Phytochemical Analysis and Antibiofilm Activity *of Allium Sativum* Against Food-Borne Microorganisms .

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Abstract: This work evaluates the chemical composition and antibiofilm potential of extracts from *Allium sativum* in the search for green and effective alternatives for overcoming menace of biofilms. The work evaluated the antimicrobial activity of the extracts against *Staphylococcus aureus* and *Pseudomonas aeruginosa,* minimum biofilm inhibitory concentration, inhibition of initial cell attachment as well as disruption of preformed biofilm. Phytochemical analysis of the plant extract was also carried out using gas chromatography-mass spectrometry. The antimicrobial activity was investigated using the Minimum Inhibitory Concentration assay (MIC) as well as Minimum Bactericidal Concentration (MBC). *Allium sativum* ethanol extract showed the highest antimicrobial activity against the test organisms with MIC and MBC values u 1 mg/ml. *P. aeruginosa* showed more susceptibility to the extracts in the antimicrobial activity test with MIC and MBC values of 0.09 mg/ml and 0.5 mg/ml respectively. However, it showed more resistance in the antibiofilm activity test with MBIC value of 1.0625 mg/ml. *Allium sativum* ethanol extract inhibited cell attachment by at least 50% against the two test organisms while the aqueous and methanol extracts showed no activity at the concentration tested. Disruption of preformed biofilms revealed that *Allium sativum* ethanol extract showed 50% inhibition against *S. aureus* but not *P. aeruginosa.* Antibiofilm potential and biosafety of bioactive compounds from *Allium sativum* reveals a prospective active principle that could be of use in biofilm associated menace.

Keywords: Antibiofilm; *Allium sativum;* extract; activity; phytoche-mical; GC-MS.

Introduction

Microorganisms are involved in the contamination and spoilage of foods. A number of these organisms produce biofilms which helps them to proliferate on surfaces of fresh produce and on food processing surfaces in the industries. Biofilms are multicellular aggregates of sessile cells that are irreversibly attached to a substratum or interface or to each other, encased in a self-produced extracellular matrix of polysaccharides, proteins and nucleic acids and exhibit an altered phenotype in terms of growth rate and gene expression as compared with planktonic bacteria (Costerton *et al.,* 1999). Biofilms can be formed by multiple species as well as single species. Those formed by multiple species predominate in the environment, while those formed by single species usually exist in infections and on medical implants (Toole *et al.,* 2000).

Bacteria embedded in biofilms have been found to be more tolerant to antibacterial compounds than their planktonic counterparts (Luppens *et al.,* 2002; Gupta *et al.,* 2013). While biofilm resistance against antimicrobial agents commences at the attachment phase, it increases greatly as the biofilm ages. Many antimicrobial agents have been used for the control or elimination of bacteria in fresh produce, households, industry and for the treatment of common bacterial infections in humans.

However, different bacterial strains with multiple antimicrobial resistance capacity have emerged over time as a result of indiscriminate use of these antibacterial agents. This has caused the antibacterial agents to be less effective against the microorganisms (Shannon and French, 2004; Dimopoulos and Falagas, 2007).

Natural compounds derived from plants have gained widespread interest in the search to identify the alternatives for microbial control (Essawi and Srour, 2000). These natural products derived from medicinal plants have proven to be an abundant source of biologically active compounds, many of which have been the basis for the development of new lead chemicals for pharmaceuticals. Studies on antimicrobial activity of plant extracts on biofilms are limited. Therefore, in this study the antibiofilm avtivity of *Allium sativum* on biofilms of *Staphylococcus aureus* and *Pseudomonas aeruginosa* were investigated.

Materials and Methods .

Collection and Identification of Plant Material

The plant material *Allium sativum* (garlic) was randomly purchased from Ubaani Main Market in Umuahia metropolis, South East Nigeria. The identification of the plant material was done in the Forestry Department of Michael Okpara University of Agriculture, Umudike, Abia State; Nigeria.

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Preparation of Plant Materials

The plant material obtained was initially air dried at room temperature and ground to a fine powder in an electric blender (SONIK R, JAPAN). The garlic bulbs were peeled to expose the flesh before cutting into small pieces and ground in a mortar with pestle.

3 Preparation of aqueous extracts

Preparation of aqueous extract was done using a modified maceration technique (Remington, 2000). The powdered plant material was macerated sorely with water by ratio of 50g of ground plant material in 150 ml of water and incubated for 3 h with shaking at intervals. Following incubation, the extract was filtered using Whatman (No. 1) filter paper in a Buchner funnel. The extraction process above was repeated twice on the residue using 100 ml and 50 ml of water at the second and third extractions, respectively. The aqueous extract obtained was aliquoted into small conical flasks, sealed with parafilm and frozen at -4°C before drying. The extract was concentrated to dryness in a hot air oven (SELLECTA CE 0505) at 45°C with constant monitoring to prevent thawing of extracts. Following drying, the extract was double sealed with parafilm and stored in a desiccator. The mass of the powdered extract was obtained and the percentage yield determined. The extract was stored at 4°C until further analysis.

4 Preparation of methanol and ethanol extracts

Both the ethanol and methanol extracts were prepared using the same process (Remington, 2000). Fifty (50) grams of the ground *Allium sativum* plant was weighed in a conical flask, followed by the addition of 150 ml of the solvent. The flask was sealed with foil and incubated at room temperature for 3 h. The extraction process was done under occasional shaking. After incubation, the extracts were filtered using Whatman (No. 1) filter paper in a Buchner funnel. The residue collected was re-suspended in another 100 ml and 50 ml of the solvent for the second and third extractions respectively. The filtrates obtained were concentrated using an oven at 50°C, and then transferred to a glass petri dish to be dried at 60°C for 24 h in a vacuum oven. The extracts were scraped out after drying, weighed to obtain percentage yields and stored in sealed vials.

Preparation of Cultures

Typed bacterial strains of *Staphylococcus aureus* and *Pseudomonas aeruginosa* were obtained from the Diagnostic Centre of National Veterinary Research Institute Vom, Plateau State, Nigeria. The strains are *S. aureus* ATCC 12540, ATCC 12600, ATCC 12660, ATCC 12732 and *P. aeruginosa* ATCC 10200, ATCC 10325, ATCC 10145, ATCC 10528.

Glycerol stock cultures of each organism were prepared and kept at -4°C prior to use. The strains were revived onto sterile Tryptone Soy Agar (Oxoid, UK) and incubated at 37°C for 18 h. The identity of the organisms was confirmed using selective media. Following incubation, the organisms were inoculated into sterile Tryptone Soy Broth (TSB) and incubated at 37°C overnight. The overnight culture was standardized to a concentration of 1.0 x 10^6 Cfu/ml. This was done by diluting the overnight cultures with TSB to obtain an absorbance (OD_{590} nm) of 0.02 (Sandasi *et al.*, 2008). The same procedure for preparing cultures was followed throughout the study.

Biofilm Formation and Quantification

The wells of a sterile 96 well flat bottomed microtitre plate were filled with 230µl TSB and thereafter, 200 µl of overnight bacterial culture standardized to a concentration of 1.0 x 10^6 Cfu/ml was poured into each well and incubated at 37°C for 24 h. The negative control wells contained TSB only. After incubation, the content of the plate was poured off and the wells were washed three times with 300 µl of sterile distilled water. The remaining attached cells were fixed with 250 µl of methanol per well and emptied after 15 min and allowed to dry at a temperature of 28 ± 2°C for 3 h. The plate was stained with 250 µl of 0.1% (w/v) crystal violet stain per well for 5 min. Excess stain was rinsed by placing the microtitre plate under running water and air dried. The dye bound to adherent cells was re-solubilized with 250 µl of 90% ethanol per well. Absorbance was measured at 570_{nm} (Stepanovic *et al.*, 2007). Experiments were performed in triplicate and repeated three times.

GC-MS Analysis

GC-MS analysis of *Allium sativum* ethanol extract was performed on the instrument GCMS-QP2010 PLUS SHIMADZU, JAPAN. The oven temperature was programmed at 80°C for 1.0 minute, and was gradually increased to 200°C at 10.0/4.0 min and then ending with 280°C at 10.05.0min. 4.0 µl of sample was injected for analysis. Helium gas 99.995% of purity was used as a carrier gas as well as an eluent. The flow rate of helium gas was set to 1.58 ml/min. The sample injector temperature was maintained at 250° C and the split ratio is 1.0 throughout the experiment periods. The ionization mass spectroscopic analysis was done with 70 eV. The mass spectra were recorded for the mass range 40-600 *m/z* for about 25 minutes. Identification of components was based on comparison of their mass spectra. As the compounds separated on elution through the column, they were detected in electronic signals. As individual compounds eluted from the Gas chromatographic column, they entered the electron ionization detector where they were bombarded with a stream of electrons causing them to break apart into fragments. The fragments were actually charged ions with a certain mass. The m/z ratio obtained was calibrated from the graph obtained which was called as the mass spectrum graph which is the fingerprint of the molecule.

Identification of Compounds

Interpretation of mass spectrum GC-MS was conducted using the database of National Institute of Standard and Technology (NIST) having more than 62,000 patterns. The spectrum of the unknown component was compared with the spectrum of the known components stored in the NIST Library 2008 WILEY8, FAME. The names, molecular weights and structures of the components of the test materials were ascertained.

Determination of Minimum Inhibitory Concentration and Minimum Bacteriocidal Concentration

The Minimum Inhibitory Concentration of *Allium sativum* was determined using the tetrazolium microplate assay as described by Eloff (1998) with slight modifications. This assay was performed using the round-bottomed polystyrene 96-well clear microtitre plates with standard plate layout as proposed by Cos *et al.*, (2006). The extracts were dissolved in Dimethyl Sulfoxide (DMSO) and an identical two-fold serial dilution was made to form 0.03125 – 4.0 mg/ml. 100 µl of the standard culture (1.0 x 10^6 cfu/ ml) was then added to all the wells. The plates were sterile sealed with sealing tape and incubated at 37°C for 24 h. The MIC of the plant extracts was detected following addition of 50µl of 0.2 mg/ml of INT (2-4-Iodophenyl-3-4-nitrophenyl-5-phenyl- 2H-tetrazoliumchloride) in all the wells and incubated for a further 30 mins at 37°C. Bacterial growth was determined by observing the colour change of INT in the microplate wells. Biologically active bacterial cells will reduce the colourless tetrazolium salt which act as an electron acceptor to a red-coloured formazan product (Berridge *et al.*, 2005). Inhibition of bacterial growth is observed when the solution in the well remained clear after incubation with INT. MIC is defined as the lowest extract concentration that completely inhibits the growth of microorganisms and it is indicated by the first clear well in the column. For the determination of minimum bactericidal concentration (MBC), 20µl of culture medium from the microtitre plate wells that showed no changes in colour was re-inoculated on Mueller Hinton (MH) agar plates. After 24 h of incubation at 37°C, MBC is determined as the lowest concentration that showed nil bacterial growth on MH agar plates. The MIC and MBC determinations was performed in duplicate. The positive and negative controls were ciprofloxacin and TSB (Oxoid, UK) respectively.

Determination of Minimum Biofilm Inhibitory Concentration (MBIC)

The MBICs of *Allium sativum* was determined according to Cernohorská and Votava (2004); Cernohorská and Votava (2008) with slight modifications. The experiments were done in 96-wells polystyrene microtitre plates with round bottoms (Sigma Aldrich, Costa, USA). 75µL of an overnight standard culture of 1.0 x 10^6 cfu/ml was added to the wells of microtiter plate and the plate incubated for 24 h at 37°C. The wells were washed three times with phosphate buffered saline (PBS, pH 7.2) under aseptic conditions to remove unattached bacteria and dried in an inverted position at a temperature of 60°C for 1 h. Volumes of 100µL of appropriate two-fold dilutions of the respective plant extracts were transferred into the dried wells with established biofilms. The microtitre plate was incubated for 18–20 h at 37°C. Following incubation, 50 µl of 0.2 mg/ml of INT (2-4-Iodophenyl-3-4-nitrophenyl-5-phenyl- 2H-tetrazoliumchloride) sample was added in all the wells and incubated for further 30 min at 37°C and the MBIC determined, which corresponds to the lowest concentration of the extracts which inhibits growth of biofilm cells as indicated by the first clear well. The positive and negative controls were ciprofloxacin (V. S. International pvt Ltd. INDIA) and Tryptone Soy Broth (TSB) (Oxoid, UK) respectively. The experiment was repeated two times.

Inhibition of cell attachment

The plant extracts were tested for their potential anti-adhension properties at a concentration of 1 mg ml^{-1}. Two hundred (200) microlitres of the extracts were added to the 96-well microtitre plates, and equal volumes of TSB and ciprofloxacin (MIC value) were added as negative and positive controls, respectively. 200 µl of standardized culture (1.0×10^6 CFU ml^{-1}) was then pipetted into the wells to yield a final volume of 400 µl in each well. The cultures were added into the wells in triplicate. The plates were sterile sealed with sealing tape and incubated at 37°C for 8 h without shaking to allow cell attachment and biofilm development. Following incubation, the modified crystal violet assay was performed to assess biofilm biomass, and the results expressed as percentage inhibition (Eqn 1).

(Eqn1) (Sandasi *et al.*, 2008)

$$\frac{OD \quad OD}{OD}$$

Assessment of biofilm biomass (Crystal violet staining assay)

Cell attachment was assessed using the modified crystal violet (CV) assay (Djordjevic *et al.*, 2002). The incubated plates were washed three times with sterile distilled water to remove loosely attached cells. The plates were air-dried and then oven-dried at 60°C for 45 min. Following drying, the wells were stained with 100 µl of 1% crystal violet and incubated at room temperature for 15 min after which the plates were washed three times with sterile distilled water to remove unabsorbed stain. The semi-quantitative assessment of biofilm formation was performed by adding 125 µl of ethanol to destain the wells. One hundred (100) microlitres of the destaining solution was then transferred to a cuvette, and the absorbance determined at 590_{nm} using a spectrophotometer (*SPECTRUMLAB S23A*). The mean absorbance ($OD_{590\,nm}$) of the samples was determined, and percentage inhibition obtained (Eqn 1) (Sandasi *et al.*, 2008).

Inhibition of biofilm growth and development

Biofilm was allowed to pre-form for 4 h prior to the addition of plant extracts at a final concentration of 1 mg ml^{-1} in the wells. Biofilm formation was achieved by aliquoting 100 µl of a standardized ($1{\cdot}0 \times 10^6$ CFU ml^{-1}) culture of the test organisms into a 96-well microtitre plate. The plates were incubated at 37°C for 4 h to allow cell attachment. Following the 4 h incubation, 100µl of each plant extract was added to yield a final concentration of 1 mg ml^{-1} in the wells, and equal volumes of TSB and ciprofloxacin were added as negative and positive controls, respectively. The plate was further incubated for 24 h before the crystal violet assay is performed (Sandasi *et al.*, 2008).

Results

Yields of Plant Extracts after Extraction with Different Solvents

Different methods abound for the successful extraction of useful and bioactive compounds from plants. In this study, extraction of plant material was performed using water, ethanol and methanol as described earlier. Quantitatively, the best extractant was water with yield of 21.86%. This is followed by methanol (16.76%) and ethanol (4.86%) as shown in table 1.

Table 1: Yield of Plant extracts after extraction with various solvents

	Allium sativum	
Solvent	Yield	Yield (%)
Water	10.93	21.86
Ethanol	2.43	4.86
Methanol	8.38	16.76

Biofilm Formation and Quantification

A total of 4 organisms each of *S. aureus* and *P. aeruginosa* collected from the diagnostic centre of National Veterinary Research Institute, Vom Plateau State were screened for biofilm formation. The results of the screening for biofilm formation and quantification are shown in table 2.

P. aeruginosa ATCC 10325 produced the highest biofilm 0.83 ± 0.006^a Biofilm Formation Index (BFI) and was quantified as strong biofilm producer. *S. aureus* ATCC 12540 produced the highest biofilm 0.70 ± 0.003^b and was quantified as strong biofilm producer.

Table 2: Biofilm formation and quantification of the test organisms

Name of organism	Biofilm (OD_{570})	Biofilm producing ability
S. aureus ATCC 12540	0.70 ± 0.003^b	Strong producer
S. aureus ATCC 12600	0.15 ± 0.009^g	Poor producer
S. aureus ATCC 12660	0.27 ± 0.003^f	Poor producer
S. aureus ATCC 12732	0.42 ± 0.012^d	Moderate producer
P. aeruginosa ATCC 10200	0.39 ± 0.003^e	Moderate producer
P. aeruginosa ATCC 10325	0.83 ± 0.006^a	Strong producer
P. aeruginosa ATCC 10145	0.41 ± 0.003^d	Moderate producer
P. aeruginosa ATCC 10528	0.62 ± 0.007^c	Moderate producer
Control	0.14 ± 0.006^g	Non producer

Values are means ±standard error of means of three replicates. Values in each column followed by different superscript within each column are significantly different at P<0.05.

GC-MS Analysis of Plant Ethanolic Extracts

GC-MS analysis of the ethanol extracts of *Allium sativum* was carried out. The Chromatogram of *Allium sativum* is shown in Figure 1. A total of nine (9) compounds were identified in *Allium sativum*. The Chromatogram shows 5 prominent peaks in the retention time range 8.675 - 26.117. The peak at 21.233 retention time is having the peak area 50.09%. This largest peak is due to the presence of Oleic acid. The Second less prominent peak at 18.517 retention time having the peak area 15.81% is due to the presence of n-Hexadecanoic acid. The third less significant peak at 12.375 retention time with the peak area 9.63% is Cyclopenta naphthalene, 5 methyl-1, 2, 3, 9b-tetraaza. The Fourth less prominent peak at 8.675 retention time with the peak area 7.86% denotes 2- Propenoic acid, 2-methyl-, 2-propenyl ester while the last prominent peak at 26.117 retention time with peak area 7.30% is vitamin E acetate. The other less prominent peaks at other retention times are given in Table 3.

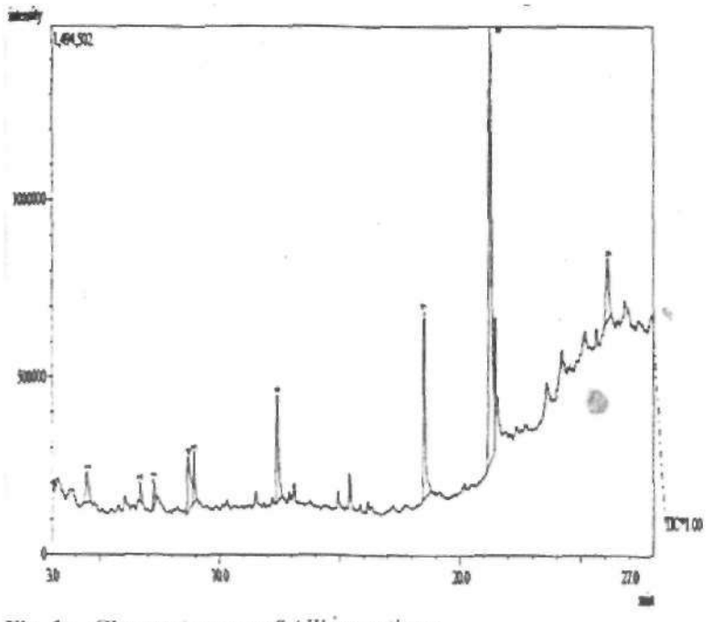

Fig. 1: Chromatogram of *Allium sativum*.

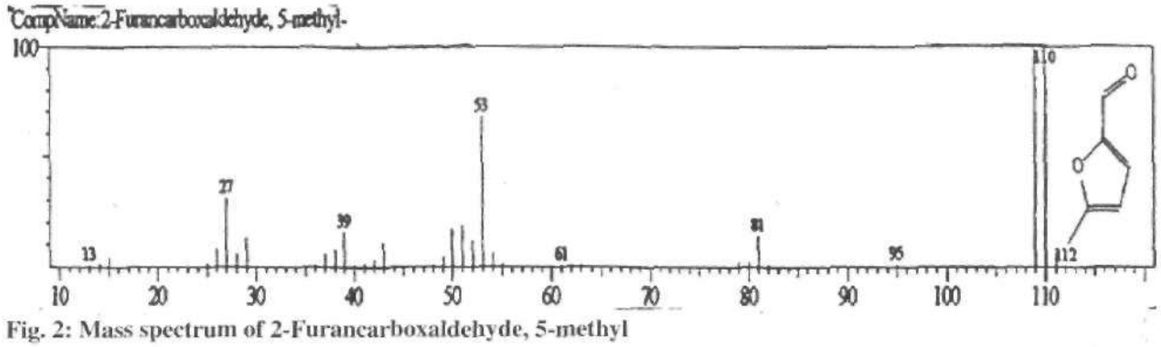

Fig. 2: Mass spectrum of 2-Furancarboxaldehyde, 5-methyl

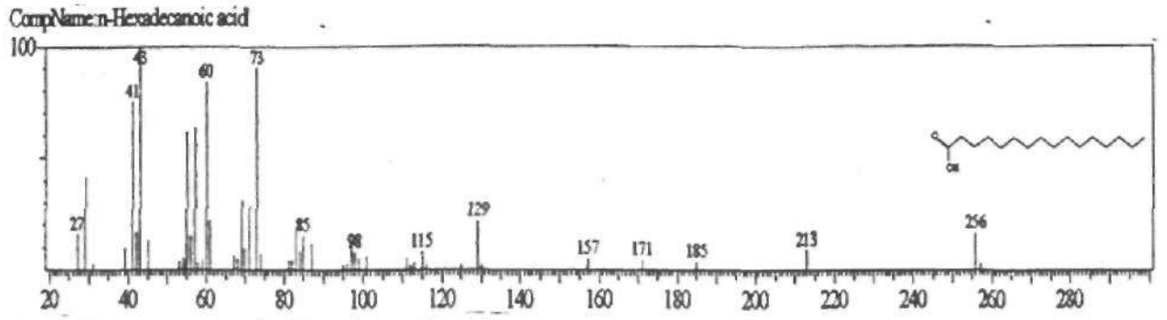

Fig. 3: Mass spectrum of n-Hexadecanoic acid

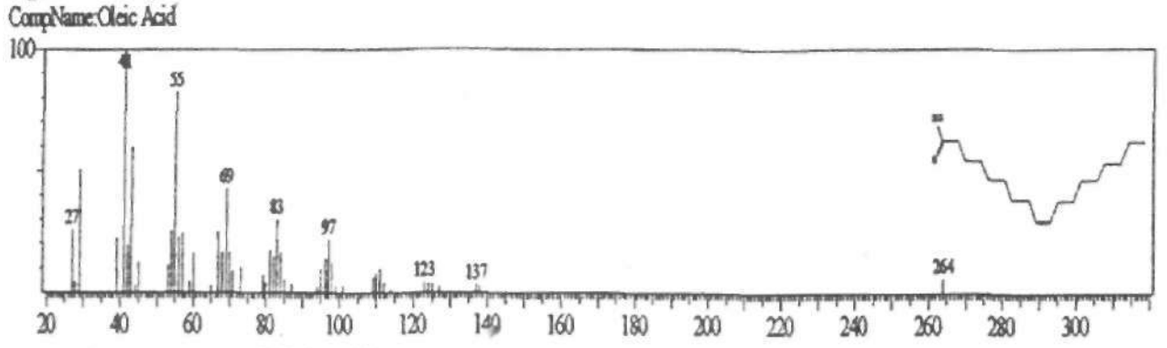

Fig. 4: Mass spectrum of Oleic acid

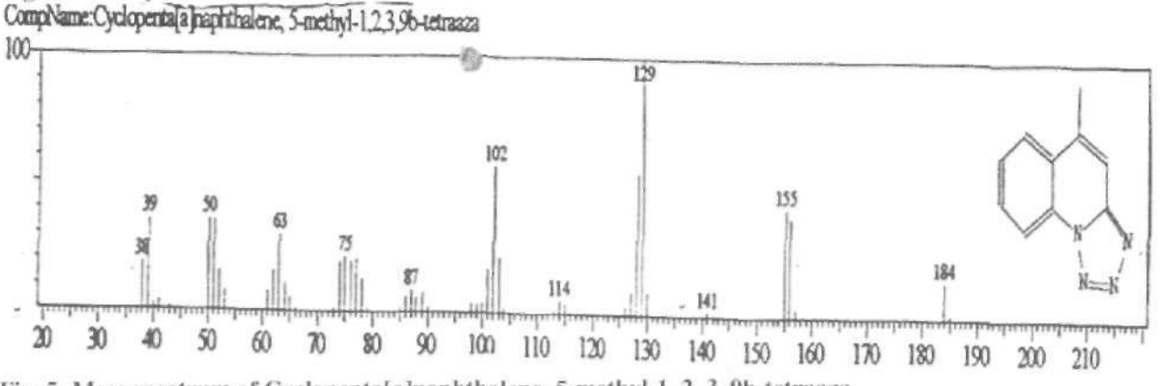

Fig. 5: Mass spectrum of Cyclopenta[a]naphthalene, 5-methyl-1, 2, 3, 9b-tetraaza

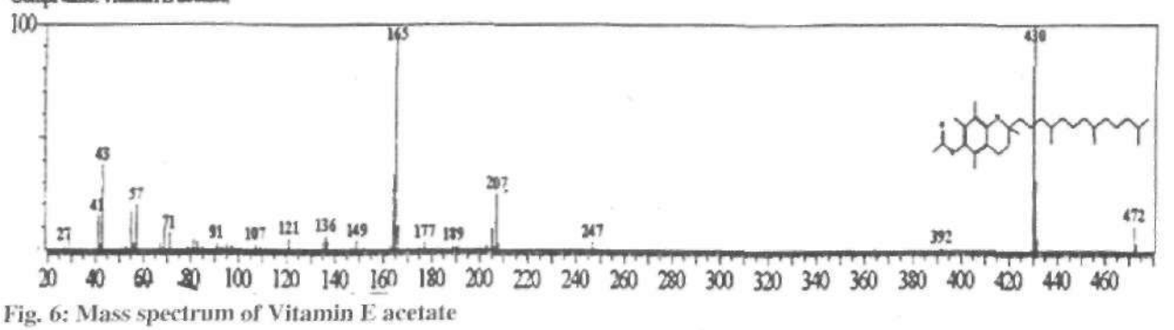

Fig. 6: Mass spectrum of Vitamin E acetate

Table 3: Identified phytochemical compounds in *Allium sativum* with their retention time, molecular weight and composition

NO	RT	Name of compound	Molecular formula	MW	Peak area %
1	4.475	2-Furancarboxaldehyde, 5 methyl	$C_6H_6O_2$	110	3.32
2	6.667	Methyl allylthioacetate	$C_6H_{10}O_2S$	146	1.18
3	7.242	4H-Pyran-4-one, 2, 3-dihydro-3, 5-dihydroxy-6-methyl	$C_6H_8O_4$	144	1.84
4	8.675	2-Propenoic acid, 2-methyl-,2-propenyl ester	$C_7H_{10}O_2$	126	7.86
5	8.917	Trisulfide, di-2-propenyl	$C_6H_{10}S_3$	178	2.95
6	12.375	Cyclopenta[a]naphthalene, 5 methyl- 1, 2, 3, 9b-tetraaza-	$C_{10}H_8N_4$	184	9.63
7	18.517	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	256	15.81
8	21.233	Oleic acid	$C_{18}H_{34}O_2$	282	50.09
9	26.117	Vitamin E acetate	$C_{31}H_{52}O_3$	472	7.30

Table 4: Identified phytochemical compounds in *Allium sativum* with their nature and activity.

No	Name of the compound	Compound Nature	Activity
1	2-Furancarboxaldehyde, 5 methyl	Aldehyde compound	Antimicrobial, Preservative
2	Methyl allylthioacetate	Sulfur compound	Antimicrobial
3	4H-Pyran-4-one, 2, 3-dihydro-3, 5-dihydroxy-6-methyl	Kojic acid	White crystalline powder, Inhibits skin melanin formation, antibacterial, antifungal
4	2-Propenoic acid, 2-methyl-,2-propenyl ester	Ester	Antimicrobial
5	Trisulfide, di-2-propenyl	Sulfur compound	Antimicrobial
6	Cyclopenta[a]naphthalene, 5 methyl- 1, 2, 3, 9b-tetraaza-	Hydrazide	Analgesic, anti-inflammatory agent
7	n-Hexadecanoic acid	Palmitic acid	Antioxidant, Hypocholesterolemic Nematicide, Pesticide,Lubricant, Antiandrogenic, Flavor, Hemolytic, 5-Alpha reductase inhibitor
8	Oleic acid	Oleic acid	Antiinflammatory, Antiandrogenic Cancer preventive, Dermatitigenic Hypocholesterolemic, 5-Alpha reductase inhibitor, Anemiagenic Insectifuge, Flavor
9	Vitamin E	Vitamin compound	Antiageing, Analgesic,Antidiabatic, Antiinflammatory, Antioxidant, Antidermatitic, Antileukemic, Antitumor, Anticancer, Hepatoprotective, Hypocholesterolemi, Antiulcerogenic, Vasodilator, Antispasmodic, Antibronchitic, Anticoronary

Activity source: Dr. Duke's Phytochemical and Ethnobotanical Databases.

Antimicrobial Screening of Plant Extracts

4.4.1 Minimum Inhibitory Concentration and Minimum Bactericidal Concentration

The ethanol, methanol and aqueous extracts of *Allium sativum* plant was assessed on growth inhibitory ability against planktonic cells of *S. aureus* and *P. aeruginosa*. The methanol extract and aqueous extracts did not show any activity within the ranges of concentration tested (03125-4 mg/ml) (Table 4). The ethanol extract of *A. sativum* was active against the test organisms with MIC values of 0.1875 and 0.10 mg/ml against *S. aureus* and *P. aeruginosa* respectively. *P. aeruginosa* was a bit more susceptible to the plant extracts than *S. aureus*. The positive control (ciprofloxacin) showed inhibitory activity with MIC values of 0.0625 and 0.05 against *S. aureus* and *P. aeruginosa* respectively.

The bactericidal activities of water, ethanol and methanol extracts of *Allium sativum* plant was also determined for *S. aureus* and *P. aeruginosa*. Generally, *P. aeruginosa* was more susceptible to the extracts than *S. aureus* as evidenced by its lower MBC values compared to that of *S. aureus*. Compared to the positive control, ciprofloxacin belonging to the β-lactam antibiotics which is inhibitor of cell wall synthesis was found to possess high inhibitory activity against the two organisms under investigation.

Also, the capability of *Allium sativum* plant extracts to attenuate biofilm formation was also shown in Table 4. The potent biofilm inhibition activity was shown by *Allium sativum* ethanol extract with MBIC value of 0.75 mg/ml and 1.0625 mg/ml against *S. aureus* and *P. aeruginosa* respectively. The remainder of the biofilms recorded the lowest resistance to the plant extracts with MBIC value greater than one (1). Within the tested MBIC range (0.03125 to 4 mg/ml), ciprofloxacin showed good biofilm inhibitory activity against the test organisms.

Table 4: Antimicrobial activity of *Allium sativum* against *S. aureus* and *P. aeruginosa*

	MIC		MBC		MBIC	
	S. aureus	*P. aeruginosa*	*S. aureus*	*P. aeruginosa*	*S. aureus*	*P. aeruginosa*
Water	NA	NA	NA	NA	NA	NA
Ethanol	0.1875	0.09	0.5	0.5	0.75	1.0625
Methanol	NA	NA	NA	NA	NA	NA
Control	0.0625	0.05	0.10	0.10	0.125	0.125

Values are means of duplicate experiments.
NA: indicates no activity within the range of concentration tested (0.03125 – 4 mg/ml)
Antimicrobial control used was ciprofloxacin

Antibiofilm Activity of Plant Extracts

Inhibition of cell attachment

The use of the extracts to inhibit the test organisms' attachment to PVC is shown in table 5. Inhibition was expressed as percentage values with the most active extract showing the highest percentage inhibition and *vice-versa*. *Allium sativum* ethanol extract successfully inhibited cell attachment of both *S. aureus* and *P. aeruginosa* by at least 50% (table 5). This is shown by the increase in biofilm biomass of the extract compared to the control biofilm which was represented as 0% inhibition. *P. aeruginosa* was more resistant than *S. aureus* as evidenced by its lower percentage inhibition values compared to that of *S. aureus*.

Table 5: Mean biofilm biomass of *S. aureus* and *P. aeruginosa* after exposure to plant extracts at a concentration of 1 mg/ml (inhibition of cell attachment).

Extracts	*S. aureus* Absorbance(OD_{590})	% Inhibition of cell attachment	*P. aeruginosa* Absorbance(OD_{590})	% Inhibition of cell attachment
A. sativum ethanol	0.13 ± 0.024^{b}	72.34	0.25 ± 0.050^{b}	50.98
A. sativum methanol	NA	-	NA	-
A. sativum aqueous	NA	-	NA	-
Ciprofloxacin	0.10 ± 0.021^{c}	78.72	0.17 ± 0.038^{c}	70.59
Control biofilm	0.47 ± 0.011^{a}	-	0.51 ± 0.042^{a}	-

Values are means±standard error of means of three replicates. NA: indicates no activity at 1 mg/ml concentration of the extract. Values in each column followed by different superscript within each column are significantly different at $P \leq 0.05$. Concentration of ciprofloxacin was 0.0625 mg/ml and 0.05 mg/ml for *S. aureus* and *P. aeruginosa* respectively.

Inhibition of growth of a preformed biofilm

The ability of the *Allium sativum* plant extract to inhibit growth of a preformed biofilm was accessed and is shown in table 6. *Allium sativum* ethanol extract was moderately active against preformed biofilm of *S. aureus* but had a very low activity against that of *P. aeruginosa* as evidenced by the percentage inhibition values of 50% and 17% against *S. aureus* and *P. aeruginosa* respectively.

Table 6: Mean Biofilm Biomass of *S. aureus* and *P. aeruginosa* after exposure to plant extracts at a concentration of 1 mg/ml.

Extracts	*S. aureus* Absorbance(OD_{590})	% Inhibition of preformed biofilm	*P. aeruginosa* Absorbance(OD_{590})	% Inhibition of preformed biofilm
A. sativum ethanol	0.35 ± 0.033^{b}	50.00	0.68 ± 0.010^{b}	17.03
A. sativum methanol	NA	-	NA	-
A. sativum water	NA	-	NA	-
Ciprofloxacin	0.31 ± 0.033^{c}	55.71	0.49 ± 0.015^{c}	40.24
Control biofilm	0.70 ± 0.014^{a}	-	0.82 ± 0.024^{a}	-

Values are means±standard error of means of three replicates. NA: indicates no activity at 1 mg/ml concentration of the extract. Values in each column followed by different superscript within each column are significantly different at $P\leq0.05$. Ciprofloxacin was used at a concentration of 0.125 mg/ml for both *S. aureus* and *P. aeruginosa*.

Discussion

Our investigation of the antibiofilm activity of *Allium sativum*, showed that the plant possess good antimicrobial properties especially when the bioactive compounds is well extracted. The lower yields obtained with methanol and ethanol compared with the aqueous extract is possibly due to the selectivity observed with such solvents. Compared to water, which is a universal solvent, these solvents selectively extract compounds from plants, resulting in lower yields of extracts (Remington, 2000). The yields however, depend on the proportions of the compounds present within a plant and hence the variations observed. However, greater yields do not necessarily depict higher antimicrobial activity.

Screening of extracts for antimicrobial activity against planktonic bacteria showed varying degrees of sensitivity of the microorganisms. The strongest inhibition was recorded against Gram negative, *P. aeruginosa* followed by moderate antibacterial activity against Gram positive *S. aureus*. The different cell wall susceptibility of bacteria is the key contributor to various MIC, MBC and MBIC values. According to Fennel *et al.* (2004), Gram positive bacteria are often found to be more susceptible to plant extracts than the Gram negative bacteria. It is well known that the outer membrane present only in the Gram negative bacteria play an important role as an effective barrier. However in this study, *S. aureus* was less susceptible to the extracts compared to *P. aeruginosa* possibly because of its thicker cell wall consisting of few peptidoglycan layers which acts as a functional barrier thus hindering the penetration of antimicrobial compound into the bacterial cell (Tian *et al.*, 2009).

In contrast to the growth inhibitory ability against planktonic cells, biofilms of *P. aeruginosa* were less susceptible to the plant extract. This is due quorum sensing which has been observed in the opportunistic pathogen *P. aeruginosa*. This organism uses quorum-sensing to coordinate the formation of biofilms, swarming motility, exopolysaccharide production and cell aggregation (De Kievit, 2009). The inhibition of *P. aeruginosa* and *S. aureus* biofilm by the *Allium sativum* extract shows that the extract displays quorum interference activity. The common and usual testing on Minimum Inhibitory Concentration (MIC) which measures only planktonic susceptibility seems to be responsible for treatment failures and resistant development among bacterial biofilms. In the present study, the results of MIC, MBC and MBIC have highlighted the interesting activity of *Allium sativum* plant.

The phytochemicals present in the crude ethanol extract of the plant plays an important role for the evident antibacterial and antibiofilm activity. Medicinal plants are rich of secondary metabolites which some of them are directly involved in plant defense mechanisms (Cowan, 1999). The GC-MS analysis of the ethanol extract of *Allium sativum* was conducted to correlate the phytochemical compounds responsible for to the antibacterial and antibiofilm activity. Among the compounds identified in the plant extract, 2-Furancarboxaldehyde, 5-methyl and 2-Propenoic acid, 2-methyl-2 propenyl ester detected in the plant have been reported to interfere with the expression of quorum sensing controlled virulence genes in *P. aeruginosa* (Adonizio *et al.*, 2008; Cady *et al.*, 2012). Quorum sensing and biofilm formation are the central and interconnected features of bacterial social life (Davey and O'Toole, 2000; Hall-Stoodley *et al.*, 2004; Bassler and Losick, 2006), which enables bacteria to organize their activities at the population level and switch from acting as individual cells to concentrated multicellular structure in the form of

biofilms (Kaufmann *et al.*, 2008). Therefore, interference with quorum sensing will definitely lead to inhibition of desired phenotypes such as biofilm formation (Dong et al., 2007). 2-Propenoic acid, 2-methyl, and 2-propenyl ester inhibit quorum sensing regulated gene expression by interacting with receptors in *P. aeruginosa* and make biofilm sensitive to antibiotics (Bjarnsholt *et al.*, 2005; Cady et al., 2012).

According to Adonizio (2008), *Allium sativum* exhibits anti-quorum sensing activities. 2-Furancarboxyaldehyde acts as an anti-quorum sensing agent (Adonizio, 2008; Abraham *et al.*, 2011). Therefore, the presence of these compounds and the possibilities of synergistic or additive effects with other compounds in the extract contributed to the antibiofilm activity observed in the plant as successfully reported throughout this study.

Conclusion

Particular attention is oriented nowadays towards eliminating microbial biofilms from surfaces of fresh produce and from food contact surfaces in the food industry using other alternatives to conventional chemical agents and physical methods. Natural products as alternatives to chemical disinfectants offer consumers safer alternatives and a broader range of choice as they possess broader spectrum of antimicrobial activity. Therefore the development of effective and safe medicine particularly plant extracts with antimicrobial properties have recently received growing interest from both academic and industrial sectors. The findings from this study seemed to validate the traditional use of plant extracts for the treatment of ailments caused by infectious agents. The interesting biofilm inhibitory activity of *Allium sativum* found against *S. aureus* and *P. aeruginosa* biofilm makes this ethnomedicinal plant an outstanding candidate for sanitizing fresh produce and food processing surfaces in food industries.

Moreover, *Allium sativum* plant may also benefit the hospitals and healthcare facilities as biofilm control agents for the prevention of contamination in the medical devises.

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