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In-vitro Antibacterial Activity of *Allium sativum* L. Clove Extract Against *Agrobacterium tumefaciens*

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Author's contribution

The sole author designed, analyzed, interpreted and prepared the manuscript.

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ABSTRACT

Agrobacterium tumefaciens is responsible for causing crown gall disease which causes major economic losses and increases the host's susceptibility to opportunistic pathogens. Although there has been much research on the effectiveness of *Allium sativum* (garlic) against human pathogenic bacteria, there are very few studies on its effect on plant bacteria, especially *Agrobacterium tumefaciens*.

Aims: To determine the *in-vitro* antibacterial activity of *A. sativum* clove crude extract against *Agrobacterium tumefaciens*

Study Design: Experimental.

Place and Duration of Study: Department of Parasitology and Microbiology, College of Veterinary Medicine, Animal Resources and Biosecurity, Makerere University between March and May 2016.

Methodology: Agar disc diffusion method was used to screen for antibiotic susceptibility. Indole test was used for the determination of minimum inhibitory concentration (MIC) of the garlic extracts. The significant antibacterial activity of the active extract was compared with the standard antibiotic, methyl bromide. Thus, methyl bromide was the positive control and distilled water was the negative control.

Results: The garlic extract had a mean inhibition zone of 12 mm and an MIC and MBC of 50 mg/ml. The inhibitory effect of the garlic clove extract was proportional to its concentration thus the higher the concentration of garlic clove extract, the more the inhibitory effects.

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Conclusion: The results indicate that garlic clove extract has the ability to both inhibit and kill *Agrobacterium tumefaciens* in a manner similar to that exhibited in other bacterial pathogens, though at higher concentrations.

Keywords: Antibacterial; crown gall disease; Agrobacterium tumefaciens; Allium sativum.

1. INTRODUCTION

Although recognised as a worldwide problem in agriculture for centuries, the negative economic impact of crown gall disease is restricted to a limited number of horticultural species, like perennial fruits, nuts, ornamentals, and vine crops [1,2]. In these crops, crown gall disease causes major economic losses due to the culling of diseased nursery stocks, decreased productivity from galled plants, and increased susceptibility to opportunistic pathogens [2]. Smith and Townsend characterised *Agrobacterium tumefaciens* as the causal agent of crown gall disease in 1907. Crown gall is a plant disease that affects a large variety of broad-leaved (dicotyledonous) plants, including tomatoes, apple, pear, cherry, almond, raspberry, and rose plants leading to several losses [3]. With the ability to infect many different plant species, *A. tumefaciens* possibly has the broadest host range as compared to other plants pathogenic bacterium. *Agrobacterium tumefaciens* cleverly transfers a genetic principle to plant host cells and integrates it into their chromosomes [4]. Therefore, the focus of crown gall disease control has typically been the elimination of *A. tumefaciens* through soil fumigation [5]. Although successful in several cases, these measures have not provided consistent and effective disease control in many crops, including English walnut. Observations by nursery operators and walnut growers suggest fumigation with methyl bromide (MeBr) is inconsistent in reducing crown gall incidence and, in some cases, actually increases crown gall incidence [6]. With the rise in *A. tumefaciens* resistance to antibiotics, there is considerable interest in the development of other classes of antimicrobials for the control of crown gall disease. One potential method of controlling this plant disease could be the use of biological substances found in plants such as *Allium sativum* [7].

Allium sativum L., garlic, is one of the most representative species of the genus, used as food, spice and medicinal plant since ancient times. The medicinal value of the plant is consistent with various biological properties such as its antimicrobial, cardiovascular and anticancer effects [8]. Garlic is still being employed in folk medicine all over the world for the treatment of a variety of diseases [9]. Evidence from several investigations suggests that the biological and medical function of garlic is mainly due to their high organo-sulphur compounds content [10]. The primary sulphur-containing constituents in the whole vegetable is the S-alk(en)yl-L-cysteine sulphoxides (ACSOs), such as alliin, and γ-glutamylcysteines, which, besides to serve as important storage peptides, are biosynthetic intermediates for corresponding ACSOs from which, and by different metabolic pathways in each vegetable, volatile, such as allicin, and lipid-soluble sulphur compounds, such as diallyl sulphide (DAS), diallyl disulphide (DADS) and others, are originated [11]. These compounds provide to garlic and onion their characteristic odour and flavour, as well as most of their biological properties [12]. This agent exhibits anti-bacterial, anti-fungal and anti-viral properties [13].

Various garlic preparations have been shown to exhibit a wide spectrum of antibacterial activity against Gram-negative and Gram-positive bacteria including species of *Escherichia*, *Salmonella*, *Staphylococcus*, *Streptococcus*, *Klebsiella*, *Proteus*, *Bacillus*, and *Clostridium* [14]. Even acid-fast bacteria such as *Mycobacterium tuberculosis* are sensitive to garlic [15]. Furthermore, garlic extracts are effective against *Helicobacter pylori*, the cause of gastric ulcers [16]. Although there has been much research on the effect that garlic has on pathogenic bacteria, [17], there are very few studies on its effect on plant bacteria including inhibitory effects of garlic on plant-associated microbes. It was observed by Timonin and Thexton [18] that applying juice from crushed garlic tissues to soil led to a drastic drop in counts of microorganisms in the soil around the roots of plants. The activity of garlic extracts against the seed-spoilage organisms *Aspergillus niger* and *Fusarium pallidorosum* was also reported by Arya et al. [19]. A study by Wei et al. [20] also indicated that crude *Allium sativum* extract inhibited the growth of *Fulvia fulva,* a tomato fungus. The current research in Uganda focuses mostly on plant pathogens and diseases of food crops. At the Department of Crop science

(Makerere University, Kampala, Uganda), National Agricultural Laboratories (Kawanda) and National Crops Resources Research Institute (Namulonge), most of the research is on Cassava, maize, sweet potatoes, and bananas [21]. This leaves out fruit crops, ornamentals, and spices. Therefore, this study determined the *in-vitro* antibacterial activity of *Allium sativum* L. (garlic) against *Agrobacterium tumefaciens*.

2. MATERIALS AND METHODS

2.1 Sample Collection and Preparation

Fresh *A. sativum* bulbs were purchased from the market. The bulbs were separated into cloves, cleaned with distilled water to remove any surface contaminants from harvesting and handling and the skin peeled off. The cloves were cut into small pieces and ground in order to increase the surface area for extraction. One hundred grams of the ground cloves were placed in a cornical flask. Four milliliters of absolute ethanol (99.9%) were added and the flask vigorously agitated for 20 minutes. After agitation, the solutions were placed in a dark cupboard for maceration. Maceration lasted 72 hours (3 days) with frequent agitation. Agitation was done three times a day to ensure even mixing of the contents. After maceration, the solution was filtered using filter paper (WHATMAN NO. 3) and the filtrate collected in a clean conical flask. The filtrate was concentrated using a rotary evaporator (BÜCHI, ROTA-VAPOR R205, SWITZERLAND) to remove excess solvent. After concentration, the extract was pasted using a water bath set at 40°C. The paste was packed in a small laboratory bottle and kept in a fridge at 4°C ready for antibacterial screening. All chemicals and reagents used in preparation and extraction were of analytical grade.

2.2 Media Preparation and Screening for Antibiotic Susceptibility

The antibiotic susceptibility test was carried out using the disc diffusion method, on Mueller Hinton Agar (MHA) media. Thirty eight grams of Mueller Hinton Agar (MHA) were weighed and mixed with 1L of sterile distilled water then sterilised by autoclaving at 120°C for 20 minutes. The media was dispensed into three pre-sterilised petri dishes to a uniform depth of 4 mm, covered and allowed to cool at 56°C undisturbed. After cooling, the dishes were allowed to set at room temperature in a running biosafety cabinet. Using an indelible marker, each of the petri dishes was divided into four quadrants. Four well isolated colonies of *A. tumefaciens* were harvested from a culture plate using a sterile platinum wire, and used to prepare a turbid suspension in 2 ml of broth and incubated for 4-8 hours in a 29℃-30℃ water bath. Two milliliters of the suspension were pipetted onto the MHA plates and distributed evenly over the media surface. Readily prepared 5 mm diameter discs were soaked, some in the garlic extract, some in methyl bromide suspension (standard), some in distilled water and others in ethanol, leaving them to absorb the substances. They were later transferred to the inoculated plates, placing the disc from different substances in the center of a separate quadrant from the other. The quadrants were then labeled with the letters X, C, W, and E (Fig. 2A), where; X represented quadrant with disc soaked with garlic extract, C represented quadrant with disc soaked in methyl bromide, W represented quadrant with disc soaked with sterile distilled water, and E represented quadrant with disc soaked with ethanol. Methyl bromide was used as a positive control, distilled water as a negative control, and ethanol was used to be sure that the activity of the extract is entirely due to the active compound in garlic and not the solvent. The plates were kept in an upright position for 8 hours to allow the extracts sufficient time to diffuse, after which they were aerobically incubated for 24 hours. The diameters of the inhibition zones were then measured.

2.3 Determination of Minimum Inhibitory Concentration (MIC)

The MIC was determined using the indole test as by Kumar et al. [22]. Thirteen grams of nutrient broth were weighed and mixed in I L of sterile distilled water. 0.005 g of phenol red indicator were added to the media, sterilised by autoclaving at 120°C for 20 minutes, and allowed to cool in a 50°C water bath. Serial concentrations of the garlic extract were prepared by first dissolving 1 g of dried *A. sativum* extract in 10 ml of sterile distilled water to give a concentration of 100 mg/ml (this was the stock solution). Serial dilutions of the stock solutions were then made by successively doubling the volume measured from the stock with distilled water to give concentrations 100, 50, 25, 12.5, and 6.25mg/ml. Eight milliliters of the melted and cooled medium were dispensed into adequate number (17) of sterile universal

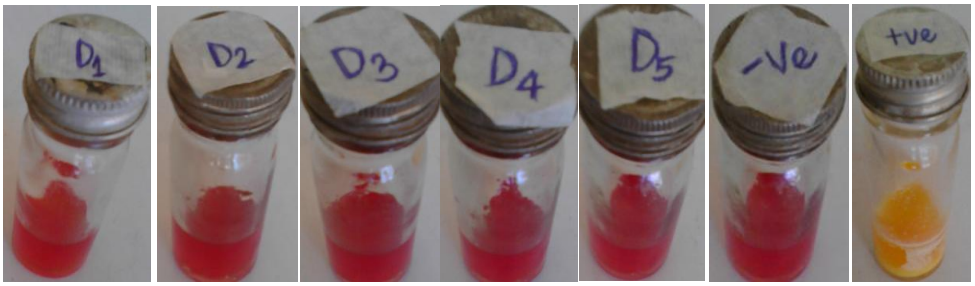

Fig. 1. Universal tubes with different concentrations of garlic extract (D_1 – D_5), positive control (methyl bromide) and negative (distilled water) controls before incubation

bottles, and 0.5 ml of bacterial suspension added to each. Two milliliters of serial concentrations of extracts were added with each concentration in triplicate, and mixed gently by inverting the bottles several times. The bottles were labelled D_1 - D_5 (Fig. 1) in the order of decreasing concentration. Two universal bottles with inoculated medium were set aside, and 2 ml of methyl bromide added to one of them, while to the other, 2 ml of distilled water were added to act as the positive and negative controls respectively. The bottles were then incubated at 29℃ for 24 hours after which the lowest concentration of extract at which no growth occurred was visually detected by no colour change.

2.4 Determination of the Minimum Bactericidal Concentration (MBC)

10-fold dilutions of the culture medium in the tube in which no apparent growth was observed (50 mg/ml) were made, and incubated at 29℃ for 24 hours on agar plates. The concentration of the extract at which the subculture from the test dilution method yielded no viable microorganisms was detected. This was the MBC.

3. RESULTS AND DISCUSSION

3.1 Bacterial Screening

After the 24 hours of incubation, only the disc soaked in garlic extract (X) and that in standard (C) had formed zones of inhibition as shown in Fig. 2 (B).

After the 24 hours of incubation, only the disc soaked in garlic extract (X) and standard (C) had formed zones of inhibition (Table 1). Discs soaked in ethanol (E) and water (W) showed no zones of inhibition. In all the three petri dishes, the standard (C) showed wider zones of inhibition.

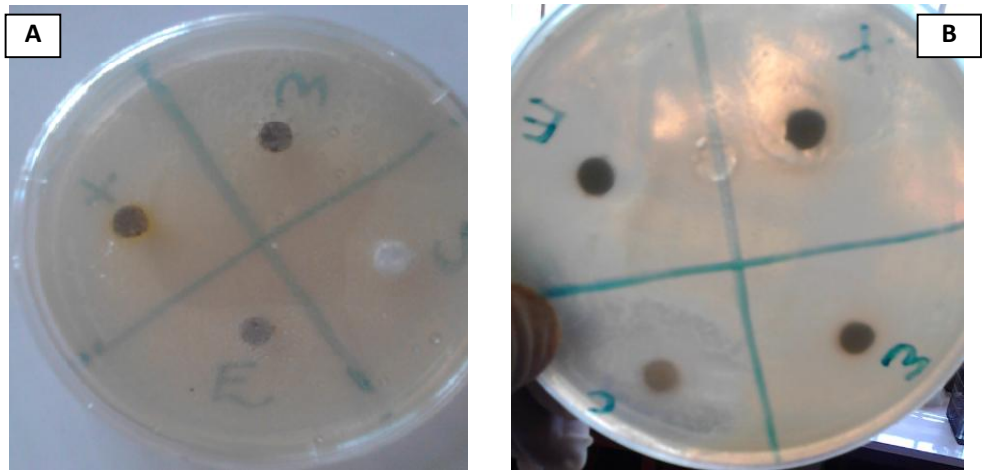

Fig. 2. A: Petri dish before incubation and B: Zones of inhibition after incubation for garlic extract X and standard C

Table 1. Diameters of the zones of inhibition

	Diameter of zone of inhibition (mm)			
	Extract (X)	**Standard/ Methyl bromide (C)**	**Ethanol (E)**	**Water (W)**
Petri dish 1	12.0	28.4	00	00
Petri dish 2	12.2	30.2	00	00
Petri dish 3	11.8	31.4	00	00

Fig. 3. Universal tubes showing changes in color of culture media due to growth of bacteria

3.2 Minimum Inhibitory Concentration and Minimum Bactericidal Concentration

At the end of the indole test, the color of the media in the universal bottles D_3, D_4, D_5, and negative control had turned from red to yellow (Fig. 3), signifying presence of bacteria. The color of the culture medium in the positive test remained yellow, while the color of the medium with concentrations D_1 and D_2 remained red, signifying absence of bacterial growth. The lowest concentration with no bacterial growth therefore was D_2 (50 mg/ml), and this was the MIC, which doubled as the MBC after serial dilution and sub-culturing.

This study demonstrated the ability of garlic clove extract to inhibit the growth of *A. tumefaciens*. The inhibitory effect is proportional to the concentration of garlic clove extract as the higher the concentration of garlic clove extract, the more the inhibitive effects. This can clearly be seen in the high concentration levels at which MIC and MBC were observed. These effects are in accordance with the results of [23,24], who reported that garlic extract had effective inhibition on *Fusarium oxysporum.* The inhibitive action of garlic clove extract on bacterial growth is attributed to the existence of allicin (diallylthiosulfinate) synthesised by enzyme catalysis in damaged garlic tissues [25]. Additionally, it has been reported that the antimicrobial substance allicin (diallylthiosulphinate) converts into oxygenated sulfur compounds, when garlic cloves are damaged and the substrate alliin (S-allyl-L-cysteine sulphoxide) mixes with the enzyme alliin-lyase (E.C.4.4.1.4). The volatile compounds act as antibacterial components that disrupt bacterial cell metabolism due to the oxidation of proteins [26]. Allicin is readily membrane-permeable and undergoes thiol-disulphide exchange reactions by the rapid reaction of thiosulfinates with free thiol groups in proteins in the bacteria. Another antibiotic mechanism of action of allicin is it's ability to inhibit thiol-containing enzymes in the microorganisms by the rapid reaction of thiosulfinates with thiol groups [27]. Pure allicin molecules have the ability to react with a model thiol compound (L-cysteine) to form the S-thiolation product, S-allylmercaptocysteine [28]. Allicin has also been found to kill bacteria through partial inhibition of DNA and protein synthesis and total inhibition of RNA synthesis [14]. The high MIC and MBC values could be due to the ability of the bacteria to tolerate lower concentrations of the active compound allicin. In addition, there is a problem on the storage environment and duration of garlic clove extract.

4. CONCLUSION

The results indicate that garlic clove extract has the ability to either inhibit or kill *Agrobacterium tumefaciens* in a manner similar to that exhibited in other pathogens, though at higher concentrations. It thus provides an insight on the use of natural plant products to treat crown gall

disease rather than relying on the synthetic and rather environmentally unfriendly fumigants. Further investigation on the mechanism of bacterial inhibition by allicin is therefore required, the study of the allicin production pathway and use of biotechnology tools to up regulate its synthesis, and study of the presence of a similar pathway in plants, or engineering the plants to produce allicin, thus protect them against attack by *Agrobacterium tumefaciens*.

COMPETING INTERESTS

Author has declared that no competing interests exist.

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