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Antimicrobial, Proximate and Phytochemical Evaluation of Garlic (*Allium sativum* L) (*Ex-Lugu* Cultivars)

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ABSTRACT

Due to the increasing rates of the evolution of resistant microorganisms to conventional antibiotics, the development of novel antimicrobials remains an area of intensive research in the field of Microbiology. The aqueous methanol and ethyl acetate extracts of *Allium sativum* var. *Ex-Lugu* were analysed for their proximate, phytochemical and antimicrobial properties. Gas chromatography-Mass spectrometry (GC-MS) was employed for the quantitative analysis of the extracts. Proximate analysis revealed the *ex-lugu* variety to be 12% richer in protein compared to the West African Food and Agricultural Organization (FAO) standard reference for composition of foods. Results of phytochemical and GC-MS analyses showed the presence of tannins, caryophyllene oxide and heptadecane-9-hexyl in the methanol extract (ME) but not in the aqueous (AE) and ethyl acetate (EE) extracts. ME (100 mg/mL) showed superior activity with zone of inhibition (ZOI) of 24 mm against *Pseudomonas aeruginosa* and *Salmonella typhimurium*. ME had a minimum inhibitory concentration (MIC) of 6.25 mg/mL against *Enterococcus faecium* and *Escherichia coli* which surpasses the other extracts while the minimum fungicidal concentration (MFC) was 25 mg/mL against *C. albicans* which was less than that of miconazole (50 mg/mL). EE had lesser MIC value (25 mg/mL) against *P. aeruginosa* compared to 50 mg/mL for ampicillin. There was significant difference ($P < 0.05$) in the antimicrobial effect of ME compared to the other extracts. This study has revealed the major and active phytochemical compounds in *Allium sativum Ex-Lugu* cultivar which might be of potential chemotherapeutic effect against infections caused by the test microorganisms.

Keywords: *Allium sativum* extract, antimicrobial activity, Phytochemical, proximate.

Introduction

Plants that possess therapeutic properties or exert beneficial pharmacological effects in humans are generally termed "medicinal plants".[1] Humans have utilized higher plants and their extracts to treat infections for thousands of years either in their crude form or purified state.[2] Therefore, the use of higher plants has become an age-long practice in traditional medicine. Historically, garlic has been used for centuries worldwide by various societies to combat infectious disease. Louis Pasteur was the first to describe the antibacterial effect of onion and garlic juices against both Gram-positive and Gram-negative bacteria.[3] In developing countries, the use of alternative medicine has been on the increase especially because of the several pre-clinical and clinical studies which provide the scientific basis for the efficacy of many local plants in treating infections.

Therefore, over the last few decades, there has been an increase in the use of natural sources as alternate medicine for the treatment of diseases. The structural differences of the bacterial strains may play a role in the bacterial susceptibility to garlic constituents, also natural products have least adverse effects due to the fact that the natural products also stimulates the functioning of the immune system.[4,5]

Garlic is a species in the onion genus "Allium" and a member of the Allium family (Liliaceae). The close relatives include the onion, shallot, leek, chive and rakkyo.[6] It has been used traditionally for ages to treat a wide array of diseases, namely, respiratory infections, ulcers, diarrhea, skin infections, etc. Previous report on the phytochemical and antimicrobial activity of garlic showed that allicin (allyl 2-propene thiosulfinate); a notable flavonoid was the major phytochemical present, and in turn, responsible for the antimicrobial activity reported.[6] In Sokoto state-Nigeria, garlic is grown extensively in Wurno, Goranyo, Gwadabawa, Kware and part of Wamakko local government areas of the state where the crop is grown under irrigation during the cool dry season (Harmattan) in November-March. Nigeria is a producer of different varieties of garlic depending on the locations grown.[7] Garlic has been reported to have antibiotic, anticancer, antioxidant, immunomodulatory, anti-inflammatory, hypoglycemic, and cardiovascular-protecting effect.[8] Systematic screening of plant materials represent an important effort to find new bioactive compounds with the needed therapeutic potential to fight against pathogenic microorganisms (for example, *Salmonella typhae*, *Klebsella pneumonia*, *Staphylococcus aureus*, etc.). Variations in composition of garlic and genetic disparity among bacteria and fungi of the same or different species have been found responsible for the few inconsistencies in the antibacterial and antifungal properties of garlic extract, necessitating the need for local antimicrobial testing of garlic.[9,10] The aim of the study is to investigate the proximate, phytochemical and antimicrobial properties of *Allium sativum* var. ex-lugu, a Nigerian variety of garlic, in view of their applications against clinical pathogens.

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Materials and Methods

Plants Procurement and Processing

Fresh bulbs of garlic (*Allium sativum* var. Ex-lugu) usually present in Wurno Local Government Area, Sokoto State[11] were purchased from a local markets in Oyingbo, Lagos, Nigeria. They were washed using distilled water, peeled, sliced into bits and sun-dried until constant weight was obtained for seven days. After drying, the garlic slices were grounded to fine powder using electric blender. Subsequently, 200 g of the ground plant material was stored in sterile bottles at room temperature and used for the extractions.[12]

Microbial Isolates

The clinical isolates used in this study included *Pseudomonas aeruginosa* (ATCC15442), *Salmonella typhimurium* (ATCC1331), *Escherichia coli* (ATCC 25922), *Enterococcus faecium* (ATCC70021), *Staphylococcus aureus* (ATCC12600). They were obtained from the Medical Microbiology Laboratory, Lagos University Teaching Hospital (LUTH), Idi-araba, Lagos while Methicillin-resistant *Stapylococcus aureus* (MRSA) (ATCC1441) and the fungus, *Candida albicans* (ATCC 90028) were obtained from the Microbiology Laboratory, University of Lagos, Akoka. The bacterial and fungal cultures were kept viable by sub-culturing on Nutrient Agar and Potato Dextrose Agar (Rapid labs, England, UK®), respectively. The test strains were maintained on Nutrient agar slants at 4ºC for the bacteria and potato dextrose agar slant for the fungus.

Preparation of plant extracts

Cold maceration method was employed in the preparation of garlic extracts.[13] Two hundred (200) grams of garlic powder was soaked in 100 mL each of distilled water, ethyl acetate and 95% methanol separately to give 200 mg/mL concentrate. The flasks were incubated at room temperature (28 ± 2°C) for 72 h with shaking at random intervals. After 72 h, the suspensions were filtered using sterile Whatman No.1 filter paper and the filtrates were concentrated using a rotary evaporator (Bibby Sterlin Ltd, England, UK) at 40°C to obtain crude extract. The extracts were labeled accordingly and stored at 4ºC for further analysis.

Proximate Analysis

The test garlic samples were evaluated for moisture, protein, fat, crude fiber, carbohydrate, total ash, according methods described by the Association of Official Analytical Chemists (AOAC).[14]

Phytochemical Analysis

The three different garlic extracts were screened for the presence of secondary metabolites using the procedure of Preshant *et al*.,[15]

Gas chromatography and Mass spectrometry (GC/MS)

For the quantitative analysis, Gas Chromatography system 7890A, Mass Spectrometer 5975C VL MSD and injector 6890N Network GC system (Agilent Technologies®) were used to analyze the methanol extract (ME) and ethyl acetate extracts (EA) of garlic. The column used was 30 m × 320 µm × 0.25 µm with helium as the carrier gas at a flow rate of 1 mL/min and a temperature of 350ºC. The initial oven temperature was 60ºC which was held at 0.5ºC/min. It ran for 10ºC/min and was later increased to 300ºC hold time for 6 min. The total run time was 30.5 min.[16]

Antimicrobial Susceptibility Test

The agar well diffusion method was adopted according to the National Committee for Clinical Standard recommendation.[17] Loopful of isolates were inoculated into Nutrient broth (Rapid labs, England, UK®) and incubated aerobically at 37°C for 18 h. The bacterial and fungal suspensions were diluted with normal saline (0.85 g/L NaCl) and adjusted to match a turbidity of 1.5×10^8 CFU/mL equivalent to the McFarland standard. The standardized suspension of each organism was used to inoculate the surfaces of Mueller Hinton agar (MHA) plates using sterile cotton swab and left to dry at room temperature for 5-10 min. Sterile cork-borer of 6 mm was used to punch holes in the seeded agar plates which were subsequently filled with 1 mL of desired concentrations (100 mg/mL, 90 mg/mL and 80 mg/mL) of each extract. The plates were then allowed to stay for 30 min in order to allow proper diffusion of the extracts into the agar. Commercial antibiotic (Ampicillin) and antifungal (Miconazole) which have been diluted appropriately to desired concentrations (100 mg/mL, 90 mg/mL and 80 mg/mL) were used as reference standards to determine the sensitivity of the isolates. The bacterial plates were incubated aerobically at 37ºC for 24 h while fungal plates were incubated aerobically at room temperature (28 ± 2^{0}C) for 48 h. After incubation, the zone of inhibition (ZOI) was determined by measuring the diameter of the clear zone around each well using a millimeter ruler.

Minimum Inhibitory Concentration (MIC)

The MIC of the various extracts and conventional antibiotics against each of the tested isolates was determined by the macrobroth dilution method. The concentration of 100 mg/mL of garlic extract was prepared for each extract. Two (2) mL of the extracts was diluted double fold with nutrient broth in a series of six test tubes labeled appropriately. The concentrations obtained included 100, 50, 25, 12.5, 6.25 and 3.13 mg/mL. Ampicillin and Miconazole served as positive controls and tubes without any extract or antibiotics (nutrient broth with inoculum) served as negative control. Each test organism was inoculated into the labeled tube by taking a loopful of the standardized bacterial and fungus suspension using a flame-sterilized wire loop. The tubes were incubated aerobically at 37ºC for 18-24 h for bacteria and 24-72 h for fungi. After incubation, the tubes were examined for evidence of growth by turbidity. The lowest concentration that did not permit any visible growth when compared with control was considered as the minimum inhibitory concentration.[18]

Minimum Bactericidal Concentration (MBC) and Minimum Fungicidal Concentration (MFC)

To determine the minimum bactericidal concentration (MBC), the MIC dilution tubes, with no visible growth and the control tubes were sub-cultured onto sterile Muller Hinton agar (MHA) (HiMedia, India®) plates using sterile inoculating loop. The plates were subsequently incubated for 24 h at 37°C and the visible colonies were counted. Similar procedure was carried out to determine minimal fungicidal concentration (MFC) but plates were incubated for 24 - 48 h at room temperature (28 ± 2ºC). The lowest concentration of extract and antimicrobial agent that prevented the growth of an organism after sub-culture onto antimicrobial-free media is considered the MBC and MFC.[18]

Statistical analysis

Data are expressed as means ± SD. One-way ANOVA was calculated with SPSS statistics for Windows, version 21.0 followed by Graph pad software calculator comparison t-tests applied for comparison between two mean values as a measure of test of significance. Difference on statistical analysis of data were considered significant at a confidence level of 95%.

Results and Discussion

The aqueous extract (AE) and methanol extract (ME) of garlic showed the presence of reducing sugars, saponins, phytosterols which were absent in the ethyl acetate extract (EA). However, volatile oils and phenols were observed in ME and EA and absent in AE. Terpenoids were present in the three extracts. Tannins were observed only in ME (Table 1). These volatile oils and phenols have been reported to inhibit the growth of microorganisms.[15] Proximate analysis showed moisture content of 9.76 ± 0.08%, ash content 3.41 ± 0.03%, fibre 1.88 ± 0.03%, protein 18.30 ± 0.02%, carbohydrate 66.20 ± 0.06% and fat 0.48 ± 0.02%. The results also showed a marked difference of 12% in the protein content of *Ex-lugu* variety (18.30 ± 0.02%) compared to the Food and Agricultural Organization of West Africa standard reference (6.80 ± 1.20%)[19] (Table 2) which might account for the different results gotten from the extracts. This difference may be due to environmental factors such as amount of sunlight. Also, there was low percentage of moisture (9.76 ± 0.08%) in the *Ex-Lugu* variety compared to the high moisture content (64.3 ± 1.3%) observed in FAO standard which could be attributed to the sun-drying method used to pulverize the *Ex-Lugu* variety in this study. The protein and carbohydrate contents were high which indicated that the *Ex-Lugu* garlic is a good source of nutrient for growth as well as for nerve tissue regulation.[20] Figures 1 and 2 show the Gas chromatograms of ME and EA, respectively. EA had 18 compounds. Comparatively, ME had 26 compounds made up of phenols, alcohols and esters amongst which are thymol, p-cymene, 1-heptadecane, and 1-octadecane which has been known to possess antimicrobial activity.[21-23] GC/MS profiling showed the presence of p-cymene with percentage composition of 14.462% in ME which probably enhanced its antibacterial activity against *P. aeruginosa* and *S. typhimurium* in this study. This is particularly noteworthy since Gram-negative organisms generally are not easily penetrated by antimicrobials due to their outer membranes.[23] Previous studies have shown that p-cymene, a constituent of essential oils found in garlic, have antimicrobial effect on *E. coli*.[20] Thymol was also present at 3.801% and

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9.822% in EA and ME, respectively. The antimicrobial effect of different concentrations of ME and conventional antibiotics against selected clinical isolates are shown in Figure 3. ME showed maximum zone of inhibition (ZOI) of 24 mm at 100 mg/mL against *P. aeruginosa* and *S. typhimurium* and minimum ZOI of 12 mm at 80 mg/mL against *S. aureus* and *E. coli*. ZOI of 20 mm and 16 mm was obtained at 100 mg/mL and at 80 mg/mL, respectively against *Candida albicans*. Previous studies reported that due to its phenolic structure, thymols have shown antibacterial activity against *S. aureus*.[21] This might have contributed to the large ZOI (23 and 19 mm) observed for ME and EA, respectively against *S. aureus* and probably also account for their better antimicrobial activity compared to the AE which was particularly demonstrated against *P. aeuriginosa*, *S. typhimurium* and *S. aureus* at 100 mg/mL. Figure 4 shows the ZOI of EA and the conventional antibiotic (Ampicillin) and the antifungal (Miconazole) against the test isolates. Maximum ZOI of 21 mm was observed against MRSA at 100 mg/mL and minimum ZOI of 10 mm at 80 mg/mL against *S. typhimurium* and *E. coli*. *Candida albicans*, had maximum ZOI of 20 mm at 100 mg/mL and minimum ZOI of 16 mm at 80 mg/mL. Figure 5 revealed that AE had maximum ZOI of 20 mm at 100 mg/mL against *S. typhimurium* and minimum ZOI of 10 mm at 80 mg/mL against MRSA and maximum and minimum ZOI of 15 mm and 11 mm against *C. albicans* and *E. faecium*, respectively. The presence of 1-heptadecene at 13.458% in EA has been reported to possess anticancer, antioxidant and antimicrobial activities which probably enhanced its antimicrobial activity.[22] The results of MIC assay presented in Figure 6 showed MIC of AE against *P. aeuriginosa, S. typhimurium, E. faecium,* MRSA and *C. albicans* to be 100 mg/mL while against *S. aureus* and *E. coli*, it was > 100 mg/mL. The ME had antimicrobial activity against all test organisms used with MIC values ranging from 6.25-50 mg/mL. The least value of 6.25 mg/mL was observed against *E. faecium* and *E. coli*. Compared to ampicillin which had MIC value of 25 mg/mL against *E. coli*, ME exerted a better MIC value of 6.25 mg/mL. The ME and ampicillin both had the same MIC values of 50 mg/mL, 25 mg/mL and 12.5 mg/mL against *P. aeruginosa*, MRSA and *S. typhimurium*. The most effective MIC of EA was observed against MRSA at 12.5 mg/mL which was better than the MIC value of 25 mg/mL when ampicillin was used. Similarly, EA showed a higher activity against *P. aeruginosa* with MIC value of 25 mg/mL than the 50 mg/mL observed in ampicillin. In Figure 7, the MBC/MFC of AE agaist the organisms showed that concentrations >100 mg/mL is required for a complete killing of the organisms. The MBC values of ME and ampicillin against *P. aeuriginosa* and *S. typhimurium* were both 50 mg/mL which infer they both have the same inhibitory effect. The MBC value of ME against *E. coli* at 12.5 mg/mL showed a better bactericidal effect compared to the 50 mg/mL obtained from the use of ampicillin. The MBC of EA on *S. typhimurium* at 25 mg/mL was observed to be more effective compared to the 50 mg/mL as seen in ampicillin. The MFC of ME against *C. albicans* was 25 mg/mL which showed better fungicidal effect than the MFC value of 50 mg/mL observed with the use of miconazole. Overall, ME showed higher activity against *P. aeruginosa, S. typhimurium, E. faecium, S. aureus*, MRSA, *E. coli* and *C. albicans*. This agrees with the findings of De Boer *et al*.,[12] The ZOI increases as the concentration of the extract increases. *Candida albicans* showed susceptibility to the three extracts used in this study which correlates with the studies of Aliyu[8] and Ameh *et al*[24] who reported that the eukaryotic nature and ergosterol availability in the fungus cell wall may also be crucial to the observed antimicrobial effect of the garlic extract. The MIC values of ampicillin and methanol extract observed against *P. aeruginosa, S. typhimurium* and MRSA were the same at 50 mg/mL, 12.5 mg/mL and 25 mg/mL, respectively while the 6.25 mg/mL MIC value of methanol extract against *E.coli* was of a higher inhibitory effect than the 25 mg/mL observed for ampicillin. EA also proved to be a better alternative to conventional antibiotics as its MIC value (25 mg/mL) against *P. aeruginosa* and *C. albicans* was better than the values obtained for ampicillin (50 mg/mL) and miconazole (50 mg/mL). The ME showed promising MBC of 12.5 mg/mL against *S. aureus* and *E. coli* and MFC of 25 mg/mL against *C. albicans*. ME and EA both showed very promising bactericidal effect while AE showed more of bacteriostatic effect as growth was seen in the MBC/MFC study. The statistical analysis showed that there is significant difference in the effect of ME, EA and AE on the test organisms. Generally, the growth of all the test organisms was inhibited though, at varying degrees. The study showed that the extracts can serve as potential alternative source of antimicrobial agents especially because it showed comparative antimicrobial activity with synthetic antimicrobial agents.[25]

Table 1: Phytochemical profile of aqueous, methanol and ethyl acetate extracts of *Allium sativum* L.

Phytochemical Constituent	Aqueous	Methanol	Ethyl acetate
Reducing sugars	+	+	-
Saponins	+	+	-
Flavonoids	+	+	+
Tannins	-	+	-
Alkaloids	+	-	+
volatile oils	-	+	+
Terpenoids	+	+	+
Phenol	-	+	+
Phytosterols	+	+	+

+ = present - = absent

Table 2: Proximate Composition (%) of Garlic as compared with FAO (2012) standard reference.

Parameters	Quantity (%)*	FAO reference
Moisture	9.76 ± 0.08	64.3 ± 1.3
Ash	3.41 ± 0.03	1.3 ± 1.2
Fat	0.48 ± 0.02	0.4 ± 0.2
crude fibre	1.88 ± 0.03	2.3 ± 1.5
Protein	18.30 ± 0.02	6.8 ± 1.2
Carbohydrate	66.20 ± 0.06	25 ± 0.0

*Values are mean ±standard deviations of duplicate determination

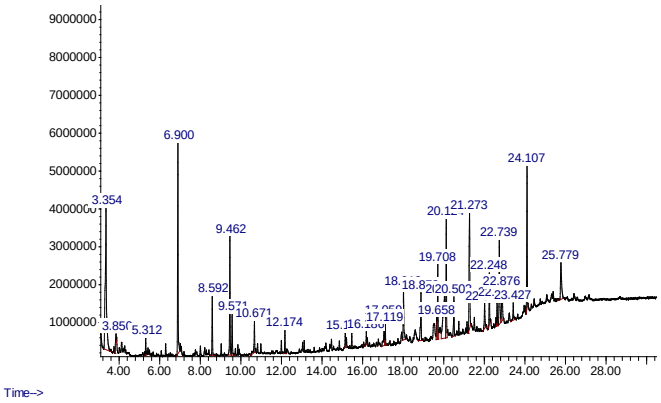

Figure 1: Gas Chromatogram-Mass Spectrometer profiling of methanolic extract of garlic (*Allium sativum*).

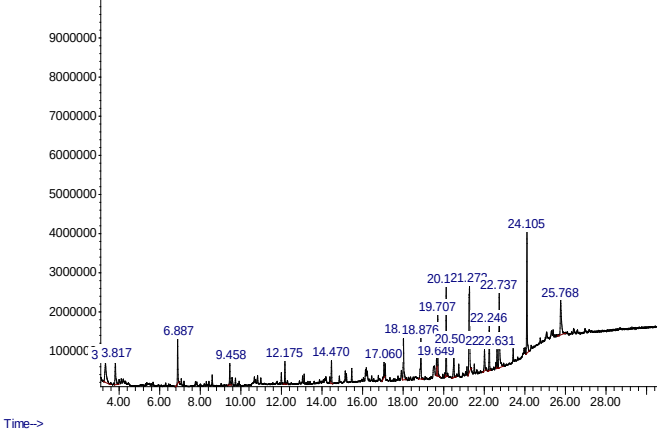

Figure 2: Gas Chromatogram-Mass Spectrometer profiling of ethyl acetate extract of garlic (*Allium sativum*) and the various peaks.

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Table 3: Quantitative chemical composition (% v/v) of ethyl acetate and methanol extract of garlic (*Allium sativum*).

Ethyl acetate Extract			Methanol Extract		
Peak no.	**Compounds**	**% comp.**	**Peak no.**	**Compounds**	**% comp.**
1	p-Cymene	4.947	1	p-Cymene	14.462
2	N-Hydroxymethylacetamide	3.341	2	N-Hydroxymethylacetamide	0.564
3	Thymol	3.801	3	Terpinen-4-ol	0.697
4	Naphtalene	2.151	4	Thymol	9.822
5	Pentadecanal-	1.949	5	Caryophyllene	2.644
6	Hexadecanoic acid, methyl ester	2.121	6	Naphthalene	5.625
				2-Isopropenyl-4a,8-dimethyl-1,2,3,	
7	Isopropyl linoleate	1.694	7	4,4a,5,6,8a-octahydronaphthalene	2.071
8	Heptadecane	5.336	8	Caryophyllene oxide	1.694
9	Tetracosane	4.592	9	1,19-Eicosadiene	0.978
10	Heptacos-1-ene	1.775	10	1-Octadecene	0.635
11	Octadecane	5.866	11	Heneicosane	0.5
12	Bis(2-ethylhexyl) phthalate	7.726	12	Isopropyl linoleate	1.834
13	Dodecane, 2,6,11-trimethyl-	3.487	13	Octadecane	0.883
14	1-Heptadecene	13.458	14	Heptadecane	2.931
15	Ethyl octacosyl ether	4.269	15	Tetracosane	2.793
16	Cyclotriacontane	1.844	16	Pentacos-1-ene	1.206
17	Eicosane	12.627	17	Heptadecane, 9-hexyl-	3.875
				1,2-Bis-[2,7-dimethoxyfluoren-9-yl	
			18	idene]hydrazine	7.533
			19	Bis(2-ethylhexyl) phthalate	5.742
			20	Heneicosane, 11-decyl-	2.067
			21	1-Heptadecene	7.942
			22	Octacosane	1.397
			23	Fumaric acid	2.19
			24	Nonacos-1-ene	1.259
				Estra-1,3,5(10)-triene-2,17-diol, 3,4-	
			25	dimethoxy-, 2TMS	2.579
			26	Tetratriacontane, 17-hexadecyl-	0.984
				1,2-Benzisothiazol-3-amine, TBDMS	
			27	derivative	3.566

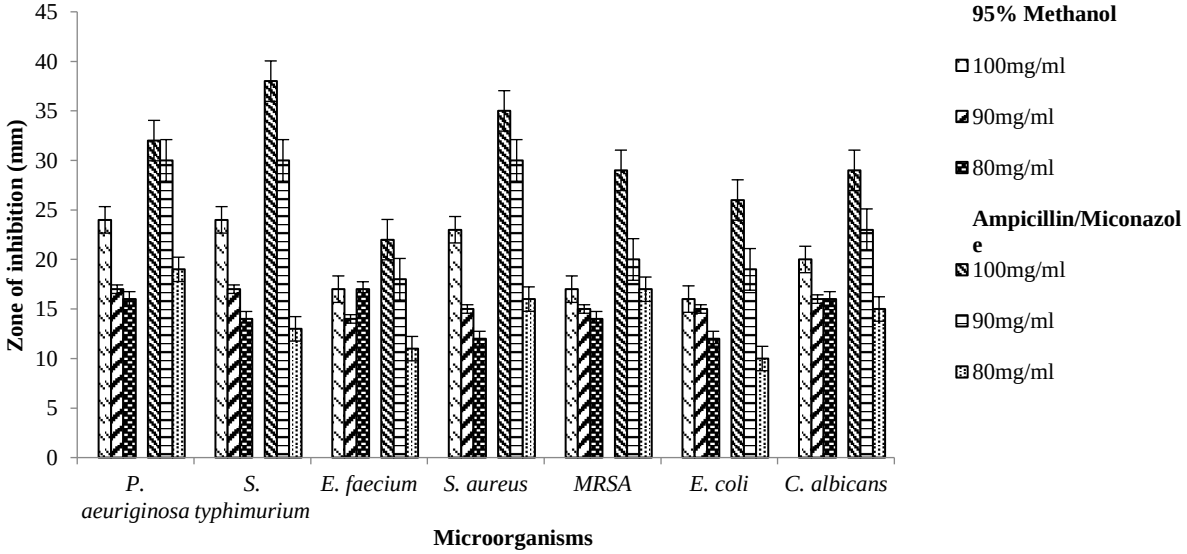

Figure 3: Zone of inhibition of methanolic extract of Garlic and Conventional antibiotics on selected clinical isolates at different concentrations.

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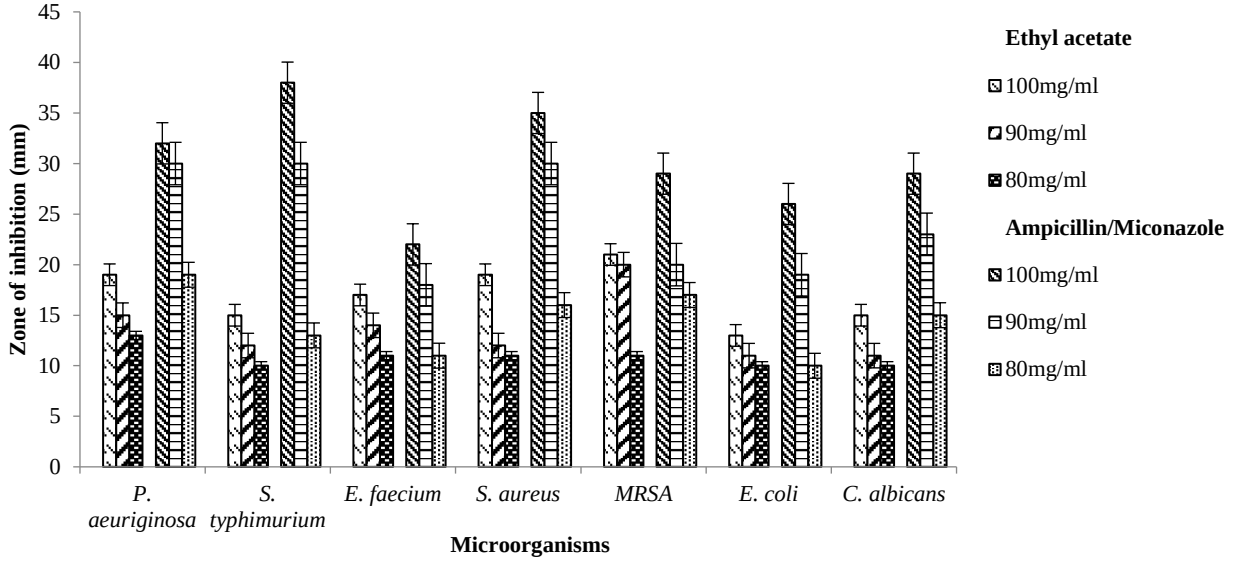

Figure 4: Zone of inhibition of ethyl acetate extract of Garlic and Conventional antibiotics on selected clinical isolates at different concentrations

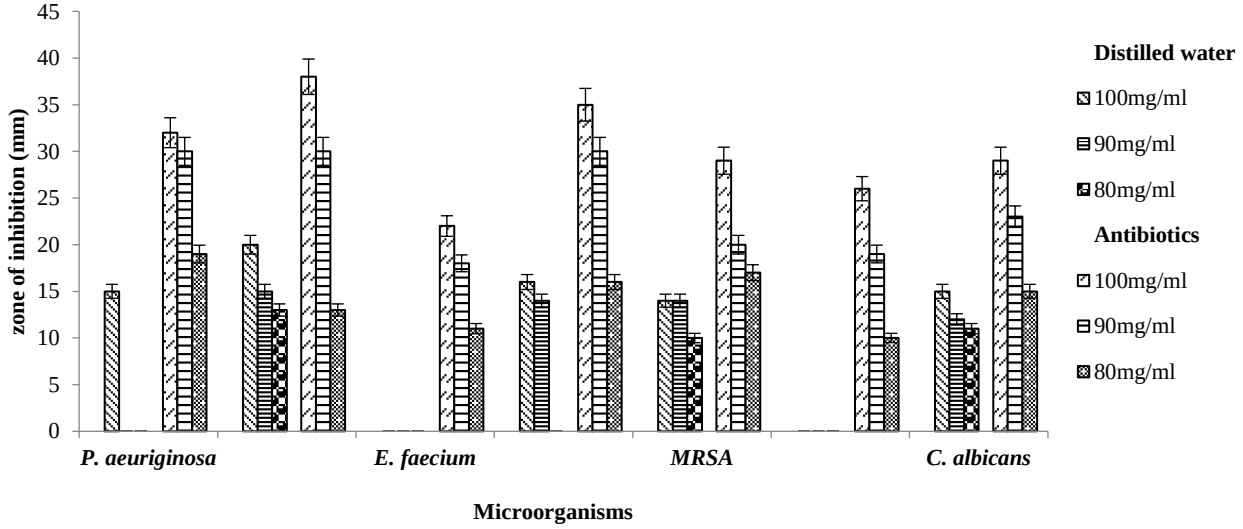

Figure 5: Zone of inhibition of distilled water extract of garlic and conventional antibiotics on selected clinical isolates at different concentrations

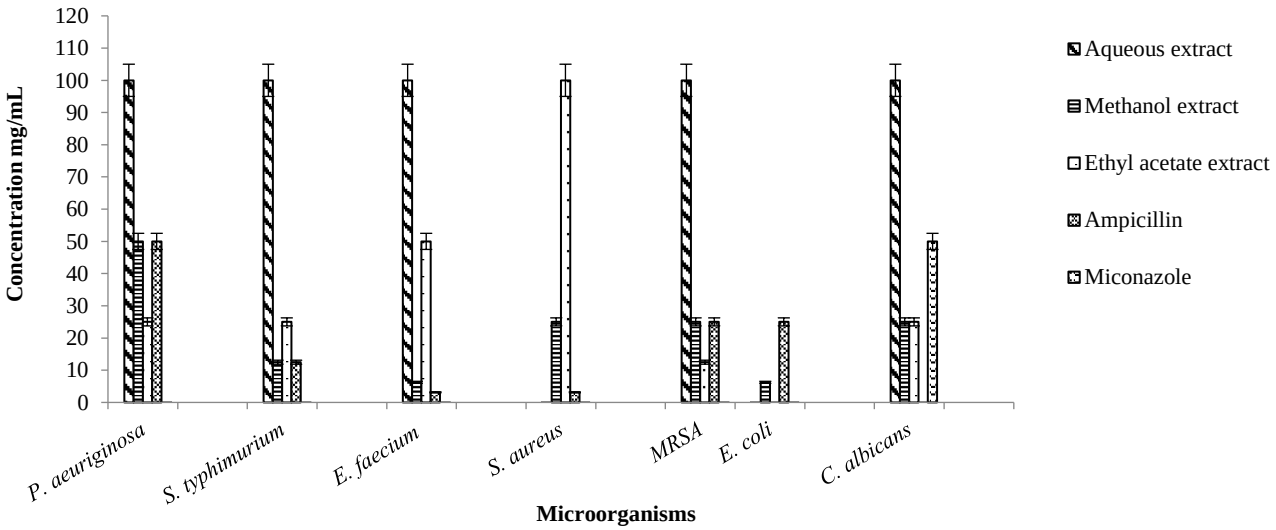

Figure 6: Minimum Inhibitory Concentration (MIC) of Garlic extracts and conventional antibiotics

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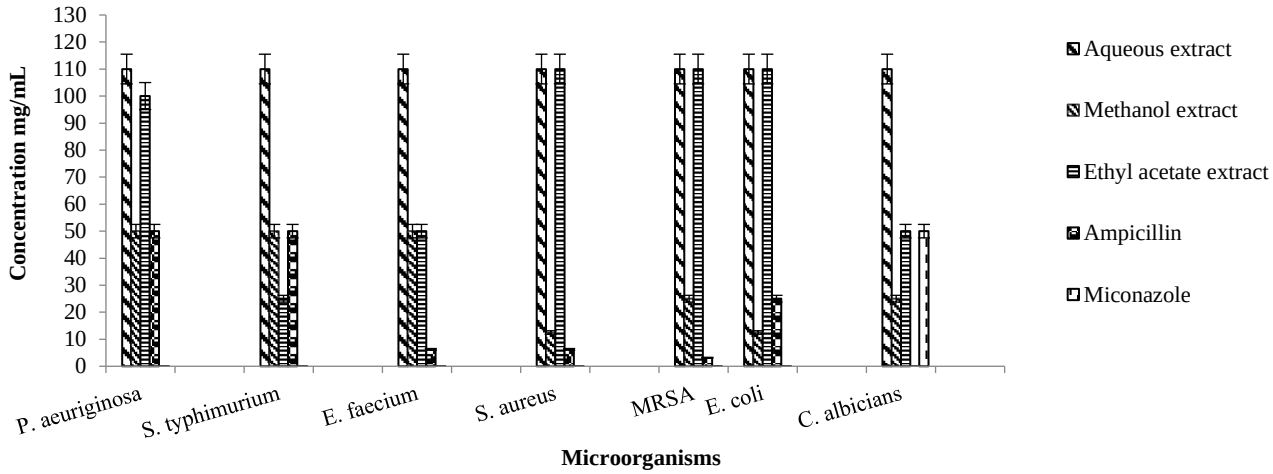

Figure 7: Minimum Bactericidal/fungicidal Concentration (MBC/MFC) of Garlic extracts and conventional antibiotics.

Conclusion

This study has shown the antimicrobial activity of *Allium sativum Ex-Lugu* cultivars and has revealed the major phytochemical compounds present in the extracts which may be responsible for its antimicrobial action.

Conflict of interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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