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Research Paper

The Antimicrobial Effects of Garlic (*Allium sativum*) extract on Bacterial and Fungal Colonies Isolated from Diseased Garden Plants

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Abstract
Garlic (Allium sativum) is a bulb which has been used since antiquity as food and to help rid us of diseases. Some plant diseases are caused by bacterial and fungal pathogens whose activity cause harm to the plant and a decline in the plants appearance and productivity. This study isolated the two (2) pathogens Serratiaspp and Aspergillus niger from plants and studied their zone of inhibition when exposed to eight (8) different concentrations (50- 0.39% w/v) of fresh, aqueous garlic extract. It was found that concentrations as low as 1.56% w/v can inhibit both microorganisms, A. niger, the fungus, is more susceptible to the antimicrobial effect of garlic than the Serratia spp. bacteria is (32mm and15mm respectively at 50% w/v concentration). Garlic extract at the 50% w/v concentration was more than twice as effective than the Ampicillin (positive control) it was tested against in the bacteria and in the fungi the garlic extract was less effective than its positive control- Carbendazim.

Key words: *Allium sativum, Serratia spp. Aspergillus niger,* antimicrobial, Ampicillin and Carbendazim

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I. INTRODUCTION

The garlic plant, *Allium sativum,* comes from the Liliaceae family; its bulb contains the pungent flavour and healing properties which allowed it to be a used since ancient times as staple in cookery and as a tool to rid us of disease and the vectors that cause them.

The earliest reference of the use of garlic in history points to ancient Asia- first to Japan, then to China and India ~ 2000 years ago. In China it was used as a food, a food preservative and to aid respiration and digestion. Also, it was prescribed for diarrhea and worm infestation and as a general healing tonic in combination therapy. The Indian Auryvedic, Tibbi and Unani medical traditions, and more specifically the medical text Charaka- Samhita (Bowers Manuscript), recommend garlic for heart disease, arthritis and digestive distress. By some of their castes, it was applied externally to repair cuts, bruises and infections. Garlic was a part of the diet of the working class, Olympians, soldiers and sailors of ancient Egypt, Greece and Rome to maintain and increase their strength. It was unearthed in Pharaoh Tutankhamun's tomb and the palace of Knossos in Crete which dates back to 1325 BCE and ~1400-1800 BC respectively. The Egyptian *Codex Ebers* prescribed garlic as the treatment of abnormal growths, abscesses, circulatory aliments and even infestations with insects and parasites. In the Bible (Numbers. 11:5), the freed Jewish slaves lamented their loss of the vegetable and it is mentioned as a treatment in The Talmud, a 2nd century AD Jewish religious text, which echoes the use by the Egyptians. Hippocrates advocated the use of garlic for uterine growths and as a cleansing and purging agent. Other Greek physicians Dioscorides and Pliny the Elder were each the authors of five-volumes documents which recommended garlic for its use maintaining cardiovascular and hepatic health respectively; also for disorders of the gastrointestinal tract, for treatment of animal bites, joint disease and seizures. In the Middle Ages and The Renaissance garlic was cultivated, studied and used by monks, scholars and physicians as it was in antiquity; being mentioned in the Hortulus manuscript of 800 AD, the work of the Abbess of Rupertsberg, St. Hildegard von Bingen, 16th century Pietro Mattioli of Siena and it was being more widely used by the ruling classes of Continental Europe. In the new world, Native Americans used garlic in a tea to treat flu- like

symptoms and Shakers medical armamentarium mentions its uses as a stimulant, expectorant and tonic (Rivlin, R. S. 2001, Block 2010).

Plant diseases are identified by the symptoms they produce on the plants. Preliminary disease identification is done by observing the symptoms, the effect on the plant and research into the cause of the disease and possible agents and pathogens which transmit the disease (Pluke, R. *et al*. 1999).

Fungal diseases are often seen like a mold on the surface of plants. They are transferred by spores, which are minute and travel by air or water and establish themselves on new host plants. Bacteria on the other hand, are microscopic and they grow best in wet conditions and are dispersed by water films and splashes of water droplets when it rains. Bacterial diseases may also be transmitted among plants by a vector, such as birds, insects or man (Pluke, R. *et al.* 1999).

Fungal disease presents itself in many different forms as there are many different types of fungi (Myxomycota, Zygomycota, Ascomycota, Basidiomycota and Deutertromycota) and the same fungus may cause symptoms that are dissimilar among different species of plants. Nonetheless, fungi generally cause local and general necrosis or death of the plant tissue, hypertrophy and hypoplasla or stunted growth and even hyperplasia or excessive growths. Necrosis may come in the form of leaf spots (localized lesions), cankers (sunken lesions), dieback, root rot, damping off (rapid death and collapse of young seedlings), basal stem rot, soft and dry rots, anthracnose (an ulcer-like lesion of the shoot system), scabs and general decline in plant health. Hypertrophy or hyplasia presents as club root (roots become enlarged), warts on tubers and stems, witches' broom, leaf curls, wilt, rust and mildew (Khan, A. 2001). The symptoms of bacterial disease in plants are quite similar to that of fungal disease. In many cases, they cause blights, cankers, leaf spots, overgrowths, soft rots of fruits, roots and storage organs and scabs (Agrios, G. N. 2005).

Plant diseases can cause poisons or toxins which when ingested cause harmful effects on the body like ergot poisoning. Farmers also suffer indirect and direct financial losses when they invest in crops which render diseased produce and finally, plant disease can limit the kinds of plants and industries that can grow in an area (Sharma, P.D. 2004).

The available options to control of disease in plants are many; they may include use of plant strains genetically resistant to pathogens in the first place, use of quarantine procedures, cultural practices like crop rotations and sanitation guidelines, but also the use of chemicals as antimicrobials. The problem with compounds on the market to kill pathogenic microorganisms (fungicides and bactericides) is that they are toxic to those organisms and they may or may not be toxic to other organisms in the environment. The full extent of all of their possible injurious effects on humans and the natural ecosystem has not been totally explored (Oku, H. 1994).

The use of eco- friendly botanicals (natural plant extracts) like those of garlic, neem, and ginger are cheaper and better for the environment, because they degrade more rapidly into substances that have less of an environmental impact or none at all (Al-Samarrai, G. et al 2012).

The garlic has its antimicrobial properties from the compound allicin. Allicin is formed when garlic tissue is destroyed, allowing sulphur containing compound alliin is acted upon by allinase. Alliin is converted into pungent allicin by allinase enzyme as soon as the garlic tissue is destroyed. This compound has evolved to allow the garlic plant to deter animals from feeding on it and to counter microbial invasion if damaged (Ankri, S. &Mirelman, and D.1999).

Allicin has shown antimicrobial activity against Gram negative and Gram positive bacteria, fungi (including *Aspergillus spp*.) and viruses (Ankri, S. &Mirelman, D.1999). Allicindisintigates into chemicals including diallylsulphide (DAS),diallyldisulphide (DADS) and diallyltrisulphide (DATS) with a half-life of approximately one (1) day at 37°. As temperature increases, the quicker the disintegration of allicin. (Block 2010).

II. MATERIALS AND METHODS

Collection of Plant Material and Preparation of Inoculum Material

Fresh, diseased leaves from the 'Forbidden Fruit/ Apple' plant and pepper plant (*Capsicum annum*) were collected from the researcher's residence in Tucber Park, New Amsterdam, Berbice, Region 6, Guyana; placed in plastic bags and transported to the University of Guyana Berbice campus (UGBC) Agricultural Science Laboratory at the John's Science Center, Corentyne, Berbice, Guyana. In the lab, the diseased parts of each leaf in turn (or the leaf entire leaf) were cut using a razor and placed into a mortar and pestle for maceration with 5ml of sterilized water. The contents of the mortar were emptied into a 25ml beaker with the aid of a spatula and 5ml of sterilized water was added. A glass rod was used to mix the contents of the beaker thoroughly. Then a small piece of aluminum foil was placed over the beaker to cover and it was set aside three (3) hours to distribute the microbes in the solution.

Preparing the Stock Cultures and Colonies

An inoculating loop was sterilized by dipping it into methylated sprits and placing it into a flame until the wire glowed red hot. It was removed from the flame, dipped into a microbe solution and streaked across the surface of potato dextrose agar medium (PDA) in a petri dish. The petri dish was sealed and labelled the method above repeat twice to prepare two (2) more petri dishes. Three (3) plates in total were prepared for each leaf stock solution. These stock culture petri dishes were inverted and left to incubate at room temperature to allow the colonies to establish and grow (1 day for bacterial colonies and 2 days for fungal colonies).

Each plate was examined after incubation and a sterilized inoculating hoop was dragged on a colony and streak it unto a fresh petri dish prepared with PDA. This process was repeated to produce pure cultures of all distinct colonies. Each plate was labelled and incubated to allow the colonies to grow (1 day for bacterial colonies and 2 days for fungal colonies). Cultures were refreshed and maintained on petri plates every 1-2days and kept incubating to avoid contamination (Mukhtar, S. &Ghori, I., 2012).

Identification of Colonies

A pink/ peach bacterial colony was selected to test the antimicrobial effects of garlic. It was observed macroscopically and short notes were made of its colony morphology. Gram stain test was done.

A black spore fungi was selected to test the antimicrobial effects of garlic. It was observed macroscopically and short notes were made of its morphology. A sterile microscope slide was dampened with water, a small sample of the fungi was placed on the slide and observed under the microscope.

Preparation of Aqueous Garlic Extract

The outer skins of the garlic bulbs were peeled; 100g was weighed out and then cleaned with sterilized water. The cloves were homogenized using a blender with 100ml of water added. The garlic pulp was sieved to separate the solid garlic pulp and the filtrate remaining was used to make up the eight dilutions of the garlic extract by a serial dilution. The 100w/v extract was measured out to 50ml and 50ml water was added to make 50 w/v and further diluted similarly to 25, 12.5, 6.25, 3.12, 1.56, 0.78, 0.39 w/v concentrations(Mukhtar, et al., 2012). Each dilution was added to a small, labelled beaker and then to a petri dish. Sterile filter paper discs (5.5mm) were added to each garlic solution plate to soak it up for an hour.

Preparing PDA Petri Plates for Antimicrobial Testing

Petri plates for garlic microbial testing were prepared following the same method to make the PDA for stock plates (after the preparation and identification of stock and pure cultures). However, following the autoclave sterilization, the agar was cooled in a water bath (cool enough that gel formation does not occur) and an antibacterial or antifungal treatment was stirred in with a glass rod. 1ml (1000µl) of fungicide Carbendazim was added to a flask with 250ml agar designated to make petri plates for the bacteria. Likewise, 1000mg (two 500mg capsules) of antibacterial Ampicillin was dissolved in 250 ml of agar. These were used to pour into the test petri plates (four for bacteria, eight for fungi). Both groups of plates were labelled and set aside to for the gel to harden.

A sterile inoculating loop was used to add fungi spores from its pure culture petri plate to the 250ml agar treated with Ampicillin. It was thoroughly distributed and poured into eight petri plates (Set 1 and Set 2) and left to harden.

The 250ml agar that was treated with 1ml Carbendazim was used to prepare four petri plates (Set 1) and four other plates were prepared using plain untreated agar (Set 2). These eight plates were left to solidify. When completely set (approximately an hour), Set 1 petri plates had its bacteria cultures added by streaking with a sterilized inoculating loop that was dipped into a pure culture and streaked across its four quadrants. Set 2 plates had their bacteria introduced in a bacteria solution. This microbe solution inoculum was made by dipping a sterile inoculating loop into colonies of the bacteria and adding it to 10ml of sterilized water; it was mixed thoroughly and set aside for about an hour. 500µl of the bacterial solution was added to each of the Set 2 plates, swirled around to cover the surface of the agar and set aside.

Control Solutions

The positive control for the bacterial plates was made up using 10 ml sterilized water and 1000mg (two 500mg capsules) Ampicillin. It was stirred to completely dissolve, poured into a petri plate, to which filter paper discs were added and left to soak. The positive control for the fungal plates was similarly made up using 10ml sterilized water and 1ml (1000µl) Carbendazim. This solution was thoroughly mixed, poured into a petri plate and filter paper discs were added. The negative control consisted of 10ml sterilized water to which the filter paper discs were added and left to soak.

Antimicrobial Testing

The eight fungal plates were divided and labeled Set 1 and 2. Each individual plate was labelled on the plate to show the placement of a total of four paper disc. For example, Set 1 plate 1 was divided quarterly with the first quadrant for the positive control, the second for the negative control, the third for the 50% w/v garlic solution and the fourth quadrant for the 25% w/v Plate 2 had the two controls and the 12.5 % w/v and 6.25% w/v concentrations and so on with Plate 3 and 4. Fungi Set 2, and the Bacteria plates were labelled in the same way.

Tweezers were used to pick up and place four (4) individual 5.5mm discs of filter paper soaked with the required positive and negative control and two concentrations of garlic solution on the surface of the agar in their designated quadrants. This was repeated with the other three Fungi Set 1 plates, the four Fungi Set 2 petri plates and the two sets of bacteria plates. The plates were left to incubate at room temperature for 48 hrs.

The plates were examined after the allotted incubation period and a ruler was used to measure and the zones of inhibition (in mm) of each disc in each plate. All data was collected in appropriate tables and photographs of the resulting plates were taken.

2.3 Data Analysis

The percentage of mycelia growth inhibition at each concentration was calculated by using the formula: P= (C – T) x 100/C, where C is the diameter of the control colony and T is the diameter of the treated colony (Perello *et. al*. 2012)

The results and discussion compares the results of the different colonies, and the effectiveness of garlic extract on fungal pathogens as opposed to bacterial pathogens.

III. RESULT:

Isolation and Identification of Colonies

TABLE 1: SHOWING THE DISEASED PLANTS AND THE TEST MICROORGANISM CULTURES ISOLATED.

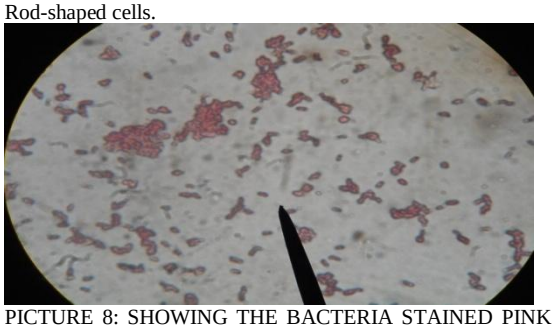
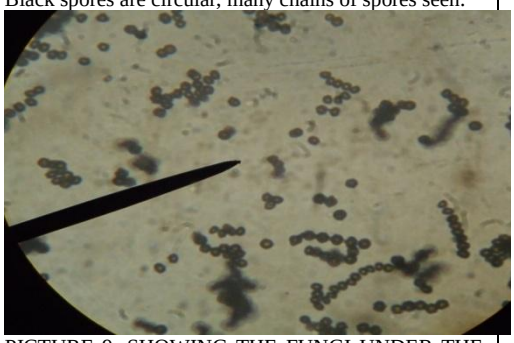
	Bacteria *(Serratiaspp)*	Fungi *(Aspergillus niger)*
Diseased Plant	Forbidden Fruit or Apple Plant	Pepper (*Capsicum annum*)

PICTURE 1: THE 'APPLE' PLANT FROM WHICH THE BACTERIA WAS ISOLATED.

PICTURE 2: SHOWING A CLOSER LOOK AT THE PLANT AND A DISEASED LEAF.

PICTURE 4: THE PEPPER PLANT FROM WHICH THE FUNGI WAS ISOLATED.

PICTURE 5: SHOWING THE DISEASED LEAF BEFORE IT WAS GROUND IN MORTAR AND

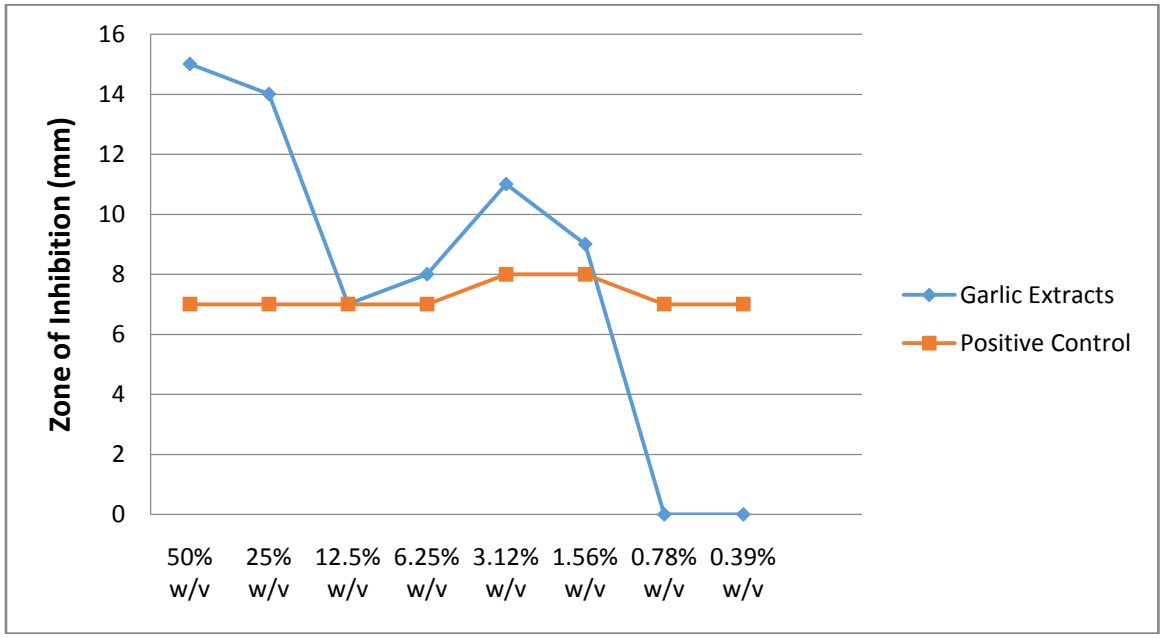
		PESTLE
Macroscopic Description of Colony	Pink/Peach coloured, colonies approx. 0.5-1mm round, entire.  PICTURE 6: SHOWING THE STOCK PLATE OF THE BACTERIA	White mycelium, black spores. Spreading over entire petri plate.  PICTURE 7: SHOWING THE STOCK PLATE OF THE FUNGI
Gram Stain	Pink (Gram Negative Bacteria)	-
Microscopic Description	Rod-shaped cells.  PICTURE 8: SHOWING THE BACTERIA STAINED PINK UNDER THE MICROSCOPE (MG 10x100.1.25)	Black spores are circular, many chains of spores seen.  PICTURE 9: SHOWING THE FUNGI UNDER THE MICROSCOPE (MG 10x100.1.25)

Antimicrobial Testing

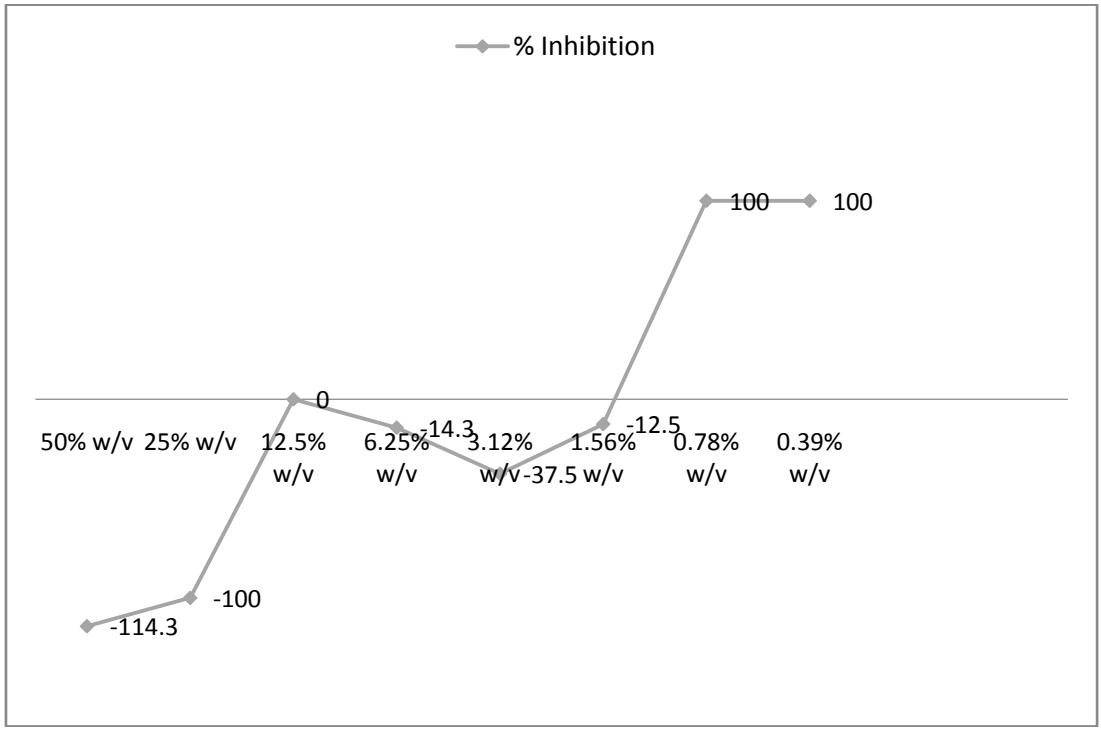
Bacteria (Set 1) plates did not produce any results, as there was no seen growth of the pink colonies. Bacteria (Set 2) did produce results (See Table 2 below).

TABLE 2: SHOWING THE INHIBITION OF BACTERIA (*SERRATIA SPP.)* IN SET 2 PLATES BY THE VARIOUS CONCENTRATIONS OF GARLIC EXTRACT, CONTROLS AND THE CALCULATED % INHIBITION.

BACTERIA (SET 2)		**Zone of Inhibition (mm)**			**% Inhibition**
		Garlic Extracts	**Positive Control (1000mg Ampicillin + 10ml Water)**	**Negative Control (10ml Water)**	
Plate 1	50% w/v	15	7	0	-114.3
	25% w/v	14			-100
Plate 2	12.5% w/v	7	7	0	0
	6.25% w/v	8			-14.3
Plate 3	3.12% w/v	11	8	0	-37.5
	1.56% w/v	9			-12.5
Plate 4	0.78% w/v	0	7	0	100
	0.39% w/v	0			100



GRAPH 1: SHOWING THE INHIBITION OF *SERRATIA SPP*.(SET 2) AT EACH CONCENTRATION OF GARLIC EXTRACT AND EACH POSITIVE CONTROL.

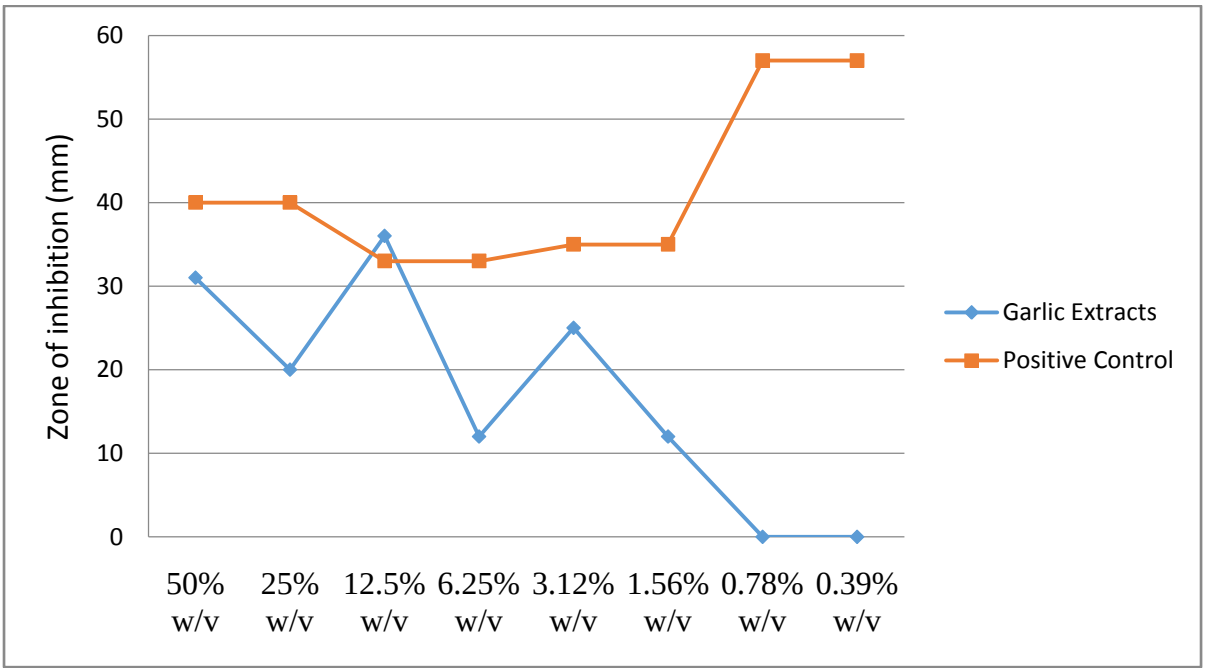


GRAPH 2: SHOWING THE % INHIBITION *OF SERRATIA SPP.* (SET 2) AT EACH CONCENTRATION OF GARLIC EXTRACT

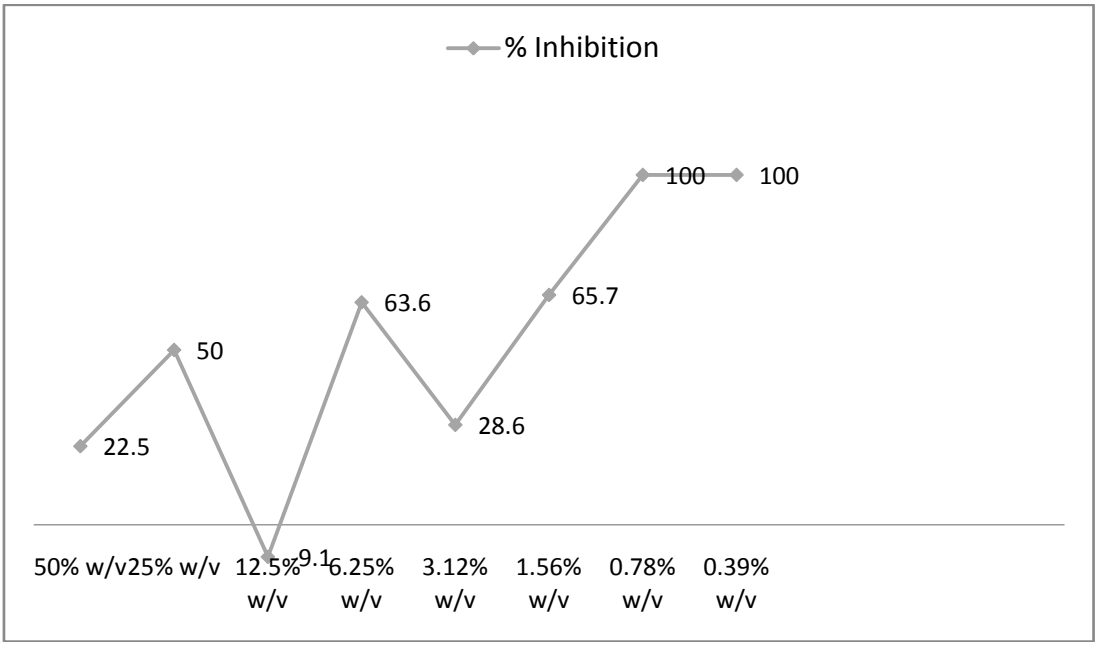
TABLE 3: SHOWING THE INHIBITION OF FUNGI (*ASPERGILLUS NIGER*) IN SET1 PLATES BY THE VARIOUS CONCENTRATIONS OF GARLIC EXTRACT, CONTROLS AND THE CALCULATED % INHIBITION.

FUNGI (SET 1)		**Zone of Inhibition (mm)**			**% Inhibition**
		Garlic Extracts	**Positive Control (1ml Carbendazim + 10ml Water)**	**Negative Control (10ml Water)**	
Plate 1	50% w/v	31	40	0	22.5
	25% w/v	20			50
Plate 2	12.5% w/v	36	33	0	-9.1

	6.25% w/v	12			63.6
Plate 3	3.12% w/v	25	35	0	28.6
	1.56% w/v	12			65.7
Plate 4	0.78% w/v	0	57	0	100
	0.39% w/v	0			100



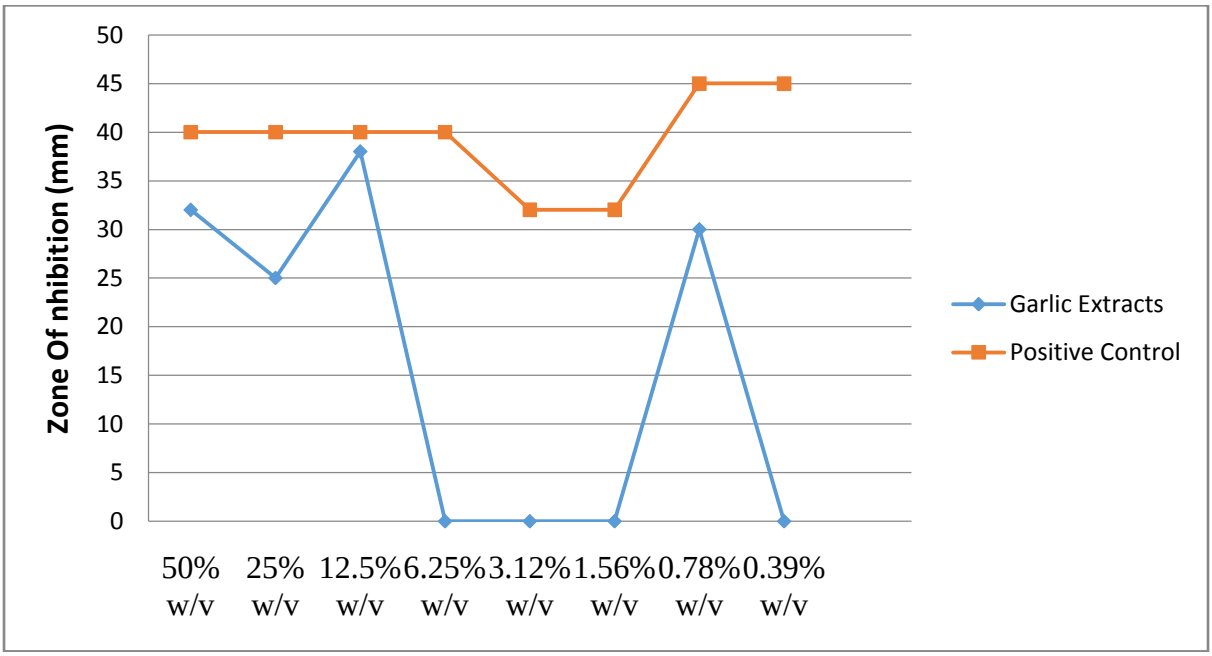
GRAPH 3: SHOWING THE INHIBITION OF *ASPERGILLUS NIGER*(SET 1) AT EACH CONCENTRATION OF GARLIC EXTRACT AND EACH POSITIVE CONTROL.



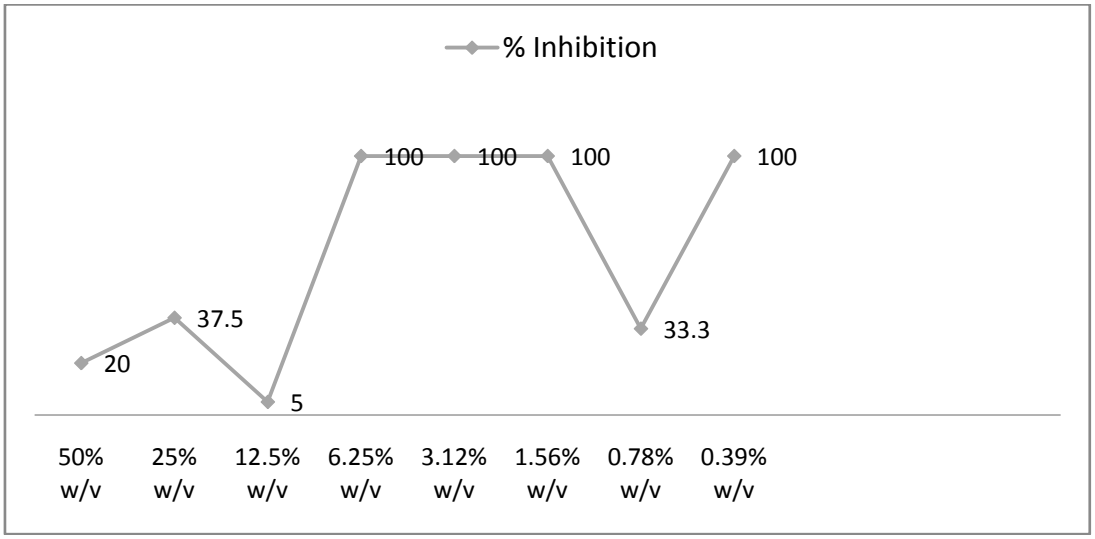
GRAPH 4: SHOWING THE % INHIBITION OF *ASPERGILLUS NIGER* AT EACH CONCENTRATION OF GARLICEXTRACT

TABLE 4: SHOWING THE INHIBITION OF FUNGI (*ASPERGILLUS NIGER*) IN SET 2 PLATES BY THE VARIOUS CONCENTRATIONS OF GARLIC EXTRACT, CONTROLS AND THE CALCULATED % INHIBITION.

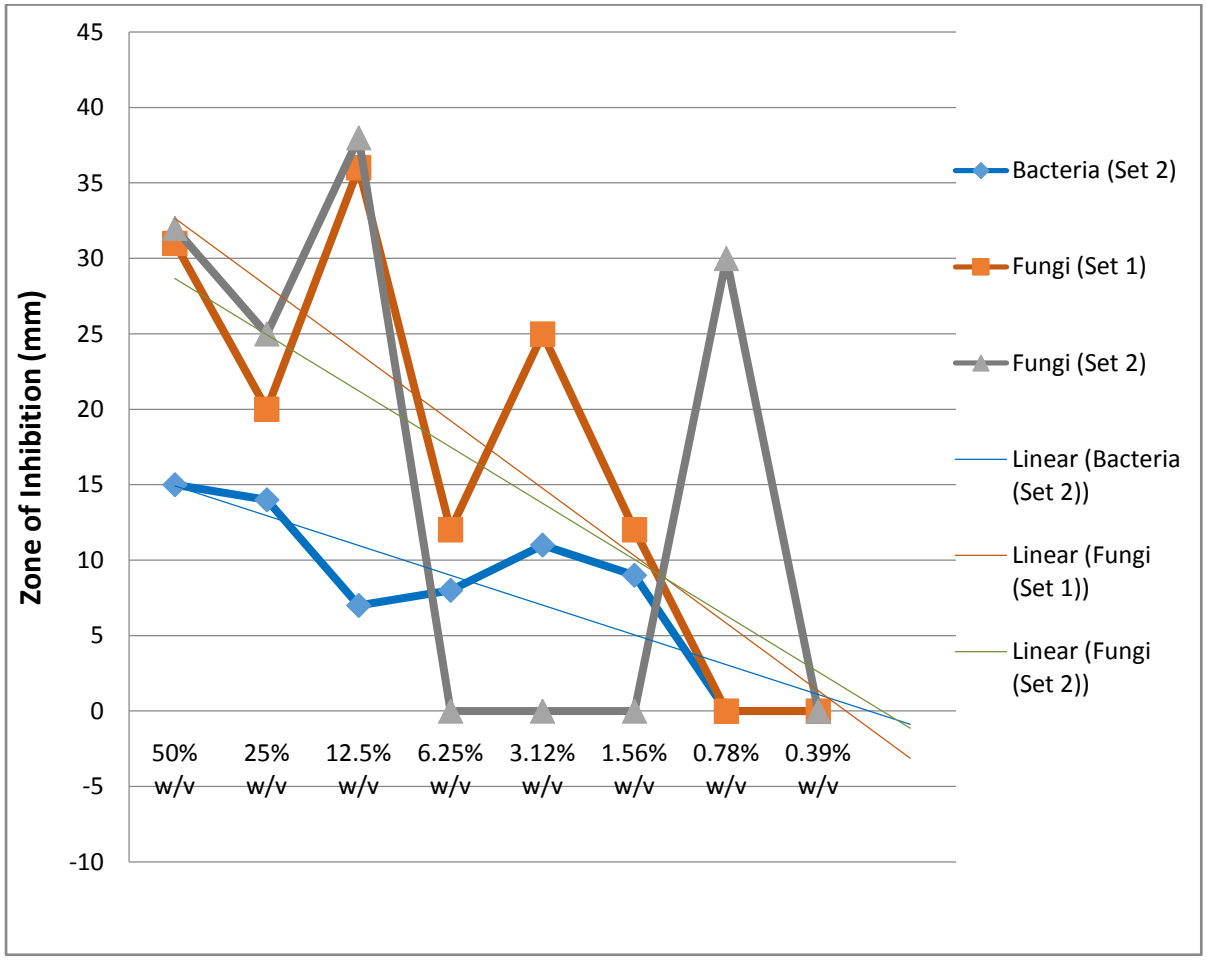
FUNGI (SET 2)		**Zone of Inhibition (mm)**			**% Inhibition**
		Garlic Extracts	**Positive Control (1ml Carbendazim + 10ml Water)**	**Negative Control (10ml Water)**	
Plate 1	50% w/v	32	40	0	20
	25% w/v	25			37.5
Plate 2	12.5% w/v	38	40	0	5
	6.25% w/v	0			100
Plate 3	3.12% w/v	0	32	0	100
	1.56% w/v	0			100
Plate 4	0.78% w/v	30	45	0	33.3
	0.39% w/v	0			100



GRAPH 5: SHOWING THE INHIBITION OF *ASPERGILLUS NIGER*(SET 2) AT EACH CONCENTRATION OF GARLIC EXTRACT AND EACH POSITIVE CONTROL.



GRAPH 6: SHOWING THE % INHIBITION OF *ASPERGILLUS NIGER* AT EACH CONCENTRATIONOF GARLIC EXTRACT



GRAPH 7: SHOWING THE ZONE OF INHIBITION OF *ASPERGILLUS NIGER* AND *SERRATIA SPP*AT EACH CONCENTRATIONOF GARLIC EXTRACT

IV. DISCUSSION AND CONCLUSION

In this Microbiology based research project, sterilization was an important and recurring practice. Microorganisms needed to be isolated from diseased leaves and they could not be mistaken for microbes found in or on any of the materials used in this study. There was constant sterilization of work surfaces with a bleach solution, heat treatments by the autoclaving of potato dextrose agar (PDA) and flaming of inoculating loops in between each use. Many other practices and precautions included using gloves, turning off the air conditioning and not talking while doing preparation works and actual testing.

The microbe solutions from each leaf were streaked on to three (3) PDA plates to increase the likelihood of obtaining colonies to do the antimicrobial testing.

The 'Forbidden Fruit/Apple' plant provided five (5) and the pepper plant (*Capisicum annum*) provided four (4) distinct colonies. The bright pink/ peach colour*Serratiaspp*colony from the 'Forbidden Fruit/Apple' plant and the *Aspergillus niger*was selected from the pepper plant because most of the other colonies were pale in colour and it would be difficult to distinguish the colony from the agar it was grown on. In the case of the pink bacteria and the fungus with black spores, a zone of inhibition would be clearly seen and easily measured.

The pure colonies were refreshed every 1-2 days until the antimicrobial testing. This was done to prevent the contamination and loss of the pure culture and to ensure the cultures stayed viable enough to inoculate the test plates. The 2-day incubation period after preparing the test plates was a time chosen considering the fact that allicin or the compound responsible for the antimicrobial effect on cultures loses its effectiveness after approximately one (1) day at 37 °C(Block, 2010).

The *Serratia spp*. bacteria when subjected to the Gram's stain, was stained pink (see Picture 8); this result was expected as the bacteria is in fact a Gram negative Enterobacteria (Grimont, F. & P.A.D. Grimont, 2006). The *A. niger* did not need to be stained to be observed under the microscope, its black spores were already clearly visible on a slide dampened with water.

The *Serratia spp.*Plates were prepared by streaking the pure culture across the surface of the agar, which was treated with Carbendazim before laying down the filter paper disc. Even after the 2-day incubation period, there was no seen growth of the characteristic pink bacteria.

However, there was a mucus- like growth along some of the streak lines and this could have been an unpigmented strain of the *Serratiaspp.*(Grimont, F. & P.A.D. Grimont, 2006). This streaking method did not prove effective at spreading the culture out on the surface of the agar so that a zone of inhibition could be identified. Only the bacteria's plates prepared with the bacteria- water inoculum on untreated agar did grow the coloured culture.

In an earlier experiment with the bacteria, both sets of bacterial plates were prepared with the Carbendazim antifungal treatment. After the incubation, there was no growth of the pink pure culture which had been streaked on. This lead to trying one set with the treatment and one without in this experiment. This method of using an untreated agar to grow the *Serratia spp*. bacteria was an idea based on research on the genus which revealed that they themselves have antimicrobial properties (De Vleesschauwer, D. &Ho¨fte, M., 2007), making the Carbendazim addition unnecessary. The Carbendazim treatment is simply a way of further ensuring than only the selected bacteria grow on the petri plate; there is no fungal contamination.

The garlic solutions in the Bacteria Set 2 plates showed a greater inhibition than its positive control, the Ampicillin solution (see Table 2). This resulted in a negative value when the % inhibition was calculated. There is an inverse relationship between the calculated % inhibition and the zone of inhibition recorded and this is seen in all of the tables. In general, there was the trend of decreasing inhibition as concentration decreased in the bacteria test plates. Plate 4 of *Serratiaspp* Set 2 did not have any inhibition shown by the last two weakest concentrations. They were too dilute to affect the cultures on the plate in any noticeable way. The same was noticed in another study with these eight (8) concentrations of garlic solution (Alzowahi, F. A. M.et. al. 2013) where they determined 1.56% w/v to be a calculated minimal inhibitory concentration (MIC) for their test bacteria.

The *Aspergillus niger*Set 1 plates did show more obvious inhibition of the fungus (Table 3). The Carbendazim solution control inhibited as much as 57mm in Plate 4. The garlic extracts were very effective also; at 50%w/v, 31mm diameter zone was seen with a recorded 22.5 % inhibition. Again, as in the bacteria's Plate 4, there was no inhibition at the two lowest concentrations. The trend of zone of inhibition decreasing as garlic extract concentration does is not as clear in the fungal plates (Set 1 or Set 2). Set 2 had its greatest inhibition of *A. niger* by a garlic concentration at 12.5 % w/v with 38mm; followed by no inhibition at the next extract concentrations 6.25- 1.56 % w/v and an uncharacteristic spike again (30mm) at 0.78% w/v. The cause of these results are unknown.

The three (3) sets of cultures grown in this study shows that the fungus *A. niger* was more greatly affected by the garlic extract solutions than the *Serratia spp*. This is clearly seen on Graph 7 which provides a visual representation of the inhibition seen at each concentration.

It can be concluded that concentrations as low as 1.56% w/v (or half of the average clove of garlic in100ml water) can inhibit *Serratiaspp*and *A.niger*. At 50% w/v, garlic solution was more than twice as effective as the positive control (15mm and 7mm respectively; with -114.3 % inhibition) in the bacteria and in the fungi it was less effective; 32mm and 40mm respectively with 20% inhibition. Even at 1.56 %, garlic was 9mm and the control had 8mm inhibition in the bacteria and 12mm and 35mm respectively in the fungi. It is also concluded that the fungus *A.niger* is more susceptible to the antimicrobial properties of garlic than the *Serratia spp.* bacteria is (32mm and15mm respectively at 50% w/v concentration).

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