts of *Phyllanthus amarus*, *P. emblica*, *Cassia auriculata*, *Acalypha indica*, *Aerva lanata* and *Caesalpinia pulcherrima* was observed to have antibacterial activity against *A. hydrophila* (de Britto *et al*., 2012a). *Aeromonas hydrophila* isolated from ornamental fish was inhibited by *Excoecaria agallocha* leaves (Dhayanithi *et al*., 2012). Crude aqueous extracts of the stem barks and fruits of *K. africana* displayed antimicrobial activity against Gram-negative and Gram-positive bacteria (Grace *et al*., 2002; Eldeen and Staden, 2008; Saini *et al*., 2009). Ethanol extracts of the stem bark and fruits of *K. africana* displayed inhibitory activity against *S. aureus* and *P. aeruginosa* (Saini *et al*., 2009)**.** Water-and-ethanol extracts of *P. granatum* were observed to contain phenolic, sterols, proanthocyanidins and flavonoid compounds which contribute to its

antibacterial activity against Gram-negative bacteria (Siri *et al*., 2008). Allicin a major component of garlic was observed to be bactericidal against *A*. *hydrophila* (Nya *et al*., 2010).

1.3.3.3 Anti-biofilm potential of phytochemicals

Phytochemicals have also been studied for their anti-biofilm and anti-fouling properties (Ponnusamy *et al*., 2010). The control of biofouling does not entail limiting bacterial growth but to block the expression of biofilm-forming phenotypes (Lade *et al*., 2014). Reduction in biofilm formation of *A*. *hydrophila* and *Pseudomonas aeruginosa* was observed on treatment with clove oil (Husain *et al*., 2013). Similarly, Millezi *et al*. (2012) reported an interference with biofilm development of *A*. *hydrophila* on stainless steel on application of essential oils of thyme and lemon grass. Majtan *et al*. (2014) observed the reduction in biofilm formation and complete biofilm detachment of *Enterobacter cloacae* and *Proteus mirabilis* at sub-inhibitory and inhibitory concentrations, respectively. Trans-cinnamaldehyde was observed to control biofilms of uropathogenic *E*. *coli* on polystyrene plates and urinary catheters at 0.1 to 1.5% concentrations (Amalaradjou *et al*., 2010). Jia *et al*. (2011) demonstrated that cinnamaldehyde detached and eradicated all methicillin resistant *Staphylococcus aureus* (MRSA) at 5× MIC and MBCs (0.0625 – 0.5% v/v). Vanillin was effective against *A*. *hydrophila* biofilm formation in a water purification system at 0.16 mg/ml concentration (Ponnusamy *et al*., 2014). Plyuta *et al*. (2013) showed vanillin enhanced biofilm production of *Pseudomonas* spp. at concentrations ranging from 40 to 400 µg/ml but inhibited biofilms at higher concentrations.

1.4 Rationale for study

Aquaculture is one of the fastest growing food producing industries and worldwide this sector has increased at an average of 9.2% per year since 1970 (FAO, 2002). Disease outbreaks in aquaculture have been reported to cause significant constraints in the growth of this sector, with losses of billions of dollars worldwide. *Aeromonas* has been highlighted as one of the primary, opportunistic microorganisms plaguing this industry causing 100% losses in some cases (Defoirdt *et al*., 2004). The increasing incidence of antimicrobial resistance and adverse drug side effects has created an opportunity for the revival of phytomedicine and many plants are being investigated for their pharmacological activities against diverse disease conditions (McGaw *et al*., 2008).

There is growing concern worldwide due to the increasing incidence of antimicrobial resistance and dissemination of antimicrobial resistance genes amongst pathogenic and non-pathogenic bacteria in varying environments. The dissemination of resistance determinants by horizontal gene transfer is responsible for antimicrobial resistance in *Aeromonas* spp. and this is facilitated by MGEs. Members of this genus play a major role as a reservoir and vector of antimicrobial resistance determinants (Goni-Urriza *et al*., 2000; Radu *et al*., 2003; Palu *et al*. 2006; Jun *et al*., 2010). Foods, especially fish and water, remain the important vectors of these infectious pathogens. Antimicrobial resistance determinants in aquaculture ecosystems may be transmitted to human pathogenic bacteria, via fish and fish products, and this can lead to serious public health issues (Jun *et al*., 2010). The danger of antimicrobial resistance in aquaculture is treatment failure, which is very expensive and may result in complete loss of fish stocks. It is, therefore, important to identify selected antimicrobial resistance genes found in *Aeromonas* spp. This will lead to an exploration of prophylactic and treatment alternatives by investigating the antimicrobial and anti-biofilm effects of phytochemicals on aeromonads isolated from aquaculture and aquatic environments.

1.5 Objectives

The following objectives have been established:

1.5.1 To determine the prevalence and diversity of β – lactam, extended spectrum β – lactam and integron-associated resistance genes associated with multi-drug resistance in *Aeromonas* spp. isolates;

1.5.2 To investigate the antimicrobial effects of selected phytochemicals on *Aeromonas* spp. isolates; and

1.5.3 To investigate the effect of these phytochemicals on aeromonad biofilm formation.

1.6 Aims

The following aims will be pursued:

1.6.1 To isolate genomic DNA of *Aeromonas* spp. isolates, using the CTAB-NaCl protocol;

1.6.2 To amplify β-lactam, and extended spectrum β-lactam resistance gene determinants by PCR;

1.6.3 To identify integron platform structures by PCR;

1.6.4 To determine the susceptibility of *Aeromonas* spp. isolates to cinnamaldehyde, vanillin and *K. africana* extracts using the disk diffusion method, and

1.6.5 To determine the effect of cinnamaldehyde, vanillin and *K. africana* extracts on biofilm formation of *A. bestiarum* isolates using micro-titre plate assays.

CHAPTER TWO

CHARACTERIZATION OF BETA-LACTAMASE RESISTANCE GENE OF *Aeromonas* spp. ISOLATED FROM FISH AND AQUATIC SOURCES

2.1 Introduction

Aeromonas spp. are aquatic, Gram-negative microorganisms that are food and ornamental fish pathogens and are emerging as human pathogens worldwide (Lamy *et al*., 2009; Verner-Jeffreys *et al*., 2009; Janda and Abbott, 2010). Antimicrobial resistance amongst this genus is increasingly being described (Janda and Abbott, 2010; Figueira *et al*., 2011; Carvalho *et al*., 2012). This resistance poses therapeutic challenge for infections caused by aeromonads and is a potential public health risk (Chen *et al*., 2012). *Aeromonas* spp. are known to be resistant to penicillins, cephalosporins and carbapenems due to chromosomal and inducible β-lactamases (Chen *et al*., 2012). Aeromonads are known to produce various β-lactamases which confer resistance to a broad-spectrum of β-lactams (Janda and Abbott, 2010).

Beta-lactams are commonly used antimicrobials in the treatment of human and animal infections (Paterson and Bonomo, 2005). They are often used at sub-therapeutic doses in fish and animal farming for the prevention of infectious diseases (Henriques *et al*., 2006). This has led to the rise in production of resistance enzymes known as β-lactamases that hydrolyze β-lactam antimicrobials (Henriques *et al*., 2006; Cabello *et al*., 2013). However, β-lactam resistant bacteria have been detected in environments with no direct antimicrobial selection pressures (Kűmmerer, 2009; Balsalobre *et al*., 2010; Chenia and Vietze, 2012; Marti *et al*., 2013). Beta-lactamases of Gram-negative bacteria are the most important mechanism of resistance against β-lactams (Henriques *et al*., 2006; De Luca *et al*., 2010), and their mode of action is hydrolysis of the β-lactam ring (Hall *et al*., 2004).

In addition to the chromosomal β-lactamases, class A ESBLs have been detected in aeromonads (Chen *et al*., 2012). In aeromonads, the majority of ESBLs belong to the TEM-, SHV- and CTX-M-type enzymes (Chen *et al*., 2012). These ESBLs are usually borne on integrons and MGEs such as plasmids and transposons (Chen *et al*., 2012). Although *Aeromonas* spp. are intrinsically resistant to β-lactams (Chen *et al*., 2012), extra-chromosomal β-lactamases have also been detected in this genus (Wu *et al*., 2011; Maravic *et al*., 2013). Maravic *et al*. (2013) identified $bla_{CTX\text{-}M\text{-}15}$, $bla_{FOX\text{-}2}$ and $bla_{SHV\text{-}1}$ genes on a conjugative plasmid (IncFIB-type) from *A*. *hydrophila* and *A*. *caviae* isolates in Croatia. In Taiwan, the $bla_{PER\text{-}3}$ gene was identified

in both chromosomal and plasmid DNA from *A. hydrophila* and *A. caviae* isolates (Wu *et al*., 2011). Neuwirth *et al*. (2007) and Libisch *et al*. (2008) observed that bla_{IMP-19} and bla_{VIM-4}, respectively, were borne on class 1 integrons.

The earliest report of aeromonads carrying ESBLs was in a faecal *A. caviae* strain harbouring $bla_{\text{TEM-24}}$ (Marchandin *et al*., 2003). Since then environmental isolates harbouring $bla_{\text{PER-1}}$, $bla_{\text{PER-6}}$, $bla_{\text{SHV-12}}$, $bla_{\text{VEB-1a}}$, and $bla_{\text{GES-7}}$ have been described (Lu *et al*., 2010; Girlich *et al*., 2011). Picào *et al*. (2008) detected the presence of $bla_{\text{PER-1}}$ from a Swiss lake *A. media* isolate, while $bla_{\text{TEM-24}}$ and $bla_{\text{CTX-M-27}}$ were identified in *Aeromonas* spp. from an urban river in China (Lu *et al*., 2010). The ESBL bla_{SHV} gene is responsible for penicillin and 2nd and 3rd generation cephalosporin resistance (Rubtsova *et al*., 2010), and has been identified in environmental *Aeromonas* spp. (Maravic *et al*., 2013). Although the CTX-M enzymes are the most prevalent ESBLs, group 1 (CTX-M-15) is increasingly being described worldwide (Haque *et al*., 2012). They hydrolyze ceftazidime efficiently, unlike the other groups of CTX-M enzymes, viz., CTX-M-2, CTX-M-8, CTX-M-9 and CTX-M-25 (Naas *et al*., 2007). In Croatia, Wu *et al*. (2011) identified $bla_{CTX-M-15}$ in environmental isolates of *A. hydrophila* and *A. caviae*.

The most common metallo-β-lactamase (MBL) found in aeromonads is CphA. CphA has a substrate-specific profile as it is only active on carbapenems and penems (Chen *et al*., 2012). CphA has been described in *A. hydrophila*, *A. veronii*, *A. jandaei* and *A. aquariorum* (Martinez-Murcia *et al*., 2008; Balsalobre *et al*., 2009). However, other MBLs have been detected in various *Aeromonas* spp., such as ImiS in *A. veronii* biovar *sobria* isolates (Walsh *et al*., 1998) and IMP-19 in a clinical *A. caviae* isolate in France (Neuwirth *et al*., 2007). A multi-resistant strain of *A. hydrophila* isolated from a stool sample was observed to produce a VIM metallo-β-lactamase (Libisch *et al*., 2008).

Characterization of β-lactamase genes in South Africa shows that TEM and SHV types are predominant (Paterson and Bonomo, 2005; Meyer *et al*., 2007). Resistance phenotype of *Aeromonas* spp. from catfish, tilapia, goldfish and seawater (Duma, 2012) shows resistance profiles common in fish and aquatic environments (Jacobs and Chenia, 2007; Balsalobre *et al*., 2010; Carvalho *et al*., 2012; Igbinosa *et al*., 2013; Marti *et al*., 2013; Ye *et al*., 2013). Duma (2012) observed that these isolates were largely resistant to penicillins such as ampicillin (92.9%; 92/99) and amoxicillin (91.9%; 91/99) across all species tested. Resistance to 2nd and 3rd generation cephalosporins (cefoxitin 9.1%; 9/99, cefuroxime 17.1%; 17/99 and cefpodoxime 20.2%; 20/99) was observed in *Aeromonas* spp., *Aeromonas* spp. 45, *A. allosaccharophila*, *A.*

bestiarum, *A. caviae*, *A. culicicola*, *A. hydrophila*, *A. jandaei* and *A. veronii* species. Although MBL production was not observed, ESBL production was detected by double disc assay for 8.08% (8/99) of isolates. It was, therefore important to assess the β-lactamase and ESBL gene content of these *Aeromonas* spp. study isolates and correlate gene presence with their resistance phenotypes.

2.2 Materials and Methods

2.2.1 Maintenance of bacterial isolates

Ninety-three *Aeromonas* spp. and six *Plesiomonas shigelloides* isolates (Table 2.1), cultured previously from moribund or healthy *Cyprinus carpio* (koi carp), *Clarias gariepinus* (catfish), *Oreochromis mossambicus* (tilapia), and sea water (Duma, 2012), were selected for study as well as two *Aeromonas* spp. type strains, viz. *A. caviae* ATCC 15468^T and *A. hydrophila* ATCC 7966^T. *Aeromonas* spp. isolates were previously characterized to species level using biochemical and physiological tests and MALDI-ToF biotyping (Duma, 2012). *Aeromonas* spp. isolates were maintained on tryptic soy agar (TSA) plates at 4 °C and for long-term storage in tryptic soy broth (TSB) containing 40 % glycerol at -70 °C (Jacobs and Chenia, 2007).

Table 2.1: Antimicrobials resistance profiles displayed by *Aeromonas* spp. isolates obtained from South African seawater and fish sources (Duma, 2012).

ISOLATES	FISH SPECIES	FISH HOST	RESISTANCE PHENOTYPES*
MI	*A. culicicola*	Catfish	NA,OX,T
M2	*A. hydrophila*	Catfish	AMP,AML,OX,W,RL
M3	*A. allosaccharophila*	Catfish	AMP,AML,OX
M4	*A. jandaei*	Catfish	AMP,AML,AMC,OX,W,RL
M5	*A. hydrophila*	Catfish	AMP,AML,OX,W,RL
M6	*A. hydrophila*	Catfish	AMP,AML,NA,OX,W
M7	*A. culicicola*	Catfish	AMP,AML,E,OX,T,W,RL
M8	*A. allosaccharophila*	Tilapia	AMP,AML,AMC,OX,T,W,RL
M9	*P. shigelloides*	Catfish	OX
M10	*A. culicicola*	Catfish	AMP,AML,CPD,OX,W,RL
M11	*Aeromonas spp.*	Catfish	AMP,AML,AMC,E,OX,W
M12	*A. bestiarum*	Catfish	AMP,AML,OX,W,RL
M13	*A. hydrophila*	Catfish	AMP,AML,OX,W,RL
M14	*A. hydrophila*	Tilapia	OX
M15	*Aeromonas* spp.	Tilapia	AMP,AML,AMC,FOX,OX
M16	*A. jandaei*	Tilapia	AMP,AMC,CPD,CXM,FOX,OX,W,RL
M17	*A. hydrophila*	Tilapia	AMP,AML,OX,W,RL

M18	*A. caviae*	Tilapia	AMP,AML
M19	*A. jandaei*	Tilapia	AMP,AML,AMC,FOX,OX,W,RL
M20	*Aeromonas* spp.	Tilapia	AMP,AML,FOX,CPD,CXM,OX,W,RL,SXT
M21	*A. allosaccharophila*	Tilapia	AMP,AML,AMC,FOX,CPD,CXM,OX,W,S
M22	*A. culicicola*	Sea water	AMP,AML,OX,W,RL
M23	*A. culicicola*	Sea water	AMP,AML,OX,RL
M24	*A. jandaei*	Sea water	AMP,AML,OX,RL
M25	*A. culicicola*	Sea water	AMP,AML,OX,RL
M26	*Aeromonas* spp. 45	Sea water	AMP,AML,OX,RL
M27	*Aeromonas* spp. 45	Sea water	AMP,E, OX, W, RL
M28	*A. jandaei*	Sea water	AMP,AML,OX,RL
M29	*A. culicicola*	Sea water	AMP,AML,OX,RL
M30	*A. jandaei*	Sea water	AMP,AML,OX,RL
M31	*A. culicicola*	Sea water	AMP,AML,OX,W,RL
M32	*A. culicicola*	Sea water	AMP,AML,OX,RL
M33	*Aeromonas* spp. 310	Sea water	AMP,AML,ATM,NA,OX,W,RL
M34	*Aeromonas* spp. 45	Sea water	AMP,AML,OX,RL
M35	*A. culicicola*	Sea water	AMP,AML,OX,RL
M36	*A. jandaei*	Sea water	AMP,AML,E,OX,RL
M37	*Aeromonas* spp. 45	Sea water	AMP,AML,OX,T,W,RL
M38	*A. culicicola*	Sea water	ATM,OX,RL
M39	*A. culicicola*	Sea water	AMP,AML,OX,RL
M40	*A. culicicola*	Sea water	AMP,AML,ATM,OX,RL
M41	*Aeromonas* spp.	Sea water	AMP,AML,OX,RL
M42	*A. culicicola*	Tilapia	AMP,AML,NA,OX,RL
M43	*Aeromonas* spp. 310	Tilapia	AMP,AML,NA,OX,RL
M44	*A. icthiosmia*	Tilapia	AMP,AML,OX,RL
M45	*P. shigelloides*	Tilapia	AMP,AML,AMC,OX,T,W,RL
M46	*P. shigelloides*	Tilapia	AMP,AML,E,NA,OX,W,S,RL,SXT
M47	*P. shigelloides*	Tilapia	AMP,AML,OX,W,RL
M48	*A. icthiosmia*	Tilapia	AMP,AML,OX,W,RL
M49	*A. sobria*	Tilapia	AMP,AML,AMC,OX,T
M50	*A. hydrophila*	Catfish	AMP,AML,CXM,E,NA,OX,W,RL
M51	*A. hydrophila*	Catfish	AMP,AML,AMC,OX,W,RL
M52	*A. hydrophila*	Tilapia	AMP,AML,AMC,CPD,CXM,E,OX,T,W,RL
M53	*A. hydrophila*	Catfish	AMP,AML,OX,T,W,RL
M54	*A. icthiosmia*	Tilapia	AMP,AML,AMC,E,OX,T,W,RL
M55	*A. veronii*	Tilapia	AMP,AML,OX,W,RL
M56	*A. culicicola*	Tilapia	AMP,AML,E,NA,OX,W,RL
M57	*A. veronii*	Tilapia	AMP,AML,AMC,CPD,CXM,C,E,OX,T,W,RL
M58	*A. culicicola*	Tilapia	AMP,AML,AMC,OX,W,RL
M59	*A. caviae*	Tilapia	AMP,AML,OX,T,W,RL
M60	*A. hydrophila*	Tilapia	AMP,AML,AMC,E,OX,T,W,RL
M61	*A. culicicola*	Tilapia	AMP,AML,OX

M62	*A. hydrophila*	Tilapia	AMP,AML,OX,RW,RL
M63	*A. veronii*	Tilapia	AMP,AML,AMC,OX,W,RL
M64	*A. hydrophila*	Tilapia	AMP,AML,CPD,FOX,OX,W,RL
M65	*A. hydrophila*	Tilapia	AMP,AML,OX,W,RL
M66	*P. shigelloides*	Tilapia	AMP,AML,OX,RL
M67	*P. shigelloides*	Tilapia	AMP,AML,OX,W,RL
M68	*A. caviae*	Koi carp	AMP,AML,AMC,CPD,OX,W,RL
M69	*A. bestiarum*	Koi carp	AMP,AML,AMC,CPD,OX,W,S,RL
M70	*A. bestiarum*	Koi carp	AMP,AML,AMC,CPD,OX,T,W,RL
M71	*A. bestiarum*	Koi carp	AMP,AML,CPD,CXM,E,OX,W
M72	*A. bestiarum*	Koi carp	AMP,AML,OX,W,RL
M73	*A. bestiarum*	Koi carp	AMP,AML,AMC,CXM,FOX,OX,W,RL
M74	*A. allosaccharophila*	Koi carp	AMP,AML,C,NA,OX,T,W,RL
M75	*Aeromonas* spp. 45	Koi carp	AMP,AML,CPD,CXM,C,E,OX,W
M76	*A. salmonicida*	Koi carp	AMP,AML,AMC,CPD,OX,W,RL
M77	*A. salmonicida*	Koi carp	AMP,AML,AMC,CPD,OX,W,RL
M78	*A. bestiarum*	Koi carp	AMP,AML,AMC,CPD,CXM,OX,W,RL
M79	*A. bestiarum*	Koi carp	APM,AML,OX
M80	*A. bestiarum*	Koi carp	AMP,AML,NA,OX,T,W
M81	*A. bestiarum*	Koi carp	AMP,AML,CXM,CTX,OX,W,RL
M82	*A. culicicola*	Koi carp	AMP,AML,AMC,ATM,CPD,CXM,E,FOX,OX,W,RL
M83	*A. bestiarum*	Koi carp	AMP,AML,OX,W,RL
M84	*A. bestiarum*	Koi carp	AMP,AML,OX,W,RL
M85	*A. allosaccharophila*	Koi carp	AMP,AML,AMC,OX,W,RL
M86	*A. hydrophila*	Koi carp	AMP,AML,AMC,OX,W,RL
M87	*A. bestiarum*	Koi carp	AMP,AML,AMC,OX,W,RL
M88	*A. bestiarum*	Koi carp	AMP,AML,AMC,CPD,CXM,E,FOX,OX,W,RL
M89	*A. allosaccharophila*	Koi carp	AMP,AML,AMC,CPD,CXM,OX
M90	*A. bestiarum*	Koi carp	AMP,AML,AMC,CXM,OX,W,RL
M91	*A. bestiarum*	Koi carp	AMP,AML,OX,W,RL
M92	*A. allosaccharophila*	Koi carp	AMP,AML,CPD,OX,W,RL
M93	*A. allosaccharophila*	Koi carp	AMP,AML,C,NA,OX,W,RL
M94	*A. hydrophila*	Koi carp	AMP,AML,AMC,E,OX,T,W,RL
M95	*A. hydrophila*	Koi carp	AMP,AML,AMC,CXM,E,OX,T,W,RL
M96	*A. bestiarum*	Koi carp	E,OX,T
M97	*A. bestiarum*	Koi carp	E,OX,T
M98	*Aeromonas* spp. 310	Koi carp	AMP,AML,OX,W,RL
M99	*A. bestiarum*	Koi carp	AMP,AML,CPD,CXM,OX

*AMP=ampicillin (AMP10), AML=amoxicillin (AML10), AMC=augmentin (AMC30), AZM=azithromycin (AZM15), ATM=aztreonam (ATM30), CPD=cefpodoxime (CPD10), CXM=cefuroxime (CXM30), E=erythromycin (E15), FOX=cefoxitin (FOX30), NA=nalidixic acid (NA30), OX=oxacillin (OX1), W=trimethoprim (W1.25), S=streptomycin (S10), RL=sulphamethoxazole (RL25), TE=tetracycline (TE30), TS=cotrimoxazole (TS25).

2.2.2 Genomic DNA isolation and analysis

Genomic DNA of *Aeromonas* spp. and *P. shigelloides* isolates was obtained according to the CTAB/NaCl protocol (Ausubel *et al.,* 1989). Bacterial cultures (1.5 ml) were pelleted, following overnight growth in LB broth, by centrifugation at 12 000 rpm for 2 min. Pellets were resuspended in 567 µl TE buffer and subjected to alkaline lysis by addition of 30 µl of 10% SDS and 3 µl of 20 mg/ml proteinase K, followed by 1 h incubation at 37 ºC. Alkaline lysis, removal of cell debris, left over protein and polysaccharide was achieved by the addition of 100 µl of cetylthrimethyl ammonium bromide CTAB and 80 µl of NaCl, followed by 10 min incubation at 65 ºC. Genomic DNA was extracted using an equal volume of chloroform/isoamyl alcohol, followed by centrifugation at 12 000 rpm for 5 min and upper aqueous phase transferred to fresh tube. Equal volumes of phenol/chloroform/isoamyl alcohol was added, followed by centrifugation for 5 min at 12 000 rpm. There after high quality DNA was precipitated out of the upper aqueous solution by the use of 0.6% of volume isopropanol, followed by centrifugation for 2 min at 12 000 rpm. Fifty µl of 70% ethanol was added to purify the DNA and pellets were resuspended in 20 µl of TE buffer, and stored at -20 ºC. Aliquots (2 µl) were subjected to electrophoresis in 1% TAE agarose gels, stained with ethidium bromide and viewed by UV transillumination, to determine the quality of the DNA samples (Jacobs and Chenia, 2007).

2.2.3 Detection of TEM type beta-lactamase resistance genes

Based on data from Duma (2012), study isolates displayed high levels of resistance to β-lactams (92.9%; 92/99), while (100%; 99/99) were susceptible to carbapenems (imipenem). Cephalosporin resistance was detected in 27.3% (27/99) of study isolates (Table 2.2), with resistance to cefoxitin (33.3%; 9/27), cefpodoxime (74.1%; 20/27) and cefuroxime (62.9%; 17/27). Thus two primers (bla_{TEM} (1) and bla_{TEM} (2)) were designed to characterise the β-lactamase gene content of study isolates. A 503 bp bla_{TEM} (1) gene fragment was amplified by PCR using β-lactamase gene primers (Table 2.2) described by Guerra *et al*. (2001). Reaction mixtures (20 µl) consisted of 100 ng template DNA, 200 µM of each dNTP (Fermentas), 50 pmol of each primer, 1.5 mM $MgCl_2$ (25mM) and 1 U Super Therm *Taq* DNA polymerase (Southern Cross Biotechnology), together with 1 × Super Therm reaction buffer. PCR cycling parameters consisted of 35 cycles of 94 ºC for 30 s; 55 ºC for 30 s; and 72 ºC for 2 min in a MJ mini thermal cycler (Biorad). An initial denaturation step of 95 ºC for 5 min and a final elongation step of 72 ºC for 10 min were included in reactions. The positive control used for this

assay was *E. coli* ATCC 35218 while *E. coli* ATCC 25922 was used as the negative control. The type strains *A. caviae* ATCC 15468^T and *A. hydrophila* ATCC 7966^T, *P. aeruginosa* ATCC 35032 and *P. aeruginosa* ATCC 27853 were also included in the assays.

Table 2.2: Primers used in beta-lactamase and extended spectrum beta-lactamase detection.

Target Gene	Sequence	Size (bp)	References
bla_{TEM} (1)	5'-TTGGGTGCACGAGTGGGT-3'	503	Guerra *et al*. (2001)
bla_{TEM}	5'-TAATTGTTGCCGGGAAGC-3'		
bla_{TEM} (2)	5'-GAGTATTCAACATTTTCGT-3'	857	Maynard *et al*. (2004)
bla_{TEM}	5'-ACCAATGCTTAATCAGTGA-3'		
bla_{SHV}	5'-GCCCGGGTTATTCTTATTTGTCGC-3'	1008	Wu *et al*. (2007)
bla_{SHV}	5'-TCTTTCCGATGCCGCCGCCAGTCA-3'		
$bla_{CTX\text{-}M\text{-}15}$	5'-TTTCCCCATTCCGTTTCCGC-3'	925	Jacoby *et al*. (2006)
$bla_{CTX\text{-}M\text{-}15}$	5'-TTCGTATCTTCCAGAATAAG-3'		
$bla_{CTX\text{-}II}$	5'-CGATGTGCAGTACCAGTAA-3'	585	Hopkins *et al*. (2007)
$bla_{CTX\text{-}II}$	5'-TTAGTGACCAGAATCAGCGG-3'		

An 857 bp bla_{TEM} (2) gene fragment was also amplified by PCR using a β-lactamase gene primer set (Table 2.2) that could detect all variants of the bla_{TEM}-type gene (Maynard *et al*., 2004). Reaction mixtures (20 µl) consisted of 100 ng template, DNA 200 µM of each dNTP (Fermentas), 50 pmol of each primer, 1.5 mM $MgCl_2$ and 1 U Super Therm *Taq* DNA polymerase (Southern Cross Biotechnology), together with 1 × Super Therm reaction buffer. PCR cycling parameters consisted of 35 cycles of 94 ºC for 30 s; 53 ºC for 30 s; and 72 ºC for 2 min in a MJ mini thermal cycler (Biorad). An initial denaturation step of 95 ºC for 5 min and a final elongation step of 72 ºC for 10 min were included in reactions. The positive control used for this amplification was *E. coli* ATCC 35218 while *E. coli* ATCC 25922 was used as the negative control.

Ten µl of both bla_{TEM} (1) and bla_{TEM} (2) PCR reaction products were subjected to electrophoresis in 1.5% agarose gels, stained in ethidium bromide, and viewed by UV transillumination to identify the 503 and 857 bp β-lactamase gene fragments, respectively, with O'GeneRuler^{TM} 100 bp DNA Ladder Plus or O'GeneRuler^{TM} 100 bp DNA Ladder as markers (Fermentas, Canada) (Jacobs and Chenia, 2007).

2.2.4 Detection of extended spectrum beta-lactamases resistance genes

PCR reaction mixtures as described in Section 2.2.3 were used to amplify ESBL resistance genes. The presence of SHV (*bla*$_{\text{SHV-}1}$) and CTX (*bla*$_{CTX\text{-}M\text{-}15}$ and *bla*$_{CTX\text{-}II}$) type ESBLs were investigated using primers described previously by Wu *et al*. (2007), Jacoby *et al*. (2006), and Hopkins *et al*. (2007) respectively (Table 2.2). Duma (2012) had previously observed eight isolates to be ESBL producers, i.e., M10, M13, M27, M37, M81, M87, M94, M95, so these isolates were screened by PCR for the presence of *bla*$_{SHV\text{-}I}$. The *bla*$_{\text{SHV-}1}$ primer set targets variants of the *bla*$_{SHV}$ gene that express FOX, CPD and CXM resistance. All variants of *bla*$_{CTX\text{-}M}$ genes were targeted by the *bla*$_{CTX\text{-}II}$ primer set, while the *bla*$_{CTX\text{-}M\text{-}15}$ primer set targets the *bla*$_{CTX\text{-}M\text{-}15}$ resistance gene. Both are plasmid-borne and mobile. *K. pneumoniae* ATCC 700603 was used as the positive control for SHV (*bla*$_{\text{SHV-}1}$) assay while *E. coli* ATCC 25922 was used as negative control. A previously identified *Salmonella typhimurium* CTX-M positive strain was used as positive control for the CTX (*bla*$_{CTX\text{-}M\text{-}15}$ and *bla*$_{CTX\text{-}II}$) assay and *E. coli* ATCC 25922 was used as negative control.

Five microliters of each PCR reaction mixture was subjected to electrophoresis in 1% agarose gels, stained in ethidium bromide, and viewed by UV transillumination with O'GeneRuler^{TM} 100 bp DNA Ladder and/or O'GeneRuler^{TM} 1kb DNA Ladder Plus as markers (Fermentas, Canada) (Jacobs and Chenia, 2007).

2.3 Results

2.3.1 Identification of TEM type beta-lactamase resistance genes

It was observed that 46.4% (46/99) of isolates possessed *bla*$_{TEM}$ genes. Using the *bla*$_{TEM}$ (1) primer set, 17% (17/99) were positive with amplification of a 503 bp fragment ((Fig. 2.1; Table 2.3). The *bla*$_{TEM}$ gene positive control *E. coli* ATCC 35218, *P. aeruginosa* ATCC 35032 and the two type strains; *A. caviae* ATCC 15468^{T} and *A. hydrophila* ATCC 7966^{T} were also positive for TEM presence with the *bla*$_{TEM}$ (1) primer set.

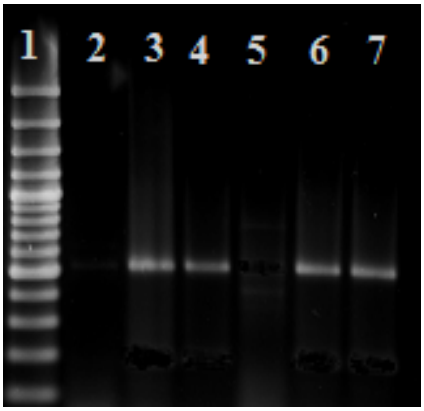

Figure 2.1: Agarose gel (1.5%) electrophoresis picture of a typical example of 503 bp *bla*$_{TEM}$ gene amplicons obtained using primer set (1). Lane 1 was O'GeneRuler^{TM} 100 bp DNA Ladder Plus (Fermentas, Canada); lane 2 was negative control *E. coli* ATCC 25922; lane 3 was positive control *E. coli* ATCC 35218; lane 4 was M63; lane 5 was M64; lane 6 was M65 and lane 7 was M66.

Since resistance profiles of isolates suggested a greater prevalence of *bla*$_{TEM}$ gene, a second primer set described by Maynard *et al*. (2004) was also used. It was observed that 29% (29/99) of study isolates were positive with the *bla*$_{TEM}$ (2) primer set. The amplification of an 857 bp fragment (Fig. 2.2; Table 2.3) was observed for all 29 positives. Figure 2.2 is a typical picture of 503 bp gene fragments obtained from *bla*$_{TEM}$ (1) primer set.

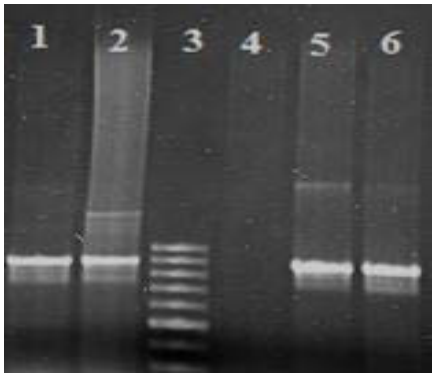

Figure 2.2: Agarose gel (1.5%) electrophoresis picture of a typical example of 857 bp *bla*$_{TEM}$ type gene amplicons obtained using primer set (2). Lane 1 was positive control *E. coli* ATCC 35218; lane 2 was M1; lane 3 was O'GeneRuler^{TM} 100 bp DNA Ladder (Fermentas, Canada); lane 4 was negative control *E. coli* ATCC 25922, lane 5 was M6 and lane 6 was M9.

Although an 857 bp *bla*$_{TEM}$ fragment was amplified from positive control *E. coli* ATCC 35218 using the *bla*$_{TEM}$ (2) primer set, it was not possible to amplify the gene fragment from *A.*

caviae ATCC 15468^T and *A. hydrophila* ATCC 7966^T. Of the isolates assayed, 12% (12/99) were positive with both bla_{TEM} (1) and bla_{TEM} (2) primer sets.

Table 2.3: Results of amplifications of bla_{TEM} gene using primer sets bla_{TEM} (1) and bla_{TEM} (2) from *Aeromonas* spp. isolated from fish and seawater.

ISOLATES	SPECIES DESIGNATION	bla_{TEM} (1)*	bla_{TEM} (2)*
MI	*A. culicicola*		+
M2	*A. hydrophila*		
M3	*A. allosaccharophila*		
M4	*A. jandaei*		
M5	*A. hydrophila*		
M6	*A. hydrophila*	+	+
M7	*A. culicicola*		
M8	*A. allosaccharophila*		
M9	*P. shigelloides*	+	+
M10	*A. culicicola*		+
M11	*Aeromonas* spp.		+
M12	*A. bestiarum*		
M13	*A. hydrophila*	+	
M14	*A. hydrophila*	+	+
M15	*Aeromonas* spp.		
M16	*A. jandaei*	+	+
M17	*A. hydrophila*		+
M18	*A. caviae*		
M19	*A. jandaei*	+	+
M20	*Aeromonas* spp.	+	+
M21	*A. allosaccharophila*		+
M22	*A. culicicola*		
M23	*A. culicicola*		+
M24	*A. jandaei*		
M25	*A. culicicola*		
M26	*Aeromonas* spp. 45		
M27	*Aeromonas* spp. 45		
M28	*A. jandaei*	+	+
M29	*A. culicicola*	+	+
M30	*A. jandaei*		
M31	*A. culicicola*		
M32	*A. culicicola*		+
M33	*Aeromonas* spp. 310		
M34	*Aeromonas* spp. 45		
M35	*A. culicicola*		
M36	*A. jandaei*		
M37	*Aeromonas* spp. 45		

M38	*A. culicicola*		
M39	*A. culicicola*		
M40	*A. culicicola*		
M41	*Aeromonas* spp.		+
M42	*A. culicicola*		+
M43	*Aeromonas* spp. 310		+
M44	*A. icthiosmia*		
M45	*P. shigelloides*	+	+
M46	*P. shigelloides*	+	
M47	*P. shigelloides*		
M48	*A. icthiosmia*		
M49	*A. sobria*		+
M50	*A. hydrophila*	+	
M51	*A. hydrophila*		
M52	*A. hydrophila*		
M53	*A. hydrophila*		
M54	*A. icthiosmia*		+
M55	*A. veronii*		
M56	*A. culicicola*		+
M57	*A. veronii*	+	+
M58	*A. culicicola*		+
M59	*A. caviae*		
M60	*A. hydrophila*		
M61	*A. culicicola*		
M62	*A. hydrophila*		+
M63	*A. veronii*	+	+
M64	*A. hydrophila*		
M65	*A. hydrophila*	+	
M66	*P. shigelloides*	+	+
M67	*P. shigelloides*		
M68	*A. caviae*		
M69	*A. bestiarum*		
M70	*A. bestiarum*		
M71	*A. bestiarum*		
M72	*A. bestiarum*		
M73	*A. bestiarum*		
M74	*A. allosaccharophila*		
M75	*Aeromonas* spp. 45		
M76	*A. salmonicida*		
M77	*A. salmonicida*		
M78	*A. bestiarum*		
M79	*A. bestiarum*		
M80	*A. bestiarum*		+
M81	*A. bestiarum*		

M82	*A. culicicola*		
M83	*A. bestiarum*		
M84	*A. bestiarum*		
M85	*A. allosaccharophila*		
M86	*A. hydrophila*		
M87	*A. bestiarum*		
M88	*A. bestiarum*		
M89	*A. allosaccharophila*		
M90	*A. bestiarum*		
M91	*A. bestiarum*		
M92	*A. allosaccharophila*		
M93	*A. allosaccharophila*		
M94	*A. hydrophila*		+
M95	*A. hydrophila*		
M96	*A. bestiarum*		
M97	*A. bestiarum*	+	
M98	*Aeromonas* spp. 310		
M99	*A. bestiarum*		
	A. hydrophila ATCC 7966	+	
	A. caviae ATCC 15468	+	
	E. coli ATCC 29522		
	E. coli ATCC 35218	+	+
	K. pneumoniae ATCC 700603		
	P. aeruginosa ATCC 27853		

2.3.2 Analysis of TEM type gene content based on species designation and source of isolation

The fourteen different species groups were analyzed with respect to presence of TEM gene within each species group (Table 2.4). Among the 17.1% (17/99) of isolates positive for the bla_{TEM} gene using the bla_{TEM} (1) primer set, the highest prevalence was observed for *A. hydrophila* (29.4%; 5/17) and *P. shigelloides* (23.5%; 4/17). Of bla_{TEM} (2) positives (29.2%; 29/99), the bla_{TEM} gene was most prevalent in *A. culicicola* (27.6%; 8/29) and *A. hydrophila* (17.2%; 5/29). The bla_{TEM} gene was amplified with both bla_{TEM} (1 and 2) primer sets (12.1%; 12/99) predominantly from *P. shigelloides* (25%; 3/12) and *A. jandaei* (25%; 3/12) (Table. 2.4).

Table 2.4: Species designation analysis of *Aeromonas* spp. bla_{TEM} gene positives using primer sets bla_{TEM} (1) and bla_{TEM} (2).

SPECIES	Total	bla_{TEM} (1)*	bla_{TEM} (2)*	bla_{TEM} (1) and bla_{TEM} (2)
Aeromonas spp.	4	25% (1/4)	75% (3/4)	25% (1/4)
Aeromonas spp. 45	5	0	0	0
Aeromonas spp. 310	3	0	33.3% (1/3)	0
A. allosaccharophila	8	0	12.5% (1/8)	0
A. bestiarum	19	5.3% (1/19)	5.3% (1/19)	0
A. caviae	3	0	0	0
A. culicicola	18	5.6 % (1/18)	44.4% (8/18)	5.6% (1/18)
A. hydrophila	17	29.4% (5/17)	29.4% (5/17)	11.8% (2/17)
A. icthiosmia	3	0	1	0
A. jandaei	7	42.9% (3/7)	42.9% (3/7)	42.9% (3/7)
A. salmonicida	2	0	0	0
A. sobria	1	0	100% (1/1)	0
A. veronii	3	66.7% (2/3)	66.6% (2/3)	66.7% (2/3)
P. shigelloides	6	66.7% (4/6)	50% (3/6)	50% (3/6)

* bla_{TEM} (1) – Guerra *et al*. (2001) primer set; bla_{TEM} (2) – Maynard *et al*. (2004) primer set.

Isolates from catfish had the highest prevalence (33.3%; 4/33) of bla_{TEM} type gene being amplified using the bla_{TEM} (1) primer set (Table. 2.5). Isolates from tilapia had the highest number of positives (51.5%; 17/33) with the bla_{TEM} (2) primer set. The amplification of bla_{TEM} gene using both bla_{TEM} (1 and 2) primer sets was most prevalent for tilapia isolates (24.2%; 8/33) (Table. 2.5).

Table 2.5: Percentage analysis of *Aeromonas* spp. bla_{TEM} gene positives using bla_{TEM} (1) and bla_{TEM} (2) primer sets, based on source of isolation.

HOST	bla_{TEM} (1)*	bla_{TEM} (2)*	bla_{TEM} (1) and bla_{TEM} (2)
Catfish (*Clarias gariepinus*) (n= 12)	33.3% (4/12)	41.7% (5/12)	16.7% (2/12)
Goldfish (*Carassius auratus*) (n= 33)	3.03% (1/33)	6.06% (2/33)	-
Sea water (n= 20)	10% (2/20)	25% (5/20)	10% (2/20)
Tilapia (*Oreochromis mossambicus*) (n= 33)	30.3% (10/33)	51.5% (17/33)	24.2% (8/33)

* bla_{TEM} (1) – Guerra *et al*. (2001) primer set,; bla_{TEM} (2) – Maynard *et al*. (2004) primer set.

2.3.3 Identification of extended spectrum beta-lactamases resistance genes

It was observed that the positive control *K. pneumoniae* ATCC 700603 was positive for bla_{SHV-1} gene presence (Fig. 2.3) as the expected 1008 bp fragment was obtained. However the 1008 bp gene fragment was not amplified from aeromonad isolates tested.

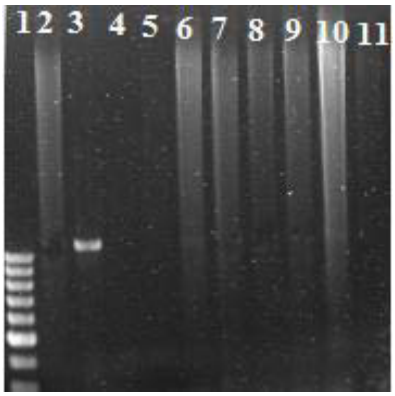

Figure 2.3: Agarose gel (1.5%) electrophoresis of 1008 bp bla_{SHV} type gene amplicon using primer set bla_{SHV-1}. Lane 1 was O'GeneRuler^{TM} 100 bp DNA Ladder (Fermentas, Canada); lane 2 was the negative control *E. coli* ATCC 25922; lane 3 was the positive control *K. pneumoniae* ATCC 700603 and lanes 4 - 11 were eight ESBL-producers (M10, M13, M27, M37, M81, M87, M94, M95).

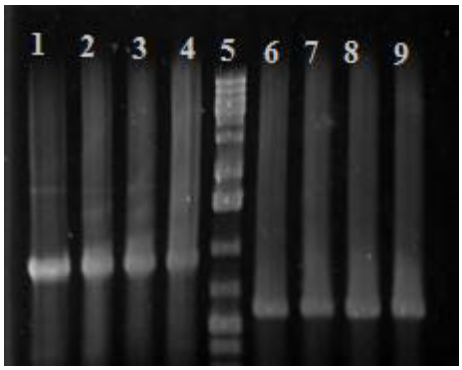

Figure 2.4: Agarose gel (1.5%) electrophoresis of $bla_{CTX-M-I5}$ (925 bp) and bla_{CTX-M} (585 bp) gene amplicons using primer sets $bla_{CTX-M-I5}$ and bla_{CTX-II}. Lane 1 was positive control *Salmonella typhimurium*; lane 2 was M81; lane 3 was M82; lane 4 was M88; lane 5 was O'GeneRuler^{TM} 1kb DNA Ladder Plus (Fermentas, Canada); lane 6 was positive control *Salmonella typhimurium*; lane 7 was M81; lane 8 was M82 and lane 9 was M88.

Second and 3rd generation cephalosporin-resistant isolates were screened for CTX genes. Expected amplicons for 925 bp ($bla_{CTX-M-15}$) and 585 bp (bla_{CTX-II}), were amplified from only three (M81, M82 and M88) cephalosporinase producers (Fig. 2.4). There were two species

group represented among the *bla*$_{CTX\text{-}M\text{-}15}$ and *bla*$_{CTX\text{-}M}$ positives viz.; *A. bestiarum* 66.7% (2/3) and *A. culicicola* 33.3% (1/3), from goldfish.

2.4 Discussion

Environmental *Aeromonas* species living in water bodies have been reported to be reservoirs of antimicrobial resistance genes (Girlich *et al*., 2011). In addition to their intrinsic antimicrobial resistance to Ambler classes B, C and D β-lactamases (Chen *et al*., 2012), they have been associated with mobile genetic elements such as integrons and plasmids (Igbinosa *et al*., 2012). This increases the incidence of extended spectrum β-lactam resistance in aeromonads (Chen *et al*., 2012). In the present study, the *bla*$_{TEM}$ primer set (1) amplified TEM-type genes from fewer aeromonads (17.1% ; 17/99) than the *bla*$_{TEM}$ primer set (2) (29.2%; 29/99). This is because while *bla*$_{TEM}$ primer set (1) was designed to amplify the classical TEM-1-like β-lactamases (Guerra *et al*., 2001), *bla*$_{TEM}$ primer set (2) was designed to detect all variants of the TEM-type β-lactamase genes (ESBLs) (Maynard *et al*., 2004). Therefore using both primer sets, a greater prevalence of *bla*$_{TEM}$ type resistance genes determinants (classical TEM-1-like and TEM-type extended β-lactamases) was identified. Data from study carried out by Duma (2012) demonstrated that isolates were resistant to ampicillin (AMP) (92.9%; 92/99), amoxicillin (AML) (91.9%; 91/99), cefpodoxime (CPD) (20.2%; 20/99), cefuroxime (CXM) (20.2%; 20/99), cefoxitin (FOX) (9.09%; 9/99) and aztreonam (ATM) (4.04%; 4/99). This is expected as TEM-1-like β-lactamase genes hydrolyze narrow spectrum penicillin while TEM-type ESBLs are known to hydrolyze narrow and extended spectrum-penicillins, monobactams and second and third generation cephalosporins (Paterson and Bonomo, 2005). Among clinical Gram-negative bacteria, *bla*$_{TEM\text{-}I}$ is the most frequently detected β-lactamases (Balsalobre *et al*., 2010; Girlich *et al*., 2011), environmental *Aeromonas* spp. have also been observed to possess *bla*$_{TEM\text{-}I}$-like genes in their chromosomes (Balsalobre *et al*., 2010). In the present study, fish and environmental isolates were observed to carry the *bla*$_{TEM}$ resistance gene determinant, with highest prevalence in tilapia isolates. The rest of the *bla*$_{TEM}$ negative isolates (53.5%; 53/99) may have other β-lactamases not tested for (Wu *et al*., 2011), perhaps the chromosomal class C β-lactamases (Chen *et al*., 2012). This may also explain the resistance of isolates to augmentin (AMC) (32.3%; 32/99).

In the present study *A. hydrophila* was found to have the highest incidence of the *bla*$_{TEM\text{-}I}$-like gene amplified with primer set (1). This is consistent with findings of Balsalobre *et al*. (2010), who observed that environmental isolates of *A. hydrophila* and *A. jandaei* possessed

bla$_{TEM-1}$-like genes. *Aeromonas culicicola* was observed to be the species harbouring the most *bla*$_{TEM}$-type gene amplified with primer set (2), although not clinically important *A. culicicola* has been isolated from mosquitoes (Piyidar *et al*., 2002). The *bla*$_{TEM}$-type (primer set 2) gene was also prevalent among *Aeromonas hydrophila*, *P. shigelloides*, *A. veronii*, *Aeromonas* spp. and *A. jandaei* isolates. Since these are clinically important species, it poses a health threat as they are known to be associated with both animal and human infections (Janda and Abbott, 2010; Dias *et al*., 2012). These mobile *bla*$_{TEM}$ resistance genes were more prevalent in isolates from tilapia than the other three sources, i.e., koi carp, catfish and seawater. Since tilapia host were not exposed to any antimicrobial agents, it is possible that fish feeds, fish farm influents from environmental water, naked DNA and resistance determinants from lysed commensal and pathogenic bacteria and biofilms forming on sediments and aquaculture structures may be sources of these resistance genes (Cabello *et al*., 2013; Marti *et al*., 2013).

The *bla*$_{SHV}$ genes were not detected on amplification, this is expected as the resistance profile of study isolates (Table 2.1) showed that they were susceptible to imipenem (100%; 99/99). However, *bla*$_{CTX-M-15}$ and *bla*$_{CTX-M}$ genes were amplified from 3 isolates, with only one isolate (*A. bestiarum*) demonstrating resistance to cefotaxime (CTX) (Fig 2.4). Resistance to cefuroxime (CXM) (6.1%; 6/99), cefoxitin (FOX) (8.1%; 8/99) and cefpodoxime (CPD) (5.1%; 5/99) may be due partly to, the presence of *bla*$_{TEM}$ gene variants that were amplified (Maynard *et al*., 2004) or presence of intrinsic class C β-lactamases (Chen *et al*., 2012).

Duma (2012) study demonstrated that isolates with β-lactam resistance also demonstrated resistance to aminoglycosides (streptomycin; 3.03%; 3/99), macrolides (erythromycin; 18.1%; 18/99), trimethoprim (68.6%; 68/99) and sulphamethoxazole (81.8%; 81/99) (Table. 2.1). This is consistent with findings of Guerra *et al*. (2001) and Maynard *et al*. (2004) that TEM positives carry class 1 integrons. Study isolates demonstrating β-lactam resistance could be correlated with presence of *bla*$_{TEM}$ and *bla*$_{CTX-M}$ genes. Data from this study suggests that *Aeromonas* spp. from fish and aquatic environment may be reservoirs and possible agents of dissemination of β-lactamase genes (Maravic *et al*., 2013; Wu *et al*., 2011, 2013).

CHAPTER THREE
IDENTIFICATION OF INTEGRONS ASSOCIATED WITH FISH AND AQUATIC *AEROMONAS* spp.

3.1 Introduction

The increasing dissemination of resistance genes among fish pathogens has become a major concern in many countries worldwide (Ndi and Barton, 2011). This problem is impacting negatively on aquaculture production worldwide, causing million dollar losses and retarding economic growth of the industry (Janda and Abbott, 2010). Antimicrobial compounds are widely used worldwide in aquaculture, agriculture farms and animal farms, and there is a tendency to abuse their use. These antimicrobials are used for prophylactic and therapeutic purposes in aquaculture usually through incorporation into fish feed (Cabello *et al*., 2013). These antimicrobial compounds are usually released into the aquaculture environment intact through uningested feed or in their unabsorbed forms in the excreta of fish and/or in absorbed forms as secretions from fish. This has accelerated the development of antimicrobial resistance genes (ARGs) and resistant bacteria (Zhang *et al*., 2009a; Cabello *et al*., 2013). The risk of contamination of human and animal food and water with resistant bacteria is an emerging problem worldwide (Cabello *et al*., 2013). The increase in the dissemination of antimicrobial resistance via resistant bacteria and antimicrobial resistance gene determinants is disturbing as it can be transferred to human commensal and pathogenic bacteria (Jacobs and Chenia, 2007). This in turn endangers humans and renders current chemotherapy for bacterial infections in aquaculture ineffective (Jacobs and Chenia, 2007).

The resistance determinants that are commonly implicated are either plasmid-borne or chromosomally-located (Janda and Abbott, 2010). Associated with these plasmids are integrons that are immobile and are only disseminated by conjugative plasmids and transposons (Pellegrini *et al*., 2010). These integrons are elements that encode a site-specific recombination system that recognizes and captures mobile gene cassettes (Pellegrini *et al*., 2010).

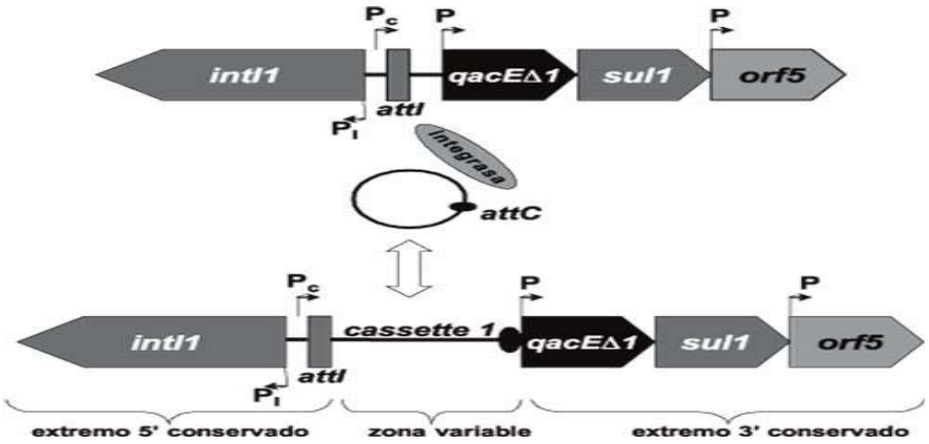

Figure 3.1: Classic integron structure diagram showing gene cassette (cassette 1), capture and integration by integrase gene *intI* at *attI* site (González *et al*., 2004).

Integrons are genetic modules associated with both pathogenic and commensal bacteria that confer the ability to capture and express promoterless DNA units called gene cassettes which confer a variety of adaptive functions including antimicrobial resistance (Binh *et al*., 2009). There are three clinically important integrons that are known:- classes 1, 2 and 3 (Stalder *et al*., 2012), with class 1 integrons being the most prevalent class (Chang *et al*., 2007; Verner-Jeffreys *et al*., 2009; Nawaz *et al*., 2010). Class 1 integrons encode an integrase (*intI*) and a recombination site (*attI*) where multiple gene cassettes may eventually be captured and expressed (Fig. 3.1) (Binh *et al*., 2009). In addition to the integrase (*intI*) and recombination (*attI*) sites, integrons possess a third integral part, the out-ward-oriented promoter (Pc) that directs transcription of the captured genes. Integration occurs downstream of the resident Pc promoter at the *attI* site, allowing the expression of genes in the cassette (Binh *et al*., 2009). The acquisition of gene cassettes occurs through a site-specific recombination mechanism catalyzed by an integron-encoded integrase (*intI*). The integrative recombination reaction occurs primarily between an integron receptor site (*attI*) and a cassette-associated sequence known as the *attC* site or 59-base element. Gene cassettes are mobile genetic elements comprised of a single gene and a recombination site (*attC*) (Binh *et al*., 2009).

Gene cassettes are the smallest mobilizable DNA, which comprise a single gene and a recombination site (*attC*), with more than 100 different gene cassettes having been reported (Binh *et al*., 2009; Nawaz *et al*., 2010). These mobile genetic elements (MGEs) are discrete genetic elements that exist as free circular, non-replicating DNA molecules when moving from one genetic site to another (Pellegrini *et al*., 2010). They exhibit different sizes that range from

500 to 1000 bp as well as different functions but have a common structure. The 3' end region of every gene is adjacent to a palindromic sequence *attC*, which constitutes a specific recombination site recognized by the integrase (Pellegrini *et al*., 2010). Gene cassettes consists of a gene immediately followed by an *attC* site, each cassette has only one *attC* site, with various elements being associated with the different resistance cassettes. Movements of the cassettes in and out of integrons are random, usually a result of circumstance and natural selection. The presence of selective pressures results in dissemination of integron-containing antimicrobial resistance cassettes (Boucher *et al*., 2007; Partridge *et al*., 2009).

Antimicrobial resistance in *Aeromonas* spp. associated with cassette-borne resistance genes and integrons are being increasingly described among food-borne, aquatic and clinical species. (Jacobs and Chenia, 2007; Lee *et al*., 2008; Verner-Jeffreys *et al*., 2009; Kadlec *et al*., 2011; Sarria-Guzmàn *et al*., 2013). Clinical isolates of *A. hydrophila*, *A. veronii*, and *A. sobria* were found to contain the *intI* gene coding for the class 1 integron (Lee *et al*., 2008). Ornamental fish and its carriage water analyzed for antimicrobial resistance gene presence were found to contain aeromonads that carried class 1 integrons (Verner-Jeffreys *et al*., 2009). Ndi and Barton (2011) observed the prevalence of class 1 integron in *A. hydrophila*, *A. veronii*, *A. veronii* bv *sobria*, *A. caviae* and *A. bestiarum* isolated from Australian farmed rainbow trout. Integron content and multidrug resistant phenotype of *Aeromonas* species have been correlated (Jacobs and Chenia, 2007; Nawaz *et al*., 2010). In Croatia, Maravic *et al*. (2013) detected the presence of class 1 and class 2 integrons in Mediterranean mussel (*Mytilus galloprovincialis*) from the Adriatic Sea.

In aeromonads, gene cassettes that are commonly described are the *dfr12* and *dfr2d* coding for trimethoprim resistance, *aadA1* and *aadA2* responsible for aminoglycoside resistance, *qacEΔ1* conferring resistance to antiseptics and detergents, *sulI* and *sulII* responsible for sulphonamide resistance, *oxa2* encoding beta-lactam resistance and *catB3* and *catB8* conferring resistance to chloramphenicol (Chang *et al*., 2007; Jacobs and Chenia, 2007; Lee *et al*., 2008; Pérez-Valdespino *et al*., 2009; Ndi and Barton, 2011). Integron-positive *Aeromonas* spp. isolates had a higher frequency of resistance compared to integron-negative isolates (Chang *et al*., 2007). Since the beta-lactam, trimethoprim and sulphonamide resistance phenotype was detected in *Aeromonas* spp. isolates from catfish, goldfish, seawater and tilapia (Duma, 2012), it was

important to determine if this resistance phenotype could be correlated with integron presence in these isolates.

3.2. Materials and Methods

3.2.1 Detection of integron and integron-associated components

The presence of integrons and associated integron structures were identified by PCR analysis using *int*, *qacEΔ1* and *sulI* primer sets (Table 3.1). Reaction mixtures (25 µl) containing 100 ng template DNA, 200 µM of each dNTP (Fermentas), 50 pmol of each primer, 1.5 mM $MgCl_2$ and 1 U SuperTherm *Taq* DNA polymerase (Southern Cross Biotechnology), together with 1× SuperTherm reaction buffer, was prepared. PCR cycling parameters consists of 35 cycles of 94 °C for 30 s; 55 °C for 1 min; and 72 °C for 2 min in a MJ Mini Thermal Cycler (BioRad). An initial denaturation step of 95 °C for 5 min and a final elongation step of 72 °C for 10 min were included in reactions.

Table 3.1: Primers used in detection of integrons and integron-associated genes

Target gene	Sequence	Size (bp)	References
intI–integrase 1	ATCATCGTCGTAGAGACGTCGG GTCAAGGTTCTGGACCAGTTGC	892	Jacobs and Chenia (2007)
intII–integrase 2	GCAAATGAAGTGCAACGC ACACGCTTGCTAACGATG	467	Jacobs and Chenia (2007)
sulI – sulI	CTTCGATGAGAGCCGGCGGC GCAAGGCGGAAACCCGCGCC	417	Jacobs and Chenia (2007)
sulII	CGGCATCGTCAACATAACC GTGTGCGGATGAAGTCAG	722	Maynard *et al*.(2004)
qacEΔ1–Qac	ATCGCAATAGTTGGCGAAGT CAAGCTTTTGCCCATGAAGC	230	Jacobs and Chenia (2007)
Class 1 integrons Conserved areas	GGCATCCAAGCAGCAAG AAGCAGACTTGACCTGA	Variable	Jacobs and Chenia (2007)
Class 2 integrons Conserved areas	CGGGATCCCCGGCATGCACGATTTGTA GATGCCATCGCAAGTACGAG	Variable	Jacobs and Chenia (2007)

The presence of *sulII* gene was identified by PCR analysis using the *sulII* primer as described by Maynard *et al*. (2004). Reaction mixtures (25 µl) containing 100 ng template DNA, 200 µM of each dNTP (Fermentas), 50 pmol of each primer, 1.5 mM $MgCl_2$ and 1 U SuperTherm *Taq* DNA polymerase (Southern Cross Biotechnology), together with 1× SuperTherm reaction buffer was prepared. PCR cycling parameters consisted of 35 cycles: 5 min

at 94°C, followed by 35 cycles of 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 1.5 min. Including a final elongation step of 72 °C for 10 min. Type strains *A. caviae* ATCC 15468^T and *A. hydrophila* ATCC 7966^T as well as *E. coli* ATCC 35218, *P. aeruginosa* ATCC 35032, *P. aeruginosa* ATCC 27853 and *K. pneumoniae* ATCC 700603 were included in assays.

3.2.2 Detection of conserved regions for class 1 and 2 integron positives

The variable regions of class 1 and class 2 *intI* and *intII* positives, respectively, were identified by PCR analysis using primers targeting the variable regions (CS and HEP) as described by Jacobs and Chenia (2007). Reaction mixtures (25 µl) containing 100 ng template DNA, 200 µM of each dNTP (Fermentas), 50 pmol of each primer, 1.5 mM $MgCl_2$ and 1 U SuperTherm *Taq* DNA polymerase (Southern Cross Biotechnology), together with 1× SuperTherm reaction buffer was prepared. PCR cycling parameters consisted of 35 cycles consisting of 5 min at 94°C, 30 cycles of 94 °C for 30 s, 53 °C for 30 s, and 72 °C for 1.5 min. A final elongation step of 72 °C for 10 min

Seven microliters of PCR reaction mixtures was subjected to electrophoresis in 1% agarose gels, stained in ethidium bromide, and viewed by UV transillumination to identify integrons and their associated genes with O'GeneRuler^{TM} 100 bp DNA Ladder and O'GeneRuler^{TM} 100 bp DNA Ladder Plus as DNA molecular weight markers (Fermentas) (Jacobs and Chenia, 2007).

3.3 Results

3.3.1 Identification of integron-associated components

It was observed that 10.1% (10/99) of study isolates were positive for *intI* gene with amplification of 892 bp amplicon (Fig. 3.2; Table 3.2). Figures 3.2 - 3.4 are typical examples of PCR products of study isolates and controls obtained with *intI* and *intII* primers. Type strain *A. caviae* ATCC 15468^T, and controls *E. coli* ATCC 35218 (Fig. 3.3), *P. aeruginosa* ATCC 27853 and *K. pneumoniae* ATCC 700603 were also positive for the *intI* gene.

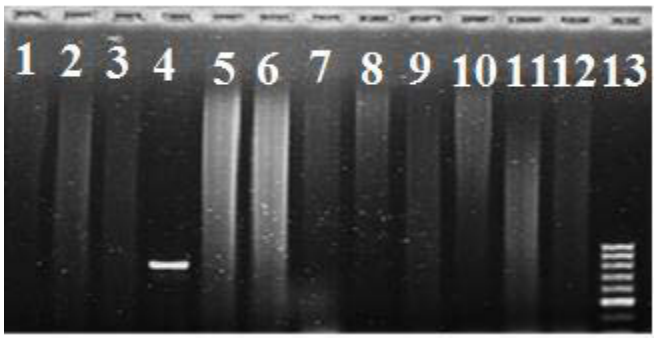

Figure 3.2: Agarose gel (1.5%) electrophoresis picture of a typical example of 892 bp *intI* gene amplicon obtained using *intI* primer. Lanes 1-3 were M42 – M44, lane 4 was M45, lanes 5 – 12 were M46 – M53 and lane 13 was O'GeneRuler^{TM} 100 bp DNA Ladder (Fermentas, Canada).

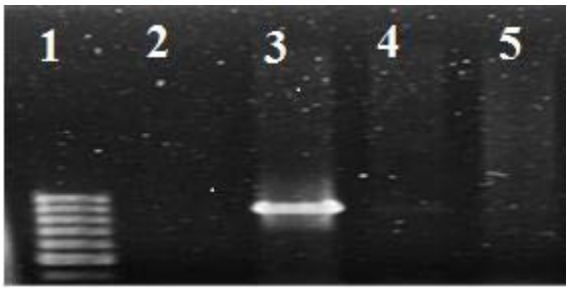

Figure 3.3: Agarose gel (1.5%) electrophoresis picture of a typical example of 892 bp *intI* gene amplicon obtained using *intI* primer. Lane 1 was O'GeneRuler^{TM} 100 bp DNA Ladder (Fermentas, Canada); lane 2 was *E. coli* ATCC 25922; lane 3 is *E. coli* ATCC 35218; lane 4 was M1 and lane 5 was M2.

The 467 bp *intII* gene was amplified from 23.2% (23/99) of isolates (Fig 3.4). *Pseudomonas aeruginosa* ATCC 27853 and *E. coli* ATCC 35218 were also positive for *intII* gene.

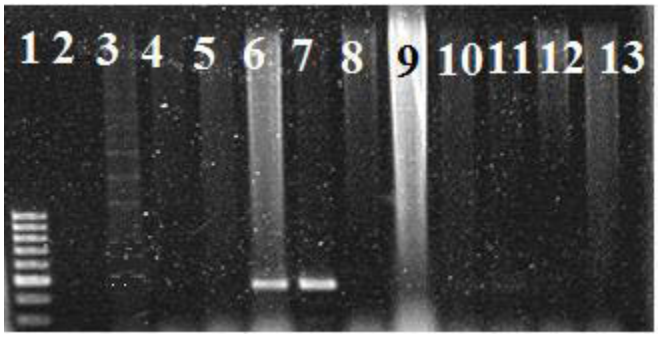

Figure 3.4: Agarose gel (1.5%) electrophoresis picture of a typical example of 467 bp *intII* gene amplicon obtained using *intII* primer. Lane 1 was O'GeneRuler^{TM} 100 bp DNA Ladder (Fermentas, Canada); lane 2 was *A. hydrophila* ATCC 7966^{T}; lane 3 was *A. caviae* ATCC 15468^{T}; lane 4 was M63; lane 5 was M64; lane 6 was M65; lane 7 was M66 and lanes 8 – 13 were M67 – M73.

Table 3.2: Characterization of *Aeromonas* spp. from fish and seawater isolates based on PCR amplification of integron and associated integron genes content and their resistance phenotypes.

Isolates	Species designation	Integrase type	*qacEΔ1**	*sulI**	*sulII**	Class 1 integron CS (bp)	Class 2 integron HEP (bp)	Resistance phenotype
M1	*A. culicicola*	*intII*	+	-	+	-	700+1500+2000+3000	NA,OX,T
M2	*A. hydrophila*	-	+	-	-	-	-	AMP,AML,OX,W,RL
M5	*A. hydrophila*	-	+	+	-	-	-	AMP,AML,OX,W,RL
M6	*A. hydrophila*	*intII*	+	-	-	-	2000	AMP,AML,OX,W,NA
M8	*A. allosaccharophila*	*intII*	+	-	+	-	2000	AMP,AML,AMC,OX,T,W,RL
M9	*P. shigelloides*	-	+	-	-	-	-	OX
M11	*Aeromonas* spp.	*intII*	+	-	-	-	2000	AMP,AML,AMC,E,OX,W
M13	*A. hydrophila*	-	+	+	-	-	-	AMP,AML,OX,W,RL
M14	*A. hydrophila*	*intII*	+	+	-	-	400	OX
M16	*A. jandaei*		+	+	-	-	700	AMP,AML,CPD,CXM,FOX,OX,W,RL
M17	*A. hydrophila*	*intII*	+	+	-	-	-	AMP,AML,OX,W,RL
M18	*A. caviae*	-	+	-	-	-	-	AMP,AML
M19	*A. jandaei*	*intII*	+	-	-	-	2000	AMP,AML,AMC,FOX,OX,W,RL
M20	*Aeromonas* spp.	-	+	-	+	-	-	AMP,AML,FOX,CPD,CXM,OX,W,RL,SXT
M21	*A. allosaccharophila*	-	+	-	-	-	-	AMP,AML,AMC,FOX,CPD,CXM,OX,W,S
M22	*A. culicicola*	-	+	-	-	-	-	AMP,AML,OX,W,RL
M23	*A. culicicola*	-	+	+	-	-	-	AMP,AML,OX,RL
M24	*A. jandaei*	-	+	-	-	-	-	AMP,AML,OX,RL
M26	*Aeromonas* spp. 45	*intI*, *intII*	+	+	-	500+600+800	400	AMP,AML,OX,RL
M28	*A. jandaei*	*intI*	-	+	-	600+800+1000	-	AMP,AML,OX,RL
M29	*A. culicicola*	-	-	+	-	-	-	AMP,AML,OX,RL
M30	*A. jandaei*	*intI*	-	-	-	600+800	-	AMP,AML,OX,RL
M31	*A. culicicola*	*intI*	-	+	-	500+600+800+1000	-	AMP,AML,OX,W,RL
M32	*A. culicicola*	-	-	+	-	-	-	AMP,AML,OX,RL

M39	*A. culicicola*	-	-	+	-	-	-	AMP,AML,OX,RL
M41	*Aeromonas* spp.	*intII*	-	+	-	-	400	AMP,AML,OX,RL
M42	*A. culicicola*	-	+	-	+	-	-	AMP,AML,NA,OX,RL
M43	*Aeromonas* spp. 310	-	+	-	-	-	-	AMP,AML,NA,OX,RL
M45	*P. shigelloides*	*intI*	-	+	-	600+900	-	AMP,AML,AMC,T,OX,W,RL
M46	*P. shigelloides*	-	+	+	-	-	-	AMP,AML,NA,E,W,S,SXT,OX,RL
M49	*A. sobria*	-	+	-	-	-	-	AMP,AMC,AML,OX,T
M52	*A. hydrophila*	-	-	+	-	-	-	AMP,AMC,AML,CPD,CXM,E,OX,T,W,RL
M53	*A. hydrophila*	*intII*	+	-	+	-	700+2000	AMP,AML,OX,T,W,RL
M54	*A. icthiosmia*	-	+	-	-	-	-	AMP,AML,AMC,E,OX,T,W,RL
M55	*A. veronii*	-	+	-	-	-	-	AMP,AML,OX,W,RL
M56	*A. culicicola*	-	+	-	+	-	-	AMP,AML,E,NA,OX,W,RL
M57	*A. veronii*	*intI*	+	-	-	500+600+800+1000	-	AMP,AML,AMC,CPD,CXM,C,E,T,OX,W,RL
M58	*A. culicicola*	-	+	-	-	-	-	AMP,AML,AMC,OX,W,RL
M61	*A. culicicola*	-	+	-	-	-	-	AMP,AML,OX
M62	*A. hydrophila*	*intI*, *intII*	+	-	+	600+900+2000	400	AMP,AML,OX,W,RL
M63	*A. veronii*	*intI*	+	-	-	500+650+800+900+1000	-	AMP,AML,AMC,OX,W,RL
M64	*A. hydrophila*	-	+	-	-	-	-	AMP,AML,OX,FOX,W,RL,CPD
M65	*A. hydrophila*	*intII*	+	-	-	-	400	AMP,AML,OX,W,RL
M66	*P. shigelloides*	*intII*	+	-	+	-	2000	AMP,AML,OX,RL
M67	*P. shigelloides*	-	+	-	-	-	-	AMP,AML,OX,RL,W
M68	*A. caviae*	-	+	-	-	-	-	AMP,AML,AMC,CPD,OX,W,RL
M69	*A. bestiarum*	-	+	-	+	-	-	AMP,AML,AMC,CPD,OX,S,W,RL
M72	*A. bestiarum*	-	+	-	-	-	-	AMP,AML,OX,W,RL
M73	*A. bestiarum*	-	+	-	-	-	-	AMP,AML,AMC,CXM,FOX,OX,W,RL
M74	*A. allosaccharophila*	*intII*	+	+	-	-	2000	AMP,AML,C,NA,OX,T,W,RL
M75	*Aeromonas* spp. 45	*intII*	+	+	-	-	1000+1500	AMP,AML,CPD,CXM,C,E,OX,W
M76	*A. salmonicida*	*intI*, *intII*	+	+	-	800+2000	2000	AMP,AML,AMC,CPD,OX,W,RL
M77	*A. salmonicida*	-	+	-	-	-	-	AMP,AML,AMC,CPD,OX,W,RL

M80	*A. bestiarum*	-	+	+	+	-	-	AMP,AML,NA,OX,T,W
M81	*A. bestiarum*	-	+	-	-	-	-	AMP,AML,CXM,CTX,OX,W,RL
M82	*A. culicicola*	-	+	-	-	-	-	AMP,AML,AMC,ATM,CPD,CXM,E,FOX,OX,W,RL
M83	*A. bestiarum*	*intII*	+	+	-	-	400	AMP,AML,OX,W,RL
M84	*A. bestiarum*	-	+	+	-	-	-	AMP,AML,OX,W,RL
M85	*A. allosaccharophila*	*intII*	+	-	+	-	-	AMC,AMP,AML,OX,W,RL
M86	*A. hydrophila*	*intII*	+	+	+	-	-	AMP,AML,AMC,OX,W,RL
M87	*A. bestiarum*	*intII*	+	+	-	-	1000+1500+2000	AMP,AML,AMC,OX,W,RL
M88	*A. bestiarum*	*intII*	+	+	+	-	-	AMP,AML,AMC,CPD,CXM,E,FOX,OX,W,RL
M89	*A. allosaccharophila*	*intII*	+	-	+	-	-	AMP,AMCL,AMC,CPD,CXM,OX
M90	*A. bestiarum*	-	+	-	-	-	-	AMP,AML,AMC,CXM,OX,W,RL
M91	*A. bestiarum*	-	+	-	-	-	-	AMP,AML,OX,W,RL
M92	*A. allosaccharophila*	*intII*	+	+	+	-	600+1500	AMP,AML,CPD,OX,W,RL
M93	*A. allosaccharophila*	-	+	-	-	-	-	AMP,AML,C,NA,OX,W,RL
M94	*A. hydrophila*	-	+	-	+	-	-	AMP,AML,AMC,E,OX,T,W,RL
M95	*A. hydrophila*	-	+	+	-	-	-	AMP,AML,AMC,CXM,E,OX,T,W,RL
M96	*A. bestiarum*	-	+	-	-	-	-	E,OX,T
M97	*A. bestiarum*	-	+	-	-	-	-	E,OX,T
M98	*Aeromonas* spp. 310	*intI*	+	-	+	500+700+800+2000+3000	-	AMP,AML,OX,W,RL
M99	*A. bestiarum*	-	+	-	-	-	-	AMP,AML,CPD,CXM,OX

**intI*=integrase class 1 gene, *intII* =integrase class 2 gene, *qacEΔ1*=quaternary ammonium metal, *sul*=sulphonamide genes, CS=integron I variable region, Hep=integron II variable region, AMP=ampicillin (AMP10), AML=amoxicillin (AML10), AMC=augmentin (AMC30), AZM=azithromycin (AZM15), ATM=aztreonam (ATM30), FOX=cefoxitin (FOX30), CPD=cefpodoxime (CPD10), CXM=cefuroxime (CXM30), CTX=cefotaxime (CTX5), E=erythromycin (E15), NA=nalidixic acid (NA30), OX=oxacillin (OX1), TE=tetracycline (TE30), W=trimethoprim (W1.25), S=streptomycin (S10), RL=sulphamethoxazole (RL25), TS=cotrimoxazole (TS25).

Only 27.2% (27/99) were positive for the *sulI* gene following amplification of 417 bp ampliner (Fig. 3.5; Table 3.2). Figures 3.5 - 3.6 are typical examples of *sulI* and *sulII* PCR gene fragments results obtained from *sulI* and *sulII* primers used. Although the *intI* and *intII* genes had been amplified, it was not possible to identify the associated *sulI* gene for all integrase-positive isolates by PCR. Therefore, *sulII* gene was targeted and a 722 bp fragment was amplified from 17.1% (17/99) of isolates (Fig. 3.6; Table 3.2). None of the type strains and controls was positive for the *sulI* gene, while type strain *A. caviae* ATCC 15468^{T} and *E. coli* ATCC 35218 were positive for the *sulII* gene.

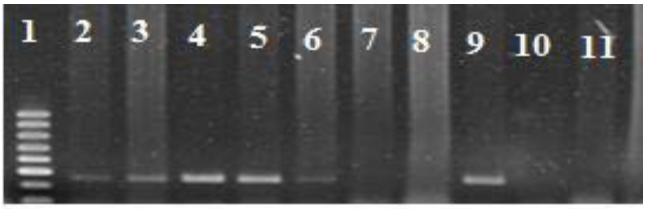

Figure 3.5: Agarose gel (1.5%) electrophoresis picture of a typical example of 417 bp *sulI* gene amplicon *sulI* primer. Lane 1 was O'GeneRuler^{TM} 100 bp DNA Ladder (Fermentas, Canada); lane 2 was M85; lane 3 was M86; lane 4 was M87; lane 5 was M88; lane 6 was M89; lane 7 was M90; lane 8 was M91; lane 9 was M92; lane 10 was M93, and lane 11 was M94.

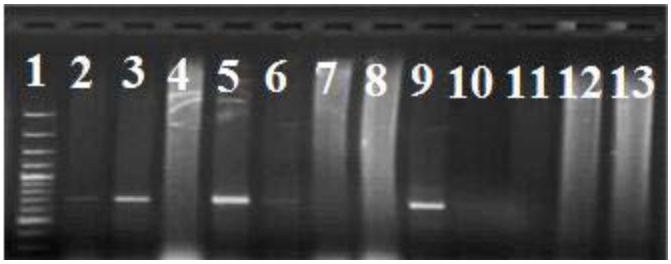

Figure 3.6: Agarose gel electrophoresis of 722 bp *sulII* gene amplicon using *sulII* primer. Lane 1 was O'GeneRuler^{TM} 100 bp DNA Ladder Plus (Fermentas, Canada); lane 2 was *A. hydrophila* ATCC 7966^{T}; lane 3 was *A. caviae* ATCC 15468^{T}; lane 4 was *E. coli* 25922; lane 5 was *E. coli* 35218; lane 6 was *P. aeruginosa* ATCC 27853; lane 7 was *P. aeruginosa* ATCC 35032; lane 8 was *K. pneumoniae* 700603; lane 9 was M1 and lane 10 was M2.

It was observed that 64.6% (64/99) of study isolates carried the *qacEΔ1* gene (Fig 3.7), with a 230 bp amplicon being obtained (Table 3.2). The *qacEΔ1* gene was also amplified from *P. aeruginosa* ATCC 35032 and *E. coli* ATCC 35218.

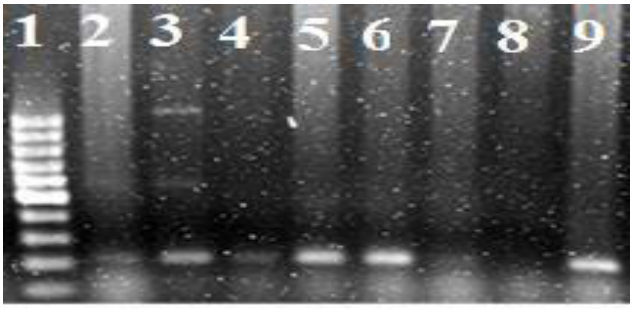

Figure 3.7: Agarose gel (1.5%) electrophoresis picture of a typical example of 230 bp *qacEΔ1* gene amplicons using Qac primer. Lane 1wasO'GeneRuler^{TM} 100 bp DNA Ladder (Fermentas, Canada); lane 2 was M54; lane 3 was M55; lane 4 was M56; lane 5 was M57; lane 6 was M58; lane 7 was M59; lane 8 was M60 and lane 9 was M61.

Sixty percent (6/10) of *intI*-positive isolates were also *qacEΔ1*-positive, while 50% (5/10) of *intI* positives carried the *sulI* gene (Table 3.2). Trimethoprim resistance was observed for 70% (7/10) of class 1 integron-positive isolates and sulphamethoxazole resistance was also observed for 90% (9/10) of class 1 integron-positive isolates. While 74% (17/23) of class 2 integron positive isolates demonstrated sulphamethoxazole resistance (Table 3.2), trimethoprim resistance was identified for 70% (16/23) of class 2 integron-positive isolates. Ampicillin resistance was observed for 91.3% (21/23) of class 2 integron-positive isolates. All five *intI* positive isolates that possessed *sulI* gene were found to carry the *qacEΔ1* gene as well. Among *intII* positives, 95.6% (22/23) were found to be *qacEΔ1*-positives, while 30.4% (7/23) of *intII* positives also possessed the *sulII* gene. The seven *intII*- *sulII*-positive isolates were observed to also possess the *qacEΔ1* gene (Table 3.2). Although 3% (3/99) of study isolates were observed to be positive for both *sulI* and *sulII* genes, 50% (32/64), 30% (3/10) and 17% (4/23) of *qacEΔ1*, *intI* and *intII* positives did not possess either *sulI* or *sulII*, respectively (Table 3.2). Three isolates: M26 *Aeromonas* spp. 45 from seawater), (M62 *A. hydrophila* from tilapia), (M76 *A. salmonicida* from goldfish) were positive for *intI*, *intII*, *qacEΔ1* and *sulI*/*sulII* genes (Table 3.2 and 3.3)

3.3.2 Analysis of integron gene content based on species designation and source of isolation

The *intI* gene was prevalent in *A. veronii* (67%; 2/3) and *A. jandaei* (29%; 2/7) isolates (Table 3.3). The *intII* gene was most prevalent in A. *allosaccharophila* and *A. hydrophila* isolates (Table 3.3). *intI* was not amplified from *Aeromonas* spp., *A. allosaccharophila*, *A. bestiarum*, *A.*

caviae, *A. icthiosmia* and *A. sobria* isolates, while *intII* was not amplified from *Aeromonas* spp. 310, *A. caviae*, *A. icthiosmia*, *A. veronii* and *A. sobria* (Table 3.3).

qacEΔ1 gene was predominantly amplified from 83% (14/17) of *A. hydrophila* and 74% (14/19) of *A. bestiarum* isolates. The *sulI* gene was not detected in *Aeromonas* spp. 310, *A. caviae*, *A. icthiosmia* and *A. veronii* (Table 3.3). Greatest prevalence for *sulI* gene was detected for *A. hydrophila* (41%; 7/17) isolates, while the *sulII* gene was predominantly amplified from *A. allosaccharophila* (Table 3.3).

Species specificity was observed primarily for *intI*, *intII*, *qacEΔ1, sulI* and *sulII* genes, with *A. hydrophila* being the species with most positives. Although *qacEΔ1* gene were amplified from *A. caviae*, *A. icthiosmia* and *A. sobria*, no corresponding *intI*, *intII*, *sulI* and *sulII* genes were amplified (Table 3.3).

Table 3.3: Species designation-based analysis of amplified integron genes

Species	*intI**	*intII**	*qacEΔ1**	*sulI**	*sulII**
Aeromonas spp. (n=4)	0	50% (2/4)	50% (2/4)	25% (1/4)	25% (1/4)
Aeromonas spp. 45 (n=5)	20% (1/5)	40% (2/5)	50% (2/5)	60% (3/5)	0
Aeromonas spp. 310 (n=3)	33% (1/3)	0	67% (2/3)	0	33% (1/3)
A. allosaccharophila (n=8)	0	63% (5/8)	88% (7/8)	25% (2/8)	50% (4/8)
A. bestiarum (n=19)	0	16% (3/19)	74% (14/19)	26% (5/19)	16% (3/19)
A. caviae (n=3)	0	0	66.7% (2/3)	0	0
A. culicicola (n=18)	6% (1/18)	6% (1/18)	44% (8/18)	28% (5/18)	17% (3/18)
A. hydrophila (n=17)	6% (1/17)	41% (7/17)	83% (14/17)	41% (7/17)	24% (4/17)
A. icthiosmia (n=3)	0	0	33% (1/3)	0	0
A. jandaei (n=7)	29% (2/7)	29% (1/7)	43% (3/7)	14% (1/7)	0
A. salmonicida (n=2)	50% (1/2)	50% (1/2)	100% (2/2)	50% (1/2)	0
A. sobria (n=1)	0	0	100% (1/1)	0	0
A. veronii (n=3)	67% (2/3)	0	100% (3/3)	0	0
P. shigelloides (n=6)	17% (1/6)	17% (1/6)	50% (3/6)	33% (2/6)	17% (1/6)

**intI*=integrase class 1 gene, *intII*=integrase class 2 gene, qacEΔ1=quaternary ammonium metal, *sul*=sulphonamide genes.

Integron-containing isolates were identified primarily from *A. hydrophila* (Table 3.3) and goldfish isolates (Table 3.3). However *A. allosaccharophila*, *A. bestiarum*, *A. jandaei* and *A. veronii* occurred frequently among positive isolates.

Table 3.4: Source of isolation-based analysis of amplified integron genes

Source	*intI**	*intII**	*qacEΔ1**	*sulI**	*sulII**
Catfish (n=13)	0	26.7% (4/15)	53% (8/15)	13% (2/15)	20% (3/15)
Goldfish (n=33)	6.1% (2/33)	30% (10/33)	85% (28/33)	33% (11/33)	85% (9/33)
Sea water (n=20)	20% (4/20)	10% (2/20)	20% (4/20)	40% (8/20)	0
Tilapia (n=33)	12% (4/33)	21% (7/33)	73% (24/33)	18% (6/33)	15% (5/33)

**intI*=integrase class 1 gene, *intII* =integrase class 2 gene, *qacEΔ1*=quaternary ammonium metal, *sul*=sulfonamide genes.

3.3.3 Identification of conserved regions for class 1 and 2 integron positives

Only 10% (10/99) of isolates examined were positive for the *intI* gene. Thus the gene cassette-containing regions of these 10 isolates were amplified using the class 1 integron CS primers. Amplicons of varying lengths (0.5 - 3 kb) were observed in CS profiles of all 10 isolates (Fig. 3.8; Table 3.4). Table 3.2 is a summary of all *Aeromonas* spp. study isolates *qacEΔ1*, *intI*, *intII*, *sulI*, *sulII* positives. The correlation between *intI* positives and CS variable regions, as well as the *intII* positives and HEP variable regions can be observed in Table 3.2. The resistance phenotypes identified by Duma (2012) were also correlated to *intI* or *intII* presence in certain cases. Figure 3.8 shows the 10 *intI* positives with the various multiple bands that ranged from 500 to 3000 kb. These bands represent various gene cassettes that were not tested for in this study.

A higher prevalence of *intII* was identified in study isolates. The conserved regions of the 22 isolates were amplified using the class 2 integron HEP primer (Fig. 3.9). Only 83.0% (19/23) of isolates yielded amplification products ranging from 0.5 – 3 kb (Table 3.2). HEP profiles were not observed for the remaining 17.3% (4/23) of isolates. Figure 3.9 shows *intII* positives and their HEP variable regions, with multiple bands representing various gene cassettes that were not tested for in this study. Lanes 19, 20 22 and 23 seemed to lack HEP regions, as there was smearing in these lanes.

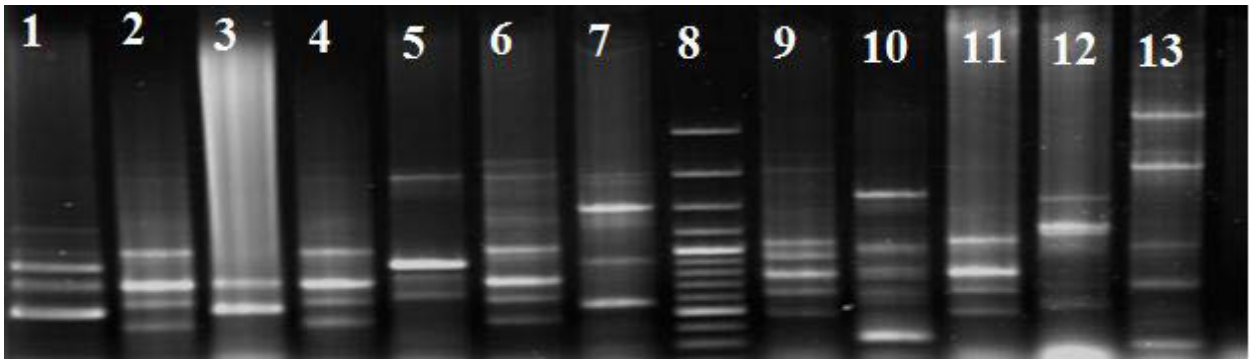

Figure 3.8: Agarose gel electrophoresis of CS variable regions (0.5 – 3 kb) of ten *intI*-positive *Aeromonas* spp. isolates. Lane 1was *A. caviae* ATCC 15468^{T}; lane 2 was *E. coli* ATCC 35218, lane 3 was M26; lane 4 was M28; lane 5 was M30; lane 6 was M31; lane 7 was M45; lane 8 wasO'GeneRuler^{TM}100 bp DNA Ladder Plus (Fermentas, Canada); lane 9 was M57; lane 10 was M62; lane 11 was M63; lane 12 was M76 and lane 13 was M98.

The CS and HEP regions were similar in the size of bands they contained (500 – 3000 kb) (Table 3.4). Both variable regions also seemed to demonstrate similar resistance phenotypes, with the commonest being the resistance to ampicillin, trimethoprim and sulphamethoxazole. However these *intI* and *intII* variable regions differ in the number of bands each isolate variable region contained. The CS variable region had an average of 3 bands each, while the HEP variable region had an average of 2 bands each (Table 3.4).

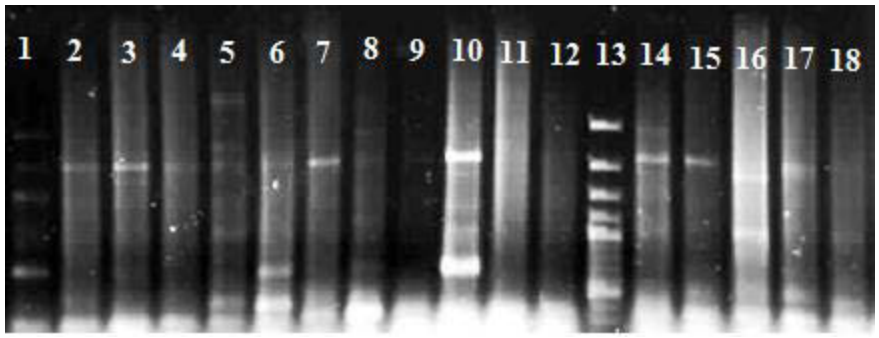

Figure 3.9: Agarose gel electrophoresis of HEP variable regions (0.5 – 3 kb) of 23 *intII*-positive *Aeromonas* sp. isolates. Lanes 1was M1; lane 2 was M6; lane 3 was M8; lane 4 was M11; lane 5 was M14; lane 6 was M17; lane 7 was M19; lane 8 was M26; lane 9 was M41; lane 10 was M53; lane 11 was M62; lane 12 was M65; lane 13 was O'GeneRuler^{TM} 100 bp DNA Ladder Plus (Fermentas, Canada); lane 14 was M66; lane 15 was M74; lane 16 was M75; lane 17 was M76; and lane 18 was M83.

3.4 Discussion

Isolates originating from aquatic sources have been shown to harbour integrons and other genes coding for resistance determinants (Ndi and Barton, 2011). A number of studies have observed the prevalence of class 1 integron among food-borne, environmental and clinical isolates of *Aeromonas* spp. (Chang *et al*., 2007; Lee *et al*., 2008; Perez-Valdespino *et al*., 2009; Čižek *et al*., 2010; Ndi and Barton, 2011). Studies by Nawaz *et al*. (2010), Kadlec *et al*. (2011) and Sarria-Guzmàn *et al*. (2013) have demonstrated that *Aeromonas* spp. in aquaculture are usually associated with class 1 integrons in the United States of America and Germany. Similarly in Australia, Ndi and Barton (2011) detected the prevalence of class 1 integrons in *Aeromonas* spp. from cultured fish. Čižek *et al*. (2010) observed that aeromonads from cultured ornamental (koi) carp (*Cyprinus carpio koi*) and common carp (*Cyprinus carpio*) were associated with class 1 integrons. In South Africa, Igbinosa *et al*. (2013) observed the prevalence of integron 1 in *Aeromonas* spp. in two South African rivers situated in the Eastern Cape region. In the present study, *Aeromonas* spp. isolated from aquatic systems and food were observed to possess both class 1 and class 2 integrons.

Class 2 integrons have previously been identified in non-pathogenic *E. coli* strains from farmed poultry and swine (Lapierre *et al*., 2008), and in resistant *E. coli* strains from wastewater treatment plants in Italy (Pellegrini *et al*., 2010). Studies by Maravic *et al*. (2013) detected the presence of both class 1 and class 2 integrons in environmental isolates of *Aeromonas* spp. In South Africa, Jacobs and Chenia (2007) detected the presence of both class 1 and class 2 integrons in *Aeromonas* spp. from aquaculture systems. This is consistent with data obtained from this study, however, in the present study there was greater incidence of class 2 integrons than class 1 integrons.

All integron-positive *Aeromonas* spp. are known to be multidrug resistant to at least three or more antimicrobials commonly in use (Kadlec *et al*., 2011; Ndi and Barton, 2011; Igbinosa *et al*., 2013). In this study, it was observed that all class 1 and 2integron-positive isolates were resistant to ampicillin, trimethoprim, sulphamethoxazole, and trimethoprim-sulphamethoxazole. Trimethoprim and sulphamethoxazole resistance among class 1 integron carrying isolates was correlated to the *sulI* and *sulII* genes presence. All of class 1 integron-containing isolates (Table 3.2) were ampicillin-resistant, with 50% (5/10) possessing either a beta-lactamase gene ($bla_{TEM\text{-}I}$) and/or extended spectrum beta-lactamase genes ($bla_{TEM\text{-}II}$) (Chapter Two). This is consistent with

findings by Tacao *et al*. (2014), where class 1 integrons found in *Aeromonas* spp. like *A. hydrophila* from aquatic systems were associated with extended spectrum beta-lactamase genes and multi-drug resistance to three or more classes of antimicrobials.

Class 2 integron positive isolates demonstrated sulphamethoxazole, trimethoprim and ampicillin resistance (Table 3.2). This is similar to findings of Maravic *et al*. (2013) and Moura *et al*. (2007), who identified that integron carrying *Aeromonas* spp. were resistant to ampicillin, cefazolin, chloramphenicol, nalidixic acid, trimethoprim, sulphamethoxazole, and trimethoprim-sulphamethoxazole. This multidrug resistance could be an effect of co-selection of several resistance genes in the same genetic platform or cross-resistance due to expression of a mechanism responsible for resistance to different compounds (Tacao *et al*., 2014).

The detection of *sulI* gene in 27.2% (27/99) and *sulII* gene in 17.1% (17/99) correlates with the high level of resistance to trimethoprim, sulphamethoxazole, and trimethoprim-sulphamethoxazole. Only 1.01% (1/99) of *sulI*-containing and 3.03% (3/99) of *sulII*-containing gene were not resistant to trimethoprim, sulphamethoxazole, and trimethoprim-sulphamethoxazole. This could be due to lack in selection pressures required for expression (Sarria-Guzmàn *et al*., 2013). Although there were isolates that were negative for both *sulI* and *sulII* genes, they presented with resistance to trimethoprim, sulphamethoxazole, and trimethoprim-sulphamethoxazole. This could be as a result of other mobile genetic elements present in study isolates that was not tested for in this study. Similarly, 3.03% (3/99) of isolates were observed to possess both integrase genes and 70.7% (70/99) of study isolates were negative for both *intI* and *intII* genes. Furthermore, 61.6% (61/99) lacking both integrase genes were observed to be resistant to either trimethoprim or sulphamethoxazole and both. This could be due to the presence of other resistance genes being associated with plasmids and transposons which present co-resistance to commonly used antimicrobials. This usually occurs when these strains are exposed to single or multiple antimicrobials. This induces production of these resistance genes through mutation or horizontal transfer (Tacao *et al*., 2014). In the present study 50% (32/64) of isolates carrying the *qacEΔ1* gene were positive for both *sulI* and *sulII* genes. This is similar to findings of Jacobs and Chenia (2007) for *Aeromonas* spp. isolates and for *E. coli* isolates by Sunde (2005).

All study isolates positive for *intI* possessed CS profiles with amplicons ranging from 500 - 3000 bp, while 17.3% (4/23) of *intII* carrying study isolates did not yield HEP profiles

(Table 3.2). This may as a result of empty variable regions (Sarria-Guzmàn *et al*., 2013), or gene cassettes present in these variable regions that are less than 300 bp or too large to be amplified (Chang *et al*., 2007).

In this study, species analysis of integron containing isolates suggests that *intI*, *intII*, *qacEΔ1*, *sulI* and *sulII* genes were prevalent among *A*. *veronii*, *A*. *jandaei*, *A*. *hydrophila*, *A*. *allosaccharophila* and *A*. *bestiarum* (Tables 3.3 and 3.5). This is similar to findings by Čižek *et al*. (2010), Nawaz *et al*. (2010), Kadlec *et al*. (2011), Ndi and Barton (2011) and Sarria-Guzmàn *et al*. (2013). Nawaz *et al*. (2010) observed that *A*. *veronii* possessing class 1 integrons were frequently isolated from catfish. However in the present study, only class 2 integrons were observed for catfish isolates. The fish pathogen *A*. *salmonicida* possessed *intI*, *intII*, *qacEΔ1* and *sulI* genes, which has been previously observed by Kadlec *et al*. (2011). Since integron-carrying motile species *A*. *veronii* biovar *sobria*, *A*. *jandaei*, *A*. *hydrophila* and *A*. *caviae* are associated with human infections and possess multi-drug resistance. This poses a threat to public health as these pathogenic aeromonads can transfer these antimicrobial resistance determinants to humans (Kadlec *et al*., 2011).

Based on data from this study, *Aeromonas* spp. from fish and seawater are associated with resistance determinants such as integrons, and are multi-drug resistant to several antimicrobials commonly in use. The prevalence of both class 1 and 2 integrons increases the possibility of horizontal gene transfer of resistance genes within the aquatic environment and to human pathogens and suggests that they may be vectors of antimicrobial resistance genes in the aquatic environments (Lupo *et al*., 2012). This presents a challenge for treatment processes, as the multidrug resistance phenotypes observed for *Aeromonas* spp. isolated from food and environmental sources can seriously cripple chemotherapeutics that are currently in use.

CHAPTER FOUR

CHARACTERISATION OF AQUATIC *Aeromonas* spp. ISOLATES' SUSCEPTIBILITY TO PHYTOCHEMICAL COMPOUNDS: CINNAMALDEHYDE, *Kigelia africana* AND VANILLIN

4.1 Introduction

According to the World Health Organization (WHO), more than 80% of the world's population depends on traditional plant medicine for their primary health care needs (Cowan *et al*., 1999). It is estimated that around 70,000 plant species, from lichens to tall trees, have been used at one time or another for medicinal purposes (De Britto *et al*., 2012b). Phytochemicals are non-nutritive plant chemicals that have protective or disease preventive properties (de Britto *et al*., 2012a). These plant chemicals are produced by plants for their protection. These phytochemicals have antimicrobial, immunostimulating, appetite increasing, growth promoting, de-stressing, and aphrodisiac properties (Citarasu *et al*., 2002; Ardo *et al*., 2008; Rajendiran *et al*., 2008; Harikrishnan *et al*., 2010; Velmurugan *et al*., 2010; Abdel-Tawwab *et al*., 2012). Plant extracts are highly efficient against resistant microbial infections. This has led to increasing interest in phytochemical pharmacology, although phytochemicals have been in use since early man (Phillipson, 2001). According to WHO, medicinal plants with various life-sustaining constituents would be the best source to obtain a variety of potential, safe and novel drugs (Natarajan *et al*., 2003).

With advances in phytochemical techniques, several active principles of many medicinal plants have been isolated and introduced as valuable drugs in modern systems of medicine (Senthilkumar *et al*., 2005). The most active components of plant phytochemicals are secondary metabolites such as alkaloids, tannins, phenolic/flavonoid compounds, sterols, terpenoids, and proanthocyanidins (Siri *et al*., 2008). The antibacterial active components of plants may lyse the cell wall, block both protein and DNA synthesis, inhibit enzyme secretions and interfere with the signaling mechanisms of the quorum sensing pathway (Chakraborty and Hancz, 2011). Tannins which are polyphenols that are obtained from various parts of different plants are toxic to bacteria, and filamentous yeast (Harborne, 1973). Phenolics are known to inactivate microbial adhesins enzymes, cell envelope, and transport proteins through non-specific forces like

hydrogen bonding, covalent bonding, and hydrophobic effects (Saleem *et al*., 2010). Flavonoids are one of the biggest classes of secondary metabolites and are distributed in various plant species. Flavonoid efficacy is attributed to their ability to complex with extracellular and soluble proteins and then alters permeability of bacterial cell walls and porins in microbial membrane (Saleem *et al*., 2010). They protect the plant from UV radiation and other environmental stresses, and significant antioxidant properties are associated with them. In comparison to antimicrobial agents, phytochemicals of plant origin are not associated with many side effects and have an enormous therapeutic potential to heal many infectious diseases (Ponnusamy *et al*., 2010).

Cinnamaldehyde or 3-phenyl-2-propenal, a natural flavouring substance and a major constituent of cinnamon essential oils, occurs naturally 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 has been shown to have antibacterial activities against a number of Gram-negative and Gram-positive organisms, e.g., *Escherichia coli* O157:H7, *Salmonella typhimurium*, *Clostridium botulinum*, *Staphylococcus aureus* (Morozumi, 1985). The important characteristic of cinnamaldehyde is its hydrophobicity, which enables it to enter and disturb the lipid bilayer of the cell membrane and cause increased permeability to protons (Jia *et al*., 2011). Cinnamaldehyde acts by uncoupling of oxidative phosphorylation, inhibition of active transport, loss of pool metabolites and disturbing the synthesis of DNA, RNA, proteins, lipids and polysaccharides by inhibiting the proton motive force, respiratory chain, electron transfer and substrate oxidation (Nuryastuti *et al*., 2009). The resulting extensive leakage from bacterial cells or the exit of critical molecules and ions leads to cell death (Denyer, 1995; Nuryastuti *et al*., 2009).

Vanillin (4-hydroxy-3-methoxybenzaldehyde) is a major component of natural vanilla, (a widely used flavouring material with generally regarded as safe status), which is a bean of the tropical orchid *Vanilla planifolia* (Walton *et al*., 2003; Bythrow, 2006; Kappachery *et al*., 2010). The mode of action of the phenylpropene phenolic aldehyde vanillin is not well understood, but it has been proposed to function as a membrane-active compound that might have intracellular targets. Being a phenolic compound, vanillin demonstrates antimicrobial and antioxidant properties (Davidson and Naidu, 2000). Vanillin's efficacy against both Gram-negative and Gram-positive bacteria yeasts and molds has been demonstrated (Cerrutti *et al*. 1997; Lopez-Malo *et al*. 1998; Fitzgerald *et al*. 2004). Vanillin inhibited respiration of *E. coli* and *Listeria*

innocua cells, and disrupted the potassium and pH homeostasis of *Lactobacillus plantarum* cells (Fitzgerald *et al*., 2004). Fitzgerald *et al*. (2004) observed that treatment with vanillin disrupted membrane integrity of only a sub-population of cells and it was proposed that although vanillin primarily is a membrane- active compound, it may also have intracellular target sites.

Kigelia africana, commonly known, as the sausage tree of the Bignoniaceae family, is commonly found in South, Central and West Africa. The semi-deciduous tree grows wild and gets up to 25 m in height (Figure 5.1). *K. africana* has been known to be effective in the therapy of primary and secondary infections of humans as well as a disinfectant. The antimicrobial properties of *K. africana* leaves, fruits and bark against Gram-negative and Gram-positive bacteria have been investigated and reported (Eldeen and Staden, 2007; Jeyachandran and Mahesh, 2007; Shai *et al*., 2008).The fruits of *K. africana* are a popular source of traditional medicine throughout Africa, and Grace *et al*. (2002) observed the antibacterial activity of *K. africana* fruits against Gram-negative and Gram-positive bacteria. Fatty acids exhibiting antibacterial activity, vermonosides γ, flavonoids, ᵞ-sitosterols and iridoids have been isolated from the fruits and this supports the traditional use of the plant in therapy for bacterial infections (Gouda *et al*., 2003; Picerno *et al*., 2005; Asekun *et al*., 2006; Olatunji and Olubunmi, 2009; Saini *et al*., 2009). Houghton (2007) demonstrated the presence of 3b, 19a-dihydroxyurs-12-ene-28oic acid, caffeic acid and chlorgeric acid in the fruits of *K. africana* and 7-0-glycoside in both the fruits and leaves. Methanolic extracts of roots and fruits of *K. africana* demonstrate the presence of naphthoquinones, kigelinone, iso-pinnatal, dehydro-α-lapachone, lapachol, phenylpropanoids, phenylethanoid, derivatives, p-coumaric acid and freulic acid (Saini *et al*., 2009).

Figure 4.1: *Kigelia africana* (Lam.) Benth. (Saini *et al*., 2009).

Aeromonas spp. are autochthonous in aquatic environments and as a result form normal flora of fish and aquatic animals. In aquaculture, over-crowding and increased stress levels predispose fish to develop infections caused by aeromonads (Yin *et al*., 2009). While prevention and treatment of these infections can be achieved using chemotherapy, the dangers of increasing incidences of drug resistance selected for by these synthetic antimicrobials have been reported (Yin *et al*., 2009). *Aeromonas* spp. have been reported to be resistant to penicillins, cephalosporins, aminoglycosides, macrolides, quinolones, and tetracyclines (Goni-Urriza *et al*., 2000; Radu *et al*., 2003; Palu *et al*. 2006; Jun *et al*., 2010). This resistance can be transmitted by resistance determinants (plasmids and integrons) through ingestion of contaminated water and foods to humans (Janda and Abbott, 2010). Phytochemicals have gained special interest as sources of natural antimicrobial and antioxidant agents because of the antimicrobial resistance of microorganisms and the toxicities of synthetic antioxidants (Ozkan *et al*., 2010). In aquaculture, phytochemicals promote various activities, including anti-stress, growth promotion appetite simulation, tonic and immune-stimulation and antimicrobial properties (Yin *et al*., 2009). Phytochemicals can be useful for the effective treatment of infectious fish diseases; enhancing fish health and food safety and quality while conserving the aquatic environment (Yin *et al*., 2009). Plant-derived phyto-medicines have great promise in the treatment of infectious disease and thus represent a vast untapped source, which has the potential to combat pathogen infection in aquaculture (Pakravan *et al*., 2011). Therefore, this study aimed to investigate the antimicrobial activity of three phytochemical compounds; cinnamaldehyde, vanillin and *Kigelia africana* fruit extracts on antimicrobial resistant aquatic *Aeromonas* spp.

4.2 Materials and methods

4.2.1 Maintenance of bacterial isolates

Ninety-three *Aeromonas* spp. and six *Plesiomonas shigelloides* isolates, cultured previously from moribund or healthy koi carp, catfish, tilapia, and sea-water, were screened as well as two *Aeromonas* spp. type strains viz; *A. caviae* ATCC 15468^T and *A. hydrophila* ATCC 7966^T. *Aeromonas* spp. isolates were maintained on tryptic soy agar (TSA) plates and stored at 4 °C and for long-term storage in tryptic soy broth containing 40 % glycerol at -70 °C (Jacobs and Chenia, 2007).

4.2.2 Preparation of crude *K. africana* fruit extracts

K. africana fruits were collected around the Westville Campus of University of KwaZulu-Natal. Material was washed, dried, chopped, and 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, and methanol 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 ºC.

4.2.3 Phytochemical antimicrobial activity test against study isolates

Antimicrobial susceptibility to cinnamaldehyde, vanillin and four crude *K. africana* extracts were determined using the disc diffusion method. Blank discs (MAST, UK) were impregnated with 10 µl (1mg/ml), 125 µl (1mg/ml), 25 µl (10 mg/ml), 50 µl (10 mg/ml) and 125 µl (10 mg/ml) of cinnamaldehyde (Sigma); 5 µl (1 mg/ml), 20 µl (1 mg/ml) and 25 µl (10 mg/ml) of vanillin (Sigma) and 40 µl (100 µg/ml) of *K. africana* ethyl acetate (EX1), 40 µl (100 µg/ml) of dichloromethane (EX2), 100 µl (100 µg/ml)of methanol (EX3), and 40 µl (100 µg/ml) of hexane (EX4) extracts and allowed to dry.

Bacterial isolates were grown overnight on TSA agar plates and the turbidity of the 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 extracts discs (CLSI, 2007). Plates were then incubated for 24 h at 30 °C. Bacterial strains *Escherichia coli* ATCC 29522, *A. hydrophila* ATCC 7966^T and *A. caviae* ATCC 15468^T were used as antimicrobial susceptibility testing controls. Testing was done in duplicate and tetracycline (TE30; 30 mg) and ampicillin (AMP10; 10 mg) discs were used as standard antimicrobial agent controls. Zone diameters were determined and averaged and the following criteria were used to assign susceptibility or resistance to phytochemicals tested: Susceptible (S) ≥ 15 mm, Intermediate (I) = 11 – 14 mm, and Resistant (R) ≤ 0 – 10 mm. Criteria for assigning susceptibility or resistance to AMP10 were 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 were calculated by comparing zones of inhibition obtained with each of the extracts with those obtained with the standard antimicrobial agents, tetracycline and ampicillin (Jeyachandran and Mahesh, 2007). The following equation was used: Activity index (AI) = Inhibition diameter (mm) with test extract / Inhibition diameter (mm) with standard antimicrobial agent.

4.3 RESULTS

4.3.1 Effect of phytochemicals on study isolates

Resistance to ampicillin was displayed by 64.6% (64/99) of isolates, while 56.5% (56/99) of isolates demonstrated susceptibility to tetracycline (Table 4.1). Differences were observed in the antimicrobial effect of cinnamaldehyde, vanillin and the *K. africana* extracts against the 93 *Aeromonas* spp., six *P. shigelloides* isolates and two type strains tested (Table 4.1; Appendix: Table A1). No zones of inhibition were observed with all concentrations of vanillin tested (5, 20 and 250 µg/ml).

While 10 µg/ml of cinnamaldehyde did not demonstrate a bactericidal effect against all isolates tested, 125, 250, 500 and 1250 µg/ml of cinnamaldehyde proved inhibitory for study isolates (Table 4.1), with zone diameters ranging from 0 to 31 mm, 0 to 32 mm, 0 to 41 mm and 0 to 40 mm, respectively (Appendix: Table A1). Susceptibility was observed for 12.1% (12/99), 57.5% (57/99), 91.9% (91/99) and 98.9% (98/99) of study isolates as well as both type strains, with 125, 250, 500 and 1250 µg/ml of cinnamaldehyde respectively (Table 4.1).

Zone diameters obtained with *K. africana* ethyl acetate extract (EX 1) ranged from 0 to 15 mm (Appendix: Table A1) with 78.7% (78/99) of isolates displaying resistance and 3.0% (1/99) of isolates being susceptible (Table 4.1). Zone /diameters for the dichloromethane extract (EX2) ranged from 0 to 13 mm (Appendix: Table A1) with 91.9% (91/99) of isolates displaying resistance and no susceptibility being observed (Table 4.1). The methanol extract (EX3) zone diameters ranged from 8 to 16.5 mm (Appendix: Table A1) with 13.1% (13/99) of isolates displaying resistance, and 34.3% (34/99) of isolates being susceptible (Table 4.1). The hexane extract (EX4) did not have an inhibitory effect on all study isolates and type strains, which appeared to have no activity as there were no zones of inhibition (Appendix: Table A1). Among study isolates the four different extracts exhibited varying ranges of activity, with the ethyl acetate extract (EX 1) being the second most inhibitory against isolates. The methanol extract

(EX 3) was the most active against study isolates, while the dichloromethane (EX 2) and hexane extracts (EX 4) were the least active against study isolates. While the two type strains *A. hydrophila* 7966^T and *A. caviae* 15468^T were susceptible to TE30, 125 to 1250 µg/ml cinnamaldehyde and the *K. africana* methanol extract (EX3), they displayed resistance to AMP10, *K. africana* dichloromethane (EX2) and hexane (EX 4) extracts, and intermediate susceptibility to *K. africana* ethyl acetate extract (EX1) (Table 4.1).

Table 4.1: Susceptibility analysis of ninety-three *Aeromonas* and six *Plesiomonas* spp. study isolates to phytochemical extracts and standard antimicrobial agent

	% Susceptibility/Resistance phenotype (number of isolates)		
Phytochemical	**Susceptible**	**Intermediate**	**Resistant**
125 µg/ml cinnamaldehyde	12 (12/99)	27 (27/99)	60 (60/99)
250 µg/ml cinnamaldehyde	57 (57/99)	26 (26/99)	16 (16/99)
500 µg/ml cinnamaldehyde	91(91/99)	7 (7/99)	1(1/99)
1250 µg/ml cinnamaldehyde	98 (98/99)	0 (99/99)	1 (1/99)
5 µg/ml vanillin	0 (99/99)	0 (99/99)	100 (99/99)
20 µg/ml vanillin	0 (99/99)	0 (99/99)	100 (99/99)
250 µg/ml vanillin	0 (99/99)	0 (99/99)	100 (99/99)
4 mg/ml *K. africana* EX 1	3 (3/99)	18 (18/99)	78 (78/99)
4 mg/ml *K. africana* EX 2	0 (99/99)	8 (8/99)	91 (91/99)
10 mg/ml *K. africana* EX 3	34 (34/99)	52 (52/99)	13 (13/99)
4 mg/ml *K. africana* EX 4	0 (99/99)	0 (99/99)	100 (99/99)
Ampicillin AMP10	8 (8/99)	27 (27/99)	64 (64/99)
Tetracycline TE30	56 (56/99)	35 (35/99)	8 (8/99)

4.3.2: Analysis of phytochemical effects on *Aeromonas* spp.

Isolates were assessed for susceptibility based upon species designation (Table 4.2). Upon exposure to 500 and 1250 µg/ml cinnamaldehyde, there was complete susceptibility observed across all *Aeromonas* species except for an *A. allosaccharophila* strain that was also resistant to all phytochemicals tested and standards used. *Aeromonas sobria* strain was observed to be resistant to all *K. africana* extracts used and 125 µg/ml of cinnamaldehyde, but was susceptible to increased cinnamaldehyde concentrations (250 µg/ml, 500 µg/ml and 1250 µg/ml) and

tetracycline. *A. bestiarum*, *A. culicicola*, *A. hydrophila*, *A. allosaccharophila*, *A. jandaei*, *Aeromonas* spp. 45 isolates displayed resistance to 250 µg/ml cinnamaldehyde, while isolates belonging to the remaining *Aeromonas* and *Plesiomonas* species exhibited susceptibility (Table 4.2).

Analysis of percentage resistance based on source (Table 4.3) shows that none of the catfish isolates were resistant to tetracycline while majority of the catfish and koi carp isolates were resistant to ampicillin. Isolates displayed increased susceptibility on exposure to increasing cinnamaldehyde concentrations, and greater susceptibility to *K. africana* EX 3 (Table 4.3).

Based on zones of inhibition obtained with phytochemicals and standard antimicrobial agents, the relative activity indices were determined (Appendix: Table A2). An extract was considered effective against an isolate if the activity index was ≥ 1 (Table 4.4). Ampicillin was regarded a poor standard for comparison since 64.6% (64/99) of the study isolates exhibited resistance (Table 4.1). Activity indices ranging from 0 to 1.286, 0 to 1.125, 0 to 1.929 and 0, relative to ampicillin for the *K. africana* extracts (EX 1, EX 2, EX 3 and EX 4) (Appendix: Table A2), respectively. Activity indices ranging from 0 to 2.385, 0 to 2.462, 0 to 2.846 and 0 to 4.353, relative to tetracycline, were obtained when using 125, 250, 500 and 1250 µg/ml cinnamaldehyde, respectively (Appendix: Table A2). Activity indices for the *K. africana* ethyl acetate extract (EX 1) ranged from 0 to 1.273, the dichloromethane extract (EX 2) had activity indices ranging from 0 to 0.909, the methanol extract (EX 3) activity indices ranged from 0 to 1.600 and the hexane extract (EX 4) had activity indices of 0 (Appendix: Table A2). Based on AI ≥ 1, 1250 µg/ml of cinnamaldehyde was the most effective.

Table 4.2 Analysis of percentage resistance of test isolates to phytochemicals based on species designation

Species	C125*	C250*	C500*	C1250*	EX 1*	EX 2*	EX 3*	EX 4*	AMP10*	TE30*
A. bestiarum	68% (13/19)	26% (5/19)	0	0	89% (17/19)	100% (19/19)	16% (3/19)	100% (19/19)	79% (15/19)	0
A. culicicola	61% (11/18)	28% (5/18)	0	0	83% (15/18)	83% (15/18)	11% (2/19)	100% (18/18)	61% (11/18)	0
A. hydrophila	65% (11/17)	12% (2/17)	0	0	76% (13/17)	88% (15/17)	12% (2/17)	100% (17/17)	65% (11/17)	12% (2/17)
A. allosaccharophila	75% (6/8)	25% (2/8)	13% (1/8)	13% (1/8)	88% (7/8)	88% (7/8)	25% (2/8)	100% (8/8)	63% (5/8)	13% (1/8)
A. jandaei	57% (4/7)	14% (1/7)	0	0	71% (5/7)	100% (7/7)	0	100% (7/7)	57% (4/7)	14% (1/7)
Aeromonas spp. 45	60% (3/5)	20% (1/5)	0	0	60% (3/5)	80% (4/5)	20% (1/5)	100% (5/5)	80% (4/5)	20% (1/5)
Aeromonas spp.	75% (3/4)	0	0	0	75% (3/4)	100% (4/4)	25% (1/4)	100% (4/4)	25% (1/4)	0
Aeromonas spp. 310	33% (1/3)	0	0	0	67% (2/3)	67% (2/3)	0	100% (3/3)	100% (3/3)	0
A. caviae	33% (1/3)	0	0	0	100% (3/3)	100% (3/3)	0	100% (3/3)	33% (1/3)	67% (2/3)
A. icthiosmia	33% (1/3)	0	0	0	33% (1/3)	100% (3/3)	0	100% (3/3)	33% (1/3)	0
A. veronii	67% (2/3)	0	0	0	67% (2/3)	100% (3/3)	0	100% (3/3)	67%(2/3)	33% (1/3)
A. salmonicida	50% (1/2)	0	0	0	50% (1/2)	100% (2/2)	0	100% (2/2)	100% (2/2)	0
A. sobria	100% (1/1)	0	0	0	100% (1/1)	100% (1/1)	100% (1/1)	100% (1/1)	100% (1/1)	0
P. shigelloides	33% (2/6)	0	0	0	83% (5/6)	100% (6/6)	17% (1/6)	100% (6/6)	50% (3/6)	0

* C125: 125 µg/ml; C250: 250 µg/ml; C500: 500 µg/ml and C1250: 1250 µg/ml cinnamaldehyde; EX 1: (4 mg/ml) *K. africana* ethyl acetate extract; EX 2: 4 mg/ml *K. africana* dichloromethane extract; EX 3: 10 mg/ml *K. africana* methanol extract; EX 4: 4 mg/ml *K. africana* hexane extract; AMP10: Ampicillin; TE30: Tetracycline.

TABLE 4.3 Analysis of percentage resistance of test isolates to phytochemicals used based on source of isolation

Source	C125*	C250*	C500*	C1250*	EX 1*	EX 2*	EX 3*	EX 4*	AMP10*	TE30*
Catfish	47% (7/15)	13% (2/15)	0	0	82% (12/15)	82% (12/15)	13% (2/15)	100% (15/15)	67% (10/15)	0
Gold fish	59% (19/32)	19% (6/32)	3% (1/32)	3% (1/32)	78% (25/32)	91 % (29/32)	16% (5/15)	100% (32/32)	78% (25/32)	94% (3/32)
Sea water	70% (14/20)	25% (5/20)	0	0	80% (16/20)	90% (18/20)	15% (3/20)	100% (20/20)	55% (11/20)	10% (2/20)
Tilapia	63% (20/32)	9% (3/32)	0	0	78% (25/32)	100% (32/32)	9% (3/32)	100% (32/32)	56% (18/32)	9% (3/32)

* C125: 125 µg/ml; C250: 250 µg/ml; C500: 500 µg/ml and C1250: 1250 µg/ml cinnamaldehyde; EX 1: 4 mg/ml *K. africana* Ethyl acetate extract; EX 2: 4 mg/ml *K. africana* Dichloromethane extract; EX 3: 10 mg/ml *K. africana* Methanol extract; EX 4: 4 mg/ml *K. africana* Hexane extract; AMP10: Ampicillin; TE30: Tetracycline.

Table 4.4 Percentage of isolates with cinnamaldehyde and *K. africana* extracts activity indices ≥1, relative to ampicillin (AMP10) and tetracycline (TE30).

Compound	% of isolates with activity indices ≥ 1			
	Study Isolates		Type Strain*	
	Ampicillin (AMP10)	Tetracycline (TE30)	Ampicillin (AMP10)	Tetracycline (TE30)
125 µg/ml cinnamaldehyde	11.1 (11/99)	13.13 (13/99)	0 (0/2)	0 (0/2)
250 µg/ml cinnamaldehyde	30.30 (30/99)	40.40 (40/99)	0 (0/2)	0 (0/2)
500 µg/ml cinnamaldehyde	40.40 (40/99)	68.69 (69/99)	0 (0/2)	0 (0/2)
1250 µg/ml cinnamaldehyde	42.42 (42/99)	81.81 (81/99)	0 (0/2)	100 (2/2)
4 mg/ml *K. Africana* EX 1	7.07 (7/99)	6.06 (6/99)	0 (0/2)	0 (0/2)
4 mg/ml *K. africana* EX 2	4.04 (4/99)	0 (0/99)	0 (0/2)	0 (0/2)
10 mg/ml *K. africana* EX 3	29.29 (29/99)	33.33 (33/99)	0 (0/2)	0 (0/2)
4 mg/ml *K. africana* EX 4	0 (0/99)	0 (0/99)	0 (0/2)	0 (0/2)

*Type strains: *A. caviae* ATCC 15468^T and *A. hydrophila* ATCC 7966^T

4.4 DISCUSSION

There have been a number of studies that have used *A. hydrophila* as an indicator organism in testing the efficacy of phytochemical extracts (Senthilkumar *et al*., 2005; MahfuzulHoque *et al*., 2007; Pachanawan *et al*., 2008; Rajendiran *et al*., 2008; Siri *et al*., 2008; Acharyya *et al*., 2009; Abdel-Tawwab *et al*., 2010; Awad and Austin, 2010; Dhayanithi *et al*., 2012). Ginger, garlic, lupin, mango and stinging nettle have been observed to prevent *A. hydrophila* disease in rainbow trout (Awad and Austin, 2010; Nya *et al*., 2010). MahfuzulHoque *et al*. (2007) observed very low levels of inhibition with both the water and ethanol extracts of guava (MICs 1 – 4 mg/ml), while neem extracts had no antibacterial activity against two *A. hydrophila* strains. Abdel-Tawaad *et al*. (2010) observed that inclusion of green tea, *Camellia sinensis*, in Nile tilapia diet increased fish health against *A. hydrophila* infection, while the use of *Psidium guajava* dry leaf powder or dried ethanol extracts in fish-feed has been suggested to reduce mortality of *A. hydrophila* infected tilapia (Pachanawan *et al*., 2008). The methanol extract of *Excoecaria agallocha* leaves demonstrated antibacterial activity against *A. hydrophila* (MIC 500 µg/ml) in *Amphiprion sebae*, a marine ornamental fish (Dhayanithi *et al*., 2012). Siri *et al*. (2008) observed a dose-dependent response by *A. caviae* to phytochemicals extracted from 20 Thai plants. Senthilkumar *et al*. (2005) observed that crude extract from three Indian medicinal plants

produced antibacterial activity against *A. hydrophila* at high concentrations of 2.5 – 10 mg/ml, while Acharyya *et al*. (2005) screened nine medicinal plant methanol extracts against *A. hydrophila* and observed MICs ranging from 1 -32 mg/ml.

All the concentrations (5, 20 and 250 µg/ml) of vanillin tested did not exhibit bactericidal activity against the study isolates. This is in keeping with findings of Kappachery *et al*. (2010) and Ponnusamy *et al*. (2009), who observed that vanillin does not have bactericidal effects at concentrations ranging from 63 to 250 µg/ml. Rather vanillin inhibits quorum sensing and biofilm development of *A. hydrophila*. Jay and Rivers (1984) have reported that vanillin was more effective against fungi and non-lactic Gram-positive bacteria than Gram-negative bacteria, while Fitzgerald *et al*. (2004) observed that vanillin had a time of exposure, concentration and target organism dependency to its antimicrobial activity. It was also observed that vanillin had bacteriostatic rather than bactericidal activity. It is possible that the resistance exhibited by *Aeromonas* spp. in the present study is due to the bacteriostatic activity of vanillin.

Most isolates in the present study were susceptible to tetracycline while displaying high level resistance to ampicillin. This is to be expected because of production of inducible chromosomal β-lactamase in *Aeromonas* spp. (Ghengesh *et al*., 2008). The phytochemicals tested in this study had varying ranges of activity indices (Appendix A.2), with 1250 µg/ml cinnamaldehyde being the most effective. This concurs with findings of Morozumi (1978), who observed that cinnamaldehyde bactericidal concentrations ranged from 100 - 200 µg/ml. Ooi *et al*. (2006) observed that cinnamaldehyde inhibited both Gram-negative and Gram-positive bacteria at concentrations from 75 - 600 µg/ml. Chang *et al*. (2001) observed that *Cinnamomum osmophloeum* essential oil extract B with cinnamaldehyde as a major component (76%) had excellent antibacterial activity against Gram-negative and Gram-positive bacteria at 250, 500 and 1000 µg/ml concentrations. Similarly, in the present study, cinnamaldehyde demonstrated excellent antimicrobial activity at 250 - 1000 µg/ml. As cinnamaldehyde is a registered flavouring and foodstuff with international food safety organizations (Zhou *et al*., 2007), its application to aquaculture may be feasible.

The use of *K. africana* tree parts for the treatment of dermal, gastrointestinal and urinary tract infections in traditional medicine has been validated by literature (Grace *et al*., 2002). Eldeen and Van Staden (2007) observed that the dichloromethane, ethyl acetate and ethanol extracts of *K. africana* leaf and stem bark demonstrated antibacterial activity against Gram-

negative and Gram-positive bacteria. Owolabi *et al*. (2007) suggested a narrow-spectrum activity of ethanolic *K. africana* stem bark extract as no activity was observed against Gram-negative bacteria. Grace *et al*. (2002) observed that the antibacterial activity of *K. africana* fruits against Gram-negative and Gram-positive bacteria was due to the antimicrobial effects of a mixture of three fatty acids (palmitic acid, nonanoic acid and 8-heptadecenoic acid) in an ethyl acetate fruit extract. However in the present study, the ethyl acetate extract only moderately inhibited study isolates while the dichloromethane extract proved less bactericidal against study isolates. The methanolic *K. africana* extract (10 mg/ml) was effective with 33.3% (33/99) of study isolates demonstrating activity indices ≥ 1 when compared to tetracycline. This is similar to the findings of Jeyachandran and Mahesh (2007), who observed that this organic solvent was more suitable to verify antibacterial activity against Gram negative bacteria. This might be as a result of methanol being an amphiphilic compound and a good solvent for extraction, since it is able solubilise polar compounds and many non-polar compounds (de Britto, 2012a and b). Studies carried out by Dhayanithi *et al*. (2012) observed that the methanolic extract *Excoecaria agallocha* used exhibited maximum activity against *A. hydrophila*. Based on survey of literature available (Eldeen and Staden, 2007; Jeyachandran and Mahesh, 2007; Shai *et al*., 2008), there is notable efficacy of the methanolic extract on Gram-negative bacteria. This efficacy is attributed to exhaustive extraction quality of the organic solvent methanol, with methanol is able to solubilise the bioactive compounds with antibacterial properties of medicinal plants (Jeyachandran and Mahesh, 2007). The antimicrobial activity of the *K. africana* extracts is most likely as a result of the synergistic action of the multiple bioactive compounds found within them. The fruits of *K. africana* is known to contain flavonoids (Gouda *et al*., 2003; Saini *et al*., 2009), which has the ability form complexes with the cell wall of bacteria thereby compromising its integrity. Methanolic extracts of roots and fruits of *K. africana* demonstrates the presence of naphthoquinones, kigelinone, iso-pinnatal, dehydro-α-lapachone, lapachol, phenylpropanoids, phenylethanoid, derivatives, p-coumaric acid and freulic acid as the compounds responsible for the observed antibacterial and antifungal activity (Saini *et al*., 2009). The ethyl acetate extract moderately inhibited study isolates while the dichloromethane extract proved less inhibitory against study isolates. There appeared to be a concentration dependent difference in the inhibitory effects of cinnamaldehyde against *Aeromonas* spp. This was observed for 500 µg/ml and 1250 µg/ml concentrations there was complete inhibition observed for test isolates except for

an *A. allosaccharophila* koi isolate that was resistant. Cinnamaldehyde exhibited greater efficacy than tetracycline as an antimicrobial agent against study isolates and the *K. africana* methanol extract was more effective than ampicillin as an antimicrobial agent against isolates.

In conclusion, the present study demonstrated that cinnamaldehyde and the methanol extract of *K. africana* are promising candidates to be tested for their efficacy in the treatment of multi-drug resistant aquatic *Aeromonas* species. Further investigations will have to be carried out to ascertain the effects of antimicrobial agent synergy with cinnamaldehyde and *K. africana* phytochemical compounds and phytotherapy of infected fish with these phytochemicals.

CHAPTER FIVE
ANTI-BIOFILM EFFECT OF CINNAMALDEHYDE, VANILLIN AND CRUDE *K. africana* FRUIT EXTRACTS AGAINST RESISTANT AQUATIC *A. bestiarum* ISOLATES

5.1 Introduction

Aeromonas species are known to be successful colonizers of diverse surfaces (Lynch *et al*., 2002; Bechet and Blondeau, 2003; Elhariry, 2011; Angeles-Morales *et al*., 2012; Vinay *et al*., 2013; Igbinosa, 2014). Their possession of pili, flagella and surface adhesins play an important role in adhesion on animal and human mucosal surfaces (Kirov *et al*., 2004) and establishing infection in hosts (Janda and Abbott, 2010). Biofilm formation is a feature of persistent infection and characterizes up to 30% of *Aeromonas* gastroenteric infections (Kirov *et al*., 2004). *Aeromonas* spp. in biofilm communities are observed to be increasingly pathogenic and resistant to many antimicrobials (Igbinosa, 2014). Biofilm formation in *Aeromonas* has been described as a virulence property of this genus, aiding survival and defence mechanisms against antimicrobials in its environment (Angeles-Morales *et al*., 2012; Desai and Desai, 2014).

There are several specific biofilm phenotypes that are activated on formation of a biofilm that makes bacteria increasingly resistant (Lopez, 2010). These phenotypes may be species-specific or general in their resistance to antimicrobials (Drenkard, 2003). Comparison of the physiology of biofilm bacteria with that of planktonic bacteria identifies an increased resistance to many antimicrobial agents that are used in medicine and industry (Simoes *et al*., 2010; Fuente-Nunez *et al*., 2013). Therefore, biofilm control is important as there is an increased risk in infections associated with biofilms and with *Aeromonas* spp. their possession of mobile genetic elements increases the risk of transfer and receipt of resistance genes within and across species in biofilms.

Since planktonic bacteria are increasingly resistant and even more so when they are in biofilms, development of new antimicrobials are required for effective biofilm control. Several studies that have explored the different stages of biofilm development as targets for biofilm control (Ponnusamy *et al*., 2009; Simoes *et al*., 2010; Fuente-Nunez *et al*., 2013; Kostakioti *et al*., 2013). The most commonly targeted are the adhesion and the dispersal stages (Ponnusamy *et al*., 2009; Simoes *et al*., 2010; Fuente-Nunez *et al*., 2013). Phytochemical compounds are thus

being investigated for their anti-adhesion and anti-biofilm properties (Borges *et al*., 2012). Phytochemical compounds and plant extracts are able to inhibit biofilm formation and disperse mature biofilms (Niu and Gilbert, 2004; Ponnusamy *et al*., 2009; Khan and Ahmad, 2011; Millezi *et al*., 2013; Nazzaro *et al*., 2013). Borges *et al*. (2014) observed that dietary phytochemical such as glucosinolates from cabbage, broccoli and cauliflower prevented adhesion and biofilm formation of *P. aeruginosa*, *S. aureus* and *Listeria monocytogenes*. Husain *et al*. (2013) observed that sub-MIC concentrations of clove oil inhibited biofilm formation of *A. hydrophila* by reducing virulence properties such as protease and EPS production while not affecting cell growth. Among the various known classes of phytochemicals, phenolic phyto-compounds are reported to have great efficacy against biofilm formation by certain bacteria (Vattem *et al*., 2007; Plyuta *et al*., 2013).

Cinnamaldehyde was observed to be bactericidal to *Aeromonas* species at 125 - 1250 µg/ml (Okolie and Chenia, 2013). It has also been observed to inhibit adhesion and biofilm formation of *Pseudomonas* spp. and *E. coli* (Niu and Gilbert, 2004; Amalaradjou *et al*., 2010). Cinnamaldehyde reduced the biofilm-forming ability of *Burkholderia* species by targeting QS with an unknown mechanism of action (Brackman *et al*., 2009). Vanillin also a phenolic phytochemical is a known QS inhibitor, able to inhibit both short and long chain AHLs in *A. hydrophila* (Ponnusamy *et al*., 2009). Antimicrobial activity was not observed at concentrations of 5, 20 and 250 µg/ml against *A. hydrophila*, *A. caviae*, *A. icthiosmia*, *A. sobria*, *A. veronii*, *A. salmonicida* and *A. bestiarum* strains (Okolie and Chenia, 2013). However Kappachery *et al*. (2010) observed that vanillin inhibited the biofilm formation of *A. hydrophila* at 0.18 mg/ml, while Ponnusamy *et al*. (2014) observed biofilm reduction of *A. hydrophila* at concentrations of 0.063 – 0.25 mg/ml.

The antimicrobial activities of *K. africana* have been studied (Higgins *et al*., 2010; Okolie and Chenia, 2013). *K. africana* fruit extracts exhibited varying levels of bactericidal and bacteriostatic activities against *Aeromonas* spp. at concentrations of 4 – 10 mg/ml (Okolie and Chenia, 2013). *K. africana* stem bark and fruit dichloromethane extracts contain antimicrobial compounds such as norviburtinal and isopinnatal (Higgins *et al*., 2010). Several studies showed that the fruit extracts contained γ-sitosterols, iridoids, phenlypropanoids, phenolics, flavonoids, terpenoids, various oleic acids and essential fatty acids (Gouda, 2006; Saini *et al*., 2009; Higgins *et al*., 2010).

Aeromonas species are successful colonizers of various biotic and abiotic surfaces (Kregiel and Niedzielska, 2014). Although *A. hydrophila* and *A. caviae* (Bechet and Blondeau, 2003; Khajanchi *et al*., 2009; Jahid *et al*., 2014) are the most common models used in studying biofilm formation in *Aeromonas* species, there is very little information available on biofilm formation by other *Aeromonas* species and their control. *A. bestiarum* is an important fish pathogen and is known to cause motile aeromonad septicemia (MAS) (Kozinska and Guz, 2004; Turska-Szewczuk *et al*., 2013). They are reported to be multi-drug resistant to several classes of antimicrobials (Kadlec *et al*., 2011). *A. bestiarum* in the present study was observed to be multidrug resistant to penicillins, first, second and third generation cephalosporins, trimethoprim, sulphamethoxazole, macrolides and aminoglycosides. It has been established that *K. africana* fruit extracts possess both antimicrobial and anti-quorum sensing properties (Higgins *et al*., 2010; Chenia, 2013), however, there is no information in literature about its anti-biofilm activities. The aim of this study was thus to determine the anti-biofilm effect of trans-cinnamaldehyde, vanillin and *Kigelia africana* fruit extracts against multi-drug resistant *A. bestiarum* isolates.

5.2 Materials and Methods

5.2.1 Bacterial isolates

Aeromonas bestiarum (n = 19; Table 5.1) were selected for study. Antimicrobial resistance phenotype (Duma, 2012), and source of isolation of the 19 *A. bestiarum* strains are shown in Table 5.1. Type strains *A. caviae* ATCC 15468^T and *A. hydrophila* ATCC 7966^T were used as controls for both the initial attachment and pre-formed, mature biofilm assays.

5.2.2 Effect of phytochemicals on initial attachment (IA) and mature biofilm (MB) by *A. bestiarum*

Trans-cinnamaldehyde, vanillin, *K. africana* fruit extracts (as described in Chapter four) and commercial 10% ethanol *K. africana* (PhytoForce, SA) extract, were used to determine their effect on initial attachment and biofilm detachment using modified microtiter plate assays (Basson *et al*., 2008). Isolates were grown overnight in TSB, washed three times with sterile distilled water and the turbidity of the cell suspensions adjusted to that equivalent to a 0.5

McFarland standard. The first assay investigated the effect of phytochemicals and/or extracts on initial attachment of aeromonad isolates.

Phytochemicals/extracts at a final concentration of 50 and 100 µg/ml trans-cinnamaldehyde (Table 5.2), 100 and 250 µg/ml vanillin, 150 and 300 mg/ml of 10% ethanolic (PhytoForce) extract of *K. africana*, and 0.5, 1, 2 and 4 mg/ml each of *K. africana* extracts (EX1, EX2, EX3, and EX4) were added to 90 µl TSB and 10 µl of respective cell suspensions and incubated for 24 h at 30 ºC with agitation.

For the second assay, biofilms were grown for 24 h without treatment at 30 ºC, following which pre-formed biofilms were exposed to respective trans-cinnamaldehyde, vanillin, 10% ethanolic extract of *K. africana* and *K. africana* fruit extracts in TSB (90 µl) and incubated for a further 24 h at 30 ºC with agitation. The negative control contained only broth, while the positive controls contained the respective cell suspensions in TSB with no phytochemicals added.

Contents of each well were aspirated, washed three times with 250 µl of sterile distilled water and the remaining cells were fixed with 200 µl of methanol for 15 min. After air-drying, wells were stained with 150 µl of 2% Hucker's crystal violet for 5 min. Excess crystal violet was removed by gently rinsing plates under running tap water and air dried. Dye bound to the adherent cells was resolubilized with 150 µl of 33% (v/v) glacial acetic acid, and the optical density (OD) of each well was obtained at 595 nm using the GloMax (Ascent F1, Thermo lab systems). Tests were done in triplicate, on three occasions and the results averaged (Basson et al., 2008). The OD595 nm of the control wells without phytochemicals were compared to wells with phytochemicals to determine the effect of these phytochemicals on biofilm formation.

A measure of efficacy called Percentage biofilm reduction was calculated from the blank (negative control), control (positive control), and treated absorbance values (Pitts *et al*., 2003): Percentage reduction $=\left[\frac{(C-B)-(T-B)}{C-B}\right] \times 100$, where B denotes the average absorbance per well for blank wells (no biofilm, no treatment), C denotes the average absorbance per well for control wells (biofilm, no treatment), and T denotes the average absorbance per well for treated wells (biofilm and treatment).

Table 5.1: *Aeromonas bestiarum* strains selected for anti-biofilm study of phytochemicals and crude extracts of *K. africana* fruit extracts.

Strains		Source	Resistance Phenotypes[#]	*bla-*$_{TEMI}$[*]	*bla-*$_{TEMII}$[*]	*intI*[*]	*intII*[*]	*qac*[*]	*sulI*[*]	*sulII*[*]	CS[*]	HEP[*]
M12	*A. bestiarum*	Catfish	AMP,AML,OX,W,RL									
M69	*A. bestiarum*	Goldfish	AMP,AML,AMC,CPD,OX,W,S,RL					+		+		
M70	*A. bestiarum*	Goldfish	AMP,AML,AMC,CPD,OX,T,W,RL									
M71	*A. bestiarum*	Goldfish	AMP,AML,CPD,CXM,E,OX,W									
M72	*A. bestiarum*	Goldfish	AMP,AML,OX,W,RL					+				
M73	*A. bestiarum*	Goldfish	AMP,AML,AMC,CXM,FOX,OX,W,RL					+				
M78	*A. bestiarum*	Goldfish	AMP,AML,AMC,CPD,CXM,OX,W,RL									
M79	*A. bestiarum*	Goldfish	APM,AML,OX									
M80	*A. bestiarum*	Goldfish	AMP,AML,NA,OX,T,W		+			+	+	+		
M81	*A. bestiarum*	Goldfish	AMP,AML,CXM,CTX,OX,W,RL					+				
M83	*A. bestiarum*	Goldfish	AMP,AML,OX,W,RL					+	+			+
M84	*A. bestiarum*	Goldfish	AMP,AML,OX,W,RL					+	+			
M87	*A. bestiarum*	Goldfish	AMP,AML,AMC,OX,W,RL				+	+	+			+
M88	*A. bestiarum*	Goldfish	AMP,AML,AMC,CPD,CXM,E,FOX,OX,W,RL				+	+	+	+		
M90	*A. bestiarum*	Goldfish	AMP,AML,AMC,CXM,OX,W,RL					+				
M91	*A. bestiarum*	Goldfish	AMP,AML,OX,W,RL					+				
M96	*A. bestiarum*	Goldfish	E,OX,T					+				
M97	*A. bestiarum*	Goldfish	E,OX,T	+				+				
M99	*A. bestiarum*	Goldfish	AMP,AML,CPD,CXM,OX					+				

**intI*=integrase class 1 gene, *intII* =integrase class 2 gene, *qacEΔ1*=quaternary ammonium metal, *sul*=sulphonamide genes, CS=integron I variable region, Hep=integron II variable region AMP=ampicillin (AMP10), AML=amoxicillin (AML10), AMC=augmentin (AMC30), AZM=azithromycin (AZM15), ATM=aztreonam (ATM30), FOX=cefoxitin (FOX30), CPD=cefpodoxime (CPD10), CXM=cefuroxime (CXM30), CTX=cefotaxime (CTX5), E=erythromycin (E15), NA=nalidixic acid (NA30), OX=oxacillin (OX1), TE=tetracycline (TE30), W=trimethoprim (W1.25), S=streptomycin (S10), RL=sulphamethoxazole (RL25), TS=cotrimoxazole (TS25).

5.2.3 Statistical analysis

Differences in adhesion between untreated and treated samples were determined by Paired *t*-tests or Wilcoxon signed rank tests if the homogeneity of variances test failed (Sigma Stat V3.5, Systat Software, Inc; San Jose, CA, USA). Differences were considered significant if $p < 0.05$.

5.3 Results

5.3.1 Effect of phytochemicals on initial attachment and biofilm formation by *Aeromonas* spp. isolates

Since cinnamaldehyde was bactericidal against study isolates at concentrations (125 - 1250 µg/ml; Chapter Four), sub-inhibitory concentrations of 50 and 100 µg/ml were used to ascertain the anti-biofilm effect. Treatment with 50 µg/ml cinnamaldehyde decreased adhesion for both *A. caviae* ATCC 15468^T and *A. hydrophila* ATCC 7966^T while 100 µg/ml increased adhesion of *A. caviae* ATCC 15468^T (Fig. 5.1). Treatment of *A. bestiarum* biofilms with 50 and 100 µg/ml concentrations of cinnamaldehyde exhibited similar anti-biofilm activities. Following treatment with 50 µg/ml cinnamaldehyde, decreased adhesion of 73.7% (14/19) of isolates, increased adhesion of 21.1% (4/19) of isolates and no effect on 5.3% (1/19) of isolates was observed. Treatment with 50 µg/ml in the initial attachment assay resulted in statistical significant altered adherence ($p = 0.025$). The 100 µg/ml concentration decreased adhesion of 63.2% (12/19) of isolates, increased adhesion of 26.3% (5/19) of isolates and had no effect on adhesion for 10.5% (2/19) of isolates (Fig. 5.1). Treatment with 100 µg/ml in the initial attachment assay resulted in statistical significant altered adherence ($p = 0.123$).

Decrease in adhesion for *A. caviae* ATCC 15468^T and *A. hydrophila* ATCC 7966^T were observed on treatment with 100 and 250 µg/ml of vanillin (Fig. 5.2). While 5, 20 and 250 µg/ml concentrations did not demonstrate antimicrobial activity against study isolates, the 100 and 250 µg/ml concentrations of vanillin exhibited similar activities against *A. bestiarum* biofilms. Where the 100 µg/ml concentration decreased adhesion of 15.8% (3/19) of isolates and increased adhesion for 84.2% (16/19) of isolates. Treatment with 100 µg/ml in the initial attachment assay resulted in statistical significant altered adherence ($p = 0.758$).Treatment with 250 µg/ml concentration decreased adhesion of 47.4% (9/19) of isolates, increased adhesion of 42.1% (8/19) of isolates and had no effect on adhesion for 10.5% (2/19) of isolates (Fig. 5.2). Treatment

with 50 µg/ml in the initial attachment assay resulted in statistical significant altered adherence ($p = 0.011$).

A. caviae isolate ATCC 15468^T and *A. hydrophila* ATCC 7966^T exhibited an increase in adhesion on treatment with both concentrations of *K. africana* ethanol extract (Fig. 5.3). Exposure to 150 µg/ml *K. africana* ethanol extract increased adhesion of 100% (19/19) of isolates. Treatment with 150 µg/ml in the initial attachment assay resulted in statistical significant altered adherence ($p = 0.001$).While 300 µg/ml decreased adhesion for 5.3% (1/19) of isolates and increased adhesion for 94.7% (18/19) of isolates (Fig. 5.3). Treatment with 300 µg/ml in the initial attachment assay resulted in statistical significant altered adherence ($p = 0.001$).

The sub-inhibitory concentrations (0.5, 1 and 2 mg/ml) and inhibitory concentration (4 mg/ml) used for the four extracts of *K. africana* (EX1, EX2, EX3 and EX4) exhibited varying levels of activities against *A. bestiarum* adhesion in the initial attachment assay (Fig. 5.1 – 5.7; Table 5.2). For *A. caviae* ATCC 15468^T, treatment with 0.5 and 2 mg/ml of the EX1 extract increased adhesion while treatment with 1 and 4 mg/ml decreased adhesion. For *A. hydrophila* ATCC 7966^T, treatment with 0.5, 1 and 2 µg/ml decreased adhesion, while 4 mg/ml had no effect on adhesion (Fig. 5.4). Reduction in adhesion was observed for 42.1% (8/19) of isolates and increased adhesion of 57.9% (11/19) of isolates on treatment with 0.5 mg/ml of EX1. Treatments with 1 mg/ml of extract resulted in decreased adhesion for 15.8% (3/19) of isolates and increase in adhesion for 84.2% (16/19) of isolates. The 2 mg/ml concentration decreased adhesion for 31.6% (6/19) isolates and increased adhesion for 68.4% (13/19). Treatment with 0.5, 1 and 2 µg/ml in the initial attachment assay resulted in statistical significant altered adherence ($p = 0.289$), ($p = 0.002$), ($p = 0.015$) respectively.While the 4 mg/ml concentration decreased adhesion for 47.4% (9/19) and increased adhesion for 52.6% (10/19) of isolates (Fig. 5.4). Treatment with 4 µg/ml in the initial attachment assay resulted in statistical significant altered adherence ($p = 0.726$).

Both *A. caviae* ATCC 15468^T and *A. hydrophila* ATCC 7966^T exhibited decreased adhesion on treatment with all concentrations of EX2 extract (0.5, 1, 2 and 4 mg/ml) (Fig. 5.5). Treatment of *A. bestiarum* isolates with 0.5 mg/ml EX2 extract decreased adhesion of 52.6% (10/19) of isolates, increased adhesion of 36.4% (7/19) of isolates and had no effect on 10.5% (2/19) of isolates. Treatment with 0.5 mg/ml in the initial attachment assay resulted in statistical

significant altered adherence (p = 0.961). Both 1 and 2 mg/ml concentrations decreased adhesion of 42.1% (8/19) isolates, increased adhesion for 52.6% (10/19) and no effect on 5.3% (1/19) of isolates. Treatment with 1 and 2 mg/ml in the initial attachment assay resulted in statistical significant altered adherence (p = 0.080) (p = 0.325) respectively. Exposure to 4 mg/ml concentration decreased adhesion of 42.1% (8/19) of isolates, increased adhesion of 47.4% (9/19) of isolates and had no effect on 10.5% (2/19) of isolates (Fig. 5.5). Treatment with 4 mg/ml in the initial attachment assay resulted in statistical significant altered adherence (p = 0.812).

For EX3 extract, treatment of *A. caviae* ATCC 15468^T with 0.5, 1 and 4 mg/ml decreased adhesion, while 2 mg/ml increased adhesion. For *A. hydrophila* ATCC 7966^T, treatment with all concentrations (0.5, 1, 2 and 4 mg/ml) decreased adhesion (Fig. 5.6). Treatment of isolates with 0.5 mg/ml decreased adhesion of 36.8% (7/19) of isolates and increased adhesion of 63.1% (12/19) of isolates. Treatment with 0.5 mg/ml in the initial attachment assay resulted in statistical significant altered adherence (p = 0.150). Decreased adhesion of 26.3% (5/19) of isolates and increased adhesion of 73.7% (14/19) of isolates was observed on treatment with 1, 2 and 4 mg/ml (Fig. 5.6). Treatment with 0.5, 1, 2 mg/ml in the initial attachment assay resulted in statistical significant altered adherence (p = 0.008) (p = 0.015) (p = 0.006) respectively.

EX4 extract treatment of *A. caviae* ATCC 15468^T at all concentrations (0.5, 1, 2 and 4 mg/ml) decreased adhesion, while for *A. hydrophila* ATCC 7966^T, treatment with 0.5, 2 and 4 mg/ml decreased adhesion of biofilms and 1 mg/ml had no effect on adhesion (Fig. 5.7). Treatment of study isolates with both 0.5 mg/ml EX4 extract decreased the adhesion of 15.8% (3/19) of isolates, increased adhesion of 73.7% (14/19) of isolates and had no effect on adhesion of 10.5% (2/19) of isolates. Treatment with 0.5 mg/ml in the initial attachment assay resulted in statistical significant altered adherence (p = 0.006).Treatment with 2 mg/ml decreased the adhesion for 21.1% (4/19) isolates and increased adhesion for 78.9% (15/19) isolates. Treatment with 2 mg/ml in the initial attachment assay resulted in statistical significant altered adherence (p = 0.001). While both 1 and 4 mg/ml decreased the adhesion for 10.5% (2/19) isolates and increased adhesion for 89.5% (17/19) (Fig. 5.7). Treatment with 1 and 4 mg/ml in the initial attachment assay resulted in statistical significant altered adherence (p = 0.001) (p = 0.001).

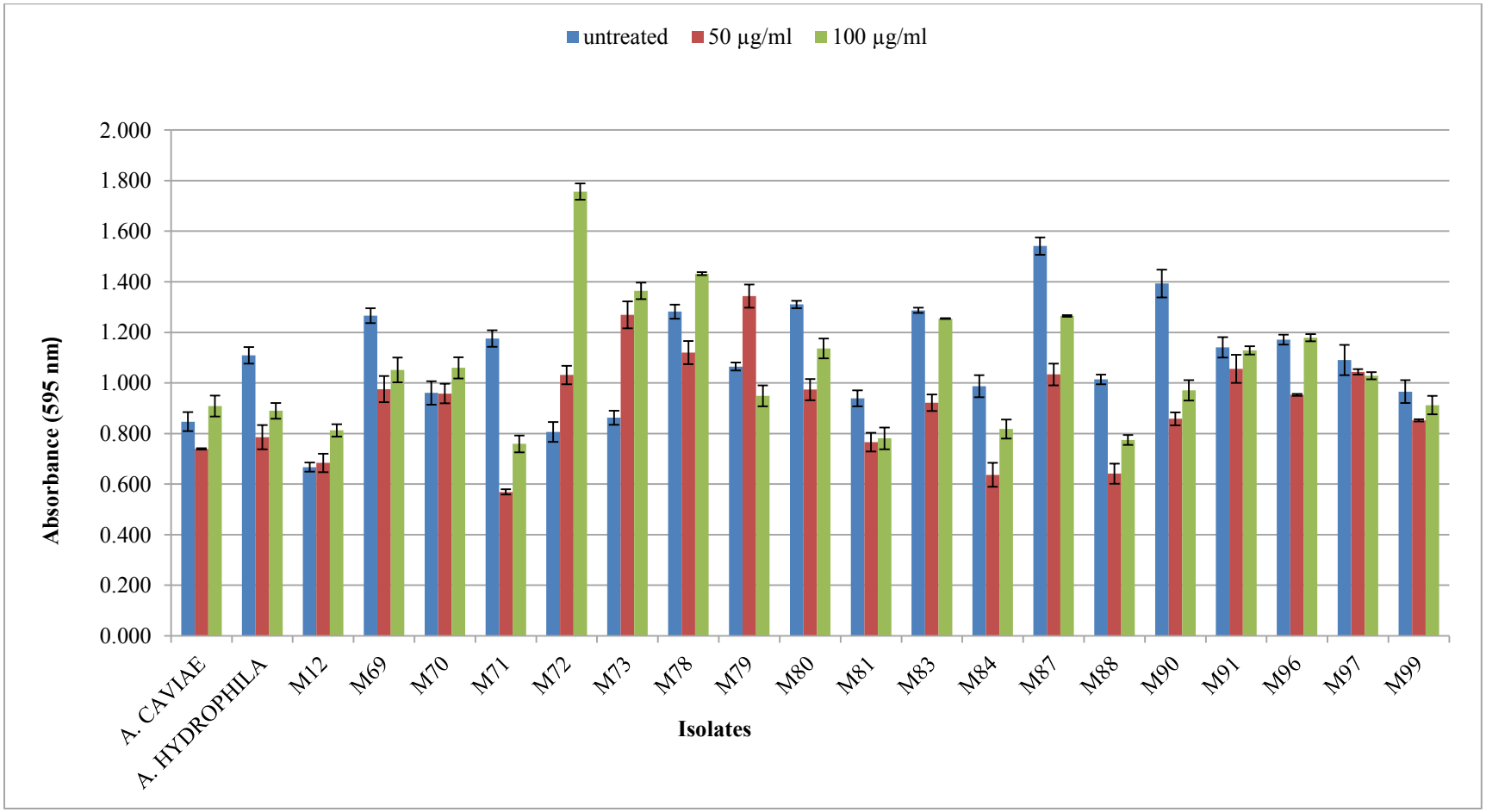

Figure 5.1: Effect of 50 and 100 µg/ml of cinnamaldehyde on initial attachment of *A. bestiarum* isolates following addition at the time of inoculation, using micro-titre plate assays. Data represents the mean standard deviations of three replicates on three separate occasions.

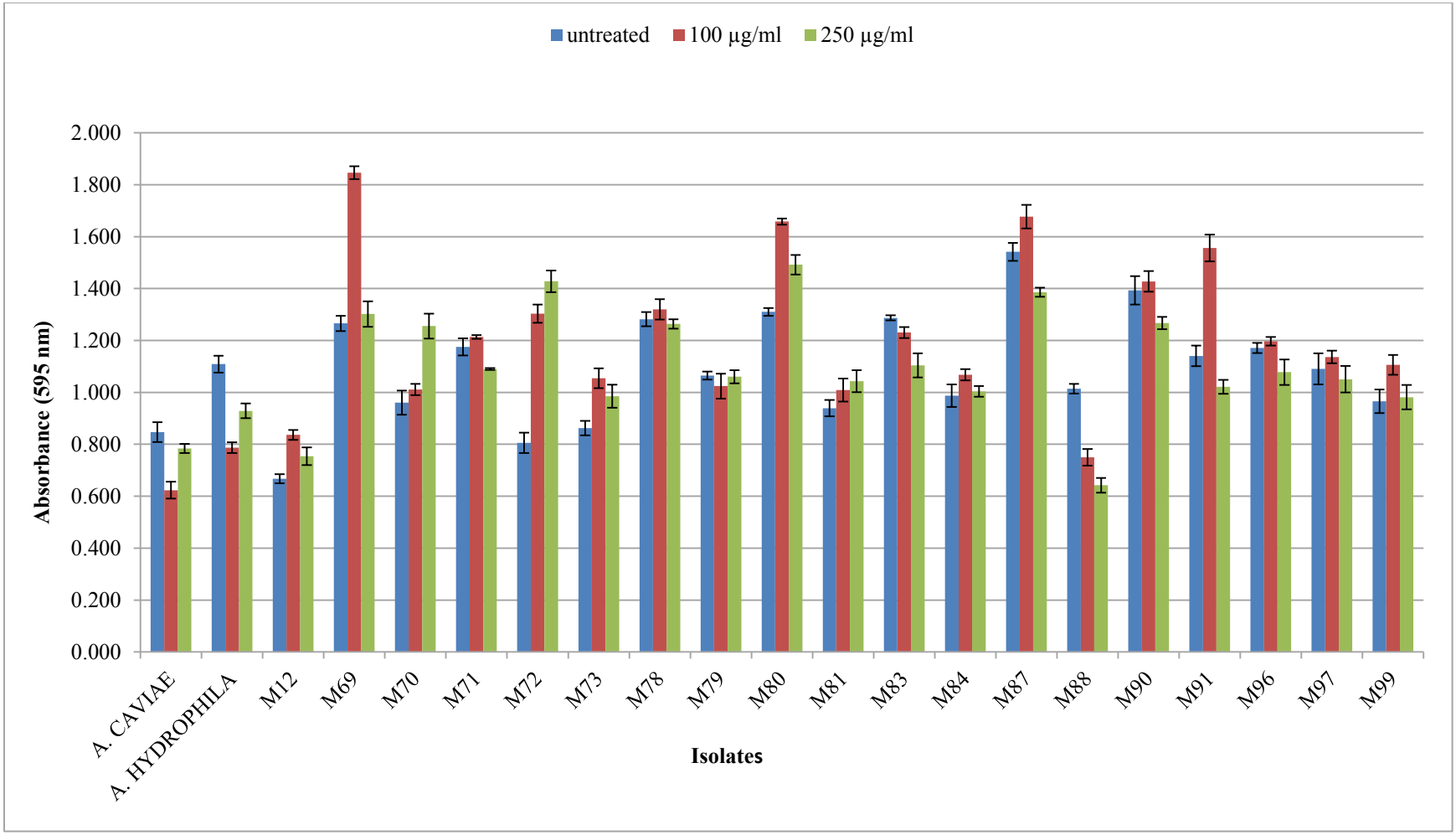

Figure 5.2: Effect of 100 and 250 µg/ml vanillin on initial attachment of *A. bestiarum* isolates following addition at the time of inoculation, using micro-titre plate assays. Data represents the mean standard deviations of three replicates on three separate occasions.

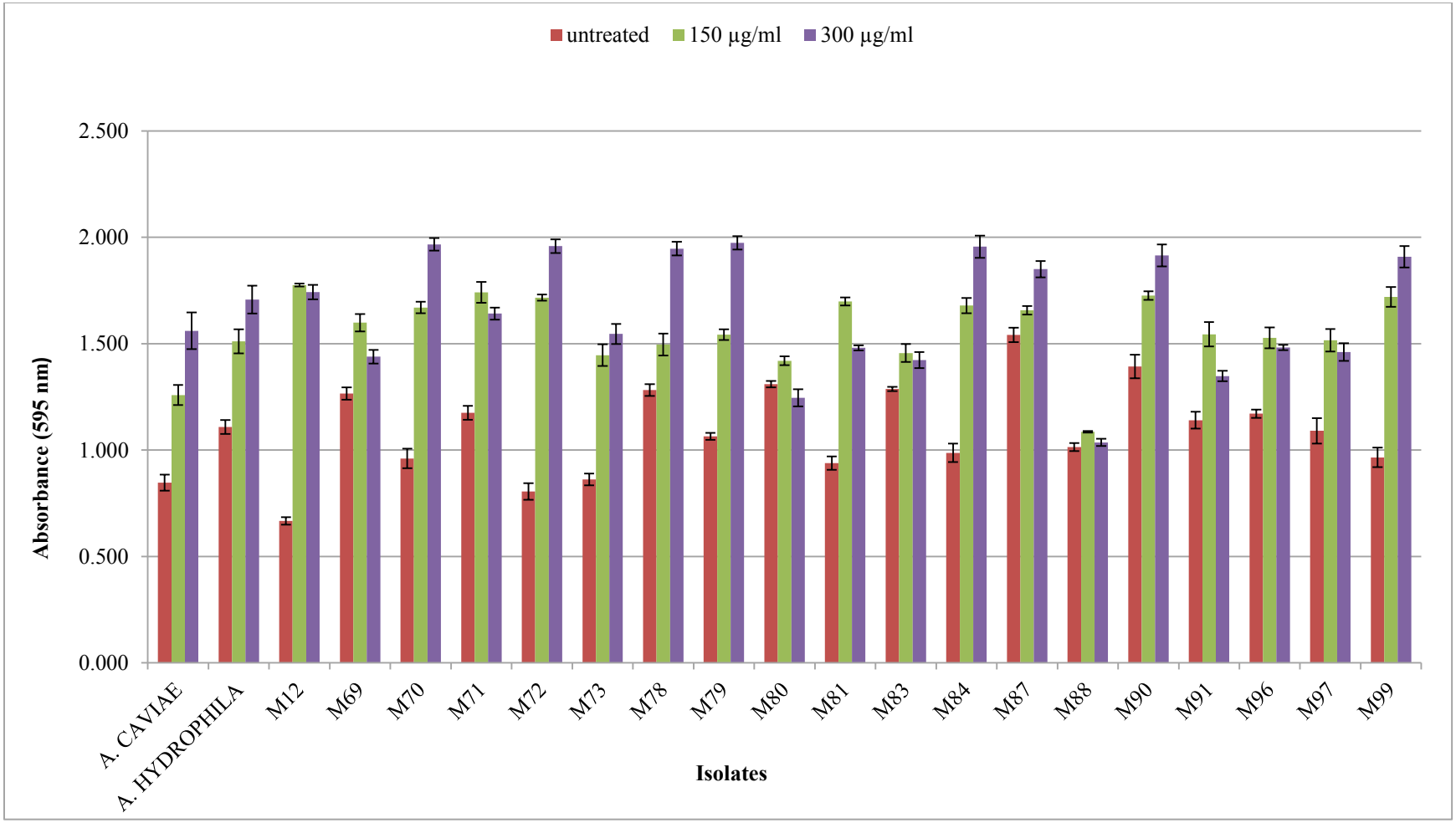

Figure 5.3: Effect of 150 and 300 µg/ml 10% *K. africana* ethanol (PhytoForce) extract on initial attachment of *A. bestiarum* isolates following addition at the time of inoculation, using the micro-titre plate assays. Data represents the mean standard deviation of three replicates on three separate occasions.

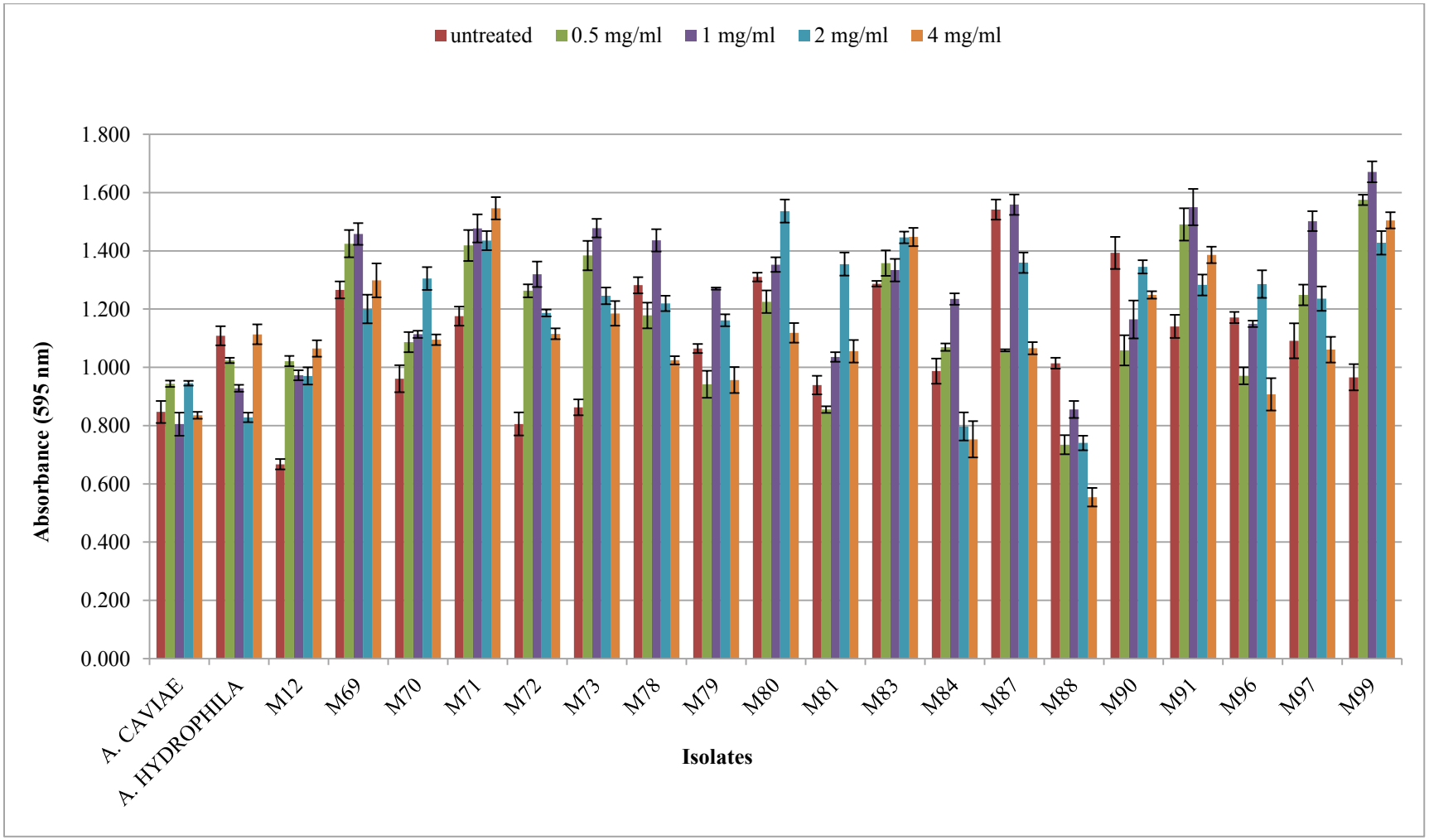

Figure 5.4: Effect of 0.5, 1, 2 and 4 mg/ml ethyl acetate EX1 *K. africana* fruit extract on initial attachment of *A. bestiarum* isolates following addition at the time of inoculation, using the micro-titre plate assays. Data represents the mean standard deviation of three replicates on three separate occasions.

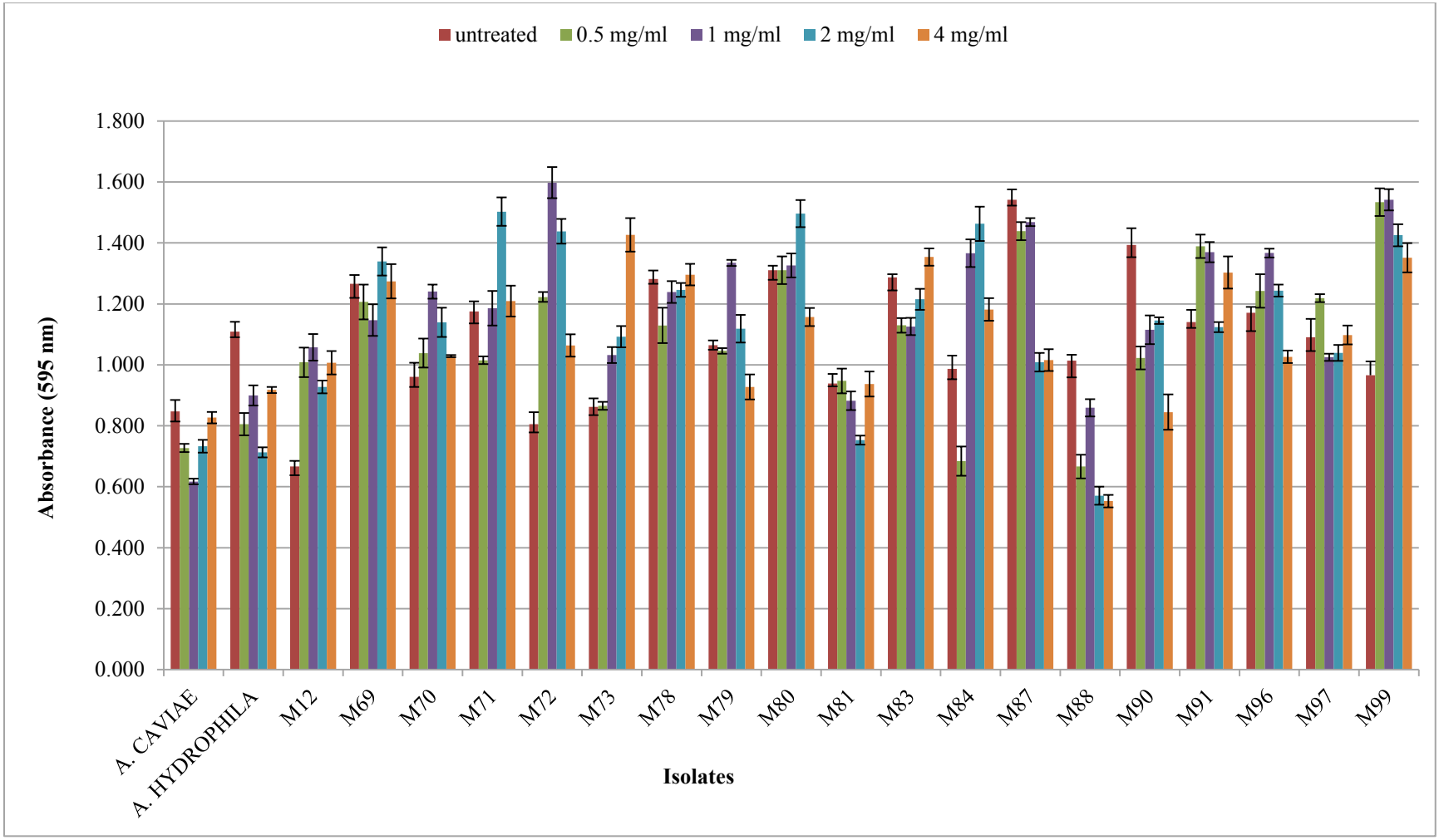

Figure 5.5: Effect of 0.5, 1, 2 and 4 mg/ml dichloromethane EX2 *K. africana* fruit extract on initial attachment of *A. bestiarum* isolates following addition at the time of inoculation, using the micro-titre plate assays. Data represents the mean standard deviation of three replicates on three separate occasions.

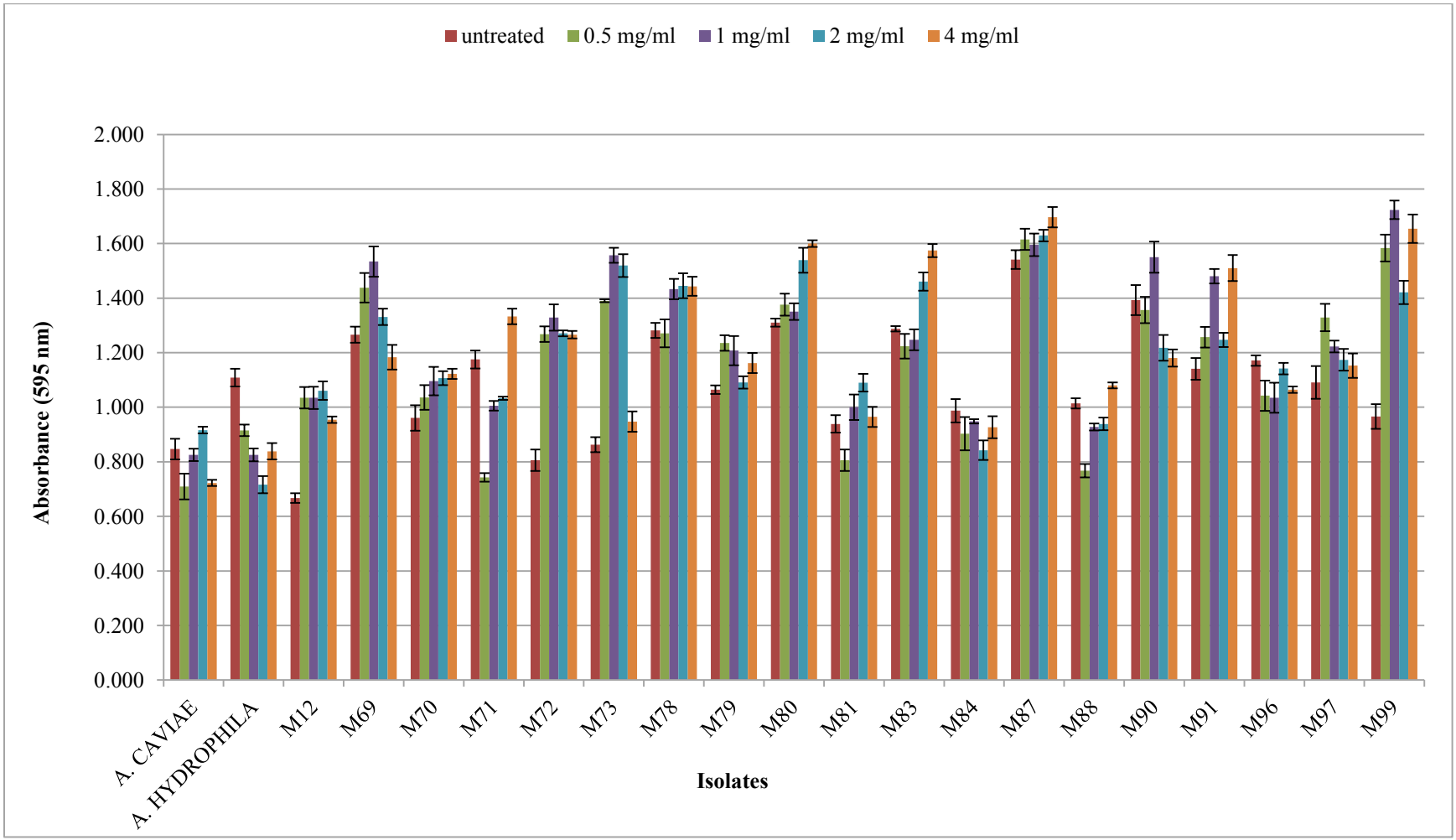

Figure 5.6: Effect of 0.5, 1, 2 and 4 mg/ml methanol EX3 *K. africana* fruit extract on initial attachment of *A. bestiarum* isolates following addition at the time of inoculation, using the micro-titre plate assays. Data represents the mean standard deviation of three replicates on three separate occasions.

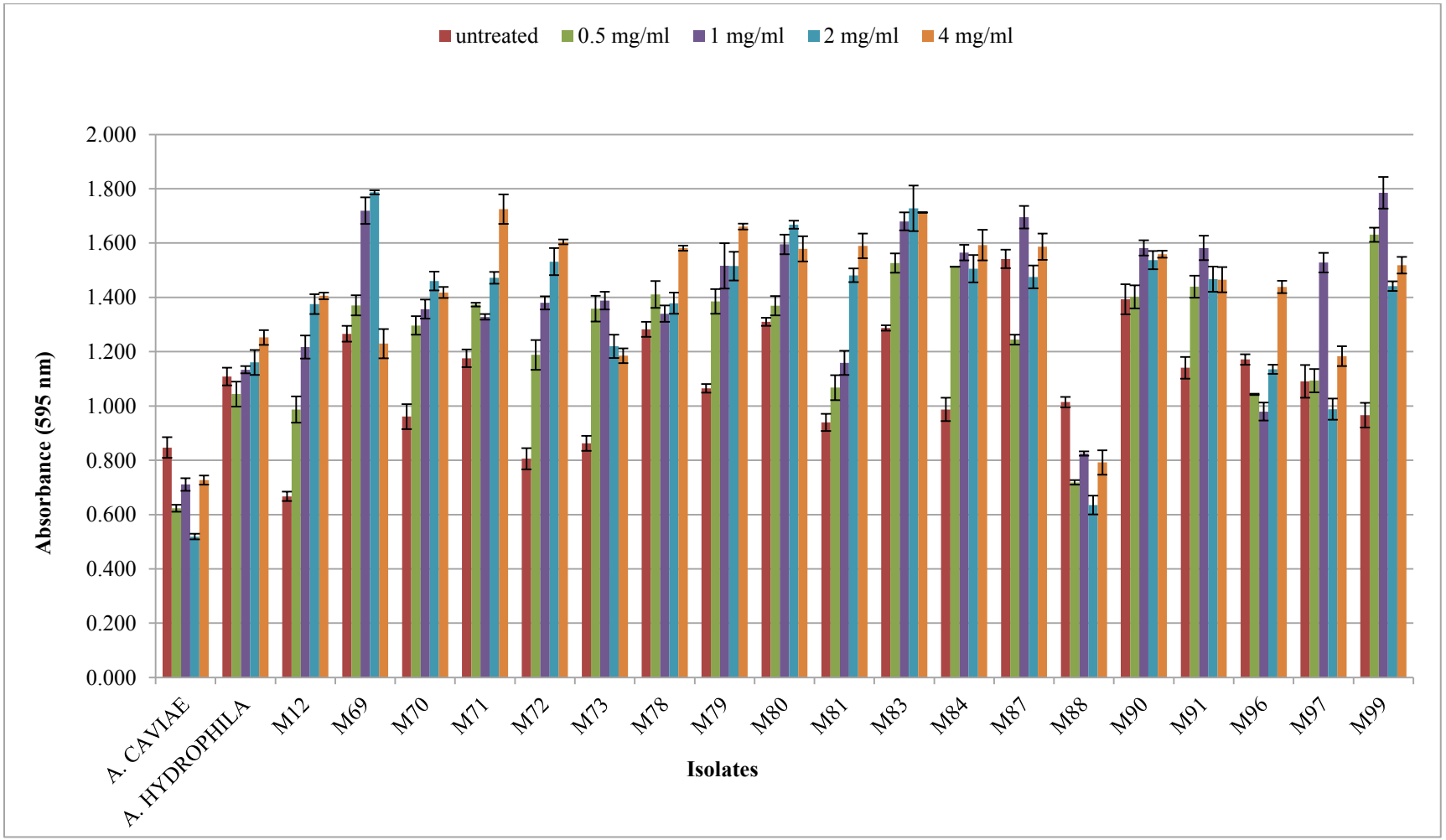

Figure 5.7: Effect of 0.5, 1, 2 and 4 mg/ml hexane EX4 *K. africana* fruit extract on initial attachment of *A. bestiarum* isolates following addition at the time of inoculation, using the micro-titre plate assays. Data represents the mean standard deviation of three replicates on three separate occasions.

5.3.2 Effect of phytochemicals on mature biofilm of *A. bestiarum* isolates

Type strains *A. caviae* ATCC 15468^T and *A. hydrophila* ATCC 7966^T exhibited similar trends where 50 and 100 µg/ml concentrations of cinnamaldehyde increased detachment of biofilm (Fig. 5.8). Treatment of *A. bestiarum* biofilms with 50 and 100 µg/ml concentrations of cinnamaldehyde exhibited varying anti-biofilm activities pre-formed biofilm assay. Following treatment with 50 µg/ml increased detachment was observed for 63.2% (12/19) of isolates (Table 5.2) and increased adhesion for 36.8% (7/19) of isolates. Treatment with 50 µg/ml in the pre-formed biofilms assay resulted in statistical significant altered adherence ($p = 0.144$). Exposure to 100 µg/ml increased detachment for 57.9% (11/19) of isolates and increased adhesion for 42.1% (8/19) of isolates (Fig. 5.8). Treatment with 100 µg/ml in the pre-formed biofilms assay resulted in statistical significant altered adherence ($p = 0.457$).

Increase in detachment of *A. caviae* ATCC 15468^T and *A. hydrophila* ATCC 7966^T biofilms was observed on exposure to 100 and 250 µg/ml concentrations of vanillin (Fig. 5.9). Treatment with 100 µg/ml in the pre-formed biofilms assay resulted in statistical significant altered adherence ($p = 0.799$). Treatment with 100 and 250 µg/ml concentrations of vanillin demonstrated similar activities against *A. bestiarum* biofilms. Exposure to 100 µg/ml increased detachment for 63.2% (12/19) of isolates and increased adhesion for 36.8% (7/19) of isolates. Treatment with 250 µg/ml increased detachment for 57.9% (11/19) of isolates and increased adhesion for 42.1% (8/19) of isolates (Fig. 5.9). Treatment with 250 µg/ml in the pre-formed biofilms assay resulted in statistical significant altered adherence ($p = 0.145$).

By contrast, *A. caviae* ATCC 15468^T and *A. hydrophila* ATCC 7966^T exhibited an increase in adhesion on exposure to both concentrations of *K. africana* ethanol extract (Fig. 5.10). Treatment with both 150 and 300 µg/ml of *K. africana* ethanol extract increased detachment for 10.5% (2/19) of isolates and increased adhesion of 89.5% (17/19) of isolates (Fig. 5.10). Treatment with 150 µg/ml and 300 µg/ml in the pre-formed biofilms assay resulted in statistical significant altered adherence ($p = 0.001$) ($p = 0.001$) respectively.

The various concentrations (0.5, 1, 2 and 4 mg/ml) used for the four *K. africana* (EX1, EX2, EX3 and EX4) extracts exhibited varying levels of inhibition against *A. bestiarum* adhesion in the pre-formed assay (Fig. 5.11 – 5.14; Table 5.2). Following treatment of *A. caviae* ATCC 15468^T and *A. hydrophila* ATCC 7966^T with 0.5, 1, 2 and 4 mg/ml EX1 extract, increased

detachment for biofilms was observed (Fig. 5.11). Treatment with 0.5 mg/ml EX1 extract exhibited increased detachment for 47.4% (9/19) of isolates and increased adhesion for 52.6% (10/19) of isolates. Treatment with 0.5 µg/ml in the pre-formed biofilms assay resulted in statistical significant altered adherence (p = 0.395). Treatment with 1 mg/ml increased detachment for 31.6% (6/19) of isolates and increased adhesion for 68.4% (13/19) of isolates. Treatment with 1µg/ml in the pre-formed biofilms assay resulted in statistical significant altered adherence (p = 0.059). Treatment with 2 mg/ml increased detachment for 31.6% (6/19) of isolates, increased adhesion for 57.9% (11/19) of isolates and had no effect for 10.5% (2/19) of isolates. Treatment with 2 µg/ml in the pre-formed biofilms assay resulted in statistical significant altered adherence (p = 0.889).Treatment of isolates with 4 mg/ml increased detachment for 42.1% (8/19) of isolates and increased adhesion for 57.9% (11/19) of isolates (Fig. 5.11). Treatment with 4 µg/ml in the pre-formed biofilms assay resulted in statistical significant altered adherence (p = 0.241).

Treatment of *A. caviae* ATCC 15468^T with 0.5 and 1 mg/ml EX2 extract increased detachment of biofilms, while 2 and 4 mg/ml had no effect. For *A. hydrophila* ATCC 7966^T 0.5, 1 and 2 mg/ml increased detachment for biofilms, while 4 mg/ml had no effect (Fig. 5.12). Following treatment with 0.5, 2 and 4 mg/ml of EX2 extract, increased detachment for 47.4% (9/19) of isolates and increased adhesion for 52.6% (10/19) of isolates was observed. Treatment with 0.5, 2 and 4 µg/ml in the pre-formed biofilms assay resulted in statistical significant altered adherence (p = 0.703) (p = 0.619) and (p = 0.609) respectively. Treatment with 1 mg/ml increased detachment for 26.3% (5/19) of isolates, increased adhesion for 63.2% (12/19) of isolates and had no effect on 10.5% (2/19) of isolate (Fig. 5.12). Treatment with 1 µg/ml in the pre-formed biofilms assay resulted in statistical significant altered adherence (p = 0.039).

For *A. caviae* ATCC 15468^T exposure to 0.5, 1, 2 and 4 mg/ml of EX3 extract increased detachment for biofilms, while for *A. hydrophila* ATCC 7966^T treatment with 1, 2 and 4 mg/ml increased detachment for biofilms and the 0.5 mg/ml had no effect (Fig. 5.13). Treatment of test isolates with 0.5 mg/ml EX3 extract increased detachment for 42.1% (8/19) of isolates and increased adhesion for 57.9% (11/19) of isolates. Treatment with 0.5 µg/ml in the pre-formed biofilms assay resulted in statistical significant altered adherence (p = 0.942). Increased detachment for 36.8% (7/19) of isolates, increased adhesion for 57.9% (11/19) of isolates and no effect against 5.3% (1/19) of isolate was observed on treatment with 1 mg/ml. Treatment with 1

µg/ml in the pre-formed biofilms assay resulted in statistical significant altered adherence (p = 0.172). Following treatment with 2 mg/ml, increased detachment for 42.1% (8/19) of isolates and increased adhesion for 57.9% (11/19) of isolates was observed. Treatment with 2 µg/ml in the pre-formed biofilms assay resulted in statistical significant altered adherence (p = 0.807). Exposure of isolates to 4 mg/ml increased detachment for 36.8% (7/19) of isolates and increased adhesion for 63.2% (12/19) of isolates (Fig. 5.13). Treatment with 4 µg/ml in the pre-formed biofilms assay resulted in statistical significant altered adherence (p = 0.414).

For *A. caviae* ATCC 15468^T treatment with all concentrations (0.5, 1, 2 and 4 mg/ml) of EX4 extract increased detachment for biofilms, while for *A. hydrophila* ATCC 7966^T treatment with 0.5, 1, and 2 mg/ml increased detachment for biofilms and the 4 mg/ml had no effect (Fig. 5.14). Treatment of test isolates with 0.5 mg/ml EX4 extract, increased detachment for 21.1% (4/19) of isolates and increased adhesion for 78.9% (15/19) of isolates. Treatment with 0.5 µg/ml in the pre-formed biofilms assay resulted in statistical significant altered adherence (p = 0.008). Exposure to 1 mg/ml increased detachment for 10.5% (2/19) of isolates, increased adhesion for 84.2% (16/19) of isolates and had no effect on 5.3% (1/19) of isolates. Treatment with 1 µg/ml in the pre-formed biofilms assay resulted in statistical significant altered adherence (p = 0.002).Treatment of isolates with 2 mg/ml increased detachment for 15.8% (3/19) of isolates and increased adhesion for 84.2% (16/19) of isolates. Treatment with 2 µg/ml in the pre-formed biofilms assay resulted in statistical significant altered adherence (p = 0.010). Exposure to 4 mg/ml increased detachment for 10.5% (2/19) of isolates, increased adhesion for 78.9% (15/19) of isolates and had no effect for 10.5% (2/19) of isolate (Fig. 5.14). Treatment with 4 µg/ml in the pre-formed biofilms assay resulted in statistical significant altered adherence (p = 0.005).

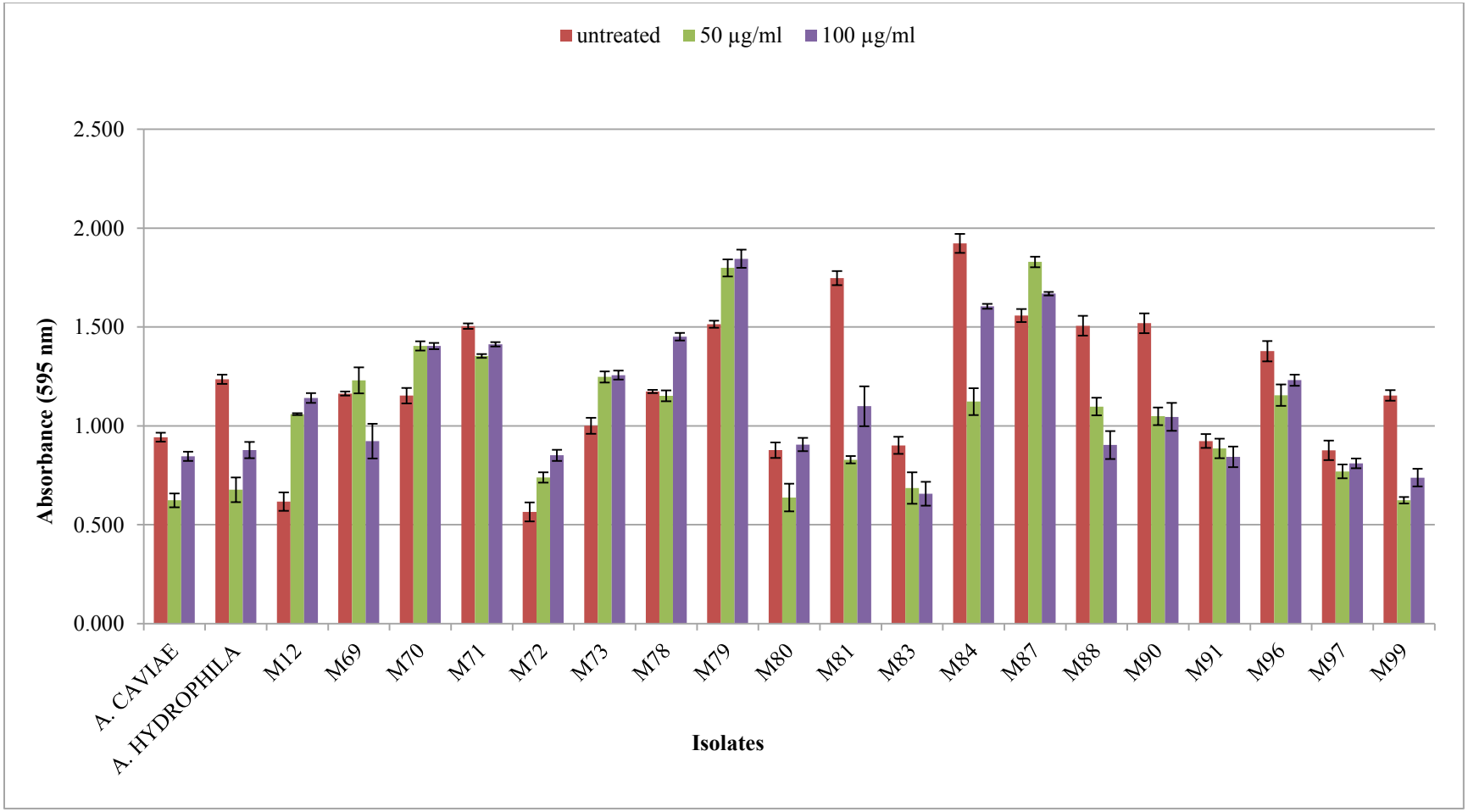

Figure 5.8: Effect of 50 and 100 µg/ml cinnamaldehyde on pre-formed biofilm of *A. bestiarum* isolates following addition to 24 h biofilm, using micro-titre plate assays. Data represents the mean standard deviation of three replicates on three separate occasions.

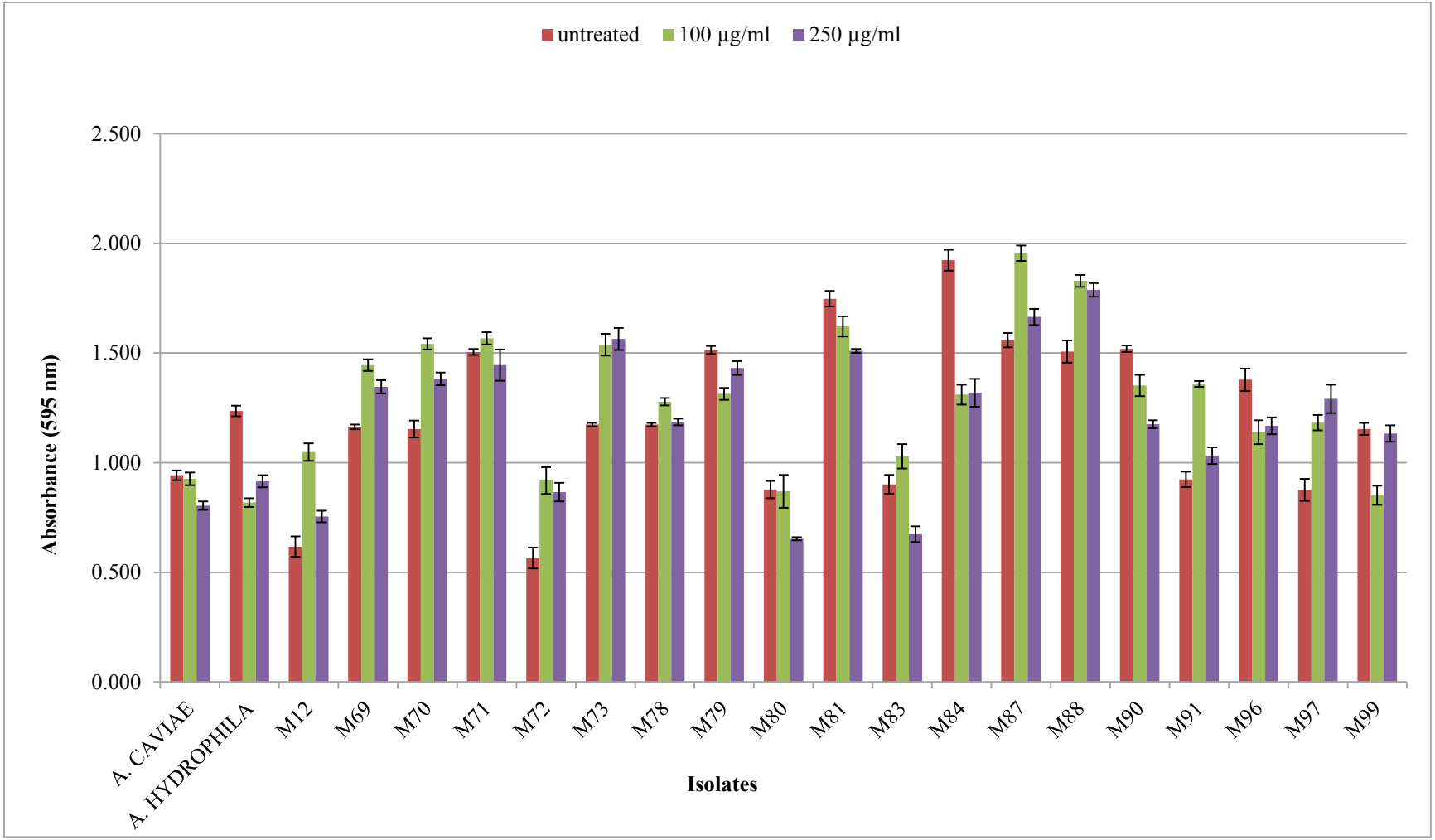

Figure 5.9: Effect of 100 and 250 µg/ml vanillin on pre-formed biofilm of *A. bestiarum* isolates following addition to 24 h biofilm, using micro-titre plate assays. Data represents the mean standard deviation of three replicates on three separate occasions.

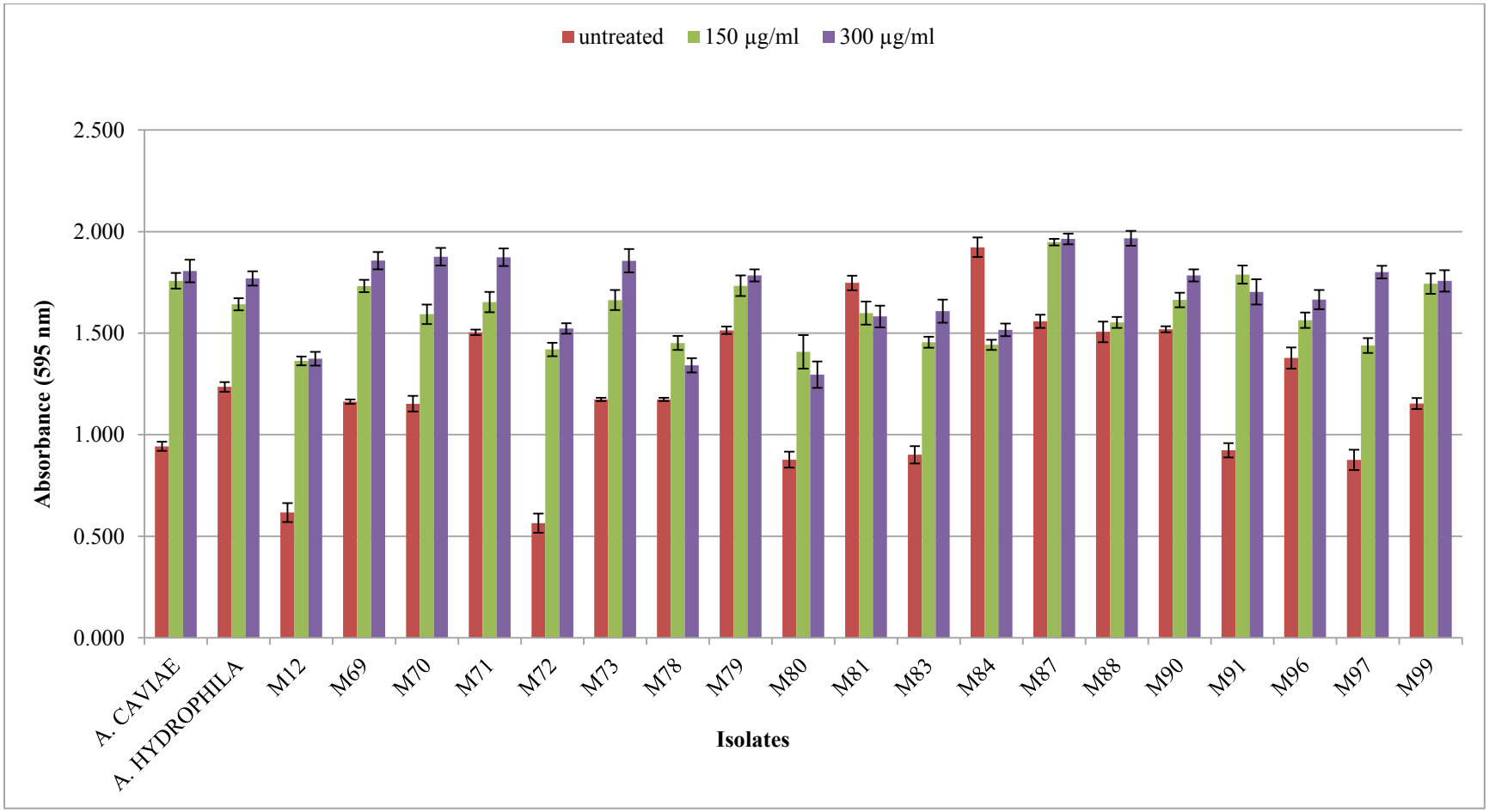

Figure 5.10: Effect of 150 and 300 µg/ml commercial ethanol *K. africana* extract (PhytoForce) on pre-formed biofilm of *A. bestiarum* isolates following addition to 24 h biofilm, using micro-titre plate assays. Data represents the mean standard deviation of three replicates on three separate occasions.

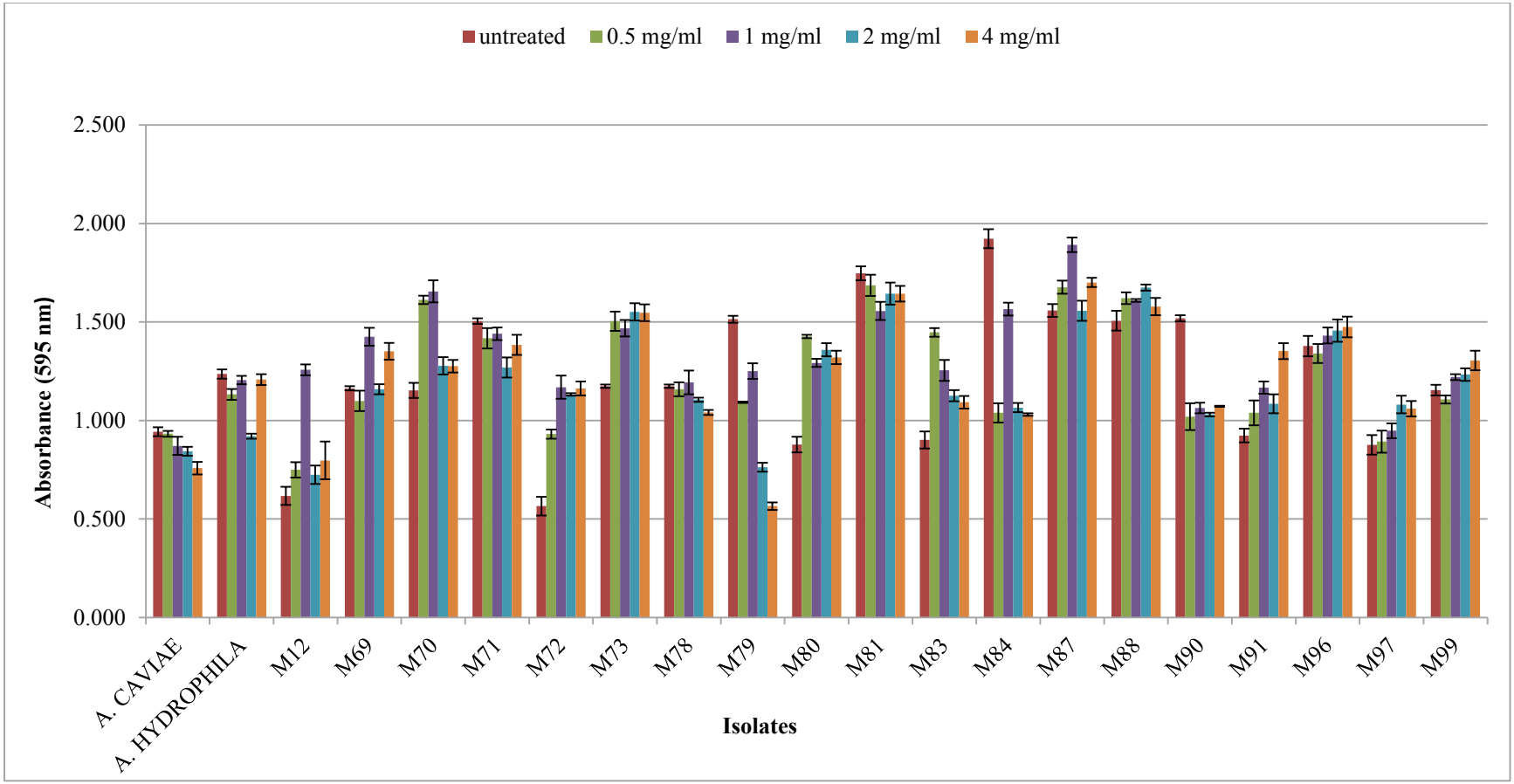

Figure 5.11: Effect of 0.5, 1, 2 and 4 mg/ml ethyl acetate EX1 *K. africana* fruit extract on pre-formed biofilm of *A. bestiarum* isolates following addition to 24 h biofilm, using micro-titre plate assays. Data represents the mean standard deviation of three replicates at three separate occasions.

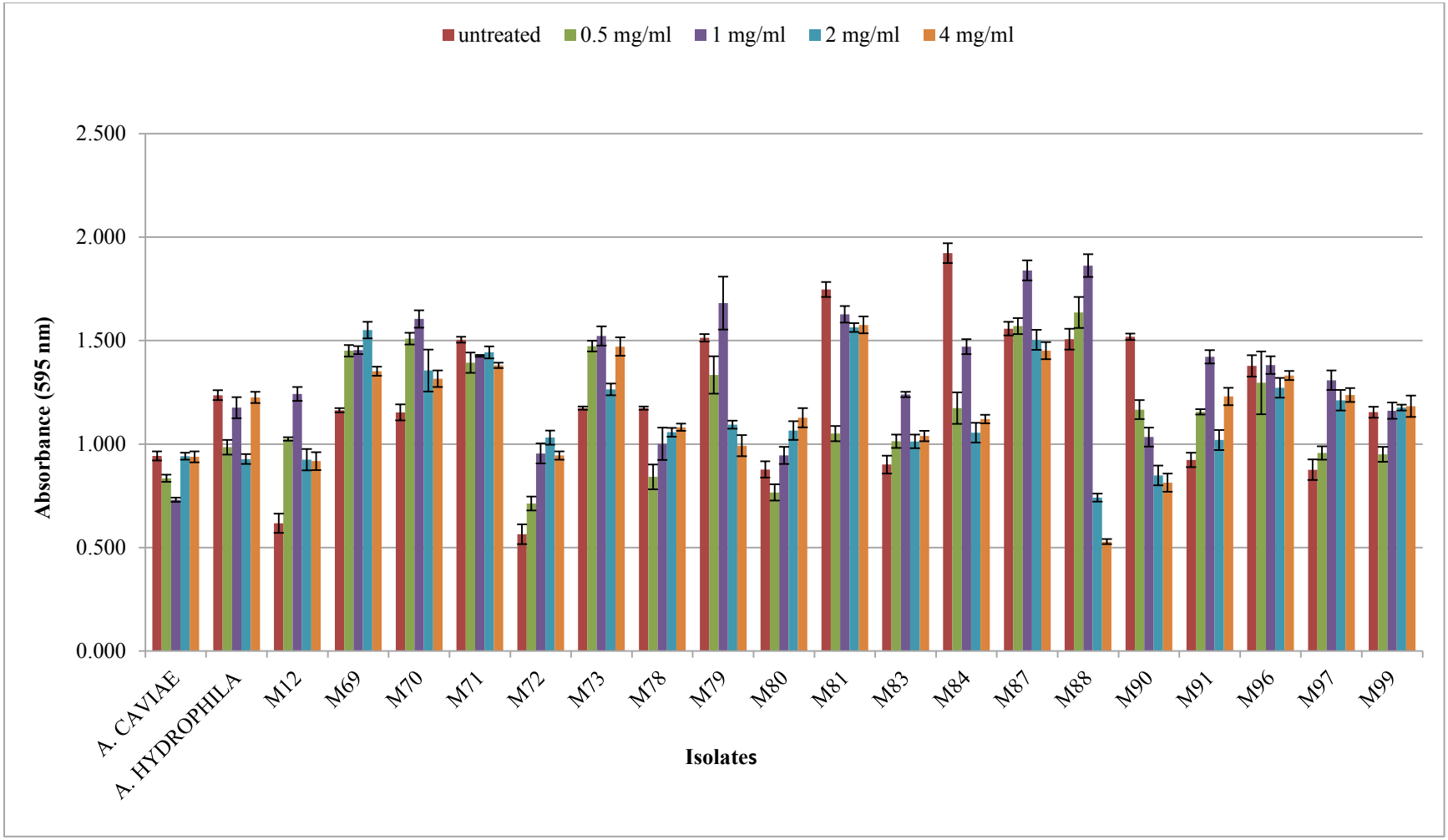

Figure 5.12: Effect of 0.5, 1, 2 and 4 mg/ml dichloromethane EX2 *K. africana* fruit extract on pre-formed biofilm of *A. bestiarum* isolates following addition to 24 h biofilm, using micro-titre plate assays. Data represents the mean standard deviation of three replicates on three separate occasions.

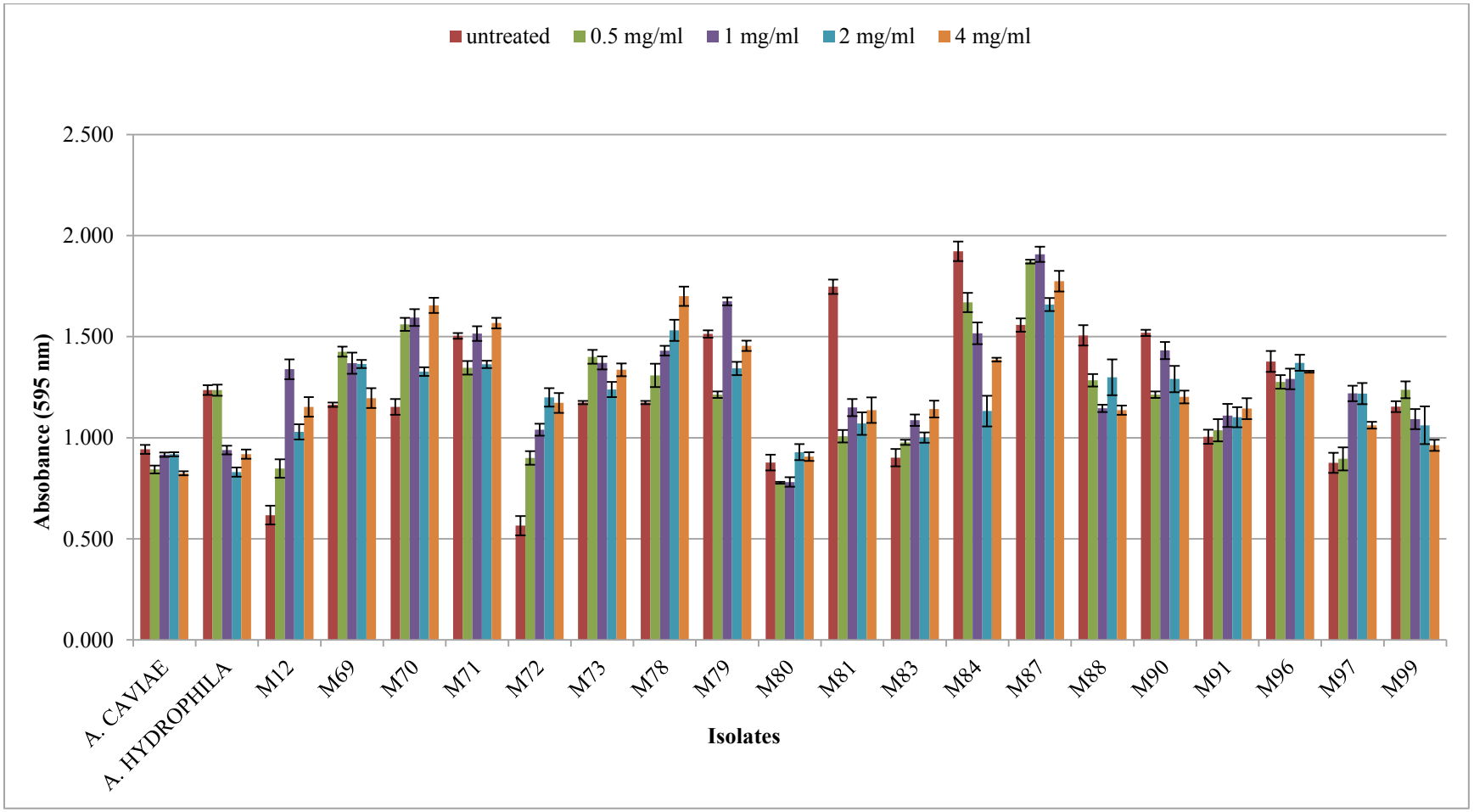

Figure 5.13: Effect of 0.5, 1, 2 and 4 mg/ml methanol EX3 *K. africana* fruit extract on pre-formed biofilm of *A. bestiarum* isolates following addition to 24 h biofilm, using micro-titre plate assays. Data represents the mean standard deviation of three replicates at three separate occasions.

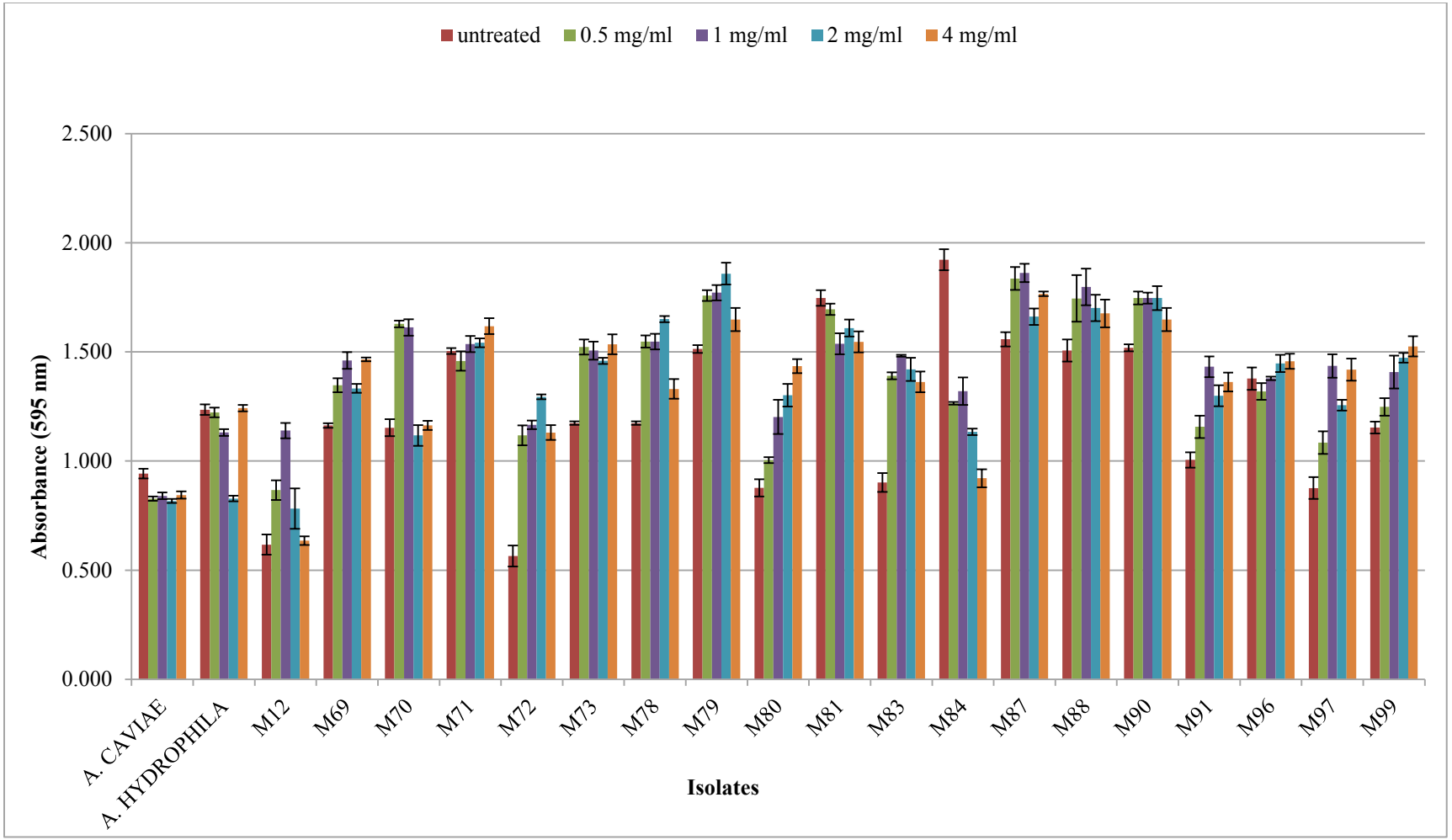

Figure 5.14: Effect of 0.5, 1, 2 and 4 mg/ml hexane EX4 *K. africana* fruit extract on pre-formed biofilm of *A. bestiarum* isolates following addition to 24 h biofilm, using micro-titre plate assays. Data represents the mean standard deviation of three replicates at three separate occasions.

Table 5.2: Effect of phytochemicals and *K. africana* extracts on initial attachment and pre-formed biofilm of *A. bestiarum* strains

Phytochemicals	Initial attachment			Pre-formed biofilm		
	% Decrease	% Increase	% No effect	% Decrease	% Increase	% No effect
Cinnamaldehyde (50 µg/ml)	21.1 (4/19)	73.7 (14/19)	5.3 (1/19)	63.2 (12/19)	36.8 (7/19)	0
(100 µg/ml)	63.2 (12/19)	26.3 (5/19)	10.5 (2/19)	57.9 (11/19)	36.8 (7/19)	5.3 (1/19)
Vanillin (100 µg/ml)	15.8 (3/19)	84.2 (16/19)	0	36.8 (7/19)	63.2 (12/19)	0
(250 µg/ml)	47.4 (9/19)	42.1 (8/19)	10.5 (2/19)	42.1 (8/19)	57.9 (11/19)	0
***Kigelia africana* ethanol (150 mg/ml)**	0	100 (19/19)	0	10.5 (2/19)	89.5 (17/19)	0
(300 mg/ml)	5.3 (1/19)	89.5 (17/19)	5.3 (1/19)	10.5 (2/19)	89.5 (17/19)	0
EX1: EA (0.5 mg/ml)	52.6 (10/19)	36.8 (7/19)	10.5 (2/19)	47.4 (9/19)	52.6 (10/19)	0
(1 mg/ml)	42.1 (8/19)	52.6 (10/19)	5.3 (1/19)	26.3 (5/19)	89.5 (12/19)	10.5 (2/19)
(2 mg/ml)	42.1 (8/19)	52.6 (10/19)	5.3 (1/19)	47.4 (9/19)	52.6 (10/19)	0
(4 mg/ml)	42.1 (8/19)	47.4 (9/19)	10.5 (2/19)	47.4 (9/19)	52.6 (10/19)	0
EX2: DCM (0.5 mg/ml)	42.1 (8/19)	57.9 (11/19)	0	47.4 (9/19)	52.6 (10/19)	0
(1 mg/ml)	15.8 (3/19)	84.2 (16/19)	0	31.6 (6/19)	68.4 (13/19)	0
(2 mg/ml)	31.6 (6/19)	68.4 (13/19)	0	31.6 (6/19)	57.9 (11/19)	10.5 (2/19)
(4 mg/ml)	47.4 (9/19)	52.6 (10/19)	0	31.6 (6/19)	68.4 (13/19)	0
EX3: MeOH (0.5 mg/ml)	15.8 (3/19)	78.9 (15/19)	5.3 (1/19)	21.1 (4/19)	78.9 (15/19)	0
(1 mg/ml)	10.5 (2/19)	89.5 (17/19)	0	5.3 (1/19)	84.2 (16/19)	10.5 (2/19)
(2 mg/ml)	21.1 (4/19)	78.9 (15/19)	0	15.8 (3/19)	84.2 (16/19)	0
(4 mg/ml)	10.5 (2/19)	89.5 (17/19)	0	10.5 (2/19)	78.9 (15/19)	10.5 (2/19)
EX4: HEX (0.5 mg/ml)	36.8 (7/19)	63.2 (12/19)	0	42.1 (8/19)	57.9 (11/19)	0
(1 mg/ml)	21.1 (4/19)	78.9 (15/19)	0	36.8 (7/19)	57.9 (11/19)	5.3 (1/19)
(2 mg/ml)	21.1 (4/19)	78.9 (15/19)	0	42.1 (8/19)	57.9 (11/19)	0
(4 mg/ml)	21.1 (4/19)	78.9 (15/19)	0	36.8 (7/19)	63.2 (12/19)	0

5.3.3 Analysis of percentage biofilm reduction data of *A. bestiarum* isolates in presence of phytochemicals/ extracts for initial attachment (IA) and mature biofilm (MB) assays

The percent reduction following phytochemical/extracts treatments at time of inoculation and on pre-formed biofilm, respectively, are shown in Table 5.3. For initial attachment assays, 50 µg/ml cinnamaldehyde treatment inhibited initial attachment of 68.4% (13/19) of isolates. Percent reduction for 50 µg/ml cinnamaldehyde ranged from 1.18 to 39.79% and percent induction ranged from 0.70 to 140.56% (Table 5.3). Cinnamaldehyde treatment (100 µg/ml) inhibited

initial attachment of 78.9% (15/19) of isolates. Percent reduction ranging from 0.30 to 57.93% and percent induction ranging from 3.10 to 55.47%.

Treatment with 100 µg/ml vanillin inhibited initial attachment of 52.6% (10/19) of isolates (Table 5.3). Its percent reduction ranged from 0.45 to 42.00% and percent induction ranged from 1.88 to 91.94%. While 250 µg/ml vanillin inhibited initial attachment of 15.8% (3/19) of isolates, its percent reduction ranged from 4.31 to 29.86% and percent induction ranged from 2.27 to 73.53%.

Exposure to 150 mg/ml *K africana* ethanol extract (PhytoForce) did not inhibit initial attachment of isolates (Table 5.3), rather it induced adhesion for isolates and percent induction ranged from 8.14 to 206.07%. Exposure to 300 mg/ml *K africana* ethanol extract inhibited initial attachment of 5.3% (1/19) of isolates.

Treatment with 0.5 mg/ml *K africana* EX1 extract inhibited initial attachment of 42.10% (8/19) of isolates (Table 5.3). Percent reduction for 0.5 mg/ml *K africana* EX1 extract ranged from 6.33 to 34.21% and percent induction ranged from 9.58 to 72.79%. Exposure of isolates to 1 mg/ml inhibited initial attachment of 15.8% (3/19) of isolates and its percent reduction ranged from 2.12 to 18.09% and percent induction ranged from 1.21 to 84.28%. Treatment with 2 mg/ml inhibited initial attachment for 31.60% (6/19) with a range of 3.81 to 22.14% reduction percent and 10.33 to 56.34% percent induction. The 4 mg/ml concentration inhibited initial attachment for 47.40% (9/19). Its percent reduction ranged from 3.10 to 51.987% and percent induction ranged from 2.89 to 73.90%. *K africana* EX2 extract 0.5 mg/ml treatment inhibited initial attachment of 47.40% (9/19) of isolates (Table 5.3). Percent reduction for 0.5 mg/ml *K africana* EX2 extract ranged from 2.03 to 39.29% and percent induction ranged from 0.03 to 67.83%.

Table 5.3: Percentage **b**iofilm reduction in the presence of phytochemicals and *K. africana* fruit extracts on initial attachment (IA) and mature biofilms (MB) of *A. bestiarum* multidrug resistant isolates.

	*% biofilm reduction													
	A. caviae		***A. hydrophila***		**M12**		**M69**		**M70**		**M71**		**M72**	
Phytochemicals	***IA**	***MB**	**IA**	**MB**	**IA**	**MB**	**IA**	**MB**	**IA**	**MB**	**IA**	**MB**	**IA**	**MB**
Cinnamaldehyde (50 µg/ml)	-8.57	12.2	22.36	32.87	-27.02	-111.43	18.87	23.63	-11.89	-24.97	39.79	6.74	-140.56	-68.53
(100 µg/ml)	15.03	40.16	32.97	51.35	-3.1	-94.01	25.54	-6.6	0.3	-25.04	57.93	11.08	-33.37	-41.75
Vanillin (100 µg/ml)	8.75	17.41	18.36	29.47	-16.14	-29.15	-3.19	-17.91	-35.43	-22.75	8.15	4.4	-91.94	-71.96
(250 µg/ml)	31.18	2.08	32.81	38.38	-31.42	-91.7	-51.08	-27.75	-6.04	-38.65	-3.67	-4.61	-73.53	-84.63
***Kigelia africana* ethanol (150 mg/ml)**	-57.4	-102.46	-41.06	-37.36	-206.07	-158.81	-29.29	-55.96	-85.3	-43.77	-54.1	-10.96	-134.61	-204.7
(300 mg/ml)	-99.45	-108.61	-61.1	-49.04	-199.95	-161.07	-15.24	-68.31	-120.97	-71.95	-44.57	-27.22	-170.37	-229.41
EX1: EA (0.5 mg/ml)	-13.52	1.31	8.68	9.49	-65.78	-28.13	-13.97	6.33	-15.15	-45.66	-23.23	6.4	-67.56	-87.63
(1 mg/ml)	5.85	9.04	18.39	2.82	-56.82	-136.24	-16.88	-25.8	-18.4	-49.95	-28.85	4.73	-75.93	-144.48
(2 mg/ml)	-13.77	12.45	28.65	28.96	-56.34	-22.76	5.79	0.45	-41.37	-12.39	-24.83	17.35	-56.31	-135.74
(4 mg/ml)	1.6	23.26	-0.47	2.59	-73.9	-38.33	-2.89	-18.48	-16.11	-12.2	-35.45	8.87	-45.7	-143.01
EX2: DCM (0.5 mg/ml)	16.66	13.57	30.93	23.06	-63.44	-86.84	5.23	-28.34	-9.36	-35.51	15.31	8.2	-61.63	-35.38
(1 mg/ml)	31.99	26.64	21.35	5.51	-72.52	-133.04	10.48	-28.62	-33.63	-44.92	-0.98	5.74	-117.06	-93.37
(2 mg/ml)	15.9	0.17	40.41	28.29	-48.41	-65.52	-6.5	-38.19	-21.55	-20.15	-31.31	4.5	-93.47	-111.7
(4 mg/ml)	2.8	0.52	19.48	0.92	-63.09	-63.96	-0.73	-18.58	-8.16	-16.24	-3.25	9.09	-38.14	-90.93
EX3: MeOH (0.5 mg/ml)	19.16	12.53	19.75	0.01	-68.35	-49.04	15.17	-25.86	-9.02	-40.63	41.34	11.64	-68.3	-80.24
(1 mg/ml)	3.03	3.3	28.91	27.29	-68.26	-153.68	-23.6	-20.29	-16.26	-43.93	16.25	-0.82	-77.33	-113.78
(2 mg/ml)	-9.67	3.03	40.05	37.27	-73.11	-87.66	-5.76	-19.83	-17.56	-17.33	13.59	10.4	-68.72	-151.98
(4 mg/ml)	17.33	14.92	27.57	29.09	-53.38	-113.86	7.23	-3.26	-19.44	-49.97	-15.05	-4.64	-68.07	-145.4
EX4: HEX (0.5 mg/ml)	31.19	14.47	6.63	1.19	-59.5	-53.15	9.23	-18.15	-40.38	-47.31	-18.89	3.33	-56.49	-132.32
(1 mg/ml)	18.95	12.85	-2.51	9.63	-102.24	-111.24	-39.88	-29.32	-47.61	-45.71	-14.61	-2.37	-84.85	-143.9
(2 mg/ml)	45.69	15.83	-5.28	37.44	-131.55	-35.11	-45.85	-16.73	-60.06	3.54	-28.34	-2.78	-107.28	-174.59
(4 mg/ml)	16.73	12.42	-14.66	-0.64	-137.19	-3.81	3.18	-29.83	-55	-1.05	-52.55	-8.39	-118.03	-135.47

*% biofilm reduction														
	M73		M78		M79		M80		M81		M83		M84	
Phytochemicals	**IA**	**MB**	**IA**	**MB**	**IA**	**MB**	**IA**	**MB**	**IA**	**MB**	**IA**	**MB**	**IA**	**MB**
Cinnamaldehyde (50 µg/ml)	-68.40	-30.04	-13.00	-27.03	12.41	-24.24	14.73	-3.87	19.54	40.50	2.84	32.35	19.74	17.95
(100 µg/ml)	-55.47	-28.96	14.06	2.06	29.78	-20.87	28.51	32.90	21.36	57.40	31.56	28.59	40.86	45.07
Vanillin (100 µg/ml)	-16.70	-38.03	1.59	-1.12	0.45	6.06	-15.38	30.73	-12.86	14.86	15.82	30.19	-1.89	34.04
(250 µg/ml)	-26.14	-35.48	-3.24	-10.14	4.31	14.62	-29.44	1.09	-8.64	7.88	4.85	-16.91	-9.37	34.52
***Kigelia africana* ethanol (150 mg/ml)**	-79.59	-47.69	-18.58	-27.09	51.02	-16.07	-9.25	-72.71	-93.82	9.24	-14.59	-73.49	-80.64	27.04
(300 mg/ml)	-93.19	-66.51	-57.67	-16.40	97.20	-19.82	5.47	-57.41	-66.86	10.32	-11.73	-93.80	-112.89	22.87
EX1: EA (0.5 mg/ml)	-71.08	-32.16	9.00	1.52	13.13	30.83	7.20	-75.24	10.36	3.82	-6.08	-72.43	-9.58	49.80
(1 mg/ml)	-83.91	-28.66	-13.36	-1.89	22.01	19.29	-3.59	-56.79	-11.92	11.96	-4.02	-46.86	-28.81	20.13
(2 mg/ml)	-52.23	-36.77	5.42	6.65	10.33	55.01	-19.15	-65.98	-51.30	6.46	-13.71	-29.79	22.14	48.32
(4 mg/ml)	-44.02	-36.34	22.36	12.94	11.59	69.44	16.24	-60.65	-14.37	6.46	-13.84	-25.37	27.31	50.28
EX2: DCM (0.5 mg/ml)	-0.44	-29.19	13.26	32.35	2.03	13.14	-0.03	15.24	-1.03	43.53	13.62	-14.94	35.34	42.20
(1 mg/ml)	-23.16	-34.04	3.73	16.79	28.84	-12.29	-1.34	-9.31	7.01	7.48	13.95	-44.89	-44.18	25.46
(2 mg/ml)	-31.39	-8.84	3.16	11.35	5.76	30.71	-15.74	-25.80	22.97	11.47	6.23	-14.85	-55.42	48.89
(4 mg/ml)	-76.85	-29.02	-1.22	8.98	14.66	38.17	12.98	-34.29	0.24	10.71	-5.77	-18.28	-22.67	45.14
EX3: MeOH (0.5 mg/ml)	-71.96	-22.10	0.97	-13.17	18.26	22.01	-5.59	13.72	16.49	46.27	5.47	-10.09	9.80	14.27
(1 mg/ml)	-94.70	-19.16	-13.12	-25.05	15.27	-11.79	-3.41	13.17	-7.53	37.36	3.47	-24.68	4.52	22.85
(2 mg/ml)	-89.57	-6.31	-14.17	-34.90	2.80	12.50	-19.38	-7.06	-18.65	42.32	-14.98	-13.26	16.90	44.54
(4 mg/ml)	-11.62	-15.83	-13.99	-51.35	10.40	4.30	-24.51	-4.02	-3.20	38.15	-24.81	-31.95	7.07	30.21
EX4: HEX (0.5 mg/ml)	-67.60	-34.02	-11.20	-36.46	34.21	-17.90	-4.98	-17.33	-15.88	3.25	-20.62	-64.96	-61.27	37.05
(1 mg/ml)	-71.63	-32.39	-5.02	-36.40	48.24	-18.87	-24.10	-44.51	-27.14	13.09	-33.89	-77.07	-67.33	33.97
(2 mg/ml)	-48.69	-27.82	-8.34	-46.49	48.12	-25.24	-30.26	-58.16	-66.93	8.60	-38.05	-68.85	-60.41	44.43
(4 mg/ml)	-43.98	-35.17	-25.95	-15.28	63.68	-9.88	-22.71	-76.39	-80.29	12.57	-36.72	-61.20	-70.50	56.43

*% biofilm reduction														
	M87		M88		M90		M91		M96		M97		M99	
Phytochemicals	**IA**	**MB**	**IA**	**MB**	**IA**	**MB**	**IA**	**MB**	**IA**	**MB**	**IA**	**MB**	**IA**	**MB**
Cinnamaldehyde (50 µg/ml)	19.60	-7.85	27.11	44.37	33.40	34.53	1.18	10.24	-0.77	11.91	6.45	9.04	6.40	41.28
(100 µg/ml)	35.96	-19.24	42.13	30.06	42.31	34.30	8.38	4.78	20.94	18.07	4.88	14.61	13.56	52.68
Vanillin (100 µg/ml)	11.02	-7.55	42.00	-20.69	9.96	25.05	11.78	-14.02	8.95	17.07	4.14	-56.93	-1.88	2.07
(250 µg/ml)	-9.58	-28.14	29.86	-23.71	-2.74	12.18	-41.14	-56.19	-2.49	19.42	-4.76	-42.09	-16.78	30.09
***Kigelia africana* ethanol (150 mg/ml)**	-8.17	-27.66	-8.14	-3.43	-26.34	-10.53	-39.93	-111.54	-34.21	-15.12	-44.25	-77.30	-90.12	-58.58
(300 mg/ml)	-21.88	-28.8	-2.46	-33.90	-41.30	-19.37	-20.53	-100.50	-29.83	-23.36	-38.05	-126.81	-112.64	-59.98
EX1: EA (0.5 mg/ml)	34.21	-8.41	31.66	-8.40	26.48	36.42	-34.59	-14.85	19.25	3.05	-16.43	-2.24	-72.79	4.69
(1 mg/ml)	-1.21	-23.63	17.94	-7.61	18.09	33.18	-40.49	-31.37	2.12	-4.31	-42.74	-9.83	-84.28	-6.59
(2 mg/ml)	12.91	0.05	30.94	-12.36	3.81	35.62	-14.05	-20.76	-11.02	-6.37	-15.14	-28.07	-55.16	-7.84
(4 mg/ml)	33.70	-10.10	51.98	-5.28	11.42	32.6	-24.27	-55.28	25.34	-7.86	3.10	-25.21	-64.35	-14.96
EX2: DCM (0.5 mg/ml)	7.27	-0.86	39.29	-9.56	29.32	25.69	-24.55	-29.98	-6.88	6.63	-13.35	-11.13	-67.83	20.18
(1 mg/ml)	5.17	-19.92	17.50	-26.20	21.99	35.36	-22.63	-64.27	-18.78	-0.35	6.83	-59.36	-68.81	-0.72
(2 mg/ml)	37.73	3.86	50.11	56.29	19.59	48.87	1.65	-12.47	-6.96	8.59	5.38	-46.06	-54.90	-2.26
(4 mg/ml)	37.23	7.57	52.07	71.92	43.36	51.46	-16.05	-39.55	13.90	3.78	-0.75	-49.59	-46.13	-2.85
EX3: MeOH (0.5 mg/ml)	-5.24	-22.25	27.85	16.32	2.89	22.30	-11.49	-1.69	12.33	8.28	-24.82	-2.65	-73.86	-8.28
(1 mg/ml)	-3.81	-24.79	9.78	26.54	-12.43	6.34	-33.63	-31.14	13.06	7.08	-13.76	47.07	-90.52	6.11
(2 mg/ml)	-6.23	-7.16	8.50	15.27	13.87	16.66	-10.53	-10.32	2.82	0.56	-8.65	47.01	-54.38	9.15
(4 mg/ml)	-11.01	-15.34	-7.47	27.19	16.82	23.13	-36.53	-21.44	10.25	4.11	-6.43	-25.48	-82.26	18.99
EX4: HEX (0.5 mg/ml)	21.04	-19.75	33.39	-17.59	-0.68	-16.65	-29.54	-21.50	12.32	4.79	-0.26	-28.63	-79.45	-9.40
(1 mg/ml)	-10.88	-21.58	21.38	-21.47	-14.94	-16.62	-43.63	-42.61	18.40	-0.11	-45.49	-76.77	-97.92	-25.23
(2 mg/ml)	4.68	-7.35	42.80	-14.38	-11.39	-16.61	-32.28	-55.69	3.46	-5.68	10.66	-52.15	-56.85	-31.72
(4 mg/ml)	-3.20	-14.76	25.13	-12.53	-13.14	-9.46	-32.08	-58.93	25.63	-6.45	-9.65	-74.45	-66.06	-36.90

*IA = initial attachment, MB = mature biofilm, %Biofilm reduction = $[\ ((C-B)-\ (T-B))/(C-B)\ ] \times 100$. Where B=average absorbance per well for blank wells, C = average absorbance per well for control wells, T = average absorbance per well for treated wells (Pitts *et al*., 2003).

Exposure of isolates to 1 mg/ml inhibited initial attachment of 42.10% (8/19) of isolates, and its percent reduction ranged from 3.73 to 21.99% while the percent induction ranged from 0.98 to 117.06%. Treatment with 2 mg/ml inhibited initial attachment for 42.10% (8/19) with a range of 1.65 to 50.11% reduction percent and 5.76 to 93.47% percent induction. The 4 mg/ml concentration inhibited initial attachment for 36.8% (7/19), while its percent reduction ranged from 0.24 to 52.07% and percent induction ranged from 0.73 to 63.04%.

Treatment with 0.5 mg/ml *K africana* EX3 extract inhibited initial attachment of 42.10% (8/19) of isolates (Table 5.3). Percent reduction for 0.5 mg/ml *K africana* EX3 extract ranged from 0.97 to 41.34% and percent induction ranged from 5.24 to 73.86%. Exposure of isolates to 1 mg/ml inhibited initial attachment of 26.30% (5/19) of isolates. Its percent reduction ranged from 3.47 to 16.25% and percent induction ranged from 3.81 to 94.70%. Treatment with 2 mg/ml inhibited initial attachment for 26.30% (5/19) with a range of 2.82 to 16.90% reduction percent and 2.80 to 89.57% percent induction. The 4 mg/ml concentration inhibited initial attachment for 21.10% (4/19). Its percent reduction ranged from 7.07 to 16.827% and percent induction ranged from 3.20 to 82.26%.

Treatment with 0.5 mg/ml *K africana* EX4 extract inhibited initial attachment of 15.80% (3/19) of isolates (Table 5.3). Percent reduction for 0.5 mg/ml *K africana* EX4 extract ranged from 12.32 to 33.39% and percent induction ranged from 0.26 to 79.45%. Exposure of isolates to 1 mg/ml inhibited initial attachment of 10.5% (2/19) of isolates. Its percent reduction ranged from 18.40 to 21.38% and percent induction ranged from 5.02 to 102.24%. Treatment with 2 mg/ml inhibited initial attachment for 21.10% (4/19) with a range of 3.46 to 42.80% reduction percent and 11.37 to 107.28% percent induction. The 4 mg/ml concentration inhibited initial attachment for 10.50% (2/19). Its percent reduction ranged from 3.18 to 25.137% and percent induction ranged from 3.20 to 137.19%.

In the pre-formed biofilm assays, 50 µg/ml cinnamaldehyde inhibited the biofilm of 74.1% (11/19) of isolates (Table 5.3). Percent reduction for 50 µg/ml cinnamaldehyde ranged from 6.74 to 44.37% and percent induction ranged from 3.87 to 111.43%. The 100 µg/ml cinnamaldehyde treatment inhibited the biofilm of 63.2% (12/19) of isolates, while the percent reduction for 100 µg/ml cinnamaldehyde ranged from 02.06 to 57.40% and percent induction ranged from 6.60 to 94.01%.

Treatment with 100 µg/ml vanillin inhibited the biofilm of 47.4% (9/19) of isolates (Table 5.3). Percent reduction for 100 µg/ml vanillin ranged from 2.07 to 34.04% and percent induction ranged from 1.12 to 71.96%, while 250 µg/ml vanillin inhibited the biofilm of 36.8% (7/19) of isolates. Percent reduction for 250 µg/ml vanillin ranged from 1.09 to 34.52% and percent induction ranged from 4.61 to 91.70%.

Exposure to 150 mg/ml *K africana* ethanol extract (PhytoForce) inhibited the biofilm of 15.8% (3/19) of isolates (Table 5.3). Its percent reduction ranged from 9.24 to 27.64% and percent induction ranged from 3.43 to 158.81. While 300 mg/ml *K africana* ethanol extract treatment inhibited the biofilm of 15.8% (3/19). Its percent reduction ranged from 10.32 to 23.36% and percent induction ranged from 16.40 to 229.41.

Treatment with 0.5 mg/ml *K africana* EX1 extract inhibited the biofilm of 47.40% (9/19) of isolates (Table 5.3). Percent reduction for 0.5 mg/ml *K africana* EX1 extract ranged from 1.52 to 49.80% and percent induction ranged from 2.24 to 87.63%. Exposure of isolates to 1 mg/ml inhibited the biofilm of 26.30% (5/19) of isolates. Its percent reduction ranged from 4.73 to 33.18% and percent induction ranged from 1.89 to 144.48%. Treatment with 2 mg/ml inhibited the biofilm for 42.10% (8/19) with a range of 0.45 to 55.01% reduction percent and 6.37 to 135.74% percent induction. The 4 mg/ml concentration inhibited the biofilm for 31.60% (6/19). Its percent reduction ranged from 6.46 to 69.447% and percent induction ranged from 5.28 to 143.01%.

Exposure to 0.5 mg/ml *K africana* EX2 extract inhibited the biofilm of 47.40% (9/19) of isolates (Table 5.3). Percent reduction for 0.5 mg/ml *K africana* EX2 extract ranged from 8.20 to 43.53% and percent induction ranged from 0.86 to 86.84%. Exposure of isolates to 1 mg/ml inhibited the biofilm of 26.30% (5/19) of isolates. Its percent reduction ranged from 5.74 to 35.36% and percent induction ranged from 0.72 to 133.04%. Treatment with 2 mg/ml inhibited the biofilm for 47.40% (9/19) with a range of 3.86 to 56.29% reduction percent and 2.26 to 111.70% percent induction. The 4 mg/ml concentration inhibited the biofilm for 47.40% (9/19). Its percent reduction ranged from 3.78 to 71.92% and percent induction ranged from 2.85 to 90.93%.

Treatment with 0.5 mg/ml of *K africana* EX3 extract inhibited the biofilm of 42.10% (8/19) of isolates (Table 5.3). Percent reduction for 0.5 mg/ml *K africana* EX3 extract ranged from 8.28 to 46.27% and percent induction ranged from 1.69 to 80.24%. Exposure of isolates to

1 mg/ml inhibited the biofilm of 36.8% (7/19) of isolates. Its percent reduction ranged from 6.11 to 37.36% and percent induction ranged from 0.82 to 153.68%. Treatment with 2 mg/ml inhibited the biofilm for 42.10% (8/19) with a range of 0.86 to 44.54% reduction percent and 6.31 to 151.98% percent induction. The 4 mg/ml concentration inhibited the biofilm for 36.80% (7/19). Its percent reduction ranged from 4.11 to 38.15% and percent induction ranged from 3.26 to 145.40%.

Exposure to 0.5 mg/ml *K africana* EX4 extract inhibited the biofilm of 21.10% (4/19) of isolates (Table 5.3). Percent reduction for 0.5 mg/ml *K africana* EX4 extract ranged from 3.25 to 37.05% and percent induction ranged from 9.40 to 53.15%. Exposure of isolates to 1 mg/ml inhibited the biofilm of 10.5% (2/19) of isolates. Its percent reduction ranged from 13.09 to 33.97% and percent induction ranged from 0.11 to 143.90%. Treatment with 2 mg/ml inhibited the biofilm for 15.80% (3/19) with a range of 3.45 to 44.43% reduction percent and 2.78 to 174.59% percent induction. The 4 mg/ml concentration inhibited the biofilm for 10.50% (2/19). Its percent reduction ranged from 12.57 to 56.43% and percent induction ranged from 1.05 to 135.47%.

5.4 Discussion

In aquaculture, antimicrobial resistance is increasingly being observed and is responsible for losses going up to millions annually (Cabello *et al*., 2013). *Aeromonas* spp. are known fish pathogens forming biofilms in their aquatic environment and are resistant to various antimicrobials used in aquaculture (Janda and Abbott, 2010; Igbinosa and Okoh, 2013). In this study the effects of cinnamaldehyde, vanillin, *K*. *africana* fruit extracts (EX1, EX2, EX3 and EX4), as well as commercially available ethanolic *K*. *africana* extract were investigated for their anti-biofilm properties against multidrug resistant *A*. *bestiarum* isolates.

There have been several studies on aeromonad biofilm formation (Bechet and Blondeau, 2003; Khajanchi *et al*., 2009; Jahid *et al*., 2014), however, none have used *A*. *bestiarum* as a study model. *A*. *bestiarum* is an important fish pathogen that is multidrug resistant to several antimicrobials presently in use (Kadlec *et al*., 2011). Data from this study shows that *A*. *bestiarum* isolates sourced from ornamental fish and catfish are multidrug resistant to ampicillin, trimethoprim, sulphamethoxazole and first, second and third generation cephalosporins (Table 5.1).

Plants are being extensively investigated for novel antimicrobial and anti-pathogenic phytochemicals that attenuate virulence properties (Chenia, 2013; Husain *et al*., 2013; Nazzaro *et al*., 2013). All concentrations of phytochemicals and *K africana* extracts used in this study seemed promising as anti-biofilm agents (Table 5.2). However these phytochemicals/extracts were more effective in treating performed biofilms than initial attachment (Tables 5.2 and 5.3).

Cinnamaldehyde is a known phytochemical, with anti-quorum sensing and anti-biofilm properties (Brackman *et al*., 2008; Niu *et al*., 2006). In the present study, the sub-inhibitory concentrations (50 and 100 µg/ml) of cinnamaldehyde were more effective in treating initial attachment than pre-formed biofilms (Tables 5.2 and 5.3). In contrast, Amalaradjou *et al*. (2010) observed an eradication of 24 h pre-formed biofilms of uropathogenic *E*. *coli* on treatment with 250 µg/ml of cinnamaldehyde. However for type strains *A*. *caviae* and *A*. *hydrophila* the sub-inhibitory cinnamaldehyde concentrations were more effective in treating pre-formed biofilms (Tables 5.2 and 5.3).

Vanillin has been shown to be a suitable and sustainable anti-biofilm agent, which inhibits biofilm formation without affecting cell growth (Ponnusamy *et al*., 2009; Kappachery *et al*., 2010). Kappachery *et al*. (2010) observed that 0.063 – 0.25 mg/ml concentrations of vanillin reduced biofilm formation and biofilm biomass thickness of *A*. *hydrophila* considerably over seven days while not inhibiting planktonic cell growth. In the present study, 100 µg/ml concentration of vanillin was more effective against initial attachment than pre-formed biofilms. However, 250 µg/ml was more effective against pre-formed biofilms than initial attachment (Tables 5.2 and 5.3). In contrast, Kappachery *et al*. (2010) observed that 0.063 – 0.25 mg/ml concentrations of vanillin had no effect on 24 h pre-formed biofilms. It is important to note the difference in aeromonad models used, as multidrug *A*. *bestiarum* biofilms may behave differently than *A*. *hydrophila* biofilms. For type strains *A*. *caviae* and *A*. *hydrophila* both concentrations of vanillin were effective in treating initial attachment and pre-formed biofilms (Tables 5.2 and 5.3).

Kigelia africana is used traditionally in the treatment of a cornucopia of infections ranging from skin infections to urinary tract infections (Saini *et al*., 2009; Dada *et al*., 2010; Higgins *et al*., 2010). In addition to their antimicrobial activities, *K*. *africana fruit extracts* have also been explored for its anti-pathogenic uses (Chenia, 2013). Sub-MIC and MIC of *K*. *africana*

fruit extracts (0.31 – 8.20 mg/ml) were active against QSing inhibition in *C. violaceum* without reducing cell growth (Chenia, 2013).

Data from this study shows that *K. africana* EX2 fruit extract was the second most effective phytochemical/extract used in treating initial attachment and pre-formed biofilms (Table 5.2 and 5.3). There is no information in current literature on anti-biofilm effects of *K. africana* fruit extracts. However, Chenia (2013) observed the efficacy of *K. africana* extracts (EX1, EX2, EX3 and EX4) against QS in *Chromobacterium violaceum* in the order EX4 > EX2 > EX1 > EX3. This substantiates results from this study as biofilm formation is a virulence property of bacteria controlled by QS phenomenon. Overall, other fruit extracts of *K. africana* used were effective against initial attachment and pre-formed biofilms in the order EX2 > EX3 > EX1 > EX4, while *K. africana* ethanolic extract (PhytoForce) (150 and 300 mg/ml), induced adhesion in both initial attachment and pre-formed biofilm assays (Tables 5.2 and 5.3). Exposure of type strains *A. caviae* and *A. hydrophila* to various concentrations of *K. africana* fruit extracts showed that they were more effective in treating preformed biofilms than initial attachment (Table 5.2 and 5.3).

In conclusion, cinnamaldehyde was more effective in inhibiting initial attachment and pre-formed biofilms of multidrug *A. bestiarum* than other phytochemical/extracts used in this study. Future work can be designed to elucidate the anti-biofilm mode of action of these phytochemicals/extracts against multidrug resistant *A. bestiarum*.

CHAPTER SIX
CONCLUSIONS

Aeromonas spp. are primary and opportunistic pathogens of humans and animals and they are responsible for great economic losses in aquaculture. They are ubiquitous in aquatic environments, this increases contact and infection rate with this genus (Janda and Abbott, 2010). Antimicrobial resistance is increasingly being described in aeromonads and they are known to be associated with resistance determinants and mobile genetic elements such as integrons, plasmids and transposons (Janda and Abbott, 2010). *Aeromonas* spp. are also known biofilm formers, although, *A*. c*aviae* and *A*. *hydrophila* are the two most studied models. Phytochemicals are being explored in the search for alternative and sustainable antimicrobial agents (Siri *et al*., 2008; Dhayanithi *et al*., 2012; de Britto *et al*., 2011; de Britto *et al*., 2012a, b). This study thus investigated the presence of genetic resistance determinants in *Aeromonas* spp. and the antimicrobial and anti-biofilm effect of phytochemicals and crude fruit *K*. *africana* extracts on resistance isolates of *Aeromonas* spp.

The bla_{TEM} gene was observed to be the most prevalent beta-lactamase gene among study isolates (34.3%; 34/99). Although ESBL resistance was observed in study isolates, correlating gene phenotypic resistance with gene content was not possible as the blaSHV gene wasn't identified among the eight ESBL producers. Three isolates (M81, M82 and M88) were positive for both $bla_{CTX\text{-}M\text{-}15}$ and $bla_{CTX\text{-}M}$ genes.

Antimicrobial resistance determinants such as integrons have been previously identified in *Aeromonas* spp. (Chang *et al*., 2007; Jacobs and Chenia, 2007; Maravic *et al*., 2013). Study isolates were observed to possess class 1 (10.1%; 10/99) and class 2 (23.2%; 23/99) integrons and 12.1% (12/99) were noted to be positive for both class 1 and 2 integrons. The *sul* gene (46.4%; 46/99) was identified with two primer sets *sulI* and *sulII*. Although not all isolates that were positive for *intI* (30%; 3/10) and *intII* (17%; 4/23) integrons were positive for ths *sul* gene. The *qacEΔ1* gene was observed to be prevalent among study isolates with 64% (64/99) being positive for this gene.

Cinnamaldehyde (250 – 1250 µg/ml) was observed to be the most effective phytochemical against study isolates. Of the four *K. africana* fruit extracts tested, the methanol extract (4 mg/ml) was more effective in inhibiting study isolates. Although vanillin possesses anti-biofilm properties, all concentrations used were observed to be ineffective in inhibiting

study isolates. Overall the *K. africana e*xtracts efficacy was in the order EX3 > EX1 > EX2 > EX4.

Cinnamaldehyde was the best phytochemical/extract used in initial attachment assay, all concentrations (50 and 100 µg/ml) used were effective against *A. bestiarum* biofilms. The second most effective phytochemical/extract used was the *K. africana* EX2 (dichloromethane), it was observed that EX2 was effective against *A. bestiarum* biofilms in both initial attachment and pre-formed biofilm assays. All of vanillin used (100 and 250 µg/ml) was effective against *A. bestiarum* biofilms in both initial attachment and pre-formed biofilm assays.

Future studies may target the elucidation of the mode of action of phytochemicals/extracts against *Aeromonas* spp., the active antimicrobial and anti-biofilm compounds in *K. africana* extracts (EX1, EX2, EX3 and EX4) and possible synergistic use of phytochemicals and *K. africana* extracts with standard antimicrobials against *Aeromonas* spp. in aquaculture.

REFERENCES

Abbot, S. L., and J. M. Janda. 2010. The genus *Aeromonas*: Taxonomy, pathogenicity and infection. Clinical Microbiology Reviews **23**:35-73.

Abd-El-Rhnan, A. M. M. 2009. Antagonism of *Aeromonas hydrophila* by propolis and its effect on the performance of Nile tilapia, *Oreochromis niloticus*. Fish and Shellfish Immunology **27**:454-459.

Abdel-Tawwab, M., Ahmad, M. H., Seden, M. E. A., and Sakr, S. F. M. 2010.Use of green tea, *Camellia sinensis* L., in practical diet for growth and protection of Nile tilapia, *Oreochromis niloticus* (L.), against *Aeromonas hydrophila* infection. Journal of the World Aquaculture Society **41**:203-213.

Acharyya, S., A. Patra, and P. K. Bag. 2009. Evaluation of the antimicrobial activity of some medicinal plants against enteric bacteria with particular reference to multi-drug resistant *Vibrio cholerae*. Tropical Journal of Pharmaceutical Research **3**:231-237.

Adeleye, I. A., R. O. Nwanze, F. V. Daniels, V. A. eyinnia, S. I. Smith, M. A. Fowora and H. A. Goodluck. 2010. Non-plasmid mediated multi-drug resistance in *Vibrio* and *Aeromonas* spp. isolated from seafoods in Lagos. International Journal of Food Safety **12**:10-15.

Akinbowale, O. L., H. Peng, and M. D. Barton. 2007. Diversity of tetracycline resistance genes in bacteria from aquaculture sources in Australia. Journal of Applied Microbiology **103**:2016-2025.

Amalaradjou, M.A.R., A. Narayanan, S.A. Baskaran, and K. Venkitanarayanan. 2010. Antibiofilm effect of trans-cinnamaldehyde on uropathogenic *Escherichia coli*. Journal of Urology **184**: 358-363.

Ardo, L., G. Yin, P. Xu, L. Váradi, G. Szigeti, Z. Jeney. 2008. Chinese herbs (*Astragalus membranceus* and *Lonicera Japonica*) and boron enhance the none-specific immune response of Nile tilapia, (*Oreochromis niloticus*), and resistance against *Aeromonas hydrophila*. Aquaculture **275**:26-33

Aravena-Romàn, M., T. J. J. Inglis, B. Henderson, T. V. Riley, B. J. Chang. 2011. Antimicrobial susceptibility of *Aeromonas* spp. isolated from clinical and environmental sources to 26 antimicrobial agents. Antimicrobial Agents and Chemotherapy **8**:3006-3008.

Asekun, O.T., E. Olusegun and O. Adebola. 2007. The volatile constituents of the leaves and flowers of *Kigelia africana* Benth. Flavor and Fragrance Journal **22**:21-23.

Asha, A., D.K. Nayak, K.M. Shankar, and C.V. Mohan. 2004. Antigenic expression in biofilm cells of *Aeromonas hydrophila* employed in oral vaccination of fish. Fish and Shellfish Immunology **16**: 429–436.

Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl.1989. Current protocols in molecular biology. John Wiley and Sons, New York, USA **2**:241-245.

Awad, E., and B. Austin. 2010. Use of *Lupinous perennis*, mango, *Mangifera indica* and stinging nettle, *Urtica dioica*, as feed additives to prevent *Aeromonas hydrophila* infection in rainbow trout, *Oncorhynchus Mykiss* (Walbaum). Journal of Fish Diseases **33**:413-420.

Azu, O. O., F. I. O. Duru, A. A. Osinobi, C. C. Naronha, S. O. Elesha, and A. O. Okanlawon. 2010. Protective agent, *Kigelia africana* fruit extract, against cisplatin-induced kidney oxidant injury in Sprague-Dawley rats. Asian Journal of Pharmaceutical and Clinical Research **2**:84-88.

Balsalobre, C. L., M. Dropa, D. E. De oliveira, N. Lincopan, E. M. Mamizuka, G. R. Matte and M. H. Matte. 2010. Presence of BLA$_{TEM_116}$ gene in environment isolates of *Aeromonas hydrophila* and *Aeromonas jandaei* from Brazil. 2010. Brazilian Journal of Microbiology **41**:718-719.

Balsalobre, L.C., M. Dropa, N. Lincopan, E.M. Mamizuka, G.R. Matte, and M.H. Matte. 2009. Detection of metallo β-lactamases-encoding genes in environmental isolates of *Aeromonas hydrophila* and *Aeromonas jandaei*. Letters in Applied Microbiology **49**:142-145.

Basson, A., L.A. Flemming, and H.Y. Chenia. 2008. Evaluation of adherence, hydrophobicity, aggregation, and biofilm development of *Flavobacterium johnsoniae*-like isolates. Microbial Ecology **55**:1-14.

Balassiano, I. T., M. C. F. Bastos, D. J. Madureira, I. G. da Silva, A. C. frietas-almeida, S. S oliveira. 2007. The involvement of *tetA* anf *tetE* tetracycline resistance genes in plasmid and chromosomal resistance of *Aeromonas* in Brazilian strains. Memorias do Instituto Oswald Cruz **102**:861-866.

Beaz-Hidalgo, R., A. Aperi, N. Bujan, J. L. Ronald, M. J. Figueras. 2010. Composition of phenotypical and genetic identification of *Aeromonas* strains isolated from diseased fish. Systematic Applied Microbiology **33**:149-153.

Beaz-Hidalgo, R., A. Martnez-Murcia and M. J. Figueras. 2013. Reclassification of *Aeromonas hydrophila* subsp. *dhakensis* Huys *et al*. 2002 and *Aeromonas aquariorum* Martinez-Murcia *et al*. 2008 as *Aeromonas dhakensis* sp. nov. comb. nov. and emendation of the species *Aeromonas hydrophila*. Systematic and Applied Microbiology **36**:171-176.

Bechet, M., and R. Blondeau. 2003. Factors associated with the adherence and biofilm formation by *Aeromonas caviae* on glass surfaces. Journal of Applied Microbiology **94**:1072-1078.

Behlau, I., and M.S. Gilmore. 2008. Microbial biofilms in ophthalmology and infectious disease. Archives of Ophthalmology **126**:1572-1581.

Bhat, R. S., and S. Al-Daihan. 2014. Phytochemical constituents and antibacterial activity of some green leafy vegetables. Journal of Tropical Biomedicine **4**: 189-193.

Binh, C. T. T., H. Heuer, M. Kaupenjohann and K. Smalla. 2009. Diverse aadA gene cassettes on class 1 integrons introduced into soil via spread manure. Research in Microbiology **160**:427-433.

Birnboim, H. C., and J. Doly. 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Resistance **7**:1513-1523.

Borges, A., M. J. Saavedra and M. Simoes. 2012. The activity of ferulic and gallic acids in biofilm prevention and control of pathogenic bacteria. Biofouling **28**:755-767.

Borges, A., L. C., M. J. Saavedra and M. Simoes. 2014. The action of selected isothiocyanates on bacterial biofilm prevention and control. International Biodeterioration and Biodegradation **86**:25-33.

Boucher, Y., M. Labbate, J. E. Koenig & H. W. Stokes. 2007. Integrons: mobilizable platforms that promote genetic diversity in bacteria. Trends in Microbiology **15**:301–309.

Brackman, G., U. Hillaert, S. Van Calenbergh, H.J. Nelis, and T. Coenye. 2009. Use of quorum sensing inhibitors to interfere with biofilm formation and development in *Burkholderia multivorans* and *Burkholderia cenocepacia*. Research in Microbiology **160**:144-151.

Cabello, F. C., H. P. Godfrey, A. Tomova, L. Ivanova, H. Dölz, A. Millanao and A. H. Buschmann. 2013. Antimicrobial use in aquaculture re-examined: its relevance to antimicrobial resistance and to animal and human health. Environmental Microbiology **213**:1-26.

Carvalho, M. J., A. Martinez-Murcia, A. C. Esteves, A. Correia and M. J. Saavedra. 2012. Phylogenetic diversity, antibiotic resistance and virulence traits of *Aeromonas* spp. from untreated waters for human consumption. International Journal of Food Microbiology **159**:230-239.

Casas, C., E. C. Anderson, K. K. Ojo, I. Keithe, D. Whelan, D. Rainnie, and M.C. Roberts. 2005. Characterisation of pRAS1-like plasmids from atypical North American psychrophilic *Aeromonas salmonicida*. FEMS Microbiology Letters **242**:59-63.

Chakraborty, S. B., and C. Hancz. 2011. Application of phytochemicals as immunostimulant, antipathogenic and antistress agents in finfish culture. Reviews in Aquaculture **3**:103-119.

Chang, S-T., P-F. Chen, and S-C.Chang. 2001. Antibacterial activity of leaf essential oils and their constituents from *Cinnamomum osmophloeum*. Journal of Ethnopharmacology **77**:123-127.

Chang, Y. C., D. Y. Shih, J. Wang, and S. Yang. 2007. Molecular characterization of class 1 integrons and antimicrobial resistance in *Aeromonas* strains from food borne outbreak-suspect

samples and environmental sources in Taiwan. Diagnostic Microbiology and Infectious Disease **59**:191-197.

Chen, P-L., W-C. Ko and C-J. Wu. 2012. Complexity of β-lactamase among clinical *Aeromonas* isolates and its clinical implications. Journal of Microbiology Immunology and Infection **45**:398-403.

Chenia, H. Y., and C. Vietze. 2012. Tetracycline resistance determinants of heterotrophic bacteria isolated from a South African tilapia aquaculture system. African Journal of Microbiology Research **6**:6761-6768.

Chenia, H. Y. 2013. Anti-qourum sensing potential of crude *Kigelia africana* fruit extracts. Sensors **13**:2802-2817.

Chikwendu, C. I., S. N. Ibe, and G. C. Okpokwasili. 2011. Detection of *bla*SHV and *bla*TEM beta-lactamase genes in multi-resistant *Pseudomonas* isolates from environmental sources. African Journal of Microbiology Research **15**:2067-2074.

Choresca, C. H., S. H. Choi, D. K. Gomez, J. H. Kim and S. C. Park. 2010. Bacteria isolated from the mucus of farmed-raised adult and juvenile charm Abalone, *Haliotis discus hannoi*. Journal of the Aquaculture Society **44**:139-144.

Chuang, H-C., Y-H. Ho, C-J. Lay, L-S. Wang, Y-S. Tsai, and C-C. Tsai. 2011. Different clinical characteristics among *Aeromonas hydrophila*, *Aeromonas veronii* biovar *sobria* and *Aeromonas caviae* monomicrobial bacteraemia. Infectious Diseases, Microbiology and Parasitology **26**:1415-1420.

Cizek, A., M. Dolejska, R. Sochorova, K. strachotova, V. Piackova, T. Vesely. 2010. Antimicrobiology resistance and its genetic determinants in aeromonads isolated in ornamental (koi) carp (*Cyprinus carpio* koi) and common carp (*Cyprinus carpio*). Veterinary Microbiology **142**:435-439.

Clinical and Laboratory Standards Institute. 2007. M100-S17. Performance Standards for Antimicrobial Susceptibility Testing, 17th informational supplement, vol. 27, No. 1, Wayne, PA.

Clinical and Laboratory Standards Institute. 2006. Methods for antimicrobial dilution and disk susceptibility testing of infrequently isolated or fastidious bacteria. Approved guideline M45-A. Clinical and Laboratory Standards Institute, Wayne, PA.

Cowan, M. M. 1999. Plant products as antimicrobial agents. Clinical Microbiology Reviews **12**:564-582.

Citarasu, T., R. R. Sekar, M. M. Babu, and M. P. Marian. 2002. Developing artemia enriched herbal diet for producing quality larvae in *Penaeus monodon*. Asia Fishery Science **15**:21-32.

Dada, A. A., E. O. Adeparusi, and O. V Alale. 2010. Dietary dried *Kigelia africana* fruits meal as fertility enhancer in female *Clarias gariepinus* (Burchell, 1822). Agriculture and Biology Journal of North America **5**:791-795.

De Britto, A. J., D. H. S Gracelin, and P. B. J. R. Kumar. 2012a. Screening and separation of bioactive compounds from *murraya koenigii* extracts. International Journal of Universal Pharmacy and Life Sciences **1**:56-63.

De Britto, A. J., P. B. J. R. Kumar, and D. Gracelin. 2012b. *Abrus precatorius* L.: A medicinal plant with potential as antibacterial agent. Journal of Pharmacy Research **2**:1207-1209.

De Carvalho C.C.C.R. 2007. Biofilms: recent developments on an old battle. Recent Patents on Biotechnology **1**:49-57.

Defoirdt, T., N. Boon, P. Bossier, and W. Verstraete. 2004. Disruption of bacterial quorum sensing: an unexplored strategy to fight infections in aquaculture. Aquaculture **240**:69-88.

De Luca, F., C. Giraud-Morm, G. M. Rossolini, J-D. Docquier and T. Fosse. 2010. Genetic and biochemical characterisation of TRU-1, the endogenous class C β-lactamase from *Aeromonas enteropelogenes*. Antimicrobial Agents and Chemotherapy **54**:1547-1554.

Del Pozo, J. L., and R. Patel. 2007. The challenge of treating biofilm-associated bacterial infection. Clinical Pharmacology and Therapeutics **82**:204-209.

Denyer, S. P. 1995. Mechanism of action of antibacterial biocides. International Biodeterioration and Biodegradation **36**:227-245.

Desai, B., and P. Desai. 2014. Biofilm formation and effect of disinfectants on the isolates obtained from aquatic ecosystem. Biomedical Research Journal **1**:1-7.

Dhayanthi, N. B., Ajit Kumar, T. T., Balasubramanian, T. 2012. Effects of *Excoecaria agallocha* leaves against *Aeromonas hydrophila* in marine ornamental fish, *Amphirion sebae*. Indian Journal of Geo-Marine Sciences **41**:76-82

Dias, C., V. Mota, A. Martinez-Murcia, M. J. Saavedra. 2012. Antimicrobial resistance patterns of *Aeromonas* spp. isolated from ornamental fish. Journal of Aquaculture Research and Development **3**:1-13.

Doughari, J. H., I. S. Human, S. Bennade, and P. A. Ndakidemi. 2009. Phytochemicals as chemotherapeutic agents and antioxidants: Possible solution to the control of the antibiotic resistant verocytotoxin producing bacteria. Journal of Medicinal Plants Research **11**:839-848.

Drenkard, E. 2003. Antimicrobial resistance of *Pseudomonas aeruginosa* biofilms. Microbes and Infection **13**:1213-1219.

Duma, S. T. 2012. Characterization of factors involved in and affecting biofilm formation by *Aeromonas* spp. isolates. University of KwaZulu Natal Masters Dissertation.

Eldeen, I. M. S., and J. Staden. 2008. Cyclooxygenase inhibition and antimycobacterial effects of extracts from Sudanese medicinal plants. South African Journal of Botany **74**:225-229.

Eldeen, I. M. S., and J. V. Staden. 2008. In vitro pharmacological investigation of extracts from some trees used in Sudanese traditional medicine. South African Journal of Botany **73**:435-440.

Elhariry, H.M. 2011. Biofilm Formation by *Aeromonas hydrophila* on green-leafy vegetables: cabbage and lettuce. Food borne Pathogens and Disease **8**:125-131.

FAO, 2002. State of World Fisheries and Aquaculture. FAO Fisheries Department, Food and Agriculture Organisation of the United Nations, Rome, Italy. 150 pp.

Farmer III, J. J., M. J. Arduino, and F. W. Hickman-Brenner. 2006. The Genera *Aeromonas* and *Plesiomonas*. The Prokaryotes **6**:564-596.

Fontes, M. C., M. J. Saavedra, C. Martins, and A. J. Martínez-Murcia. 2011. Phylogenetic identification of *Aeromonas* from pigs slaughtered for consumption in slaughter houses at the North of Portugal. International Journal of Food Microbiology **146**:118-122.

Fosse, T., C. Giraud-Morin, and I. Madinier. 2003. Phénotypes de résistance aux β-lactamines dans le genre *Aeromonas*. Pathological Biology (Paris) **51**:290–296.

Figuera, V., I. Vaz-Moreira, M. Silva, C. M. Manaia. 2011. Diversity and antibiotic resistance of *Aeromonas* spp. in drinking and waste water treatment plants. Water Research **45**:5599-5611.

Fitzgerald, D. J., M. Stratford, M. J. Gasson, J. Ueckert, A. Bos and A. Norbad. 2004. Mode of antimicrobial action of vanillin against *Escherichia coli*, *Lactobacillus platarum* and *Listeria innocua*. Journal of Applied Microbiology **97**:104-113.

Fuente-nunez, C., F. Reffuville, L. Fernandez and R.EW. Hancock. 2013. Bacterial biofilm development as a multicellular adaptation: antibiotic resistant and new therapeutic strategies. Current Opinion in Microbiology **16**:580-589.

Gavin, R., A. A. Rabaan, S. Merino, J. M. Tomas, I. Gryllos, and J. G. Shaw. 2002. Lateral flagella of *Aeromonas* species are essential for epithelial cell adherence and biofilm formation. Molecular Microbiology **43**:383-397.

Gavin, R., S. Merino, M. Altarriba, R. Canals, J. G. Shaw, and J. M Tomas. 2003. Lateral flagella are required for increased cell adherence, invasion and biofilm formation by *Aeromonas* spp. FEMS Microbiology Letters **224**:77-83.

Gabriel, O. A., and A. Olubunmi.2009. Comprehensive scientific demystification of *Kigelia africana*: A review. African Journal of Pure and Applied Chemistry **3**:158 -164.

Ghenghesh, K. S., S. F. Ahmed, R. A. ElKhalek, A. Al-Gendy and J. Klena. 2008. *Aeromonas*-associated infections in developing countries. Journal of Infections in Developing Countries **2**:81-98.

Godoy, M., V. Gherardelli, A. Heisinger, J. Fernandez, P. Olmos, L. Ovalle, P. Llardi and R. Avendano-Herrera. 2010. First description of atypical furunculosis in freshwater farmed Atlantic salmon, *Salmo salar* L., in Chile. Journal of Fish Diseases **33**:441-449.

Goni-Urriza, M., L. Pineall, M. Capedepy, C. Roques, P. Caumette, and C. Quentin. 2000. Antimicrobial resistance of mesophilic *Aeromonas* spp. isolated from two European rivers. Journal of Antimicrobial Chemotherapy **46**:297-301.

Gonzalez, G., S. Mella and R. Zemelmann. 2004. Integrons and resistance gene cassettes: structure and role against antimicrobials. Revista Medica de Chile **32**:619-626.

Gomez-garcés, J. L., D. Saez, M. Almagro, M. Fernandez-Romero, S. merino, F. Campos, J. Oteo. 2011. Osteomyelitis associated to CTX-M-15-producing *A*. *hydrophila*: first description in the literature. Diagnostic Microbiology and Infectious Disease **70**:420-422.

Gouda, Y. G., A. M. Abdel-Baky, F. M. Darwish, K. M. Mohammed, R. Kasai and K. Yamasaki. 2003. Iridoids from *Kigelia pinnata* DC. fruits. Phytochemistry **63**:887-892.

Gouda, Y. G., A. M. Abdel-Baky, K. M Mohammed, F. M. Darwish, R. Kasai and K. Yamasaki. 2006. Phenylpropanoid and phenylethanoid derivatives from *Kigelia pinnata* DC. fruits. Natural Product Research **20**:935-939.

Girlich, D., L. Poirel and P. Nordmann. 2011. Diversity of clavulanic acid-inhibited extended spectrum β-lactamase in *Aeromonas* spp. from the Seine river, Paris, France. Antimicrobial Agents and Chemotherapy **55**:1256-1261.

Grace, O. M., M. E. Light, K. L Lindsey, D. A. Mulholland, J. Van Staden and A. K. Jagar.2002. Antibacterial activity and isolation of active components from fruits of the traditional African medicinal tree *Kigelia africana*. South African Journal of Botany **68**:220-222.

Guerra, I. M. F., R. Fadamelli, M. Figuerio, F. Schreimer, A. P. L. Delaware, C. wollheim, S. O. P. Costa, S. Echeverrigaray. 2007. Aeromonas associated diarrhoeal disease in South Brazil: prevalence, virulence and antimicrobial resistance. Brazilian Journal of Microbiology **38**:638-643.

Hall, B. G., S. J. Salipante, M. Barlow. 2004. Independent origins of subgroup B1 and B2 and subgroup B3 metallo-β-lactamases. Journal of Molecular Evolution **59**:133-141.

Haque, S. F., S. S. Ali, T. P. Mohammed and A. U. Khan. 2012. Prevalence of plasmid mediated bla_{TEM-1} and $bla_{CTX-M-15}$ extended spectrum beta-lactamase in patients with sepsis. Asian Pacific Journal of Tropical Medicine **12**:98-102.

Harikrishnan, R., and C. Balasundaram. 2005. Modern Trends in *Aeromonas hydrophila* disease management with fish. Reviews in Fish Science **13**:281-320.

Harikrishanan, R. C. Balasundaram, and M-S. Heo. 2010. Herbal supplementation diets on hematology and innate immunity in goldfish against *Aeromonas hydrophila*. Fish and Shellfish Immunology **28**:354-361.

Harborne, J. B. 1973. Method of plant analysis. Phytochemical methods. London: Chapman and Hall **pp** 17.

Henriques, I. S., F. Fonseca, A. Alves, M. J. Saavedra, A. Correia. 2006. Occurrence and diversity of integrons and β-lactamase genes among ampicillin-resistant isolates from estuarine waters. Research in Microbiology **157**:938-947.

Hernould, M., S. Gagné, M. Fournier, C. Quentin, and C. Arpin. 2008. Role of the Ahe ABC pump in *Aeromonas hydrophila* intrinsic multidrug resistance. Antimicrobial Agents and Chemotherapy **52**:1559-1563.

Hoa, P. T. P., S. Mnagolia, N. Nkada, H. Takada, A. Shimizu, D. H. Anh, P. H. Viet and S. Suzuki. 2011. Antibiotic contamination and occurrence of antibiotic-resistant bacteria in aquatic environments of northern Vietnam. Science of the Total Environment **409**:2894-2901.

Houghton, P. J. 2002. The sausage tree (*Kigelia africana*): ethnobotany and recent scientific work. South African Journal of Botany **68**:14-20.

Huddleston, J.R., J.C. Zak, and R.M. Jeter. 2006. Antimicrobial susceptibilities of *Aeromonas* spp. isolated from environmental sources. Applied and Environmental Microbiology **72**:7036-7042.

Husain, F. M., J. Ahmad, A. Mohammed and Q. Tahseen. 2013. Influence of clove oil on certain quorum-sensing regulated functions and biofilm of *Pseudomonas aeruginosa* and *Aeromonas hydrophila*. Journal of Bioscience **38**:835-844.

Hopkins, K. L., L. Wootton, M. R. Dey, and E. J. Threfall. 2007. Plasmid-mediated quinolone resistance determinants *qnrS*I found in *Salmonella enteric* strains isolated in the UK. Journal of Antimicrobial Chemotherapy **59**:1071-1075.

Igbinosa, I. H., E. Igumbor, F. Aghdasi, T. Mvuyo, and A. I. Okoh. 2012. Emerging *Aeromonas* species infections and their significance in public health. The Scientific World Journal **2012**:1-13.

Igbinosa, I. H., and A. I. Okoh. 2012. Antibiotic susceptibility profile of *Aeromonas* species isolated from wastewater treatment plant. The Scientific World Journal **2012**:1-6.

Igbinosa, I. H., V. N. Chigor, E. O. Igbinosa, l. C. obi, and A. I. Okoh. 2013. Antibiogram, adhesive characteristics and incidence of class1 integron in *Aeromonas* species isolated from two South African rivers. Biomedical Research International **2013**:1-8.

Igbinosa, I. H. 2014. Antibiogram profiling and pathogenic status of *Aeromonas* spp. recovered from chicken. Saudi Journal of Biological Sciences **21**:481-485.

Jacobs, L., and H. Y. Chenia. 2007. Characterization of integrons and tetracycline resistance determinants in *Aeromonas* spp. isolated from South African aquaculture systems. International Journal of Food Microbiology **114**:295-306.

Jacoby, G. A., K. E. Walsh, D. M. Millis, V. J. Walker, H. Oh, A. Robicsek and A. C. Hooper,. 2006. *qnrB*, another plasmid-mediated gene for quinolone resistance. Antimicrobial Agents and Chemotherapy **50**:1178-1182.

Jahid, I. K., N. Han and S-D. Ha. 2104. Inactivation kinetics of cold oxygen plasma depends on incubation conditions of *Aeromonas hydrophila* biofilm on lettuce. Food Research International **55**:181-189.

Jay, J. M., and G. M. Rivers. 1984. Antimicrobial activity of some food flavoring compounds. Journal of Food Safety **6**:129-139.

Jeyachandran, R., and A. Mahesh. 2007. Antimicrobial evaluation of *Kigelia africana* (Lam). Research Journal of Microbiology **8:**645-649.

Jia, p., Y. J. Xue, X. J. Duan, and S. H. Shao. 2011. Effect of cinnamaldehyde on biofilm formation and *sarA* expression by methicillin-resistant *Staphylococcus aureus*. Letters in Applied Microbiology **53**:409-416.

Jun, W.J., J. H. Kim, D. K. Gomez, C. H. Choresca jnr, J. E. Han, S. P. Shin, and S. C. Park. 2010. Occurrence of tetracycline-resistant infection in Korean cyprinid loach (*Misgumus anguillicaudatus*). Journal of Microbiology Research **9**:849-855.

Kadlec, K., E. V. Czapiewski, H. Kaspar, J. Wallmann, G. B. Micheal, U. Steinacker, S. Schwarz. 2011. Molecular basis of sulphonamide and trimethoprim resistance in fish-pathogenic *Aeromonas* isolates. Applied and Environmental Microbiology **77**:7147-7150.

Kappachery, S., D. Paul, J. Yoon, and J. H. Kweon. 2010. Vanillin, a potential agent to prevent biofouling of reverse osmosis membrane. The Journal of Bioadhesion and Biofilm Research **26**:667-672.

Kaplan, J. B. 2010. Biofilm dispersal: Mechanisms, clinical implications, and potential therapeutic uses. Journal of Dental Research **89**:205-218.

Khajanchi, B. K., A. A. Fadl, M. A. Borchardt, R. L. Berg, R. L. Hornemon, M. E. Joseph, S. W. Moyer, J. Sha and A. A. Chopra. 2010. Distribution of virulence factors and molecular

fingerprinting of *Aeromonas* species isolates from water and clinical sample suggestive evidence of water-to-human transmission. Applied and Environmental Microbiology **7**:2313-2325.

Khan, M. S. A., and I. Ahmad. 2011. Anti-biofilm activity of certain phytochemicals and their synergy with flucanazole against Candida albicans biofilms. Journal of Antimicrobial Chemotherapy **67**:618-621.

Kiplimo, J. J., N. A. Koorbanally, and H. Y. Chenia. 2011. Triterpernoids from *Vernonia auriculifera* Hiern exhibit antimicrobial activity. African Journal of Pharmacy and Pharmacology **5**:1150–1156.

Kirov, S. M., B. C. Tassel, A. B. T. Semmler, L. A. O'Donovan, A. A. Rabaan, and J. G. Shaw. 2002. Lateral flagella and swarming motility in *Aeromonas* species. Journal of Bacteriology **184**: 547-555.

Kostakioti, M. M. Hadjifrangiskou and S. J. Hultgreen. 2013. Bacterial biofilms: development, dispersal and therapeutic strategies in the dawn of the post-antibiotic era. Cold Spring Harbour Perspectives in Medicine **2013**:1-24.

Kozinska, A., and L. Guz. 2004. The effect of various *Aeromonas bestiarum* vaccines on non-specific immune parameters and protection of carp (*Cyprinus carpio* L.). Fish Shellfish Immunology **6**:437-445.

Kregiel, D., and K. Niedzielska. 2014. Effect of plasma processing and organosilone modifications of polyethylene on *Aeromonas hydrophila* biofilm formation. Biomedical Research International **214**:1-5.

Kreth, J., Y. Zhang, M. C. Herzberg. 2008. Streptococcal antagonism in oral biofilms: *Streptococcus sanguinis* and *Streptococcus gordonii* interference with *Streptococcus mutans*. Journal of Bacteriology **190**:4632-4640.

Kümmerer, K. 2009. Antibiotics in the Aquatic Environment. Chemosphere **75**:435-441.

L'Abee-Lund, T. M., and H. Sorum. 2001. Class 1 integrons mediate antibiotic resistance in the fish pathogen *Aeromonas salmonicida* worldwide. Microbial Drug Resistance **7**:263-272.

Lamy, B., A. Kodjo, the colBVH Study Group, and F. Laurent. 2009. Prospective nationwide study of *Aeromonas* infections in France. Journal of Clinical Microbiology **47**:1234–1237.

Lapierre, L., J. Cornejo, C. Borie, C. Toro and B. S. Martin. 2008. Genetic characteristics of antibiotic genes linked to class 1 and class 2 integrons in commensal strains of *Escherichia coli* isolated from poultry and swine. Microbiological Drug Research **14**:265-272.

Lee, M. F., C. F. Peng, Y. H. Lin, S. R. Lin, and Y. H. Chen. 2008. Molecular diversity of class 1 integrons in human isolates of *Aeromonas* spp. from Southern Taiwan. Japan Journal of Infectious Diseases **61**:343-349.

Lewis, K. 2005. Persister cells and the riddle of biofilm survival. Biochemistry **70**:267-274.

Levy, S. B., and B. Marshall. 2004. Antibacterial resistance worldwide: causes, challenges and responses. Nature Medical **10**:S122–9.

Libisch, B., C. G. Giske, B. Kovács, T. G. Tóth, and M. Füzi. 2008. Identification of the first VIM metallo-β-lactamase-producing multiresistant *Aeromonas hydrophila* strain. Journal of Clinical Microbiology **46**:1878–1880.

Lopez, D., H. Vlamakis and R. Kolter. 2010. Biofilms. Cold Spring Harbour Perspectives in Biology. **256**:1-15.

Lu, S-Y., Y-L. Zhang, S-N. Geng, T-Y. Li, Z-M. Ye, D-S. Zhang, F. Zou and H-W. Zhou. 2010. High diversity of extended-spectrum beta-lactamase producing bacteria in an urban river sediment habitat. Applied and Environmental Microbiology **76**:5972-5976.

Lu, C., H. Wang, W. L. P. Xu, J. Zhu, J. Xie, B. Liu, and Z. Lou. 2011. Antibacterial properties of anthraquinones extracted from rhubarb against *Aeromonas hydrophila*. Fisheries Science **77**:375–384.

Lupo, A., S. Cogne, and T. U. Berendonk. 2012. Origin and evolution of antibiotic resistance: The common mechanisms of emergence and spread in water bodies. Frontiers in Microbiology **3**: 1-13.

Lynch, M. J., S. Swift, D. K. Kirke, C. W. Keevil, C. E. R. Dodd, and P. Williams. 2002. The regulation of biofilm development by quorum sensing in *Aeromonas hydrophila*. Environmental Microbiology **4**:18-28.

Mah, T.-F.. C., and G.A. O'Toole. 2001. Mechanisms of biofilm resistance to antimicrobial agents. Trends in Microbiology **9**:34-39.

MahfuzulHoque, M. D., Bari, M. L., Inatsu, Y. Juneja, V. K., and Kwaamoto, S. 2007. Antibacterial activity of guava (*Psidium guajava* L.) and neem (*Azadirachta indica* A. Juss.) extracts against food-borne pathogens and spoilage bacteria. Food-borne Pathogens and Disease **4**:481-488.

Majumbar, T., S. Ghosh, J. Pal, and S. Mazumder. 2006. Possible role of a plasmid in the pathogenesis of a fish disease caused by *Aeromonas hydrophila*. Aquaculture **256**:95-104.

Matyar, F., T. Akkan, Y. Ucak, B. Eraslan. 2010. *Aeromonas* and *Pseudomonas*: antibiotic and heavy metal resistant species from Iskenderun bay, Turkey (northeast Mediterranean Sea). Environmental Monitoring Assessment **167**:309-320.

Maravic, A., M. Skocibusic, I. Samanic, Z. Fredotovic, S. Cvjetan, M. Jutronic, J. Puizina. 2013. *Aeromonas* spp. simultaneously harbouring $bla_{CTX\text{-}M\text{-}15}$, $bla_{SHV\text{-}12}$, $bla_{PER\text{-}1}$ and $bla_{FOX\text{-}2}$, in wild

growing Mediterranean mussel (*Mytilus galloprovincialis*) from Adriatic Sea, Croatia. International Journal of Food Microbiology **166**:301-308.

Marchandin, H., S. Godreuil, H. Darbes, H. Jean-Pierre. 2003. Extended spectrum beta-lactamase tem-24 in an *Aeromonas* clinical strain: acquisition from the prevalent *Enterobacter aerogenes* clone in France. Antimicrobial Agents and Chemotherapy **47**:3994-3995.

Marti, E., J. Jofre, J. I. Balcazar. 2013. Prevalence of antibiotic resistance genes and bacterial community composition in a river influenced by a waste water treatment plant. PLOS One **10**:234-233.

Martınez-Murcia, A. J., M. J. Saavedra, V. R. Mota, T . Maier, E. Stackebrandt, S. Cousin. 2008. *Aeromonas aquariorum* sp. nov., isolated from aquaria of ornamental fish. International Journal Systematic and Evolutionary Microbiology **58**:1169–1175.

Mayanard, C., S. Bekal, F. Sanschagrin, R. C. brousseau, L. Masson, S. Lariviere and J. Harel. 2004. Heterogeneity among virulence and antimicrobial resistant gene profiles of extraintestinal *Escherichia coli* isolates of animals and human origins. Journal of Clinical Microbiology **42**:5444-5452.

Meyer, L., C. D. J. Labuschagne, M. M. Ehlers, M. G. Dove, G. F. Weldhagen. 2007. Diversity of *bla*-type genes in extended-spectrum β-lactamase-producing *Klebsiella pneumoniae* isolated during 2003-2004 at Pretoria academic hospital. South African Journal of Epidemiology and Infection **22**:5-7.

McGaw, L. J., N. Lall, J. J. M. Meyer, and J. N. Eloff. 2008. The potential of South African plants against *Mycobacterium* infections. Journal of Ethnopharmacology **119**:482-500.

Mcintosh, D., M. Cunningham, B. Ji, F. A. fekete, E. M. Parry, S. E. Clark, Z. B. zalinger, I. C. Gilg, G. R. Danner, K. A. Johnson, M. Bealtie and R. Ritchie. 2008. Transferable, multiple antibiotic and mercury resistance in Atlantic Canadian isolates of *Aeromonas salmonicida* subsp. *salmonicida* is associated with carriage of an IncA/C plasmid similar to the salmonicida enteric plasmid PSN254. Journal of Antimicrobial Chemotherapy **61**:1221-1228.

Millezi, A. F., M. G. Cardoso, E. Alves, R. H. Piccolo. 2013. Reduction of *Aeromonas hydrophila* biofilm on stainless steel surface by essential oils. Brazilian Journal of Microbiology **44**:73-80.

Molyneaux, R. J., S. T. Lee, D. R. Gardner, K. E. Panter, and J. F. Jones. 2007. Phytochemicals: The good, the bad and the ugly? Phytochemistry **68**:2973-2985.

Morozumi, S. 1985. Isolation, purification, and antibiotic activity of o-methoxycinnamaldehyde from cinnamon. Applied and Environmental Microbiology **36**:577-582.

Moura, A., I. Henriques, R. Ribeiro, A. Correia. 2007. Prevalence and characteristics of integrons from bacteria isolated from a slaughterhouse wastewater treatment plant. Journal of Antimicrobial Chemotherapy **60**:1243-1250.

Naas, T., C. Oxaceky and P. Nordmann. Identification of CTX-M-type extended spectrum-β-lactamase genes using real-time PCR and pyro-sequencing. Antimicrobial Agents and Chemotherapy **51**:223-230.

Nagar, V., R N. Shashidhar and J. R. Bondekar. 2011. Prevalence, characterization and antimicrobial resistance of *Aeromonas* strains from various retail food products in Mumbai, India. Journal of Food Science **76**:486-492.

Najimi, M., M. L. Lemos, and C. R. Osorio. 2008. Distribution of small plasmids in *Aeromonas salmonicida* strains isolated from NW Spain and Portugal: evidence of clonality in strains isolated from turbot *Psetta maxima* (L.). Journal of Fish Diseases **31**:469-472.

Nawaz, M., S. A. Khan, A. A. Khan, K. Sung, Q. Tran, K. Kerdahi, R. Steele. 2010. Detection and characterization of virulence genes and integrons in *Aeromonas veronii* isolated from catfish. Food Microbiology **27**:327–331.

Nascimento, G. G. F., L. Juliana, P. C. Freitas, G. L. Silva. 2000. Antibacterial activity of plant extracts and phytochemicals on antibiotic resistant bacteria. Brazilian Journal of Microbiology **31**:247-256.

Naviner, M., L. Gordon, E. Giraud, M. Denis, C. Mangion, H. Le-Bris, and J. Ganiere. 2011. Antimicrobial resistance of *Aeromonas* spp. isolated from the growth pond to the commercial product in a rainbow trout farm following a flumequine treatment. Aquaculture **315**:236-241.

Nazzaro, F., F. Fratianni and R. Coppola. Quorum sensing and phytochemicals. International Journal of Molecular Science **14**:12607-12619.

Neuwirth, C., E. Siebor, F. Robin, R. Bonnet. 2007. First occurrence of an isolate from france. Antimicrobial Agents and Chemotherapy **51**:4486-4488.

Ndi, O. L., and Barton, M. D. 2011.Incidence of class 1 integron and other antibiotic resistance determinants in *Aeromonas* spp. from rainbow trout farms in Australia. Journal of Fish Diseases **34**:589-599.

Niu, C. and E. S. Gilbert. 2004. Colorimetric method for identifying plant essential oil components that affect biofilm formation and structure. Applied and Environmental Microbiology **12**:6951-6956.

Niu, C., S. Afre and E. S. Gilbert. 2006. Subinhibitory concentrations of cinnamaldehyde interference with quorum sensing. Letters in Applied Microbiology **43**:489-494.

Nuryastuti, T., Van der Mei, H. C., Bussher, H. J., Iravati, S., Aman, A. T., and Krom B. P. 2009. Effect of cinnamon oil on *ica*A expression and biofilm formation by *Staphylococcus epidermidis*. Applied and Environmental Microbiology **75**:6850-6855.

Nya, E. J., Dawood, Z., and Austin, B. 2010. The garlic component Allicin prevents disease caused by *Aeromonas hydrophila* in rainbow trout, *Oncorhynchus mykiss* (Walbaum). Journal of Fish Disease **33**:293-300.

Obi, C. L., J. Ramalivhana, A. Samie, J. O. Igumbor. 2007. Prevalence, pathogenesis, antibiotic susceptibility profiles and in-vitro activity of selected medicinal plants against *Aeromonas* isolates from stool samples of patients in the Venda region of South Africa. Journal of Health Population and Nutrition **4**:428-435.

Obi, C. L., J. Ramalivhana, M. N. B. Momba, B. Onabolu, J. O. Igumbor, M. Lukoto, T. B. Mulaudzi, P. O. Bessong, E. L. Jansen Van Rensburg, E. Green, and S. Ndou. 2007. Antibiotic resistance profiles and relatedness of enteric bacterial pathogens isolated from HIV/AIDS patients with and without diarrhoea and their household drinking water in rural communities in Limpopo province South Africa. African Journal of Biotechnology **6**:1035-1047.

Ooi, L. S. M., Li, Y., Kam S-L., Wang, E. Y. L., and Ooi, V. E. C. 2006. Antimicrobial activities of cinnamon oil and cinnamaldehyde from the Chinese medicinal herb *Cinnamomum cassia* Blume. American Journal of Chinese Medicine **34**:511-522.

Owolabi, O. J., Omogbai, E. K. I., Obasuyi, O.2007. Antifungal and antibacterial activities of ethanolic and aqueous extract of *Kigelia africana* (Bignoniaceae) stem bark. African Journal of Biotechnology **6**:1677-1680.

Ozkan, G., O. Sagdic, R. S. Gokturk, O. Unalis, and S. Albayrak. 2010. Study on chemical composition and biological activities of essential oil and extract from *Salvia pisidia*. Food Science and Technology **43**:186-190.

Pachanawan, A., P. Phumkhachorn, and P. Rattanachaikunsopon. 2008. Potential of *Psidium guajava* supplemented fish diets in controlling *A. hydrophila* infection in tilapia (Oreochromis niloticus). Journal of Bioscience and Bioengineering **106**:419-424.

Palu, A. P., M. A. L. Miguel, I. I. Balassiano, M. L. P. Queiroz, A. C. Freitas-Almeida, and S. S. De-Oliveira. 2006. Antimicrobial resistance in food and clinical *Aeromonas* isolates. Food Microbiology **23**:504-509.

Partridge, S. R., G. Tsafnat, E. Coiera and J. R. Iredell. 2009. Gene cassette arrays in mobile resistance integrons. FEMS Microbiology Reviews **33**:757-784.

Paterson, D. L. and R. A. Bonomo. 2005. Extended spectrum β-lactamases: a clinical update. Clinical Microbiology Reviews **18**:657-686.

Pakravan, S., A. Hajimoradloo and R. Ghorbani. 2011. Effect of dietary willow herb, *Epilobium hirsutum* extract on growth performance, body composition, haematological parameters and *Aeromonas hydrophila* challenge on common carp. *Cyprinus carpio*. Aquaculture Research **2011**:1-9.

Parker, J. L., and J. C. Shaw. 2011. *Aeromonas* spp. clinical microbiology and disease. Journal of Infection **62**:109-118.

Pérez-Valdespino, A., E. Férnāndez-Rendõn and E. Curiel-quesada. 2009. Detection and characterisation of class 1 integrons in aeromonas spp. isolated from human diarrheic stool in Mexico. Journal of Basic Microbiology **49**:572-578.

Pellegrini, C., G. Celenza, B. Segatore, P. Beslio, D. Setacci, G. Amicosante and M. Perili. 2010. Occurrence of class 1 and class 2 integrons in resistance Enterobacteriaceae collected from a urban wastewater treatment plant: fruit report from central Italy. Microbiology and Drug Resistance **17**:229-234.

Phillipson, J. D. 2001. Phytochemistry and medicinal plants. Phytochemistry **56:**237-243.

Ponnusamy, K., D. Paul, and J.H. Kweon. 2009. Inhibition of quorum sensing mechanism and *Aeromonas hydrophila* biofilm formation by vanillin. Environmental Engineering Science **26**: 1359-1363.

Ponnusamy, K., D. Paul, Y. Sam Kim, and J.H. Kweon. 2010. 2(5H)-furanone: a prospective strategy for biofouling-control in membrane biofilm bacteria by quorum sensing inhibition. Brazilian Journal of Microbiology **41**:227-234.

Ponnusamy, K., S. Kappachery, M. Thekeetle, J, H. Song, J. H. Kweon. 2013. Anti-biofouling property of vanillin on *A. hydrophila* initial biofilm on various membrane surfaces. World Journal of Microbiology and Biotechnology **29**:1695-1703.

Poole, K. 2004. Resistance to β-lactam antibiotics. Cellular and Molecular Life Sciences **61**:2200-2223.

Picerno, P., C. Antere, S. Marzocco, M. Mekni, R. Sanogo and R. P. Aquino.2005. Anti-inflammatory activity of vermonosides from *Kigelia africana* and evaluation of cutaneous irritation in cell cultures and reconstituted human epidermis. Journal of Natural Products **68**:1610-1614.

Picào, R. C., L. Poirel, A. Demarta, C. S. F. Silva, A. R. Corvaglia, O. Petrini, and P. Nordmann. 2008. Plasmid-mediated quinolone resistance in *Aeromonas allosaccharophila* recovered from a Swiss lake. Journal of Antimicrobial Chemotherapy **62**:948-950.

Picào, R. C., J. P. Cardoso, E. H. Campana, A. G. Nicoletti, F. V. Petrolini, D. M. Assis, L. Juliano and A. C. Gales. 2013. The role of antimicrobial resistant from the hospital effluent to

the environment: focus on the occurrence of KPC producing *Aeromonas* spp. and *Enterobacteriaceae* in sewage. Diagnostic Microbiology, Infection and Disease **76**:80-85.

Pitts, B., M. A. Hamilton, N. Zelver, P. S. Stewart. 2003. A microtiter-plate screening method for biofilm disinfection and removal. Journal of Microbiology Methods **54**:269-276.

Piyidar, V., A. Kaznowski, N. B. Narayan, M. Patole and Y. S. Shouche. 2002. *Aeromonas culicicola* sp. nov., from midgut of *Culex quinquefasciatus*. International Journal of Systematic and Evolutionary Microbiology **52**:1723-1728.

Radu, S., N. Almad., F. H. Ling, and A. Reezal. 2003. Prevalence and resistance to antibiotics for *Aeromonas* spp. from retail fish in Malaysia. International Journal of Food Microbiology **81**:261-266.

Rajendiran, A., Natarajan, E., Subramanian, P. 2008. Control of *Aeromonas hydrophila* infection in spotted snakehead, *Chana puntatus*, by *Solanum nigrum*, L., a medicinal plant. Journal of the World Aquaculture Society **39**:375-383.

Rossolini, G. M., A. zanchi, A. Chiesurin, G. Amicosante, G. Salta and P. Guglielmelti. 1995. Distribution of CphA or related carbapenemases activity in members of the genus *Aeromonas.* Antimicrobial Agents and Chemotherapy **39**:346-349.

Rubtsova, M. T., M. M. Ulyashova, T. T. Bachmann, R. D. Schimd and A. M. Egorov. Muliparametric determination of genes and their point mutations for identification of beta-lactamase. Biochemistry **75**:1628-1649.

Saini, S., H. Kaur., B. Verma., Ripudaman, and S. K Singh. 2009. *Kigelia africana* (Lam) Beth. - An overview. Natural Product Radiance **2**:190-197.

Saleem, M., M. Nazir, M. S Ali, H. Hussain, Y. S. Lee, N. Riaz and A. Jabbar. 2010. Antimicrobial natural products: An update on future antibiotic drugs. Nature Product Reports **27**:238-254.

Sànchez-céspedes, J., M. J. Figueras, C. aspiroz, M. J. aldea, M. Toledo, A. Alperi, F. Marco and J. Vila. 2009. Development of impenem resistance in an *A*. *veronii* biovar *sobria* clinical isolate recovered from a patient with cholangitis. Journal of Medical Microbiology **58**:451-455.

Saria-Guzmàn, Y., A. P. Lõpez-ramirez, Y. Chàvez-romero, E. Ruiz-romero,L. Dendooven and J. M. Bello-lopez. 2013. Identification of antbiotic resistance cassettes in class 1integrons in aeromonas spp. strains isolated from fresh fish (*Cyprinus carpio* L.). Current Microbiology **68**:581-586.

Senthikumar, M., Gurumoorthi, P., and Janardhana, K. 2005.Antibacterial potential of some plants used by trials in Maruthamalai hills, Tamil Nadu. Natural Product Radiance **4**:27-34.

Santos, P.G., P.A. Santos, A.R. Bello, and A.C. Freitas-Almeida. 2010. Association of *Aeromonas caviae* polar and lateral flagella with biofilm formation. Letters in Applied Microbiology **52**:49–55.

Samaha-Kfoury, J. N., and G. F. Araj. 2003. Recent developments in β-lactamases and extended spectrum β-lactamases. Clinical Reviews **327**:1209-13.

Shai, L. J., L. J McGraw, P. Masoko, and J. N Eloff. 2008. Antifungal and antibacterial activity of seven traditionally used South African plant species active against *Candida albicans*. South African Journal of Botany **74**:677-684.

Sorum, H., T.M. L' Abee-Lund, A. Solberg, and A. Wold. 2003. Integron-containing IncU R plasmids pRAS1 and pAr-32 from the fish pathogen *Aeromonas salmonicida*. Antimicrobial Agents and Chemotherapy **47**:1285-1290.

Simões, M., L.C. Simões, and M.J. Viera. 2010. A review of current and emergent biofilm control strategies. Food Science Technology **43**:573-583.

Siri, S. P., P. Wadbua, W. Wangphathanakul, N. Kitancharoen and P. Chantaranothai.2008. Antibacterial and phytochemical studies of 20 Thai medicinal plants against catfish-infectious bacteria *Aeromonas caviae*. Khon Kaen University Science Journal **36**:1-10.

Stalder, T., O. Barraud, M. Casellas, C. Dagot, M-C. Ploy. 2012. Integron involvement in environment spread of antibiotic resistance. Frontiers in Microbiology **3**:1-14.

Sunde, M. 2005. Prevalence and characterisation of class 1 and class 2 integrons in Escherichia coli isolated from meat and meat products of Norwegian origin. Journal of Antimicrobial Agents and Chemotherapy **58**:741-747.

Tacao, M., A. Moura, A. Correia and I. Henriques. 2014. Co-resistance to different classes of antibiotics among extended-spectrum-beta-lactamase-producers from aquatic systems. Water Research **1448**:100-107.

Talavera, B. M. M., F. O. Benassi, M. H. Von Specht, M. I. Quiroga, M. A. A. Garcia, A. B. Pucciarelli, E. Zubreski, M. E. Laczeski, and G. Gutkind. 2006. Susceptibilities to carbepenams and presence of *cphA* gene on food borne *Aeromonas*. Brazilian Archives of Biology and Technology **40**:677-682.

Tenover, F. C. 2006. Mechanisms of antimicrobial resistant in bacteria. The American Journal of Medicine **6A**:S3-S10.

Tristram, S. G., R. Haves and J. Souprounov. 2005. Variation in selected regions of bla_{TEM} genes and promoters *Haemophilus influenza*. Journal of Antimicrobial Chemotherapy **56**:481-484.

Tsai, M.-S., C.-Y. Kuo, M.-C. Wang, H.-C. Wu, C.-C. Chien, and J.-W. Liu. 2006. Clinical features and risk factors for mortality in *Aeromonas* bacteremic adults with hematologic malignancies. Journal of Microbiology, Immunology and Infections **39:**150–154.

Turska-szewczuk, A., B. Lindner, I. Komaniecka, A. Kozinska, A. Pekala, A. Choma and O. Holst. 2013. Structural immunochemical studies of the lipopolysaccharide from the fish pathogen *Aeromonas bestiarum* strain K296, serotypes 016. Marine Drugs **235**:1-10.

Vega-Sanchez, V., J. Acosta-Dibarrat, F. Vega-Castillo, G. Castro-Escarpulli, G. Aguilera-Arreola and E. Soriano-Vargas. 2014. Phenotypical characteristics, genetic. Identification and antimicrobial sensitivity of *Aeromonas* species isolated from farmed rainbow trout (Onchorynchus Mykiss) in Mexico. Acta Tropica **130**:76-79.

Velmurugan, S., S. M. J. Punitha, M. M. Babu, T. Selvaraj, and T. Citarasu. 2010. Indian Herbal Medications to replace antibiotics for shrimp *Penaeus monodon* post larvae. Journal of Applied Aquaculture **3**:230-239.

Verner-Jeffreys, D. W., T. J. Welch, T. Schwarz, M. J. Pond, M. J. Woodward and S. J. Haig. 2009. High prevalence of multidrug-tolerant bacteria and associated antimicrobial resistance genes isolated from ornamental fish and their carriage water. PLOS One. **234**:1-6.

Vladimir, P., Z. Julia, L. Elena, Z. Natalia, K. Alexander, K. Inessa. 2103. Effect of plant phenolic compounds on biofilm formation by *Pseudomonas* Acta Pathologica Microbiologica et Immunologica Scandinavica. **121**:1073-1081.

Vinay, T. N., R. P. Patil, B. P. P. Suresh, R. Rajesh, and K. M. Shankar. 2013. Evaluation of the efficiency of *Aeromonas hydrophila* biofilm vaccine in *Labeo rohita* employing monoclonal antibody based ELISA. Scientific Reports **2**:1-5.

Walton, N. J., M. J. Mayer, and A. Narbad. 2003. Vanillin. Phytochemistry **63**:505-515.

Walsh, T .R., W. A. Neville, M. H. Haran, D. Tolson, D. J. Payne, J. H. Bateson, A. P. Mac Gowan and P. M. Bennett. 1998. Nucleotide and amino acid sequence of the metallo-β-lactamase, Imis, from *Aeromonas veronii* bv. *sobria*. Antimicrobial Agents and Chemotherapy **42**:436-439.

WHO (World Health Organization). 2004. Joint FAO/OIE/WHO second workshop on none-human antimicrobial usage and antimicrobial resistance: Management Options. pp 1-31.

Wu, C.J., J. J. Wu, J. J. Yan, H. C. Lee, N. Y. Lee, C. M. Chang, H. I. Shih, H. M. Wu, L. R. Wang, W. C. Ko. 2007. Clinical significance and distribution of putative virulence markers of 116 consecutive clinical *Aeromonas* isolates in southern Taiwan. Journal of Infections **54**:151-158.

Wu, C-J., Y-C. Chuang, M-F. Lee, C-C. Lee, H-C. lee, N-Y. Lee, C-M. Chang, P-L. Chen, Y-T. Lin, J-J. Yan, and W-C Ko. 2011. Bacteraemia due to extended-spectrum-β-lactamase producing *Aeromonas* spp. at a medical centre in southern Taiwan. Antimicrobial Agents and Chemotherapy **55**:5813-5818.

Xanthopoulos, v., n. Tzanetakise. Litopoulou-tzanetaki. 2010. Occurrence and characterisation of *Aeromonas hydrophila* and *Yersina enterocolitica* in minimally processed fresh vegetable salads. Food Control **21**:393-398.

Ye, Y., X-H. Xu and J-B. Li. 2010. Emergency of CTX-M-3, TEM-1 and a new plasmid-mediated MOX-4 Amp C in a multiresistant *A. caviae* isolate from a patient with pneumoniae. Journal of Medical Microbiology **59**:843-847.

Yin, G., L. Ardo, K. D. Thompson, A. Adams, Z. Jeney, and G. Jeney. 2009. Chinese herbs (*Astragalus radix* and *Ganoderma lucidum*) enhance immune response of carp, *Cyprinus carpio*, and protection against *Aeromonas hydrophila*. Fish and Shellfish Immunology **26**:140-145.

Zaky, A. M. M., F. A. Mansour and K. M. Person. 2010. Factors influencing multi-drug resistant and plasmid harbouring *Aeromonas hydrophila* isolated from Lake Manzala, Eqypt. Journal of Bacteriology Research **2**:30-40.

Zhang, X., T. Zhang, and H. H. P. Fang. 2009. Antibiotic resistance genes in water environments. Applications of Microbiological Biotechnology **82**:397-414.

Zhou, F., Ji, B., Zhang, H., Jiang, H., Yang, Z., Li, J., and Yan, w. 2007.The antibacterial effect of cinnamaldehyde, thymol, carvacrol and their combinations against the food-borne pathogen *Salmonella typhimurium*. Journal of Food Safety **27**:124-133.

APPENDIX

Table A1: Zones of inhibition (mm) obtained with cinnamaldehyde and *K. africana* extracts as well as standard antimicrobial agents, ampicillin (AMP10) and tetracycline (TE30), against water-and fish-associated *Aeromonas* spp. isolates

Isolates	Source	Species ID	C 125*	C 250*	C 500*	C 1250	EX 1*	EX 2*	EX 3*	EX 4*	AMP10*	TE30*
A. caviae		ATCC 15468^T	9	16	22	34	11	9	15	0	0	28
A. hydrophila		ATCC 7966^T	14	17	23	33	14	10	17	0	0	27
E. coli		ATCC 25922	0	0	0	40	8	8	10	0	22	28
M1	Catfish	*A. culicicola*	9	16	21	21	8	0	14	0	0	11
M2	Tilapia	*A. hydrophila*	8	14	19	20	11	7	18	0	14.5	18
M3	Catfish	*A. allosaccharophila*	14	19	27	28	8.5	0	10.5	0	0	28
M4	Catfish	*A. jandaei*	0	9	17	20	9	0	11	0	13	27.5
M5	Catfish	*A. hydrophila*	0	12	14	19	9	10.5	14	0	0	12.5
M6	Catfish	*A. hydrophila*	0	0	17	20	0	0	13.5	0	14	14.5
M7	Catfish	*A. culicicola*	19	30	41	21	0	0	11.5	0	0	26
M8	Tilapia	*A. allosaccharophila*	0	12	17	25	9	8	15	0	0	14
M9	Catfish	*P. shigelloides*	10	14	23	20	6	7	9	0	0	13.5
M10	Catfish	*A. culicicola*	14	19	26	32	7	0	12	0	0	16
M11	Catfish	*Aeromonas* spp.	11	16	21	23	11.5	0	13.5	0	0	17
M12	Koi carp	*A. bestiarum*	0	0	13	21	9	9	13	0	12	18
M13	Catfish	*A. hydrophila*	14	17	25	27	9	8	12.5	0	0	25
M14	Tilapia	*A. hydrophila*	10	14	27	29	12.5	10	18	0	19	12
M15	Tilapia	*Aeromonas* spp.	9	13	17	19	0	9.5	13	0	12	27
M16	Tilapia	*A. jandaei*	13	15	20	28	10	0	11	0	0	14.5
M17	Tilapia	*A. hydrophila*	12	15	20	25	9	9	14	0	0	15.5
M18	Tilapia	*A. caviae*	10	17	23	30	10	9	15	0	11	15
M19	Tilapia	*A. jandaei*	0	16	18	34	12	10	16	0	0	17.5
M20	Tilapia	*Aeromonas* spp.	0	12	16	30	8	7	8.5	0	12	29

M21	Tilapia	*A. allosaccharophila*	0	12	19	37	8	0	12.5	0	11	13
M22	Sea water	*A. culicicola*	14	19	23	28	9	8	12.5	0	11.5	13
M23	Sea water	*A. culicicola*	8	11	18	26	11	0	13	0	0	14
M24	Sea water	*A. jandaei*	0	13	24	24	10	0	13	0	0	14.5
M25	Sea water	*A. culicicola*	0	10	19	31	8	8	14	0	13	16
M26	Sea water	*Aeromonas* spp. 45	0	0	14	20	7	7	13	0	12	13
M27	Sea water	*Aeromonas* spp. 45	0	15	19	35	13	10	16	0	0	33
M28	Sea water	*A. jandaei*	8	14	21	33	12	10	14.5	0	11.5	13.5
M29	Sea water	*A. culicicola*	11	26	30	34	0	0	10	0	18	32
M30	Sea water	*A. jandaei*	13	18	27	36	7	8	11	0	0	16
M31	Sea water	*A. culicicola*	0	0	19	30	0	8	14.5	0	12	12
M32	Sea water	*A. culicicola*	8	15	21	21	0	0	10	0	0	14.5
M33	Sea water	*Aeromonas* spp. 310	9	12	17	28	8	0	10.5	0	0	12.5
M34	Sea water	*Aeromonas* spp. 45	0	14	16	31	8	0	11	0	0	15
M35	Sea water	*A. culicicola*	0	12	15	33	0	11	16	0	0	34
M36	Sea water	*A. jandaei*	21	24	34	30	10	8	14	0	12	0
M37	Sea water	*Aeromonas* spp. 45	13	17	23	30	0	0	10	0	0	10
M38	Sea water	*A. culicicola*	20	23	27	33	0	0	13	0	0	12
M39	Sea water	*A. culicicola*	0	0	15	30	9	8	14.5	0	14	12
M40	Sea water	*A. culicicola*	0	9	12	31	15	12	13	0	0	30
M41	Sea water	*Aeromonas* spp.	7	14	18	30	9	0	16	0	12	11
M42	Tilapia	*A. culicicola*	0	9	17	33	10	9	16.5	0	0	34
M43	Tilapia	*Aeromonas* spp. 310	18	24	28	36	8	0	13	0	0	31.5
M44	Tilapia	*A. icthiosmia*	11	14	19	35	14	9	15	0	11.5	14
M45	Tilapia	*P. shigelloides*	13	16	25	35	0	0	16	0	13	14
M46	Tilapia	*P. shigelloides*	9	16	27	34	8	0	11.5	0	0	17
M47	Tilapia	*P. shigelloides*	13	15	26	31	10	9	16.5	0	9	12
M48	Tilapia	*A. icthiosmia*	31	32	37	19	10.5	9	13.5	0	8.5	13
M49	Tilapia	*A. sobria*	0	17	26	28	7	8	10	0	0	15.5
M50	Catfish	*A. hydrophila*	0	16	21	25	7	0	12	0	0	12.5
M51	Catfish	*A. hydrophila*	14	18	23	35	8.5	9	9	0	14	15
M52	Tilapia	*A. hydrophila*	0	12	16	25	9	9	14	0	17	0

M53	Catfish	*A. hydrophila*	10	15	21	30	10	9	13	0	8	17
M54	Tilapia	*A. icthiosmia*	8	14	18	28	0	0	15	0	11.5	14
M55	Tilapia	*A. veronii*	11	16	24	26	9.5	9.5	14.5	0	0	16
M56	Tilapia	*A. culicicola*	0	0	16	29	11	11.5	20	0	0	18
M57	Tilapia	*A. veronii*	0	21	23	31	10	8	13	0	18	0
M58	Tilapia	*A. culicicola*	0	16	20	31	9.5	10	14.5	0	14	15
M59	Tilapia	*A. caviae*	11	17	20	30	6	7	13.5	0	0	10
M60	Tilapia	*A. hydrophila*	9	14	18	29	7.5	9	14.5	0	13.5	14
M61	Tilapia	*A. culicicola*	13	16	19	31	10	9	14	0	0	12
M62	Tilapia	*A. hydrophila*	0	13	17	30	9.5	10	15	0	0	12
M63	Tilapia	*A. veronii*	10	15	19	28	11.5	0	14.5	0	0	31.5
M64	Tilapia	*A. hydrophila*	17	24	33	32	0	0	9	0	10	13.5
M65	Tilapia	*A. hydrophila*	0	0	13	18	6.5	0	12	0	0	16
M66	Tilapia	*P. shigelloides*	12	16	27	30	0	6	11	0	11	16.5
M67	Tilapia	*P. shigelloides*	11	20	31	40	12	9	15.5	0	12	12
M68	Koi carp	*A. caviae*	15	30	36	32	0	8	13	0	11	0
M69	Koi carp	*A. bestiarum*	0	14	27	31	9	10	14	0	0	12
M70	Koi carp	*A. bestiarum*	8	15	26	28	9	0	12	0	13	16
M71	Koi carp	*A. bestiarum*	14	19	30	30	9	9.5	17	0	10	13
M72	Koi carp	*A. bestiarum*	0	12	16	31	0	0	9	0	0	25
M73	Koi carp	*A. bestiarum*	9	15	21	27	7	9	10	0	15	28
M74	Koi carp	*A. allosaccharophila*	9	15	18	30	0	0	8	0	12	11
M75	Koi carp	*Aeromonas* spp. 45	12	18	28	35	15	13	11	0	0	28
M76	Koi carp	*A. salmonicida*	11	14	25	33	12	9	14	0	0	10.5
M77	Koi carp	*A. salmonicida*	10	14	21	34	10	0	16	0	0	19
M78	Koi carp	*A. bestiarum*	18	19	30	15	10	0	16	0	0	19
M79	Koi carp	*A. bestiarum*	15	19	21	32	12	0	13	0	0	37
M80	Koi carp	*A. bestiarum*	0	0	16	35	0	0	14	0	0	13
M81	Koi carp	*A. bestiarum*	0	14	20	33	7.5	9	10	0	0	29
M82	Koi carp	*A. culicicola*	12	15	19	30	7	7.5	10.5	0	23	31.5
M83	Koi carp	*A. bestiarum*	0	16	21	27	9	0	13	0	10	18
M84	Koi carp	*A. bestiarum*	0	0	25	31	0	0	13	0	0	16

M85	Koi carp	*A. allosaccharophila*	12	20	22	31	15	12	16	0	0	33
M86	Koi carp	*A. hydrophila*	21	26	28	29	11	0	16	0	0	10
M87	Koi carp	*A. bestiarum*	17	23	29	31	0	0	14	0	0	11.5
M88	Koi carp	*A. bestiarum*	10	19	21	33	8	10	12.5	0	20	29
M89	Koi carp	*A. allosaccharophila*	0	0	0	0	9	0	15	0	14	19
M90	Koi carp	*A. bestiarum*	9	16	19	32	9	8	15	0	0	17
M91	Koi carp	*A. bestiarum*	0	0	13	18	10	0	12	0	0	12
M92	Koi carp	*A. allosaccharophila*	8	15	20	30	0	0	14.5	0	9	13.5
M93	Koi carp	*A. allosaccharophila*	0	0	11	17	8	0	9	0	0	10
M94	Koi carp	*A. hydrophila*	0	16	18	33	9	7	13.5	0	7	22
M95	Koi carp	*A. hydrophila*	11	16	18	30	14	12	15	0	0	34
M96	Koi carp	*A. bestiarum*	0	9	16	30	7	0	14.5	0	0	17
M97	Koi carp	*A. bestiarum*	13	16	21	30	14	10	15.5	0	0	11
M98	Koi carp	*Aeromonas* spp. 310	11	18	24	31	14	10.5	14	0	0	40
M99	Koi carp	*A. bestiarum*	15	17	21	31	8	8	12	0	0	16

* C125: 125 µg/ml; C250: 250 µg/ml; C500: 500 µg/ml and C1250: 1250 µg/ml cinnamaldehyde; EX 1: (4 mg/ml) *K. africana* ethyl acetate extract; EX 2: (4 mg/ml) *K. africana* dichloromethane extract; EX 3: (10 mg/ml) *K. africana* methanol extract; EX 4: (4 mg/ml) *K. africana* hexane extract; AMP10: Ampicillin; TE30: Tetracycline.

TABLE A2 Activity indices of cinnamaldehyde and *K. africana* extracts in relation to the standard antimicrobial agents, ampicillin (AMP10) and tetracycline (TE30), against *Aeromonas* spp. isolates from seawater and fish

Isolates	Activity index AMP10								Activity index TE30							
	C125*	C250*	C500*	C1250*	EX 1*	EX 2*	EX 3*	EX 4*	C125* EX 4*	C250*	C500*	C1250*	EX 1*	EX 2*	EX 3*	
A. caviae ATCC 15468^T	0	0	0	0	0	0	0	0	0.333	0.593	0.815	1.259	0.393	0.321	0.536	0
A. hydrophila ATCC 7966^T	0	0	0	0	0	0	0	0	0.500	0.607	0.821	1.179	0.519	0.370	0.630	0
E. coli ATCC 25922	0	0	0	1.818	0.364	0.364	0.455	0	0	0	0	1.429	0.286	0.286	0.357	0
M1	0	0	0	0	0	0	0	0	0.818	1.455	1.909	1.909	0.727	0	1.273	0
M2	1.379	0.552	0.966	1.310	0.759	0.483	1.241	0	0.444	0.778	1.056	1.111	0.611	0.389	1.000	0
M3	0	0	0	0	0	0	0	0	0.500	0.679	0.964	1.000	0.304	0	0.375	0
M4	1.538	0	0.692	1.308	0.692	0	0.846	0	0	0.327	0.618	0.727	0.327	0	0.400	0
M5	0	0	0	0	0	0	0	0	0	0.960	1.120	1.520	0.720	0.840	1.120	0
M6	1.429	0	0	1.214	0	0	0.964	0	0	0	1.172	1.379	0	0	0.931	0
M7	0	0	0	0	0	0	0	0	0.731	1.154	1.577	0.808	0	0	0.442	0
M8	0	0	0	0	0	0	0	0	0	0.857	1.214	1.786	0.643	0.571	1.071	0
M9	0	0	0	0	0	0	0	0	0.741	1.037	1.704	1.481	0.444	0.519	0.667	0
M10	0	0	0	0	0	0	0	0	0.875	1.188	1.625	2.000	0.438	0	0.750	0
M11	0	0	0	0	0	0	0	0	0.647	0.941	1.235	1.353	0.676	0	0.794	0
M12	1.750	0	0	1.083	0.750	0.750	1.083	0	0	0	0.722	1.167	0.500	0.500	0.722	0
M13	0	0	0	0	0	0	0	0	0.560	0.680	1.000	1.08	0.360	0.320	0.500	0
M14	1.526	0.526	0.737	1.421	0.658	0.526	0.947	0	0.833	1.167	2.25	2.417	1.042	0.833	1.500	0
M15	1.583	0.750	1.083	1.417	0	0.792	1.083	0	0.333	0.481	0.630	0.704	0	0.352	0.481	0
M16	0	0	0	0	0	0	0	0	0.897	1.034	1.379	1.931	0.690	0	0.759	0
M17	0	0	0	0	0	0	0	0	0.774	0.968	1.29	1.613	0.581	0.581	0.903	0
M18	2.727	0.909	1.545	2.091	0.909	0.818	1.364	0	0.667	1.133	1.533	2.000	0.667	0.600	1.000	0
M19	0	0	0	0	0	0	0	0	0	0.914	1.029	1.943	0.686	0.571	0.914	0
M20	2.500	0	1.000	1.333	0.667	0.583	0.708	0	0	0.414	0.552	1.034	0.276	0.241	0.293	0
M21	3.364	0	1.091	1.727	0.727	0	1.136	0	0	0.923	1.462	2.846	0.615	0	0.962	0

M22	2.435	1.217	1.652	2.000	0.783	0.696	1.087	0	1.077	1.462	1.769	2.154	0.692	0.615	0.962	0
M23	0	0	0	0	0	0	0	0	0.571	0.786	1.286	1.857	0.786	0	0.929	0
M24	0	0	0	0	0	0	0	0	0	0.897	1.655	1.655	0.690	0	0.897	0
M25	2.385	0	0.769	1.462	0.615	0.615	1.077	0	0	0.625	1.188	1.938	0.500	0.500	0.875	0
M26	1.667	0	0	1.167	0.583	0.583	1.083	0	0	0	1.077	1.538	0.538	0.538	1.000	0
M27	0	0	0	0	0	0	0	0	0	0.455	0.576	1.061	0.394	0.303	0.485	0
M28	2.870	0.696	1.217	1.826	1.043	0.87	1.261	0	0.593	1.037	1.556	2.444	0.889	0.741	1.074	0
M29	1.889	0.611	1.444	1.667	0	0	0.556	0	0.344	0.813	0.938	1.063	0	0	0.313	0
M30	0	0	0	0	0	0	0	0	0.813	1.125	1.688	2.250	0.438	0.500	0.688	0
M31	2.500	0	0	1.583	0	0.667	1.208	0	0	0	1.583	2.500	0	0.667	1.208	0
M32	0	0	0	0	0	0	0	0	0.552	1.034	1.448	1.448	0	0	0.69	0
M33	0	0	0	0	0	0	0	0	0.720	0.960	1.360	2.240	0.640	0	0.84	0
M34	0	0	0	0	0	0	0	0	0	0.933	1.067	2.067	0.533	0	0.733	0
M35	0	0	0	0	0	0	0	0	0	0.353	0.441	0.971	0	0.324	0.471	0
M36	2.500	1.750	2.000	2.833	0.833	0.667	1.167	0	0	0	0	0	0	0	0	0
M37	0	0	0	0	0	0	0	0	1.300	1.700	2.300	3.000	0	0	1.000	0
M38	0	0	0	0	0	0	0	0	1.667	1.917	2.250	2.750	0	0	1.083	0
M39	2.143	0	0	1.071	0.643	0.571	1.036	0	0	0	1.250	2.500	0.750	0.667	1.208	0
M40	0	0	0	0	0	0	0	0	0	0.300	0.400	1.033	0.500	0.400	0.433	0
M41	2.500	0.583	1.167	1.500	0.750	0	1.333	0	0.636	1.273	1.636	2.727	0.818	0	1.455	0
M42	0	0	0	0	0	0	0	0	0	0.265	0.500	0.971	0.294	0.265	0.485	0
M43	0	0	0	0	0	0	0	0	0.571	0.762	0.889	1.143	0.254	0	0.413	0
M44	3.043	0.957	1.217	1.652	1.217	0.783	1.304	0	0.786	1.000	1.357	2.500	1.000	0.643	1.071	0
M45	2.692	1.000	1.231	1.923	0	0	1.231	0	0.929	1.143	1.786	2.500	0	0	1.143	0
M46	0	0	0	0	0	0	0	0	0.529	0.941	1.588	2.000	0.471	0	0.676	0
M47	3.444	1.444	1.667	2.889	1.111	1.000	1.833	0	1.083	1.250	2.167	2.583	0.833	0.75	1.375	0
M48	2.235	3.647	3.765	4.353	1.235	1.059	1.588	0	2.385	2.462	2.846	1.462	0.808	0.692	1.038	0
M49	0	0	0	0	0	0	0	0	0	1.097	1.677	1.806	0.452	0.516	0.645	0
M50	0	0	0	0	0	0	0	0	0	1.280	1.68	2.000	0.56	0	0.960	0
M51	2.500	1.000	1.286	1.643	0.607	0.643	0.643	0	0.933	1.200	1.533	2.333	0.567	0.600	0.600	0
M52	1.471	0	0.706	0.941	0.529	0.529	0.824	0	0	0	0	0	0	0	0	0

M53	3.750	1.250	1.875	2.625	1.25	1.125	1.625	0	0.588	0.882	1.235	1.765	0.588	0.529	0.765	0
M54	2.435	0.696	1.217	1.565	0	0	1.304	0	0.571	1.000	1.286	2.000	0	0	1.071	0
M55	0	0	0.00	0	0	0	0	0	0.688	1.000	1.500	1.625	0.594	0.594	0.906	0
M56	0	0	0	0	0	0	0	0	0	0	0.889	1.611	0.611	0.639	1.111	0
M57	1.722	0	1.167	1.278	0.556	0.444	0.722	0	0	0	0	0	0	0	0	0
M58	2.214	0	1.143	1.429	0.679	0.714	1.036	0	0	1.067	1.333	2.067	0.633	0.667	0.967	0
M59	0	0	0	0	0	0	0	0	1.100	1.700	2.000	3.000	0.600	0.700	1.350	0
M60	2.148	0.667	1.037	1.333	0.556	0.667	1.074	0	0.643	1.000	1.286	2.071	0.536	0.643	1.036	0
M61	0	0	0	0	0	0	0	0	1.083	1.333	1.583	2.583	0.833	0.750	1.167	0
M62	0	0	0	0	0	0	0	0	0	1.083	1.417	2.500	0.792	0.833	1.250	0
M63	0	0	0	0	0	0	0	0	0.317	0.476	0.603	0.889	0.365	0	0.460	0
M64	3.200	1.700	2.400	3.300	0	0	0.900	0	1.259	1.778	2.444	2.370	0	0	0.667	0
M65	0	0	0	0	0	0	0	0	0	0	0.813	1.125	0.406	0	0.750	0
M66	2.727	1.091	1.455	2.455	0	0.545	1.000	0	0.727	0.970	1.636	1.818	0	0.364	0.667	0
M67	3.333	0.917	1.667	2.583	1.000	0.750	1.292	0	0.917	1.667	2.583	3.333	1.000	0.750	1.292	0
M68	2.909	1.364	2.727	3.273	0	0.727	1.182	0	0	0	0	0	0	0	0	0
M69	0	0	0	0	0	0	0	0	0	1.167	2.250	2.583	0.750	0.833	1.167	0
M70	2.154	0.615	1.154	2.000	0.692	0	0.923	0	0.500	0.938	1.625	1.750	0.563	0	0.750	0
M71	3.000	1.400	1.900	3.000	0.900	0.950	1.700	0	1.077	1.462	2.308	2.308	0.692	0.731	1.308	0
M72	0	0	0	0	0	0	0	0	0	0.480	0.640	1.240	0	0	0.360	0
M73	1.800	0.600	1.000	1.400	0.467	0.600	0.667	0	0.321	0.536	0.750	0.964	0.250	0.321	0.357	0
M74	2.500	0.750	1.250	1.500	0	0	0.667	0	0.818	1.364	1.636	2.727	0	0	0.727	0
M75	0	0	0	0	0	0	0	0	0.429	0.643	1.000	1.250	0.536	0.464	0.393	0
M76	0	0	0	0	0	0	0	0	1.048	1.333	2.381	3.143	1.143	0.857	1.333	0
M77	0	0	0	0	0	0	0	0	0.526	0.737	1.105	1.789	0.526	0	0.842	0
M78	0	0	0	0	0	0	0	0	0.947	1.000	1.579	0.789	0.526	0	0.842	0
M79	0	0	0	0	0	0	0	0	0.405	0.514	0.568	0.865	0.324	0	0.351	0
M80	0	0	0	0	0	0	0	0	0	0	1.231	2.692	0	0	1.077	0
M81	0	0	0	0	0	0	0	0	0	0.483	0.690	1.138	0.259	0.310	0.345	0
M82	1.304	0.522	0.652	0.826	0.304	0.326	0.457	0	0.381	0.476	0.603	0.952	0.222	0.238	0.333	0
M83	2.700	0	1.600	2.100	0.900	0	1.300	0	0	0.889	1.167	1.500	0.500	0	0.722	0

M84	0	0	0	0	0	0	0	0	0	0	1.563	1.938	0	0.00	0.813	0
M85	0	0	0	0	0	0	0	0	0.364	0.606	0.667	0.939	0.455	0.364	0.485	0
M86	0	0	0	0	0	0	0	0	2.100	2.600	2.800	2.900	1.100	0	1.600	0
M87	0	0	0	0	0	0	0	0	1.478	2.000	2.522	2.696	0	0	1.217	0
M88	1.650	0.500	0.950	1.050	0.400	0.500	0.625	0	0.345	0.655	0.724	1.138	0.276	0.345	0.431	0
M89	0	0	0	0	0.643	0	1.071	0	0	0	0	0	0.474	0	0.789	0
M90	0	0	0	0	0	0	0	0	0.529	0.941	1.118	1.882	0.529	0.471	0.882	0
M91	0	0	0	0	0	0	0	0	0	0	1.083	1.500	0.833	0	1.000	0
M92	3.333	0.889	1.667	2.222	0	0	1.611	0	0.593	1.111	1.481	2.222	0	0	1.074	0
M93	0	0	0	0	0	0	0	0	0	0	1.100	1.700	0.800	0	0.900	0
M94	4.714	0	2.286	2.571	1.286	1.000	1.929	0	0	0.727	0.818	1.500	0.409	0.318	0.614	0
M95	0	0	0	0	0	0	0	0	0.324	0.471	0.529	0.882	0.412	0.353	0.441	0
M96	0	0	0	0	0	0	0	0	0	0.529	0.941	1.765	0.412	0	0.853	0
M97	0	0	0	0	0	0	0	0	1.182	1.455	1.909	2.727	1.273	0.909	1.409	0
M98	0	0	0	0	0	0	0	0	0.275	0.450	0.600	0.775	0.350	0.263	0.350	0
M99	0	0	0	0	0	0	0	0	0.938	1.063	1.313	1.938	0.500	0.500	0.750	0

* C125: 125 µg/ml; C250: 250 µg/ml; C500: 500 µg/ml and C1250: 1250 µg/ml cinnamaldehyde; EX 1: 4 mg/ml *K. africana* ethyl acetate extract; EX 2: 4 mg/ml *K. africana* dichloromethane extract; EX 3: 10 mg/ml *K. africana* methanol extract; EX 4: 4 mg/ml *K. africana* hexane extract; AMP10: Ampicillin; TE30: Tetracycline.

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Assessment of Aquatic *Aeromonas* spp. Isolates' Susceptibility to Cinnamaldehyde, Vanillin, and Crude *Kigelia africana* Fruit Extracts

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Abstract

***Aeromonas* spp. are primary and opportunistic fish pathogens that are responsible for severe economic losses in aquaculture, and are exacerbated by their increasing incidence of antimicrobial resistance. Phytochemicals are being explored as alternatives to conventional antimicrobial agents since they have destressing, growth-promoting, immune-stimulating, and antimicrobial properties. The susceptibility of 93 aquatic *Aeromonas* spp. and six *Plesiomonas shigelloides* isolates (from cultured catfish, koi carp, tilapia, and seawater) to three phytochemicals, viz.: cinnamaldehyde (10–1250 µg/mL), vanillin (5–250 µg/mL), and crude *Kigelia africana* fruit extracts (4–10 mg/mL ethyl acetate, dichloromethane, methanol, and hexane) were assessed using the disk diffusion assay and compared to ampicillin and tetracycline, using activity indices. No zones of inhibition were obtained with 10 µg/mL of cinnamaldehyde, or with 5–250 µg/mL of vanillin. However, varying degrees of inhibition were observed with higher concentrations of cinnamaldehyde, as well as with the *K. africana* methanol extract. Cinnamaldehyde (≥500 µg/mL) and the *K. africana* methanol extract displayed better antimicrobial activity against study isolates in comparison to vanillin and ampicillin. They appear to be promising and sustainable phytochemicals that might be useful as alternatives to the antimicrobial agents currently in use in aquaculture.**

Aeromonas spp. are Gram-negative, facultatively anaerobic, motile, rod-shaped bacteria belonging to the family Aeromonadaceae. Aeromonads are widely distributed in fresh water, marine water, sewage-contaminated water, sludge, soil, and foods (Ghenghesh et al. 2008). *Aeromonas* spp. are important fish and shellfish pathogens responsible for several fish diseases such as hemorrhagic septicemia, fin and tail rot disease, and furunculosis. These infections have caused high mortality in cultured freshwater fish, resulting in extensive losses worldwide (Jacobs and Chenia 2007; Lu et al. 2011). In humans, *Aeromonas hydrophila*, *Aeromonas caviae*, and *Aeromonas veronii* biotype *sobria* are associated with food-borne gastroenteritis, wound infections, and infections in immuno-compromised patients. Both veterinary, aquaculture, and human clinical infections have been treated with effective chemotherapy using antimicrobial agents, although there is an increased frequency of antimicrobial resistance in aeromonads, especially those from fish (Jacobs and Chenia 2007; Ghenghesh et al. 2008; Jun et al. 2010).

Antimicrobial agents are used extensively in aquaculture systems as a prophylactic (Park et al. 2012) to prevent fish disease in addition to their therapeutic use, as well as being used as feed additives (Ghenghesh et al. 2008). Although these infections may be resolved using synthetic antimicrobial agents, the dangers of multidrug resistance, environmental pollution, residues in fish and the push toward organic aquaculture has made researchers look for bioactive materials from plants that have potent antimicrobial activity. The most active components of phytochemicals are alkaloids, phenolic/flavonoid compounds, sterols, terpenoids, and proanthocyanidins (Siri

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