ISSN (Print): 2476-8316
ISSN (Online): 2635-3490
Dutse Journal of Pure and Applied Sciences (DUJOPAS), *Vol. 5 No. 2b December 2019*

Antibacterial Activity of Crude Extracts of *Allium sativum* on Selected bacteria from Urine of Pregnant Women Attending Barau Dikko General Hospital, Kaduna State

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Abstract
This study was conducted to determine the antibacterial activity of Allium sativum extracts on Staphylococcus aureus and Escherichia coli isolated from urine samples of pregnant women attending Barau Dikko General Hospital, Kaduna, Kaduna State. Isolation was carried out using Manitol salt agar and Eosin Methylene Blue agar. Identification of S. aureus and E. coli was done using conventional standard methods. Aqueous and ethanolic extracts of Allium sativum were prepared by maceration while phytochemical screening was carried out using standard methods. Agar well diffusion method was used for the susceptibility test. Phytochemicals detected include flavonoids, tannins, steroids and alkaloids for both the ethanolic and aqueous extracts. Susceptibility test result revealed zones of inhibitions of 06.00 ± 0.09 to 16.00 ± 0.09 mm and 06.20 ± 0.09 to 15.00 ± 0.09 mm for S. aureus and E. coli respectively, while aqueous extract had zones of inhibition of 06.00 ± 0.05 to 14.00 ± 0.05 mm and 06.00 ± 0.05 to 13.00 ± 0.05 mm for S. aureus and E. coli respectively. There was significant difference (P<0.05) between the antibacterial activity of ethanolic and aqueous extracts. The Minimum Inhibitory Concentration (MIC) of ethanolic extract were 20 mg/ml and 30 mg/ml, while aqueous extract had 30 mg/ml and 40 mg/ml on S. aureus and E. coli respectively. The Minimum Bactericidal Concentration (MBC) of ethanolic extract on S. aureus and E. coli were 40 mg/ml and 50 mg/ml, while that of aqueous extract were 50 mg/ml each respectively. The result showed a significant (P<0.05) antibacterial activity of the A. sativum extract, indicating that these extracts can be further developed as chemotherapeutic agents for the treatment of Urinary tract infections.

Keywords: *Allium sativum* extract, Antibacteria, *Escherichia coli*, Phytochemicals, *Staphylococcus aureus*,

INTRODUCTION
Since ancient time, plants have played a vital role in the discovery of new therapeutic agents (Adesuyi *et al.,* 2011). Many spices that are used daily possess antimicrobial properties and medicinal values (Tepe *et al*., 2004). Most bacteria are sensitive to the extracts from some plants such as garlic, mustard, bitter kola, clove, guava and turmeric (Odebunmi *et al.,* 2009).

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Allium sativum (Garlic) is a bulbous plant that grows up to 1.2m in height, belonging to the family Amaryllidaceae and genus Allium (Adesuyi *et al.,* 2011; Gebreselema and Mebrahtu, 2013).*A. sativum* has many important dietary and medicinal roles which include effects on the cardiovascular system, antibiotic, anticancer, anti-inflammatory, hypoglycaemic, and hormone-like effects (Benkeblia 2004; Ifra and Sana, 2012). It is effective against many Gram positive and Gram negative bacteria and also possesses antiviral and antifungal activity (Rosss *et al*., 2001; Martin and Ernst 2003; Ifra and Sana, 2012). The oil, water solubility, organosulfur compounds, thiosulfinates and numerous phenolic compounds of garlic are responsible for its therapeutic property (Ifra and Sana, 2012).The pungent odour and antibacterial activity of *A. sativum* are due to the presence of allicin (Adesuyi *et al.,* 2011).

S. aureus is a Gram-positive, coccus bacterium, it appears as grape-like clusters when viewed under the microscope, and has large, round, golden-yellow colonies, often with haemolysis when grown on blood agar plates or nutrient agar. *S. aureus* is a member of the Firmicutes, and is frequently found in the nose, urinary tract, respiratory tract and on the skin. It is often positive for catalase and nitrate reduction and is a facultative anaerobe (Masalha *et al.,* 2001*).* Pathogenesis is by production of virulence factors such as potent protein toxins, and the expression of a cell-surface protein(GP24) that binds and inactivates antibodies.

Escherichia coli is a Gram-Negative, facultative anaerobic, rod-shaped, coliform bacterium of the genus *Escherichia*, commonly found in the lower intestine of warm-blooded organisms (Tenaillon *et al.,* 2010*).* Most *E. coli* strains are harmless, but some serotypes can cause Urinary Tract Infection (UTI) and serious food poisoning in their hosts (Vogt and Dippold, 2005; Yu *et al.,* 2015*).* The harmless strains are normal flora of the gut, and can benefit their hosts by producing vitamin K_2, and preventing colonization of the intestine with pathogenic bacteria(Yu *et al.,* 2015). *E. coli* is expelled into the environment with faecal matter and grows massively in the faecal matter under aerobic conditions for 3 days, but its numbers decline slowly afterwards (Vogt and Dippold 2005). *E. coli* and other facultative anaerobes constitute about 0.1% of gut flora, and faecal–oral transmission is the major route through which pathogenic strains of the bacterium cause disease which makes them potential indicator organisms to test environmental samples for fecal contamination (Tenaillon *et al.,* 2010*).*

UTIs are a significant cause of morbidity in infant boys, older men and females of all ages, and there is a high recurrence rates and increasing antimicrobial resistance among uropathogens which greatly increase the economic burden of these infections (Flores-Mireles *et al*., 2015). Chemotherapeutic potentials of plants have shown that they can be sources of antimicrobial compounds (Sibanda *et al.,* 2010; Njume *et al.,* 2011). The problem of antibacterial resistance to commonly used antibiotics has led to the presence of microorganisms in the urinary tract. This study therefore determined the antibacterial activity of crude ethanol and aqueous extract of *Allium sativum* on *S. aureus* and *E. coli* isolated from urine of pregnant women attending Barau Dikko General Hospital, Kaduna, Nigeria.

MATERIALS AND METHODS

Area of study

This study was carried out in the Department of Microbiology, Kaduna State University, Tafawa Balewa Road, Angwan Rimi, Kaduna. Kaduna state is located on latitude 10.53^0 N and longitude 7.44^0E with an elevation of 626m above sea level. It has an annual rainfall range of between 200mm to 600mm. Kaduna State is in the North-Western part of Nigeria. The vegetation cover is Sudan Savannah type, characterized by scattered short trees, shrubs and

grasses. Soil type is mostly loamy to sandy type and substantial amount of clay is found also. Kaduna State consists of 23 Local Government Areas and 59 ethnic groups with a population of 1652800 (Nigeria Population Census 2015). Their major occupation is trading, farming and rearing. Kaduna State share boundary with Nasarawa State, Plateau state, Katsina State, Niger State and Federal Capital Teritory (Abaji *et al.,* 2010).

Sample Collection

Fifty (50) urine samples were collected from pregnant women between the ages of 18-35yrs attending antenatal care clinic of Barau Dikko Hospital, Kaduna. About10-15ml of mid-stream urine sample was collected into sterile, transparent universal bottle containing 0.15g of Boric acid crystals (1% w/v) (Cheesbrough, 2009). The procedure was repeated for all other samples collected after which they were transported on ice pack immediately to the Microbiology Laboratory of Kaduna State University, Kaduna for analyses.

Three kilogram (3kg) of garlic (*Allium sativum)* was purchased from Kaduna central market and packaged in a clean polythene bag. It was identified by botanist (Plant taxonomist) in the Department of Biological Sciences, Kaduna State University, Kaduna. Samples were kept in the herbarium unit of Department of Biological Sciences of the University.

Isolation of *Staphylococcus aureus* and *E. coli*

Ten milliliter (10ml) of urine sample was centrifuged at 2000rpm for 10 minutes and the supernatant discarded. About 0.5ml of the deposit was transferred using sterile pasture pipette onto the prepared Manitol Salt Agar, Eosin Methylene blue and MacConkey agar. Sterile bent glass rod was used to spread the samples on the surface of the media and allowed to adsorb for 1 hour before incubation at 37^{o}C for 24 hour. This was done in duplicate. A suspected discrete colony was picked from a mixed culture plate of MSA, Eosin Methylene blue and MacConkey agar using a sterilize wire loop (Cheesbrough, 2006) and streaked on sterile freshly prepared MSA and EMB agar, and incubated at 37^{o}C for 24 hours. Discrete colonies were then inoculated into a sterile fresh nutrient agar slant to form pure culture stocks of *S. aureus* and *E. coli* respectively (Cheesbrough 2006). Gram's staining was used to determine the morphology of the isolates. Biochemical tests such as catalase, coagulase and haemolysis were carried out for *S. aureus* while methyl red, Voges-proskauer, urease, citrate and indole test were carried out for *E. coli* as described by Cheesbrough (2009).

Preparation of Extracts and Phytochemical Screening of *A. sativum*

Allium sativum was peeled using sterile knife and washed with sterile distilled water and oven dried at 40^{o}C for 3 days. It was milled using ceramic mortar and pestle, and sieved with 0.2 mm sieve to obtain a fine powder (Adesuyi *et al.,* 2011). Fifty grams (50g) of the fine powder was soaked in 250ml of 95% ethanol and water respectively for 72 hours with constant agitation for proper extraction. The mixtures were centrifuged at 3000rpm and filtered using a muslin cloth then suctioned using Suction machine over Whatman number1 filter paper. The filtrate was dried using rotary evaporator and the dried extracts were stored at 4^{o}C in a refrigerator for further analyses (Handa *et al.,* 2008).

Phytochemical Screening of the Extract

The extracts were screened for tannins, flavonoids, saponins, alkaloids, phlobotannins, steroid and glycoside using the method described by Ingle *et al*. (2017).

Determination of Extract Concentration
Ten gram (10g) each of the dried ethanol and aqueous extracts of *A. sativum* were reconstituted with 90 ml of 10% Dimethyl sulfoxide (DMSO) to get a concentration of 100 mg/ml as stock solution. 10 mg/ml, 20 mg/ml, 30 mg/ml, 40 mg/ml and 50 mg/ml were prepared from the stock solution.

Antibacterial Activity of the Extracts on *S. aureus* and *E. coli*
The activity of each crude extract of the plant was determined using the agar well diffusion method; 0.5 McFarland turbidity standard inocula were prepared and 0.5 ml of each was inoculated onto sterile Mueller-Hinton agar using pasture pipette and spread using sterile bent glass rod. Six milliliter (6mm) diameter, 4 mm deep wells were bored into the agar medium using a sterile cork borer, and 0.5ml of the respective extract concentrations were dispensed into the agar wells having sealed the bottom of the holes with a drop of molten agar (Njume *et al*., 2011). The plates were allowed to stand on the laboratory bench for 30 minutes to allow diffusion of the extract into the medium and incubated at 37^{o}C for 24 hours. The zones of inhibition produced by the extracts were measured using transparent meter rule according to the method of Clinical and Laboratory Standards Institute (2016). Streptomycin (10µg/ml) was used as a positive control, and Dimethyl sulphoxide was used as the negative control.

Determination of Minimum Inhibitory Concentration (MIC) of the Extracts
Minimum inhibitory concentration was determined using the broth dilution method as described by Clinical and Laboratory Standards Institute. (2016). About 0.1ml of concentration of each extract that showed antibacterial activity and 0.1ml of inocula were inoculated into five test tubes containing 2ml of Mueller Hinton broth and mixed, and the initial absorbance of each broth tube determined using spectrophotometer at 650 nm wavelength. The tubes were incubated at 37^{o}C for 24 hours, and the final absorbance measured after incubation. The least concentration that gives a reduction in absorbance was termed the MIC.

Determination of Minimum Bactericidal Concentration (MBC) of the Extracts
From each MIC tube, a loopful of the cultures were streaked onto Mueller-Hinton agar plate and incubated at 37^{o}C for 24 hours and observed for the presence or absence of colony. The MBC was considered to be the lowest concentration that demonstrates a pre-determined reduction (99.9%) in Colony Forming Unit (CFU)/ml (CLSI, 2016)

Statistical Analysis
All data collected for this research were subjected to simple analysis of variance (ANOVA), using two way classification, least significant difference (LSD) test was carried out at ($P<0.05$) to determine significant difference between the means (Mukhtar, 2012; Kim, 2014)

RESULTS

Colonial and Morphological Characteristics of *S. aureus* and *E. coli* isolated from urine of pregnant women in Kaduna

Table 1 showed the appearance of golden yellow colonies of *S. aureus* on Manitol Salt Agar (MSA). Green metallic sheen colonies were seen on Eosin Methylene Blue (EMB) agar revealing *E. coli*. Gram examination showed Gram negative short-rods when viewed under x100 objective lens (table 1).

The biochemical characteristics of the isolates were catalase, coagulase positive and produced β-haemolysis on sheep blood agar (table 1). Isolate from EMB agar was found to be methyl red, indole and citrate positive producing gas in triple sugar iron agar. It was also oxidase, Voges-Proskauer and urease negative (table 1).

Phytochemical Compounds of *A. sativum* Extracts

Qualitative phytochemical analysis of ethanolic and aqueous extracts of *A. sativum* (Table 2) revealed the presence of tannins, alkaloids, flavonoids, and steroids in both aqueous and ethanolic extracts. However, phlobotanins and glycosides were detected in ethanol extract and saponin in aqueous extract (table 2).

Table 1: Morphological and Biochemical Characteristic of the Isolates

Sample	Colonial appearance	Gram reaction	Biochemical Characteristic He Ca Co In Mr Vp Ox Ur Ci S/B Gs H_2S	TSI	Inference
US_1 – US_{10}	Yellow colonies	Gram+ve cocci	+ + + - - - - - - - - -		*S. aureus*
UE_1 – UE_{10}	Green metallic sheen	Gram -ve rods	+ - + + + - - + -	A/A + -	*E. coli*

Key:+=Positive, He=Haemolysis, Ca=Catalase, Co=Coagulase, In=Indole, Mr=Methyl Red,Gs=Gas Vp=Voges Proskauer, Ox=Oxidase, Ur=Urease, Ci=Citrate, , S/B=Slant/ Butt. A=Acid (yellow)

Table 2. Phytochemical constituents of Aqueous and Ethanol Extracts of *A. sativum*

Phytochemical	Aqueous Extract	Ethanol Extract
Tannins	+	+
Alkaloids	+	+
Flavonoids	+	+
Phlabotannins	_	+
Saponins	+	_
Steroids	+	+
Glycosides	_	+

Key: (+) = Detected, (-) = Not detected

Antibacterial Susceptibility of *S. aureus* and *E. coli* to crude ethanolic and aqueous extracts of *A. sativum*

The results showed that ethanolic extract had susceptibility of 6.00 ± 0.05 mm to 16.00 ± 0.09 mm and aqueous extract had 6.00 ± 0.05 mm to 14.00 ± 0.09 mm on *S. aureus* respectively. It was 6.20 ± 0.05 mm to 15.00±0.09 mm and 6.10 ± 0.05 mm to 13.00±0.05 mm for ethanolic and aqueous extracts respectively on *E. coli* (table 3).

Table 3. Antibacterial Sensitivity Pattern of Aqueous and Ethanol Extracts of *A. sativum* on *S. aureus* and *E. coli* isolated from urine of pregnant women in Kaduna

Organisms Concentration *Staph aureus* (mg/ml)	Aqueous Extracts (mm)	Ethanol Extracts (mm)	Control; Streptomycin (10µg/ml)
10	06.00±0.05[a]	06.00±0.09[a]	20.01±0.01
20	06.20±0.05[a]	06.20±0.09[b]	
30	08.20±.0.05[b]	10.00±0.09[a]	
40	11.25±0.05[b]	13.50±0.09[b]	
50	14.00±0.05a	16.00±0.09[a]	
P. Values	0.002	0.003	
E. coli			
10	06.10±0.05[a]	06.20±0.09[a]	20.01±0.01
20	06.20±0.05[b]	06.80±0.09[b]	
30	08.20±.0.05[b]	09.00±0.09[a]	
40	11.25±0.05[b]	12.50±0.09[b]	
50	13.00±0.05[a]	15.00±0.09a	
P. Values	0.002	0.003	

Values obtained are mean of triplicate reading.ab, Mean with difference superscript are significantly different at ($P<0.05$).

Minimum Inhibitory Concentration and Minimum Bactericidal Concentration of Aqueous and Ethanol Extracts of *A. sativum on S. aureus* and *E. coli*

The ethanol extracts of *A. sativum* showed MIC at 30mg/ml on both *S. aureus* and *E. coli* (Table 4), while that of aqueous extract was 40mg/ml against *S. aureus* and *E. coli* (table 4). The MBC of ethanol extract against *S. aureus* was 40 mg/ml, and 50 mg/ml on *E. coli*, while that of aqueous extract of *A. sativum* was 50 mg/ml against *S. aureus* but not bactericidal on *E. coli* (table 4).

Table 4: Minimum Inhibitory Concentration (MIC) and Bactericidal Concentration (MBC) of *A. sativum* on *S. aureus* and *E. coli*

Organism	MIC (mg/ml)		MBC (mg/ml)	
	A	E	A	E
Staphylococcus aureus	40	30	50	40
Escherichia coli	40	30	-	50

KEY: A= Aqueous Extract, E= Ethanol Extract, Conc = Concentration, - =No effect

DISCUSSION

The appearance of golden yellow colonies on Manitol Salt Agar (MSA) (Table 1) revealed the presence of *S. aureus*. The production of the yellow colonies on MSA has been reported by Fitzgeerald (2014) to be as a result of fermentation of mannitol salt and consequent production of acid. Microscopic examination of the isolates revealed Gram positive cocci appearing in grape-like cluster. Tong *et al.* (2015) reported similar appearances for *S. aureus.* Also, the observation of Green metallic sheen colonies on Eosin Methylene Blue (EMB) agar revealed the isolate to be *E. coli*. Ibrahim *et al*. (2019) reported the formation of green metallic sheen by *E coli*. In a similar studies in Chiken in Egypt. Gram examination showed Gram negative short-rods when viewed under x100 objective lens (table 1).

The biochemical characteristics of the organism were catalase, coagulase positive and produced β-haemolysis on sheep blood agar (table 1). Studies has reported that *S. aureus* isolated from humans have bound and free coagulase (Holt *et al.,* 1993) and form haemolysis on blood agar plate. The presence of the enzyme coagulase is phenotypically used to differentiate between strain of virulent and less virulent *S. aureus*. It was found to be methyl red, indole and citrate positive produced gas on triple sugar iron plate, and it was oxidase, Voges-Proskauer and urease negative (Whitman *et al.,* 2012). Holt *et al.* (1993) reported similar phenotypic characteristics. Isolation of the *S. aureus* and *E. coli* indicated that the organisms are urinary tract pathogens (Grozdenov *et al.,* 2004; Lindsay, 2010).

Qualitative phytochemical analysis of ethanolic and aqueous extracts of *A. sativum* (Table 2) revealed the presence of tannins, alkaloids, flavonoids, and steroids, on both aqueous and ethanolic extracts. However, phlabotanins and glycosides were detected in ethanol extract and saponin in aqueous extract. Gulsen and Erol (2010), Keerthi and Kumaresan (2014) reported similar findings. According to Das *et al.* (2010) phytochemical compound are secondary metabolites synthesized by plants and has been found to be major part of plants defense mechanism. Findings from this study revealed the presence of saponins only in aqueous extract. Possible reasons could be solvent polarity and nature for better solubility of active agent. Mule *et al.* (2012) reported that solvent used for extraction has an effect on the nature of the compounds extracted. This clearly implies that polarity of solvents (non-polar, polar and less polar) plays crucial role on the types of bioactive compound that can be extracted from plant parts.

The aqueous and ethanolic extracts of *A. sativum* in this study exhibit a good antibacterial activity on *S. aureus* and *E. coli.* The results (Table 4 and 5) of the susceptibility test of the extracts against *S. aureus* revealed zones of growth inhibitions with ethanolic extract having higher values ranged (6.00±0.05mm -16.00±0.09mm) and aqueous extract having lower range (6.00±0.05mm - 14.00±0.09mm). The antibacterial activity of the two extracts showed significance difference ($P<0.05$). Similarly, the result of susceptibility of test against *E. coli* revealed antibacterial activity of the extract having higher zone of growth inhibition values ranged (6.20±0.05mm - 15.00±0.09mm) and lower value range (6.10±0.05mm -13.00±0.05mm); and the antibacterial activity of these extracts on the test organism showed significant difference ($P<0.05$). This pattern of inhibition could be attributed to the fact that the *A. sativum* are more soluble in ethanol than water (Oboh and Masodje 2009; Evbuomwan *et al*., 2018). These findings indicated an antibacterial activity of *A. sativum* extracts against both Gram positive and Gram negative bacteria. Keerthi and Kumareson (2014) reported similar findings to this study. Several studies have attributed the antibacterial and therapeutic activities of *A. sativum* extracts to the presence of flavonoids, biflavonoids, mixture of phenolic compounds and tannins (Anegbeh *et al.,* 2006). The phenolic compounds are said to act as protoplasmic poison which penetrate and disrupt bacterial cell wall in addition to precipitation of cell proteins (Gebreselema and Melbratu, 2013). It implies that the available secondary metabolite is attributed to the antibacterial activity of *A. sativum* extracts recorded in this study; and it can be deduced from the results that *A. sativum* is also a source of bioactive compounds with potential therapeutic benefits.

The zones of inhibition of all the extracts used in this study was higher in Gram positive *Staphylococcus aureus* than Gram negative *Escherichia coli.* This could be attributed to their cell wall composition where by *E. coli* has somatic lipid bilayer, lipopolysaccharide and thin peptidoglycan while *S. aureus* has thick peptidoglycan on their cell wall hence this causes a variation in the penetration of these extracts on their cell walls.

The ethanol extracts of *A. sativum* against *S. aureus* (Table 4) showed MIC at 30mg/ml likewise against *E. coli.* However, aqueous extract showed MIC at high concentration of 40mg/ml against *S. aureus* and *E. coli.* This finding was in agreement with the work of Gulsen and Erol (2010). The low MIC value of ethanol extract could be due to the fact that ethanol extracts contain more phytochemicals than aqueous extract and the ability of ethanol to dissolve and extract more phytochemicals. The MBC of ethanol extract against *S. aureus* occurred at 40mg/ml whereas *E. coli* occurred at 50mg/ml. on the other hand, aqueous extract of *A. sativum* showed MBC at 50mg/ml against *S. aureus* but was not bactericidal to *E. coli* even at the highest concentration used in this study. This finding depicts that aqueous and ethanol extract of *A. sativum* is bactericidal to *S. aureus* likewise the ethanol extract extract is bactericidal to *E. coli* but aqueous extract was not, this could be that, the phytochemicals present in the aqueous extract were not significant enough to cause bactericidal effect on *E. coli.* This result is in agreement with the work of Keerthi and Kumareson (2014) where he report that Extract of Garlic is more effective against *S. aureus, P. aeruginosa* and *E. coli* respectively.

CONCLUSION

The phytochemicals detected in ethanolic and aqueous extracts of *A. sativum* includes tannins, alkaloids, flavonoids, and steroids, on both aqueous and ethanolic extracts. However, phlobotanins and glycosides were detected in ethanol extract and saponin in aqueous extract. Solvent polarity and solubility of active agent is responsible for the differences in phytochemicals observed. There is also a significance difference in antibacterial activity of the two extracts on both *S. aureus* and *E. coli.* Further purification of the extract to determine the exact phytochemical with the exact antimicrobial effects on the isolates. Studies should be carried out to determine the chronic toxicity level of the extracts.

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