

## Elucidation of Bactericidal Effects Incurred by *Moringa oleifera* and Chitosan

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### ABSTRACT

The aim of this investigation was to elucidate the potential of two natural, cost-effective, and nontoxic antimicrobial agents, *Moringa oleifera* and chitosan, for use in rural areas chronically plagued with polluted water. Using high-throughput bactericidal assays, the efficacy of the two biocides were evaluated both individually and in combination as a novel disinfectant against *Pseudomonas putida* and *Bacillus subtilis*. Results established *Moringa oleifera* and chitosan as reliable and nontoxic water disinfecting agents at low doses and under typical environmental conditions. Antibacterial activity increased with biocide concentration while increased ionic strength decreased bactericidal activity by reducing antimicrobial agent solubility. Acidic solutions increased the antibacterial activity of chitosan. A novel disinfectant consisting of both *Moringa oleifera* and chitosan demonstrated consistent bactericidal activity but was not significantly greater in efficacy than *Moringa oleifera* and chitosan solutions alone.

**KEYWORDS:** chitosan, *Moringa oleifera*, water purification, biocide, antimicrobial, bactericidal, drumstick tree, Flo peptide, mucoadhesive polymer, high-throughput screening.

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## INTRODUCTION

Water contamination, which plagues the human race with 80% of all diseases (Wilson, 2009), is a recurring hazard worldwide. Advanced water treatment techniques are too costly for many developing nations and many currently-used disinfectants can produce harmful products. Promising organic alternatives for water treatment include the natural disinfectants, *Moringa oleifera* and chitosan. Naturally abundant, biodegradable, and non-toxic, *Moringa oleifera* and chitosan hold great promise as highly effective antibacterial agents. The implementation of these two substances could provide an affordable, effective drinking water disinfection process.

Commonly known and used in rural areas of Africa for water clarification, the *Moringa oleifera* tree, also known as the ‘drumstick’ tree, is easily propagated and widely grown throughout the tropics. The Flo peptide is a specific polypeptide found in *Moringa oleifera* that is both a flocculant and a biocide. The cationic Flo peptide supposedly serves as a highly efficacious immunity response, interacting with the anionic cell membranes of bacterium (Suarez, *et al.*, 2005). This interaction destabilizes the bacterial membrane, causing leakage of cytoplasmic content and killing the bacterial cell. Antimicrobial peptides, such as the Flo peptide, have been reported to act directly and non-specifically upon bacterial membranes, thus hindering their ability to develop resistance. Antimicrobial peptides rarely affect the membranes of cells in multicellular species (Fisch, 2004).

Derived from chitin, an essential and abundant component of exoskeletons, the mucoadhesive polymer chitosan holds potential as a highly effective biocide. Similar to the Flo polypeptide in *Moringa oleifera*, the amine groups in chitosan particles induce a positive charge when in acidic solution (Rhoades and Rastall, 2003) The cationic nature of the chitosan particle disrupts the anionic bacterial cell membranes resulting in leakage of cytoplasmic content and death of the bacterial cell (Rabea, *et al.*, 2003). Chitosan has only recently been studied in terms of bactericidal activity (Rhoades and Rastall, 2003). These studies identified chitosan as an excellent disinfectant because of its low toxicity toward mammalian cells and efficacy as a biocide. Studies of the antibacterial activity of chitosan in food preservation, have been made (Rhoades and Rastall, 2003) but no successful application of chitosan in contaminated aquatic environments has been made.

While separate studies have been conducted on identifying the antibacterial activity of *Moringa oleifera* and chitosan, few attempts have been made to elucidate their combined bactericidal effects (Rabea, *et al.*,

2003). This study is based on the hypothesis that a solution of these two antibacterial agents together will have a greater range of bactericidal activity than solutions of the individual biocides

## **I. OBJECTIVE/HYPOTHESIS**

The focus of the first part of this research was to ascertain optimum conditions for bactericidal activity with respect to pH, biocide concentration and ionic strength for both *Moringa oleifera* and chitosan. Antimicrobial properties were hypothesized to be most prominent at high concentrations and low ionic strengths for both *Moringa oleifera* and chitosan because a low ionic strength resulting in a minimal amount of salt dissolved in solution other dissolved particles. The antimicrobial activity of chitosan was hypothesized to be highest at low pH levels due to its tendency to be more soluble in acidic solutions while *Moringa oleifera* was hypothesized to have an optimum pH level of seven because use of the seeds by African village people appeared to only involve *Moringa oleifera* and water (Folkard, *et.al.*, 1999). The second part of this study evaluated the combined efficacy of *Moringa oleifera* and chitosan in a single solution. It was hypothesized that the two antimicrobial agents together would have a greater bactericidal activity than their bactericidal effects alone.

## **II. METHODOLOGY**

### ***Part I – Preliminary Experiment***

#### **I. Preparation of stock solutions**

A preliminary experiment was developed to determine optimum *Moringa oleifera* and chitosan concentrations. Four 2L dispersing media were prepared, yielding NaCl solutions with the following ionic strengths: 1M, 0.1M, 0.01M, and 0.0M (DI water). Chitosan stock solutions were subsequently prepared and adjusted to 100 mg/L (Folkard, Shaw) by dissolving chitosan in the dispersing media together with acetic acid (HAc). This resulted in 1% HAc stock solutions of 100 mg chitosan/L with ionic strengths of 1M, 0.1M, 0.01M, and 0.0M (DI water). *Moringa oleifera* stock solutions were prepared with the dispersing media to produce a final concentration of 1000 mg/L. *Moringa oleifera* seeds were obtained from Mother Herbs Inc. (Delhi, India) and were husked then crushed with a mortar and pestle. The seed powder produced was sieved through a 0.045 mm metal sieve and dissolved in the NaCl solutions. The resulting solutions were filtered through a 0.45 µm membrane filter (Millipore Corp., Billerica, Massachusetts).

## II. Preparation of bacterial suspension

Two species of bacteria were used. These were the Gram-positive, *Bacillus subtilis*, and the Gram-negative, *Pseudomonas putida*. The bacteria were cultured in growth media for 24 hours at 150 rpm at 25°C on a shaker plate (Lab-line Orbit, Environ Shaker, Melrose Park, Illinois). Luria-Bertani Broth (LB) was used for *B. subtilis* and Tryptic Soy Broth (TSB) for *P. putida*. Subsequently, the bacterial cells were harvested and washed by centrifugation at 4,000X g for eight minutes in autoclaved centrifuge tubes using a Marathon 21K Centrifuge (Fisher Scientific, Pittsburgh, Pennsylvania). Supernatants were discarded and the remaining pellet was resuspended in 3mL of the required dispersing medium. The bacterial cell counts were determined with a hemacytometer and adjusted to  $10^{7.7}$  bacteria/mL using PBS.

## III. Bactericidal Assay of *Moringa oleifera* and chitosan

Using high throughput screening (HTS), samples of chitosan and *Moringa oleifera* solutions were prepared in 96-well black, clear-bottomed microplates using the previously created stock solutions. Row 2 wells were filled with 100% non-viable bacteria to serve as a negative control. Subsequently, Row 11 wells were filled with 100% viable bacteria and served as a positive control. First, 80  $\mu$ L of dispersing medium was dispersed into each well from Columns 2-11 of a 96-well microplate. pH and ionic strength adjusted Chitosan or *Moringa oleifera* solutions (160  $\mu$ L) were dispensed into each well of Column 3. Next, 80  $\mu$ L of solution from each well of Column 3 was dispensed into Column 4. The solutions from Column 4 (80  $\mu$ L) were dispensed into the Column 5 wells and subsequently mixed. This procedure was repeated so that the solutions from Columns 3-10 had half the *Moringa oleifera* or chitosan concentration of the preceding column. A volume of 20  $\mu$ L live *P. putida* suspension was dispensed into the top half of the microplate in Columns 3-11 while *B. subtilis* suspension was dispensed into the bottom half. In Column 2, dead *P. putida* suspension was dispensed in the top 4 wells while dead *B. subtilis* suspension was dispensed into the bottom 4 wells. After incubation for 1h at room temperature, 20  $\mu$ L of PI stain (stains dead cells fluorescent red) and SYTO 9 stain (stains live cells fluorescent) were each dispensed into all wells from Columns 2-11. The microplates were again incubated at room temperature for 15 minutes in the dark and immediately placed in the plate reader. The plates were prepared in the following manner:

- 1) Ionic strength kept constant at 0.01M NaCl, pH varied (3, 5, 7, 9)
- 2) pH kept constant at 7, ionic strength varied (0.00, 0.01M, 0.1M, 1.0M)

The concentrations of *Moringa oleifera* and chitosan were arranged as follows:

	1	2	3	4	5	6	7	8	9	10	11	12
A	Positive control	<i>P. putida</i>									Negative control	
B												
C		1000	500	250	125	62.5	31.625	15.625	7.8125			
D		<i>B. subtilis</i>										
E												
F		1000	500	250	125	62.5	31.625	15.625	7.8125			
G												
H												

**Figure 1. 96-well Microplate** – various concentrations of *Moringa oleifera* (mg/L) were tested using the designated setup

	1	2	3	4	5	6	7	8	9	10	11	12
A	Positive control	<i>P. putida</i>									Negative control	
B												
C		100	50	25	12.5	6.25	3.125	1.5625	0.78125			
D		<i>B. subtilis</i>										
E												
F		100	50	25	12.5	6.25	3.125	1.5625	0.78125			
G												
H												

**Figure 2. 96-well Microplate** – various concentrations of chitosan (mg/L) were tested using the designated setup

#### IV. Construction of Live/Dead Assay

A live/dead assay was constructed in order to create a calibration curve to correspond fluorescence ratios of live to dead bacteria as given by the Fluorescence Microplate Reader to a percentage of live bacteria.

Bacterial cultures of *P. putida* and *B. subtilis* were grown in four 150 mL flasks at 25 °C for 24 hours. The bacterial culture was concentrated through centrifugation at 4,000X g for eight minutes. A volume of 1 mL of bacterial suspensions was added to two 50 mL autoclaved centrifuge tubes containing either 20 mL of 0.85% NaCl (for live bacteria) or 20mL of 70% isopropyl alcohol (for dead bacteria). Samples were then incubated at room temperature for one hour, mixing every 15 minutes. Subsequently, samples were pelleted again by centrifugation under the same conditions. The pellets were resuspended in 20 mL of 0.85% NaCl and centrifuged again. The resulting pellets were resuspended in separate tubes with 2 mL of DI water. Next, 0.5 mL of suspension was distributed in eight 10 mL volumetric flasks (four flasks for live bacteria, four flasks for dead bacteria). Each flask was filled with 0.01M NaCl because only one calibration curve was constructed. Bacterial cell counts were carried out using a hemacytometer and the concentration of the suspension was adjusted to  $10^{7.7}$  bacteria/mL using phosphate buffer saline (PBS). Different proportions of the suspensions were mixed in a 96-well microplate.

Ratio of live:dead cells	Live-cell suspension (μL)	Dead-cell suspension (μL)
0:100	0	200
10:90	20	180
50:50	100	100
90:10	180	20
100:0	200	0

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B		0:100	10:90	50:50	90:10	100:0						
C		0:100	10:90	50:50	90:10	100:0						
D		0:100	10:90	50:50	90:10	100:0						
E							0:100	10:90	50:50	90:10	100:0	
F							0:100	10:90	50:50	90:10	100:0	
G							0:100	10:90	50:50	90:10	100:0	
H												
			<i>P. putida</i>									
			<i>B. subtilis</i>									

**Figure 3.** Volumes corresponding to proportions of live/dead bacteria

**Figure 4.** Arrangement of live/dead assay in one 96-well microplate

### V. Analysis of Microplates

After preparation, the microplates were placed in the FlexStation II Fluorescence Microplate Reader (Molecular Devices, Life Science Instrumentation, Sunnyvale, California) and their fluorescence measure using an excitation wavelength of 485 nm and an emission wavelength for green fluorescence of 530 nm and for red fluorescence of 630 nm. The results from the microplates were compared to the previously-

constructed calibration curve and the percentage of live bacteria in each well was determined to identify the optimum conditions for bactericidal activity.

**Part II – Determining the combined effect of chitosan and *Moringa oleifera***

I. Preparation and Analysis of Samples

Using HTS, one 96-well microplate of combined *Moringa oleifera* and chitosan solutions was prepared at 0.01M and pH 7, the conditions of a realistic aquatic environment. Preparation of the microplate followed the previously described procedures except that instead of 80 µL of one disinfectant solution in each well, 40 µL each of 500 mg/L *Moringa oleifera* solution and 50 mg/L chitosan solution in Column 4 were mixed. Final preparation of the microplate resulted in the solutions as follows:

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B			500	250	125	62.5	31.25	15.625	7.8125	3.90625		
C			+	+	+	<i>P. putida</i>		+	+	+		
D			50	25	12.5	6.25	3.125	1.5625	0.78125	0.390625		
E		Positive control										
F			500	250	125	62.5	31.25	15.625	7.8125	3.90625		
G			+	+	+	<i>B. subtilis</i>		+	+	+		
H			50	25	12.5	6.25	3.125	1.5625	0.78125	0.390625		
											Negative control	

**Figure 5. 96-well plate** – Combined *Moringa oleifera* (top concentration) and chitosan (bottom concentration solutions) in mg/L. Copyright © 2009 Water Environment Federation. All rights reserved.

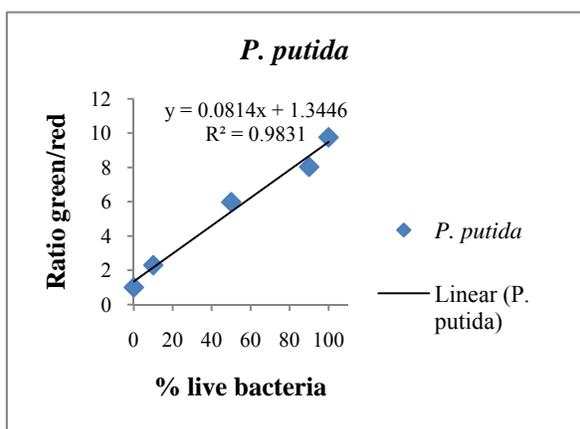
After preparation the microplates were placed in the Flex Station II Fluorescence Microplate Reader and fluorescence measured as described above. Results were compared to the calibration curves to determine the percentage of live bacteria in each sample.

## RESULTS & DISCUSSION

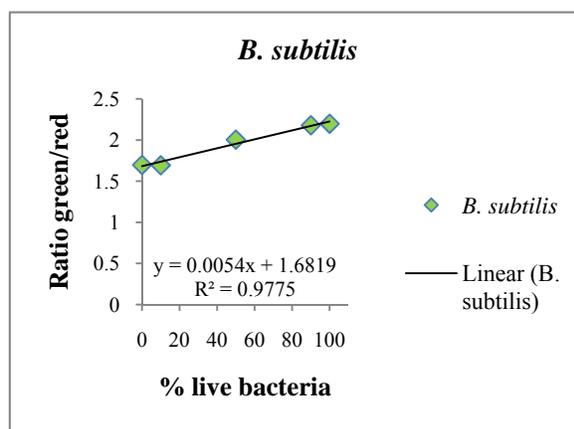
Two sets of results were obtained: (1) bactericidal assay of *Moringa oleifera* and chitosan as separate disinfectants, (2) bactericidal assay of *Moringa oleifera* and chitosan together as a novel disinfectant.

### Part I – Preliminary Experiment

Because the data resulting from analysis by the Fluorescent Microplate reader calculated the ratio of red/green fluorescence (live/dead bacteria), it was necessary to quantify the data by obtaining the percentage of live bacteria and comparing these values to the calibration curves. Data corresponding to the live/dead calibration microplate was compiled into two graphs, one for *P. putida* and one for *B. subtilis*. These graphs provided a correlation between the ratio of green/red fluorescence and percentage of live bacteria remaining in the solution.

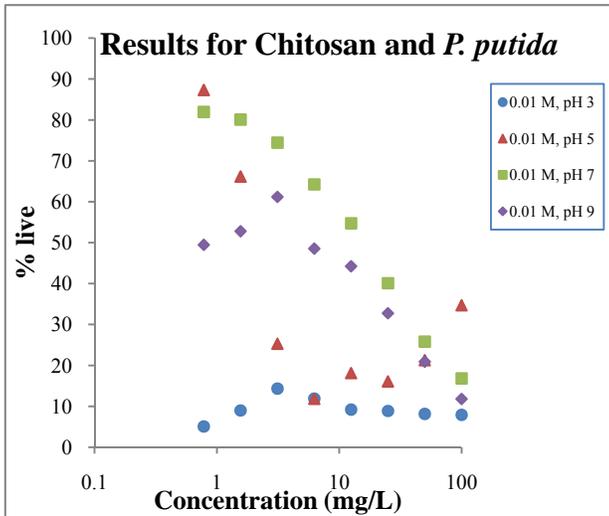


**Figure 6.** Calibration curve for *Pseudomonas putida* in 0.01M NaCl solution

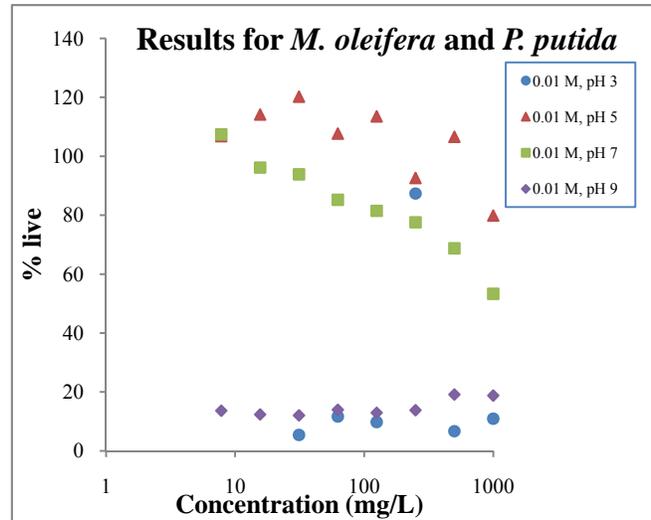


**Figure 7.** Calibration curve for *Bacillus subtilis* in 0.01M NaCl solution

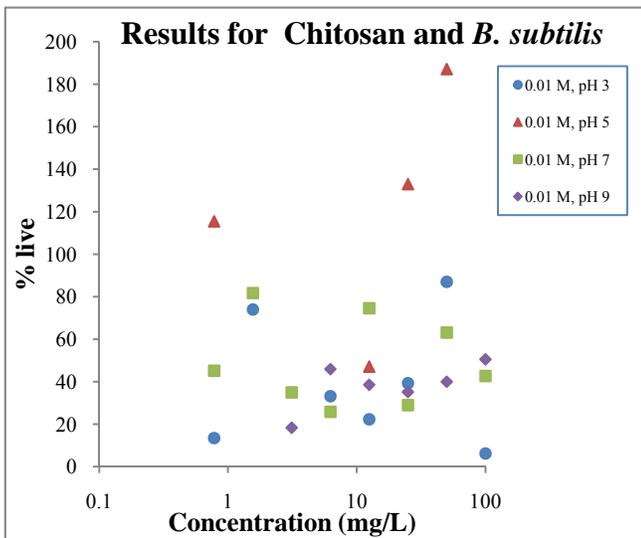
Subsequently, the percentages of live bacteria for the remaining data values were calculated using the calibration curve equation Percentages were tabulated into graphs and compared.



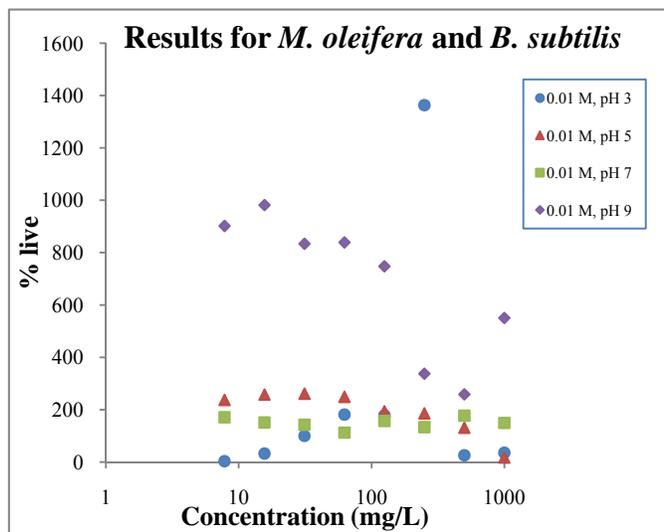
**Figure 8.** Percentages of live bacteria for 0.01M solutions of chitosan and *P. putida*, with variation in pH levels.



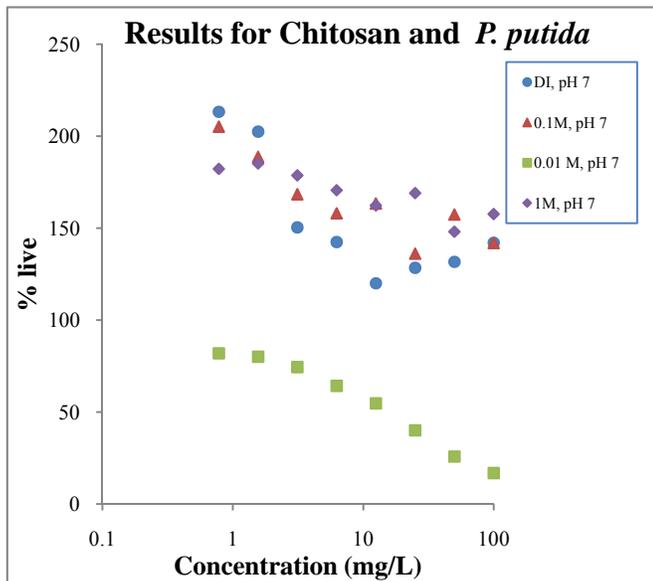
**Figure 9.** Percentages of live bacteria for 0.01M solutions of *Moringa oleifera* and *P. putida*, with variation in pH levels.



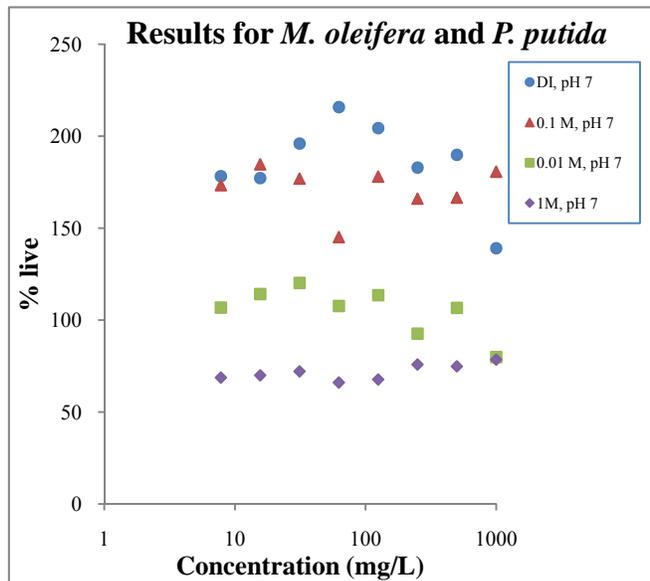
**Figure 10.** Percentages of live bacteria for 0.01M solutions of chitosan and *B. subtilis*, with variation in pH levels.



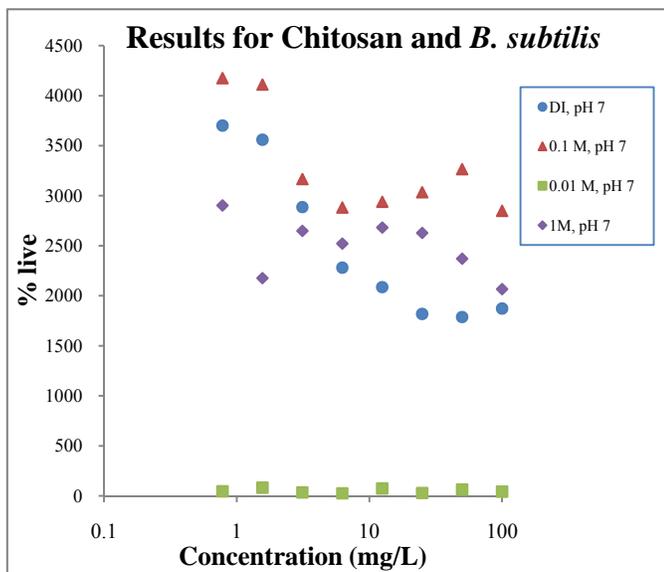
**Figure 11.** Percentages of live bacteria for 0.01M solutions of *Moringa oleifera* and *B. subtilis*, with variation in pH levels.



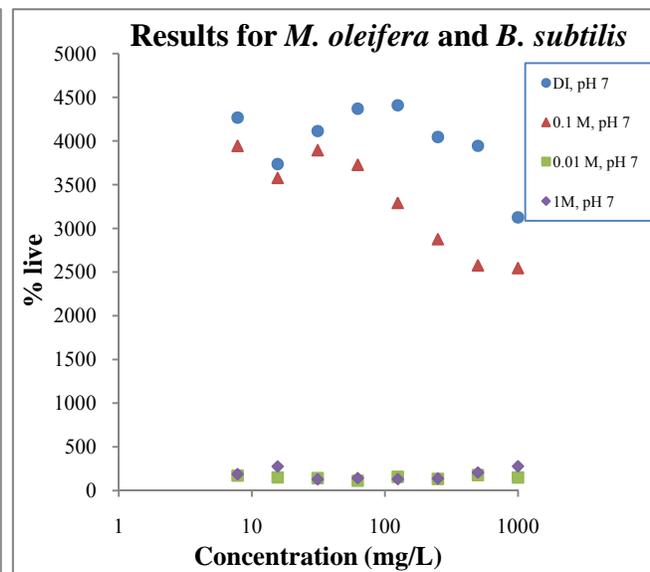
**Figure 12.** Percentages of live bacteria for 0.01M solutions of chitosan and *P. putida*, with variation in ionic strengths.



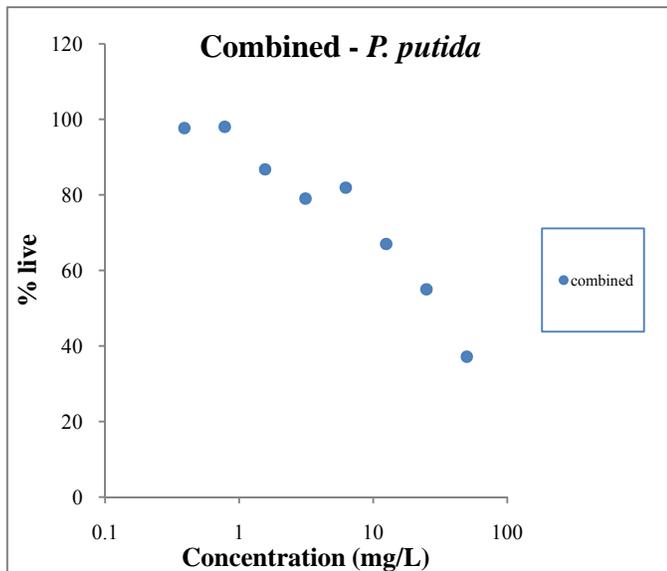
**Figure 13.** Percentages of live bacteria for 0.01M solutions of *Moringa oleifera* and *P. putida*, with variation in ionic strengths.



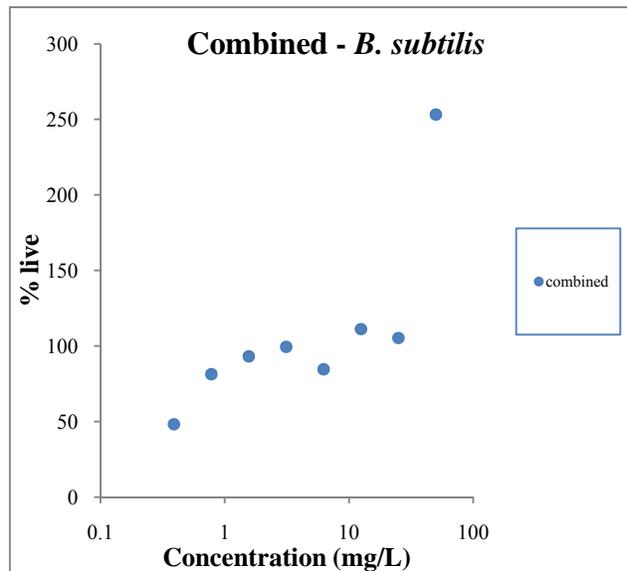
**Figure 14.** Percentages of live bacteria for 0.01M solutions of chitosan and *B. subtilis*, with variation in ionic strengths.



**Figure 15.** Percentages of live bacteria for 0.01M solutions of *Moringa oleifera* and *B. subtilis*, with variation in ionic strengths.



**Figure 16.** Percentages of live *P. putida* bacteria for a combined solution of *Moringa oleifera* and chitosan, prepared at 0.01M NaCl and a pH level of 7.



**Figure 17.** Percentages of live *B. subtilis* bacteria for a combined solution of *Moringa oleifera* and chitosan, prepared at 0.01M NaCl and a pH level of 7.

The experimental data showed that the antimicrobial agents were more active against *P. putida* than against *B. subtilis* because the Gram-negative *P. putida* has a thinner layer of peptidoglycan within their membranes than the Gram-positive *B. subtilis*; it is possible that the *Moringa oleifera* and chitosan particles were able to more effectively rupture its membranes. Another cause for this difference may be experimental error in the *B. subtilis* calibration curve. Thus Figure 5 shows that the ratio of live to dead bacteria constant at between approximately 2-2.5 for all percentages of live bacteria. Because the bacteria/ bacteria ratio for 0% live bacteria was similar to the ratio for 100% live bacteria, the validity of the calibration curve is questioned.

As expected, increases in the concentration of disinfectants appeared to increase bactericidal activity. Optimum pH for disinfection was in the range of pH 5 to pH 7. Within this range the bactericidal activity of chitosan appeared to be greater at pH 5 than at pH 7 possibly because of its higher solubility in acidic conditions. Solutions at pH 3 or 9 appeared to negate the effect of the antibacterial agents by prematurely killing most of the bacteria. This was determined by comparing the percentage of live bacteria in the positive control (initially 100% live bacteria with no disinfectant) with the remainder of the data. Because the trend of

relatively low live bacteria percentages extended to both the positive and negative controls, bactericidal activity could not be attributed to the disinfectants. Ionic strength had no consistent effect on disinfection. At an ionic strength of 0.01M chitosan had a higher bactericidal effect than *Moringa oleifera*. For the remainder of the ionic strengths and pH levels, chitosan and *Moringa oleifera* appeared to exhibit similar bactericidal effects overall. Bacterial viabilities of >100% were observed on several occasions possibly because of a delay in fluorescence analysis or an error in the *B. subtilis* calibration curve.

### ***Part II - Bactericidal Assay of a Novel Antibacterial Agent***

A novel disinfectant was developed combining solutions of *Moringa oleifera* and chitosan. A bactericidal assay was conducted in which the percentage of live bacteria was assessed at a range of disinfectant concentrations, pH values and ionic strengths. There was a consistent increase in bactericidal activity towards *P. putida* as the *Moringa oleifera* and chitosan concentrations increased. The disinfectant had little bactericidal activity on *B. subtilis*.

## **CONCLUSION**

The objective of this investigation was to 1) ascertain optimal conditions for bactericidal activity incurred by *Moringa oleifera* and chitosan and 2) to quantify the potential of a novel disinfectant consisting of combined solutions of *Moringa oleifera* and chitosan.

### ***Part I – Preliminary Experiment***

The bactericidal assay using HTS supported the hypotheses that “higher disinfectant concentrations increase bactericidal activity, low pH levels increase the antimicrobial effects of chitosan, and neutral pH levels enhance the antimicrobial effects of *Moringa oleifera*. There was no correlation between the antimicrobial effects of *Moringa oleifera* and chitosan and ionic strength. *Moringa oleifera* and chitosan had greater antimicrobial effects upon Gram-negative bacteria. The tabulated results provide valuable information that could potentially be applied to the implementation of *Moringa oleifera* and chitosan in rural areas affected by water contamination.

## ***Part II – Bactericidal Assay of a Novel Antibacterial Agent***

Part II of this study focused on the development of a novel antibacterial agent. The efficacy of a combination of *Moringa oleifera* and chitosan particles in one solution was quantified through a bactericidal assay using HTS. The combined compounds had bactericidal activity to *P. putida* but were less effective for *B. subtilis* however the combined disinfectant was no more effective than *Moringa oleifera* and chitosan on their own. Thus, the hypothesis that a combined disinfectant consisting of *Moringa oleifera* and chitosan is superior to the individual compounds alone” was not supported.

### ***Future Research***

The bactericidal effects of *Moringa oleifera* and chitosan in a water filtration system should be evaluated. Additional research could focus on investigation of physiochemical interactions between *Moringa oleifera* particles and chitosan particles to explain their limited functionability when combined. Using the bactericidal assay methods developed in this investigation, the efficacy of other organic antibacterial agents, such as carbon nanotubes or chitosan nanoparticles, should be studied.

### **ABBREVIATIONS AND ACRONYMS**

**DI** – deionized water

**LB** – Luria Bertani

**HAc** – acetic acid

**TSB** – Tryptic Soy Broth

**PI** – propidium iodide

**HTS** – High throughput screening

### **ACKNOWLEDGMENTS**

#### **Credits**

Most importantly, I would like to acknowledge the Civil and Engineering Department at the University of Los Angeles (Calif.) for allowing me to conduct my investigation at its laboratories and to use the instrumentation located there. Professor Eric M.V. Hoek, PhD, and Catalina Marimbio-Jones have proven to be invaluable mentors and I am forever grateful for their guidance, compassion, and willingness to dedicate

time out of their busy schedules to work with me. Peter Starodub, my Science Research teacher for two years now, has been invaluable to my investigation as he initially introduced me to Dr. Hoek. I am deeply grateful for his direction and great sense of humor during frustrating times. Lastly, I will never be able to thank my mother enough for driving me to UCLA and dedicating so much time toward my research goals.

### **Author**

As a sophomore at Palos Verdes Peninsula High in Palos Verdes, Calif., I have had a keen interest in science. Thus, I have participated in the science research program at my school for two years. Alarmed by the various environmental hazards that continue to make headlines, I have focused my scientific research to environmental issues in hopes of improving the conditions of people everywhere. Besides possessing an interest in science, I enjoy playing the violin and piano and am a member of my school's Advanced Orchestra. Additionally, I am a member of the Spanish National Honor Society, the Mu Alpha Theta Math Society, and a two-year member of the California Scholarship Federation. In the future, I hope to pursue a medical career as a research scientist.

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