Invitro Antibacterial Activity of Garlic Cloves and Lemon Juice Extracts on Clinical Isolates of Streptococcus pyogenes

JUMARE, F.M[a], YAKUBU, S.E[b], AMEH, J.B[b]*

[a]First affiliation, Department of Integrated Science, Federal College of Education, Zaria, Kaduna, Nigeria.

[b]First affiliation, Department of Microbiology, Ahmadu Bello University Zaria, Kaduna, Nigeria.

[a]Email: fatimajumare@gmail.com

ABSTRACT

The lack of scientific backup for the use of some herbal preparations as well as the fight against antibiotic resistance indicates a strong need for continuous effort to validate the use of plant materials as alternative therapy regimens with similar or higher antibiotics beneficial properties. The present study describes the antibacterial activity of aqueous and acetone extracts of garlic cloves (Allium sativum) and lemon juice (Citrus limon) on clinical isolates of Streptococcus pyogenes. The antibacterial potency was initially determined by the agar well diffusion method followed by quantitative evaluation of antibacterial activity by MIC and MBC method as well as fractionation of crude plant extracts. The results showed that acetone extracts of garlic cloves and lemon juice were more effective than the aqueous extracts. Acetone extract of lemon juice and garlic cloves had the highest antibacterial activity on the isolates with mean inhibition zone diameter ranging from 16.8 ± 0.40 to 37.8 ± 0.34 mm higher than the control antibiotic, Erythromycin (10µg) with mean inhibition zone diameter of 22.7 ± 0.71mm. The aqueous extract of lemon juice also had good antibacterial activity on the isolates with mean inhibition zone diameter ranging from 7.5 ± 0.44 to 24.7 ± 0.87mm while the aqueous extract of garlic cloves recorded the lowest antibacterial activity. Thus, this study has proven the effectiveness of garlic cloves and lemon juice in inhibiting S. pyogenes pathogenic bacteria of clinical origin with a suggestion that further studies should identify and screen the chemical compounds responsible for the efficacy of these plants for development of alternative regimens.

***Key words**: Antibacterial activity, Garlic cloves, Lemon juice, Extract and S. pyogenes.*

INTRODUCTION

The use of plant materials to prevent and treat infectious diseases successfully over the years has attracted the attention of scientist world-wide leading to many investigations being conducted on medicinal plants [10]. Garlic (*Allium sativum*) among the *Allium* vegetables (onion, shallot, leek, chive, and rakkyo) in the family *Liliaceae* is a perennial bulb- forming plant known worldwide [16] and for several centuries, it has been used for dietary and medicinal purposes in the form of capsules and powders [20]. In Africa, particularly in Nigeria, it is used to treat abdominal discomfort, diarrhoea, otitis media and respiratory tract infections [18].While *Citrus limon* commonly called lemon of the family *Rutaceae* is an acidic fruit which acts as a fabulous source of vitamins and essential nutrients required by the body [5]. Fresh fruits and their juice or industrially processed juices, contain mostly flavonoids [15]. However, flavonoids can function as direct antioxidants and free radical scavengers, as well as exhibiting the capacity to modulate enzymatic activities and inhibit cell proliferation. Thus in plants, they appear to play a defensive role against invading pathogens, including bacteria, fungi and viruses [24].

In Nigeria as well as in most countries of Sub Saharan Africa, traditional healers prescribe herbal preparations for the cure of many diseases such as diarrhoea, intestinal tract infections, sore throat, ear infections, fever and skin diseases with the claim that plant-derived medicine is relatively safer than synthetic alternatives offering profound therapeutic benefits and more affordable treatment [12]. However, some of these herbal preparations have little or no scientific basis for their application and could be dangerous to human health [13]. Thus, in order to substantiate the claims of cure made by the traditional healers and provide scientific basis for their efficacy, the need to evaluate plant resources for therapeutic potential is important [9] of which this work is aimed at evaluating the in vitro antibacterial activity of garlic cloves (*Allium sativum*) and lemon juice (*Citrus limon)* extracts on clinical isolates of *Streptococcus pyogenes*.

MATERIALS AND METHODS

Identification and Characterization of Test Organism

Clinical isolates of *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 further identified and characterized by Gram Staining technique, Microscopy, and Conventional biochemical test in accordance with the procedures of Cheesbrough [6] as well as Microgen* *S. pyogenes* Identification Kit.

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 1.5×10^8 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 (1.5×10^8CFU/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].

Preparation of crude plant extracts

Fresh garlic and lemon were purchased at Tudun-wada market, Zaria, Kaduna state. These were subsequently authenticated at the Herbarium unit, Department of Biological Sciences, Ahmadu Bello University Zaria, Kaduna State, Nigeria.

Garlic cloves

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. Aqueous and acetone extracts of the garlic were prepared by soaking resultant garlic paste of each of the chosen solvents contained in sterile Erlenmeyer flasks. The flasks were 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(s) were filtered using muslin cloth and then Whatman no. 1 filter paper in to sterile Erlenmeyer flasks. The filtrate(s) were condensed in water bath and used as stock. The extracts were stored in the refrigerator at 4°C for subsequent use [4].

Lemon juice

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. Aqueous and acetone extracts of the lemon were prepared by soaking resultant lemon juice each of the chosen solvents contained in sterile Erlenmeyer flasks. The flasks were covered with cotton wool plug and then wrapped with aluminium foil. Homogenization of the mixture and saturation of the solvents were achieved by shaking mechanically for 24hrs on a shaker (lab-line orbit, Melrose Park, ILL) at 100rpm. The mixture(s) were filtered using Whatman no. 1 filter paper in to sterile Erlenmeyer flasks. The filtrate(s) were condensed in water bath and used as stock. The extracts were stored in the refrigerator at 4°C for subsequent use [1].

Preliminary Phytochemical Screening

The extracts were subjected to various phytochemical tests to identify the chemical constituents present using standard methods as described by Sofowora [23] 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 [23].

Preparation of Extract Concentration

This was carried out as described by Srinivasan *et al.*, [25]. 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.

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 (Aqueous and Acetone) against the test organism and a standard strain was evaluated by using agar well diffusion method of sensitivity test described by Srinivasan *et al*., [25]. 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 [25]. Standard strain of *S. aureus* (ATCC 25923) was sourced from Faculty of Vetenary Medicine, Department of Microbiology, ABU, Zaria for comparison with the test organisms in terms of susceptibility to the extracts and also to serve as a control. In order to check the activity of the extracts, the reconstituting solvent (DMSO) was used as negative control. Commercially available standard antibiotics; Erythromycin (10µg) and Ampicillin (30µg) were used as positive control parallel with the extracts. For these antibiotics, inhibition zones were interpreted in accordance with the CLSI (Clinical Laboratory Standards Institute) interpretation guideline [7].

Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bacteriocidal Concentration (MBC)

Extracts which exhibited activity against the test organisms were further assayed for their 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]. Minimum Bacteriocidal Concentration (MBC) was determined from the MIC tube and the other tubes following the MIC tube by sub-culturing an aliquot from the tubes on to nutrient agar plates and incubated at 37^0C for 24hrs. The lowest concentration of the extracts that yielded no growth was recorded as the MBC [3]. Negative controls were set up as nutrient agar only and nutrient agar with extract only. Positive control was set up to contain nutrient agar and the test organism only [3].

Thin Layer Chromatography (TLC) of Crude Extracts

Thin Layer Chromatography was used to separate the chemical components of *A.sativum* and *C.limon* extracts using silica gel TLC plates in accordance with the procedure adopted from Patra *et al*. [19]. The silica gel plates (Merck F_{254} Darmstadt Germany) of 5×10 cm each were prepared for each extract. The two plates were spotted with the garlic and lemon extracts at 1.5cm origin line using a micro capillary tube; and then subjected to separation in the developing chamber containing Ethylacetate- Methanol (10:2 v/v) developing solvent. After development, the plates were removed, air dried, and sprayed with p-anisaldehyde sulphuric acid. This was placed for 5 seconds in an oven to reveal the separated components. The following formula

was used to measure the retention factor (Rf) which is the distance the compound travels to the distance the solvent travels [19].

R*f* =Distance moved by the compound

Distance moved by the solvent

Fractionation of Crude Plant Extracts

The active crude extracts of *A.sativum* and *C.limon* were fractionated in accordance with the procedures of Venskutonis *et al.* [27]. The extraction solvents were; petroleum ether, ethyl acetate, n- butanol and water. The procedure was carried out in a separating funnel in which fractions obtained were evaporated to dryness on a water bath to remove the solvent. Detail of the procedure steps for the fractionation is represented in figure 1 as a schematic diagram.

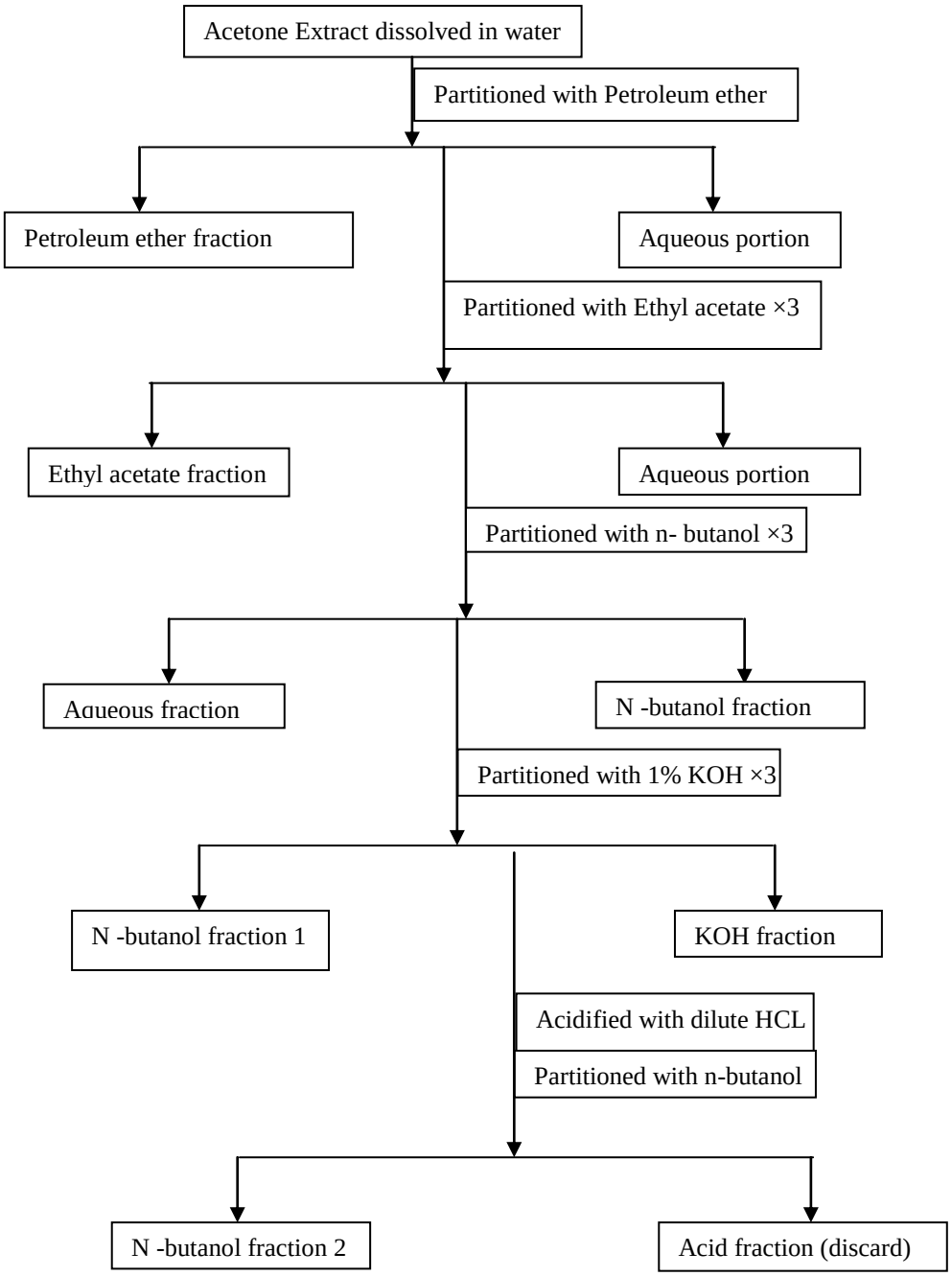

Figure 1: Schematic diagram of procedure steps for fractionation of crude plant extracts.

Phytochemical Screening of Extracts Fractions on TLC Plates

The phytochemical constituents of all the fractions of *A.sativum* and *C.limon* crude extracts obtained were evaluated according to Stahl [26] on Thin Layer Chromatography plates with the aid of specific spray reagents that reveals different compounds. Four TLC plates (5×20cm) were spotted with all the fractions obtained from the extracts and developed using Ethylacetate- Methanol (10:2 v/v) developing solvent. After development, the plates were removed, air dried, and sprayed with different reagents [26]. The retention factor (Rf) was then calculated for each fraction. The following reagents were used;

Ferric chloride for phenolic compounds: This was heated to 100-105°C in oven until maximal visualisation of the spots [26].

Dragendorff's reagent for alkaloids and other nitrogen-containing compounds: After spraying, alkaloids and some other compounds containing nitrogen show orange spots [26].

Aluminium chloride for flavonoids: This was sprayed with 1% ethanolic solution of aluminium chloride. Fluorescence in long-wave UV light is indicative of flavonoid [26].

Anisaldehyde-sulphuric acid for sugars, steroids and terpenes: This was heated to 100-105°C in oven until maximal visualisation of the spots. The background may be brightened by water vapour. Lichen constituents, phenols, terpenes, sugars and steroids turn violet, blue, red, grey or green [26].

Antibacterial Activity Assay of Extracts Fractions

The antibacterial activity of each fraction of *A. sativum* and *C. limon* crude extracts against the test organism was evaluated by using agar well diffusion method of sensitivity test described by Srinivasan *et al*., [25]. Mueller Hinton agar plates were inoculated with 100μl of standardized inoculum of each bacterium using a micropipette of 100μl size and spread uniformly with sterile swab sticks. Wells of 4 mm size were made with sterile cork borer into the agar plates containing the bacterial inoculum. Using the micropipette, 100μl volume of the various fractions; petroleum ether, ethylacetate, n-butanol 1, n-butanol 2 and aqueous fraction 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 [25].

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, the standard strain, and the positive control antibiotics 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.

RESULTS

The cultural and physiologic properties of clinical isolates of *Streptococcus pyogenes* as presented in Table 1 shows that out of 47(100%) clinical isolates of *Streptococcus pyogenes* obtained from the hospital, 44(93.6%) appeared as Small, colourless, dry colonies on blood agar, gram positive, cocci under the microscope and catalase negative. Among the 44 (93.6%) isolates, 7(15.9%) were in chains under the microscope, sensitive to bacitracin, beta haemolytic and identified as *S. pyogenes* while 13(29.5%) were in

pairs/chains under the microscope, resistant to bacitracin, alpha haemolytic and identified as *S. pneumonae.*

Table 1: Cultural and Physiologic Properties of Clinical Isolates of *Streptococcus pyogenes*

Isolates	Colonial morphology	Gram reaction	Cell morphology	Cell arrangement	Catalase	Bacitracin test	Haemolysis	Identification
B29	Small, colourless, dry	Positive	Cocci	Chains	-	S	β	*S. pyogenes*
B28	Small, colourless, dry	Positive	Cocci	Chains	-	S	β	*S. pyogenes*
B27	Small, colourless, dry	Positive	Cocci	Chains	-	S	β	*S. pyogenes*
B32	Small, colourless, dry	Positive	Cocci	Chains	-	S	β	*S. pyogenes*
B33	Small, colourless, dry	Positive	Cocci	Chains	-	S	β	*S. pyogenes*
B34	Small, colourless, dry	Positive	Cocci	Chains	-	S	β	*S. pyogenes*

Key: B- isolate code, - = negative, S- sensitive, β- beta

Table 2 shows the results of biochemical characterization and identification of clinical isolates of *S. pyogenes* using Microgen* *S. pyogenes* Identification Kit.

Table 2: Biochemical Characterization and Identification of Clinical Isolates of *S. pyogenes* using Microgen* *S. pyogenes* Identification Kit

IS	H	A	B	M	S	I	L	A	R	E	V	P	B	PYR	A	% probability	Profile No	Final identification
B29	-	-	+	-	-	-	+	-	-	-	-	+	-	+	+	100%	10413	*S. pyogenes*
B28	-	-	+	-	-	-	+	-	+	+	-	+	-	+	+	99.92%	10653	*S. pyogenes*
B27	-	-	+	-	-	-	+	-	-	+	-	+	-	+	+	99.92%	10453	*S. pyogenes*
B32	-	-	+	-	-	-	+	+	-	-	+	+	-	+	+	99.98%	10633	*S. pyogenes*
B33	-	-	+	-	-	-	+	+	-	+	-	+	-	+	+	99.92%	10653	*S. pyogenes*
B34	-	-	+	-	-	-	+	-	-	-	-	+	-	+	+	100%	10413	*S. pyogenes*
B36	-	-	+	-	-	-	+	-	-	-	-	+	-	+	+	100%	10413	*S. pyogenes*

KEY

IS=Isolate No.
H= Hippurate test
A=Alpha haemolysis
B=Beta haemolysis
P= Alkaline Phosphatase
A=Arabitol
R=Ribose
E=Esculin
V= Voges Proskauer (VP)
β = β- Glucuronidase
M=Melibiose
S=Sorbitol
I=Inulin
L=Lactose
PYR=Pyrrolidonyl arylamidase
A=Arginine

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 3 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.

Table 3: Phytochemical Constituents of Aqueous and Acetone Extracts of garlic cloves and lemon juice

Metabolites	Test type	AQGC	AQLJ	ACGC	ACLJ
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
AQGC = Aqueous extracts of garlic cloves
AQLJ = Aqueous extracts of lemon juice
ACGC = Acetone extracts of garlic cloves
ACLJ = Acetone extracts of lemon juice

The susceptibility of clinical isolates of *Streptococcus pyogenes* to different concentrations of aqueous and acetone extracts of garlic cloves and lemon juice where means with different superscripted alphabets along a column are significantly different at $P < 0.05$ is presented in Table 4, figure 2 and plate 1 shows indicating that the isolates of *S. pyogenes* exhibited varying degrees of resistance and susceptibility to different concentrations of aqueous and

acetone extracts of garlic cloves and lemon juice as well as the antibiotics used as positive control in the susceptibility analysis (Erythromycin-10µg and Ampicillin-30µg). Thus, Different concentrations of aqueous extracts of garlic cloves yielded no inhibitory activity on all the isolates and the acetone extract of garlic cloves yielded low inhibitory activity with mean inhibition zone diameter ranging from 7.5 ± 1.52 mm to 24.7 ± 0.87mm while the different concentration of aqueous and acetone extracts of lemon juice yielded the highest inhibitory activity with mean inhibiting zone diameter ranging from 16.8 ± 0.40mm to 37.8 ± 0.34mm which is even higher than the inhibitory activity of all the antibiotics; Erythromycin and Ampicillin, with mean inhibition zone diameter of 22.7 ± 0.71mm and 3.9 ± 1.38mm respectively.

Table 4: Susceptibility of Clinical Isolates of *S. pyogenes* to Different Concentrations of Aqueous and Acetone Extracts of Garlic cloves and Lemon juice

Extracts	Dosages (mg/ml)	Mean Inhibition Zone Diameters (mm) ± SEM
AQG	100.0	0.0 ± 0.00^{i}
AQG	50.0	0.0 ± 0.00^{i}
AQG	25.0	0.0 ± 0.00^{i}
AQG	12.5	0.0 ± 0.00^{i}
AQL	100.0	24.7 ± 0.87^{d}
AQL	50.0	17.1 ± 0.40^{e}
AQL	25.0	13.7 ± 0.45^{f}
AQL	12.5	7.5 ± 1.52^{g}
ACG	100.0	36.5 ± 0.36^{ab}
ACG	50.0	35.1 ± 0.36^{bc}
ACG	25.0	23.5 ± 0.86^{d}
ACG	12.5	17.3 ± 0.44^{e}
ACL	100.0	37.8 ± 0.34^{a}
ACL	50.0	33.3 ± 0.57^{c}
ACL	25.0	24.5 ± 0.47^{d}
ACL	12.5	16.8 ± 0.40^{e}
Erythromycin	10µg	22.7 ± 0.71^{d}
Ampicillin	30µg	3.9 ± 1.38^{h}

Key: Means with the different superscripted alphabets along a column are significantly different at $P < 0.05$. AQG=Aqueous garlic extract, AQL=Aqueous lemon extract, ACG=Acetone garlic extract, ACL=Acetone lemon extract.

Plate 1: Antibacterial susceptibility plates of *S. pyogenes* and extracts after 18-24hrs incubation

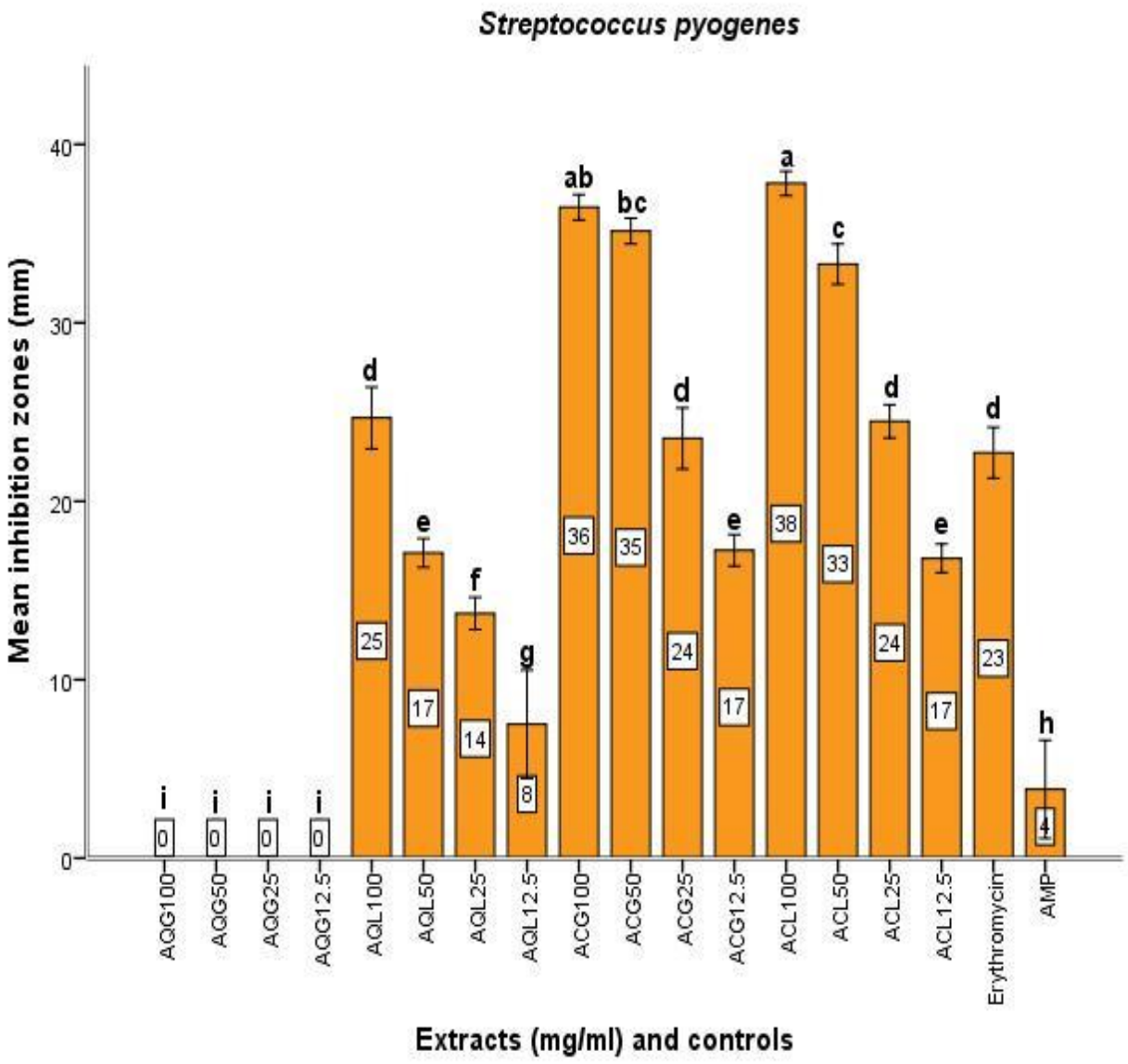

Figure 2: Susceptibility of Clinical Isolates of *S. pyogenes* to Different Concentrations of Aqueous and Acetone Extracts of Garlic cloves and Lemon juice.

Key: AQG=Aqueous garlic extract, AQL=Aqueous lemon extract, ACG=Acetone garlic extract, ACL=Acetone lemon extract.

The MIC and MBC of aqueous and acetone extracts of garlic cloves and lemon juice on susceptible *S. pyogenes* isolates is presented in Table 5 where aqueous garlic cloves extract has no MIC and MBC due to resistance of all the isolates. Aqueous lemon juice extract has the same MIC and MBC on all the isolates as 1.56mg/ml and 3.125mg/ml respectively except on isolate 33 which has MIC and MBC as 3.125mg/ml and 6.25mg/ml respectively. Acetone garlic cloves extract has the same MIC and

MBC on isolates 27, 29, 33 and 34 as 3.12mg/ml and 6.25mg/ml respectively while it also has the same MIC and MBC on isolates 28, 32, and 36 as 1.56mg/ml and 3.125mg/ml respectively. Acetone lemon juice extract has the same MIC and MBC on all the isolates as 1.56mg/ml and 3.125mg/ml respectively.

Table 5: Minimum Inhibitory Concentrations (MIC) and Minimum Bacteriocidal Concentrations (MBC) of Aqueous and Acetone Extracts of Garlic cloves and Lemon juice on Susceptible *S. pyogenes* Isolates.

ISOLATES	MIC and MBC of Extracts on *S. pyogenes* Isolates (mg/ml)							
	AQG		AQL		ACG		ACL	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
B27	-	-	3.125	6.25	1.56	3.125	1.56	3.125
B28	-	-	1.56	3.125	1.56	3.125	1.56	3.125
B29	-	-	3.125	6.25	1.56	3.125	1.56	3.125
B32	-	-	1.56	3.125	1.56	3.25	1.56	3.125
B33	-	-	3.125	6.25	3.125	6.25	1.56	3.125
B34	-	-	3.125	6.25	1.56	3.125	1.56	3.125
B36	-	-	1.56	3.25	1.56	3.125	1.56	3.125

Key: AQG=Aqueous garlic cloves extract, AQL= Aqueous lemon juice extract, ACG=Acetone garlic cloves extract, and ACL=Acetone lemon juice extract. MIC= Minimum Inhibitory Concentration, MBC= Minimum Bacteriocidal Concentration. B = Isolate code.

The result of thin layer chromatography (TLC) of crude acetone extracts of garlic cloves and lemon juice is presented in plate 2 where both Acetone extract of garlic cloves and Acetone extract of lemon juice had four spots on the TLC plate developed with ethylacetate methanol (10:2 v/v) solvent. The R_F value for each spot was calculated. Spot c and d of both extracts appear to be close to each other and has low R_F values of (0.06 and 0.13) and (0.07 and 0.16) respectively while the other two spots, a and b were more separated with R_F values of (0.64 and 0.80) and (0.68 and 0.82) respectively.

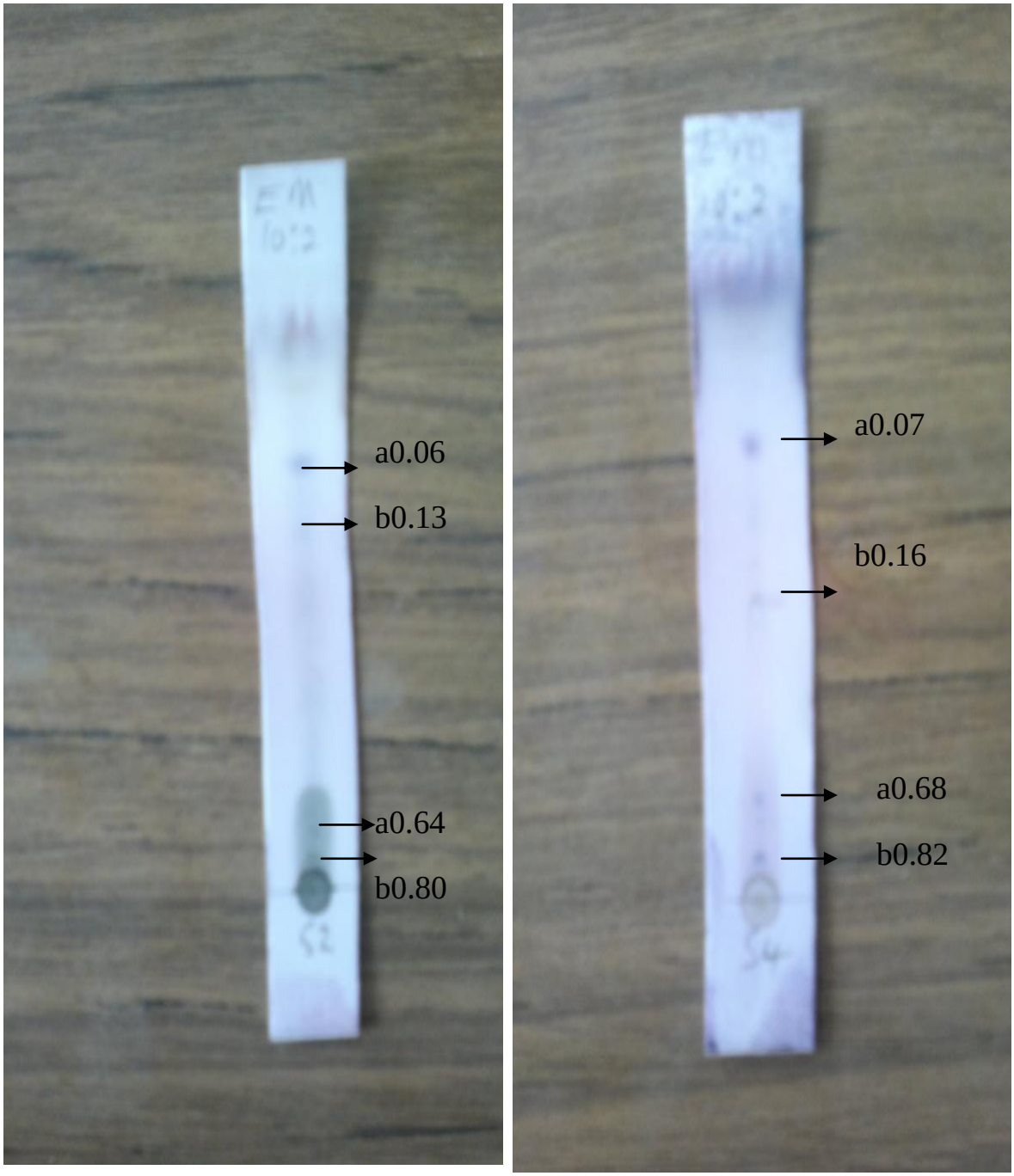

Plate 2: Thin Layer Chromatography (TLC) of Crude Acetone Extracts of Garlic cloves (left) and Lemon juice (right)

Key: Rf value= retention factor, a-d= spot position on TLC plate

The result of phytochemical constituents of fractionated acetone extracts of garlic cloves and lemon juice on thin layer chromatography (TLC) plates is presented in Table 6 and plate 3. Table 6 shows Rf values of various fractions of acetone extracts of garlic cloves and lemon juice based on phytochemical test conducted using TLC plates and specific spray reagents. The result shows that the fractionated acetone extract of the garlic cloves and lemon juice revealed some spots on the TLC plate based on the type of phytochemical test. Ferric chiloride test for phenolic compounds revealed the presence of phenols in two fractions i.e. EL and EG where EL has two spots with R_f values less than 1. Dragendorff's reagent for alkaloids revealed the presence of Alkaloid in four fractions which includes, EG, PG, BIL and B2L with of values also less than 1. Alluminium chloride test for flavonoids revealed the

presence of flavonoids in six fractions including four fractions of lemon juice extract and two fractions of garlic cloves extract with R_f values also less than 1. P-anisaldehyde general spray revealed the presence of two to three spots representing unknown compounds in each of the fractions with R_f values less than 1.

Table 6: Rf Values of various fractions of Acetone Extracts of Garlic cloves and Lemon juice based on phytochemical test

Plants Acetone Extracts Fractions	Ferric chloride test(Phenolic compounds)	Dragendorff's reagent (Alkaloids)	Alluminiun chloridetest (Flavonoids)	P-anisaldehyde (General spray)
EL	a=3.9/10=0.39		a=3.8/7.0=0.54	a=3.3/7.3=0.45
	b=4.2/10=0.42			b=3.8/7.3=0.52
				c=5.3/7.3=0.73
EG	a=6.9/10=0.69	a=3.9/7.1=0.55	a=5.8/7.0=0.83	
PL			a=6.5/7.0=0.93	a=4.6/7.3=0.63
				b=5.5/7.3=0.75
PG		a=3.9/7.1=0.55	a=6.5/7.0=0.93	a=2.2/7.3=0.30
				b=4.0/7.3=0.55
				c=5.3/7.3=0.73
				d=6.0/7.3=0.82
B1L		a=6.5/7.1=0.92	a=5.8/7.0=0.83	a=5.3/7.3=0.73
B1g				a=5.6/7.3=0.73
				b=6.4/7.3=0.88
B2L		a=4.2/7.1=0.59		a=0.9/7.3=0.12
				b=6.5/7.3=0.89
B2g				a=5.6/7.3=0.77
AL			a=3.3/7.0=0.47	a=3.1/7.3=0.42
				b=5.2/7.3=0.30
AG				a=5.5/7.3=0.75

Key: EL=Ethylacetate lemon juice fraction, EG=Ethylacetate garlic cloves fraction, PL=Petroleum ether lemon juice fraction, PG=Petroleum ether garlic cloves fraction, B1L=n-Butanol 1 lemon juice fraction, B1g= n-Butanol 1 garlic cloves fraction, B2L=n-Butanol 2 lemon juice fraction, B2g= n-Butanol 2 garlic cloves fraction, AL=Aqueous lemon juice fraction, AG=Aqueous garlic cloves fraction.

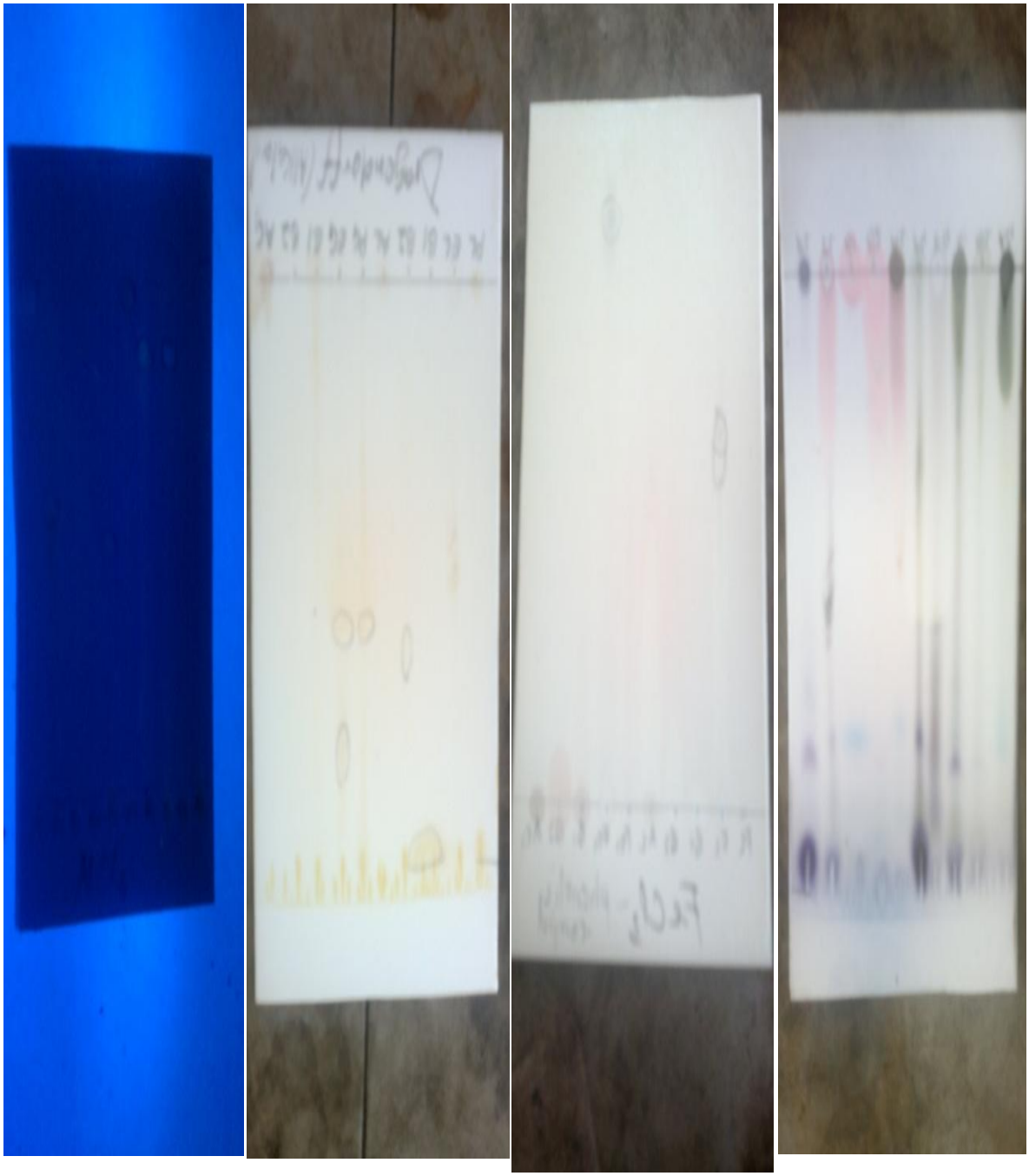

Plate 3: Phytochemical Constituents of Fractionated Acetone Extracts of Garlic cloves and Lemon juice on Thin Layer Chromatography (TLC) Plates.

The result of antibacterial susceptibility test of various fractions of garlic cloves and lemon juice acetone extracts to *S. pyogenes* clinical isolates is presented in Table 7. The result shows that ethylacetate fraction of acetone extract of lemon juice had the highest susceptibility on *S. pyoyenes* isolate with mean inhibition zone diameter of 32.0± 0.00mm and its aqueous and n-butanol 1 fraction also has susceptibility on the isolates with mean inhibition zone diameter of 16.0± 0.00mm. The aqueous fraction of acetone extract of garlic cloves has susceptibility on *S. pyogenes* isolate with mean inhibition zone diameter of 15.0± 0.00mm

.

Table 7: Antibacterial Susceptibility test of various fractions of Acetone Extracts of Garlic cloves and Lemon juice on *S. pyogenes* Clinical Isolates

Garlic and Lemon Fractions	Mean Inhibition Zone Diameter of *S.pyogenes* Isolate (mm)
EL(1)	NZ
EG(1)	NZ
EL(2)	32. 0±0.00
PG(2)	NZ
B1L(3)	NZ
B1g(3)	NZ
B2L(4)	NZ
B2g(4)	NZ
AL(5)	16. 0±0.00
AG(5)	15. 0±0.00

Key: EL=Ethylacetate lemon juice fraction, EG=Ethylacetate garlic cloves fraction, PL=Petroleum ether lemon juice fraction, PG=Petroleum ether garlic cloves fraction, B1L=n-Butanol 1 lemon juice fraction, B1g= n-Butanol 1 garlic cloves fraction, B2L=n-Butanol 2 lemon juice fraction, B2g= n-Butanol 2 garlic cloves fraction, AL=Aqueous lemon juice fraction, AG=Aqueous garlic cloves fraction, NZ= no zone of inhibition.

DISCUSSION

The results of the current study reveals the efficacy of aqueous and acetone extracts of garlic cloves and lemon juice based on in vitro evaluation of antibacterial activity of aqueous and acetone extracts of garlic cloves (*Allium sativum*) and lemon juice (*Citrus limon*) on clinical isolates of *Streptococcus pyogenes.* Acetone extracts of both garlic cloves and lemon juice recorded higher antibacterial activity than the aqueous extracts. This may be attributed to the fact that acetone as an extraction solvent extracts highly polar and non polar components from the plant material; it is miscible with water, very volatile, has a low toxicity in antimicrobial bioassays and is easily removed from the plant material at a low temperature [21].

Among the acetone extracts of garlic cloves and lemon juice, highest antibacterial activity was recorded with acetone extract of lemon juice which inhibited the *S. pyogenes* isolates with mean inhibiting zone diameter ranging from 16.8 ± 0.40mm to 37.8 ± 0.34mm higher than the inhibitory activity of the control antibiotics; Erythromycin and Ampicillin, with mean inhibition zone diameter of 22.7 ± 0.71mm and 3.9 ± 1.38mm respectively. This result may be due to the antioxidant activities of citrus fruits and the ability of *Citrus* flavonoids to complex with bacterial cell walls and disrupt microbial membrane [14]. The acetone extract of lemon juice also inhibited the standard strain (*S. aureus*-ATCC 25923) with mean inhibiting zone diameter ranging from 2.8 ± 1.07 to 21.9 ± 6.46mm but not as high as the inhibition of the acetone extract of lemon juice on *S. pyogenes* isolates. The differences in

susceptibility between *Staphylococcus aureus* and *Streptococcus pyogenes* can be explained by differences in the nature and extent of cell membrane damage [33].

The antibacterial effects of lemon juice were previously studied. Indeed, it was reported 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 [11] 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, Sengun, and Karapinar [22] also reported that Citrus juices of *lemon* and *bitter orange* (*C. limon* and *C. auran-tium*) showed good antibacterial activities against gram positive and gram negative microorganisms where they related the stage of microbial growth to the production of phenolic compounds by fruits which showed to be powerful anti-oxidants and free radical scavengers, those compound being able to induce reactions of electron transfer and do react with vital nitrogen compound in the microbial cell such as nucleic proteins and acids [30]. The complicated mixtures of those compounds represented the strongest barrier to infection and may contribute to the differences in their bactericidal activity. *Citrus* flavonoids have also been reported to act as antioxidants in various biological systems. These findings and the results obtained in the present study clearly confirm the effectiveness of lemon juice on inhibition of microbial activity.

Similarly, acetone extract of garlic cloves being second in terms of highest antibacterial activity on *S. pyogenes* isolates with mean inhibition zone diameter ranging from 17.3±0.44 mm to 36.5±0.36 mm. The order of inhibition followed same pattern exhibited by lemon juice acetone extract on *S. pyogenes* isolates. The susceptibility of *S. pyogenes* isolates to acetone extract of garlic cloves supports the claim that crushed garlic can be used as home remedy to help speed recovery from sore throat and other minor ailments [31;34]. This finding on garlic cloves serves to concretize the findings of Ankri and Mirelman [4] on the report that garlic cloves when crushed yields *allicin*, a thiosulfinate compound that interferes with RNA synthesis. If RNA cannot be produced or produced in fewer amounts then protein synthesis will be greatly affected [32]. It would be stopped at every stage due to the absence of messenger RNA, ribosomal RNA and transfer RNA, if amino acids and proteins cannot be produced then growth and development of the organism will not occur as they are essential for all parts of cell structure [28; 29].

Other research works on different garlic extracts has demonstrated activity against both Gram-positive and Gram-negative bacteria including *Staphylococcus aureus, Streptococcus pyogenes, Salmonella typhi, Psuedomonas aeruginosa, Escherichia coli, Klebsiella pneumoniae, and Bacillus subtilis* [20; 18; 25; 8]. All these findings on garlic cloves agree with the findings in the present study on the antimicrobial activity of garlic cloves. However, the finding on the aqueous extract of garlic cloves seems to contradict other findings showing little or no inhibition on the two tested organisms which could be based on source of isolates or behaviour of the extract at that particular point in time although the present study strictly adhered to findings of Srinivasan *et al*. [25] on the *invitro* antibacterial activity and stability of garlic aqueous extract at different pH and temperature where the results revealed that the antimicrobial efficacy of garlic aqueous extract is time and temperature dependent.

The MIC and MBC assay procedures are frequently used to evaluate some diverse agents such as antibiotics, antiseptics, disinfectants and chemotherapeutic agents [3]. Antimicrobial agents with low activity against an organism usually gives a high MIC and MBC values, while those that are highly

effective give low MIC and MBC values. In this study, the MIC values of both aqueous and acetone extracts of garlic cloves and lemon juice was as low as 1.56mg/ml and 3.125mg/ml respectively. This implies that the efficacy of both garlic cloves and lemon juice aqueous and acetone extracts could be as low as the MIC and MBC obtained in this study but this also confirms the strong efficacy of lemon juice as was revealed by Mishra and Behal [17] that the broad-spectrum of activity of lemon should be due to its acidic pH and the presence of essential oils α-pinene, camphene, β-pinene, sabinene, myrcene, α-terpinene, linalool, β-bisabolene, limonene, trans-α-bergamotene, nerol and neral that contains proven antimicrobial properties.

The thin layer chromatography (TLC) of the crude acetone extracts of garlic cloves and lemon juice revealed four spots for each extract using ethylacetate methanol (10:2 v/v) as the developing solvent after several trials with other solvents. Each of the four spots represents a compound whose retention factor (R_f) was calculated and these compounds could be fractionated from the crude plant extract. This serves as an indication that the nature of the antibacterial activity of the crude extracts can be identified after fractionation, to be the activity of only one compound, two or even all the compounds present in the crude extract. Patra *et al*. [19] reported that TLC is firstly used for the separation of the crude plant extract in to its constituents components predetermined by phytochemical screening. The developed chromatogram provides not only a fingerprint of the plant constituents but also a template for the analysis of sample properties. Further more, the crude plants extracts were fractionated and fractions were further characterized on TLC plates and sprayed with specific phytochemical spray reagents that revealed some compounds like alkaloids, phenols and flavonoids in the fractions.

The susceptibility pattern of the fractions to *S. pyogenes* isolates shows that not all the fractions of garlic cloves and lemon juice were active on the isolates except three fractions of lemon juice and two fractions of garlic cloves out of which the highest antibacterial was obtained from ethylacetate fraction of lemon juice on the isolate with mean inhibition zone diameter of 32. 0±0.00mm followed by n-Butanol 2 fraction of garlic cloves with mean inhibition zone diameter of 16.0±0.00mm. This finding indicates that not all the components of the plant extracts acted on the isolates in crude form but rather some of the components.

CONCLUSION

In conclusion, the results obtained in this study has clearly demonstrated the effectiveness of aqueous and acetone extracts of lemon juice as well as broad spectrum antibacterial activity of acetone extracts of Lemon juice and garlic cloves on *S. pyogenes* pathogenic bacteria of clinical origin. Thus, this work is part of an effort to validate the use of garlic cloves and lemon juice in traditional medicine and to explore the use of these plants as a source for future discovery of antibacterial drugs.

RECOMMENDATION

In view of the findings in this study, the following recommendations were made. The future work on this study should be to determine actual chemical compounds responsible for the antibacterial activity of these plants, their toxicity, side effects and pharmacokinetic properties for new drug development. There should also be further investigation to identify molecular targets in the bacterial cells such as the active sites of enzymes involved in cell division and then developing the bioactive compounds in plants to serve as inhibitors of the specific target molecule.

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