

## In-vitro Sensitivity of Selected Enteric Bacteria to Extracts of *Allium sativum* L.

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### Abstract

Garlic has been used throughout all of recorded history for culinary and medicinal reasons. The portion of the plant most often consumed is an underground storage structure called a head. The antimicrobial effects of *Allium sativum* (garlic) against some bacterial isolates were investigated using the agar diffusion well method. Standard methods were used to carry out the investigation. Photochemical analyses of the ethanolic extracts showed the presence of many secondary metabolites such as saponins, tannins, alkaloid steroids and glycosides. The minimum inhibitory concentration and minimum bactericidal concentration of the agent (garlic) was determined for both the aqueous and ethanolic extract. The ethanolic extract was more effective than the aqueous extract, inhibiting all the test organisms. While the aqueous extracts was effective against *Escherichia coli*, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*. Garlic extracts are strictly broad-spectrum with immune boosting phytonutrients from *Allium* 'family'. Further research will need to be done to carry out the purification of the active ingredients which have potential for combating human disease. Also, toxicological studies need to be evaluated.

**Keywords:** extract, *in vitro*, garlic, phytonutrient, test organism

### Introduction

Garlic belongs to the family *Alliaceae* and its scientific name is *Allium sativum*. Other members of the family are leek, onion, and shallot. Garlic is popularly used in culinary and medicine (Karuppiyah and Rajaram, 2012). Herbs and spices are very essential and indispensable as therapeutic agents against many pathological infections (Gull *et al.*, 2012). Phytotherapeutic agents such as *Allium sativum* which are commonly utilized in alternative medicine have gained immense interest in medical literature (Iwalokun *et al.*, 2004). Garlic is present in various forms namely, garlic oil, garlic powder, raw juice and many types of extracts such as, aged garlic extract, aqueous garlic extract (AGE), methanolic extract, ethanolic extract and many more (Jabar and Al-Mossawi, 2007).

The therapeutic effect of garlic is possible because of its oil-water soluble organosulfur compounds, which are responsible for its typical flavor and odour. Thiosulfinates play a major role in the antibiotic activity of garlic (Durairaj *et al.*, 2009). In recent times a number of studies have been conducted to verify the effectiveness of plant extracts against bacterial infections (Prashanth *et al.*, 2006; Ung-Kyu *et al.*, 2010). A daily dose of 1 mL/kg body weight of garlic extract for six months can result in significant reduction in oxidant stress in the blood of patients with arteriosclerosis and cholesterol circulating in the blood stream. The ability of garlic to prevent these oxidation reactions may explain some of its useful effects in arteriosclerotic heart diseases (Karuppiyah and Rajaram, 2012).

The enteric bacteria are members of the family enterobacteriaceae which is large heterogeneous group of Gram negative rods whose natural habitat is the intestinal tracts of humans and some other animals (Ilori *et al.*, 1996). They are facultative anaerobes that ferment a wide range of carbohydrates, possess a complex antigenic structure produce a variety of toxin and other virulent factors. The family includes members of the genera: *Shigella*, *Escherichia*, *Salmonella*, *Proteus*, *Serratia*, *Arizona*, *Enterobacter* (Pelczar *et al.*, 1993).

This study seeks to determine *in vitro* the minimum inhibitory concentration, minimum bactericidal concentration of some enteric bacteria to extracts of *Allium sativum* and the dosage at which this is achieved.

### Materials and methods

#### Collection and treatment of plant materials

Samples of *Allium sativum* were used in this study. They were purchased fresh from Ipata market, Ilorin, Nigeria. The samples were then sun dried for two weeks after which they were milled into coarse powder with a Qlink electric blender; the milled samples were kept in tight bottles.

#### Extraction procedure

Aqueous and ethanolic extracts of *Allium sativum* were prepared using the method described by Barry and Thornsberry (1991).

*Aqueous extract*

Twelve grams of the blended sample was measured into a conical flask and 100 ml of sterile distilled water was added, covered with a cork, mixed together properly and left in the shaker at 100 revolution per minute for 24 hours. The sample was filtered and squeezed through four layers of muslin cloth. The filtrate was then centrifuged at 2,000 revolutions per minute (r.p.m.) for 5 minutes after which it was decanted. The pellet was discarded and the supernatant was sterilized by using the membrane filtration unit with type HC filters. The filtrate obtained was stored in sterile McCartney bottles and kept in the refrigerator at 4°C and later used for antimicrobial tests.

*Ethanollic extract*

Twelve grams of the blended sample was measured into a conical flask and 100 ml of 95% ethanol was added; covered with a cork, mixed together and left on the shaker at 100 r.p.m. for 24 hours after which the extract was filtered and squeezed through four layers of muslin cloth. The filtrate was then centrifuged at 2,000 r.p.m. for 5 minutes after which it was decanted. The pellet was discarded and the supernatant was sterilized by using the membrane filtration unit with type HC filters. The filtrate obtained was stored in sterile McCartney bottles and kept in the refrigerator at 4°C and later used for antimicrobial tests.

*Sterility test of the plant extract*

Each of the above extract (aqueous extract and ethanolic extract) was tested for growth or contaminant. This was carried out by inoculating 1 ml of each of them on Nutrient agar and incubated at 37°C for 24 hours (Barry and Thornsberry, 1991). The plates were observed for growth. Absence of growth in the extracts after incubation indicated that the extracts were sterile. The extracts were then assessed for antimicrobial activity.

*Sterilization of materials*

All glassware used were washed with detergent, rinsed properly with tap water and dried. They were then sterilized by wrapping in Aluminium foil in a hot air oven at 160°C for 90 minutes. All the media, distilled water, and McCartney bottles used were sterilized in the autoclave at 121°C for 15 minutes. The laboratory bench was swabbed with 70% alcohol before and after each round of experiment.

*Collection and maintenance of test organisms*

Pure cultures of bacteria isolated from clinical specimens were obtained from the microbiology and parasitological laboratory of University of Ilorin Teaching Hospital (UIITH), Kwara State, Nigeria, and were used in this study. The organisms are *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Bacillus subtilis* and *Klebsiella pneumoniae*. The organisms were maintained on Nu-

trient agar slants at 4°C and were routinely sub-cultured during storage.

*Preparation and standardization of bacterial inoculum*

Preparation and standardization of each bacterium inoculum was done using the method described by Odebiyi and Sofowora (1979). This was carried out by picking test organism growing as a pure culture on solid media and then transferred into sterile nutrient broth and incubated for 18-24 hours to produce a growth of the same turbidity. Each standardized inoculum was used for antimicrobial test.

*Preparation of culture media*

The materials used for the preparation of culture media were spatula, conical flasks, aluminum foil, Bunsen burner and tripod stand, autoclave, weighing balance, cotton wool, and measuring cylinder. The culture media used in this project work were Nutrient agar, Mueller Hinton agar, and Nutrient broth. All media were prepared according to manufacturers specifications. For example, Nutrient agar powder (14 g) was dissolved in a sterile conical flask containing 500 ml of sterile distilled water. The suspension was heated to boiling and was autoclaved at 121°C for 15 minutes. After autoclaving, the media was allowed to cool to a temperature of about 45°C before pouring into Petri dishes

*Phytochemical screening of the extract of Allium sativum*

The methods described by Odebiyi and Sofowora (1978) were used to determine the presence of saponins, tannins, phenolics and alkaloids, Lieberman Burchard reaction as described by Harborne (1973) was used to test for Steroids, while the Salkowski test was used to test for the presence of glycosides.

*Antibacterial test*

The antibacterial properties of the aqueous and ethanolic extracts were determined using the agar well diffusion method of Bauer *et al.* (1996). Twenty-four hour old broth cultures of each test organism (standardized inoculum) were swabbed onto sterile Mueller Hinton agar in Petri dishes using sterile cotton swab. A sterile stainless steel cork borer of size 5mm in diameter was used to make wells on the plates. The holes were filled with 10 mg/ml, 20 mg/ml and 30 mg/ml of the extracts. Each well was labeled appropriately. Control experiment was also set up where the holes were filled with sterile distilled water. The sterile distilled water and the extract were incorporated into the holes by the use of sterile 2 ml syringes.

The inoculated Petri dishes were left for an hour at room temperature for the extract to diffuse before the growth of organism commenced. The plates were incubated at 37°C for 24 hours after which the result were read by measuring

the diameters of zones of inhibition around the wells with the aid of a metric ruler and recorded. The antimicrobial studies were done in duplicates and diameters of zones of inhibition (mm) are expressed as means and standard errors on means. A larger zone of inhibition indicated the test substance was very active against the bacteria. A small zone may mean that the substance was only slightly active against the organism, and no zone may mean no activity, but this might also mean that the substance was unable to diffuse through the agar.

#### Minimum inhibitory concentration (MIC)

The MIC of ethanolic extract was determined using agar dilution method described by Mitscher *et al.* (1972) and Baker and Breach (1980). One milliliter of different concentration of each of the extract was added to 14 ml of Nutrient agar to make the final concentration ranging from 10 mg/ml to 30 mg/ml of the extract in the agar. Standardized inoculum was streaked in a radial pattern on the agar in Petri dishes containing various concentrations of the extracts. The plates were incubated at 37°C for 24 hours. The lowest concentration of each extract inhibiting the growth of test organism was taken as the minimum inhibitory concentration.

#### Minimum bactericidal concentration (MBC)

Visual observation of growth inhibition on solid medium was used to determine MBC as described by Mitscher *et al.* (1972). The agar plates of different extracts of different concentrations inoculated with organisms were observed for growth after incubation for 24 hours at 37°C. The plates that show no visible growths were sub-cultured on fresh Nutrient agar plates by picking inoculum from the line of streaking. The lowest concentration of the extracts that does not yield any growth on the solid medium after the incubation period was taken as the minimum bactericidal concentration.

#### Comparison of the antimicrobial effectiveness of the ethanolic extracts with those of pure conventional antibiotics.

Three antibiotics, chloramphenicol, ampicillin and co-trimoxazole were used. The antimicrobial activity of 12 mg/ml concentrations of each of the antibiotics were compared with that of plant extracts at the same concentration since the initial concentration of the extract used

was 12 mg/ml. Sterile distilled water was used as diluent for the antibiotics while ethanol was used for the extracts. Antibacterial assay was carried out by agar diffusion technique (Leong and Yang, 1981). Zones of inhibition, which are measures of antibacterial activities, were determined around the well.

## Results

The phytochemical screening of *Allium sativum* showed the presence of saponins, trace amount of tannins and alkaloids and absence of steroids and glycosides this is presented in Tab. 1.

Tab. 1. Phytochemical screening of ethanolic extracts of *Allium sativum*

Phytochemicals	Ethanolic extract of <i>Allium sativum</i>
Saponins	+
Tannins	±
Alkaloids	±
Steroids	-
Glycosides	-

Key: + = Present, - = Absent, ± = Trace amount

The aqueous and ethanolic extracts of *Allium sativum* possess appreciable antibacterial activity against some Gram-negative test bacteria. The aqueous extract was found to exhibit differential activity against *Escherichia coli*, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*. Two bacteria, *Staphylococcus aureus* and *Bacillus subtilis* however showed resistance to the aqueous extract. Ethanolic extract was active in all cases with the most activity against *Escherichia coli* while *Staphylococcus aureus* showed least sensitivity. This is presented in Tab. 2 and 3.

The minimum inhibitory concentration (MIC) and concentration minimum bactericidal concentration (MBC) of both aqueous and ethanolic extract of *Allium sativum* are presented in Tab. 4 and 5. For aqueous extracts *E. coli* was inhibited at 20 mg/ml while *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* was inhibited at 30 mg/ml but *Bacillus subtilis* and *Staphylococcus aureus* show no zone of inhibition. The aqueous extracts of *Allium sativum* has no bactericidal effects on *Staphylococcus aureus*, *Bacillus subtilis*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Escherichia coli*.

Tab. 2. Antimicrobial activity of the aqueous extract of *Allium sativum* against some selected microorganisms

Organisms	Zone of inhibition (mm) Concentration			
	Control	10mg/ml	20mg/ml	30mg/ml
<i>Staphylococcus aureus</i>	0.00±0.00 <sup>a</sup>	0.00±0.00 <sup>a</sup>	0.00±0.00 <sup>a</sup>	0.00±0.00 <sup>a</sup>
<i>Klebsiella pneumoniae</i>	0.00±0.00 <sup>a</sup>	2.50±0.12 <sup>b</sup>	4.50±0.21 <sup>c</sup>	6.00±0.21 <sup>d</sup>
<i>Escherichia coli</i>	0.00±0.00 <sup>a</sup>	3.00±0.20 <sup>b</sup>	7.00±0.20 <sup>c</sup>	9.50±0.40 <sup>d</sup>
<i>Bacillus subtilis</i>	0.00±0.00 <sup>a</sup>	0.00±0.00 <sup>a</sup>	0.00±0.00 <sup>a</sup>	0.00±0.00 <sup>a</sup>
<i>Pseudomonas aeruginosa</i>	0.00±0.00 <sup>a</sup>	2.50±0.10 <sup>b</sup>	5.00±0.27 <sup>c</sup>	7.00±0.21 <sup>d</sup>

Key: Each value is a mean of three determinations ± standard deviation. Different superscripts across the table are significantly different ( $p < 0.05$ )

Tab. 3. Antimicrobial activity of ethanolic extracts of *Allium sativum* against some selected microorganisms

Organisms	Zone of inhibition (mm) Concentration			
	Control	10 mg/ml	20 mg/ml	30 mg/ml
<i>Staphylococcus aureus</i>	0.00±0.00 <sup>a</sup>	2.50±0.12 <sup>b</sup>	3.00±0.10 <sup>c</sup>	4.50±0.10 <sup>d</sup>
<i>Klebsiella pneumoniae</i>	0.00±0.00 <sup>a</sup>	5.50±0.12 <sup>b</sup>	7.00±0.15 <sup>c</sup>	9.50±0.21 <sup>d</sup>
<i>Escherichia coli</i>	0.00±0.00 <sup>a</sup>	7.00±0.21 <sup>b</sup>	9.50±0.10 <sup>c</sup>	12.50±0.20 <sup>d</sup>
<i>Bacillus subtilis</i>	0.00±0.00 <sup>a</sup>	2.50±0.21 <sup>b</sup>	3.50±0.10 <sup>c</sup>	5.00±0.15 <sup>d</sup>
<i>Pseudomonas aeruginosa</i>	0.00±0.00 <sup>a</sup>	6.50±0.21 <sup>b</sup>	8.50±0.15 <sup>c</sup>	11.00±0.20 <sup>d</sup>

Key: Each value is a mean of three determinations ± standard deviation. Different superscripts across the table are significantly different ( $p < 0.05$ )

Tab. 4. The bacteriostatic effects of aqueous and ethanolic extracts of *Allium sativum*

Organisms	Concentrations of extracts (mg/ml)					
	Aqueous extracts			Ethanolic extracts		
	10	20	30	10	20	30
<i>Staphylococcus aureus</i>	-	-	-	-	-	+
<i>Klebsiella pneumoniae</i>	-	-	+	-	-	+
<i>Escherichia coli</i>	-	+	+	-	+	+
<i>Bacillus subtilis</i>	-	-	-	-	-	+
<i>Pseudomonas aeruginosa</i>	-	-	+	-	-	+

Key: + = Growth inhibition by the extracts. - = No growth inhibition by the extracts.

Tab. 5. The bactericidal effects of aqueous and ethanolic extracts of *Allium sativum*

Organisms	Concentrations of extracts (mg/ml)					
	Aqueous Extracts			Ethanolic Extracts		
	10	20	30	10	20	30
<i>Staphylococcus aureus</i>	-	-	-	-	-	-
<i>Klebsiella pneumoniae</i>	-	-	-	-	-	-
<i>Escherichia coli</i>	-	-	-	-	-	+
<i>Bacillus subtilis</i>	-	-	-	-	-	-
<i>Pseudomonas aeruginosa</i>	-	-	-	-	-	+

Key: + = Growth inhibition of the organism by the extracts. - = No growth inhibition of the organism by the extracts

Tab. 6. Comparison of the antimicrobial efficacy of the ethanolic extracts of *Allium sativum* with standard antibiotics

Test Organisms	Zone of inhibition (mm)			
	<i>Allium sativum</i>	Chloramphenicol	Ampicillin	Cotrimoxazole
<i>Staphylococcus aureus</i>	4.50	19.50	6.00	0.00
<i>Klebsiella pneumoniae</i>	9.50	0.00	18.00	26.50
<i>Escherichia coli</i>	12.50	23.00	19.50	18.00
<i>Bacillus subtilis</i>	5.00	17.50	6.00	0.00
<i>Pseudomonas aeruginosa</i>	11.00	0.00	9.00	0.00

Key: \* = Concentrations of extract and antibiotics used = 30 mg/ml.

Also the bactericidal effects of ethanolic extracts were shown at 30 mg/ml for *Escherichia coli* and *Pseudomonas aeruginosa* but no bactericidal effects was shown on *Klebsiella pneumoniae*, *Bacillus subtilis* and *Staphylococcus aureus*.

The comparison of the antibacterial efficacy of the ethanolic extracts with standard antibiotics is presented in Tab. 6, the antibiotics tested were more effective than the extracts in most cases.

## Discussion

The Phytochemical screening of ethanolic extract of *Allium sativum* shows that it contains alkaloids, saponins, tannins ( Tab. 1). Many plants that are rich in phenolics compound have been reported to exhibit antibacterial effects.

Okerulu and Ani (2001) reported that extracts of the seeds of *Tetracarpidium canophorum* contains tannins and flavonoids and were found to have inhibitory effects

on the growth of *Streptococcus viridans*, *Bacillus* spp. and *Staphylococcus aureus*. Therefore the presence of tannins in the ethanolic extract of *Allium sativum* could partly be accountable for the observed antibacterial effects in this work. They exhibited their antibacterial action through effects on membrane permeability and inhibition of membrane bound enzymes such as the ATPase and Phospholipase A2 (Hausteen, 1983). The result presented above indicates that the extract of *Allium sativum* had antimicrobial properties, thus justifying their traditional use as a medicinal plant.

The present study showed that the aqueous and ethanolic extracts of *Allium sativum* exhibited antibacterial activity against some medically important bacteria. The extract showed a differential activity against the test organisms. The bioactive extraction potential of the solvents was determined in terms of the extent to which the aqueous or ethanolic extract inhibited the growth of all the test organisms at a concentration of 30 mg/ml. In this study

water was observed to be a poor extractant while ethanol seemed to be better, as its extracts in most cases possessed the widest spectra of activities and was more effective on the test bacteria than the aqueous extract. This observation suggests that the active substances for *Allium sativum* are more soluble in ethanol than in water. This is in harmony with the work of Ibekwe *et al.* (2000).

The work of Brain and Turner (1995) indicated that the efficacy of an extraction procedure depends upon the accessibility of the constituent to the solvents. The result is also in accord with the report of Majekodunmi *et al.* (1996) and Martinez *et al.* (1996) that a higher activity of extrac Tab. natural products was obtained in ethanol compared with aqueous extracts. Ethanol extract of *Allium sativum* inhibited the growth of all the test organism while aqueous extracts was effective only on *Escherichia coli*, *Pseudomonas aeruginosa* and *Klebsiella pneumonia* (Tab. 2). The increase in the antimicrobial effectiveness observed with increase in concentration of the extract (Tab. 2 and 3) is in agreement with the work of Boakye – Yiedom (1979) who reported that higher concentration of anti-bacterial substances showed appreciable growth inhibition of bacteria.

The extracts with low activity against an organism had high MIC while the extract with high activity gave a low MIC (Emeruwa, 1982; El-Faraley *et al.*, 1983). Investigation into the antibacterial effectiveness of ethanolic extracts of *Allium sativum* revealed that the sample have lower activity compared with those of the conventional antibiotics ( Tab. 6).

It has been shown in this work that the use of extracts of *Allium sativum* for curing cholera, dysentery, and urinary tract infections is justified by the effectiveness of its extract on *Escherichia coli*, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* which are known pathogens especially in immunocompromised persons. Some researchers have described the antibacterial activity of garlic extract against microorganisms. Bulbs belonging to the *Allium* genus had the most antibacterial activity against *Streptococcus mutans* (Ohara *et al.*, 2008) and against *Streptococcus agalactiae* (Alsaid *et al.*, 2010). Garlic has antimicrobial activity against *Escherichia coli*, *Salmonella typhi*, *Shigella flexneri*, *Proteus mirabilis* (Shobana *et al.*, 2009); *Streptococcus olaris*, *Streptococcus mitis*, *Staphylococcus aureus* (Silva and Fernandes, 2010; Daka, 2011); and *Vibrio parahaemolyticus*, *Escherichia coli* and *Staphylococcus aureus* (Vuddhakul *et al.*, 2007). Studies have shown some bacteria to be resistant towards garlic extract and these include *Escherichia coli* and *Staphylococcus aureus* (Esimone *et al.*, 2010). This is an indication that when further subjected to in vivo trials after isolation and characterization of the active components; as well as elucidation of the structure of such components, it can be used to treat diseases such as typhoid fever, urinary tract infections (UTI), pneumonia and a host of other. Efforts should include the determination of their relative toxicity to patients, suitability and

possible contra-indication in oral use after they could be incorporated into drugs for the treatment of diseases.

## Conclusions

The use of the extracts of *Allium sativum* for curing cholera, dysentery and urinary tract infections is justified by the effectiveness of its extract on *Escherichia coli*, *Pseudomonas aeruginosa* and *Klebsiella pneumonia*. Nevertheless, further work is still required to purify the active ingredients of *Allium sativum* which have potential for combating human disease. Clinical evaluation of spices in *in vivo* experiments is required to be carried out for low cost treatment with few side effects. In addition toxicological studies need to be evaluated.

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