Antibacterial Property of ripe *Averrhoa bilimbi* juice against *Staphylococcus aureus*

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by

John Amstel M. Barrios
Ian Louise O. Celestino
Fourth Year - Photon

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APPROVAL SHEET

This paper herein entitled:

Antibacterial Property of ripe *Averrhoa bilimbi* juice against *Staphylococcus aureus*

Prepared and submitted by John Amstel M. Barrios and Ian Louise O. Celestino in partial fulfillment of the requirements in Science Research 2, has been examined and recommended for acceptance and approval.

FLORDELIZA T. REMONDE
Science Research 2 Adviser

Approved by the members of the Science Research Committee on March 2012.

EDWARD C. ALBARACIN

MIALO C. LACADEN

ARIS C. LARRODER

HAROLD P. MEDIODIA

ERIKA EUNICE P. SALVADOR

Accepted in partial fulfillment of the requirements in Science Research 2.

EDGAR F. ALMERO, Ph.D.
OIC – Director- PSHSWVC

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Barrios J.A.M. and Celestino I.L.O.

Philippine Science High School-Western Visayas Campus, Bitoon, Jaro, Iloilo City

ams_jhn@yahoo.com, il_celestino@yahoo.com

ABSTRACT

This study was conducted to determine the antibacterial effects of ripe Averrhoa bilimbi juice on *Staphylococcus aureus* thru computing the bacterial reduction after a contact time of 36 hours.The ripe bilimbi samples were collected and then extracted to obtain the juice for the study. The pure *Staphylococcus aureus* culture was then prepared using the dilution method, 3 replications were made and labelled after the dilution. The extracted juice was then mixed with the bacteria in the labelled culture plates. The plates were incubated.Results showed that all replications of the culture plates in contact with the ripe bilimbi juice inhibited the growth of *Staphylococcus aureus* after 36 hours of incubation. The culture plates were placed under a colony counter to count the colony forming units. The juice exhibited a 100% inhibition in bacterial population in the culture plates.Through the observation of the culture plates under a colony counter, it was determined that ripe *Averrhoa bilimbi* juice has an antibacterial property against *Staphylococcus aureus*.

Keywords: colony forming units, serial plate dilution, bacterial reduction

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Ian Louise O. Celestino

John Amstel M. Barrios

Fourth Year - Photon

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CHAPTER 1
INTRODUCTION

A. Background of the Study

Averrhoa bilimbi is a small tree growing 5 to 12 meters high. Its leaves are pinnate, 20-60 cm long, with hairy rachis and leaflets. Its leaflets are opposite to each other, has 10 to 17 pairs, oblong, and 5 to 10 cm in length. The flowers are about 1.5 cm long, and slightly fragrant. The fruit is green and edible, about 4 cm long, subcylindric with 5 obscure, broad, rounded, longitudinal lobes (www.stuartxchange.org).

The components of bilimbi are: 94.2-94.7 g Moisture, 0.61 g Protein, 0.6 g Fiber, 0.31-0.40 Ash, 3.4 mg Calcium, 11.1 mg Phosphorus, 1.01 mg Iron, 0.035 mg Carotene, 0.010 mg Thiamine, 0.026 mg Riboflavin, 0..302 mg Niacin and 15.5 mg Ascorbic acid *food value per 100 g of edible portion. Bilimbi has up to 65 to 75% alcohol in the juice of its fruit (waynesword.palomar.ed). *Averrhoa bilimbi* has pH, titratable acidity and total soluble solids of 1.94, 2.9% and 3.00 respectively (South Indian Horticultural Association "Value addition to under exploited *Averrhoa* fruits.").

In the Philippines, leaves are applied as a paste when itching and on pimples, swellings of mumps and rheumatism, and on skin eruptions. Leaves are applied on bites of poisonous creatures. Malayans take fresh, or fermented leaves as a treatment for venereal diseases. A leaf infusion is a remedy for cough and also taken after childbirth as a tonic. A leaf decoction is taken to be effective against cough and thrush. The fruit is also used for piles. A paste of pickled bilimbi is smeared all over the body to hasten recovery after fever. The fruit conserve is used as a treatment for cough, beri-beri and biliousness. The syrup prepared from the fruit is taken as a cure for fever and inflammation and to stop rectal bleeding and alleviate internal hemorrhoids (Jayaweera, 1982, Kirtikar, et al, 1935).

Pushparaj et al. (2000) reported on the hypoglycemic, hypotriglyceridemic, anti-lipid peroxidative and anti-atherogenic properties of ethanol extracts of bilimbi. A suggestion for

bilimbi leaves as a potential source for the isolation of active principle(s) for oral anti-diabetic therapy was made by Tan et al. (2005).

Staphylococcus aureus is a non-motile, spherical, Gram positive microscopic bacterium (coccus) 0.5 to 1.0 μm in diameter which on microscopic examination appears in pairs, short chains, or grapelikeclusters. *Staphylococcus aureus* is often found closely associated with the human body. It may also be found in many parts of our environment, including dust, water, air and faeces and on clothing or utensils. Although *S. aureus* is an important pathogen, many healthy people carry it as part of the normal population of micro-organisms associated with the nose, throat, perineum or skin. The carrier rate varies in different populations. The nasal passages are reported to harbour *S. aureus* in 10-50% of the healthy population. *Staphylococcus aureus* causes several infections that compromise food safety because of their frequency and the fact that they do not necessarily prevent the infected person from working. Various types of skin eruptions and inflammations (boils, acne, styes, etc.) and wounds, sometimes as small as minor damage around fingernails, can harbour large numbers of the organisms. *Staphyloccocus aureus* can also cause respiratory infections or may become established in the gut, causing enteritis.*Staphylococcus aureus* is a hardy organism that withstands desiccationwell, and can survive in dust and on dry metal, glass, or porcelain surfacesfor long periods of time.

This organism has a high heat resistance for anon-sporing bacteria, with D values at 60°C being reported to range from 2to 50 minutes depending on the food.*S. aureus* grows between 7 and 47°C, with an optimum of 30-37°C. Enterotoxins are produced between 10 and 46°C, with an optimum of35-45°C. Enterotoxin production is substantially reduced at 20-25°C. It isgenerally accepted that enterotoxin production is unlikely to occur at temperatures below 10°C.Bactericidal soaps and creams can be useful in reducing the carriage of *S.aureus*on hands. Antibiotic or antiseptic cream may be of use to treatworkers who have high levels of *S. aureus* in their nose. In some instances,limiting the contact that chronic carriers have with food may be the bestoption.Using utensils and disposable gloves is certainly advantageous (Bremer P.J., et al. 2004).

Bilimbi has been tested and known to have antimicrobial properties against Staphylococcus aureus. The components of its leaves and fruits extract have been tested by

means of the Kirby-Bauer method against gram-positive bacteria such as *Escherichiacoli, Staphylococcus aureus, and Salmonella enteriditis*(Andales et al). Although its antimicrobial properties have been investigated, the method used does not show how fast the test material reacts with the test organism. The Time Kill study is such method that can describe how fast a test material reduces the population of the test organism after time materials.

B. Statement of the Problem

This study aims to test the antibacterial property of ripe *A. bilimbi* juice against *S. aureus*.

C. Objectives

Specifically, this study aims to:

1. count the Colony Forming Units in the culture plates with and without ripe *Averrhoa bilimbi* juice.
2. compute for the microbial reduction of the culture plates with and without ripe *Averrhoa bilimbi* juice.
3. compare the microbial reduction of the culture plates with and without ripe *Averrhoa bilimbi* juice.

D. Scope and Delimitations

The focus of this study is to determine the antibacterial property of ripe *Averrhoa bilimbi* juice by determining the microbial reduction in the colony forming units. Only ripe fruits were used instead of a combination of ripe and unripe fruits. This was to eliminate variables that could interfere with the results of this study. In addition, all the fruits that were used in this study were taken from a single tree rather than buying from a local market. This was also to eliminate variables that could affect the quality of the fruits.

The Time Kill Study will not be used in this study because of the availability of a neutralizing broth. The contact time of ripe *A. bilimbi* on *S. aureus* will be limited to the incubation time of 36 hours. A neutralizing broth will not be used to stop the reaction between the ripe bilimbi juice and *S. aureus* due to the unavailability of the chemical.

E. Significance of the study

The ripe *Averrhoa bilimbi* juice was proven to have antibacterial properties against *Staphylococcus aureus.* Thus the juice of ripe bilimbi fruits can be used to treat S. aureus infections on the surface of the skin. In areas where resources such as alcohol, antiseptics and disinfectants are scarce, the juice of ripe bilimbi fruits could be used as a substitute. Furthermore, products such as hand sanitizers, lotions and skin moisturizers can be formulated from the juice of the ripe bilimbi fruits.

Definition of Terms

Averrhoa bilimbi – is a small tree growing 5 to 12 meters high, closely related to the carambola but quite different in appearance, manner of fruiting and uses. The fruit, is green and edible, about 4 cm long, subcylindric with 5 obscure, broad, rounded, longitudinal lobes.

Time Kill study – is a basic microbiology method for assessment of Antimicrobial Activity of An Antimicrobial Test Material or Disinfectant. The Kill Time Test is carried out to evaluate the microbial reduction by a disinfectant against selected bacteria or fungi. Various organisms are studied depending upon the type of analysis and test material, however, most common organisms tested include:*Staphylococcus aureus, Salmonella cholerasuis, Pseudomonas aeruginosa, E.coli, Aspergillusniger,*and *Trichophytonmentagrophytes*.

CFU or Colony Forming Unit – is a measure of viable cells in which a colony represents an aggregate of cells derived froma single progenitor cell. It is used to determine the number of viable bacterial cells in a sample per milliliter. Hence, it tells the degree of contamination in samples of water, vegetables, soil or fruits, or the magnitude of the infection in humans and animals.

Antimicrobial – is any substance that possesses the property of being lethal to bacteria and other unicellular organisms.

Staphylococcus aureus –is a non-motile, spherical, Gram positive microscopic bacterium (coccus) 0.5 to 1.0 μm in diameter which on microscopic examination appears in pairs, short chains, or grapelikeclusters.

CHAPTER 2

REVIEW OF RELATED LITERATURE

A. *Averrhoa Bilimbi*

A.1. Physical properties of *A. bilimbi*

Fruits are fairly cylindrical with five broad rounded longitudinal lobes, and produced in clusters. During maturity stage occurs the maximum increase in fruits weight and dimensions, and their external green colour changes into light yellow (de Lima et al 2001).

Figure 1. Averrhoa bilimbi

A.2. Chemical properties of *A. bilimbi*

The components of bilimbi are: 94.2-94.7 g Moisture, 0.61 g Protein, 0.6 g Fiber, 0.31-0.40 Ash, 3.4 mg Calcium, 11.1 mg Phosphorus, 1.01 mg Iron, 0.035 mg Carotene, 0.010 mg Thiamine, 0.026 mg Riboflavin, 0..302 mg Niacin and 15.5 mg Ascorbic acid *food value per 100 g of edible portion. Bilimbi has up to 65 to 75% alcohol in the juice of its fruit (waynesword.palomar.ed). *Averrhoa bilimbi* has pH, titratable acidity and total soluble solids of 1.94, 2.9% and 3.00 respectively (South Indian Horticultural Association "Value addition to under exploited *Averrhoa* fruits.").

A.3. Uses of *A. bilimbi*

In the Philippines, leaves are applied as a paste when itching and on pimples, swellings of mumps and rheumatism, and on skin eruptions. Leaves are applied on bites of poisonous creatures. Malayans take fresh, or fermented leaves as a treatment for venereal diseases. A leaf infusion is a remedy for cough and also taken after childbirth as a tonic. A leaf decoction is taken

to be effective against cough and thrush. The fruit is also used for piles. A paste of pickled bilimbi is smeared all over the body to hasten recovery after fever. The fruit conserve is used as a treatment for cough, beri-beri and biliousness. The syrup prepared from the fruit is taken as a cure for fever and inflammation and to stop rectal bleeding and alleviate internal hemorrhoids (Jayaweera, 1982, Kirtikar, et al, 1935).

Bilimbi is generally too acidic to be eaten as a fresh fruit. The green, uncooked fruits are prepared as a delicacy which is served with rice. Sometimes it is an accompaniment for fish and meat. Fruits yield about 60-76% juice having a low pH. Bilimbi is used to make chutney, salad, jam, sherbet, pickle and curry. To reduce acidity, it maybe first pickled and soaked in water overnight, or in salted water for a shorter time, then boiled with sugar to make jam, or an acid jelly.

Bilimbi, due to its high acidity is used to clean the blade of *kris*(dagger), and serves as mordants in the preparation of an orange dye for silk fabrics. It's juice, because of its oxalic acid content, is useful for bleaching stains from the hands and rust from white cloth, and tarnish from brass. Billimbi leaves are traditionally used to accelerate the ripening of banana fruits. Bilimbi fruits are also used as toothpaste (Pushpakumara D.K.N.G.).

A.4. Difference between mature and young *A. bilimbi*

Ripe bilimbi fruits have thin skin, yellowish-green colour, soft texture and a peculiar smell, which resembles the one of carambola, a fruit of the same botanical family. Half-ripe fruits have firm texture and imperceptible smell. Ripe bilimbi fruits had higher levels of total soluble solids than half-ripe fruits, independently of the season in which they were harvested. Half-ripe bilimbi fruits are more acidic than ripe bilimbi fruits (de Lima et al 2001).

B. *Staphylococcus Aureus*

B.1. Characteristics of *S. aureus*

Staphylococcus aureus is a non-motile, spherical, Gram positive microscopic bacterium (coccus) 0.5 to 1.0 μm in diameter which on microscopic examination appears in pairs, short chains, or grapelike clusters.

Staphylococcus aureus is often found closely associated with the human body. It may also be found in many parts of our environment, including dust, water, air and faeces and on clothing or utensils. Although *S. aureus* is an important pathogen, many healthy people carry it as part of the normal population of micro-organisms associated with the nose, throat, perineumorskin. The carrier rate varies in different populations. The nasal passages are reported to harbour*S. aureus* in 10-50% of the healthy population (http://www.textbookofbacteriology.net/staph.html).

B.2. Culturing of *S.aureus*

In general, *S. aureus* grows between 7 and 47°C, with an optimum of 30-37°C (Bremer P.J. et al 2004).*S. aureus* can also grow at a temperature range of 15 to 45 degrees and at NaCl concentrations as high as 15 percent(www.textbookofbacteriology.net/staph.html). One mL of Staphylococcus aureus diluted solution is to be dispensed into a petri dish and mixed gently with culture media until the agar settles.

C. Antimicrobial tests

C.1 Antimicrobial Efficacy — In Vitro — Time Kill Study

A 4.5mL aliquot of undiluted KIMCARE* Moisturizing Instant Hand Sanitizer will be placed in a sterile glass jar at 25C. An inoculum of 0.5mL of a broth culture containing approximately 108 CFU/mL of the test organism is to be added to the jar. The contents of the jar will be then mixed.

After the appropriate test time, neutralizer will be added to the jar to stop the activity of the antiseptic and the contents stirred for 60 seconds. Serial dilutions are prepared, plated

and incubated. This process is completed for each of the organisms (Kimberly-Clark Professional).

C.2 Antimicrobial Efficacy — In Vitro —Minimum Inhibitory Concentration (MICs)

The minimum concentration of the product which will inhibit growth of bacterial or fungal organisms in a laboratory study will be evaluated as an objective of this study.

For each organism to be tested, serial dilutions of KIMCARE* Moisturizing Instant Hand Sanitizer are prepared. Each dilution within a series is to be exposed to approximately 5 x 104 colony forming units of the organism. Theplates are then incubated. The Minimum Inhibitory Concentration (MIC) reported will be the lowest dilution in which growth of the organism is completely inhibited (Kimberly-Clark Professional).

C.3 Skin Moisturization

The moisturization potential of aKIMCARE* Moisturizing Instant Hand Sanitizer (Kimberly-ClarkProfessional) is to be evaluated as an objective to this study.

Skin conductance values are to be obtained from testing sites on the volar forearms atbaseline (prior to hand sanitizer application), 30 minutes and at 2 hours using askin conductance meter. Each subject had an untreated control and they also each tested KIMCARE* Moisturizing Instant Hand Sanitizer and a leading instant hand sanitizer.

C.4. Antimicrobial Efficacy — in vivo — Glove Juice Test

The ability of the product to reducetransient microbial flora on the skin is to be determined as an objective of this study.

Test Description:

1) Pre-Test Period

Subjects will refrain from using any antibacterial products for one week prior to the study and will wear rubber gloves while doinghousehold chores.

2) Baseline

Three 1.5mL aliquots of Serratiamarcescens (minimum of 108 organisms per mL) are to be added toeach subjects hands. After eachaliquot is added, the suspension will be rubbed thoroughly over the surface of both hands for 20 seconds.(applicationand rubbing)Between each aliquot, hands are then allowed to dry.Plastic bags withlow bioburdenwill be placed on each of the subject's hands. A 75mL aliquot ofstripping solution will be added to eachbag. The bag will be secured and massaged for 1 minute. An aliquot of the fluid will then be aseptically obtained to determine the baselinebacterial count. The subjects hands will be washed thoroughly with anon-medicated soap and dried.

3) Treatment Procedure

Hands will be contaminated with *Serratiamarcescens*. After the hands have been contaminated, they will be treated with the test product. Five mLof the product will be applied to the subject's hands and rubbedvigorously over hands and lowerforearms until dry. A second 2.5mL aliquot will be applied and allowed to dry. Particular attention is paid to the nails andinterdigital spaces. Water and ortowelling are will not used in this process.This procedure will be repeated 10 times with atleast 5 minutes between eachtreatment. Within 5 minutes of completion of thefirst ,third, seventh and tenth treatments, hands will be sampled.

C.5. Antimicrobial Susceptibility testing

C.5.1. Kirby – Bauer disk diffusion test

The Kirby Bauer test is aqualitative assay whereby discs of paper will be impregnated with a singleconcentration of different antibiotics. The discs will be placed on thesurface of anagar plate that has been inoculated with test bacteria. During incubation, theantibiotics will diffuse outward from the discs creating a concentration gradient. After18-24 hours, the zone diameter (zone of inhibition) will be measured and referencetables are to be used to determine if the bacteria are Sensitive(S), Intermediate (I) orResistant (R) to the antimicrobial drugs.

D. Obtaining Colony Forming Units per mL

$$\left(\frac{Colony\ forming\ units}{ml}\right) x\ dilution\ factor = \frac{cfu}{ml}$$

Figure 2. Formula for Colony Forming Units

E. Plate Dilution Method

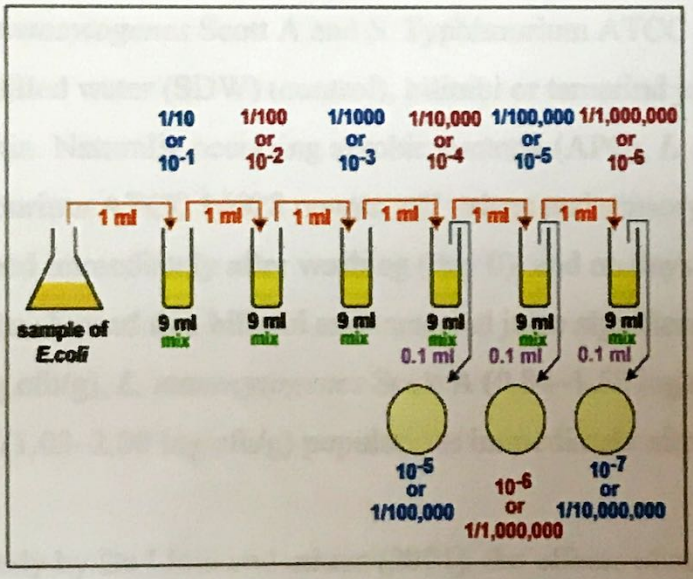

Figure 3. Plate Dilution Method

Six dilution tubes are prepared, containing 9mL of distilled water. Each tube will be labelled with its dilution factor (1/10, 1/100, 1/1000, 1/10000, 1/100000, 1/1000000). 1.0mL of the *S .aureus* broth culture issyringed into the dilution tube labelled 1/10. The liquid is drawn up and down in the syringe several times to rinse the syringe and help mix. The tube is then flamed and capped. The tube is mixed by holding the tube in one hand and tapping the bottom. This was to assure an even distribution of the bacteria. The same procedure is used to transfer 1mL of the 1/10 dilution tube into the 1/100 dilution tube. This procedure was then repeated until 1 mL of the 1/100000 dilution tube was dispensed to the 1/1000000 dilution tube.

F. Related Studies

In a study done by Mackeen and others (2009), the potential of using juice of bilimbi (*Averrhoa bilimbi L.*) and tamarind (*Tamarindusindica L.*) to reduce *Listeriamonocytogenes* Scott A and *Salmonella* Typhimurium ATCC 14028 populations on raw shrimps after washing and during storage (4 °C) was investigated. Bilimbi (*A. bilimbi*) significantly reduced , *L. monocytogenes* Scott A and and *S.* Typhimurium ATCC populations immediately after washing. . The uninoculated raw shrimps and those inoculated with ~ 9 log cfu/ml of *L. monocytogenes* Scott A and *S.* Typhimurium ATCC 14028 were washed (dipped or rubbed) in distilled water (SDW) (control), bilimbi or tamarind juice at 1:4 (w/v) concentrations for 10 and 5 min. Naturally occurring aerobic bacteria (APC), *L. monocytogenes* Scott A and *S.* Typhimurium ATCC 14028 counts, pH values and sensory analysis of washed shrimps were determined immediately after washing (day 0), and on days 3 and 7 of storage. Compared to SDW, results showed that bilimbi and tamarind juice significantly ($p < 0.05$) reduced APC (0.40–0.70 log cfu/g), *L. monocytogenes* Scott A (0.84–1.58 log cfu/g) and *S.* Typhimurium ATCC 14028 (1.03–2.00 log cfu/g) populations immediately after washing (0 day).

In a study by De Lima and others (2001), the effects of maturity stages on the physicochemical characteristics ofbilimbi (*Averrhoa bilimbi* L.) were investigated. Ripe bilimbi fruits have higher vitamin C content thanhalf-ripe ones. This pattern has also been observed onguava(Esteves et al., 1984) and camu-camu (*Myrciariadubia*) (Zapata&Dufour, 1993). Inother

fruits, like acerola (*Malpighiasp*), theopposite happens: the highest levels of vitamin C arefound inhalf-green and green fruits (Carvalho&Manica, 1993). The levelsof vitamin C in ripeand half-ripe bilimbi fruits varied from 20.82to 60.95 mg/100g, as shown on Table 1. Thevitamin C levels inripe and half-ripe bilimbi harvested in the same season werestatistically different. Ripe fruits harvested during dry season hadthe highest vitamin C level. This result may have been influencedby climatic factors. As expected, during the dry season, anincrease of photosynthetic activity (induced by rising solarradiation and reduced average seasonal rainfall) produces higherlevels of vitamin C, since this vitamin is synthesised from hexosesugarprecursors (Harris, 1977). In spite of the low levels ofvitamin C in bilimbi, the ripe fruit has significant amount of thisvitamin. Therefore, the medicinal use of this fruit against scurvy, which was recommended by Corrêa (1926) and Wong & Wong(1995), can be justified.

In a study done by Andales et al. (2008), they used the leaf and fruit extracts of *Averrhoa bilimbi* as an antibacterial agent that is more or equally effective than the commercialized one and at the same time, available in our environment. Ten Replicates on ten Petri dishes were prepared for each bacteria. Pure cultures of the three bacteria were spread on the replicates. After extracting the needed substances from the kamias leaves and fruit extract, the researchers were able to begin the experimentation. Six treatments were made in the set-up-Treatment A as 100% water served as the negative control; Treatment B as 100% Kamias leaves extract; Treatment C as 50% Distilled water and 50% Kamias leaves extract; Treatment D as 100% antibiotics was used as the positive control because of its known antibacterial properties; Treatment E as 100% Kamas fruit extract and Treatment F as 50% Distilled water and 50% Kamias fruit extract. The disk-diffusion test was admonished to the replicates and the data were recorded after 24 hours of incubation at 35.5 degrees Centigrade of all the treated dishes. The data gathered were subjected to Analysis of Variance (ANOVA) and Scheffe's Test. Results of statistical analyses show that Treatment B and Treatment E is equally effective with Treatment D for Escherichia coli, Staphylococcus aureus and Salmonella enteritidis. This study led to the conclusion that Kamias leaves and fruit extract is an effective antibacterial against Escherichia coli, Staphylococcus aureus and Salmonella enteritidis.

CHAPTER 3

METHODOLOGY

A. Overview of the Study

The objective of the study was to count the colony forming units of *Staphylococcus aureus* with and without exposure to ripe *Averrhoa bilimbi* juice was counted.

After 36 hours of incubation and contact time, the colony forming units of the bacteria were counted under a colony counter. The bacterial reduction was computed by comparing the colony forming units of the culture plates of pure *Staphylococcus aureus* with the culture plates exposed to ripe *Averrhoa bilimbi* juice.

The study was conducted at the West Visayas State University Biology laboratory during the month of December 2011.

B. Materials and Equipment

Autoclave
12 test tubes
test tube rack
alcohol lamp
2 metal basins
7 plastic culture plates
16 5mL sterile syringes
4 gallon of distilled water
2 sterile surgical gloves
2 surgical masks
towel
incubator

C. Gathering of Materials

C.1. Collection of *A. bilimbi fruits*

The ripe *Averrhoa bilimbi* fruits have been gathered from a tree in Celestino's house. The unripe *A. bilimbi* fruits were separated from the mature ones. The maturity of the *A. bilimbi* fruits was subjectively determined by color development. Unripe fruits have a light green hue, while ripe fruits are yellowish green (de Lima et al 2001).

C.2 Collection of *Staphylococcus aureus*

The *S. aureus* culture was provided by Prof. Celia P. Magno of West Visayas State University. The S. aureus culture was in a liquid suspension in a test tube.

C.3. Collection of other materials

The autoclave, test tubes, test tube rack, alcohol lamp, metal basins, towel, and incubator were provided by the West Visayas State University Biology laboratory.

The sterile plastic culture plates and 5ml sterile syringes were bought from Roest Marketing. The distilled water, sterile surgical gloves and surgical masks were bought from Rose Pharmacy.

The Mannitol Salt Agar was bought from the West Visayas State University laboratory.

D. Preparation of materials

D. 1. Preparation of Equipment

All glass materials were autoclaved at 121°C for 15 minutes including the towel for it is to be used in extracting the juice from ripe *A. bilimbi*.

D. 2. Preparation of *Averrhoa bilimbi*

All fruits were placed on a table and sorted according to color. All areas of imperfection on the fruits were removed using a cutter. The ripe fruits were placed in a metal basin and washed with distilled water. The ripe fruits were then rinsed in another metal basin with distilled water.

D. 3. Preparation of Solutions

D. 3. 1 Preparation of Mannitol Salt Agar

Eleven grams of the Mannitol Salt Agar powder were suspended in 100 mL of purified water in a 1L glass beaker. The mixture was then mixed. The mixture was heated with frequent agitation and boiled for 1 minute in a hot plate. Each of the 6 petri dishes was dispensed with 9 mL of MSA.

E. Ripe *Averrhoa bilimbi* juice

The ripe A. bilimbi fruits was placed in a sterilized towel and squeezed of its juice. The extracted juice was then placed in a sterile plastic petri dish.

F. *Staphylococcus aureus*

F. 1. Culture of *S. aureus*

The *S. aureus* was cultured by inoculating the bacterial suspension in the culture media. Müller-Hinton agar was used for the culture of pure *S. aureus* while Mannitol Salt agar was used for the *S. aureus* culture with ripe *A. bilimbi* juice.

F. 2. Determining CFU/ mL

Six dilution tubes were prepared, containing 9mL of distilled water. Each tube will be labelled with its dilution factor (1/10, 1/100, 1/1000, 1/10000, 1/100000, 1/1000000). 1.0mL of the *S .aureus* broth culture wassyringed into the dilution tube labelled 1/10. The liquid was drawn up and down in the syringe several times to rinse the syringe and help mix. The tube was then flamed and capped. The tube was then mixed by holding the tube in one hand and tapping the bottom. This was to assure an even distribution of the bacteria. The same procedure was used to transfer 1mL of the 1/10 dilution tube into the 1/100 dilution tube. This procedure was then repeated until 1 mL of the 1/100000 dilution tube was dispensed to the 1/1000000 dilution tube.

A new syringe was used to transfer 1mL from each of the last three dilution tubes to three petri dishes containing 9mL of MSA each. Each of the plates was labelled according to its dilution factor. The plate was spun for 30 seconds to distribute the 1mL of dilution evenly over the entire surface. This procedure was then repeated for the other petri dishes. The petri dishes were covered and incubated upside down at 37°C for 48 - 36 hours. After 36 hours, the number of colonies in each dish was recorded. The total colony forming unit/ ml in the pure broth culture was determined by multiplying the number of colonies by the dilution factor.

G. Determining the Microbial Reduction

The *S. aureus* cultures will be in contact with the ripe *A. bilimbi* juice for the whole incubation period of 24 – 36 hours. After 36 hours of incubation, the plates were placed under a colony counter to count the colony forming units (CFU) of bacteria. All circular dots of the same color and size were counted as one colony forming unit.

H. Handling and Disposal

All plates will be put in a basin with Zonrox and boiled at 100°C until all agar melts. The plates will be then placed in a ziplock bag prior to its disposal in the garbage can.

CHAPTER 4

RESULTS AND DISCUSSION

This study was conducted to determine whether the juice of ripe *Averrhoa bilimbi* fruits have antibacterial effects on *Staphylococcus aureus*. The results were obtained by determining the number of viable cells in which a colony represents an aggregate of cells derived from a single progenitor cell. Hence it tells the degree of contamination in the samples of agar.

Based on the data acquired from the experiment, the results show that there is a significant result in the bacterial reduction of *S. aureus* colonies. All culture plates exposed to ripe *A. bilimbi* juice exhibited no signs of *S. aureus* habitation after being exposed for the incubation period of 36 hours. For the two dilution test tubes of dilution factor 1:100000 and 1:1000000, there were three replicates for plating from each test tube. *Staphylococcus aureus* colonies would appear to be yellow dots of varying sizes on Mannitol Salt Agar medium. There were no signs of *S. aureus* on the culture plates based on the color and selective medium.

	Pure *S. aureus* culture (cfu per mL)	*S. aureus* culture with ripe *A. bilimbi* juice (cfu per mL)	Bacterial reduction (%)
1: 100 000 Dilution factor	8,500,000	0	100%
	11,400,000	0	100%
	8,300,000	0	100%
1: 1 000 000 Dilution factor	3,500,000	0	100%
	2,600,000	0	100%
	3,000,000	0	100%

Table 1. Bacterial reduction of *A. bilimbi* against *S.aureus*

De Lima and others found out the physicochemical properties of ripe and unripe *A. bilimbi* fruits during the dry and wet season. Their study showed that ripe *A. bilimbi* fruits have varying levels of total soluble solids, ascorbic acid, and oxalic acid. It is also known that the fruits also contain alcohol. Its use of a bleaching agent implicate that it is capable of neutralizing bacterial populations apart from it being poisonous to cells. Alcohols kills bacteria by first making the lipids that are part of the outer protective cell membrane of each bacteria cell more soluble in water so that the cell membrane begins to lose its structural integrity and fall apart. As the cell membrane disintegrates, alcohol can then enter the cell and denature proteins within each bacteria thus disrupts important cell functions which causes the cell to die.

In the study conducted by Mackeen and others, the potential of using juice of bilimbi (*Averrhoa bilimbi L.*) and tamarind (*Tamarindusindica L.*) to reduce *Listeria monocytogenes* Scott A and *Salmonella* Typhimurium ATCC 14028 populations on raw shrimps after washing and during storage (4 °C) was investigated. Their results showed that bilimbi significantly reduced naturally occurring anaerobic bacteria *L. monocytogenes* Scott A and *S.* Typhimurium ATCC 14028 populations immediately after washing with bilimbi juice. Andales and others also used bilimbi in the form of leaf and fruit extracts as an antibacterial agent that is more or equally effective than the commercialized one and at the same time, available in our environment. After their experiment of different concentrations of water, pure fruit extract, pure leaf extract and combinations of both, they concluded that *A. bilimbi* extracts were good antibacterial agents against Escherichia coli, Staphylococcus aureus and Salmonella enteritidis.

CHAPTER 5

SUMMARY, CONCLUSIONS AND RECOMMENDATIONS

In this study, the researchers aimed to find out if the juice of the ripe *Averrhoa bilimbi* fruits has an antibacterial effect on *Staphylococcusaureus*. The bacteria was collected from a university laboratory. The juice was extracted from the fruits by means of squeezing with the use of a cotton cloth sanitized in an autoclave. The antibacterial test of *A. bilimbi* on *S. aureus* was done by mixing the juice with 1mL of pure *S. aureus* and diluting the samples in test tubes to a dilution factor of 1:100 000 and 1: 1 000 000, both positive and negative. The positive control was the ripe *A. bilimbi* juice while the negative control was distilled water. Both controls were plated on agar and left to incubate for 36 hours. Through the observation under the colony counter, it was determined that the ripe fruit juice had a significant effect on the test bacteria.

A. Summary

1. The plates of *S. aureus* exposed to ripe *A. bilimbi* juice exhibited no bacterial growth on the culture plates.

2. There is a significant difference among the pure *S. aureus* culture plates and the culture plates exposed to ripe *A. bilimbi* juice.

B. Conclusion

Therefore the researchers conclude that ripe *Averrhoa bilimbi* juice has antibacterial properties against *Staphylococcus aureus*.

C. Recommendations

For further research, the researchers recommend the following:

1. Use of a neutralizer broth to limit the contact time between the juice and the bacteria to minute and specific values. This experiment with neutralizer broth can provide more specific data on how bacteria the juice is able to kill after a preset time.

2. The formulation of a hand sanitizer based on the fruit extract of ripe *A. bilimbi* fruits. A hand sanitizer from the juice would be a good application of the findings of this study.

LITERATURE CITED

Bremer P.J., Fletcher G.G., Osborne C. April 2004. Staphylococcus aureus.New Zealand Institute for Crop & food Research Limited.pages 1-8.

De Lima V.L.A.; Melo E. D. A.; Lima L. D. S. August 2001. Physicochemical characteristics of bilimbi Averrhoa bilimbi L.)Rev. Bras. Frutic.,Jaboticabal v. 23. p. 421-423

Kimberly-Clark® KIMCARE Moisturizing Instant Hand Sanitizer. Technical Bulletin C2008-1168

Wan Norhana et al. 2009.Effects of bilimbi (*Averrhoa bilimbi L.*) and tamarind (*Tamarindus indica L.*) juice on *Listeria monocytogenes* Scott A and *Salmonella* Typhimurium ATCC 14028 and the sensory properties of raw shrimps. School of Land, Crop and Food Sciences, University of Queensland, St. Lucia, Queensland 4067, Australia. Fisheries Research Institute, 11960, BatuMaung, Penang, Malaysia.Queensland Primary Industries and Fisheries, Hamilton, Queensland 4007, Australia.CSIRO Food and Nutritional Sciences, PO Box 3312 Tingalpa DC, Queensland 4173, Australia.

South Indian Horticultural Association. September 2009. Value addition to under exploited Averrhoa fruits.

Textbook of Bacteriology[Internert]. [cited 2011 Aug 11]. Available from: http://www.textbookofbacteriology.net/staph.html

APPENDIX

APPENDIX A: RAW DATA

Table.1 Bacterial reduction

	Pure *S. aureus* culture (cfu per mL)	*S. aureus* culture with ripe *A. bilimbi* juice (cfu per mL)	Bacterial reduction (%)
1: 100 000 Dilution factor	8,500,000	0	100%
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	3,000,000	0	100%

APPENDIX B: PLATES

Plates 1&2.Collection and separation of ripe and unripe bilimbi fruits.

Plate 3. Preparation of bilimbi samples

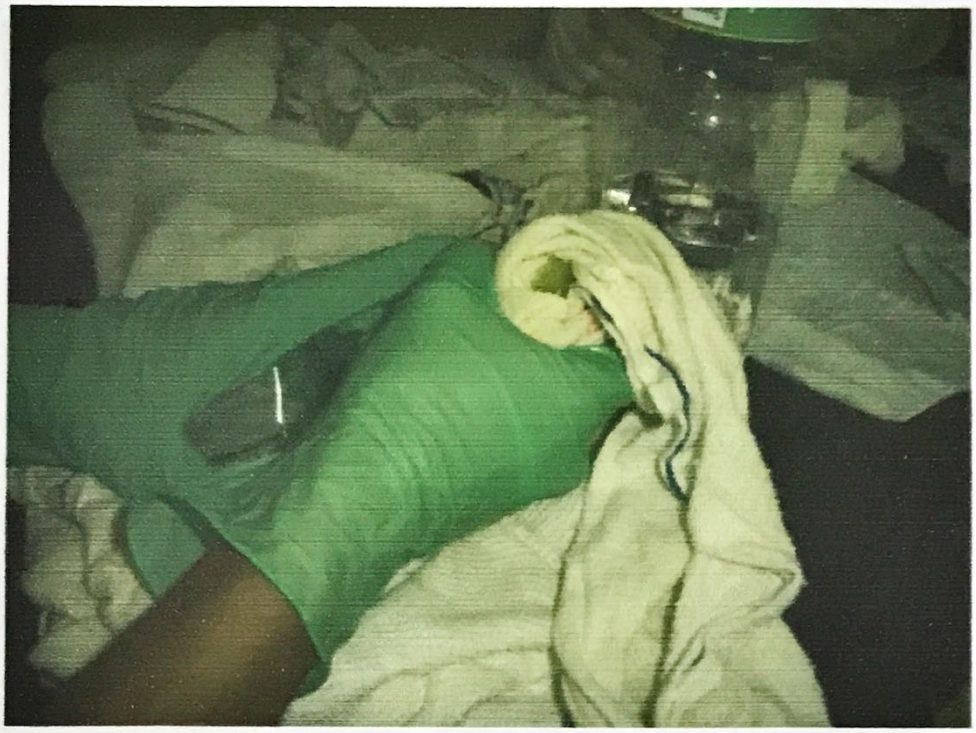

Plate 4.Extracting of bilimbi juice.

Plate 5. Serial Dilution

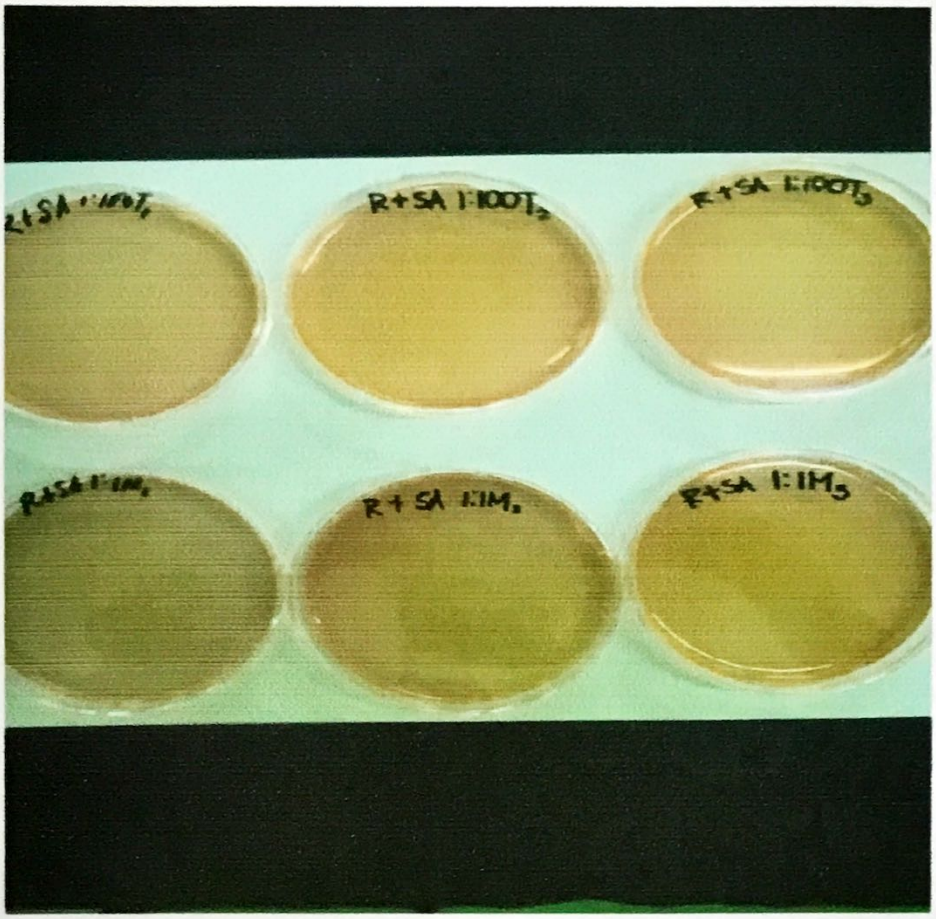

Plate 6. Plating of samples