Antimicrobial Potentials of Ayurvedic Products Extracts and Streptomycin Against Necrosis Causing Bacteria in the Saguaro Cactus.

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Abstract

Bacterial soft rot is the initial stage of the saguaro cactus' natural disintegration. Necrosis is the process of cellular death caused by a significant injury and followed by an invasion of microorganisms. The objective of the present study was to investigate the antimicrobial potential of ayurvedic products extracts; while we tested their inhibitory effects on the growth of likely pathogenic microorganisms found in the necrotic tissue of the saguaro cactus. The effects of oregano, garlic, clove, and cinnamon extracts- known to inhibit the growth of pathogenic bacteria- were compared to the antimicrobial potential of the antibiotic, streptomycin. Extracts of the treated plants were tested in samples of the necrotic tissue of three saguaro cactus. A PCR test was ran to identify the predominant bacterial colonies from each sample. Fifty to seventy-five percent of the plant's extracts were found to significantly exhibit an antimicrobial potential compared to streptomycin. One of the four tested extracts exhibited a significant difference to streptomycin; while another extract presented a higher inhibitory effect and a significant difference on two of the necrotic tissue samples.

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Introduction

The saguaro cactus is a tropically derived cold-intolerant cactus species, occurring throughout the latitudinal and elevational limits of the Sonoran Desert (Steenberg et, al.,1983). A defining element of this ecosystem, the long-lived (125-300yrs) Saguaro cactus is one of the representative species in the southwest area of Arizona, California, and the Northwest region of Sonora, Mexico (Dettman, 2011). The largest cactus in the United States and Mexico has long been the center of scientific interest since Shreve (1910) (Brum, 1973).

Saguaro is not troubled by many pests and diseases. However, the "soft rotting disease of the saguaro" first described by Hubbard in 1899 (Schuyler, 1943) is a common cause of death. Lightle (1918) provided additional observations, occurrence, and progress of the disease caused by the bacterial pathogen *Erwinia carnegiana* Standring. Data presented by these early researches showed the decline rates of plants caused by bacterial necrosis, and the estimations suggested that if the disease were not controlled, the population of this specific cactus species would have become extinct in the late 1990s.

Erwinia carnegiana has been highlighted as the prevalent cause of the disease, originally described as "a gram positive, peritrichous, encapsulated, nonpyrogenic rod" (Schuyler, 1968). Attributed to being similar to *E. cartovora* that belongs to Erwinia a genus of bacterial plant pathogens, recognized as closely related to the *Escherichia-Aerobacter* group.

Soft root producing coliform bacteria[1] possess enzymes that destroy pectic substances[2] in the middle lamella of the cell walls of parenchymatous tissue[3], resulting in water-like and soft tissue (Graham, 1964). The physiological and cultural similarities between the *Erwinia* species lead to misconceptions and the poor understanding of the relationship between them and coliform bacteria (Elrod, 1942). Boyle's investigations lead him to conclude that *Erwinia carnegiana* belonged to the soft rot bacteria, considering the similarities this species shared with *Erwinia cartovora*. Sutton and Graham (1964) study contradicted the results of Boyle (1948), after they examined the cultures of *E. carnegiana* and observed this bacterium did not cause pectolytic and therefore could not belong to the soft rot bacteria (Schuyler, 1968). The literature regarding the taxonomy of *E. carnegiana* is indeed confusing, misguiding the correlation between the *Erwinia* species and their effects as pathogenic bacterium.

Symptoms of *E. carnegiana* infection first appear as a small circular, light-colored spot usually surrounded by water-soaked anywhere on the surface. After bacterial invasion beneath the surface the tissues become water-soaked and brown or black in color. As the infection progresses the area infected enlarges and takes a purple coloration. As the infection advances the tissue will break down, releasing brown liquid, resulting in rapid decay of tissue accompanied by "bleeding," and dry but slower internal decay. The rotted tissues dry and break up into pieces (Boyle, 1948). Boyle proposed different possible modes of transmission of the soft rot disease: root-to-root

[1] Indicators of the presence of pathogenic microorganisms
(eg. *E. coli*, *Erwinia*)
[2] Group of polysaccharides in plant cell walls that protect them from pathogens, gelling, etc.
[3] Tissue typically comprised of living cells that are thin-walled, unspecialized in structure, and therefore adaptable, with differentiation, to various functions (photosynthesis, food storage, sap secretion, and gas exchange).

transmission, soil contamination, collapse of infected plants against healthy plants, and other living organisms.

Gene transfer within bacterial communities has been recognized as the prevalent contributor in the evolution of antibiotic resistance on a larger scale. The distribution of *strAB* genes, which encode streptomycin-inactivating enzymes, confer resistance. Antibiotic therapy has played a key role in curing diseases and conserving the life of living organisms. However, the concerning antibiotic use has been eroded by the widespread development of antibiotic resistance and the expense involved. Rezzonico, Stockwell, and Duffy (2009), demonstrated that 18 strands of commercial streptomycin lack detectable intact strA, meaning they are not harmful to the environment. However, a single copy of the resistance gene would be enough to acquire antibiotic resistance (Rezzonico et al., 2009). Unfortunately, the effects of the microbiome of commercial organisms on the potential of plant pathogens to evolve antibiotic resistance are unknown to us. The possibility of discovering potential antibiotic solutions to ensure an effective and inexpensive method to inhibit the growth of pathogenic microorganisms is critical to the conservation of the saguaro cactus population unique to the Sonoran Desert.

a. Antibiotics: Ayurvedic products and streptomycin

Clove. *Eugenia caryophyllata,* an evergreen tree, has been traditionally used as a spice for thousands of years. It is commonly used in Indian ayurvedic and Chinese medicine for its antimicrobial properties. Clove has been found to have a great inhibitory effect on fungi, nematodes, human and animal pathogens and one plant pathogenic bacterium, *Erwinia cartovora* (Huang and Lakshman, 2010). Previous research has reported the inhibition potential of clove essential oil in the growth of mold, yeasts, and bacterial (Nuñez and D'Aquino, 2012).

Oregano. From the labiate family, Oregano (*Origanum vulgare*) has been widely used in gastronomy and traditional healing (Fournomiti et al., 2015). Chemical analysis of oregano reveals the presence of different ingredients, the majority possessing antimicrobial properties (Fournomiti et al., 2015). This leaf is widely known for its antimicrobial potential but it also possesses antiviral and antifungal properties.

Cinnamon. The properties of cinnamon as a curative plant, antimicrobial against microbial pathogens in both humans and plants, is widely known (Parisa et al., 2019), its antimicrobial activity has been tested on several pathogenic microorganisms including Escherichia coli and Staphylococcus aureus. According to previous researches the antimicrobial activity of cinnamon is associated with its ingredients: cinnamaldehyde, alkaloids, flavonoids, eugenol, coumarin, steroids, saponins, tannins, and phenols.

Garlic. *Allium sativum L*. belongs to the Liliaceae family and falls within the group of onion. Antibacterial activity of garlic against many common pathogenic bacteria has been experimentally demonstrated (Nejad et al., 2014). Previous research has shown the inhibitory effects of garlic on plant-associated microorganisms. Besides the antimicrobial effects of allicin on pathogens in plants, it is possible that garlic extract contains chemicals that can induce systemic acquired resistance (SAR) in the host plant, garlic extract is used in the host, its components might be a contributing factor to the reduction of the disease (Curtis et al., 2004).

Streptomycin. Streptomycin is used in plant agriculture for bacterial disease control, a compound produced by a microbe with killing or growth-inhibiting activity against other microbes (Rezzonico et al., 2009).

Problem Statement

The purpose of this research was fourfold:

1. To investigate and test the antimicrobial potential of ayurvedic products extracts that could potentially exhibit inhibitory effects against the growth of likely pathogenic microorganisms found in the necrotic tissue of saguaro cactus.
2. To compare the effects of the prepared extracts to streptomycin, and observe if bacteria acquired antibiotic resistance to the antibiotic.
3. To determine if *Erwinia cargeniana* is present in any necrotic tissue sample collected from the infected saguaros.
4. If the bacterium was/was not found in the infected tissue, then determine what relationship exists between the presence of the bacterium and other bacterial colonies found in the collected samples.

Hypothesis

H_1: if the plant extract from different ayurvedic products is applied to the microorganisms found in samples, then it will inhibit the growth of bacteria because plant extracts have antibacterial effects.

Methodology

a. Field work.

(Reserva de la Biosfera Del Pinacate in the Gran Desierto de Altar) In an area of approximately 62,500m^2, 87 saguaro cactuses were found; withing this area we estimated that 80% of the population was infected and decaying. Prior to sample collection, materials were sterilized

with a hypochlorite solution (1:9 concentration). Samples were collected from 4 different saguaros within 70-100m one from another. An incision was made in the infected area of the cacti, water-like internal tissue was sampled using a sterile cotton swab and stroked across the Muller-Hinton agar plate. The samples were sealed with parafilm and transported to the lab. Samples previously collected were stored in an incubator (35^{o}C) for a period of 8 days to allow bacterial colonies to form.

b. Preparation of bacterial dilution

Samples were examined, one bacterial colony was drawn from each plate, as a saline solution of 0.9 was prepared by dissolving a packet of saline in 240ml of distilled water with the help of a magnetic stirrer. Using a vortex to obtain a homogeneous bacterial dilution, 2ml of the saline solution were poured into test tubes; while bacterial colonies were separately added to the solution using a sterile inoculating loop. Using a sterile cotton swab the bacterial dilutions were streaked on new agar plates, and left in the incubator.

c. Preparation of antibiotic disks.

The ayurvedic products: oregano, garlic, clove, and cinnamon were each soaked in 50ml of ethanol and let to rest for four days. The pure extract of each plant was obtained using the rotary evaporator. To extract the pure solution the rotary evaporator was set to 100rmp at 60° (boiling point of ethanol). Sterile paper disks were soaked in 10mg/ml of the obtained extracts.

d. Kirby-Bauer Sensitivity Assay

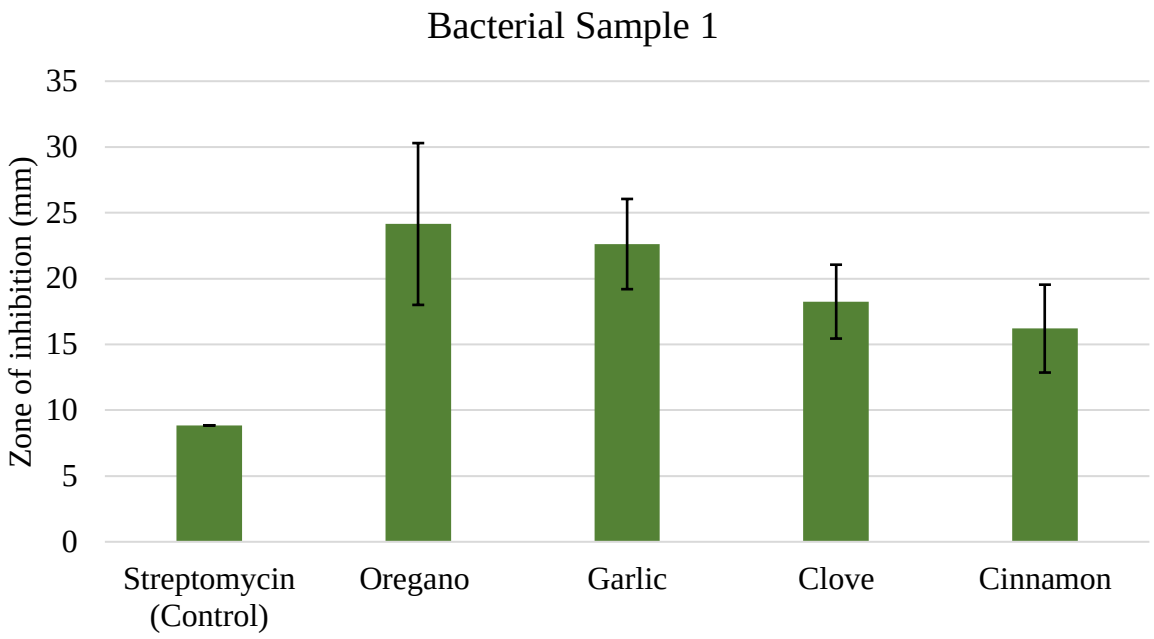
Muller-Hinton agar plates were inoculated with newly prepared bacterial dilutions using a sterile cotton swab (Following the 0.5 McFarland Standard). The experimental conditions were as follows:(1) 12 Sterile paper disks were soaked in the oregano extract; (2) 12 disks were soaked in

garlic extract; (3) 12 disks were soaked in clove extract; (4) 12 disks were soaked in cinnamon extract; and lastly (5) 12 sterile paper disks were soaked in streptomycin The prepared antibiotic disks were placed in the inoculated agar plates and stored in the incubator at 37° overnight. The diameter of the zones of inhibition was measured using a digital caliper to determine the antibiotic potential of each extract against the likely pathogenic bacteria. The data collected will be analyzed and a T.TEST was conducted to verify if the antimicrobial potential of the ayurvedic products extracts is significant or not compared to streptomycin.

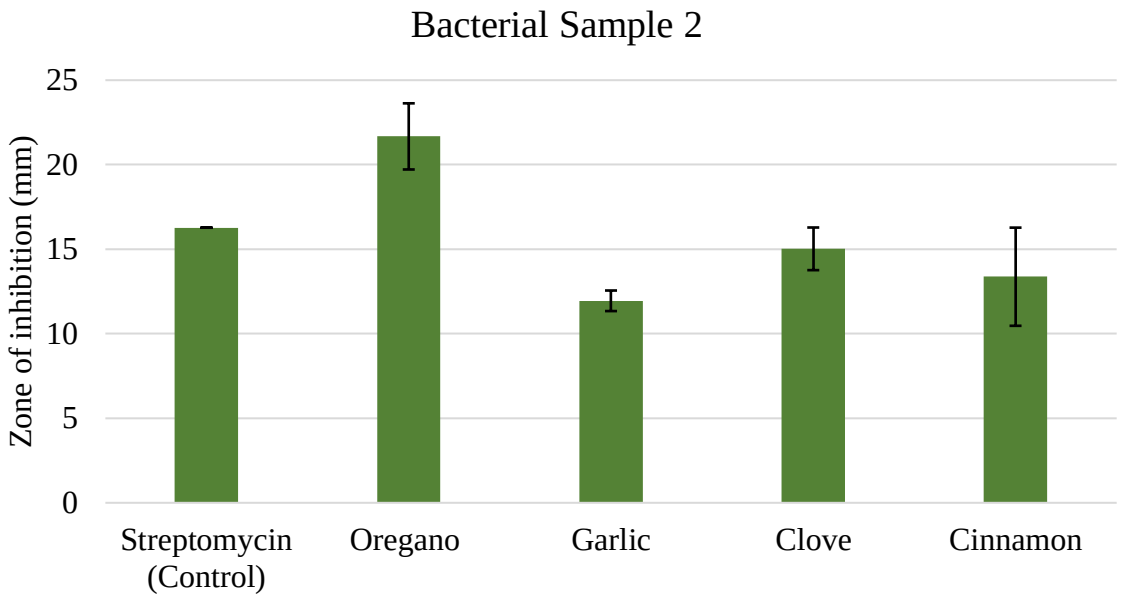
e. Identification of microorganisms

Muller-Hinton agar plates were inoculated with newly prepared bacterial dilutions using a sterile cotton swab, plates were sealed and sent to the lab for DNA testing. Three predominant bacterial colonies were selected from each plate, and a Polymerase chain reaction test was conducted. Short sequences (600 bare pairs) were placed in a machine in charge of decoding the nucleotide and results were uploaded into a digital database. To analyze these results, the platform DNA subway was used as a median to find similarities between the sequences of microorganisms and the sequences NCBI houses. To determine the sequence relationship, the lab results were uploaded to the platform and trimmed. The trimmed sequences were blastn to compare the sequences to previously published bacterium.

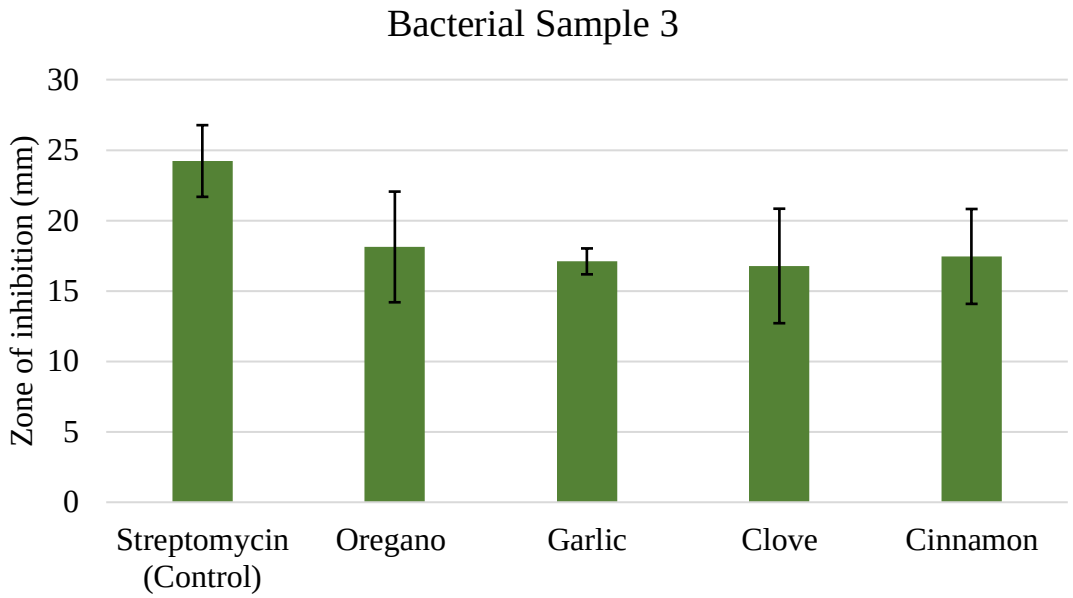
Results



* The results show the average zones of inhibition present on sample 1 of infected tissue by oregano, garlic, clove, cinnamon extract, and streptomycin (control) and the difference among the extracts compared to streptomycin.



* The results show the average zones of inhibition present on sample 2 of infected tissue by oregano, garlic, clove, cinnamon extract, and streptomycin (control) and the difference among the extracts compared to streptomycin.



* The results show the average zones of inhibition present on sample 3 of infected tissue by oregano, garlic, clove, cinnamon extract, and streptomycin (control) and the difference among the extracts compared to streptomycinn

BLASTN			
Name	Results	Mismatches	Quality of Sequence
Sample1a-16SF	N/A	N/A	⃠
Sample1a-16SR	N/A	N/A	⃠
Sample1b-16SF	*Enterobacter sp. | Bacillus sp.*	25	⃠
Sample1b-16SR	*Pseudescherichia vulneris strain* *Klebsiella grimontii strain* *Enterobacter sp. strain*	1	liable
Sample2a-16SF	*Micrococcus sp. strain*	0	⃠
Sample2a-16SR	*Micrococcus sp. strain*	0	⃠
Sample2b-16SF	N/A	N/A	⃠
Sample2b-16SR	*Planococcus ruber strain*	22	⃠
Sample2c-16SF	*Isoptericola variabilis strain*	2	liable
Sample2c-16SR	*Isoptericola variabilis strain*	5	⃠
Sample3a-16SF	*Bacillus sp.*	0	⃠
Sample3a-16SR	*Uncultured Planococcus sp.*	3	liable
Sample3b-16SF	N/A	N/A	⃠
Sample3b-16SR		3	⃠

*Results of bacteria that exhibits a similar/exact sequence compared to bacterial colonies samples

BLASTN			
Name	Results	Mismatches	quality of sequence
Sample1b-16SR	*Pseudescherichia vulneris strain* *Klebsiella grimontii strain* *Enterobacter sp. strain*	1	liable
Sample2c-16SF	*Isoptericola variabilis strain*	2	liable
Sample3a-16SR	*Uncultured Planococcus sp.*	3	liable

*Results of bacteria that exhibits a similar/exact sequence compared to bacterial colonies samples with quality sequences

Discussion

Successfully isolated bacterial colonies from saguaro lesion were identified, finding liable DNA sequences to be like soft rotting producing bacteria. Sample1b-16SF and Sample1b-16SR colonies were matched to coliform bacteria that are commonly found in soils, on plants, and water. According to the obtained results, bacteria from sample 1 developed antibiotic resistance to streptomycin, while the tested extracts significantly inhibited the growth of the bacterium. The antimicrobial properties of the ayurvedic products tested have been found to have a significant inhibitory effect on the coliform bacteria found in the samples.

The extracts of oregano, garlic, clove, and cinnamon exhibited antimicrobial potential against different bacteria found in the predominant colonies from each sample. Among the extracts tested, oregano exhibited the highest antimicrobial potential with an average zone of inhibition of 21.32. However, oregano extract showed a significant difference to streptomycin in two of the three tested bacteria. Garlic followed with an average of 20.45, and showing a significant difference in the three samples. While clove, cinnamon, and streptomycin showed an average zone of inhibition less than 17.

The genus, *Erwinia*, has long been recognized to have two distinct kinds of organisms being morphologically similar; that differ in their pathogenicity, nutritional, and biochemical properties (Graham, 1964). One group is responsible for dry necrosis, and the second group is responsible for the true soft rots, considering their secret pectolytic enzyme, undetectable in the first group (Graham, 1964). The widespread of these organisms in nature has led to confusion with members of the *Enterobacteriaceae*. Among the bacterium samples, we find coliform bacteria that indicate the presence of pathogenic bacteria. Referring to the literature provided by Graham, the taxonomy of these bacterium has been debated by previous results, and the inconsistency of these led to more confusion. Bacterial sequences analyzed did not match a pathogenic bacterium directly

affecting the saguaro cactus. The prevalent colonies found in these samples belonged to bacteria that could have been transferred from a human being, considering that these species are prevalent in humans.

As of the sampled colonies, the identification of microorganisms was not as expected. *Erwinia carnegiana* did not match any of the sequences drawn from the PCR (Polymerase Chain Reaction) test, nor did we find a clear correlation between the bacterium identified and bacterial necrosis. These observations indicate that the bacterial samples were contaminated, or were not prevalent. The presence of these bacteria is not clear, considering the factors influencing the sampling and identification of samples.

Conclusion

The successfully isolated microorganisms from the saguaro lesion matched the sequences of coliform bacteria found in humans, roots, and plants. The correlation between necrosis and these microorganisms is unclear. After results were collected and analyzed, the data suggests that all antibiotic solutions used in this research significantly inhibited the bacteria found in saguaro necrotic tissue samples. The results were as followed: (1) oregano extract significantly inhibited the bacterium found in the infected tissue with an average zone of inhibition of 21.32mm, (2) garlic extract showed an average zone of inhibition of 17.23mm, (3) clove exhibited an average zone of inhibition of 16.68mm, (4) cinnamon with an average of 15.68mm, and lastly (5) streptomycin showed an average zone of inhibition of 16.45mm. From these observations, we can conclude that

oregano showed the greatest antibiotic activity in this assay. The antimicrobial potential of these plants has been demonstrated by the zones of inhibition, and their significant difference when compared to streptomycin. I have concluded that the presence of coliform bacteria is unclear. Although the results were not as expected, we have found that the treatments in this research can potentially inhibit the growth of microorganisms identified in this investigation

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