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Degree : MASTER OF SCIENCE IN MARINE SCIENCE

Department/School : The University of the South Pacific

Institution/University : DEPARTMENT OF MARINE STUDIES, SCHOOL OF ISLANDS AND OCEANS, FACULTY OF SCIENCE, TECHNOLOGY AND ENVIRONMENT.

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Contact Address

LOT 29, POORVA PL,
MAKOI, NASINU,
FIJI.

e-mail : ajaymsp1@gmail.com

Permanent Address

LOT 2, POORVA PL,
MAKOI, NASINU,
FIJI.

e-mail: ajay_msp1@yahoo.com.

Antibacterial Effects of Selected Plant Extracts Against Histamine Producing Bacteria, Heterotrophic Bacteria and Coliforms

By

Ashneel Ajay SINGH

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of the

Master of Science

in

Marine Science

Division of Marine Studies
School of Islands and Oceans
Faculty of Science, Technology and Environment
The University of the South Pacific

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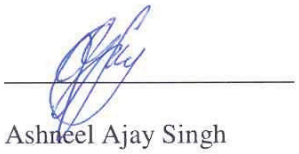

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Date

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ABSTRACT

Plants have been known for their medicinal properties since antiquity and research interest on their medicinal properties is gaining momentum. Plant extracts from noni (*Morinda citrifolia*), citrus (*Citrus aurantium*), tamarind (*Tamarindus indica*), black tea and green tea (*Camellia sinensis*) were assessed for their antibacterial activity against three groups of bacteria. These included fifteen species of histamine producing bacteria (HPB), total heterotrophic bacteria and total coliforms. Heterotrophic bacteria and coliform studies were conducted on fresh chub-mackerel (*Rastrelliger brachysoma*) and assessed *ex vivo* at seven different incubation temperatures. HPB were tested for their individual histamine producing capabilities. All species of HPB demonstrated significant histamine production ability. The plant extracts exhibited substantial inhibitory activity against HPB while exhibiting various degrees of growth inhibition against heterotrophic bacteria and coliforms. These plant extracts can be used as potential antibacterial agents in seafood industries to control seafood spoilage and biotoxin formation.

Key words: plant extracts, antibacterial activity, histamine producing bacteria, heterotrophic bacteria, coliforms, salala.

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DEDICATION

Dedicated to my parents, Uday Singh and Madhu Kiran Devi, for their vision and courage without which this work would not be possible. Also dedicated to my wife Swastika, for her unconditional love, support and constant encouragement.

I. Introduction

The first humans appeared on earth approximately a million years ago. In these ancient times a lot of people died due to diseases caused by food consumption. They had few means to fight against these health problems and finding cures. Things have changed with time as technology and knowledge has advanced, which has enabled humans to find medicines and cures for many food associated health problems. At first, cures were mostly herbal in nature but most of the remedies used were undocumented or simply lost through time.

The past few centuries have seen a revolutionary change in medicine from herbal to mostly synthetic drugs. Although a number of these drugs are able to provide cure and prevention, some of them are not readily assimilated in the body. Some synthetic components and their derivatives tend to persist in the body and cause a variety of side effects (Ernst, 2002; Patrono *et al.*, 2004). The advantage of utilizing herbs and plant materials as medicine is their low cost, wide availability, their ability to be easily assimilated and also their ability to cure or prevent many illnesses with less side effects.

1.1 Medicinal properties of plant extracts

Over the past few decades, extracts from plants have captured research interest as alternative remedies to cure and treat many forms of infectious diseases caused by bacteria associated with contaminated food. (Craig, 1999; Dorman and Deans, 2000; Prabuseenivasan *et al.*, 2006). Medicinal properties exhibited by plant extracts

demonstrate their potential use as alternative medicines for primary healthcare (Prabuseenivasan *et al.*, 2006).

1.1.1 Medicinal properties of Tamarind (*Tamarindus indica*)

Tamarind is the common name for *Tamarindus indica*. It is native to India and its plant components have been utilized as treatment for various ailments. In the United States, more than 90 tonnes of shelled tamarind fruits are imported annually from Lesser Antilles, Mexico, India and Egypt for use in pharmacological drugs (Nyadoi, 2004). Although tamarind is widely used in India and the United States, it is used on a much lower scale in the South Pacific. Tamarind is widely distributed but little literature is available on its uses in the South Pacific.

Applications for tamarind include its use to allay thirst and reduce fevers. The fruit is also quite nutritive and forms therapeutic drinks when given to persons recuperating from sickness to keep their bowels regular (Ghelardi *et al.*, 2004). In Samoa, the pulp of tamarind is made into a cooling drink and used as a laxative, which is suitable for fevers and a variety of illnesses. In the South Pacific, the leaves of tamarind are also made into a tea for jaundice (McGrath, 1996). It is also used in digestive disorders, as a remedy for bile disorders. In Southeast Asia, tamarind is used to heal inflammation, sore throats, and is administered to alleviate alcoholic intoxication (Ghelardi *et al.*, 2004; Sidhu *et al.*, 2007). The plant has been shown to possess significant antibacterial and anti-inflammatory properties (Nyadoi, 2004; Muthu *et al*., 2005). Tamarind has been used for

a long time not only as a flavoring agent but also for its medicinal properties (Ayres *et al.*, 1980).

1.1.1.1 Antibacterial properties of Tamarind

Scientific evidence on medicinal properties of tamarind is limited, but there has been significant investigations performed for it to be recognized as a medicinal plant. The fruit extracts demonstrate antibacterial activity against *Pseudomonas aeruginosa* and *Staphylococcus aureus* (Ghelardi *et al.*, 2004). Methanolic extracts of *T. indica* exhibit significant anti-*Burkholderia pseudomallei* activity at different concentrations (Muthu *et al.*, 2005). Ethanolic extracts from tamarind also possesses inhibitory properties against multiple drug-resistant *Helicobacter pylori* (Voravuthikunchai and Mitchell, 2008). In Bauchi, Nigeria, decoction from tamarind plant is used to treat ulcers and sore eyes. It is known to lessen inflammation, relieve pains, reduce secondary bacterial infections and promote healing (Ghelardi *et al.*, 2004). Aqueous and organic extracts of tamarind have been tested for their antimicrobial activity using the disc diffusion method. Inhibitory activity was demonstrated against *Escherichia coli, Proteus mirabilis, P. aeruginosa, Salmonella typhi, Salmonella paratyphi, Shigella flexnerri, S. aureus, Bacillus subtilis* and *Streptococcus pyogenes* (Doughari, 2006).

1.1.2 Medicinal properties of Citrus (*Citrus aurantium*)

The bitter orange *C. aurantium* (Rutaceae) is commonly known as citrus. It is also known by other names in the South Pacific such as moli'aina (Samoa), moli kai (Tonga), moli (Futuna, Niue), moli'anani (Cook Islands), moli'arani (Society Islands) and molidawa

(Fiji) respectively. Citrus is native to tropical Asia, and has provided food and medicine for thousands of years. In Polynesia, the tree has got a number of medicinal uses. In Tonga, citrus leaves are brewed with mango (*Mangifera indica*) and other leaves to form a potion for treating relapse sickness (*kita*), while in Samoa, the bark is used for treating postpartum sickness (*failele gau*) (Whistler, 1992; WHO, 1998). Citrus leaves are also used as eye medicine and the fruits are used to treat yaws and breathlessness (Uhe, 1974). In Tahiti, the leaves of citrus are used as remedies for internal ailments and fractures (WHO, 1998). In the Cook Islands, citrus juice is mixed with coconut oil and taken as a pugative (Whistler, 1992; WHO, 1998). Citrus is also used in Polynesia to treat abdominal pain (*tupito*), against tropical ulcers and as a remedy for obesity (Singh and Sawatibau, 1980; Whistler, 1992). Studies performed on citrus demonstrate that its extracts and oil possess antibacterial activity against many bacterial species including human pathogens (Deng *et al*., 1999; Prabuseenivasan *et al.*, 2006).

1.1.2.1 Antibacterial properties of Citrus

There have been several scientific studies conducted on citrus extracts but knowledge of its medicinal benefit remains limited. The majority of studies performed on this plant has focused on the antibacterial properties of the essential oils derived from the fruit and leaves. There may be other components of the plant, which may also possess strong medicinal properties. Essential oil from citrus has been shown to possess antimicrobial properties against a number of bacteria. The disc diffusion method was utilized to determine the *in vitro* antibacterial activity of essential oil from citrus fruit against four gram-negative bacteria *E. coli*, *Klebsiella pneumoniae*, *P. aeruginosa* and *Proteus*

vulgaris and two gram-positive bacteria *B. subtilis* and *S. aureus*. Citrus oil exhibited strong bactericidal activity against all bacterial species tested (Prabuseenivasan *et al.*, 2006). Essential oil extracted from citrus has been of great interest to researchers in terms of its antibacterial properties.

Citrus may also owe part of its bactericidal property to the low pH of its extract as most bacterial species are not able to survive below a pH of 3.0 (Rocelle *et al*., 1996; Rhee *et al*., 2003). Citric acid is a major constituent of citrus and it is mainly responsible for the low pH (2.64) of juice extracted from the fruit. The acid is quite effective in inhibiting the growth of pathogenic bacteria. The effectiveness of citric acid in comparison to some other acids was tested against food borne pathogens. Results demonstrated varying effectiveness of the acids. They can be placed in the order of effectiveness: acetic acid > citric acid > malic acid (Deng *et al*., 1999).

Food borne pathogens such as *E. coli* O157:H7 (causative agent for hemorragic colitis), *Salmonella enterica* serovar typhimurium (causes salmonellosis) and *Listeria monocytogenes* (causes listeriosis, meningitis and encephalitis) have been tested for their death kinetics as affected by the lowering of pH. With the exception of *E. coli* O157:H7, other bacterial species were quite sensitive to low pH (<3.0) (Rocelle *et al*., 1996; Rhee *et al*., 2003). This is in contrast to another study, which demonstrates that the pH limit for growth of *E. coli* is between pH 3.4 and 3.6 (Presser *et al.*, 1998). According to Jordan *et al.*, (1999), even the most acid tolerant isolates of *E. coli* O157:H7 have been found to be sensitive to a pH of <3.0. Citrus is a well-known preservative when applied to food products. Citric acid has shown to be effective in reducing the risk of diseases caused by

E. coli and *Salmonella* spp (Beuchat and Scouten, 2002). According to these studies, low pH of citrus extract plays a significant role in its antibacterial properties.

1.1.3 Medicinal properties of Noni (*Morinda citrifolia*)

Noni or Indian Mulberry is the common name for *Morinda citrifolia* and is also known as Kura (Fiji), te non (Kiribati), nonu (Tonga, Samoa, Futuna, Niue, Tokelau, Tuvalu), nono (Tahiti, Cook Islands), dilo-K (Solomon Islands), Ba Ji Tian, Cheese Fruit and Nhau by different cultures around the world (Croft and Tu'ipulotu, 1980; WHO, 1998; Wang *et al.*, 2002; Nelson, 2006). It is a tropical and subtropical plant grown in the Pacific Islands and has been used in Polynesia traditionally as folk medicine to treat a broad range of diseases for over 2000 years (Solomon, 2001; McClatchey, 2002).

The leaves, bark and fruits of noni are shared as a traditional remedy by all Polynesians (Croft and Tu'ipulotu, 1980). In Fiji and Hawai'i, the leaves are used for treating cuts, inflammations and abscesses of various types. In Samoa, Tonga and Futuna, the fruit is crushed or chewed to treat infections of the mouth and gums as well as for toothaches and sore throat (Uhe, 1974; Whistler, 1992; Aalbersberg, 1999). The Samoans use noni leaves for treating gastric ulcers, fevers, constipation and breast inflammations. The fruit is used to treat cough, worms, tuberculosis, fever and as eye lotion (Uhe, 1974; Singh and Sawatibau, 1980; WHO, 1998). In Hawai'i, a commonly prepared tonic from the fruit is used for ailments such as diabetes, high blood pressure, and loss of appetite (Whistler, 1992; WHO, 1998). The Tongans use noni to treat stomachache and sties. In Micronesia, the fruit is used as a remedy to treat ulcerated sores on the feet (WHO, 1998). The

Tahitian healers use the plant for treating diabetes, fish poisoning, tonsillitis, abdominal swelling, burns, ranula, and inflammation of the fingers or toes (Petard, 1972; WHO, 1998). In Fiji, the leaves are used as a poultice for broken bones and sprains, the roots are used for treating urinary disorders and the fruits are used to treat high blood pressure (WHO, 1998). In the Cook Islands, noni fruit, combined with *miro* (*Thespesia populnea*) fruits and *tou* (*Cordia subcordata*) leaves, are used for treating urinary tract ailments. The fruit alone is used sometimes in remedies for abdominal swelling and diaphramic hernia (Whistler, 1992; WHO, 1998).

Noni fruit gives off a rancid, cheesy off odor due to its content of carboxylic acids (Duke *et al.*, 2002). The plant possesses a wide range of therapeutic effects, including antibacterial, antiviral, antifungal, antitumour, anti-inflammatory and immune enhancing properties (Solomon, 2001; Wang *et al.*, 2002; Thomson, 2007). Even though noni has been used widely as traditional medicine there has been very little human clinical research to support these claims (Zin *et al.*, 2006).

A number of *M. citrifolia* products are widely sold commercially in the market as 'Noni' preparations including juices, health tonics, encapsulated noni powder and applications for skin related problems (Aalbersberg, 1999; Nelson, 2006). Medicinal properties of noni may be linked to the antioxidative properties of some of its constituent compounds (Zin *et al.*, 2006).

Various biological compounds have been isolated from noni fruits, leaves and roots such as morindin, scopoletin, anthraquinones, alkaloids, glycosides, polysaccharides, minerals and vitamins which may be responsible for the wide range of medicinal properties of the plant (Croft and Tu'ipulotu, 1980; Liu *et al*., 2001; Wang *et al.*, 2002; Martin, 2005; Zin *et al.*, 2006). The primary active compounds present in both the fruit and the leaves of noni appear to be glycosides, which have been shown to be effective in inhibiting growth of tumor cells which could be effective in cancer prevention (Liu *et al*., 2001).

1.1.3.1 Antibacterial properties of Noni

Plant extracts from noni have been used traditionally in the Pacific region to treat infectious diseases, deep cuts, bruises, sores and wounds (Nelson, 2006). The properties of noni are responsible for its ability to treat skin infections, colds, fevers and other bacterial-related health problems (Atkinson, 1956; Solomon, 2001; Thomson, 2007). The Jah Hut people in Malaysia and Polynesians often use noni to treat boils by topical application of the leaves or fruits (Whistler, 1972; Lin, 2005). This coincides with a pharmacological validation; since boils (impetigo, folliculitis, furuncles, or carbuncles) are often caused by *S. aureus* and noni is able to inhibit growth of this bacterium (Wang *et al.*, 2002). Noni fruit extract exbibits positive bioactivity against *Streptococcus* spp (Aalbersberg, 1999). Microbiological analysis of Panamanian Noni Juice reveals that coliforms including *E. coli* and other bacteria such as *Listeria* spp and *S. aureus* are unable to survive in noni juice (Martin, 2005).

Noni was tested for its antibacterial activity using the disc diffusion method, where it showed significant positive inhibitory activity against *E. coli* and *P. aeuruginosa* (Singh and Sawatibau, 1980). Methanol, ethyl acetate and hexane extracts from noni fruit were tested for their antibacterial activity against pure bacterial cultures *in vitro*. The extracts were able to inhibit the growth of *B. subtilis, S. aureus, Lactobacillus lactis, S. aureus, P. aeruginosa, S. typhi, E. coli, Vibrio harveyi, K. pneumoniae, S. flexneri, S. paratyphi, Aeromonas hydrophila, Vibrio cholerae, Chromobacterium violaceum* and *Enterobacter faecalis* (Jayaraman *et al.*, 2008). In another study, the ethanol, methanol and ethyl acetate extracts of noni were found to demonstrate inhibitory antimicrobial activity against *E. coli, S. aureus, Candida albicans, Aspergillus niger* and *Trichophyton mentagrophytes* (Gerson *et al.*, 2003).

1.1.4 Medicinal properties of Black tea and Green tea (*Camellia sinensis*)

Tea is made from the leaves of the plant *Camellia sinensis* and is also known as chai in India as well as other names by different cultures around the globe. After water, tea is the most popularly consumed beverage worldwide with a per capita consumption of 120 ml/day (McKay and Blumberg, 2002; Jazani *et al.*, 2007a). It is classified into three groups depending upon the level of fermentation: black (fully fermented), oolong (partially fermented) and green (unfermented) (Jazani *et al.*, 2007a,b). Black tea is consumed principally in the South Pacific, Europe, North America and North Africa, while green tea is drunk throughout Asia (McKay and Blumberg, 2002; Kovacs, 2006).

Although the health benefits of tea have been acknowledged since the beginning of its history, scientific research on it has captured interest only in the last few decades. Tea has

extensive biological properties which helps to reduce risk of diseases and promote human health (Craig, 1999). Water-soluble extracts from tea have a wide range of medicinal properties and have been shown to cure and prevent many human diseases including cancer (Craig, 1999). Further investigation of medicinal properties of tea is needed to identify its full range of benefits.

1.1.4.1 Antibacterial properties of Black tea and Green tea

Research on black tea and green tea has shown that both types of tea possess antibacterial properties. Recently, in a study black tea was extracted for 2, 8 and 18 h with absolute acetone, *N,N*-dimethylformamide (DMF), ethanol and methanol. The extracts were screened for their antibacterial activities against four bacterial species. *S. aureus* was found to be the most sensitive to all tea extracts, except for the methanol extract. Black tea extracts were found to be ineffective against *Yersinia enterocolitica*, *L. monocytogenes* and *E. coli* O157:H7 (Turkmen *et al.*, 2007).

The antibacterial activity of water-soluble green tea extracts was tested against 43 multi-antibiotic resistant isolates of *P. aeruginosa*. Results demonstrated significant inhibitory activity of tea against all multi-drug resistant strains of *P. aeruginosa* (Jazani *et al.*, 2007b). Tea extracts were also evaluated against multi-antibiotic resistant isolates of *Acinetobacter* spp. Results showed significant bactericidal activity of green tea against all 20 strains of *Acinetobacter* spp (Jazani *et al.*, 2007a). Derivatives of green tea demonstrated antibacterial activity against carcinogenic *Helicobacter pylori* (Carlson *et al.*, 2007). Methanolic extracts from green tea has also been shown to possess

antibacterial activity against the cariogenic bacteria *Streptococcus sanguinis* (Tsai *et al*., 2008).

Medicinal properties of tea is attributed to its high antioxidant capacity and rich content of phenolic compounds such as chlorogenic acid, caffeic acid, rutin and kaempferol (Tsai *et al*., 2008), polyphenolics, particularly flavonoids such as epicatechin, epicatechin gallate, epigallocatechin and epigallocatechin gallate, which are the most abundant and probably the most pharmacologically active (Kovacs, 2006; Tsai *et al.*, 2007).

1.1.5 Application of plant extracts in food

Plants have naturally occurring compounds possessing properties that are of interest in food processing and production. Plant extracts provide alternative ways of controlling agricultural pests. Crude plant extracts have been prepared as botanical insecticides and tested on various agricultural crops. Extracts from citrus (*C. aurantium*) and tamarind (*T. indica*) were found to be effective against various pests including moths, weevils and bagworms (Singh and Saratchandra, 2005). Essential oil from citrus has been found to effective in increasing the shelf life of rye bread by controlling the growth of rye bread fungi including *Penicillium roqueforti*, *Penicillium corylophilum*, *Eurotium* spp, *Endomyces fibuliger*, and *Aspergillus flavus* (Suhr and Nielsen, 2003). It has been determined that extracts form citrus forms a suitable food preservative as it exhibits antibacterial properties against important various food spoilage bacteria including *Acinetobacter baumanii*, *Aeromonas veronii*, *Candida albicans, Enterococcus faecalis,*

E. coli, *K. pneumoniae*, *P. aeruginosa*, *S. enterica, Serratia marcescens* and *S. aureus* (Hammer *et al*., 1999).

Anderson and Rapp, (1969), developed edible and nutritive nutrient blocks with higher nutritive value for animal feed. Citric acid extract from citrus was added to improve the taste and nutrient value of the nutrient blocks (Anderson and Rapp, 1969). Acids such as citric acid, tartaric acid and malic acid extracted from citrus and tamarind have been used in varying concentrations as natural acidulants to enhance the flavor of fruit products. These acids are used in products such as aspartame-sweetened beverages, orange flavored beverages and grape flavored beverages to improve their taste (Sortwell *et al*., 1996). A mixture of d-limonene extracted from citrus fruits, beeswax and monohydric alcohol has shown to be a preservative for beverage products such as orange juice, fruit punch, grape drink, strawberry kiwi flavoured drink, margherita mix and fruit punch beverage syrup (Winniczuk, 2004).

Noni fruit juice is a very healthy and at the same time a distasteful drink. When added together with a complementary blend of fruits including Luo Han Guo, raspberry, blueberry, lemon and citric acid from citrus, it forms preferable dietary supplement with improved taste. Noni extract in the drink acts as a nutritive natural preservative and improves the shelf life of the dietary supplement (Mower *et al*., 2006). Citric acid from citrus and tartaric acid from tamarind have been used in various compositions for protein containing food products to provide flavoring and sufficient acidity for protein thickening. The acids are used for food products such as acid thickened orange juice pie,

acid thickened orange and tangarine pie. Other pies that have been made using citric acid and tartaric acid include orange and vanilla, tangarine, tangelo, peach, pineapple, grape, mango, tamarind, plum and sugar water pie (Weisberg, 2006).

1.2 The Problem of Histamine

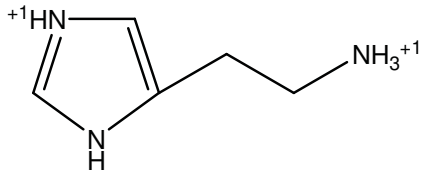
In the Pacific Island countries, seafood associated health problems are a major cause of concern. Most of these problems are due to microbiological contamination and chemical intoxication of food. The fish associated biotoxin histamine is a major cause of histamine or scromboid poisoning in the South Pacific (Quested, 2002).

1.2.1 Properties of Histamine

A number of common bacteria collectively called histamine-producing bacteria (HPB) are responsible for the production of toxic levels (>50 ppm) of histamine in fish (Quested, 2002). Consumption of these fish results in histamine poisoning (Chamberlain, 2000). Incidence of histamine poisoning has been known since the beginning of twentieth century and is a major cause of concern throughout the world including the South Pacific region (Chamberlain, 2000). The high incidence of toxicity has been attributed to the inability to determine histamine content through sensory or organoleptic methods (Chamberlain, 2001). It is also called scombroid poisoning as most incidences result from consumption of mishandled and temperature abused scombroid fish (Niven *et al*., 1981; Chamberlain, 2000).

Histamine is a chemical compound that belongs to a group of toxins called biogenic amines. These are organic bases with aliphatic, aromatic or heterocyclic structures, which can be present in several foods including fish, where they are mainly formed by microbial decarboxylation of amino acids (Kimata *et al.*, 1958; USFDA, 2001). Biogenic amines are low molecular weight organic bases that are required by the body in small amounts. They are essential for carrying out several human physiological functions such as regulation of brain activity, body temperature, stomach volume, immune responses, heart activity and stomach pH. In high concentrations histamine can cause several adverse allergic reactions and illnesses called histamine poisoning (Chamberlain, 2000; Bremer *et al.*, 2003). The most common symptoms of histamine toxicity are rashes, diarrhea, nausea, flushing, tingling, itching skin and headaches (Niven *et al*., 1981; Chen *et al*., 2008). Serious complications, such as abdominal discomfort described as "violent illness" and "enormous" amount of diarrhea have occurred in individuals but no fatalities have been reported so far (Lerke *et al*., 1978).

Chemically, histamine is *2-(4-imidazolyl)ethylamine* and has the formula $C_5H_9N_3$ (Abel and Kubota, 1919).



Histamine structure

Figure 1.1: Chemical structure of histamine ($C_5H_9N_3$) as determined by Abel and Kubota, (1919)**.**

It is synthesized by decarboxylation of the amino acid histidine, a reaction catalyzed by the exogenous enzyme L-histidine decarboxylase which is released by HPB (Lehane and Olley, 1999; Chamberlain, 2000). The formation of histamine starts from degradation of proteins in the presence of urocanic acid to free histidine amino acid (Baranowski, 1985). Histidine is present in larger quantities in scombroid fish as compared to others (Yoshinaga and Frank, 1982). Histamine is formed by removing the alpha-carboxyl group from histidine and named by its originating amino acid. Its structure consists of an aromatic organic and basic nitrogenous compound with low molecular weight (Abel and Kubota, 1919; Lehane and Olley, 1999).

1.2.2 Occurrence of Histamine in fish

Histamine occurs in various foods and its content ranges from 50 ppm to 500 ppm in fish (Chamberlain, 2001). Histamine fish poisoning (HFP) is caused by consumption of spoiled fish and it happens throughout the world (Chamberlain, 2000; Allen, 2004). In Japan many cases of histamine poisoning have been reported involving fish containing elevated levels (>50 ppm) of histamine (Taylor, 1985). The implicated fish (saury, tuna, mahi-mahi, amberjack, bonito, butterfly kingfish and mackerel) are mainly of the families *Scomberesocidae* and *Scombridae* (the so-called scombroid fish) (Chamberlain, 2000; Bremer *et al.*, 2003). Ingestion of 70-1000 ppm of histamine in a single meal is necessary to elicit any symptoms of histamine poisoning depending on the age and health of the individual (Taylor *et al*., 1978). The Australian New Zealand Food Standards Code – Volume 2, states that fish or fish products with histamine levels of less than 200 ppm is acceptable for human consumption (Bremer *et al.*, 2003). In the Turkish Food Codex,

200ppm of histamine is accepted as a defect indicator for fish (FDA, 1998). On the other hand, 50ppm is the allowable limit suggested by the United States Food and Drug Administration (USFDA, 2001). Therefore, fish containing more than 50ppm of histamine can be considered as unsafe for human consumption.

1.2.3 Histamine producing bacteria

There are many species of HPB and many research works have been published to show their histamine producing ability. *Clostridium perfringens, Enterobacter aerogenes, Hafnia alvei, Klebsiella pneumoniae*, *Vibrio alginolyticus, Photobacterium damselae, Photobacterium phosphoreum*, *Raoultella planticola*, *Proteus mirabilis, Proteus vulgaris* and *Morganella morganii* have been isolated as HPB from raw and decomposed fish and found to be positive for showing histidine decarboxylase (HDC) activity (Taylor *et al.*, 1979; Niven *et al*., 1981; Yoshinaga and Frank, 1982; Lehane and Olley, 1999; Jorgensen *et al*., 2000; Kanki *et al*., 2002; Takahashi *et al*., 2003). *Enterobacter* spp have been isolated from tuna recently and found to be positive for producing histamine (Chen *et al*., 2008). An incidence of histamine poisoning took place in San Francisco in September 1977, where various bacteria were isolated from the implicated fish and identified as *Enterobacter cloacae, H. alvei, K. pneumoniae* and *P. vulgaris* (Taylor *et al.*, 1979). *M. morganii*, *P. damselae, P. phosphoreum* and *Raoultella ornithinolytica* have been reported as strong histamine producers while *Citrobacter youngae* and *R. planticola* have also been demonstrated to be able to exhibit some degree of HDC activity (Niven *et al.*, 1981; Lehane and Olley, 1999; Jorgensen *et al*., 2000; Wauters *et al*., 2004). In a different study *R. ornithinolytica* and *R. planticola* have been reported to be important

HPB and found to be equivalent to each other in their histamine producing ability (Kanki *et al*., 2002).

K. pneumoniae is considered to be the best-known HPB to date by some researchers, and *Klebsiella oxytoca* is also well known as a HPB in fish (Taylor *et al.*, 1979; Lopez-Sabater *et al*., 1996). *K. pneumoniae* and *K. oxytoca* have been considered to be the most important HPB isolated from fish (Lopez-Sabater *et al*., 1996). In contrast, other research shows *Proteus morganii* (also known as *M. morganii*) to be present in large quantities in scombroid fish and produce the most quantity of histamine (Niven, 1981; Chamberlain, 2001). Histamine-positive cultures have been isolated from raw tuna, mahi mahi and mackerel. These cultures were identified by using the API system, where *P. morganii* proved to be the most common species, followed by *K. pneumoniae*, *E. coli*, *E. aerogenes*, *E. cloacae*, *Proteus* spp, *Edwardsiella* spp and *Vibrio* spp (Kimata *et al*., 1958). Since *P. morganii* is present in the largest quantity in fish, it can be considered as the most effective species of HPB.

While these studies demonstrate histamine production by the various bacterial species, other studies show contrasting results. Recent works have shown that *K. pneumoniae* and *K. oxytoca* do not produce any histamine at all (Kanki *et al*., 2002; Wauters *et al*., 2004). Taylor's decarboxylation broth was used to detect the histamine producing ability of various strains of enterobacteriaceae. Results illustrated the absence of histidine decarboxylation by *E. cloacae, E. coli, H. alvei, P. mirabilis* and *P. Vulgaris* (Wauters *et al*., 2004).

Review of previous research works show many differences in the reported histamine producing ability of different bacterial species. While some studies claim *P. morganii* (Kimata *et al*., 1958; Niven, 1981; Chamberlain, 2001) to be strongest histamine producer in fish, others consider *K. pneumoniae* and *K. oytoca* (Taylor *et al.*, 1979; Lopez-Sabater *et al*., 1996) to be the best histamine producers known so far. Some research works claim certain bacterial species to be histamine producers (Kimata *et al*., 1958; Taylor *et al.*, 1979; Lopez-Sabater *et al*., 1996) while other studies claim that they don't produce any histamine (Kanki *et al*., 2002; Wauters *et al*., 2004). With such contrasting reports, the true histamine producing ability of bacterial species cannot be established.

1.3 Fish spoilage bacteria

In comparison to all flesh foods, fish are most susceptible to autolysis and spoilage. They spoil quickly and are among the world's most perishable commodities. Fish spoilage begins soon after the organism's death. The body of any fish comprises mostly of muscle and as soon as fish die, blood circulation stops and this leads to a series of changes within the fish muscle. Spoilage of fresh fish is a combination of several interrelated processes where no single factor is responsible. These processes act together and lead to the breakdown of protein. This causes the softening of flesh, loss of cellular fluid and development of undesirable odors, at which point fish is considered spoilt and unsafe for human consumption (Chamberlain and Tilili, 2001).

All food types have a bacterial flora which has the potential to cause spoilage and safety problems. Fish spoilage can occur through bacterial spoilage, enzymatic spoilage and chemical spoilage or a combination of the three but it is mostly bacterial in nature (FDA, 1998). Bacteria are found mostly on the surface of a live fish and are also introduced during mishandling after the fish dies (USFDA, 2001). Bacterial populations present on fish are mostly heterotrophic bacteria, which rely on other organisms for their requirement of reduced, pre-formed organic molecules as carbon sources. Growth of these bacteria is generally responsible for spoilage and putrefaction of fish (Crapo and Himelbloom, 1999). Coliforms are mostly gram-positive mesophilic bacteria and comprise of important human pathogens but are also important in terms of promoting fish spoilage (Shewan, 1971; Gram, 1993; Niemi *et al*., 2001; Kahlmeter, 2003). Coliforms are indicator organisms for the presence of pathogenic bacteria; the higher the number of coliforms the higher the number of pathogens (Prescott *et al*., 2005). Some coliforms are naturally present on fresh fish while others are introduced when fish are exposed to contamination from a variety of sources such as workers hands and clothing, equipment and contact surfaces of fishing vessel (USFDA, 2001).

1.3.1 Chilling Fish

Reducing the temperature of fish immediately after being caught is critical. All types of fish tend to spoil rapidly if they are not chilled right away. Block ice is the best choice for chilling fish, especially during long fishing trips as it lasts for a longer period. Low temperature reduces bacterial activity and slows down spoilage. In many areas of the South Pacific, especially in the villages where fish are caught primarily for household

consumption, chilling fish is not a common practice. In many areas electricity and ice machines are not available; therefore fish are not chilled and are at high risk of being spoilt prior to consumption.

Chilling may not work adequately for all types of fish. Fish adapted to low temperatures will have bacterial flora which are also adapted to cold temperatures. These fish will detoriorate rapidly at refrigerator temperatures (1-4°C). Certain psychrophilic bacteria have been shown to cause spoilage of fish and fish products at refrigerator and even freezing temperatures (Shaw and Shewan, 1968). *Pseudomonas* spp has shown to cause spoilage of North Sea Cod at 0.6°C. Spoilage by *Pseudomonas* spp and *Moraxella* spp was evident even at -3°C (Shaw and Shewan, 1968). Lactic acid bacteria were isolated from spoiled, vacuum packed 'gravad' rainbow trout stored at 3°C. *Lactobacillus sakei*, *Lactobacillus curvatus* and *Carnobacterium piscicola* were the three major species identified. *Carnobacterium divergens* was also detected, but as a minor species (Lyhs *et al*., 2000). Gram-negative spoilage bacteria were isolated from cod stored at 2°C. The bacterial species were identified as *Pseudomonas putrefaciens*, *Pseudomonas* spp, *Pseudomonas fragi*, *Pseudomonas putida* and *Pseudomonas fluorescens* (Herbert *et al*., 1971).

Bacteria were enumerated and identified from spoiled vacuum-packed, cold-smoked and 'gravid' rainbow trout and semi-preserved marinated herring. These products spoiled at 3-8°C and the spoilage bacteria were identified as *L. sakei, Lactobacillus curvatus, Leuconostoc mesenteroids, Leuconostoc citreum* and *Lactobacillus alimentarius* (Lyhs,

2002). The growth of specific spoilage bacteria on the Mediterranean fish boques (*Boops boops*) at different temperatures was studied. *Pseudomonas* spp, *Shewanella putrefaciens*¸ *Brochothrix thermosphacta* and members of Enterobacteriaceae caused spoilage of fish at 0°C and 3°C (Taoukis *et al*., 1999). In another study, the growth of natural microflora of marine cultured, air-packed, sea bass (*Dichentrachus labrax*) was studied at 0-15°C. At 0°C, development of the microflora of sea bass comprised of Pseudomonads, Enterobacteriaceae and lactic acid bacteria (Koutsoumanis *et al*., 2002).

These studies show that chilling alone may not be an effective method for controlling the spoilage of all types of fish. Also, using ice is not practical for all areas of the South Pacific. In such areas other alternatives to ice are needed to control fish spoilage.

1.4 Importance of Chub mackerel (*Rastrelliger brachysoma*)

Salala is the common name for *Rastrelliger brachysoma* in Fiji and is also known as chub-mackerel in other parts of the globe. Salala belongs in the suborder Scombriodei, family Scombridae; therefore it is classified as a scombroid fish capable of causing scombrotoxicosis (Bremer *et al.*, 2003).

There are three species of mackerel that can be found in the waters of Fiji: the chub mackerel (*Rastrelliger faughni*), the slender chub mackerel (*Rastrelliger kanagurta*) and the stout chub mackerel (*R. brachysoma*). *R. kanagurta* and *R. brachysoma* are quite abundant in the Fiji waters and are important to fisheries (Sharma and Adams, 1990). *R. brachysoma* is widely distributed in the Indo-Pacific Region and is generally found in

waters close to the shore (Lewis, 1985). It is most important to fisheries out of all three species as *R. brachysoma* is processed and marketed in the highest quantity (Lewis, 1985). The catchable sizes of salala are approximately 15-25 cm in length and are generally consumed fresh in Fiji (Lewis, 1985). The flesh of salala is quite dark and oily, and is marketed fresh, frozen, smoked, salted, dried and canned (Richards *et al.*, 1994).

Salala is a very important food commodity in the South Pacific, both as an export product and as food for the locals. It is an easily available and cheap food product that is consumed by a large South Pacific community. Salala is sold for FJD2.00-3.00 per kg (Anonymous, 1993), which is quite cheap and affordable for people of all classes. The catches are mostly sold fresh to the consumers through municipal and non-municipal markets, while some are also sold by roadsides. These suppliers do not follow proper hygienic procedures, storage and handling; therefore fish are at high risk of becoming contaminated with bacterial load, including HPB, heterotrophic bacteria and coliforms. Processed salala is also sold canned and frozen in local supermarkets and fish stores (Richards *et al.*, 1994). Despite its importance, there have been no studies conducted on salala to ensure its safety for human consumption.

In the South Pacific, seafood related diseases are an increasingly important public health problem. The importance of microbiological hazards and toxins such as histamine in fish has been highlighted in news headlines (Quested, 2002). Fresh fish is consumed at high levels and is an important food commodity to the Pacific Islanders. Therefore, it is important to develop simple and affordable methods for controlling fish spoilage,

pathogenicity and histamine formation to make it safe for human consumption. Bacteria are the main agents of fish spoilage and histamine formation. Thus, developing a method to control growth of HPB, heterotrophic bacteria and coliforms will enable the control of fish spoilage, histamine formation and risk of diseases. To control growth of these bacteria, an antibacterial agent is required that is cost effective, natural and easily available.

In spite of all the information available on the fifteen species of HPB selected for this study, there is no research work found which has attempted to inhibit the growth of all fifteen species using a single antibacterial agent. These fifteen species comprise approximately 88%[1] of all histamine producers in fresh fish identified so far. Therefore controlling the growth of these HPB will allow the control of histamine production to a great extent. Antibacterial agents that permit growth control of HPB can be used to control histamine formation in seafood industries for commercial purposes.

Noni, citrus and tamarind are plants that are quite common and widely distributed in the South Pacific. These plants grow in most types of soil and can be grown just about anywhere. They are not seasonal, therefore will bear fruits in all seasons throughout the year. Tea is widely consumed in the Pacific on a daily basis; therefore it is easily obtainable as well. Many research works have been conducted on various extracts from these plants but these methods use sophisticated equipment, are expensive and the

[1] A total of seventeen HPB have been identified from unprocessed fish so far. This study focuses on fifteen species of HPB. Although more than seventeen names can be found in literature, some of these HPB have now been identified as the same species. More information about this can be obtained from the American Type Culture Collection (ATCC) located in the United States of America or from their website: www.atcc.org.

methods employed for utilizing the extracts are beyond the skills of local people. Also, there has been no work done on utilizing the antibacterial properties of these extracts for fish safety. Hence, it is important to investigate the application of noni, citrus, tamarind and tea extracts in fish safety using methods that can be easily replicated with little skill and effort. Through review of literature there is no doubt that plant extracts contain strong antibacterial properties. Therefore, it is appropriate to suggest that crude extracts from noni, citrus, tamarind, black tea and green tea may possess the ability to control the growth of HPB, heterotrophic bacteria and coliforms.

1.5 Hypothesis of study

This study was undertaken with the intention of determining the histamine forming potential of fifteen species of histamine producing bacteria (HPB); to test the *in vitro* antibacterial activity of noni extract, water soluble extracts from black tea and green tea, citrus extract and tamarind extract against these species; to observe the *ex vivo* effect of these five plant extracts on growth of total heterotrophic bacteria and total coliforms present in post-harvest salala (*R. brachysoma*) at different incubation temperatures.

1.6 Specific objectives of investigation

- To assess the histamine forming potential of *Clostridium perfringens, Citrobacter youngae, Escherichia coli, Enterobacter aerogenes, Enterobacter cloacae, Enterobacter amnigenous, Hafnia alvei, Klebsiella oxytoca, Klebsiella pneumoniae, Morganella morganii, Proteus vulgaris, Proteus mirabilis,*

Photobacterium damselae, Raoultella ornithinolytica and *Vibrio alginolyticus* and characterize them as histamine producing bacteria (HPB).

- To assess the *in vitro* bactericidal potential of noni extract, black tea, green tea, tamarind extract and citrus extract against the following fifteen species of HPB; *Clostridium perfringens, Citrobacter youngae, Escherichia coli, Enterobacter aerogenes, Enterobacter cloacae, Enterobacter amnigenous, Hafnia alvei, Klebsiella oxytoca, Klebsiella pneumoniae, Morganella morganii, Proteus vulgaris, Proteus mirabilis, Photobacterium damselae, Raoultella ornithinolytica* and *Vibrio alginolyticus.*

- To investigate the *ex vivo* effect of noni extract, black tea, green tea, citrus and tamarind extract on growth response of total heterotrophic bacteria in salala at different incubation temperatures.

- To evaluate the *ex vivo* antibacterial properties of noni, black tea, green tea, tamarind and citrus extract against total coliforms present in salala at different incubation temperatures.

II. Materials and Methods

2.1 Histamine production by bacterial cultures

Fifteen species of selected pure bacterial cultures were assessed for their histamine producing capability. The cultures included *Clostridium perfringens, Citrobacter youngae, Escherichia coli, Enterobacter aerogenes, Enterobacter cloacae, Enterobacter amnigenous, Hafnia alvei, Klebsiella oxytoca, Klebsiella pneumoniae, Morganella morganii, Proteus vulgaris, Proteus mirabilis, Photobacterium damselae, Raoultella ornithinolytica* and *Vibrio alginolyticus.* The sources of the bacterial species are given in the materials and methods section, chapter 2.5.

2.1.1 Preparation of media

Nutrient tuna infusion broth[2] (NTIB) medium was prepared by supplementing nutrient broth with 10% tuna meat extract. Tuna fish infusion broth (TFIB) was prepared from fresh tuna muscle by a procedure previously described by Taylor *et al.*, (1979) (Appendix 7.6).

2.1.2 Preparation of inoculum

15 x 10 ml NTIB were inoculated with 15 bacterial pure cultures from stock stored at -70°C and incubated at 32°C for 24 hours. 0.2 ml aliquots of 24-hour culture were taken and inoculated in another 15 NTIB tubes and subsequently incubated overnight at 32°C.

[2] In case of *P. damselae*, nutrient broth was supplemented with 1:10 ratio of marine agar. This overnight culture served as the inoculum for the histamine production experiment

2.1.3 Production of histamine

A 2 ml amount of the 15 overnight cultures were inoculated into 70 ml of TFIB in 300 ml nepheloculture flasks. 1 ml of sample was immediately withdrawn from each flask for histamine analysis. All flasks were incubated at 32°C in a shaking incubator (New Brunswick Scientific) at 100 rpm. The rest of the procedure has been previously described by Taylor *et al.*, (1979) (Appendix 7.7). Additional 1 ml samples were withdrawn for histamine analysis at 7 hours and 24 hours following incubation. All tests were done in triplicates and results were expressed as mean values ± SD.

An uninoculated sample was used as a control. Also, *Aeromonas hydrophila* (obtained as a clinical isolate from the Microbiology Lab, Colonial War Memorial Hospital, Suva, Fiji) was used as a negative control for the experiment. *A. hydrophila* is a non-histamine producing species.

2.1.4 Histamine analysis

All samples used for histamine analysis were diluted 5-fold in ethanol. Histamine was analyzed by western blotting paper electrophoresis using the JBB histamine checker set-up. The rest of the analytical procedure has been previously described by Sato *et al.*, (2006) (Appendix 7.8). The histamine blot analysis was carried out by comparison of blot intensity, size and coloration with external standards treated identically.

2.1.5 Evaluation of Histamine content

On the dried H-type Filter Paper (Electrophoresis paper), there appeared color developments, which were reddish in color if histamine was contained in the test samples. To quantify the test samples, colors developed by test samples were compared with colors developed for known standard samples treated identically. Initially, standards were prepared from 10 ppm to 300 ppm. Upon further testing, it was observed that histamine standards as low as 5 ppm could be detected and quantified. Histamine standard liquids were prepared, ranging from 5 ppm to 300 ppm at every 1 ppm interval. The standards were prepared from Histamine Dihydrochloride $C_5H_9N_3.2HCl$ crystals. All stadards ranging from 5 ppm to 300 ppm were analysed by JBB Histamine Checker and histamine blots obtained. All blots were reddish in color.

A large number of standards were prepared to get better accuracy of results. A total of 295 standards were prepared. A single run on the JBB Histamine Checker electrophoresis vessel takes only 30 minutes. In a single run, 7 samples can be tested. This means that 295 standards will take a total of 22 hours to run. The standards can be easily run in the JBB Histamine Checker in a week.

The analysis for each histamine standard was replicated 5 times and blot pattern recorded. Blots at each histamine concentration gave unique blot pattern with respect to color, intensity and size. These blot patterns were used as measurement criteria for test samples. Histamine blots obtained from unknown test samples were compared to standard blots. Test sample blot pattern in each case matched to only one standard blot pattern at a

particular concentration. This was taken as the concentration of histamine in the test sample. Test samples in each case were analysed in triplicates.

The JBB histamine checker was calibrated using the standard addition method previously described by Singh and Bowry, (2005) (Appendix 7.9).

2.2 Preparation of plant material

Green tea (China Ever-Green Green Tea) and black tea (China Lichee Black Tea) were used for this study. Both types of tea were bought from a local supermarket in Suva, Fiji. They were purchased in sealed tin containers with green tea being 100 g of tea leaves per container and black tea being 227 g of tea leaves per container. Ripe (yellow skin) citrus and tamarind (black shell) fruits were hand picked by the researcher directly from the tree and brought to the laboratory within 1 hour for further processing. 100% pure Noni Miracle noni juice was bought from a local pharmacy. The juice was prepared from ripe yellow/pale skin noni fruit only after 2 to 6 hours of harvesting. Information was obtained from the manufacturers (footer number 3, page 42). Aseptic techniques were followed to avoid contamination from other unwanted microorganisms. Aseptic techniques does not remove contaminants that are present, it only avoids contamination from unwanted sources, such as from the environment, contact surfaces, equipment, human body and clothing.

In this study, the different extracts were treated differently due to their practicality in real situations. This may have affected and contributed to the differences in the concentrations

of each plant extract. For example, tamarind does not contain any juice, so water needs to be added to get the water soluble extracts out. Enough water was added to tamarind pulp to get the soluble extracts, which came to 1 g flesh/4 ml water. Tamarind extract was used at high concentration as literature was not available on the concentration of crude extract from tamarind that would be effective against bacteria. Similarly, for tea, water needs to be added to get the soluble extracts out. It is not practical to use tea in the ratio of 1 g leaves/4 ml water, as this will be highly uneconomical, the cost of tea leaves will be very high. Also, this concentration of tea will cause a lot of undesired organoleptic change to the food item. Literature was available on the concentration of tea that would be effective against bacteria. The concentration of 1 g leaves/25 ml water mentioned in literature was used (Jazani *et al.*, 2007a,b). Noni and citrus contain a lot of juice, so addition of water was not required. The juices were not diluted since literature was not available on the crude extracts from noni and citrus. Also, these fruits are not expensive and are easily available; therefore they could be used without dilution. The aim of the study was to use concentrated extracts where literature was not available. Future studies could be done on determining the Minimum inhibitory concentrations (MIC) of the extracts used. The methodology used in this investigation can be compared with studies by Gerson *et al.*, (2003); Ghelardi *et al.*, (2004); Muthu *et al.*, (2005); Doughari, (2006); Carlson *et al.*, (2007); Jazani *et al.*, (2007a,b); Turkmen *et al.*, (2007); Prabuseenivasan *et al.*, (2006); Jayaraman *et al.*, (2008); Tsai *et al*., (2008) and Voravuthikunchai and Mitchell, (2008) as well as future studies as the concentration of the active ingredients in each of the analyte was determined.

All extracts used for the study were pasteurized prior to the experiment. During pasteurization, bacterial cells are heated to 72°C and maintained at this temperature for a period of time. Most bacteria are unable to survive and function at this temperature. Heat causes bacterial protein and enzymes to denature and the cells to rupture. The bacterial cells are then quickly brought down from 72°C to almost 0°C. A small number of cells are able to survive at 72°C. Bringing down the temperature suddenly provides heat shock to the remaining bacterial cells, resulting in their death.

Extracts were placed in enclosed sterile Duran bottles and placed in a water bath maintained at 72°C for seven minutes. Following this, the bottles were immediately taken out and immersed in ice and water slurry maintained as close to 0°C as possible to remove heat rapidly and provide heat shock to kill bacterial cells. 1 ml from each extract was withdrawn and subjected to bacteriological analysis by pour plate method as described by Gilchrist *et al.*, (1973) to test for bacterial contaminants. Tests were done in triplicates. Plant extracts containing less than 2 cfu/ml of bacterial contaminants were selected for the experiment.

2.2.1 Tea extraction

Tea containers were aseptically opened and extracts were prepared by suspending 1 g of dried tea leaves in 25 ml of boiling water and boiled for 2 minutes. Leaves were separated from liquid by filtering through sterile multiple sieves. Size of the smallest sieve was 0.1-0.2 mm.

2.2.2 Citrus extraction

The fruits were washed in tap water followed by washing in sterile distilled water (SDW). Subsequently, the skin was wiped with 75% ethanol and allowed to dry for 5 minutes. After drying, fruits were cut in halves and the juice was squeezed out. The extracted juice was filtered through multiple sterile sieves.

2.2.3 Tamarind extraction

Fruits were washed in tap water followed by washing in sterile distilled water and then the fruit skin was wiped with 75% ethanol and allowed to dry for 5 minutes. After drying, the shell and seed were removed and the flesh was soaked in SDW for 24 hours. A ratio of 1 g of tamarind flesh in 4 ml of SDW was used. The liquid extract was then filtered through sterile multiple sieves.

2.2.4 Noni extract

Noni Miracle noni juice[3] was bought from a local pharmacy. The purity and sterility of the juice has previously been analyzed and confirmed by the University of the South Pacific's laboratories (Personal Communication with Professor William Aalbersberg). The liquid extract was filtered through multiple sterile sieves.

[3] Bottled and locally produced in Fiji by Noni Miracle, Koro Island, Fiji. It is 100% pure, undiluted and free from any additives.

2.3 Treatment of Plant extracts

Water soluble extracts from green tea leaves (*Camellia sinensis*), black tea leaves (*Camellia sinensis*), tamarind (*Tamarindus indica*), noni (*Morinda citrifolia*) and citrus (*Citrus aurantium*) were screened for their *in vitro* antibacterial activity using the pour plate method as described by Gilchrist *et al.*, (1973) against 15 species of histamine producing bacteria (HPB) pure cultures. These five extracts were also tested for their *ex vivo* inhibitory activity against total heterotrophic bacteria by total plate counts (TPC) and coliforms by total coliform count (TC) in headed and gutted chub-mackerel (*Rastrelliger brachysoma*). These methods are explained in detail in chapters 2.5, 2.6 and 2.7. Aseptic techniques were followed to avoid contamination from other unwanted microorganisms.

2.4 Test organisms

A total of fifteen species of histamine producing bacteria (HPB) were used to assess the antibacterial properties of the five plant extracts. The organisms *Escherichia coli, Enterobacter aerogenes, Enterobacter cloacae, Proteus vulgaris*, *Proteus mirabilis*, *Citrobacter youngae, Klebsiella oxytoca, Klebsiella pneumoniae* and *Raoultella ornithinolytica* were obtained as clinical isolates from the Microbiology Lab, Colonial War Memorial Hospital, Suva, Fiji. Other bacterial cultures used were *Clostridium perfringens* (ATCC 13124), *Enterobacter amnigenous* (NZ isolate ERL00/748), *Hafnia alvei* (CDC 5190-70), *Morganella morganii* (ATCC 25830), *Photobacterium damselae* (ATCC 33539) and *Vibrio alginolyticus* (NCTC 10675). The bacterial cultures were maintained on the following agar slants at 4°C throughout the study which were used as stock cultures. *E. coli* (Nutrient Agar (NA)), *E. aerogenes* (NA), *E. cloacae* (NA), *P.*

vulgaris (NA), *P. mirabilis* (NA), *C. youngae* (NA), *K. oxytoca* (NA), *K. pneumoniae* (NA), *R. ornithinolytica* (NA), *E. amnigenous* (Tryptic Soy Agar (TSA)), *H. alvei* (TSA), *M. morganii* (TSA), *P. damselae* (Marine Agar) and *V. alginolyticus* (TSA). In the case of the *C. perfringens*, Blood Agar was used as growth medium. The inoculated growth medium was placed in an enclosed anaerobic jar with a deoxygenating sachet to create anaerobic conditions and permit growth of *C. perfringens*. All bacterial cultures were sub-cultured every two weeks. Permanent cultures were also made and stored at -70°C (refer to Appendix 7.10).

2.5 Antibacterial activity of plant extracts against HPB

HPB cells from stock cultures were inoculated into 10 ml nutrient broth and incubated overnight at 32°C. In the case of the *C. perfringens*, 10 ml of Brain Heart Broth was used as a growth medium and was inoculated with a loopful of growth from stock. The cultures were adjusted to approximately 10^6 cfu/ml with sterile peptone-physiological saline solution (0.1% peptone + 0.85% NaCl). These cultures were used as inoculum within 1 hour after adjustment. Subsequently, 1 ml of each of the fifteen cultures was inoculated into 99 ml of the plant extracts to obtain a final bacterial concentration of 10^4 cfu/ml. The analytes were incubated at 32°C and 1 ml sample was taken immediately after inoculation from each analyte for bacteriological analysis. The samples were serially diluted with sterile peptone-physiological saline solution and plated at 10^0, 10^{-1} and 10^{-2} dilutions. Sterile peptone-physiological saline solution was used as the diluent for all microbiological assays.

All HPB samples were enumerated using the pour plate method described by Gilchrist *et al.*, (1973) on their mentioned agar medium (Section 2.4). 1 ml samples were taken from each bottle at five different time intervals for up to 48 hours. Tests were carried out at 32°C since the growth of HPB and histamine formation is particularly rapid near this temperature (Taylor *et al.*, 1979; FDA, 2001). 32°C was selected as the experimental temperature to measure the inhibitory effect of the plant extracts against HPB at their optimum growth and histamine production. All plates were incubated at 32°C for 24 hours and the results expressed as Log_{10} colony forming units per milliliter of sample ± standard deviation (Log_{10} CFU/ml ± SD).

The inocolumn of 10^4 cfu/ml was chosen for HPB analysis based on the concentration present on freshly caught fish. Histamine production in fish can only be slowed down if bacterial growth can be inhibited at the initial stages of fish capture. Bacterial cells will not be able to produce the enzyme that converts histidine to histamine. The experimental procedure in this study is intended to be used on freshly caught fish. Fish caught within 1 hour were obtained and the total bacterial loads present on the fish were enumerated using the pour plate method described by Gilchrist *et al.*, (1973). In all cases, bacterial populations were either less than or close to 10^4 cfu/ml. Therefore, this value was selected as the inoculum for the experiment.

A recovery test was conducted to observe the residual effect of the plant extracts on the growth of HPB, TPC and TC. In each case, the extracts were inoculated with a known concentration (~10^4 cfu/ml) of bacteria and plated at 10^0, 10^{-1} and 10^{-2} dilution using the

pour plate method described by Gilchrist *et al.*, (1973). In all cases, cell recovery was above 90%. Such a high recovery indicates acceptable level of residual effect of the plant extracts on the growth of HPB, TPC and TC.

2.6 Preparation of fish material

Fresh unchilled whole chub-mackerel were purchased within four hours after death from local seafood markets of Nasinu, Suva and Nausori, Fiji. Fish were headed, gutted, packed into polythene bags and brought to the lab.

2.7 Antibacterial activity of plant extracts against TPC and TC

TPC and TC levels were determined *ex vivo* in salala at seven different incubation temperatures. For each temperature and plant extract 5 whole headed and gutted salala were taken. Each fish was immersed into the extract with a ratio of 1 g of fish to 9 ml of plant extract. The contents were incubated in sterile, airtight containers. Each preparation consisted of five containers containing identical ratio of fish and plant extract. For each plant extract seven preparations (one for each temperature) were made with the ratio of fish to plant extract kept constant. For each plant extract a total of 35 fish were used for incubation at seven different temperatures. The preparations were incubated subsequently at 5ºC, 10ºC, 15ºC, 20ºC, 25ºC, 30ºC and 32ºC. From each preparation, a fish sample was taken immediately for TPC and TC analysis. Fish samples were taken subsequently from each preparation for TPC and TC analysis at five different time intervals in a 48-hour period. Each fish sample taken for analysis was filleted and cut into 1-1.5 cm slices. These fillets were added to sterile peptone-physiological saline solution with a ratio of 1

g of fish to 9 ml of saline solution and homogenized in a stomacher for 5 minutes to abtain a homogenized mixture. This homogenate was used for TPC and TC analysis. All tests were done in triplicates.

Bacterial populations of TPC and TC were determined at 10^0, 10^{-1} and 10^{-2} dilution using the pour plate method described by Gilchrist *et al.*, (1973). For TPC analysis, nutrient agar was used as the growth medium. McConkey agar was utilized as a differential plating medium for TC analysis. Presence of pink to red colonies was considered as positive for presence of coliforms. All plates were incubated at 32^{o}C for 24 hours and the results expressed as Log_{10} colony forming units per milliliter of sample ± standard deviation (Log_{10} CFU/ml ± SD).

2.8 Determination of active constituents of plant extracts

The microbiologically active constituents of green tea, black tea, citrus, tamarind and noni were determined using HPLC. Methodologies were partially adapted from studies by Castillo *et al.*, (1992); Joseph *et al.*, (2005); Turkmen *et al*., (2007); Yang *et al.*, (2007) and Tsai *et al*., (2008).

2.8.1 Analysis of total polyphenols in green tea and black tea

Green tea and black tea leaves were prepared separately. 1 g of dried tea leaves were added to 25 ml of boiling water (SDW) and boiled for 2 minutes. Leaves were separated from liquid by filtering through sterile multiple sieves. Size of the smallest sieve was 0.1-0.2 mm. After cooling the polyphenol concentration in both types of tea were then

measured by HPLC with a reverse phase C-18 column (Phenomenex, Torrance, CA, 300 mm × 4.6 mm × 5 µm). A mobile phase of trifluoroacetic acid (0.1%) in distilled water and a UV detector operating at 280 nm was used. The flow rate was 1 ml/min and the injected volume was 10 µl. Concentrations were determined by comparison with standard curves.

2.8.2 Analysis of total flavonoids in citrus

Citrus fruits were halved and the juice squeezed out, followed by filtration through multiple sterile sieves. The total flavonoid was measured by HPLC with a reverse phase C-18 column (Phenomenex, Torrance, CA, 300 mm × 4.6 mm × 5 µm). A mobile phase of water, methanol, acetonitrile and acetic acid in the ratio of 15:2:2:1 and a UV detector operating at 280 nm were used. The flow rate was 1 ml/min and the injected volume was 10 µl. Concentrations were determined by comparison with standard curves.

2.8.3 Analysis of acid contents in tamarind

Tamarind flesh was soaked in SDW for 24 hours in a ratio of 1 g of tamarind flesh to 4 ml of SDW. The liquid extract was then filtered through sterile multiple sieves. Acid contents were measured by HPLC with a reverse phase C-18 column (Phenomenex, Torrance, CA, 300 mm × 4.6 mm × 5 µm). A mobile phase of trifluoroacetic acid (0.1%) in distilled water and a UV detector operating at 254 nm was used. The flow rate was 1 ml/min and the injected volume was 10 µl. Concentrations were determined by comparison with standard curves.

2.8.4 Analysis of total polyphenols and ascorbic acid in noni

Pure noni juice was filtered through sterile multiple sieves. The polyphenol concentration was measured by HPLC with a reverse phase C-18 column (Phenomenex, Torrance, CA, 300 mm × 4.6 mm × 5 µm). A mobile phase of trifluoroacetic acid (0.1%) in distilled water and a UV detector operating at 280 nm was used. The flow rate was 1 ml/min and the injected volume was 10 µl.

Ascorbic acid concentration was measured by HPLC with a reverse phase C-18 column (Phenomenex, Torrance, CA, 300 mm × 4.6 mm × 5 µm). A mobile phase of trifluoroacetic acid (0.1%) in distilled water and a UV detector operating at 254 nm was used. The flow rate was 1 ml/min and the injected volume was 10 µl. Concentrations were determined by comparison with standard curves.

III. Results

3.1 Production of histamine in TFIB by bacterial cultures

Pure bacterial cultures representing fifteen species were tested for their histamine producing capacity in TFIB over a 24-hour phase. The results are presented in Figures 3.1 and 3.2. The results are also summarized in Table 7.1 (Appendix 7.1). Among the fifteen species tested, *C. youngae* produced the highest amount of histamine after 7 hours of incubation followed by *M. morganii, P. damselae* and *C. perfringens*. Other bacterial species produced less than 50 ppm histamine (Figure 3.1). After 24 hours of incubation (Figure 3.2), histamine production by all fifteen species increased, with *M. morganii* producing the highest amount. *C. perfringens, C. youngae, E. aerogenes, E. cloacae, H. alvei, K. oxytoca, M. morganii, P. damselae,* and *V. alginolyticus* produced more than 50 ppm of histamine after 24 hours of incubation, while *E. coli, E. amnigenous, K. pneumoniae, P. vulgaris, P. mirabilis* and *R. ornithinolytica* produced less than 50 ppm of histamine. There was no histamine produced in the control and negative control (*A. hydrophila*) samples.

3.2 Antibacterial activity of plant extracts against HPB

Antibacterial activity of five selected plant extracts against fifteen species of histamine producing bacteria (HPB) is summarized in Table 3.1 to Table 3.6. Plant extracts showed antibacterial activity with varying magnitudes. The inhibition of bacterial growth within 24 hours of incubation was taken as excellent inhibitory activity by the plant extract tested. Growth inhibition between 24 and 48 hours of incubation was taken as moderate

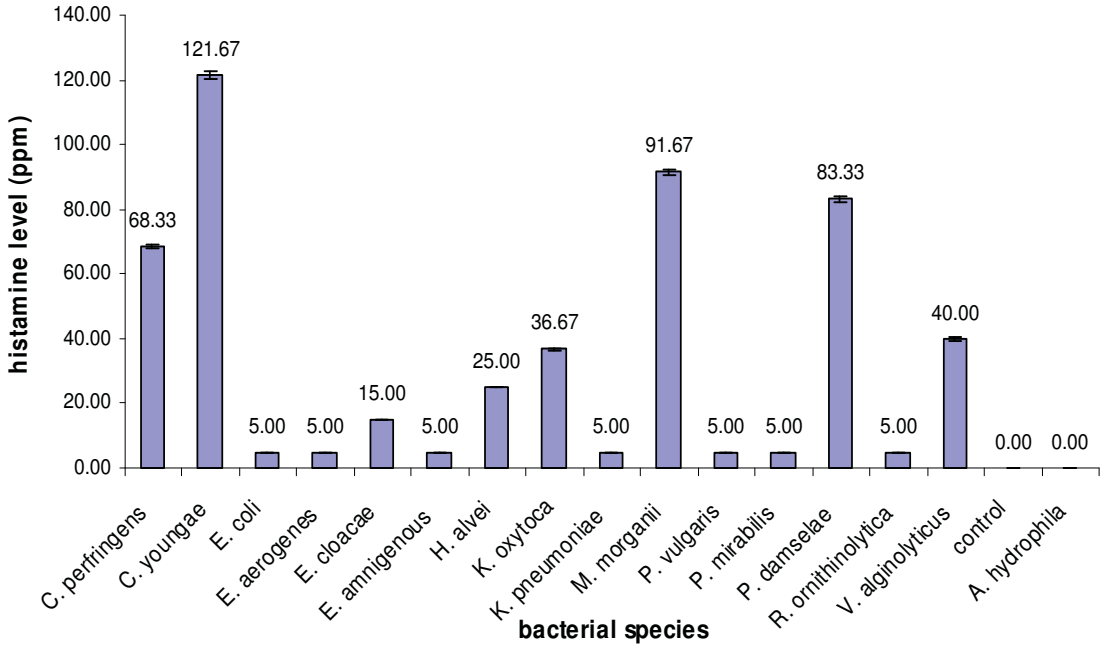


Figure 3.1: Histamine production in tuna fish infusion broth by fifteen bacterial species after 7 hours of incubation. Histamine standards from 5 ppm to 300 ppm were taken as reference standards for comparison.

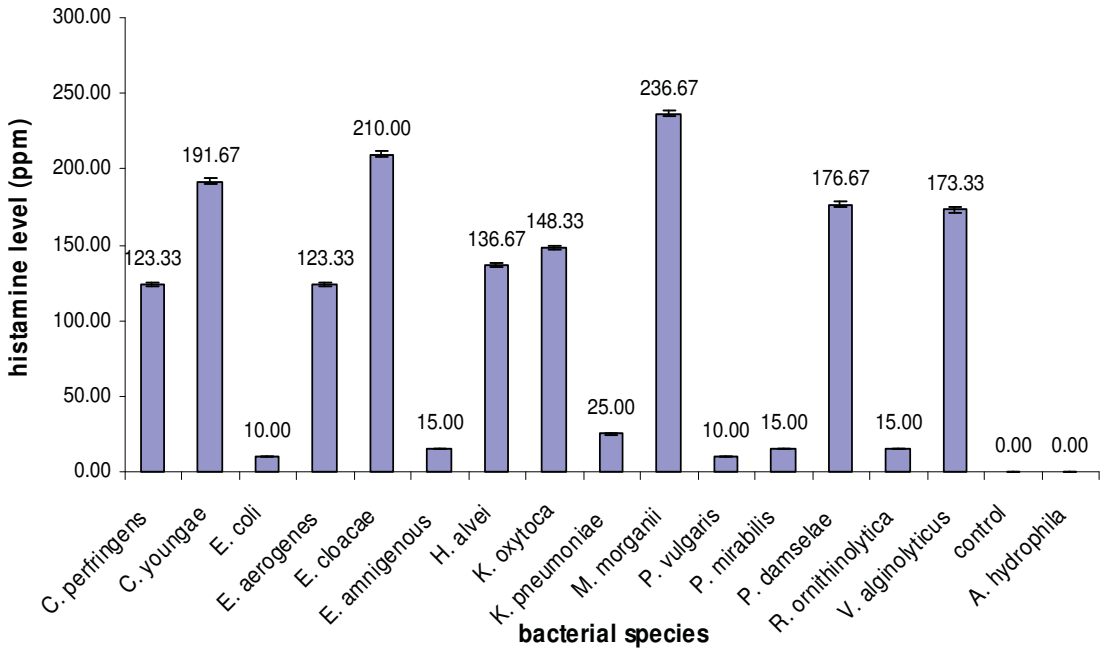


Figure 3.2: Histamine production in tuna fish infusion broth by fifteen bacterial species after 24 hours of incubation. Histamine standards from 5 ppm to 300 ppm were taken as reference standards for comparison.

antibacterial activity. Generally all plant extracts tested demonstrate antibacterial activity against one or more bacteria.

The time intervals were chosen dependent on the growth behavior and experimental parameters. The time interval of 0 hour was chosen to determine the total actual bacterial load transferred to the analytes before they were subjected to being affected by the extracts. The interval of time 2 hours was chosen to observe the immediate effect of the extracts on the bacterial cells. The extracts will either have an immediate and rapid effect on the growth of the bacterial cells, or a slow effect. The rapid effects can be observed at two hours, while the slow effect can be determined aver a longer period of 24 hours. Bacterial cells evolve quite rapidly. Within the same species of bacteria there may be individuals present, who may possess resistance against the extracts. At 24 hours, the bacterial cells may be below detection limit, but there may be a small number of cells that may have developed resistance to the extracts. It takes time for these cells to recover and grow above detection limit. The time interval of 48 hours was chosen to observe this.

Noni extract showed maximum antibacterial activity against all fifteen HPB species tested. As shown in Table 3.1 growth of all fifteen bacterial species is inhibited within 2 hours of incubation, bringing the Log_{10} CFU/ml surviving cells to below undetectable limits.

Table 3.1: Antibacterial activity of noni extract against histamine producing bacteria

HPB	Log_{10} CFU/ml ± SD bacterial cells remaining at each time interval (hours)				
	0:00	2:00	24:00	26:00	48:00
C. perfringens	3.96 ± 0.006	-	-	-	-
C. youngae	3.98 ± 0.006	-	-	-	-
E. coli	3.97 ± 0.000	-	-	-	-
E. aerogenes	3.96 ± 0.005	-	-	-	-
E. cloacae	3.97 ± 0.006	-	-	-	-
E. amnigenous	3.97 ± 0.006	-	-	-	-
H. alvei	3.97 ± 0.007	-	-	-	-
K. oxytoca	3.98 ± 0.006	-	-	-	-
K. pneumoniae	3.98 ± 0.000	-	-	-	-
M. morganii	3.97 ± 0.006	-	-	-	-
P. vulgaris	3.97 ± 0.006	-	-	-	-
P. mirabilis	3.96 ± 0.005	-	-	-	-
P. damselae	3.97 ± 0.007	-	-	-	-
R. ornithinolytica	3.98 ± 0.006	-	-	-	-
V. alginolyticus	3.96 ±0.006	-	-	-	-

- Below detection limit

Table 3.2: Antibacterial activity of tamarind extract against histamine producing bacteria

HPB	Log_{10} CFU/ml ± SD bacterial cells remaining at each time interval (hours)				
	0:00	2:00	24:00	26:00	48:00
C. perfringens	3.97 ± 0.006	1.56 ± 0.007	-	-	-
C. youngae	3.96 ± 0.006	-	-	-	-
E. coli	3.96 ± 0.007	-	-	-	-
E. aerogenes	3.97 ± 0.007	-	-	-	-
E. cloacae	3.97 ± 0.006	-	-	-	-
E. amnigenous	3.97 ± 0.006	4.14 ± 0.006	4.48 ± 0.006	4.54 ± 0.006	3.47 ± 0.006
H. alvei	3.97 ± 0.005	4.11 ± 0.006	4.43 ± 0.007	4.36 ± 0.006	-
K. oxytoca	3.97 ± 0.006	-	-	-	-
K. pneumoniae	3.97 ± 0.006	-	-	-	-
M. morganii	3.98 ± 0.006	4.13 ± 0.006	2.56 ± 0.007	2.56 ± 0.006	-
P. vulgaris	3.97 ± 0.000	-	-	-	-
P. mirabilis	3.97 ± 0.007	-	-	-	-
P. damselae	3.98 ± 0.007	1.68 ± 0.005	1.60 ± 0.006	1.46 ± 0.005	3.74 ± 0.007
R. ornithinolytica	3.98 ± 0.005	-	-	-	-
V. alginolyticus	3.97 ± 0.006	4.11 ± 0.006	4.48 ± 0.005	4.51 ± 0.006	3.23 ± 0.006

Tamarind extract showed excellent antibacterial activity against ten out of the fifteen bacterial species and moderate activity against two bacterial species tested (Table 3.2). *E. coli, E. aerogenes, E. cloacae, P. vulgaris*, *P. mirabilis, C. youngae, K. oxytoca, K. pneumoniae, R. ornithinolytica* and *C. perfringens* were inhibited within 24 hours of incubation while inhibition of *M. morganii* and *H. alvei* occurred between 24 and 48 hours of incubation. *E. amnigenous, P. damselae* and *V. alginolyticus* were not inhibited after 48 hours but bacterial cell numbers were reduced to some extent. *P. damselae* cell population reduced to almost undetectable limit within two hours of incubation and remained at this level for 24 hours before increasing rapidly at 48 hours.

Table 3.3: Antibacterial activity of citrus extract against histamine producing bacteria

HPB	**Log_{10} CFU/ml ± SD bacterial cells remaining at each time interval (hours)**				
	0:00	2:00	24:00	26:00	48:00
C. perfringens	3.97 ± 0.006	-	-	-	-
C. youngae	3.96 ± 0.000	1.15 ± 0.006	1.32 ± 0.006	1.89 ± 0.005	2.42 ± 0.005
E. coli	3.97 ± 0.006	1.56 ± 0.007	1.46 ± 0.005	1.18 ± 0.007	4.24 ± 0.007
E. aerogenes	3.97 ± 0.005	1.89 ± 0.007	4.57 ± 0.005	4.61 ± 0.007	4.71 ± 0.007
E. cloacae	3.98 ± 0.005	-	-	-	-
E. amnigenous	3.96 ± 0.006	-	-	-	-
H. alvei	3.97 ± 0.006	1.77 ± 0.006	1.49 ± 0.006	1.64 ± 0.006	3.75 ± 0.006
K. oxytoca	3.97 ± 0.006	4.34 ± 0.006	4.55 ± 0.006	4.56 ± 0.006	4.70 ± 0.005
K. pneumoniae	3.96 ± 0.005	4.11 ± 0.007	4.34 ± 0.007	4.42 ± 0.005	4.60 ± 0.006
M. morganii	3.97 ± 0.007	1.83 ± 0.006	-	-	-
P. vulgaris	3.97 ± 0.006	3.54 ± 0.005	4.41 ± 0.006	4.44 ± 0.005	4.52 ± 0.006
P. mirabilis	3.97 ± 0.006	-	-	-	-
P. damselae	3.96 ± 0.005	-	-	-	-
R. ornithinolytica	3.96 ± 0.005	3.81 ± 0.000	-	-	-
V. alginolyticus	3.98 ± 0.006	3.92 ± 0.006	4.21 ± 0.005	4.27 ± 0.006	4.48 ± 0.006

Table 3.3 shows the bactericidal activity of citrus extract against fifteen species of documented HPB. Citrus shows excellent bactericidal activity against seven bacterial species tested. *C. perfringens, E. cloacae, E. amnigenous, M. morganii, P. mirabilis, P. damselae* and *R. ornithinolytica* are reduced to undetectable Log_{10} CFU/ml cells in less than 24 hours. *C. youngae* is sensitive to citrus extract to some extent but a small quantity of cells persists from 2 to 48 hours. *E. aerogenes* is observed to decrease initially to almost undetectable limit in two hours followed by a rapid increase to above 3.97 Log_{10} CFU/ml after 24 hours of incubation. *K. oxytoca*, *K. pneumoniae*, *P. vulgaris* and *V. alginolyticus* are not sensitive to citrus extract.

Table 3.4: Antibacterial activity of black tea against histamine producing bacteria

HPB	**Log_{10} CFU/ml ± SD bacterial cells remaining at each time interval (hours)**				
	0:00	2:00	24:00	26:00	48:00
C. perfringens	3.97 ±0.006	-	-	-	-
C. youngae	3.97 ± 0.005	4.11 ± 0.007	4.35 ± 0.005	4.39 ± 0.006	4.50 ± 0.006
E. coli	3.98 ± 0.005	3.92 ± 0.005	-	-	-
E. aerogenes	3.96 ± 0.006	3.95 ± 0.005	-	-	-
E. cloacae	3.98 ± 0.006	3.98 ± 0.006	3.88 ± 0.005	3.96 ± 0.006	-
E. amnigenous	3.97 ± 0.006	4.22 ± 0.006	4.28 ± 0.007	4.32 ± 0.005	4.46 ± 0.007
H. alvei	3.96 ± 0.007	4.20 ± 0.005	0.78 ± 0.006	0.70 ± 0.000	4.09 ± 0.007
K. oxytoca	3.97 ± 0.007	3.97 ± 0.007	-	-	-
K. pneumoniae	3.98 ± 0.006	3.90 ± 0.006	-	-	-
M. morganii	3.98 ± 0.006	4.22 ± 0.006	4.21 ± 0.006	4.14 ± 0.006	-
P. vulgaris	3.96 ± 0.005	3.89 ± 0.007	-	-	-
P. mirabilis	3.97 ± 0.005	3.92 ± 0.007	-	-	-
P. damselae	3.97 ± 0.006	-	-	-	-
R. ornithinolytica	3.98 ± 0.006	4.11 ± 0.006	4.36 ± 0.007	4.38 ± 0.006	4.46 ± 0.006
V. alginolyticus	3.97 ± 0.007	4.11 ± 0.006	4.35 ± 0.006	4.37 ± 0.007	4.45 ± 0.006

Black tea extract demonstrates excellent inhibitory action against eight species of HPB (Table 3.4). Moderate antibacterial activity is expressed against two bacterial species. Inhibition of *C. perfringens, E. coli, E. aerogenes, K. oxytoca, K. pneumoniae, P. vulgaris, P. mirabilis* and *P. damselae* happens within 24 hours. Inhibition of *M. morganii* and *E. cloacae* occurs between 24 and 48 hours. *R. ornithinolytica* and *H. alvei* show some degree of sensitivity against black tea extract between 0 and 26 hours. *C. youngae, E. amnigenous* and *V. alginolyticus* do not show any sensitivity to black tea extract.

Table 3.5: Antibacterial activity of green tea against histamine producing bacteria

HPB	**Log_{10} CFU/ml ± SD bacterial cells remaining at each time interval (hours)**				
	0:00	2:00	24:00	26:00	48:00
C. perfringens	3.97 ± 0.006	-	-	-	-
C. youngae	3.97 ± 0.007	4.11 ± 0.006	4.34 ± 0.006	4.37 ± 0.006	4.47 ± 0.007
E. coli	3.96 ± 0.006	3.94 ± 0.006	-	-	-
E. aerogenes	3.97 ± 0.006	3.66 ± 0.005	-	-	-
E. cloacae	3.96 ± 0.006	3.92 ± 0.006	-	-	-
E. amnigenous	3.96 ± 0.007	4.09 ± 0.006	4.37 ± 0.006	4.41 ± 0.006	4.51 ± 0.006
H. alvei	3.98 ± 0.007	4.14 ± 0.006	0.60 ± 0.000	0.48 ± 0.007	-
K. oxytoca	3.97 ± 0.006	3.95 ± 0.007	-	-	-
K. pneumoniae	3.96 ± 0.005	3.94 ± 0.006	-	-	-
M. morganii	3.98 ± 0.006	4.14 ± 0.005	1.88 ± 0.007	3.80 ± 0.007	-
P. vulgaris	3.97 ± 0.006	-	-	-	-
P. mirabilis	3.96 ± 0.007	-	-	-	-
P. damselae	3.97 ± 0.006	1.57 ± 0.007	-	-	-
R. ornithinolytica	3.96 ± 0.005	4.12 ± 0.006	4.08 ± 0.007	4.12 ± 0.006	4.41 ± 0.005
V. alginolyticus	3.96 ± 0.006	3.98 ± 0.006	4.06 ± 0.006	4.10 ± 0.006	4.39 ± 0.006

Table 3.5 summarizes the antibacterial activity of green tea extract against HPB. *E. coli, E. aerogenes, E. cloacae, P. vulgaris*, *P. mirabilis, K. oxytoca, K. pneumoniae, P. damselae* and *C. perfringens* show excellent sensitivity against green tea extract. For *M. morganii* and *H. alvei* inhibition of growth occurred between 24 and 48 hours. *C. youngae, E. amnigenous, R. ornithinolytica* and *V. alginolyticus* were not inhibited at all.

Table 3.6: Effect of control (absence of plant extracts) against histamine producing bacteria

HPB	**Log_{10} CFU/ml ± SD bacterial cells remaining at each time interval (hours)**				
	0:00	2:00	24:00	26:00	48:00
C. perfringens	3.96 ± 0.007	3.96 ± 0.006	3.96 ± 0.007	3.96 ± 0.006	3.95 ± 0.006
C. youngae	3.97 ± 0.005	4.01 ± 0.005	4.04 ± 0.007	4.06 ± 0.006	4.19 ± 0.005
E. coli	3.96 ± 0.006	3.96 ± 0.006	3.96 ± 0.005	3.96 ± 0.006	3.97 ± 0.006
E. aerogenes	3.97 ± 0.006	3.97 ± 0.007	3.97 ± 0.005	3.97 ± 0.006	3.96 ± 0.006
E. cloacae	3.97 ± 0.006	3.97 ± 0.005	3.97 ± 0.006	3.97 ± 0.005	3.97 ± 0.007
E. amnigenous	3.97 ± 0.005	3.99 ± 0.005	4.14 ± 0.006	4.17 ± 0.006	4.24 ± 0.006
H. alvei	3.96 ± 0.005	4.02 ± 0.006	4.21 ± 0.006	4.22 ± 0.007	4.29 ± 0.006
K. oxytoca	3.96 ± 0.006	3.96 ± 0.006	3.96 ± 0.006	3.96 ± 0.007	3.95 ± 0.007
K. pneumoniae	3.98 ± 0.006	3.97 ± 0.006	3.97 ± 0.006	3.97 ± 0.006	3.97 ± 0.005
M. morganii	3.96 ± 0.007	4.03 ± 0.006	4.06 ± 0.007	4.15 ± 0.006	4.17 ± 0.007
P. vulgaris	3.98 ± 0.007	3.97 ± 0.007	3.96 ± 0.007	3.96 ± 0.006	2.53 ± 0.007
P. mirabilis	3.97 ± 0.005	3.97 ± 0.007	3.97 ± 0.005	3.97 ± 0.007	3.18 ± 0.007
P. damselae	3.97 ± 0.005	3.96 ± 0.005	3.96 ± 0.006	3.96 ± 0.005	3.96 ± 0.000
R. ornithinolytica	3.98 ± 0.006	4.06 ± 0.006	4.13 ± 0.006	4.16 ± 0.005	4.27 ± 0.006
V. alginolyticus	3.97 ± 0.006	4.06 ± 0.005	4.17 ± 0.005	4.20 ± 0.007	4.30 ± 0.006

Table 3.6 is the control and shows bacterial growth behavior in the absence of the plant extracts. There is no significant reduction in bacterial cells in the absence of the extract against the fifteen species of HPB.

3.3 Antibacterial activity of plant extracts against TC

Tables 3.7 to Table 3.12 summarize the *ex vivo* antibacterial activity of selected plant extracts against TC in post harvest chub mackerel *R. brachysoma* at different incubation temperatures. Generally, results revealed that the antibacterial activity of the plant extracts is affected significantly by change in temperature. The extracts showed antibacterial activity against coliforms with varying magnitudes. Citrus, noni, tamarind and black tea demonstrated inhibitory activity against TC at one or more temperatures. Green tea extract failed to show any inhibitory activity at any of the seven temperatures tested. The inhibition of bacterial growth within 24 hours of incubation was taken as excellent antibacterial activity while growth inhibition between 24 and 48 hours of incubation was taken as moderate antibacterial activity.

Table 3.7: Antibacterial activity of noni extract against TC at different temperatures

Temperature (°C)	**Log_{10} CFU/ml ± SD bacterial cells remaining at each time interval (hours)**				
	0:00	2:00	24:00	26:00	48:00
5	3.98 ± 0.005	-	-	-	-
10	3.98 ± 0.006	-	-	-	-
15	3.99 ± 0.007	-	-	-	-
20	3.98 ± 0.006	-	-	-	-
25	3.99 ± 0.006	-	-	-	-
30	3.98 ± 0.006	-	-	-	-
32	3.99 ± 0.006	-	-	-	-

Noni extract shows utmost antibacterial activity against TC at all seven incubation temperatures. Noni extract is able to inhibit the growth of TC flora of *R. brachyosoma*

tested within 2 hours of incubation and bring down the population of TC to zero (Table 3.7).

Tamarind extract exhibit excellent antibacterial activity against TC at six out of seven temperatures tested (Table 3.8). At 5°C, 10°C, 15°C, 20°C, 30°C and 32°C coliforms were inhibited within 24 hours of incubation. TC show insignificant sensitivity against tamarind extract at 25°C.

Table 3.8: Antibacterial activity of tamarind extract against TC at different temperatures

Temperature (°C)	**Log_{10} CFU/ml ± SD bacterial cells remaining at each time interval (hours)**				
	0:00	2:00	24:00	26:00	48:00
5	3.99 ± 0.006	-	-	-	-
10	3.98 ± 0.006	-	-	-	-
15	3.99 ± 0.006	-	-	-	-
20	3.99 ± 0.007	-	-	-	-
25	3.98 ± 0.007	4.02 ± 0.007	4.39 ± 0.005	4.41 ± 0.006	4.55 ± 0.006
30	3.99 ± 0.005	4.22 ± 0.006	-	-	-
32	3.99 ± 0.006	1.77 ± 0.006	-	-	-

Citrus extract demonstrates excellent bactericidal activity against TC at 10°C and 15°C and moderate activity at 20°C (Table 3.9). Coliforms show some level of sensitivity to citrus extract at 5°C, 30°C and 32°C. At 30°C and 32°C TC population decrease rapidly to less than 0.30 and 2.98 Log_{10} CFU/ml in 2 hours followed by an increase after 24 hours. At 5°C TC cells numbers decrease rapidly to almost undetectable limit in 2 hours followed by an increase above 3.99 Log_{10} CFU/ml after 26 hours. TC shows no sensitivity to citrus extract at 25°C.

Table 3.9: Antibacterial activity of citrus extract against TC at different temperatures

Temperature (°C)	Log_{10} CFU/ml ± SD bacterial cells remaining at each time interval (hours)				
	0:00	2:00	24:00	26:00	48:00
5	3.99 ± 0.005	-	3.11 ± 0.007	4.42 ± 0.005	4.57 ± 0.005
10	3.99 ± 0.005	-	-	-	-
15	3.99 ± 0.006	-	-	-	-
20	3.98 ± 0.007	-	2.82 ± 0.006	2.71 ± 0.000	-
25	3.98 ± 0.007	3.99 ± 0.006	4.38 ± 0.006	4.40 ± 0.007	4.48 ± 0.005
30	3.98 ± 0.006	0.30 ± 0.000	4.26 ± 0.006	4.39 ± 0.006	4.55 ± 0.006
32	3.99 ± 0.006	2.98 ± 0.005	4.21 ± 0.005	4.26 ± 0.006	4.39 ± 0.007

Table 3.10: Antibacterial activity of black tea extract against TC at different temperatures

Temperature (°C)	Log_{10} CFU/ml ± SD bacterial cells remaining at each time interval (hours)				
	0:00	2:00	24:00	26:00	48:00
5	3.98 ± 0.006	3.97 ± 0.005	3.95 ± 0.008	3.95 ± 0.006	3.48 ± 0.006
10	3.98 ± 0.005	3.73 ± 0.000	4.19 ± 0.006	4.28 ± 0.006	-
15	3.99 ± 0.006	4.09 ± 0.006	4.37 ± 0.006	4.44 ± 0.006	4.57 ± 0.005
20	3.99 ± 0.006	3.65 ± 0.006	4.41 ± 0.007	4.45 ± 0.005	4.52 ± 0.007
25	3.98 ± 0.000	3.34 ± 0.007	-	-	-
30	3.98 ± 0.007	3.87 ± 0.007	4.18 ± 0.006	4.20 ± 0.007	4.49 ± 0.006
32	3.99 ± 0.006	4.05 ± 0.007	4.41 ± 0.006	4.43 ± 0.000	4.52 ± 0.006

Black tea extract demonstrates excellent inhibitory effect against TC at 25°C incubation temperature and moderate activity at 10°C temperature (Table 3.10). Inhibition of TC growth at 25°C is observed within 24 hours and at 10°C growth inhibition occurs between 24 and 48 hours. At 20°C and 30°C TC show some degree of sensitivity against black tea initially. At 5°C, 15°C and 32°C black tea has no effect on TC.

Table 3.11: Antibacterial activity of green tea extract against TC at different temperatures

Temperature (°C)	**Log_{10} CFU/ml ± SD bacterial cells remaining at each time interval (hours)**				
	0:00	2:00	24:00	26:00	48:00
5	3.99 ± 0.006	3.98 ± 0.006	3.48 ± 0.006	3.47 ± 0.005	3.45 ± 0.008
10	3.99 ± 0.007	3.84 ± 0.006	4.23 ± 0.006	4.29 ± 0.006	4.55 ± 0.007
15	3.99 ± 0.006	3.77 ± 0.006	4.37 ± 0.008	4.44 ± 0.007	4.54 ± 0.006
20	3.98 ± 0.006	3.94 ± 0.006	4.18 ± 0.007	4.21 ± 0.005	4.45 ± 0.006
25	3.99 ± 0.006	3.98 ± 0.006	4.25 ± 0.007	4.28 ± 0.006	4.45 ± 0.006
30	3.99 ± 0.005	4.09 ± 0.005	4.31 ± 0.005	4.37 ± 0.005	4.52 ± 0.005
32	3.99 ± 0.007	4.05 ± 0.005	4.43 ± 0.006	4.48 ± 0.006	4.53 ± 0.000

Table 3.11 summarizes the antibacterial activity of green tea extract against TC flora of *R. brachysoma* at seven temperatures. Green tea does not exhibit antibacterial activity at any of the seven temperatures tested. TC shows some degree of sensitivity to green tea at 10°C and 15°C

Table 3.12: Effect of control (absence of plant extracts) against TC at different temperatures

Temperature (°C)	**Log_{10} CFU/ml ± SD bacterial cells remaining at each time interval (hours)**				
	0:00	2:00	24:00	26:00	48:00
5	3.98 ± 0.006	3.91 ± 0.005	3.51 ± 0.006	3.42 ± 0.006	3.39 ± 0.005
10	3.98 ± 0.005	3.94 ± 0.005	3.91 ± 0.006	3.93 ± 0.000	4.12 ± 0.006
15	3.98 ± 0.007	3.94 ± 0.006	4.21 ± 0.007	4.22 ± 0.005	4.47 ± 0.006
20	3.98 ± 0.000	3.95 ± 0.005	4.14 ± 0.006	4.20 ± 0.006	4.42 ± 0.006
25	3.99 ± 0.004	3.96 ± 0.007	4.28 ± 0.005	4.32 ± 0.006	4.53 ± 0.007
30	3.98 ± 0.006	4.25 ± 0.006	4.38 ± 0.006	4.39 ± 0.007	4.53 ± 0.007
32	3.99 ± 0.006	3.85 ± 0.006	4.08 ± 0.006	4.16 ± 0005	4.42 ± 0.005

Table 3.12 serves as a control and shows growth behavior of TC at different temperatures in the absence of plant extracts tested. There is insignificant reduction in TC growth and survival in the absence of tested plant extracts.

3.4 Antibacterial activity of plant extracts against TPC

Tables 3.13 to Table 3.18 summarize the antibacterial effect of selected plant extracts against TPC heterotrophic flora present in post harvest chub mackerel *R. brachysoma* at seven different temperatures. Generally, the results demonstrate that antibacterial activity of the plant extracts is affected significantly by change in temperature. The plant extracts expressed antibacterial activity against TPC with varying magnitudes. Citrus, tamarind and noni extracts demonstrate excellent inhibitory activity against TPC at one or more temperatures. Green tea and black tea extracts failed to demonstrate any significant bactericidal effect against TPC.

Table 3.13: Antibacterial activity of noni extract against TPC at different temperatures

Temperature (°C)	**Log_{10} CFU/ml ± SD bacterial cells remaining at each time interval (hours)**				
	0:00	2:00	24:00	26:00	48:00
5	3.99 ± 0.005	-	-	-	-
10	3.98 ± 0.006	-	-	-	-
15	3.99 ± 0.007	-	-	-	-
20	3.98 ± 0.006	-	-	-	-
25	3.98 ± 0.007	-	-	-	-
30	3.99 ± 0.006	1.30 ± 0.007	3.20 ± 0.006	-	-
32	3.99 ± 0.005	-	-	-	-

Noni extract demonstrates maximum antibacterial activity against TPC flora of *R. brachysoma* at all seven incubation temperatures (Table 3.13).

Tamarind extract expresses excellent inhibitory activity against TPC at four incubation temperatures (Table 3.14). At 10°C, 15°C, 30°C and 32°C growth TPC was suppressed within 24 hours of incubation. Growth of TPC at 5°C and 25°C was not inhibited after 48 hours but bacterial cells did express some degree of sensitivity to tamarind. This is in agreement with an earlier work which showed that most common bacterial species are able to survive around 5°C but do not show any significant growth for up to 72 hours (Masette, 1999). Insignificant growth inhibition was observed at 20°C.

Table 3.14: Antibacterial activity of tamarind extract against TPC at different temperatures

Temperature (°C)	**Log_{10} CFU/ml ± SD bacterial cells remaining at each time interval (hours)**				
	0:00	2:00	24:00	26:00	48:00
5	3.99 ± 0.006	4.01 ± 0.005	3.62 ± 0.006	3.64 ± 0.006	3.68 ± 0.005
10	3.98 ± 0.008	0.95 ± 0.003	-	-	-
15	3.99 ± 0.007	-	-	-	-
20	3.99 ± 0.005	4.44 ± 0.005	3.98 ± 0.007	4.28 ± 0.007	4.45 ± 0.006
25	3.99 ± 0.007	2.95 ± 0.007	4.41 ± 0.005	4.45 ± 0.007	4.53 ± 0.007
30	3.98 ± 0.006	3.91 ± 0.006	-	-	-
32	3.99 ± 0.005	-	-	-	-

Table 3.15 demonstrates the bactericidal activity of citrus extract against TPC flora of *R. brachysoma* at seven incubation temperatures. Citrus expresses excellent bactericidal activity at 10°C and 15°C. At 5°C, 25°C, 30°C and 32°C TPC cells show some degree of

sensitivity to citrus extract. At 20°C citrus does not express any bactericidal activity against TPC.

Table 3.15: Antibacterial activity of citrus extract against TPC at different temperatures

Temperature (°C)	**Log_{10} CFU/ml ± SD bacterial cells remaining at each time interval (hours)**				
	0:00	2:00	24:00	26:00	48:00
5	3.98 ± 0.006	4.32 ± 0.007	3.24 ± 0.007	3.71 ± 0.008	4.70 ± 0.007
10	3.99 ± 0.000	-	-	-	-
15	3.99 ± 0.006	-	-	-	-
20	3.99 ± 0.007	4.06 ± 0.006	4.50 ± 0.005	4.52 ± 0.008	4.57 ± 0.006
25	3.98 ± 0.007	3.83 ± 0.006	3.63 ± 0.007	4.12 ± 0.007	4.15 ± 0.006
30	3.99 ± 0.005	1.72 ± 0.005	3.31 ± 0.006	4.11 ± 0.007	4.38 ± 0.000
32	3.98 ± 0.006	3.65 ± 0.008	4.16 ± 0.005	4.19 ± 0.006	4.40 ± 0.005

Table 3.16: Antibacterial activity of black tea against TPC at different temperatures

Temperature (°C)	**Log_{10} CFU/ml ± SD bacterial cells remaining at each time interval (hours)**				
	0:00	2:00	24:00	26:00	48:00
5	3.98 ± 0.007	4.06 ± 0.007	4.31 ± 0.007	4.33 ± 0.006	4.46 ± 0.006
10	3.99 ± 0.006	4.02 ± 0.006	4.33 ± 0.007	4.34 ± 0.006	4.54 ± 0.005
15	3.98 ± 0.007	4.30 ± 0.007	4.44 ± 0.005	4.45 ± 0.006	4.53 ± 0.006
20	3.99 ± 0.006	4.04 ± 0.006	4.36 ± 0.006	4.42 ± 0.005	4.57 ± 0.007
25	3.99 ± 0.005	4.12 ± 0.006	4.32 ± 0.006	4.36 ± 0.005	4.49 ± 0.007
30	3.99 ± 0.006	3.99 ± 0.000	4.36 ± 0.006	4.25 ± 0.007	4.46 ± 0.006
32	3.98 ± 0.005	4.05 ± 0.005	4.26 ± 0.007	4.31 ± 0.006	4.48 ± 0.000

According to Table 3.16 extract from black tea does not demonstrate any bactericidal activity against TPC flora of *R. brachysoma*. Table 3.17 summarizes the antibacterial

activity of green tea extract against TPC. No inhibitory activity is demonstrated by green tea at any of the incubation temperatures.

Table 3.17: Antibacterial activity of green tea against TPC at different temperatures

Temperature (°C)	**Log_{10} CFU/ml ± SD bacterial cells remaining at each time interval (hours)**				
	0:00	2:00	24:00	26:00	48:00
5	3.98 ± 0.005	3.99 ± 0.005	4.46 ± 0.000	4.48 ± 0.007	4.56 ± 0.005
10	3.98 ± 0.006	3.77 ± 0.006	4.43 ± 0.006	4.45 ± 0.005	4.54 ± 0.007
15	3.98 ± 0.006	3.44 ± 0.006	4.40 ± 0.006	4.44 ± 0.006	4.55 ± 0.006
20	3.99 ± 0.006	3.70 ± 0.007	4.12 ± 0.005	4.12 ± 0.006	4.35 ± 0.006
25	3.99 ± 0.007	4.34 ± 0.005	4.49 ± 0.008	4.49 ± 0.006	4.56 ± 0.007
30	3.99 ± 0.007	3.42 ± 0.008	4.06 ± 0.007	4.11 ± 0.007	4.42 ± 0.006
32	3.98 ± 0.000	4.42 ± 0.006	4.48 ± 0.006	4.49 ± 0.007	4.54 ± 0.006

Table 3.18: Effect of control against TPC at different temperatures

Temperature (°C)	**Log_{10} CFU/ml ± SD bacterial cells remaining at each time interval (hours)**				
	0:00	2:00	24:00	26:00	48:00
5	3.99 ± 0.006	4.04 ± 0.008	4.41 ± 0.005	4.41 ± 0.005	4.48 ± 0.005
10	3.99 ± 0.006	4.25 ± 0.008	4.28 ± 0.006	4.28 ± 0.006	4.32 ± 0.006
15	3.98 ± 0.000	3.95 ± 0.007	4.15 ± 0.004	4.17 ± 0.006	4.39 ± 0.007
20	3.98 ± 0.004	4.03 ± 0.008	4.33 ± 0.006	4.31 ± 0.007	4.48 ± 0.000
25	3.98 ± 0.006	3.97 ± 0.006	4.11 ± 0.007	4.15 ± 0.006	4.35 ± 0.006
30	3.98 ± 0.006	4.01 ± 0.006	4.23 ± 0.006	4.25 ± 0.006	4.38 ± 0.006
32	3.99 ± 0.006	3.04 ± 0.005	4.26 ± 0.006	4.28 ± 0.006	4.37 ± 0.007

Table 3.18 serves as a control and demonstrates the behavior of TPC in the absence of the plant extracts. There is no significant reduction in bacterial growth and survival of TPC at any of the incubation temperatures.

3.5 Active constituents of plant extracts

The microbiologically active constituents of the plant extracts used in this study were determined.

3.5.1 Total polyphenols in green tea and black tea

The active compounds present in tea belong to a group of chemicals collectively called polyphenol compounds. The polyphenol content of the prepared tea extracts used for this study were as follows: Green tea total polyphenols - 9.16 mg/ml of extract. Black tea total polyphenols – 4.56 mg/ml of extract.

3.5.2 Total flavonoids in citrus

The groups of active compounds that are mostly responsible for the antibacterial properties of citrus are collectively known as flavonoids. The total flavonoid content of the prepared extract used for this study was 2.56 mg/ml of extract.

3.5.3 Acid contents of tamarind

Tamarind owes it antibacterial properties mostly due to its low pH brought about by high content of acids (tartaric acid, malic acid and citric acid). Tamarind also contains potassium bi-tatrate, catechin, nasturtium, tamarind, alkaloids, inositol, serine, polysaccharides, balsamine, phosphatidic acid and its choline and ethanolamine, which may also be adding to its antibacterial properties. The acid contents of the prepared extract used for this study were; tartaric acid – 13.9 mg/ml, citric acid – 12.5 mg/ml,

malic acid – 31.3 mg/ml, potassium bi-tartrate – 11.7 mg/ml. Concentrations of other unidentified constituents were – 40.0 mg/ml.

3.5.4 Total polyphenols and ascorbic acid in noni

The most active compounds responsible for noni's antibacterial activity are phenols and ascorbic acid, which lowers its pH. The content of total phenols in noni extract used for this study was 2.10 mg/g of extract and ascorbic acid was 2.50 mg/g of extract. Phenols are classified into two major groups, phenol carboxylic acids and flavonoids. These include catechins, anthocyanidins, flavonols, seven flavones and proanthocyanidins (Zin *et al.*, 2006). There may be other compounds present in noni juice, which are suspected to have antibacterial properties but these have not been proven scientifically to date.

IV. Discussion

Various plant extracts have been utilized as alternative medicine, pharmaceuticals, natural therapies and in food preservation for thousands of years (Dorman and Deans, 2000; Prabuseenivasan *et al.,* 2006). Plant extracts contain many novel antibacterial compounds that are effective against many species of harmful bacteria including pathogens (Dorman and Deans, 2000; Prabuseenivasan *et al.,* 2006). Utilization of traditional medicines against food borne pathogens needs to be scientifically investigated both *in vitro* and *in vivo* in order to improve the quality of food and reduce food related health problems. Natural plant extracts can be employed to prevent spoilage and toxicity of fish and other seafood by controlling the growth of spoilage microorganisms such as histamine producing bacteria (HPB), coliforms and heterotrophic bacteria.

Bacteria are normally present on the skin, guts and gills of fish but do not cause harm to a living fish as the natural defense system of the body keeps them under control. The one place bacteria are not found is in the flesh of a live, healthy fish. After the fish die, bacterial cells grow quickly in number. Bacteria together with the enzymes that they produce begin to penetrate the flesh through the skin, the lining of the body cavity or a break in the skin. Once in the flesh, bacteria secrete enzymes, which break down complex organic molecules into simpler substances resulting in the spoilage of fish (Shewan, 1971).

The rate of bacterial spoilage depends on temperature and the number and types of bacteria present on fish (Shewan, 1961). Generally, the higher the initial bacterial load

present, the faster the rate of fish spoilage. Bacterial reproduction and growth rates normally increase above 4°C and go up to about 45°C (Prescott *et al.,* 2005). Similarly, bacterial growth is affected by a decrease in temperature, as the temperature decreases bacterial growth rate also decreases, and normally becomes stationary below 4°C (Chamberlain, 2001; Prescott *et al.,* 2005). The growth of heterotrophic bacteria (TPC) and coliforms (TC) is affected by temperature as most heterotrophic bacteria and coliforms are mesophiles, which have their optimum growth between 20°C and 40°C (Prescott *et al.,* 2005). This is in agreement with the results of this study. The initial bacterial load is higher in post-harvest fish if it is handled with dirty hands, exposed to dirty surfaces, handled and gutted without proper care and stored at high temperatures. Fish can generally spoil within 12 to 20 hours post-harvest depending on the method of capture and the species of fish (FDA, 1998).

Bacterial fish spoilage is mostly caused by the growth of heterotrophic bacteria and to some extent by coliforms (Crapo and Himelbloom, 1999). Coliforms comprise mostly of members belonging to the family enterobacteriaceae and include mostly pathogenic bacteria such as *E. coli, E. aerogenes*, *H. alvei* and *K. pneumoniae* that are responsible for causing a wide range of diseases and infections in humans when taken in through consumption of contaminated or mishandled food (Niemi *et al*., 2001; Kahlmeter, 2003; Prescott *et al.,* 2005). Growth of coliforms also aids in the spoilage of fish (Gram, 1993; Crapo and Himelbloom, 1999; Niemi *et al*., 2001).

During spoilage of fish, bacteria break down the fish protein (proteolysis) and break the bonds that hold amino acids together, thus increasing the concentration of free amino acids in the flesh such as histidine (Chamberlain, 2000). HPB also increase in number during spoilage and produce the enzyme histidine decarboxylase, which converts histidine into histamine (Figure 4.1) (Kimata *et al.*, 1958; USFDA, 2001). Control of histamine formation in fish is very important, because histamine level in fish cannot be determined organoleptically. Although 50 ppm of histamine can cause poisoning, fish may develop from 200 ppm to 500 ppm of histamine before they appear organoleptically unsuitable or spoiled (Chamberlain, 2001).

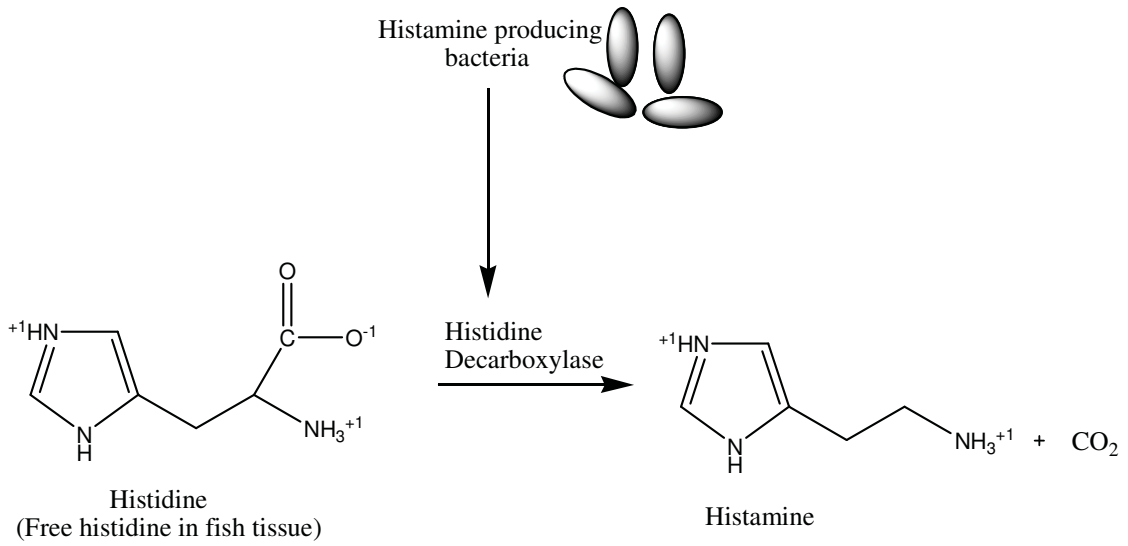


Figure 4.1: Formation of histamine from free histidine amino acid molecules in fish tissue. Histamine producing bacteria produces the exogenous enzyme histidine decarboxylase, which is responsible for removing the alpha carboxyl group from histidine resulting in the formation of histamine in fish muscle (Kimata, 1958; Lucas *et al*., 2005).

Through review of literature, it was determined that *in vitro* studies have been conducted on extracts from noni, citrus, tamarind and tea to assess their antibacterial properties. There have not been any reports of *in vivo* studies conducted on these plant extracts in

their natural matrices. This investigation is the first of its kind that has been conducted *ex vivo* on whole fish to study the antibacterial properties of unmodified natural extracts from noni, tamarind, citrus, green tea and black tea against total coliforms (TC) and total heterotrophic bacteria (TPC) on chub-mackerel. This makes the present study unique and highly applicable for use by fish handlers of all backgrounds, as it requires little skill, time and money to replicate the procedure employed.

4.1 Production of histamine by selected bacterial species

All fifteen species of bacteria under investigation have been identified as significant histamine producers in fish. Figures 3.1 and 3.2 show that all fifteen species of bacteria are able to produce histamine in TFIB with varying magnitudes. *P. morganii* was identified as the strongest histamine producer, which is in agreement with previous, studies by Kimata *et al*., (1958); Niven, (1981); Chamberlain, (2001). *C. perfringens, C. youngae, E. aerogenes*, *E. cloacae H. alvei*, *K. oxytoca, P. damselae* and *V. alginolyticus* can also be considered as strong histamine producers as they are able to produce over 100 ppm of histamine in 24 hours. 50 ppm histamine in fish is considered to be toxic to humans (USFDA, 2001), therefore these bacterial species can be considered as important histamine producers. *E. coli, E. amnigenous, K. pneumoniae, P. vulgaris, P. mirabilis* and *R. ornithinolytica* cannot be ruled out, as they are able to produce moderate levels (<50 ppm) histamine in TFIB. Even though they produce lower levels of histamine, these species are quite important, as they possess histamine producing ability and can contribute significantly to the build up of toxic levels of histamine (>50 ppm) in fish tissue.

Despite the fact that their capabilities vary, all fifteen species of bacteria undertaken for this study show histamine producing ability, therefore they are all characterized as histamine producing bacteria (HPB). No single bacterial species is solely responsible for production of toxic levels of histamine in fish. It is a combination of different HPB bacterial species acting together and leading eventually to the build up of high levels of histamine (Takahashi *et al.*, 2003).

In nature there are many different factors that come into effect and may alter the histamine producing capacity of the individual bacterial species. In a laboratory controlled environment the bacterial species studied were tested individually. They did not have any other bacterial species competing for space and nutrients. In nature however, there is a large flora of microorganisms growing together at the same time. Some bacterial species are stronger competitors than others and can have negative influence on the growth and survival of other bacterial species. HPB species selected for this study can grow effectively on implicated fish species as they have been previously isolated from scromboid fish containing high levels of histamine (Kimata *et al*., 1958; Taylor *et al.*, 1979; Niven *et al*., 1981; Yoshinaga and Frank, 1982; Lopez-Sabater *et al*., 1996; Lehane and Olley, 1999; Jorgensen *et al*., 2000: Chamberlain, 2000; Kanki *et al*., 2002; Takahashi *et al*., 2003; Wauters *et al*., 2004).

Since, all the bacterial species studied were significant histamine producers (Figure 3.1 and 3.2), it was very important to find a simple and affordable method for inhibiting their

growth. Natural plant extracts are the simplest and most readily available antibacterial agents in the South Pacific region.

4.2 Antibacterial activity of Noni extract

All five plant extracts selected for this study demonstrated some level of antibacterial activity against HPB, TPC and TC. Noni, tamarind and citrus extracts exhibited strong antibacterial activity against the three selected groups of bacteria. The results demonstrate that noni was the most effective and consistent antibacterial agent against all three groups of microorganisms. It inhibited the growth of all fifteen species of HPB tested quite rapidly. Inhibiting the growth of HPB in the initial stages of fish capture will not allow them to produce the enzyme histidine decarboxylase (Figure 4.1). The chain reaction of histamine formation in the initial stages will be repressed and histamine will not build up in fish tissue. Therefore, it will take much longer for histamine content of fish to reach the critical limit of 50 ppm at which point it will be considered unacceptable for human consumption (USFDA, 2001).

When evaluated *ex vivo* against TC present in salala, noni extract demonstrated excellent inhibitory activity at all seven incubation temperatures. Its inhibitory activity was not affected by a rise or fall in temperature. Therefore, incubation temperature between 5°C and 32°C will not affect the bactericidal properties of noni against TC present in *R. brachyosoma*. The ability of noni extract to inhibit TC growth in salala can be applied for home use as well as at industrial fisheries level. It will keep harmful human enteric pathogenic bacteria under control by inhibiting their growth. In the South Pacific, salala

eaten fresh is not a problem as the bacterial load is initially quite low. However, fish in the Pacific are not always fresh when eaten, which increases the chances of adverse health effects upon consumption (Chamberlain, 2000). The number of viable bacterial cells required to cause disease is called the minimum infective dose (MID). People are at risk of getting bacterial associated illnesses from fish consumption if the population of pathogenic bacterial species exceeds their MID. MID is reached faster if the fish is handled with dirty hands, comes from polluted waters and is stored at high temperatures (Chamberlain and Titili, 2001). The application of noni extract at initial stages after fish (*R. brachyosoma*) capture will ensure that TC are kept under control and ensure that the fish remains safe for human consumption.

Noni extract displayed excellent *ex vivo* bactericidal activity against TPC in salala at all seven incubation temperatures. Spoilage bacteria comprise of heterotrophs and coliforms (Gram, 1993; Crapo and Himelbloom, 1999; Niemi *et al*., 2001) and noni is able to inhibit both groups of bacteria quite effectively. Therefore, noni can significantly delay bacterial spoilage and putrefaction of fish. However, noni should be applied soon after fish is harvested as mishandling and temperature abuse will increase spoilage rate of fish. Higher bacterial load will take longer to inhibit compared to lower bacterial load. The application of noni extract to fresh fish will also eliminate the problem of incubation temperature, as the bactericidal activity of the extract is not significantly affected by increase in temperature up to 32°C. Even though noni can be used as a natural post-harvest preservative for fish, the organoleptic properties, especially taste and odour may be affected. Further investigation on the organoleptic properties of noni is required.

Antibacterial activity of noni fruit extract may be attributed to the presence of the chemical constituents acubin, *L*-asperuloside, alizarin and glycosides in the fruit and anthraquinone compounds such as nordamnacanthal, moridone, rubiadin, rubiadin 1-methylether and soranjidiol which are all antibacterial agents (Atkinson, 1956; Liu *et al*., 2001; Wang *et al*., 2002). These compounds have been shown to inhibit pathogenic bacterial strains such as *P. aeruginosa, P. morganii, S. aureus, B. subtilis, E. coli, Salmonella* spp and *Shigella* spp quite effectively (Atkinson, 1956; Wang *et al*., 2002). The cumulative affect of these components and low pH may account for the strong antibacterial properties of noni.

4.3 Antibacterial activity of Tamarind extract

After noni, tamarind was the second most effective as an antibacterial agent against HPB, TPC and TC. It managed to inhibit growth of twelve species of HPB with its antibacterial effectiveness ranging from moderate to excellent. According to these results tamarind may not be able to completely inhibit histamine production when applied to fresh fish but it will significantly delay the process, as it is able to inhibit most species of HPB. Tamarind inhibits the growth of *M. morganii*, *C. perfringens, C. youngae, E. aerogenes*, *E. cloacae* and *K. oxytoca*, which have been classified as strong histamine producers in this study. Extract from tamarind is also able to inhibit *K. pneumoniae*, which is considered to be a major histamine producer in scombroid fish and fisheries products (Lopez-Sabater *et al*., 1996; Lehane and Olley, 1999; Chamberlain, 2000). Fewer species of histamine producers present will take much longer to build up histamine in fish muscle

and delay the time taken for histamine to reach the critical limit of 50ppm (USFDA, 2001).

Excellent *ex vivo* inhibitory effect was exhibited by tamarind extract against TC present in salala at six of the seven incubation temperatures with the only exception being 25°C. Tamarind can be an effective antibacterial agent against TC if the temperature can be maintained below 25°C. It will significantly inhibit TC growth when applied to fresh salala. It can be utilized as a pickle or prepared as marinade for fish fillets giving a tasty sour flavor. Pathogenic bacteria won't be able to thrive in the presence of tamarind extract.

TPC bacteria comprise the majority of the bacterial population present in fish, so they will be more difficult to inhibit as compared to TC and HPB. Tamarind extract showed excellent inhibitory effect against TPC at four temperatures. If tamarind is to be used for inhibiting growth of TPC in salala, then incubation temperature has to be critically considered. The bactericidal activity of tamarind extract varies with temperature between 5°C and 32°C. At 20-25°C tamarind extract is ineffective against TPC and TC growth. TPC and TC are mesophiles and the temperatures of 20-25°C is in their optimum growth range (Prescott *et al.,* 2005). TPC and TC may be able to combat against the inhibitory effect of tamarind extract at these temperatures. At 10°C to 15°C and 30°C to 32°C, tamarind will demonstrate excellent inhibitory effect against TPC and delay spoilage, keeping salala fresh and acceptable.

The bactericidal activity of tamarind may be due to its low pH (which in this case was measured to be 2.47) brought about by tartaric acid, which is its main acidulant (Nyadoi, 2004). Most bacteria are neutrophiles, which are unable to survive at pHs below 5.5 (Prescott *et al.,* 2005). The constituents of tamarind fruit are tartaric acid, citric acid, malic acid, potassium bitartarate, pectin, gum, water and parenchymatous fiber which may also be playing a role in tamarind's antibacterial properties (Nyadoi, 2004). Tamarind fruit has been shown to have antibacterial activity against *S. aureus, B. cereus, E. coli, P. aeruginosa* and *A. hydrophila*, which is in agreement with the results presented here (Srinivasan *et al.*, 2001; Ghelardi *et al.*, 2004).

4.4 Antibacterial activity of Citrus extract

Fruit extracts from citrus demonstrated moderate bactericidal properties against HPB, TPC and TC. It showed excellent inhibitory activity against seven species of HPB tested. Citrus fruits are quite cheap and widely distributed in Fiji, as well as in other South Pacific countries and preparation of the fruit extract is also quite simple. Citrus may prove to be a good antibacterial agent against HPB as it is able to inhibit growth of *C. perfringens, E. cloacae* and *P. damselae* which have been characterized as strong histamine producers in this study (Figure 3.1 and 3.2.). It will be able to inhibit the growth of some of the histamine producers, therefore delaying histamine build up in fish muscle. Citrus extract is able to inhibit the growth of *M. morganii*, which is a major histamine producer in most fisheries products (Chamberlain, 2000). Thus, citrus extract will increase the shelf life of fresh fish and fisheries products by keeping their scombroid toxicity at an acceptable level.

Citrus extract was able to inhibit TC growth at three out of the seven storage temperatures tested. It exhibits its best antibacterial potential between incubation temperatures of 10°C to 20°C. In table 3.9, the bacterial growth behavior pattern at 5°C can be observed. Bacterial population initially decreases to undetectable limits followed by a significant increase in bacterial population. A small number of TC cells surviving below the detection limit may have developed resistance against low temperature and citrus extract. These cells may have grown and multiplied in number, resulting in an increase in bacterial population. At 10, 15 and 20°C, none of the cells seemed to have developed any resistance against the extract. Also, there may be some chemical components of citrus that are active and masking the antibacterial activity of the extract against TC at 5°C. Further work is required to determine the true reason behind such behavior by TC. While not being able to exhibit temperature stability, citrus can still prove to be a good antibacterial agent against TC in fresh salala if the storage temperature is monitored carefully. Citrus extract is a natural product; therefore it will exhibit little to no side effects in humans when consumed through food. Also, citrus extract can be used as a sour flavoring agent in preparation of a pickle or in marinades together with other ingredients to preserve salala or other fish fillets. Application of citrus extract to fresh salala under controlled temperature will decrease the pathogenicity of TC by hindering their growth.

Citrus extract showed excellent antibacterial activity against TPC at two out of the seven storage temperatures, showing it to be the next most effective as an antibacterial agent following noni and tamarind extracts. Citrus extract will be able to reduce TPC load quite

effectively when applied to fresh salala if the storage temperature is kept between 10°C and 15°C. Inhibiting growth of heterotrophic bacteria will increase the shelf life of fish (Crapo and Himelbloom, 1999). Citrus will be a good antibacterial agent for delaying spoilage of salala provided the storage temperature is kept under control. In fishing industries citrus extract can be mixed with water to form a wash for gilled and gutted fish. This would remove more bacteria than just using water alone. Further work is required to determine the minimum inhibitory concentration (MIC) of citrus so that the ratio of citrus to water for washing fish can be determined.

Citrus may owe part of its antibacterial properties to the presence of flavonoids in their fruits which are anti-inflammatory, antibacterial and antifungal (Robards *et al.*, 1997; Zayachkivska *et al.*, 2005). Oils, vapours and their components from citrus have shown significant reduction in bacterial populations against a number of common food borne pathogens including *Listeria monocytogenes*, *Bacillus cereus*, *E. coli* O157 and *Campylobacter jejuni* (Fisher and Philips, 2007). These factors together with citric acid and the low pH of citrus extract (measured to be 2.64) may account for its antibacterial properties.

Essential oil from citrus has been shown to have antibacterial activity against four gram-negative bacteria *E. coli*, *K. pneumoniae*, *P. aeruginosa*, *P. vulgaris* and two gram-positive bacteria *B. subtilis* and *S. aureus* (Prabuseenivasan *et al.*, 2006). This does not agree with the results presented here (Table 3.3), which show that the growth of *K. pneumoniae*, *E. coli* and *P. vulgaris* were not inhibited by citrus extract. Other

components of the crude extract used in the present study may be masking the antibacterial effect of the essential oil(s) within the extract.

At 5°C the TPC cells were observed to decrease initially followed by increasing significantly in number. This is not in agreement with earlier studies, which show that most bacterial species are able to survive at low temperatures but do not show any significant growth for up to 72 hours (Masette, 1999). The low pH resistant cells surviving may also be psychrophiles, which have their optimum growth at 0-12°C, which would explain their growth (Prescott *et al.,* 2005).

4.5 Bactericidal activity of Black tea and Green tea extracts

Tea extracts investigated in this study proved to be good antibacterial agents against HPB but not against TPC and TC. Both extracts illustrated lower inhibitory properties against TPC and TC in comparison to noni, tamarind and citrus extracts. Tea extracts formed strong antibacterial agents against HPB. Green tea was able to inhibit the growth of eleven HPB while black tea inhibited ten species of HPB. Both extracts inhibited the growth of *K. pneumoniae*, *M. morganii* and *P. vulgaris*, which are all enterobacteriaceae species and have been identified as the important species of histamine formers in fish (Niven, 1981; Lopez-Sabater *et al*., 1996; Chamberlain, 2001; Takahashi *et al*., 2003). Tea extracts also inhibited growth of *C. perfringens, E. aerogenes, E. cloacae, K. oxytoca, P. damselae* and *H. alvei* which have all been determined as strong histamine producers in this study (Figure 3.1 and 3.2). Black tea and green tea extracts will form good antibacterial agents against histamine formers and significantly reduce the rate of

histamine formation in fish muscle when applied to fresh fish. Although green tea is expensive and not readily available in Fiji, it does possess stronger antibacterial potential against HPB in comparison to black tea and citrus extracts. Green tea can be used to control histamine production in export or sashimi quality whole fresh tuna and other more expensive fisheries products.

Tea extracts formed weak antibacterial agents against TC. Black tea was able to inhibit the growth of TC present in salala *ex vivo* at only two out of seven incubation temperatures while green tea was not able to inhibit coliform growth at all. Black tea is widely consumed in Fiji by nearly all racial groups within the population. It is easily available and its preparation is quite simple. Black tea extract can be used as a significant TC inhibiting agent in fresh whole salala. However, the storage temperature has to be critically monitored as the anti-coliform activity of black tea extract varies with temperature. It is only affective at 10°C and 25°C, while not being effective at other temperatures between 5°C and 32°C. All biological compounds need certain conditions including certain temperatures to function. The antibacterial activity of active compounds in black tea may be affected by temperature. The compounds may be exhibiting their antibacterial activity only at 10°C and 25°C and not at other temperatures tested.

Tea may owe its antibacterial properties to its high content of polyphenolics, particularly flavonoids that possess strong medicinal properties (Miller, 1995). Tea extract has been shown to possess *in vitro* antibacterial activity against *E. coli* (Mbata, 2007), but there have been no *in vivo* studies reported so far. Significant inhibitory effect of tea has been

shown against *Acinetobacter* spp., *E. coli, S. aureus, V. cholerae, H. alvei, Proteus* spp., and *P. aeruginosa* (Jazani *et al.*, 2007a, b; Mbata, 2007; Turkmen *et al.*, 2007). This is in agreement with the results presented here for green tea and black tea against *E. coli, P.vulgaris* and *P. mirabilis* (Tables 3.4 and 3.5).

Both types of tea failed to exhibit any *ex vivo* antibacterial activity against TPC at any of the incubation temperatures. It was observed that black tea extract actually promoted the growth of TPC while green tea promoted the growth of both TC and TPC at all seven temperatures. It is advisable that tea extracts should not be used to delay spoilage of salala. Instead of delaying spoilage it will increase the rate of TPC growth and cause the fish to spoil even faster. Tea extracts may be containing certain compounds which may be causing an increase in the metabolic or growth rate of TPC and TC. Black tea aqueous extracts have been shown to be unable to inhibit the growth of *Y. enterocolitica*, *L. monocytogenes* and *E. coli* O157:H7 (Turkmen *et al.*, 2007). Similarly, Kenyan and Nigerian tea extracts have been shown to be unable to inhibit growth of *Salmonella* spp and *P. aeruginosa* due to their lower content of active ingredients such as phytochemicals (Mbata, 2007). In this study 'China Ever-Green Green Tea' and 'China Lichee Black Tea' were used which are different brands of tea compared to other studies. The low antibacterial activity of tea used in this study may be due to lower phytochemical and polyphenol content as compared to the tea extracts used for other studies.

According to the results of this study, green tea exhibits stronger antibacterial activity against HPB in comparison to black tea. TPC showed some degree of sensitivity against

green tea while not showing any sensitivity to black tea. Chemical components such as polyphenols and catechins account for the antibacterial properties of tea and green tea has got much higher content of these compounds in comparison to black tea (Gardner *et al.*, 2007).

4.6 Application of Antibacterial properties of extracts

Three important groups of fish spoilage bacteria were studied in this investigation with the aim of using natural antibacterial agents to control of their growth. HPB growth can be quite effectively controlled by all five extracts, with the most dominant extract being noni. This method has an advantage over previously used methods as the plant extracts were used in this study without altering their natural matrices. This makes it quite easy to obtain an antibacterial agent from the five plants used for this study.

Growth of TPC and TC in whole salala was effectively controlled *ex vivo* by noni extract, which makes it an excellent bactericidal agent for controlling fish spoilage and pathogenicity. Tamarind also demonstrated highly effective growth control of TPC; therefore it will also form a suitable antibacterial agent. Citrus will form a moderately effective antibacterial agent for TPC and TC control in salala. Tea extracts are strong antibacterial agents against HPB but are unsuitable for controlling growth of TC and TPC. The major advantage of this study over previous works is that the investigations against TPC and TC are conducted on fish using natural plant extracts. The method used in this study is highly applicable for fishermen, fisheries industries and all types of fish

handlers as there are no special skills and knowledge required to employ these procedures for everyday use.

Fish is consumed all over the world and fish safety is a major concern globally. The antibacterial properties of the five extracts undertaken in this study can be effectively employed by fishermen and fisheries industries in the Pacific Island countries as well as in other parts of the globe as antibacterial agents to improve the processing and handling of post-harvest fish, especially salala.

These extracts can be utilized in the preparation of ice for chilling of freshly caught fish. Chilling is done to retain the freshness of fresh fish mainly by delaying bacterial growth (Graham *et al*., 1993). Some species of fish spoilage bacteria are able to survive and grow at chill temperatures between 0°C and 5°C such as *E. coli, Shewanella putrefaciens, Aeromonas sobria, Pseudomonas fluorescens, Listeria monocytogenes,* and *S. aureus* (Gram, 1993). Addition of noni, tamarind and citrus extracts to ice and water slurry will improve its bactericidal properties and may inhibit the growth of these bacteria and extend the shelf life of fish. Noni extract can also be used in the absence of ice, as its bactericidal activity is not affected by change in temperature. Noni, tamarind and citrus extracts can also be utilized at other stages of fish processing such as during washing to remove even more bacterial load than just using water alone.

Extracts of tamarind and citrus have strong bactericidal properties, possess low pH and are used as flavoring agents in food. These properties can be utilized in preservation

techniques for fish. Preservation methods may cause changes to the taste, texture and the physical appearance of the fish, which is usually made in a positive way to suit the needs of the market. Marinating, salting, drying and smoking are the most common, simple and widely used methods of fish preservation in the South Pacific. Utilizing tamarind and citrus for marinade preparation will ensure that the fish are kept safe from spoilage bacteria, histamine development and pathogens.

C. botulinum is a bacterium that causes food poisoning called botulism upon consumption of food in which it has grown and produced its toxin. Its spores are highly heat resistant and can survive in anaerobic conditions. *C. botulinum* are usually present in fish but their growth can be inhibited by a pH of <4.6 (Shapiro *et al.*, 1998). Tamarind and citrus have got a pH of less than 2.7; therefore using them in marinades will ensure that fish is safe from *C. botulinum.* Before fish or fish fillets are subjected to salting, drying or smoking processes, they undergo brining. Tamarind, noni or citrus extract can be added to the brining solution depending on taste preference. The bactericidal properties of the extracts will significantly reduce the bacterial load on the fish and fish fillets. After smoking, salting or drying and proper storage, the fish can be kept safe from HPB, TPC and TC and their storage life can also be extended.

Utilization of the antibacterial properties of the extracts should not be restricted for fish safety only. It can be applied to other foods as well to ensure their microbiological and toxilogical safety. Commercial food and drinks such as cheese and wine also possess HPB flora and the risk of causing histamine poisoning. The five extracts studied here can

be used as natural antibacterial agents to control histamine formation in these foods. Packed commercial seafood products such as clams, shrimps and other fish are quite vulnerable to microbial spoilage. Tamarind, noni and citrus can be used as flavoring agents as well as antibacterial agents to control bacterial growth and delay spoilage of these products.

V. Conclusion and future work

There have been numerous studies performed on extracts from plants undertaken for this study to assess their antibacterial properties. Majority of these works show promising results as outlined in the literature review. It should also be realized that most studies on the antibacterial properties of the extracts have been conducted *in vitro*. *In vitro* studies are mostly conducted in a laboratory-controlled environment and there is no guarantee that the procedure will work outside laboratory conditions such as on a freshly caught fish on board a fishing vessel. *In vitro* procedures rarely give the same results in *in vivo* or *ex vivo* situations. Application of these procedures and extracts for ensuring microbiological safety of fish by fisheries industries and fish handlers is quite difficult as they use sophisticated procedures and equipments. These procedures require expertise knowledge and skills, which are not possessed by local fishermen and fish handlers in the South Pacific, and it is uneconomical as well. Therefore it was important to conduct *ex vivo* studies on the unmodified extracts from plants so that the procedures are directly and easily applicable for fisheries industries and by fish handlers at all levels, especially in the South Pacific.

It can be concluded that all fifteen species of HPB selected for this study possess significant histamine producing ability with varying magnitudes. It was established that tamarind, citrus and noni extracts can be utilized as safe organic antibacterial agents against HPB, TC and TPC in fish with noni demonstrating the strongest and most outstanding antibacterial potential. Tamarind and citrus extracts have their greatest potential use as food preservatives as they are aromatic and improve the taste of food in

most cases. The low pH of these extracts will cause significant organoleptic changes to foodstuffs and would delay the onset of spoilage by retarding bacterial contamination. The five extracts studied here also possess strong antioxidant properties**,** which delays organoleptic spoilage by combating free radical-mediated spoilage of foods (Robards *et al.*, 1997; Dorman and Deans, 2000; Nyadoi, 2004; Muthu *et al*., 2005; Zayachkivska *et al.*, 2005; Kovacs, 2006; Zin *et al.*, 2006; Tsai *et al.*, 2007).

The employment of noni extract with fish may be questionable. Although it can form an excellent antibacterial agent against all three groups of bacteria, noni does give a rancid, cheesy off odor and has a bitter taste. Further work needs be done on noni extracts to make it an acceptable antibacterial agent for fish and other seafood products, especially regarding the aroma and taste.

In this study, a ratio of 1 gram of fish to 9 ml of plant extract was used. This ratio may not be practical for fishermen and fish handlers to use. The possibility of using the plant extracts at a lower concentration needs to be investigated. Further work is required to determine the MIC of the extracts. The concentration at which these extracts can be used effectively with fish while having an acceptable level of organoleptic change needs to be determined.

Exhibition of antimicrobial properties by the five extracts suggests that they all possess one or more bioactive compounds, which are responsible for their bactericidal effectiveness. This study forms the basis for numerous research works in studying,

determining and isolating the active components of the extracts and their potential use as antimicrobial agents in the field of medicine, food safety and food preservation.

Apart from containing bioactive compounds, the extracts of tamarind, citrus and noni also have a low pH, which may be playing a significant role in their antibacterial properties exhibited in this study. The impact of pH may be the major reason for these extracts to exhibit antibacterial properties. Low pH of the extracts will also cause significant organoleptic change to food items. Whether it is low pH or bioactive compounds, there is no doubt that the extracts used in this study will form good natural antibacterial agents.

In this study, investigations on TPC and TC were conducted *ex vivo* on fresh salala, which makes the results applicable to real situations. All species of HPB could not be tested due to their unavailability in pure form; also the tests were carried out on individual pure cultures of bacteria *in vitro* in a laboratory-controlled environment. Their behavior and interaction on fish and other food may be quite different. Additional *ex vivo* studies need to be conducted in order to justify and further develop the potential use of extracts from noni, citrus, tamarind and tea against histamine formation in fish.

VI. References:

1. **Aalbersberg, B.** 1999. Traditional and modern medicine: Harmonizing the two approaches in the South Pacific. Technical report. Institute of Applied Sciences, University of the South Pacific, Suva, Fiji. IAS technical report number 99/07.

2. **Abel, J.J., and S. Kubota.** 1919. The presence of histamine (β-iminazolylethylamine) in the hypophysis cerebri and other tissues of the body and its occurrence among the hydrolutic decomposition products of proteins. *J. Pharmacol. Exp. Ther.* **13:**243-300.

3. **Allen, D.G.** 2004. Regulatory control of histamine production in North Carolina harvested mahi-mahi (*Coryphaena hippurus*) and yellowfin tuna (*Thannus albacares*): A HACCP-based industry survey. Thesis. Department of Food Science, Graduate Faculty of North Carolina Raleigh, North Carolina State University, NC.

4. **Anderson, P.C., and J.L.C. Rapp.** 1969. Nutrient blocks containing gum arabic. *United States Patent.* 3476565.

5. **Anonymous.** 1993. Fiji Fisheries Division Annual Report. Fisheries Division, Suva, Fiji.

6. **Atkinson, N.** 1956. Antibacterial substances from flowering plants. Antibacterial activity of dried Australian plants by a rapid direct plate test. *Aust. J. Exp. Biol.* **34:**17-26.

7. **Ayres, J.C., J.O. Mundt, and W.E. Sandine.** 1980. Microbiology of Foods. H. H. Freeman, San Francisco.

8. **Baranowski, J.D**. 1985. Low-temperature production of urocanic acid by spoilage bacteria isolated from mahi-mahi (*Coryphaena hippurus*). *Appl. Environ. Microbiol*. **50(2):**546-547

9. **Beuchat, L.R., and A.J. Scouten.** 2002. Combined effects of water activity, temperature, and chemical treatments on survival of *Salmonella* spp and *Escherichia coli* O157:H7 on alfalfa seeds. *J. Appl. Microbiol.* **92:**382-395.

10. **Bremer, P.J., G.C. Fletcher, and C. Osborne.** 2003. Scombrotoxin in seafood. New Zealand Institute for Crop and Food Research Limited, Christchurch, New Zealand.

11. **Carlson, J.R., B.A. Bauer, A. Vincent, P.J. Limburg, and T. Wilson.** 2007. Reading the tea leaves: Anticarcinogenic properties of (-)-epigallocatechin-3-gallate. *Mayo Clin. Proc*. **82:**725-732.

12. **Castillo, J., O. Benavente, and J.A.D. Rio.** 1992. Naringin and Neohesperidin levels during development of leaves, flower buds, and fruits of *Citrus auratium*. *Plant Physiol*. **99:**67-73.

13. **Chamberlain T., and G. Titili.** 2001. Seafood spoilage and sickness. Community fisheries training Pacific series 4. Star Printery Limited, Suva, Fiji.

14. **Chamberlain, T.** 2000. Histamine in artisinal tuna fisheries in the Pacific Islands. Technical report. Marine Studies Program. The University of the South Pacific, Suva, Fiji. Series number: 2000/06.

15. **Chamberlain, T.** 2001. Histamine levels in long-lined tuna in Fiji: A comparison of samples from two different body sites and the effect of storage at different temperatures. *S. Pac. J. Nat. Sci*. **19:**30-34.

16. **Chen, H.C., H.F. Kung, W.C. Chen, W.F. Lin, D.F. Hwang, Y.C. Lee, and Y.H. Tsai.** 2008. Determination of histamine and histamine-forming bacteria in tuna dumpling implicated in a food-borne poisoning. *Food Chem.* **106:**612-618.

17. **Craig, W.J.** 1999. Health-promoting properties of common herbs. *Am. J. Clin. Nutr.* **70:**491-499.

18. **Crapo, C., and B. Himelbloom.** 1999. Spoilage and histamine in whole Pacific herring (*Clupeaharengus pallasi*) and Pink Salmon (*Oncorhynchus gorbuscha*) fillets. *J. Food Saf.* **19:**45–55.

19. **Croft, K.D., and P. Tu'ipulotu.** 1980. A survey of Tongan medicinal plants. *S. Pac. J. Nat. Sci.* **1:**45-57.

20. **Deng, Y., J.H. Ryu, and L.R. Beuchat.** 1999. Tolerance of acid-adapted and non-adapted *Escherichia coli* O157:H7 cells to reduced pH as affected by type of acidulant. *J. Appl. Microbiol*. **86:**203-210.

21. **Dorman, H.J.D, and S.G. Deans.** 2000. Antimicrobial agents from plants: Antibacterial activity of plant volatile oils. *J. Appl. Microbiol.* **88:**308–316.

22. **Doughari, J.H.** 2006. Antimicrobial activity of *Tamarindus indica* Linn. *Trop. J. Pharm. Res.* **5:**597-603.

23. **Duke, J.**, **M. Bogenschutz, and P. Duke.** 2002. Handbook of medicinal plants. 2nd Edn, CRC Press, Boca Raton, Florida.

24. **Ernst, E.** 2002. Adulteration of Chinese herbal medicines with synthetic drugs: a systematic review. *J. Intern. Med*. **252:**107–113.

25. **FDA.** 1998. FDA and EPA guidance levels. Appendix 5. Fish and fish products hazards and controls guide, 2nd edition, Department of Health and Human Services, Public Health Service, Food and Drug Administration, Center for Food Safety and Applied Nutrition, Office of Seafood, Washington, DC.

26. **FDA.** 2001. Scombrotoxin (histamine) formation (a chemical hazard) chapter 7. Fish and fish products hazards and controls guide, 3rd edition, Department of Health and Human Services, Public Health Service, Food and Drug Administration, Center for Food Safety and Applied Nutrition, Office of Seafood, Washington, DC.

27. **Fisher, K., C. Rowe, and C.A. Phillips.** 2007. The survival of three strains of *Arcobacter butzleri* in the presence of lemon, orange and bergamot essential oils and their components *in vitro* and on food. *Lett. Appl. Microbiol.* **44:**495–499.

28. **Gardner, E.J., C.H.S. Ruxton, and A.R. Leeds.** 2007. Black tea - helpful or harmful? A review of the evidence. *Eur. J. Clin. Nutr.* **61:**3–18.

29. **Gerson, S., K. Palu, B.N. Zhou, C. Su, C.J. Jensen, S.P. Story, and R.V. Ogden.** 2003. Antifungal effects of *Morinda citrifolia. United States Patent.* 0225005.

30. **Ghelardi, E., A. Tavanti, P. Davini, F. Celandroni, S. Salvetti, E. Parisio, E. Boldrini, S. Senesi, and Mario Campa1.** 2004. A Mucoadhesive polymer extracted from tamarind seed improves the intraocular penetration and efficacy of rufloxacin in

topical treatment of experimental bacterial keratitis. *Antimicrob. Agents Chemother.* **48:**3396–3401.

31. **Gilchrist, J.E., J.E. Campbell, C.B. Donnelly, J.T. Peeler, and J.M. Delaney.** 1973. Spiral plate method for bacterial determination. *Appl. Microbiol.* **25:**244-252.

32. **Graham, J., W.A. Johnston, and F.J. Nicohlson.** 1993. Ice in fisheries. FAO fisheries technical papers – T331. Food and Agriculture Organization of the United Nations, Viale delle Terme di Caracalla, 00100 Rome, Italy.

33. **Gram, L.,** 1993. Inhibitory effect against pathogenic and spoilage bacteria of *Pseudomonas* strains isolated from spoiled and fresh fish. *Appl. Environ. Microbiol.* **59:**2197-2203.

34. **Hammer, K.A., C.F. Carson, and T.V. Riley.** 1999. Antimicrobial activity of essential oils and other plant extracts. *J. Appl. Microbiol.* **86:**985-990.

35. **Herbert, R.A., M.S. Hendrie, D.M. Gobson, and J.M. Shewan.** 1971. Bacteria active in the spoilage of cetain seafoods. *J. Appl. Bact.* **34:**41-50.

36. **Jayaraman, S.K., M.S. Manoharan, and S. Illanchezian.** 2008. Antibacterial, antifungal and tumor cell suppression potential of *Morinda citrifolia* fruit extracts. *Int. J. Integr. Biol.* **3:**44-49.

37. **Jazani, N.H., S. Shahabi, A.A. Ali, and M. Zartoshti.** 2007a. Antibacterial effects of water soluble green tea extracts on multi-antibiotic resistant isolates of *Acinetobacter* sp. *Pak. J. Biol. Sci.* **10:**1477-1480.

38. **Jazani, N.H., S. Shahabi, and A.A. Ali.** 2007b. Antibacterial effects of water-soluble green tea extracts on multi-antibiotic resistant isolates of *Pseudomonas aeruginosa*. *Pak. J. Biol. Sci.* **10:**1544-1546.

39. **Jordan, S.L., J. Glover, L. Malcolm, F.M.T. Carter, I.R. Booth, and S.F. Park.** 1999. Augmentation of killing of *Escherichia coli* O157 by combinations of lactate, ethanol, and low-pH conditions. *Appl. Environ. Microbiol.* **65:**1308–1311.

40. **Jorgensen, L.V., H.H. Huss, and P. Dalgaard.** 2000. The effect of biogenic amine production by single bacterial cultures and metabiosis on cold-smoked salmon. *J. Appl. Microbiol.* **89:**920-934.

41. **Joseph, K.C., B.B. Parekh, and M.J. Joshi.** 2005. Inhibition of growth of urinary type clacium hydrogen phosphate dihydrate crystals by tartaric acid and tamarind. *Curr. Sci.* **88:**1232-1238.

42. **Kahlmeter, G.** 2003. An international survey of the antimicrobial susceptibility of pathogens from uncomplicated urinary tract infections: the ECO·SENS project. *J. Antimicrob. Chemother.* **51:**69-76.

43. **Kanki, M., T. Yoda, T. Tsukamoto, and T. Shibata.** 2002. *Klebsiella pneumoniae* produces no histamine: *Raoultella planticola* and *Raoultella ornithinolytica* strains are histamine producers. *Appl. Environ. Microbiol.* **68:**3462–3466.

44. **Kimata, M., A. Kawai, and M. Akamatsu.** 1958. Classification and identification of the bacteria having an activity which can produce a large amount of histamine. *Mem. Res. Inst. Food Sci. Kyoto Univ.* **14:**33-41.

45. **Koutsoumanis, K., M.C. Giannakourou, P.S. Taoukis, and G.J.E. Nychas.** 2002. Application of shelf life dicision system (SLDS) to marine cultured fish quality. *Int. J. Food Microbiol.* **73:**375-382.

46. **Kovacs, E.M.R., and D.J. Mela.** 2006. Metabolically active functional food ingredients for weight control. *Obes. Rev.* **7:**59–78.

47. **Lehane, L., and J. Olley.** 1999. Histamine (scombroid) fish poisoning: a review in a risk-assessment framework. National Office of Animal and Plant Health, Canberra, Australia.

48. **Lerke, P.A., S.B. Werner, S.L. Taylor and L.S. Guthertz.** 1978. Scromboid poisoning – report of an outbreak. *West J. Med.* **129:**381-386.

49. **Lewis, A.D.,** 1985. Fishery resource profiles: information for development planning. Fiji Fisheries Division, Ministry of Primary Industries, Suva, Fiji.

50. **Lin, K.W**. 2005. Ethnobotanical study of medicinal plants used by the Jah Hut peoples in Malaysia. *Indian J. Med. Sci.* **59:**156-161.

51. **Liu, G.M., A. Bode, W.Y. Ma, S.M. Sang, C.T. Ho, and Z.G. Dong.** 2001. Two novel glycosides from the fruits of *Morinda citrifolia* (noni) inhibit AP-1 transactivation and cell transformation in the mouse epidermal JB6 cell line. *Cancer Res.* **61:**5749–5756.

52. **Lopez-Sabater, E.I., J.J. Rodriguez-Jerez, M. Hernandez-Herrero, and M.A.T. Mora-Ventura.** 1996. Incidence of histamine-forming bacteria and histamine content in scombroid fish species from retail markers in the Barcelona area. *Int. J. Food Microbiol*. **28:**411–418.

53. **Lucas, P.M., W.A.M. Wolken, O. Claisse, J.S. Lolkema, and A.L. Funel.** 2005. Histamine-producing pathway encoded on an unstable plasmid in *Lactobacillus hilgardii. Appl. Environ. Microbiol.* **71:**1417-1424.

54. **Lyhs, U.** 2002. Lactic acid bacteria associated with spoilage of fish products. Thesis. Department of Food and Environmental Hygiene, Faculty of Veterinary Medicine, University of Helsinki, Finland.

55. **Lyhs, U., H. Korkeala and J. Björkroth.** 2000. Identification of lactic acid bacteria from spoiled, vacuum-packed 'gravad' rainbow trout using ribotyping. Report. Department of Food and Environmental Hygiene, Faculty of Veterinary Medicine, University of Helsinki, Finland.

53. **Martin, P.D.** 2005. Application claiming substantial equivalence of "leap of faith farms noni juices" (skin- and seed-free expressed juices of *Morinda citrifolia L*) to 'Tahitian Noni Juice" of morinda Inc. Peter D Martin, Perspekt Consulting, Faringdon, Oxfordshire SN7 7WX, UK. Email: peter.martin@perspektprocess.com.

54. **Masette, M.** 1999. A comparative study of storage time of warm and cold-water fish in view of the current market demands. Final projects. Food, Science and Technology Research Institute. Kampala – Uganda. Phone/Fax 0256-41–222657.

55. **Mbata, T.I.** 2007. Preliminary studies of the antibacterial activities of processed Kenyan and Nigerian tea. *Afr. J. Biotechnol.* **6:**278-279.

56. **McClatchey, W.** 2002. From Polynesian healers to health food stores: Changing perspectives of *Morinda citrifolia* (Rubiaceae). *Integr. Cancer Ther.* **1:**110–120.

57. **McGrath, W.R.** 1996. Healing plants of the Pacific Isles. Ethanobotany Books, U.S.A.

58. **McKay, D.L., and J.B. Blumberg.** 2002. Review; the role of tea in human health: an update. *J. Am. Coll. Nutr.* **21:**1–13.

59. **Miller, J.M.T.H.** 1995. Antimicrobial properties of tea (*Camellia sinensis* L.). Minireview. *Antimicrob. Agents Chemother.* **39:**2375–2377.

60. **Mower, W., J.C. Bawden, M.C. Harmon, C.R. Stutz, and C. Wang.** 2006. Compositions and methods using *Morinda citrifolia. United States Patent.* 0286188.

61. **Muthu, S.E., S. Nandakumar, and U.A. Rao.** 2005. The effect of methanolic extract of *Tamarindus indica* Linn. on the growth of clinical isolates of *Burkholderia pseudomallei*. *Indian J. Med. Res.* **122:**525-528.

62. **Nelson, S.C.** 2006. *Morinda citrifolia* (noni), ver. 4. In: Elevitch, C.R. (ed.). Species profiles for Pacific island agroforestry. Permanent Agriculture Resources (PAR), Hōlualoa, Hawai‘i. http://www.traditionaltree.org.

63. **Niemi, R.M., M.P. Heikkila, K. Lahti, S. Kalso and S.I. Niemela.** 2001. Comparison of methods for determining the numbers and species distribution of coliform bacteria in well water samples. *J. Appl. Microbiol.* **90:**850-858.

64. **Niven, C.F., M.B. Jeffrey, and D.A. Corlett.** 1981. Differential plating medium for quantitative detection of histamine-producing bacteria. *Appl. Environ. Microbiol.* **41:**321-322.

65. **Nyadoi, P.** 2004. Population structure and socio economic importance of *Tamarindus indica* in Tharaka, eastern Kenya. Master of Science thesis. Makerere University, Uganda, Africa.

66. **Patrono, C., B. Coller, G.A. FitzGerald, J. Hirsh, and G. Roth.** 2004. Platelet-active drugs: The relationships among dose, effectiveness, and side effects: The seventh ACCP conference on antithrombotic and thrombolytic therapy. *Chest.* **126:**234-264.

67. **Petard, P.** 1972. Raau Tahiti: The use of Polynesian medicinal plants in Tahitian medicine. *So. Pac.Tech. Paper*. **167:**1-66.

68. **Prabuseenivasan, S., M. Jayakumar, and S. Ignacimuthu.** 2006. *In vitro* antibacterial activity of some plant essential oils. *BMC Complement. Altern. Med.* **6:**39-46.

69. **Prescott, L.M., J.P. Harley, and D.A. Klein.** 2005. Microbiology. 6th edition. McGraw-Hill Companies, Inc., 1221 Avenue of the Americas, New York, NY 10020, U.S.A.

70. **Presser, K.A., T. Ross, and D.A. Ratkowsky.** 1998. Modelling the growth limits (growth/no growth interface) of *Escherichia coli* as a function of temperature, pH, lactic acid concentration, and water activity. *Appl. Environ. Microbiol.* **64:**1773–1779.

71. **Quested, C.** 2002. FAO/SPC/WHO Pacific Islands food safety and quality consultation. Report. World Health Organization, Regional Office for the Western Pacific. Manila, Philippines. Report series number: RS/2002/GE/30(FIJ). Ph: +685 2122 ext 228, Email: nutrition@health.gov.ws.

72. **Rhee, M.S., S.Y. Lee, R.H. Dougherty, and D.H. Kang.** 2003. Antimicrobial effects of mustard flour and acetic acid against *Escherichia coli* O157:H7, *Listeria monocytogenes*, and *Salmonella enterica* Serovar Typhimurium. *Appl. Environ. Microbiol.* **69:**2959–2963.

73. **Richards, A., M. Lagibalavu, S. Sharma, and K. Swamy.** 1994. Fiji Fisheries Resources Profiles. F.F.A. Report No.94/4, Fiji.

74. **Robards, K., X. Li, M. Antolovich, and S. Boyd.** 1997. Characterisation of citrus by chromatographic analysis of flavonoids. *J. Sci. Food Agric*. **75:**87-101.

75. **Rocelle, M., S. Clavero, and L.R. Beuchat.** 1996. Survival of *Escherichia coli* O157:H7 in broth and processed salami as influenced by pH, water activity, and temperature and suitability of media for its recovery. *Appl. Environ. Microbiol.* **62:**2735–2740.

76. **Sato, M., Z. Tao, K. Shiozaki, T. Nakano, T. Yamaguchi, T. Yokohama, N. Kanno, and E. Nagahisa.** 2006. A simple and rapid method for the analysis of fish histamine by paper electrophoresis. *Fisheries Sci.* **72:**889-892.

77. **Shapiro, R.L., C. Hatheway and D.L. Swerdlow.** 1998**.** Botulism in the United States: A clinical and epidemiologic review. *Ann. Internal Med.* **129:**221-228.

78. **Sharma, S.P., and T.J.H. Adams.** 1990. The Fiji tuna baitfishery. In: Blaber, S.J.M. and Copland, J.W. (ed.). 1990. Tuna baitfish in the Indo-Pacific region: proceedings of a workshop, Honiara, Solomon Islands, 11-13 December 1989. ACIAR Proceedings No.30.

79. **Shaw, B.G., and J.M. Shewan.** 1968. Psychrophilic spoilage bacteria of fish. *J. Appl. Bact*. **31:**89-96.

80. **Shewan, J.M.** 1971. The microbiology of fish and fishery products – a progress report. *J. Appl. Bact.* **34:**299-315.

81. **Shewan, J.M.** 1961. The microbiology of sea-water fish. In Borgstrom, G. (ed), Fish as Food – Vol 1. New York, Academic Press.

82. **Sidhu, K., J. Kaur, G. Kaur, and K. Pannu.** 2007. Prevention and cure of digestive disorders through the use of medicinal plants. *J. Hum. Ecol.* **21:**113-116.

83. **Singh, A., and S. Sawatibau.** 1980. Medicinal plants in Fiji and other South Pacific Islands, Part 1. School of Natural Resources and Center for Applied Studies, University of the South Pacific, Suva, Fiji. Research report.

84. **Singh, A.A., and V. Bowry.** 2005. Detection and quantitation of histamine in fish tissue by western blotting electrophoresis. Unpublished work. University of the South Pacific, Fiji.

85. **Singh, R.N., and B. Saratchandra.** 2005. The development of botanical products with special reference to seri-ecosystem. *Caspian J. Env. Sci.* **3:**1-8.

86. **Solomon, N.** 2001 Noni, (*Morinda citrifolia*) The tropical fruit with 101 medicinal uses. Woodland Publishing. Pleasant Grove, Utah, U.S.A.

87. **Sortwell, D., S.A. Nutriquim, C.V. De, and A. Woo.** 1996. Improving the flavour of food products with acidulants. Bartek Ingredients, Inc., Expotecnoalimentaria, Mexico City, Mexico.

88. **Srinivasan, D., S. Nathan, T. Suresh, and T. Lakshmanaperumalsamy.** 2001. Antibacterial activity of neem (*Azadirachta indica*) and tamarind (*Tamarindus indica*) Leaves. *Asian J. Microb. Biotechnol. Environ. Sci.* **3:**67-73.

89. **Suhr, K.I., and P.V. Nielsen.** 2003. Antifungal activity of essential oils evaluated by two different application techniques against rye bread spoilage fungi. *J. Appl. Microbiol.* **94:**665-674.

90. **Takahashi, H., B. Kimura, M. Yoshikawa, and T. Fujii.** 2003. Cloning and sequencing of the histidine decarboxylase genes of gram-negative, histamine-producing bacteria and their application in detection and identification of these organisms in fish. *Appl. Environ. Microbiol*. **69:**2568–2579.

91. **Taoukis, P.S., K. Koutsoumanis, and G.J.E. Nychas.** 1999. Use of time-temperature integrators and predictive modelling for shelf life control of chilled fish under dynamic storage conditions. *Int. J. Food Microbiol.* **53:**21-31.

92. **Taylor, S.L., L.S. Guthertz, M. Leatherwood, and E. Lieber.** 1979. Histamine production by *Klebsiella pneumoniae* and an incident of scombroid fish poisoning. *Appl. Environ. Microbiol.* **37:**274–278.

93. **Taylor, S.L.** 1985. Histamine poisoning associated with fish, cheese, and other foods. Monograph. Geneva: World Health Organization (VPH/FOS/85.1).

94. **Taylor, S.L., M. Leatherwood, and E.R. Cieber.** 1978. Histamine in Sauerkraut. *J. Food Sci.* **43:**1030-1032.

95. **Thomson, G.E.** 2007. The health benefits of traditional chinese plant medicines: Weighing the scientific evidence. A report for the Rural Industries Research and Development Corporation. Project No.DAV-227A. RIRDC Publication, No. 06/128. Kingston.

96. **Tsai, P.J., T.H. Tsai, C.H. Yu, and S.C. Ho.** 2007. Comparison of NO-scavenging and NO-suppressing activities of different herbal teas with those of green tea. *Food Chem.* **103:**181–187.

97. **Tsai, T.H., T.H. Tsai, Y.C. Chien, C.W. Lee and P.J. Tsai.** 2008. *In vitro* antimicrobial activities against cariogenic streptococci and their antioxidant capacities: A comparative study of green tea versus different herbs. *Food Chem.* **110:**859-864.

98. **Turkmen, N., Y.S. Velioglu, F. Sari, and G. Polat.** 2007. Effect of extraction conditions on measured total polyphenol contents and antioxidant and antibacterial activities of black tea. *Molecules.* **12:**484-496.

99. **Uhe, G.** 1974. Medicinal plants of Samoa. A preliminary survey of the uses of plants for medicinal purposes in the Samoan islands. *Econ. Bot.* **28:**1-30.

100. **USFDA (U.S. Food and Drug Administration).** 2001. Scombrotoxin (histamine) formation in fish and fishery products hazards and controls guide (3rd ed., pp. 73-93). Washington, DC: Department of Health and Human Services, Public Health Service, Food and Drug Administration, Center for Food Safety and Applied Nutrition, Office of Seafood.

101. **Voravuthikunchai, S.P., and H. Mitchell.** 2008. Inhibitory and killing activities of medicinal plants against multiple antibiotic-resistent *Helicobacter pylori. J. Health Sci.* **54:**81-88.

102. **Wang, M.Y., B.J. West, C.J. Jensen, D. Nowicki, C. Su, A.K Palu, and G. Anderson.** 2002. *Morinda citrifolia* (Noni): A literature review and recent advances in Noni research. *Acta. Pharmacol. Sin.* **23:**1127-41.

103. **Wauters, G., V. Avesani, J. Charlier, M. Janssens, and M. Delmee.** 2004. Histidine decarboxylase in *Enterobacteriaceae* revisited. *J. Clin. Microbiol.* **42:**5923-5924.

104. **Weisberg, M.D.** 2006. Acid-thickened food compositions and products. *United States Patent*, 0286254.

105. **Whistler, W.A.** 1992. Polynesian herbal medicine. Everbest Printing Company Limited, Hong Kong, China.

106. **WHO.** 1998. Medicinal plants in the South Pacific. Information on 102 commonly used medicinal plants in the South Pacific. WHO Regional Publications. Western Pacific Series No. 19.

107. **Winniczuk, P.** 2004. Food and beverage preservative. *United States Patent.* 0161517.

108. **Yang, J., R. Paulino, S.J. Stedronsky, and F. Abawi.** 2007. Free-radical-scavenging activity and total phenols of noni (*Morinda citrifolia* L.) juice and powder in processing and storage. *Food Chem.* **102:**302-308.

109. **Yoshinaga, D.H., and H.A. Frank.** 1982. Histamine-producing bacteria in decomposing skipjack Tuna (*Katsuwonus pelamis*). *Appl. Environ. Microbiol.* **44:**447-452.

110. **Zayachkivska, O.S., S.J. Konturek, D. Drozdowicz, P.C. Konturek, T. Brzozowski, and M.R. Ghegotsky.** 2005. Gastroprotective effects of flavonoids in plant extracts. *J. Physiol. Pharmacol.* **56:**219-231.

111. **Zin, Z.M., A.A. Hamid, A. Osman, and N. Saari.** 2006. Antioxidative activities of chromatographic fractions obtained from root, fruit and leaf of Mengkudu (*Morinda citrifolia* L.). *Food Chem.* **94:**169–178.

VII. Appendices

Appendix 7.1: Histamine production results

Table 7.1: *Histamine production in TFIB by fifteen bacterial pure cultures*

Species	**Net histamine production (ppm) at time:**	
	7hours	**24hours**
C. perfringens	68.33 ± 1.67	123.33 ± 1.67
C. youngae	121.67 ±1.67	191.67 ± 1.67
E. coli	5.00 ± 0.00	10.00 ± 0.00
E. aerogenes	5.00 ± 0.00	123.33 ± 1.67
E. cloacae	15.00 ± 0.00	210.00 ± 3.33
E. amnigenous	5.00 ± 0.00	15.00 ± 0.00
H. alvei	25.00 ± 0.00	136.67 ± 1.67
K. oxytoca	36.67 ± 1.67	148.33 ± 1.67
K. pneumoniae	5.00 ± 0.00	25.00 ± 0.00
M. morganii	91.67 ± 1.67	236.67 ± 3.33
P. vulgaris	5.00 ± 0.00	10.00 ± 0.00
P. mirabilis	5.00 ± 0.00	15.00 ± 0.00
P. damselae	83.33 ± 1.67	176.67 ± 1.67
R. ornithinolytica	5.00 ± 0.00	15.00 ± 0.00
V. alginolyticus	40.00 ± 0.00	173.33 ± 1.67
Control	0.00 ± 0.00	0.00 ± 0.00
A. hydrophila	0.00 ± 0.00	0.00 ± 0.00

Histamine standards from 5 ppm to 300 ppm were taken as reference standards for comparison; Values are mean histamine content ± S.D of three replicates.

Appendix 7.2:

Table 7.2: Results for histamine producing bacteria (HPB) against different plant extracts (cfu/ml)

		Species of Histamine Producing Bacteria						
Test substance	**Time (hours)**	*E. coli*	*E. aerogenes*	*E. cloacae*	*P. vulgaris*	*P. mirabilis*	*K. oxytoca*	*K. pneumoniae*
Green tea pH= 5.57 Temp= 32°C	Initial	0	0	0	0	0	0	0
	0	9158	9664	9196	9324	9223	9420	9044
	2	8664	4577	8436	0	0	8826	8778
	24	0	0	0	0	0	0	0
	27	0	0	0	0	0	0	0
	48	0	0	0	0	0	0	0
Black tea pH= 5.05 Temp= 32°C	Initial	0	0	0				
	0	9405	9216	9600	9196	9300	9310	9520
	2	8382	8898	9500	7827	8397	9387	8006
	24	0	0	7600	0	0	0	0
	27	0	0	9120	0	0	0	0
	48	0	0	0	0	0	0	0
Noni pH= 3.84 Temp= 32°C	Initial	0	0	0	0	0	0	0
	0	9229	9135	9412	9390	9178	9454	9523
	2	0	0	0	0	0	0	0
	24	0	0	0	0	0	0	0
	27	0	0	0	0	0	0	0
	48	0	0	0	0	0	0	0
Tamarind pH= 2.47 Temp= 32°C	Initial	0	0	0	0	0	0	0
	0	9190	9378	9280	9338	9234	9320	9418
	2	0	0	0	0	0	0	0
	24	0	0	0	0	0	0	0
	27	0	0	0	0	0	0	0

	48	0	0	0	0	0	0	0
Sterile Distilled Water (SDW) control pH= 6.78 Temp= 32°C	Initial	0	0	0	0	0	0	0
	0	9214	9390	9424	9500	9348	9176	9462
	2	9210	9377	9409	9362	9317	9164	9410
	23	9190	9335	9390	9120	9272	9089	9364
	26	9038	9297	9361	9080	9234	9020	9348
	48	9411	9186	9314	342	1520	9005	9296
Citrus pH= 2.64 Temp= 32°C	Initial	0	0	0	0	0	0	0
	0	9388	9428	9531	9340	9368	9420	9210
	2	36	78	0	3492	0	21915	12952
	24	29	37456	0	25747	0	35084	21722
	26	15	41068	0	27623	0	36432	26317
	48	17513	50943	0	33216	0	50677	39664

The line labelled "initial" containing 0 in the tables 7.2 and 7.3 in Appendices 7.2 and 7.3 refers to the bacterial load present in the extract before it was inoculated. To show the level of bacteria initially present in the extract prior to experiment.

Table 7.2: Results for histamine producing bacteria (HPB) against different plant extracts (cfu/ml)

		Species of Histamine Producing Bacteria							
Test substance	**Time (hours)**	*C. perfringens*	*P. damselae*	*M. morganii*	*E. amnigenous*	*H. alvei*	*C. youngae*	*V. alginolyticus*	*R. ornithinolytica*
Green tea pH= Temp= 32°C	Initial	0	0	0	0	0	0	0	0
	0	9244	9427	9546	9134	9478	9230	9176	9560
	2	0	37	13963	12310	13806	12802	9624	13166
	24	0	0	76	23464	4	21850	11589	12074
	26	0	0	6308	25970	3	23409	12567	13093
	48	0	0	0	32591	0	29237	24586	25615

Black tea pH= Temp= 32°C	Initial	0	0	0	0	0	0	0	0
	0	9313	9361	9472	9322	9138	9305	9296	9568
	2	0	0	16605	16428	15983	13022	12782	13156
	24	0	0	16275	18975	6	22552	22499	23157
	26	0	0	13897	20687	5	24735	23392	24076
	48	0	0	0	29126	12296	31519	28133	28956
Noni pH= Temp= 32°C	Initial	0	0	0	0	0	0	0	0
	0	9176	9368	9241	9278	9271	9489	9233	9576
	2	0	0	0	0	0	0	0	0
	24	0	0	0	0	0	0	0	0
	26	0	0	0	0	0	0	0	0
	48	0	0	0	0	0	0	0	0
Tamarind pH= Temp= 32°C	Initial	0	0	0	0	0	0	0	0
	0	9360	9508	9480	9258	9345	9178	9347	9543
	2	36	48	13514	13696	12932	0	12760	0
	24	0	40	1213	30408	26903	0	30455	0
	26	0	29	363	34738	23075	0	32493	0
	48	0	5498	0	2982	0	0	1698	0
Sterile Distilled Water (SDW) control pH= Temp= 32°C	Initial	0	0	0	0	0	0	0	0
	0	9168	9373	9211	9364	9134	9257	9476	9322
	2	9114	9217	10759	9684	10379	10182	11438	11448
	24	9059	9205	11595	13648	16316	10910	13531	14820
	26	9053	9162	14223	14630	16771	11526	14530	15827
	48	8960	9029	14778	17391	19503	15322	18443	20075
Citrus pH= Temp= 32°C	Initial	0	0	0	0	0	0	0	0
	0	9280	9180	9358	9120	9238	9158	9518	9220
	2	0	0	68	0	59	14	8360	6397
	24	0	0	0	0	31	21	16159	0
	26	0	0	0	0	44	77	18687	0
	48	0	0	0	0	5623	266	30205	0

Appendix 7.3:

Table 7.3.: Results for total plate count against different plant extracts (cfu/ml)

Temperature (ºC)	Time (hours)	Test substances					
		Citrus	Tamarind	Noni	Black Tea	Green Tea	SDW
		TPC	TPC	TPC	TPC	TPC	TPC
5ºC	Initial	0	0	0	0	0	0
	0	9798	9866	9674	9596	9516	9683
	2	20996	10164	0	11424	9874	11033
	24	1718	4185	0	20526	28548	25445
	25	5107	4335	0	21380	30045	25700
	48	49763	4783	0	29022	36527	30337
10ºC	Initial	0	0	0	0	0	0
	0	9823	9630	9582	9864	9557	9761
	2	0	9	0	10530	5867	17644
	24	0	0	0	21187	27057	18879
	26	0	0	0	22004	28209	19153
	48	0	0	0	34838	34940	20759
15ºC	Initial	0	0	0	0	0	0
	0	9782	9737	9713	9532	9512	9634
	2	0	0	0	20017	2774	8961
	24	0	0	0	27314	25365	14077
	26	0	0	0	27950	27242	14683
	48	0	0	0	34097	35633	24690
20ºC	Initial	0	0	0	0	0	0
	0	9665	9841	9590	9734	9686	9564
	2	11614	28027	0	10844	4967	10696
	24	31525	9474	0	22897	13136	21519
	26	33373	18934	0	26210	14230	24547
	48	37491	28139	0	37252	22472	29972
25ºC	Initial	0	0	0	0	0	0
	0	9629	9671	9600	9814	9726	9560
	2	4256	883	0	13053	21973	9241
	24	13133	25973	0	21064	30597	12821
	26	14040	27907	0	23035	31202	14050
	48	24723	33532	0	30682	35977	22304
30ºC	Initial	0	0	0	0	0	0
	0	9680	9564	9888	9736	9828	9560
	2	52	8197	20	9736	2632	10204
	24	2057	0	1599	22834	11373	16853
	26	13025	0	0	17970	12980	17794
	48	23974	0	0	28999	26460	24192

32ºC	Initial	0	0	0	0	0	0
	0	9540	9736	9861	9656	9604	9881
	2	4452	0	0	11302	26552	1154
	24	14621	0	0	18067	30020	18234
	25	15373	0	0	20613	30998	18904
	48	24989	0	0	30289	34699	23672

Appendix 7.4:

Table 7.4.: Results for total coliforms(TC) against different plant extracts (cfu/ml)

Temperature (ºC)	Time (hours)	Test substances					
		Citrus	Tamarind	Noni	Black Tea	Green Tea	SDW
		TC	TC	TC	TC	TC	TC
5ºC	Initial	0	0	0	0	0	0
	0	9715	9682	9561	9653	9832	9548
	2	1	0	0	9289	15362	8200
	24	1295	0	0	20357	27652	20327
	25	26554	0	0	25461	29815	26319
	48	37564	0	0	30507	35633	29934
10ºC	Initial	0	0	0	0	0	0
	0	9751	9540	9658	9566	9714	9532
	2	0	0	0	5466	6901	8714
	24	0	0	0	15536	17212	8091
	26	0	0	0	19132	19428	8442
	48	0	0	0	0	35618	13078
15ºC	Initial	0	0	0	0	0	0
	0	9842	9681	9743	9796	9820	9537
	2	0	0	0	12346	5892	8771
	24	0	0	0	23265	23240	16149
	26	0	0	0	27854	27496	16776
	48	0	0	0	37064	34589	29748
20ºC	Initial	0	0	0	0	0	0
	0	9630	9815	9601	9846	9610	9605
	2	1	0	0	4450	8736	8873
	24	666	0	0	25674	15224	13721
	26	518	0	0	28083	16113	15687
	48	0	0	0	33166	28187	26530
25ºC	Initial	0	0	0	0	0	0
	0	9552	9652	9852	9532	9852	9852
	2	9877	10584	0	2166	9572	9180
	24	24305	24569	0	0	17911	19121
	26	25185	25555	0	0	19032	20963
	48	30045	35252	0	0	28322	33782
30ºC	Initial	0	0	0	0	0	0
	0	9648	9835	9567	9631	9668	9528
	2	2	16755	0	7352	12227	17790
	24	22816	0	0	15110	20633	23817
	26	24772	0	0	16009	23504	24413
	48	35219	0	0	30728	33360	34040

32ºC	Initial	0	0	0	0	0	0
	0	9828	9732	9764	9836	9756	9836
	2	948	59	0	11294	11296	7153
	24	16063	0	0	25554	26957	12141
	25	18170	0	0	26979	30256	14423
	48	24366	0	0	33037	33955	26040

Appendix 7.5:

Table 7.5: pH of the different plant extracts

Common name	Scientific name	pH
Citrus	*Citrus aurantium*	2.64
Tamarind	*Tamarindus indica*	2.47
Noni or Kura	*Morinda citrifolia*	3.84
Green tea	*Camellia sinensis*	5.57
Black tea	*Camellia sinensis*	5.05
Sterile distilled water (SDW)	-	6.78

Appendix 7.6: Preparation of Tuna Fish Infusion Broth.

Tuna fish infusion broth (TFIB) was prepared from fresh tuna muscle. Tuna meat was cut in small pieces and homogenized with 2 volumes (wt/vol) of water. The homogenate was steamed at 100°C for 1 hour, followed by filtration with Whatman no.1 filter paper. The filtrate was supplemented with 1% glucose, and sterilized at 121°C for 15 minutes. The final pH of TFIB was pH 5.7.

Procedure adapted from Taylor *et al.*, (1979).

Appendix 7.7: Production of histamine

In the histamine production experiment a 2 ml amount of each of the overnight bacterial pure cultures were inoculated into 70 ml of TFIB in 300 ml nepheloculture flasks (Bellco). Flasks were incubated at 32°C in a shaking incubator (New Brunswick Scientific) at 100 rpm. Immediately after inoculation of the TFIB, 1 ml was withdrawn for histamine analysis. After a 7 hour incubation period, 1 ml was again withdrawn for histamine analysis. An additional 1ml aliquot was removed for histamine analysis after completion of 24 hour incubation.

Procedure adapted from Taylor *et al.*, (1979).

Appendix 7.8: Histamine analysis by electrophoresis

A small volume of liquid sample of TFIB (1.0 ml) was placed in a mortar and 5.0ml of 80% ethyl alcohol (ethanol) solution was poured into the mortar (the volume of alcohol should be five times as much as the sample is). The sample was brayed for exactly two minutes until the sample got melted into alcohol. The mixture solution (sample and alcohol) was placed into a Micro Tube. If six kinds of samples were to be tested, the above procedure was repeated another five times. For histamine standards preparation was not required. Six micro tubes were taken and each micro tube surface was marked with a number from one to six. Six mini round (Whatman No. 1) filter papers were taken and each was marked with the corresponding number marked on each micro tube surface.

Extraction of histamine from fish meat matrix by filtration

By the micropipette, 10 µl of supernatant was taken into the pipette tip; this was the transparent liquid from the micro tube No. 1 on the micro tube rack. A small volume from the pipette was placed on the Mini-round Filter Paper No. 1 and it was allowed to dry for 15-20 seconds. After this the remaining liquid of the pipette tip was placed again on the same filter paper. These steps were repeated for the other mini round filter papers (the used pipette tip was replaced with a new one for each different sample). For the standards, 10 µl of Histamine Standard Liquid (specification given in the last table of this section) was taken into the pipette tip directly from the bottle of Histamine Standard Liquid and the same procedure as the previous paragraph was followed.

Isolation of histamine from interfering substances by Western Blotting Electrophoresis using the JBB histamine checker set-up.

The filter paper holder was placed in the electrophoresis vessel with the plus and minus sides of the holder corresponding to the plus and minus sides of the vessel. Some buffer solution (a mixture of pure water, pyridine and glacial acetic acid) was placed in a clean tray. Some buffer solution was also poured in both sides of the electrophoresis vessel.

A H-type Filter Paper was soaked a in the Solution in the tray. The wet H-type Filter Paper was placed on the Filter-Paper Holder with the line of Hs exactly on the center bar of the Filter-Paper Holder (Both sides of the filter paper should be soaked in Buffer Solution in both sides of the electro-phoresis Vessel). With a pair of Stainless Tweezers each liquid-penetrated Mini-round Filter Papers were placed on the center of the "H" on the Filter Paper. The Vessel Cover was placed on the Electro-phoresis Vessel.

The driving timer on Voltage Adjuster was set at 10 minutes and the "ON/OFF" button was turned on. While "ON/OFF" button was green the "START" button was pressed. When the timer indication was turned off, the vessel cover was opened and all the Mini-round Filter Papers were removed from the H-type Filter Paper. The H-type Filter Paper was taken out from the Electro-phoresis Vessel with Stainless Tweezers and it was dried by putting it in a dryer until fully dry.

Preparation of sprayer liquids for color development

Two spraying bottles were taken and were marked as "**Sprayer A**" and **"Sprayer B"**. 0.5ml of Liquid A-1 was taken by 1ml pipette and this was placed in Sprayer A**.** 5.0ml of

Liquid A-2 was taken by 5.0ml pipette and this was also placed in Sprayer A**.** 5ml of Liquid B was taken by 5.0ml pipette and this was placed in Sprayer B. The mixture liquid in Sprayer A was sprayed uniformerly over the "Histidine/Histamine Band" which was an area 5 cm wide from the end of the "H". Liquid B in Sprayer B was sprayed over the same "Histidine/Histamine Band". The sprayed H-type Filter Paper was placed into a dryer until dry. Precautions were taken as Liquid A is poisonous.

Table 7.6: Specification of equipment and chemicals employed in the procedure

Instruments and Chemicals
JBB histamine checker set-up
Voltage adjuster, model JB 1080
Step down transformer (AC110V, 220V, or 240 to AC 100V)
Electrophoresis Vessel with cover and filter-paper holder
Filter-paper dryer
H-type filter paper
Buffer solution (mixture of pure water, pyridine and glacial acetic acid).
Micro Tube 25µl
Micro Tube 50µl
Histamine standard liquids 10 ppm – 300 ppm
Liquid A-1 (mixture of pure water, sulfanillic acid and hydrochloric acid,).
Liquid A-2 (mixture of pure water and sodium nitrate solution).
Liquid B (mixture of pure water, sodium carbonate anhydride and ethanol).

Methodology adapted from Sato *et al*., (2006) and Singh and Bowry, (2005)

Appendix 7.9: Calibration of JBB Histamine Checker

The JBB histamine checker equipment was calibrated using the standard addition method to observe matrix effects.

Preparation of samples for standard addition method

Seven mini round filter papers were taken and marked from 1 to 7. To each filter paper the following combinations of liquids as shown in the table given below were added to be absorbed. The same fish sample was used for filter papers 3 to 7. Analysis was done in triplicates and results were presented as average values.

Filter paper no.	1	2	3	4	5	6	7
Components added	10ppm standard	50ppm standard	Fish sample	Fish sample + 10ppm standard	Fish sample + 20ppm standard	Fish sample + 50ppm standard	Fish sample + 100ppm standard

Histamine analysis was done following the same procedure as for other samples.

Calibration using standard addition method

The standard addition method was done to observe whether there was interference in the samples due to matrix effects (Figure 5). To a fixed volume of sample aliquots, increments of standards with increasing concentration were added. As the concentration of the standards was increased, the matrix of the sample became smaller in comparison to the matrix of the standards. At higher concentrations of standards, the sample matrix becomes irrelevant, therefore matrix effects due to sample matrix becomes insignificant. Therefore the degree of linearity of the trend line provides a measure of matrix affects; the higher the linearity the smaller is the matrix effects. According to the results obtained

for the standard addition method a graph of *volume of histamine added against concentration of histamine* as detected by the JBB histamine checker was constructed as shown by figure 7.1.

In the graph R^2 is the coefficient of determination or correlation coefficient, it provides a measure for the degree of linearity. If R^2 equals 1, this would mean that there is zero matrix effect (which is rarely the case). In this case R^2 was found to be 0.9984 (Figure 7.1), which suggests that there is a good degree of linearity or correlation between the points or analytes. It also suggests that there is some matrix effect involved but that it is quite small. Therefore, although results obtained by the JBB histamine checker will not be a hundred percent accurate, it will still be quite close to the actual value, so it will provide dependable results.

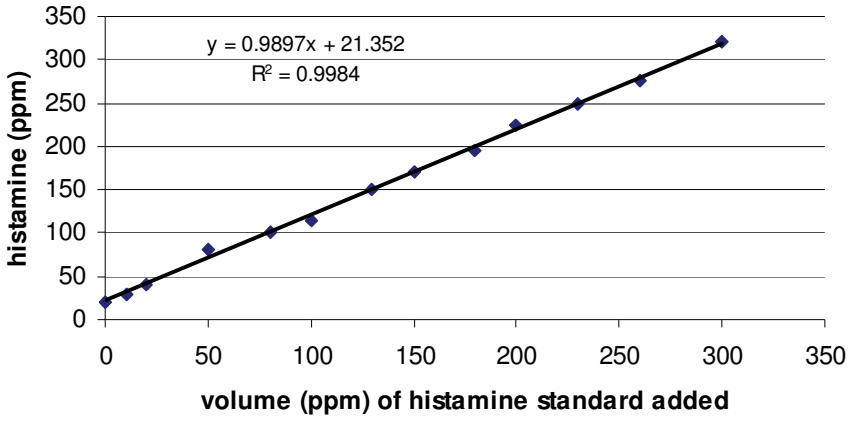


Figure 7.1: Graph of standard addition method for calibration of the JBB histamine checker

Procedure adapted from Singh and Bowry, (2005).

Appendix 7.10: Glycerol preservation technique

Materials required were sterile-autoclaved glycerol at the concentration of 25-80% (working range). It is recommended to use 25-30% concentration of glycerol because it is more economical and easy to work with.

Materials used were:

1. 30% sterile Glycerol
2. 24-hour-old microbial broth cultures
3. Double sterilized (autoclaved and UV treated) 1.5ml Eppendorf tubes
4. Sterile 1000 microlitre pipette tips (with filter is better)
5. Pipette filler: 1000microlitre volume
6. Fine tip permanent marker (for labelling)
7. Sterile double distilled water, or sterile MilliQ water or sterile RO water

Procedure:

The preservation is based on the working ratio of microbial broth culture: 30% glycerol of 2:1,,,,,that is,,,,200 µl of broth culture is added to a tube with 100 µl of 30% glycerol.

However, the volumes added could be altered as long as the ratio is maintained. For example, it is advisable to use 600 µl of broth culture with 300 µl of 30% glycerol (ratio; 600:300= 2:1).

Therefore, the final volume of sample and glycerol was kept to about 1ml only in the tube before storing at –20ºC or –70ºC. Also, the tubes should always be properly with fine tip permanent markers as labels will come off and degrade at such low temperatures.

All the cultures were stored in duplicates at –70ºC and a third set at –20ºC.

Appendix 7.11: Subculturing methodology

Imported bacterial cultures were received in powdered form in an ampoule. The following procedure was employed in reviving the cultures.

Opening an ampoule

A file cut was made on the ampoule near the middle of the cotton wool plug. A broken off Pasteur pipette was heated in a Bunsen flame till red hot and molten and quickly placed on the file cut to crack the glass. The pointed end of the ampoule was discarded and the cotton wool plug placed into disinfectant. The open end of the ampoule was flamed and with a pasteur pipette, about 0.5 ml (15 drops) of broth (cooked meat broth for *C. perfringens* and nutrient broth/heart or brain heart infusion broth for others) was added and the contents was mixed carefully. The suspension was transferred to suitable solid or liquid media, placing the numbered strip on the surface of the solid medium. The media was incubated at appropriate temperature and atmospheric conditions

Clostridium perfringens

0.5 ml of chocolate broth/brain heart broth was used to extract the cultures. Using a micropipette the cultures were transferred and streaked with a loop on blood agar. After streaking the cultures were placed in an anaerobic jar with a deoxygenating sachet to remove O_2. The plates were incubated at 37^oC.

Method of preparing blood agar

Blood agar was used as a plating medium for *C. perfringens.* 100 ml of nutrient agar was prepared and autoclaved; consequently its temperature was brought down to 48^{o}C and 10 ml of Ox blood was added. The contents were mixed well taking precaution not to damage the blood cells. The mixture was poured into petri plates and McCartney bottles for agar slants and left to cool and solidify.

Method of preparing chocolate broth

50 ml of nutrient broth was prepared and into it 5 ml of Ox blood was poured. The contents were mixed, poured into McCartney bottles and autoclaved.

Morganella morganii, *Vibrio alginolyticus*, *Hafnia alvei* and *Enterobacter aminigenus*

10 Tryptic Soy Agar (TSA) agar plates were prepared and 15 TSA slants and 5 nutrient broth. 0.5 ml nutrient broth was used to extract the cultures. Cultures were streaked with a loop on TSA plates. Plates were incubated at 37^{o}C.

Photobacterium damselae

For reviving *P. damselae* marine agar was used. 5 marine agar plates, 5 marine agar slants and 3 nutrient broths were prepared. 0.5 ml nutrient broth was used to extract the cultures the cultures were streaked with a loop on marine agar. Plates were incubated at 25^{o}C.