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Synergistic Antibacterial Activity among Crude Extracts of *Allium sativum* and *Citrus Limon* Using Checkerboard Microdilution Method

Jumare F.M[1], Suleiman A.B[2], Afegbua S.L[3], Anchau Z.G[4]

[1]Department of Integrated Science,Federal College of Education Zaria, Kaduna, Nigeria.

[2, 3, 4]Department of Microbiology, Ahmadu Bello University Zaria, Kaduna, Nigeria

Abstract: ***The present study describes the synergistic antibacterial activity among crude acetone extracts of A. sativum and C. limon on clinical isolates of Staphylococcus aureus and Streptococcus pyogenes. The antibacterial activity was initially determined by the agar well diffusion method followed by determination of Minimum Inhibitory Concentration (MIC) and the synergistic effect among the two extracts using checkerboard Microdilution method. The results showed that the extract of C. limon had the highest antibacterial activity on the isolates with mean inhibition zone diameter ranging from 37.8±0.34 to 2.8±1.07 mm followed by the extract of A. sativum with mean inhibition zone diameter ranging from 24.7±0.8 to 0.0±0.0 mm. The control antibiotics, Erythromycin (10µg) recorded higher antibacterial activity on S. aureus isolate than the extracts with mean inhibition zone diameter of 32.1±0.86mm while it had a lower antibacterial activity than C. limon extract on S. pyogenes isolate with mean inhibition zone diameter of 22.7±0.71mm. There was synergistic antibacterial activity among the extracts on S. pyogenes isolate at equal proportions (0.39mg/ml A. sativum and 0.39mg/ml C. limon) and when A. sativum concentration was slightly higher than C. limon in the combination (0.78mg/ml A. sativum and 0.39mg/ml C. limon). While there was no synergistic activity among the extracts on S. aureus isolate but rather an indifferent effect was observed. This study has proven the efficacy of A. sativum and C. limon extracts in inhibiting S. aureus and S. pyogenes pathogenic bacteria of clinical origin and most importantly, it has revealed the synergistic effect of the extracts against S. pyogenes pathogenic bacteria.***

Keywords*:* Synergy, Antibacterial activity, Allium sativum, Citrus limon, Checkerboard method

1. Introduction

Synergistic treatment of ailments has been a long aged practice in both orthodox and traditional medicines. In Sub-saharan Africa, especially West Africa, including Ghana and Nigeria, herbal medicines are believed to be more effective when taken in combination [(11)]. However, the synergism between phytochemicals and antibiotics has been well documented like the report of Olgica *et al.* [(16)] on synergistic antibacterial activity of *Salvia officinalis* and *Cichorium intybus* extracts and antibiotics, but very little research has been done to evaluate combined effect of spices and herbs against bacterial human pathogens. Thus, in order to add to existing literature on antibacterial synergy, it is important to determine the combined effect of herbs and spices [(8)].

Allium sativum (garlic) contains the highest sulfur content of any member of the genus *Allium*. Two trace elements, germanium and selenium, have detectable quantities and have been postulated to play a role in the herb's antitumor effect. It contains approximately 0.5% of a volatile oil composed of sulfur-containing compounds (diallyldisulfide, diallyltrisulfide, methylallyltrisulfide) [(14)]. The bulbs contain an odorless, colorless, non-volatile sulfur-containing amino acid called alliin (S-allyl-L-cysteine sulfoxide) [(15)]. *Citrus limon* (lemon) is an excellent preventative medicine and have a wide range of uses in the domestic medicine chest. The fruit is rich in vitamin C which helps the body to fight off infections and also to prevent or treat scurvy [(12)] [(13)].

Previous study on the synergistic effect of *Allium sativum* and *Gongronema latifolium* on *Escherichia coli* and *Staphylococcus aureus* has been reported by Eja *et al.* [(8)] where the combination of the two herbs in equal proportions against the test organisms was not effective due to antagonism between the plants. Although, individual plant when combined with ciprofloxacin in equal proportions completely suppressed the effect of the antibiotic on the organisms. The study didn't vary the proportions of each extract to determine if there will be any activity in which the current investigation has sought to explore. This work is also an attempt to prove or otherwise, the combined effectiveness of *A. sativum* and *C. limon* extracts in inhibiting bacterial pathogens of clinical origin.

2. Experimental

2.1. Identification and Characterization of Test Organisms

Clinical isolates of *Staphylococcus aureus* and *Streptococcus pyogenes* were obtained from Microbiology Laboratory of Hajiya Gambo Sawaba General Hospital, Kofan Gayan, Zaria City, Kaduna State, Nigeria. Ethical Clearance was sought from the Scientific Ethical Committee (SEC), Kaduna State Ministry of Health as well as the hospital. The isolates were identified on the basis of morphological, biochemical and physiological characteristics.

2.2. Preparation of Turbidity Standard and Standardization of Bacterial Inoculum

McFarland standards are used as a reference to adjust the turbidity of microbial suspension so that number of bacteria will be within a given range. Firstly, (1% w/v) $BaCl_2$ and

(1% v/v) H_2SO_4 were prepared by dissolving 1g of $BaCl_2$ in 100ml of sterile distilled water and 1ml of concentrated H_2SO_4 in 99ml of sterile distilled water respectively to serve as stock solutions for the preparation of the McFarland standard. From the stock solutions, 0.5 McFarland scale was prepared by adding 9.95ml of (1% v/v) H_2SO_4 to 0.05ml of (1% w/v) $BaCl_2$ with constant stirring to maintain a suspension of (1% w/v) $BaSO_4$ whose density is equivalent to 1.5 $\times 10^8$ CFU /ml or 150million/ml approximate cell density of bacteria. The barium sulphate suspension in 4- to 6-ml aliquots were transferred in to screw-cap tubes, tightly sealed, and stored in the dark at room temperature to prevent loss by evaporation. This was subsequently used for comparison with the turbidity of the bacterial inoculum [6].

For inoculum standardization, density of isolated cultures was adjusted equal to that of 0.5 McFarland standards (1.5 x 10^8CFU/ml) by suspending some quantity of the bacterial culture in to 2ml of sterile physiological saline as suspending medium. The physiological saline was prepared by dissolving 8.5g of $NaCl_2$ in 1L of distilled water and sterilised. To aid comparison, the test organisms and standard were compared against a white background with contrasting black lines [6].

2.3. Preparation and Extraction of Crude Plant Extracts

Fresh garlic and lemon were purchased at Tudun-wada market, Zaria, Kaduna state, Nigeria. These were subsequently authenticated at the Herbarium unit, Department of Biological Sciences, Ahmadu Bello University Zaria, Kaduna State, Nigeria. The fresh garlic cloves were peeled, weighed in to a beaker and cleaned. Cleaned cloves were surface-sterilized with 70% sodium hypochlorite for 2 minutes, rinsed twice with sterile distilled water and crushed using sterile mortar and pestle. Acetone extracts of the garlic were prepared by soaking resultant garlic paste in the solvent contained in sterile Erlenmeyer flask. The flask was covered with cotton wool plug and then wrapped with aluminium foil. Homogenization of the mixture and saturation of the solvents was achieved by shaking mechanically for 24hrs on a shaker (lab-line orbit, Melrose Park, ILL) at 100rpm. The mixture was filtered using muslin cloth and then Whatman no. 1 filter paper in to sterile Erlenmeyer flask. The filtrate was condensed in water bath and used as stock. The extract was stored in the refrigerator at 4°C for subsequent use [5].

The fresh lemon balls were weighed, washed in running tap water in the laboratory, surface sterilized with 70% sodium hypochlorite for 2 minutes, rinsed twice with sterile distilled water and cut open with a sterile knife and the juice was asceptically squeezed into a sterile universal container and then filtered into another sterile Erlenmeyer flasks to remove the seeds and other tissues. Acetone extract of the lemon was prepared by soaking resultant lemon juice in the solvent contained in sterile Erlenmeyer flask. The flask was covered with cotton wool plug and then wrapped with aluminium foil. Homogenization of the mixture and saturation of the solvent was achieved by shaking mechanically for 24hrs on a shaker (lab-line orbit, Melrose Park, ILL) at 100rpm. The mixture was filtered using Whatman no. 1 filter paper in to sterile Erlenmeyer flask. The filtrate was condensed in water bath and used as stock. The extract was stored in the refrigerator at 4°C for subsequent use [1].

2.4. Preliminary Phytochemical Screening

The extracts were subjected to various phytochemical tests to identify the chemical constituents present using standard methods as described by Sofowora [19] as follows;

Test for Carbohydrates.
(a) Molisch's Test
To a portion of each extract in a test tube, few drops of molisch reagent was added and concentrated sulphuric acid was added down the side of the test tube to form a lower layer, a reddish coloured ring at the interphase indicates presence of carbohydrates.

Test for Unsaturated Steroid and Triterpenes

(a) Liebermann-Bucchard Test
To a portion of each extract, equal volume of acetic acid anhydride was added and mixed gently. 1ml of concentrated sulphuric acid was added down the side of the test tube to form a lower layer. Colour changes were observed immediately and over a period of one hour. Blue to blue-green colour in the upper layer and a reddish, pink or purple colour indicate the presence of triterpenes.
(b) Salkowski Test for Unsaturated Sterols
To a portion of each extract, 2-3 drops of concentrated sulphuric acid was added at the side of the test tube. Immediate colour change at the interphase of the extract and sulphuric acid was noted as well as colour change over one hour period (cherry red colour usually indicates the presence of unsaturated sterols.

Test for Cardiac Glycosides
(a) Keller- Kiliani Test
A portion of each extract was dissolved in 1ml of glacial acetic acid containing traces of ferric chloride solution. This was then transferred in to a dry test tube and 1ml of concentrated sulphuric acid was added down the side of the test tube to form a lower layer at the bottom. This was observed carefully at the interphase for purple-brown ring that indicates the presence of desoxy sugars and a pale green colour in the upper acetic acid layer indicating the presence of cardiac glycosides.

Test for Saponin Glycoside
(a) Frothing Test
About 10ml of distilled water was added to a portion of each extract and was shaken vigorously for 30seconds. The tube was allowed to stand in a vertical position and was observed for 30mins. A honeycomb froth that persists for 10-15mins indicates presence of saponins.

Test for Tannins
(a) Lead Sub-acetate Test
To a portion of each extract, 3-5 drops of lead sub-acetate solution was added. A colored precipitate indicates the presence of tannins.

Test for Flavonoids

(a) Shinoda's Test

A portion of the extract was dissolved 1-2ml of 50% methanol in the heat Metallic magnesium chips and few drops of concentrated hydrochloric acid were added. Appearance of red colour indicates presence of flavonoids.

Test for Alkaloids

(a) Mayer's Test

To test tubes containing 1ml of each extract, few drops of Meyer's reagent were added. A cream precipitate indicates presence of alkaloids.

(b) Dragendoff's Test

To test tubes containing 1ml of each extract, few drops of Dragendorff's reagent were added. A reddish brown precipitate indicates presence of alkaloids.

(c) Wagner's Test

Few drops of wagner's reagent were added to a portion of each extract, whitish precipitate indicates presence of alkaloids.

Test for Free Anthracene Derivatives (Bontrager's Test)

To a portion of the extract in a dry test tube, 5ml of chloroform was added and was shaken for at least 5 minutes. This was filtered and the filtrate shaken with equal volume of 10% ammonia solution, bright pink colour in the aqueous (upper) layer indicates the presence of free anthraquinones [19].

2.5. Preparation of Extract Concentration

This was carried out as described by Srinivasan *et al*., [20]. Stock solution of the plant extracts were prepared by adding 1g of each crude plant extract in 10ml of 10% dimethylsulphuroxide (DMSO) as reconstituting solvent to make 100mg/ml stock solution. From the stock solution, 50mg/ml, 25mg/ml, and 12.5mg/ml concentrations were prepared using Two-fold serial dilution method. These concentrations were labelled and kept in bijou bottles for subsequent use.

2.6 Antibacterial Susceptibility Test

Mueller Hinton Agar (Titan Biotech Ltd. Bhiwadi- 301 019, Rajasthan, India.) was used for the antibacterial susceptibility testing and prepared according to manufacturer's instructions. The antibacterial activity of *A. sativum* and *C. limon* crude extracts (Acetone) against the test organisms was evaluated by using agar well diffusion method of sensitivity test described by Srinivasan *et al*., [20]. Mueller Hinton agar plates were inoculated with 100μl of standardized inoculum of each bacterium (in triplicates) using a micropipette of 100μl size and spread uniformly with sterile swab sticks. Wells of 8 mm size were made with sterile cork borer into the agar plates containing the bacterial inoculum. Using the micropipette, 100μl volume of the various concentrations; 100mg/ml, 50mg/ml, 25mg/ml, and 12.5mg/ml each of the extracts were poured into wells of inoculated plates. The plates thus prepared were left at room temperature for ten minutes allowing the diffusion of the extracts into the agar and then incubated for 18 to 24 hrs at 37^0C. The diameter of inhibition zone (DIZ) was measured and expressed in millimetres. The mean values of the diameter of inhibition zones were calculated to the nearest whole number [20]. In order to check the activity of the extracts, the reconstituting solvent (DMSO) was used as negative control. Commercially available standard antibiotic; Erythromycin (10µg) was used as positive control parallel with the extracts. For this antibiotic, inhibition zones were interpreted in accordance with the CLSI (Clinical Laboratory Standards Institute) interpretation guideline [7].

2.7 Determination of Minimum Inhibitory Concentration (MIC)

The Extracts were further assayed for minimum inhibitory concentrations (MIC). The Broth Dilution was employed using Mueller Hinton broth as described by Andrews [3]. Two- fold serial dilutions of the reconstituted extracts were made to obtain the following concentrations; 25mg/ml, 12.5mg/ml, 6.25mg/ml, 3.125mg/ml and 1.56mg/ml. From the Mueller Hinton broth prepared, 9ml broth was added to each of the test tubes labelled as containing 1ml of 25mg/ml, 12.5mg/ml, 6.25mg/ml, 3.125mg/ml and 1.56mg/ml concentrations of each extract resulting in two sets of five different test tubes for the two organisms that were then inoculated with 100μl inoculum size of the test organisms. Mueller Hinton broth samples with 100μl of active inoculum of standardized bacterial isolates in tubes were incubated for 24hrs at 37^0C. The MIC determined as the lowest concentration of the extract which inhibited the organism and results were observed in the form of turbidity [3]. Negative controls were set up to contain Mueller Hinton broth only and Mueller Hinton broth with extract only. Positive control was set up to contain Mueller Hinton broth and the test organism only [3].

2.8 Determination of Synergistic Effect among Plant Extracts

From the stock solutions, twofold dilutions of each plant extract starting at four times the MIC and ending at zero MIC were prepared using the Broth Microdilution Method prior to the synergy test by the Checkerboard Method in accordance with the method described by Olgica *et al.* [16] and Gani *et al.* [9]. Then the two plant extracts were combined such that garlic extract was in ascending concentrations while lemon extract was in descending concentrations. A total of 50 µl of Mueller-Hinton broth was distributed into each well of the microdilution plate containing the combination, inoculated with 100 µl of the standardized innoculum and then incubated at 37°C for 24 hour. The resulting checkerboard contained combination of two extracts (garlic and lemon), with microtitre wells that contain the highest concentration of each extract at opposite corners are shown in Table 1 and Table 2 below. The test included two growth controls consisting of the microtiter well with the solvent (DMSO) alone as negative control and microtiter well with bacterial suspension, equal volumes of the extract (0.25μg/ml) and Erythromycin (0.25μg/ml) as positive control [16].

Table 1: Broth microdilution checkerboard panel before incubation with *S. aureus* isolate.

A1	A2	A3	A4	A5	A6	A7
No extract MHB only	G0.39	G0.78	G1.56	G3.125	G6.25	G12.5 (x 4MIC)
B1	B2	B3	B4	B5	B6	B7
L 0.39	G0.39 +L 0.39	G0.78 + L 0.39	G1.56 +L 0.39	G3.125 + L 0.39	G6.25 + L 0.39	G12.5 +L0.39
C1	C2	C3	C4	C5	C6	C7
L 0.78	G0.39 +L0.78	G 0.78 + L0.78	G 1.56 +L0.78	G3.125 +L 0.78	G 6.25 + L0.78	G 12.5 + L 0.78
D1	D2	D3	D4	D5	D6	D7
L 1.56	G0.39 +L 1.56	G0.78 + L1.56	G 1.56 + L 1.56	G3.125+ L 1.56	G 6. 25 + L 1.56	G 12.5 + L1.56
E1	E2	E3	E4	E5	E6	E7
L 3.125	G 0.39 +L 3.125	G 0.78 +L 3.125	G 1.56 +L 3.125	G3.125+ L3.125	G6.25 + L3.125	G 12.5+ L 3.125
F1	F2	F3	F4	F5	F6	F7
L 6.25	G 0.39 + L6.25	G0.28 + L6.25	G 1.56+ L 6.25	G3.125 +L6. 25	G 6.25 + L6.25	G 12.5 + L 6.25
G1	G2	G3	G4	G5	G6	G7
L12.5 (x 4MIC)	G 0.39 + L 12.5	G 0.38 + L 12.5	G 1.56 +L12.5	G3.125+L l2.5	G 6.25 + L 12.5	G 1.25 + L l2.5

NOTE: G = garlic cloves acetone extract, L = Lemon juice acetone extract, Well A1= Extract free (growth control) Containing Mueller Hinton Broth and organism, Isolate 16 was selected because it was the most susceptible isolate.

Table 2: Broth microdilution checkerboard panel before incubation with *S. pyogenes* isolate

A1	A2	A3	A4	A5	A6	A7
No extract MHB only	G0.39	G0.78	G1.56	G3.125	G6.25	G12.5 (x 4MIC)
B1	B2	B3	B4	B5	B6	B7
L 0.39	G0.39 +L 0.39	G0.78 + L 0.39	G1.56 +L 0.39	G3.125 + L 0.39	G6.25 + L 0.39	G12.5 +L0.39
C1	C2	C3	C4	C5	C6	C7
L 0.78	G0.39 + L0.78	G 0.78 + L0.78	G 1.56 +L0.78	G3.125 +L 0.78	G 6.25 + L0.78	G 12.5 + L 0.78
D1	D2	D3	D4	D5	D6	D7
L 1.56	G0.39 +L 1.56	G0.78 + L1.56	G 1.56 + L 1.56	G3.125+ L 1.56	G 6. 25 + L 1.56	G 12.5 + L1.56
E1	E2	E3	E4	E5	E6	E7
L 3.125	G 0.39 +L 3.125	G 0.78 + L 3.125	G 1.56 +L 3.125	G3.125+ 3.125	G6.25 + L3.125	G 12.5+ L 3.125
F1	F2	F3	F4	F5	F6	F7
L6.25(x 4MIC)	G 0.39 + L6.25	G0.28 + L6.25	G 1.56+ L 6.25	G3.125 +L6. 25	G 6.25 + L6.25	G 12.5 + L 6.25

NOTE: G = garlic cloves acetone extract, L = Lemon juice acetone extract, Well A1= Extract free (growth control) Containing Mueller Hinton Broth and organism, Isolate 27 was selected because it was the most susceptible

The summation of fractional inhibitory concentrations (ΣFICs) were calculated as follows: ΣFIC = FIC garlic + FIC lemon, where FIC garlic is the MIC of garlic extract in the combination/MIC of garlic extract alone, and FIC lemon is the MIC of lemon extract in the combination/MIC of lemon extract alone. The combination is considered synergistic when the ΣFIC is ≤0.5, indifferent when the ΣFIC is >0.5 to <4, and antagonistic when the ΣFIC is ≥4 (13). Therefore, the action of antimicrobial agents is considered to be synergistic if their joint effect is stronger than the sum of effects of the individual agents; or indifferent if their joint effect is equal to the effect of either individual agent; or Antagonistic if their joint effect is weaker than the sum of effects of the individual agents or weaker than the effect of either individual agent [9].

2.9. Statistical Analysis

The data generated are presented in tables and charts and were analysed statistically using the S.P.S.S (Statistical Package for Social Sciences) package- SPSS 18. ANOVA was used to compare means of the plant extracts at different concentrations and the positive control antibiotic if there is any statistically significant difference in the diameter of zones of inhibition and P values < 0.05 is considered significant. Subsequently, these were further ranked by the Duncan's multiple range tests.

3. Results

The cultural and physiologic properties of clinical isolates of *Staphylococcus aureus* and *Streptococcus pyogenes* are presented in Table 3 which shows that the *Staphylococcus aureus* isolate obtained from the hospitals appeared as golden yellow, slightly raised colonies on mannitol salt agar, gram positive, cocci and clustered under the microscope, catalase positive, coagulase positive, ferment mannitol, positive for latex agglutination, revealed biochemical characteristics using Microgen* *S. aureus* Identification Kit, and thus, was identified as *S. aureus.* While Table 4 shows that the *Streptococcus pyogenes* isolate appeared as Small, colourless, dry colonies on blood agar, gram positive, cocci in chains under the microscope, sensitive to bacitracin, beta haemolytic, revealed biochemical characteristics using Microgen* *S. pyogenes* Identification Kit and thus identified as *S. pyogenes.*

Table 3: Cultural/ Physiologic Properties and Biochemical Characteristics of clinical isolate of *Staphylococcus aureus*

Cultural/ Physiologic Properties	Observation
Colonial morphology	Golden yellow, slightly raised
Gram reaction	Positive
Cell Morphology	Cocci
Cell arrangement	Clusters
Catalase	+
Coagulase	+

Mannitol	+
Latex agglutination	+
Identification	*S. aureus*
Biochemical Characteristics	**Observation**
Latex agglutination test	+
Colony pigmentation	+
Nitrate	+
Sucrose	+
Trehalose	+
Mannitol	+
N -Acetyl Glucosamine	+
Mannose	+
Turanose	+
Alkaline Phosphatase	+
β- Glucosidase	-
β- Glucuronidase	+
Urease	+
Arginine	+
Pyrrolidonyl arylamidase (PYR)	-
% probability	100%
Profile No	77776
Final identification	*S. aureus subsp aureus*

KEY
-= Negative, += Positive

Table 4: Cultural/ Physiologic Properties and Biochemical Characteristics of clinical isolate of *Streptococcus pyogenes*

Cultural/ Physiologic Properties	Observation
Colonial morphology	Small, colourless, dry
Gram reaction	Positive
Cell Morphology	Cocci
Cell arrangement	Chains
Catalase	Negative
Bacitracin test	Sensitive
Haemolysis	Beta Haemolytic
Identification	*S. pyogenes*
Biochemical Characteristics	**Observation**
Hippurate test	-
Alpha haemolysis	-
Beta haemolysis	+
Melibiose	-
Sorbitol	-
Inulin	-
Lactose	+
Arabitol	-
Ribose	-
Esculin	-
Voges Proskauer (VP)	-
Alkaline Phosphatase	+
β- Glucuronidase	-
Pyrrolidonyl arylamidase (PYR)	+
Arginine	+
% probability	100%
Profile No	10413
Final identification	*S. pyogenes*

KEY
-= Negative, += Positive

Table 5: Phytochemical Constituents of Acetone Extracts of *Allium sativum* and *Citrus limon*

Metabolites	Test type	A	B
Carbohydrate	Molish	+	+
Unsaturated Sterols	Salkowski	+	+
Triterpenes	Liebermann-Bucchard	+	+
Cardiac Glycoside	Keller-Kiliani	+	+
Saponin Glycoside	Frothing	+	+
Tannins	Lead sub-acetate	+	+
Flavonoids	Sodium hydroxide	+	+
Alkaloids	Mayer's	+	+
	Dragendorff's	+	+
	Wagner's	+	+
Free Anthracene Derivatives	Bontrager's	+	+

Key:
+ = Positive
- = Negative
A = Acetone extracts of *Allium sativum*
B = Acetone extracts of *Citrus limon*

The susceptibility of *S. aureus* and *S. pyogenes* isolates to graded doses of crude acetone extracts of *Allium sativum* and *Citrus limon* as well as the antibiotic where means with different superscripted alphabets along a column are

Garlic was identified as *Allium sativum L* (voucher no. 990) belonging to the familiy Liliaceae while lemon was identified as *Citrus limon L* (voucher no. 2196) of the Rutaceae family. Based on the phytochemical screening conducted on the aqueous and acetone extracts of garlic cloves and lemon juice, the results presented in Table 5 shows the presence of Carbohydrate, Cardiac Glycoside, Tannins, Saponin Glycoside, Flavonoids and Alkaloids in both aqueous and acetone extracts of garlic cloves and lemon juice. Unsaturated Sterols is present in acetone extracts of both garlic cloves and lemon juice while it is absent in the aqueous extracts of both garlic cloves and lemon juice. Triterpenes and Free Anthracene Derivatives were indicated present in acetone extracts of both garlic cloves and lemon juice as well as aqueous extract of lemon juice while they were absent in the aqueous extract of garlic cloves.

significantly different at $P < 0.05$ is presented in Table 6. The result indicates that the isolates exhibited varying degrees of resistance and susceptibility to different concentrations of the extracts as well as the antibiotic used as positive control in the susceptibility analysis (Erythromycin (10µg)). The different concentrations of *Allium sativum* extract yielded high inhibitory activity with mean inhibition zone diameter ranging from 7.5 ± 1.52mm to 17.1 ± 0.40mm on *S. pyogenes* isolate than it yielded on *S. aureus* isolate with mean inhibition zone diameter ranging from 5.3 ± 1.04mm to 0.0 ± 0.0mm. While the extract of *Citrus limon* at different concentrations exhibited higher inhibitory activity on *S. pyogenes* isolate with mean inhibition zone diameter ranging from 16.8 ± 0.40mm to 36.5 ± 0.36mm than on *S. aureus* isolate with mean inhibition zone diameter ranging from 9.9 ± 1.13mm to 21.9 ± 0.46mm. The antibiotic used as control exhibited higher inhibitory activity on *S. aureus* isolate with mean inhibition zone diameter of 32.1 ± 0.86mm than on *S. pyogenes* isolate with mean inhibition zone diameter of 22.7 ± 0.71mm. However, the different concentrations of *Allium sativum* and *Citrus limon* extracts exhibited the highest inhibitory activity on *S. pyogenes* isolate with mean inhibition zone diameter of 21.9 ± 0.46mm and 37.8 ± 0.34mm respectively.

Table 6: Susceptibility of *S. aureus* and *S. pyogenes* Isolates to Graded Dosages of Crude Acetone Extracts of *A. sativum* and *C.limon*

Extracts and Dosages (mg/ml)	*Mean Inhibition Zone Diameters (mm) ± SEM*	
	Staphylococcus aureus	*Streptococcus pyogenes*
A. sativum extract (100.0)	5.3±1.04[a]	24.7±0.87[a]
A. sativum extract (50.0)	0.9±0.65[b]	17.1±0.40[a]
A. sativum extract (25.0)	5.4±1.34[b]	13.7±0.45[a]
A. sativum extract (12.5)	0.0±0.0[b]	7.5±1.52[a]
C.limon extract (100)	20.4±0.36[b]	37.8±0.34[a]
C.limon extract (50)	16.0±0.51[b]	33.3±0.57[a]
C.limon extract (25.0)	13.6±0.44[b]	23.5±0.86[a]
C.limon extract (12.5)	2.8±1.07[b]	17.3±0.44[a]
E(10 µg)	32.1±0.86[a]	22.7±0.71[b]

Key: Means with the different superscripted alphabets across rows are significantly different at $P < 0.05$. E=Erythromycin.

The result of minimum inhibitory concentrations (MIC) values of acetone extracts of *Allium sativum* and *Citrus limon* on *S. aureus* and *S. pyogenes* isolates shows that the MIC value of *Allium sativum* and *Citrus limon* on *S. aureus* isolate was 3.125mg/ml for both extracts and the MIC value of *Allium sativum* and *Citrus limon* on *S. pyogenes* isolate was 3.125mg/ml and 1.56mg/ml respectively.

The result of synergistic activity among the extracts of *Allium sativum* and *Citrus limon* on *S. aureus* clinical isolate using the checkerboard microdilution method is presented in Table 7 and Table 8. Table 7 shows the inhibitory activity of the extracts combinations on the isolate in the microtitre plate after 18 to 24 hours of incubation in terms of turbidity and clearity of the wells while the result in Table 8 shows the interpretation of the combined inhibitory activity among the extracts on the isolate based on the clear wells where the combination of *A. sativum* and *C. limon* at (1.56mg/ml *A. sativum* and 0.39mg/ml *C. limon*) (3.125mg/ml *A. sativum* and 0.39mg/ml *C. limon*) (1.56mg/ml *A. sativum* and 0.78mg/ml *C. limon*), (3.125mg/ml *A. sativum* /0.78mg/ml *C. limon*), (1.56mg/ml *A. sativum* and 1.56mg/ml *C. limon*) and (3.125mg/ml *A. sativum* and 1.56mg/ml *C. limon*) were all indifferent based on the summation of fractional inhibitory concentration (ΣFIC) value that is > 0.5 to < 4 [(16)].

Table 7: Broth microdilution checkerboard panel after 24 hrs of incubation with *S. aureus* isolate

A1	A2	A3	A4	A5	A6	A7
T	T	T	T	C	C	C
B1	B2	B3	B4	B5	B6	B7
T	T	T	C	C	T	T
C1	C2	C3	C4	C5	C6	C7
T	T	T	C	C	T	T
D1	D2	D3	D4	D5	D6	D7
T	T	T	C	C	T	T
E1	E2	E3	E4	E5	E6	E7
C	T	T	T	T	T	T
F1	F2	F3	F4	F5	F6	F7
C	T	T	T	T	T	T
G1	G2	G3	G4	G5	G6	G7
C	T	T	T	T	T	T

NOTE: T=Turbid well and C=Clear well

Table 8: Synergistic Activity among Acetone Extracts of Garlic cloves and Lemon juice on Clinical Isolate of *S. aureus* using Checkerboard Microdilution method

	Garlic		Lemon		ΣFIC	Interpretation
Clear well	Conc (mg/ml)	Fic of garlic	Conc. (mg/ml)	Fic of lemon	FicG+FicL	
A5	3.125	Mic garlic	0	Mic garlic	Mic garlic	Mic garlic
B4	1.56	1.56/3.125 =0.4992	0.39	0.39/1.25=0.1248	0.624	IND
B5	3.125	3.125/3.125 =1	0.39	0.39/3.125 =0.01248	1.1248	IND
C4	1.56	1.56/3.125= 0.4992	0.78	0.78/3.125=0.2496	0.7488	IND
C5	3.125	3.125/3.125 =1	0.78	0.78/3.125=0.2496	1.2496	IND
D4	1.56	1.56/3.125 =0.4992	1.56	1.56/3.12=0.4992	0.9984	IND
D5	3.125	3.125/3.125 =1	1.56	1.56/3.125 =0.4992	1.4992	IND
E1	0	Mic lemon	3.125	Mic lemon	Mic lemon	Mic lemon

Key: When ΣFIC is ≤ 0.5 = synergistic (SYN), When ΣFIC is >0.5 to <4 = Indifferent (IND), When ΣFIC is ≥4 = Antagonistic (ANT) [(16)], A-E= Microtitre plate well number.

The result of synergistic activity among the extracts of *Allium sativum* and *Citrus limon* on *S. pyogenes* clinical isolate using the checkerboard microdilution method is presented in Table 9 and Table 10. Table 9 shows the inhibitory activity of the extracts combinations on the isolate in the microtitre plate after 18 to 24 hours of incubation in terms of turbidity and clearity of the wells while the result in Table 10 shows the interpretation of the combined inhibitory activity among the extracts on the isolate based on the clear wells where the combination of *A. sativum* and *C. limon* at (0.39mg/ml *A. sativum* and 0.39mg/ml *C. limon*) and (0.78mg/ml *A. sativum* and 0.39mg/ml *C. limon*) where synergistic based on summation of fractional inhibitory (**Σ**FIC) value that is ≤ 0.5 while the other combinations where all indifferent based on **Σ**FIC value that is > 0.5 to < 4 [(16)].

Table 9: Broth microdilution checkerboard panel after 24 hrs of incubation with *S. pyogenes* isolate.

A1	A2	A3	A4	A5	A6	A7
T	T	T	T	C	C	C
B1	B2	B3	B4	B5	B6	B7
T	C	C	C	T	T	T
C1	C2	C3	C4	C5	C6	C7
T	C	C	T	T	T	T
D1	D2	D3	D4	D5	D6	D7
T	C	T	T	T	T	T
E1	E2	E3	E4	E5	E6	E7
C	C	T	T	T	T	T
F1	F2	F3	F4	F5	F6	F7
C	C	T	T	T	T	T

G1	G2	G3	G4	G5	G6	G7
C	C	T	T	T	T	T

NOTE: T=Turbid well and C=Clear well

Table 10: Synergistic Activity among Acetone Extracts of Garlic cloves and Acetone Extracts of Lemon juice on Clinical Isolate of *S. pyogenes* using Checkerboard Microdilution method

	Garlic		Lemon		ΣFIC	Interpretation
Clear well	Conc (mg/ml)	Fic garlic	Conc. (mg/ml)	Fic lemon	FicG+FicL	
A5	3.125	Mic garlic	0	Mic garlic	Mic garlic	Mic garlic
B2	0.39	0.39/3.125 = 0.1248	0.39	0.39/1.56=0.25	0.3748	SYN
B3	0.78	0.78/3.125 =0.2496	0.39	0.39/1.56=0.25	0.4496	SYN
B4	1,56	1.56/3.125 =0.4992	0.39	0.39/1.56 =0.25	0.7492	IND
C2	0.39	0.39/3.125=0.1248	0.78	0.78/3.156 =0.5	0.6248	IND
C3	0.78	0.78/3.125 =0.5	0.78	0.78/1.56 =0.5	1	IND
D1	0	Mic lemon	1.56	Mic lemon	Mic lemon	Mic lemon

Key: When ΣFIC is ≤ 0.5 = synergistic (SYN), When ΣFIC is >0.5 to <4 = Indifferent (IND), When ΣFIC is ≥4 = Antagonistic (ANT) [16], A-E= Microtitre plate well number.

4. Discussion

The results of the current study reveals the efficacy of crude acetone extracts of *Allium sativum* and *Citrus limon* based on antibacterial activity and synergistic effect on clinical isolates of *Staphylococcus aureus* and *Streptococcus pyogenes*. Highest antibacterial activity was recorded with *Citrus limon* extract which inhibited the *S. pyogenes* isolate with mean inhibition zone diameter of 37.8±0.34mm higher than the inhibitory activity of the control antibiotic; Erythromycin(10 µg) with mean inhibition zone diameter of 32.1±0.86mm . Indeed, the antibacterial effects of *Citrus limon* were previously studied by Al-ani *et al.* [1] on the evaluation of antibacterial activity of citrus fruit juices against *Staphylococcus aureus*, *Proteus vulgaris* and *Pseudomonas aeruginosa* and concluded that the use of different concentrations of Citrus juice extracts had an effective antibacterial activity against the tested organisms. Hayes and Markovic [10] investigated the antimicrobial properties of *lemon* and found that *lemon* possesses significant antimicrobial activity against *Candida albicans*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Proteus vulgaris*. AL- Jedah *et al.* [2] analyzed the action of combined spices including *lemon* in its mixture and found that the spices mixture were able to exert static effect on *Shigella sonnei* and *Salmonella paratyphi*. In the same vein, the susceptibility of *S. pyogenes* and *S. aureus* isolates to the extract of *Allium sativum* supports the claim that crushed garlic can be used as home remedy to help speed recovery from sore throat and other minor ailments.

From the result of synergistic activity conducted using the microdilution checkerboard panel among acetone extracts of *Allium sativum* and *Citrus limon* on *S. aureus* and *S. pyogenes* clinical isolates, the result indicated that the combination of acetone extracts of garlic cloves and lemon juice on *S. aureus* registered an indifferent effect compared to when both extracts acted singly on the *S. aureus* isolates. This implies that acetone extract of garlic cloves and lemon juice either in combination or singly inhibits the growth of *S. aureus* in the same pattern meaning that synergistic effect dose not exist among the two plants on *S. aureus* isolates. This finding contradicts with the synergistic study where the introduction of lemon juice in the combination of garlic and sweet basil augmented the inhibitory activity against *S. paratyphi*-B [18]. However, there were conflicting reports on the combined effects of some plant extracts on some microbial species, including *E. coli* and *S. aureus* in a study of the antimicrobial activity of Nigerian medicinal plants potentially usable as hop substitute [17]. In the report [17], high concentrations were used besides combining two to three plant extracts. Even then, the report of Oshodi *et al* [17] did not deviate much from the findings of this study since synergy also depends on the susceptibility of the organism coupled with the fact that not much work has been done in the combination of garlic cloves and lemon juice on *S. aureus* isolate of clinical origin. Thus the current study should serve as basis for other research.

On the contrary, the result of synergistic activity conducted on *S. pyogenes* clinical isolates showed that the combination of acetone extracts of garlic cloves and lemon juice on *S. pyogenes* isolate indicated synergistic effect at equal proportions (0.39mg/ml garlic and 0.39mg/ml lemon) and when garlic concentration is slightly higher than lemon juice in the combination (0.78mg/ml garlic and 0.39mg/ml lemon) while other combinations indicated an indifferent activity. This agrees with the findings of Andy *et al.* [4] reporting that desired effect is mostly achieved when combinations are used on Gram positive bacteria in which case, the effect may be synergistic or indifferent. Synergistic effect as applied in this study means an enhanced effect produced by the combination of two or more plant extracts. This synergy exerted by garlic cloves and lemon juice also proves the claim of traditional healers in combining garlic cloves and lemon juice as regimen for sore throat in some areas of northern Nigeria.

5. Conclusion

In conclusion, the results obtained in this study has shown clear potency of crude acetone extracts of *Allium sativum* and *Citrus limon* against *S. aureus* and *S. pyogenes* pathogenic bacteria of clinical origin. The study has also demonstrated a non synergistic effect among the extracts on *S. aureus* isolate while synergistic antibacterial activity was established among the extracts on *S. pyogenes* isolate. Thus; this work has formed part of an effort to validate the use of *Allium sativum* and *Citrus limon* in traditional medicine

particularly as a combination therapy against *S. pyogenes* infections.

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