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Molecular Profiles of Resistant Gram Negative Bacteria Isolated from *Azadirachta Indica* and *Psidium Guajava* Rhizosphere

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Abstract The aim of this study was to investigate the antibacterial activity of Psidium guajava and Azadirachta indica leaf extracts against gram negative bacteria. The extracted plants were rinsed with water to remove dust and air dried at normal room temperature (27°C) for two weeks and the grinded plants extracts preparation was divided into four different concentrations. Bacteria were isolated from the plants rhizosphere and the leaves extracts were diluted serially in different milliliters with discs soaked into the extracts differently and placed on the Mueller Hinton agar by using antibiotic sensitivity according to the Kirby-Bauer technique. The sensitivity of both extracts was initially tested with Escherichia coli and Salmonella spp. Identification of bacterial isolates from the rhizosphere where the two plants grow was subjected to conventional biochemical and molecular sequencing technique. The statistical analysis of the results showed that there was no significant inhibition on the tested organisms at (P>0.05). All bacteria isolates were catalase (+) and oxidase (+). Antibiotic Susceptibility test showed that Pseudomonas aeruginosa and Serratia marcescens were resistant to both plant extracts but yielded remarkable susceptibility to Ofloxacin, gentamycin and Ceftriaxone. Molecular characterization of Pseudomonas aeruginosa strain P1 (KF530797) and Serratia marcescens strain HA 517(KJ535328) indicated the presence of specific amplicons of ampG, ampC, ampD genes and gyrA respectively. Consequently, exchange of resistance genes could have been acquired horizontallybetween both plants and the bacteria population in the rhizosphere.

Keywords: *Serratia marcescens, rhizosphere, Kirby-Bauer, Ceftrizone, amplicons*

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1. Introduction

About 80% of individuals from developed countries use traditional medicine, which has compounds derived from medicinal plants. Therefore, such plants should be investigated to better understand their properties, safety and efficiency. Plants of various origins have been exploited effectively over many generations for therapeutic purposes. The selection procedure was often haphazard to the extent that some valuable errors are caused [1]. Thus, the discard or otherwise of such plant depends on its being beneficial or hazardous. In the local traditional settings, plant parts such as the roots or leaves are used without recourse to photochemical isolates [2]. The argument is that the synergy of the combined substances enhances the efficacy and dilutes toxicity [3]. Modern pharmacy, however, prefers single ingredients on the grounds that dosage can be more easily quantified [1]. Medicinal plants are important with respect to new drug and pharmacological research development.

Plants are very good sources of medicinal compounds that have continued to play a dominant role in the maintenance of human health since ancient times as reported by [4]. Medicinal plants represent a rich source of antimicrobial agents. These plants possess potent medicinal value that is due to the presence of a variety of phytochemical constituents in the plant tissues which cast a definite physiological action on the human body [5]. They are used medicinally in different countries as well a source of many potent and powerful drugs [6]. Phytotherapy (herbal medicine) have a long-standing history in Egypt. Scientific investigations for medicinal plants have been initiated in many countries because of their contributions to health care. It was cleared that the primary benefits of using plant derived medicines are relatively safer than synthetic alternatives, offering

profound therapeutic benefits and more affordable treatment. A lot of supplementary treatment strategies were tried. Current social trends in health care showed a definite movement towards the use of natural remedies like medicinal plants away from chemotherapeutic regimens [4].

The use of crude extracts of plants parts and phytochemicals, of known antimicrobial properties, can be of great significance in the therapeutic treatments. The use of natural plant antimicrobial compounds is important in the control of microbial growth in the diseases condition [7]. Antimicrobial agents are effective in curing diseases because of their selective toxicity against pathogenic microbes without causing any harm to the cells of the host [8]. The antimicrobial activity of plant extracts and phytochemicals was evaluated with antibiotic susceptible and resistant microorganisms as cleared [9]. Some studies concluded that the spices may be very valuable because bacteria develop resistance to conventional antibiotics [10]. Antibiotic resistance has become a global concern. There has been an increasing incidence of multiple resistances in human pathogenic microorganisms largely due to indiscriminate use of commercial antimicrobial drugs commonly employed in the treatment of infectious diseases [11].

Neem plant is used in traditional medicine as a source of many therapeutic agents and grows well in the tropical countries. Its twigs provide a chewing stick and are widely used in the Indian sub-continent. Neem leaves has antibacterial properties and could be used for controlling airborne bacterial contamination in the residential premise [12,13]. The popularity of the plant products is increasing because of their biodegradability, least persistence and least toxic to non-target organisms, economic and easy availability. Today about two hundred plants with insecticidal activities are known. Among the natural products, one of the most promising natural compounds is Azadirachtin, an active compound extracted from the *Azadirachta indica* A. Juss (neem) tree (Meliaceae) whose antiviral, antifungal, antibacterial and insecticidal properties have been known for several years. Neem elaborates a vast array of biologically active compounds that are chemically diverse and structurally complex. Medicinal properties of the plant *Azadirachta indica* were studied by several workers. They were anti-pyretic, anti-malarial and anti-tumor effect, anti-ulcer effect, anti-diabetic effect, anti-fertility effect, effect on central nervous system and antioxidant activity.

P. guajava, belongs to the family *Myrtaceae* which is considered to have originated from tropical South America, Guava tree grown in tropical and sub-tropical area of the world like Asia, Africa and Hawaii etc [14]. *Psidium guajava* common names are Guava (English), Gwaiba (Hausa), Goifa (Yoruba), Ugova (Igbo): Gutiba (Spanish), Goyave (French) and Goejaaba (Dutch) [15]. Many parts of the plant have been used in traditional medicine to manage conditions like malaria, Gastroenteritis, vomiting, diarrhea, dysentery, wound, ulcer, toothache, cough, sore throat and a number of other conditions [16]. The leaves are particularly rich in flavonoids and distinctly in quercetin [17]. The Ethanolicleaf extract also showed in vitro antibacterial activity against *E. coli, S. typhii, Staphylococcus aureus* and a strong antibacterial action have been reported on Gram positive and gram negative organism [16]. Therefore, the basic phytochemical investigation of its extract for major phyto-constituents is also vital. The phytochemical analyses of Guava plant shows that its extracts contain over twenty compounds [18].

Plant rhizosphere is a distinctive soil environment which is under the direct influence of soil micro flora and different secretions of roots. This region of soil contains many microorganisms including bacteria, which feed on dead cells of plant as well as sugars and proteins released by roots of plant. All biological and biochemical activities in rhizosphere region of soil are overwhelmed by the interactions between plant roots, associated flora and root microorganism [19]. Rhizosphere also known as “bio-influenced zone” [20] is the richest environment for the isolation of industrially important microorganisms especially Actinomycetes, which are slow growing, branching filamentous and gram positive bacteria [20]. Their presence with great diversity in particular soil depends on many factors like type of soil, geographical location, cultivation and organic matter. Their industrial importance is based on their capabilities to produce biologically active compounds including enzymes, antibiotics and vitamins [21,22]. With the advent of modern technology and increased demand for microbial enzymes in international enzyme market, scientists are nowadays more interested in discovery of new enzymes with novel properties. Some of the reported extracellular enzymes produced by Actinomycetes are cellulase [23,24], amylase [25,26], protease and chitinase [27]. Proteases of microbial origin are among the most vital hydrolytic enzymes which have been extensively studied. Producer microorganisms are easy to grow in bulk quantities by sub-merged fermentation techniques for production of desired products within minimum time. Although many microorganisms have inherent capability of production of secondary metabolites, the strains producing extracellular enzymes have been employed for production of enzymes on industrial scale. Among hydrolytic enzymes, proteases are of most importance because of convenient methodologies of purification [28].

2. Materials and Methods

2.1. Collection of Samples

The medical plant studiedincluded *Psidium guajava* and *Azadirachta indica* and their soils (rhizosphere of the plants). The leaves were collected from the plants and the soil samples were collected from the rhizosphere of the root plants and were taken inside clean and sterilized polythene bags. The sample were moved directly to the laboratory and allowed to air dried. Within 24 hours, the collected rhizosphere sample was used to isolate the bacteria. They were collected from their natural habitat in a village known as Sabe Community from ATISBO Local Government Area, Oyo State, Nigeria.

2.2. Test Organisms

Test organisms were isolated from water and milk samples, such as *Escherichia coli, Salmonella*

typhii which were obtained from microbiology laboratory in the Oke-Ogun Polytechnic Saki, identification and characterization of the isolates were conducted by using three procedures namely, gram staining, cultural characterization, using selective or indicative media and biochemical characterization with reference to [29]. The pure isolates of each test organism were inoculated and sub-cultured into another plates containing Eosin Methylene Blue Agar (EMB) and MacConkey agar, then, refrigerated at 4°C before use.

2.3. Preparation of Plant Extracts

The plants samples were washed with water to remove dust and rinsed with distilled water. Samples were air dried under a room temperature of 27°C for two weeks and pulverized into fine powder using sterile mortar and pestle in the laboratory. The powdered samples were bagged into sterile black polythene bags and store in air tight container for further work.

2.4. Extract Preparation

During the preparation of plant extracts, four types of solvents were used which are n- hexane, ethanol, ethanol+ aqueous and aqueous. The plants powders were soaked into each solvent separately for three (3) days. For aqueous, this was done by weighing 150g of the dried powder was poured into 750ml of distilled water, for ethanol, 150g of the dried powder was poured into 750ml of ethanol, for ethanol + aqueous, 100g into 250ml of ethanol and 250ml of water, for n- hexane, 100g of the powder was poured into 500ml of n- hexane. The filtrate was kept in a rotary evaporator for complete evaporation of the solvent. After running this procedure, a gummy extraction was obtained (concentrated) in which the extract of *Psidium guajava* in ethanol + aqueous weighed 8.633g and *Azadirachta indica* weighed 6.698g, for n- hexane, *Psidium guajava* weighed 1.413g and *Azadirachta indica* weighed 2.07g, for ethanol, *Psidium guajava* weighed 11.685g and *Azadirachta indica* weighed 5.043g for aqueous, *Psidium guajava* weighed 10.433g and *Azadirachta indica* weighed 8.499g was preserved in the refrigerator before use.

2.5. Preparation of Concentration or Antibacterial Assay of Extracts

2.5.1. Serial Dilution

Each plant extract was subjected to a serial dilution using each solvent and sterile distilled water for the control known as chloramphenicol as a diluent. Serial dilution was done to determine the standard solution of the gummy extract by dilution of the gummy extracts into a round bottom flask and 100ml of each solvents was poured into the 1000ml of the round bottom flask covered with foil paper and shaken gently until it dissolved and 75ml from the 100ml was poured into another round bottom flask, 25ml of the solvent was poured to make it 100ml, 50ml from the 75ml was poured into another round bottom flask to make it 100ml, 25ml from the 50ml was poured into another round bottom flask and 75ml of each solvents was poured to make it 100ml. for the aqueous, 0.2g, 0.4g, 0.6g, 0.8g, and 1g of the gummy extract of each plants was diluted seriallyinto 1ml each of the solvents.

2.5.2. Bacteria Inoculation and Disc-Diffusion Method

Media specify for each strain that was sub-cultured (EMB for *salmonella* and MacConkey for *E.coli*) and on each labeled plate that contains the medium, was inoculated 40 µl of standardized broth culture of the bacteria. The swab stick spread the bacteria on the plate of Mueller Hinton Agar which was used to ensure uniform distribution of the microorganisms on surface of plates.This disc-diffusion was described by Kirby-Bauer. Normally, this method is used for testing the effect of chemical drugs on bacteria; therefore the same method was used in order to compare the effectiveness of neem and guava extracts concentrations from leaves; obtained by using aqueous, ethanol, n- hexane, and ethanol+ aqueous as solvents. Filter paper discs were prepared and sterilized, these discs were then soaked in different concentration of extracts, and then they were aseptically placed over the media (Mueller Hinton Agar which is known as MHA) with specific bacteria. The plates were incubated in an upright position at 37 °C for 24 hours. The diameters of inhibition zones were measured in cm.

2.6. Isolation of Rhizosphere's Microorganisms

Serial dilution method was used to isolate the bacteria. The soil sample was serially diluted in the sterilized water under the sterilized conditions (laminar air flow chamber). Serial dilutions were made up to 10^{-3} and 10^{-5} and 0.1 ml of each dilution was poured on different sterile Petri dish having antibacterial agent (50 µg/ml streptomycin) [34] Nutrient Agar (NA) was heated in the autoclave at 121°C for 15minutes, it was allow to cool for 5minutes and then poured on the Petri dishes filled with 0.1ml of d diluted samples, then, it was allowed to solidify for 15min. Plates were incubated at the°C37in the incubator for 24 hours. Morphologically different bacteria colonies were separated by inoculating each colony on the different selective media such as Eosin Methylene Blue Agar (EMB), MacConkey Agar, to maintain the pure culture. The same processes were carried out for the isolation of bacteria from milk and water.

2.7. Gram Staining Techniques

A thin smear of each of the pure 24 h old culture was prepared on clean grease-free slides, fixed by passing over gentle flame. Each heat-fixed smear was stained by addition of 2 drops of crystal violet solution for 60 sec and rinsed with water. The smear was again flooded with Lugol's Iodine for 30 sec and rinsed with water, decolorized with 70% alcohol for 15 sec and were rinsed with distilled water. They were then counter stained with 2 drops of Safranin for 60 sec and finally rinsed with water, then allowed to air dry. The smears were mounted on a microscope and observed under oil immersion objective lens. Gram negative cells appeared pink or red while gram positive organisms appeared purple.

2.7.1. Oxidase Test

A piece of filter paper was soaked with few drops of oxidase reagent. Sterile inoculating loop was used to pick a colony of the test organismandsmeared on the filter paper. If the organism is oxidase producing, the Phenylenediamine in the reagent will be oxidized to a deep purple colour [30].

2.8. Molecular Identification of Organism Isolated from Plants Rhizosphere

One (1kb) plus ladder from NEB was used as the molecular weight ladder. The thermal cycler used is ARKTIK thermal Cycler from Thermo fisher. The centrifuge used is MIKRO 200 from Hittich Zentrufugen. Sanger sequencing methodology was used for sequencing and the genetic analyzer used is ABI 3500 from Thermo fisher Scientific. The cat log for kits used is included in the methodology.

2.8.1. DNA Extraction Using ZR Fungal/ Bacterial DNA Mini prep (Manufactured By Zymo Research Cat Number: D6005)

Two milliliters (2mls) of bacterial cells broth was added to a ZR BashingTM lysis Tube. 750µl lysis solution was added to the tube. It was secure in a bead fitted with 2ml tube holder assembly and was processed at maximum speed for >5 minutes. The ZR bashing BeadTM Lysis Tube was centrifuged in a microcentrifuge at >10,000 x g for 1 minutes. It was transferred up to 400µl supernatant to a Zymo-spin TM IV Spin filter (orange top) in a collection tube and was centrifuged at 7,000 x g for 1minute. 1,200µl of fungal/bacterial DNA binding buffer was added to the filtrate in the collection tube from step 4. 80l of the mixture from step 5 was transferred to a Zymo-spin TM IIC column in a collection tube and was centrifuged at 10,000 x g for 1 minute. The flow through from the collection tube was discarded and step 6 was repeated. 200µl DNA pre- wash buffer was added to the Zymo-spin TM IIC Column in new collection tube and was centrifuged at 10,000 x g for 1 minute. 500µl fungal/bacterial DNA wash buffer was added to the Zymo-spin TM IIC column and centrifuged at 10,000 x g for 1 minute. The Zymo-spin TM IIC column was transferred to a clean 1.5ml microcentrifuge tube and 100ul (35µl minimum) DNA elution buffer was added directly to the column matrix. It was centrifuged at 10,000 x g for 30 seconds to elute the DNA.

2.8.2. Electrophoresis for DNA and PCR

One gram (1g) of agarose (for DNA) and 2g of agarose for PCR was measured. Agarose powder with 100mL 1 TAE was mixed in a microwavable flask. It was microwavedfor 1-3 minutes until the agarose is completely dissolved. Agarose solution is allowed to be cooled down to about 50°C (about when I can comfortably keep hand on the flask), for 5minutes. 10µl EZ vision DNA stain was added. EZ vision binds to the DNA and it allows to visualize the DNA under ultraviolet (UV) light. The agarose was poured into a gel tray with the well comb in place, newly poured gel was placed at 4°C for 10-15 minutes or allowed to sit at room temperature for 20-30 minutes until it has completely solidified.

2.8.3. Loading Samples and Running Agarose Gel

Loading buffer is added to each of the DNA samples or PCR products. When it was solidified, the agarose gel was placed into the gel box (electrophoresis unit). The gel box was filled with 1 TAE (or TBE) until the gel is covered. A molecular weight ladder is carefully load into the first lane of the gel. The samples were carefully load into the additional wells of the gel. The gel was run at 80-150 V for about1-1.5 hours. The power was turned OFF, the electrode was disconnected from the power source, and the gel was carefully removed from the gel box. The DNA fragments or PCR product was visualized under UV transilluminator.

2.8.4. PCR Mix Components

The PCR mix is made up of 12.5µl of Taq 2X master Mix from new England Bio-labs (m0270); 1ul each of 10µlM forward and reverse primer; 2µl of DNA template and then made up with 8.5µl nuclease free water.

Primer Sequences for Bacterial identification

27F: AGAGTTTGATCMTGGCTCAG

1525R: AAGGAGGTGWTCCARCCGCA

Cycling Conditions

Initial denaturation at 94°C for 5mins, followed by 36 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for 30seconds and elongation at 72°Cfor 45sec. followed by a final elongation step at 72°C for 7 minutes and hold temperature at 10°C forever. The lids are replaced and after solidification. The agar plates are incubated 30°C for 24- 48 hours in bacterial, and fungal colony forms counter and expressed as CFU × 106/g.

2.9. Statistical Analysis

The antimicrobial activity of the mean of *Azadirachta indica* and *Psidium guajava* having applied for statistical analysis for Social Sciences data (SPSS) for data Analysis.

3. Results

Table 1. The mean Concentration of Azadirachta indica and Psidium guajava on Escherichia coli

Concentration	Ethanol	N-hexane	Ethanol + water	Aqueous
25 µm/g	9.67±9.67[a]	11.67±10.00[a]	8.33±10.00[a]	9.33±10.00[a]
20 µm/g	8.33±9.00[a]	10.00±8.67[a]	7.67±8.67[a]	7.33±8.33[a]
15 µm/g	7.67±8.33[a]	9.00±8.00[a]	5.00±8.00[a]	5.33±5.00[a]
10 µm/g	5.00±5.00[a]	7.33±6.00[a]	4.00±4.33[a]	4.00±4.67[a]
5 µm/g	3.33±4.33[a]	5.33±5.00[a]	3.33±3.67[a]	3.33±3.33[a]

NOTE: ([a]) means not significant

Table 2. The mean of concentration of Azadirachta indica and Psidium guajava on Salmonella species

Concentration	Ethanol	N-hexane	Ethanol + water	Aqueous
25 µm/g	9.33±10.00 [a]	11.67±11.00 [a]	11.67±11.67 [a]	9.67±9.67 [a]
20 µm/g	8.33±9.00 [a]	9.00±8.00 [a]	8.33±6.67 [a]	8.33±8.00 [a]
15 µm/g	7.33±7.67 [a]	7.67±7.33 [a]	6.67±8.33 [a]	5.33±7.33 [a]
10 µm/g	6.67±6.67 [a]	6.00±5.67 [a]	5.00±7.33 [a]	4.67±6.00 [a]
5 µm/g	6.00±4.00 [a]	5.00±4.67 [a]	4.00±5.00 [a]	4.00±4.00 [a]

NOTE: ([a]) means not significant

Table 3. Susceptibility of Synthetic Antibiotics disc (mm) on the Plants Rhizosphere Isolates

Test Antibiotic	(Conc.)	PS	SM
Augmentin (AUG)	30µg	R	R
Erythromycin (ERY)	5 µg	R	R
Ofloxacin (OFL)	5 µg	23(S)	20(S)
Ceftazidime (CAZ)	30 µg	R	R
Gentamycin (GEN)	10 µg	13(I)	14(I)
Cloxacillin (CXC)	5 µg	R	R
Ceftriaxone (CTR)	30 µg	R	R
Cefuroxime (CRX)	30 µg	R	R

Source: field work, 2019.
PS= *Pseudomonas species*, SM=*Serratia marcescens,* PM= *Pseudomonas*
Resistant (R) which ranges from (0-11), Susceptible (S) <15, (I) = Intermediate which ranges from (12-14)

Plate 1. Inhibition Zone (mm) of antibiotic against *Pseudomonas aeruginosa*

Plate 2. Inhibition Zone (mm) of antibiotic against *Serratia marcescens*

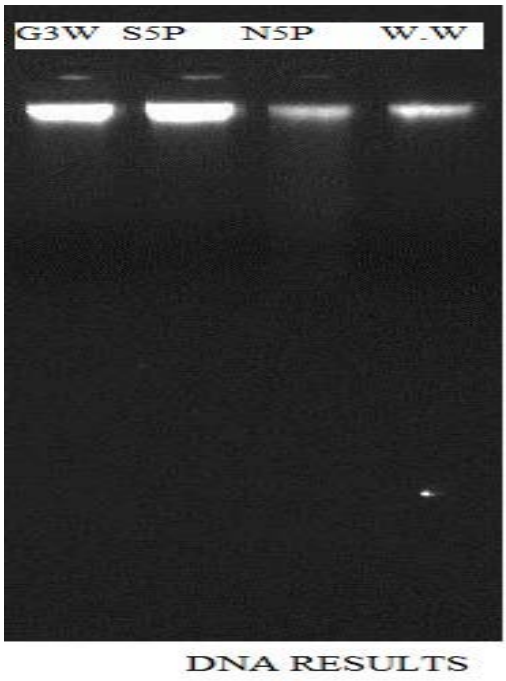

Plate 3. Gel electrophoresis purification of DNA Extraction amplicons of representative's bacteria isolates from plants rhizosphere samples (G3W& N5P) using *Pseudomonas aeruginosa* and *Serratia marcescens* type specific primers.

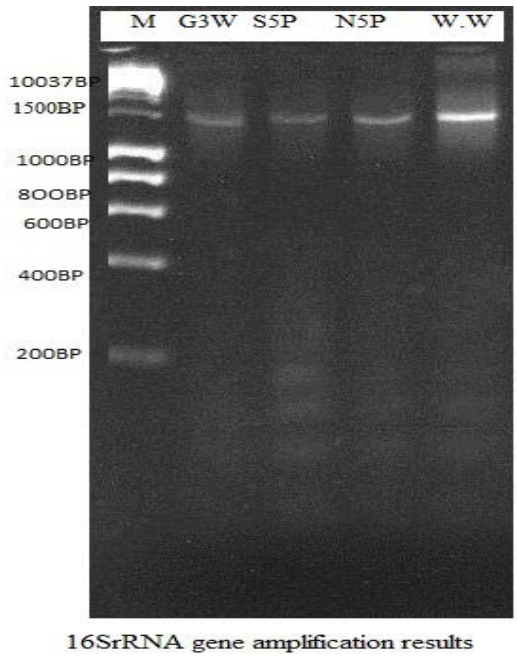

Plate 4. Gel electrophoresis purification of polymerase chain reaction amplicons of representative's bacteria isolates from plants rhizosphere samples (G3W& N5P) using *Pseudomonas aeruginosa* and *Serratia marcescens* type specific primers.

IDENTIFIED ORGANISM: *Serratia marcescens* strain HA 517

% PAIRWISE IDENTITY: 85.70%

NCBI ACCESSION NUMBER: KJ535328

E Value: 0

Isolate Sequences:

AGGTGATCTCTGGTATATGTGTGATCATGGCTCA
GGTGCCGGTGTGGTATCACCTTCTTCTATATTTTG
ACCAGGGCCCAGAGGGGGAGTAAGACTCCTTAA
AAGCCTGATGGAGGCGATAACTACTGGAAACTGT
TACTAATACCGCATAGCGTCACAAGACCAAGGAT
CTTCACCTTCGGGCCTCTTGCCATCACATGTGCCC
AGATGGGATTAGCTAGTAGGTGGGGTAATGGCTC
ACCTAGGCCACGATCCGTAGCTGGTCTGAGAGGA
TGACCACCCACACTGAAACTGAGACACGGTCCAT
ACTCCTACGGGAGGCGGCAGTGGGTAATATTGCA
CAATGGGCGCAAGCCTGATGCGCCCATGCCGCGT
GTGTGGACAACGCCTTCGGGTTGTATAGCACTTT
CAGCGAGGAGGAAGGTGGTGATCTTAATACGTTC
ATCCATTGACGTTACTCGCAGAACAAGCACCGGC
TATGTCCGTGCCAGCAGCCGCGGTAATACTGAGA
GTGCAAGCGTTAATCACAGTTACGTGAGGCGTAA
AGCGCACGCATGCGGTTTGTTAAATGCAGATGTG
AAATCCCCCGGGCTCAACAATGGGCATCTGCATT
CTGAATACTGGTAAGCTACGAGTCTCGGTAGAGG
GGGGGTTAGCAATGTCCTAGCTGTAGCGGTGCAA
ATGACGTAGAGATGTAGCACCACAGCCGGATGG
GCGACCGCCGTGGCACCTGATACTGAACACTGAC
GCTGCAAGATGCGAAGGGCGATGCGGAGCGAAT
CAGGATTCACATGACTCCTGCCTAGTCCACAGAC
TGTAAAATCGATGTACGCACTTGGAAGTCGTGCC
ATCGAAGCCGTGGACTAGCAGGAGCTATCGCGTT
AACTGCGCATCGGCCATGGACGATTACGGACGCT
TAGACTCAAATCTGCAATGACTCGGACGCGGATC
AGCGAAGCAGCTGGATGCAG

Identification of *Pseudomonasaeruginosa* strain P1

IDENTIFIED ORGANISM: *Pseudomonas aeruginos a*strain P1

% PAIRWISE IDENTITY:82.30%

NCBI ACCESSION NUMBER: KF530797

E Value: 3.29E-07

IsolateSequences:

GGCGGTATACTATGATGACATGGCTCGGTGCGGT
TGGATCCCTCCTAATATTTTGACCAGAGGCCAGT
GGATGCCTATGACTCTACCAGGGCCTGGGGGACG
GCGTTTCCTAAGGGAGCTGATACCGCTTACGTCC
TACGGCACAAAACAGGGGACCTTCACCTTTTGCC
CTCTCGCATGAGCATGGGTCCATTTAGCTTTTTGT
TGGGGTGGGGCTCACCATCGCCTCGATCACTATC
TGTTCTTAGTCTATGATCATTCCCACTGACACTGA
AACTGGGACAAGACCCCTACTCCTGGCGGAGGTG
GCGAATATTGATATTTCGCCAAAAGCCTGATCCT
GACGCGCCCCTGGCGTGAATGTGGTCATCGCCTT
CAAATTCACTTTAACTTGCGGCGAAGAGCAGGGA
GTTAATACCTTGCTGTTTTGACTTTAACGACACTA
TAAGAACAAGCTAACTCTGTCTCCGCGCCCGCGG
CCATACTAATGGGGGCAAGTGTTAATCTTAATTA
CAGTTCCGTAAACGCTCCCCCCCCGTGCTTTTGTT
TTTTTTAAATGTAGATACCCACGTGCCTCCGGGCT
TGAACACGTGTTTCCGTATTTTGTCACTGCTAGAG
CTCCAGTACTCGGCAGAGGGGATTTTTTCCTTTTC
CCGCGGTATCACTGCAATATATATCACAGATGGA
ACCACCAATCCCCCGAGGGCCACCCCCCTCCCCC
CTGATACCATACACTGACGTCCCAGACGCCGGTG
GGGGGCGCCACAAGATTTATTTAGCATTTCCCTG
GCCCTCCCTCCGCTCCCACGAGCGGATACCCCCT
CTCTCCACACTTGTGGAAATTCTCCTTTGCGACAA
TTTTTTGACTCGTCGGCTTCACGAGAACGC

4. Discussion

The antimicrobial activity of the *Psidium guajava* and *Azadirachta indica* shows that the plants extract such as ethanol, aqueous, ethanol + aqueous and N-Hexane are resistant to the bacterial isolates tested i.e *Escherichia coli* and *Salmonella species tested.* The concentration of the plant's extracts were diluted serially into different concentrations of standard solutions (extracts/ml) into 100, 75, 50 and 25 mg/ml. The statistical analysis of the extracts was not significant to inhibit the bacteria isolates. The plants extracts showed visible remarkable resistance to the two bacterial isolates (*Escherichia coli* and *Salmonella species).* Pharmacological studies of *Psidium guajava and Azadirachta indica* plants are at the preliminary level requiring studies to delineate the mechanism of actions. The study provides an outlook on various aspects that need to be done based on earlier studies in developing suitability or otherwise clinical therapeutics of *Psidium guajava and Azadirahcta indica* plants. Literature search indicated that although the extracts possesses natural remedies solution for painful menses and diarrhea. They are used traditionally for several ailments including diabetes mellitus, diarrhea, cough and hypertension. They also used to treat tooth decay, gum infection, sore throat, to disinfect wounds and also antiseptic. Those leaves are digestive, carminative, gives vigor and strength to heart, lungs and whole body. *Psidium guajava* improves blood circulation and when eaten with seeds it gives roughage to the diet helps in the normal evacuations of the bowels. *Psidium guajava* is a rich source of antioxidants and fiber. It is a poor man's fruit because they are quite cheap. The antibacterial activity of aqueous and Ethanolic, N-Hexane, Ethanol + Aqueous leaf extracts shows the mean diameter of zone of inhibition of extract on the test isolate with *Escherichia coli and Salmonella species* is resistant. Minimum inhibitory concentration (MIC) of the plants extracts shows that both aqueous and Ethanolic N-Hexane, Ethanol + Aqueous leaves extracts of the plants cannot inhibit the growth of the test isolates. This shows that Ethanolic N-Hexane, Ethanol + Aqueous, Aqueous extracts are less effective against the test isolates. The *E. coli* was not affected by any of the used extracts. This might be due to the fact that *E.coli* can alter their genetic makeup with astonishing rapidity. In general, gram negative bacteria show resistance to antibiotics because of their cell wall. Resistant bacteria change their cell walls slightly, so the antibiotics cannot attach or they produce enzymes to disable the antibiotics, so the *E.coli* might have done the same and consequently, *Azadirachta indica* (neem) extracts did not show any effect on it [31]. Our results are different to those obtained during a study carried out by [32] whereby *E.coli* was the most susceptible bacterium to aqueous and ethanol extracts of *Azadirachta indica* [31]. As per the observations on cultured Mueller Hinton agar

plates, antibacterial activity of leaf extract of neem was evaluated against both gram negative bacteria. Leaf extract of *Azadirachta indica* showed less inhibition zone against *E. coli* and *S. typhi* are less susceptible to neem extract. The antibacterial properties of *Azadirachta indica* leaves reported in this study is in line with the report by [34]. In accordance with the present findings, [33] had reported *Azadirachta indica* in the treatment of vaginal infections. Similarly, in a 2-week double-blind, placebo-controlled clinical trial of 55 women with abnormal vaginal discharge due to bacterial vaginosis, treatment Also, *Azadirachta indica* leaves has been reported to possessed good anti-bacterial activity and this led [33] to conclude it confirmation as a great potential of bioactive compounds and is useful for rationalizing the use of this plant in primary health care. These antibiotic principles are actually the defensive mechanism of the plants against different pathogens [33,34].

The bacterial isolates from rhizosphere soil of *Azadirachta indica* and *Psidium guajava* of Sabe location clearly reveal that diversity of bacterial population in the rhizosphere are not much significant. The types of bacterial colony isolated from the soil samples of the site show uniformity in their exo-morphological characters on the basis of which bacterial types have been discriminated. Though the types of bacterial isolates in the rhizosphere soil of this plant are more or less similar but the total quantity per gram of soil is different [14].

In strict sense therefore, the rhizosphere isolates of neem and guava plant might have sufficient bio prospective potentiality in terms of suitable bio fertilizer formulations for better crop production. This rapidly growing colony forms cloudy mass of cells on the growth medium merely within overnight period of incubation (24 hours). The colony of other isolates show moderate expansion or growth in cultural environment. Such vigorously growing bacteria in the rhizosphere may be responsible for the suppression of growth of other microorganisms in the rhizosphere by making the environment adverse for their growth. Root exudates from the plant itself and its inhibitory constituents may also be the other attributes for low microbial diversity. The exact reasons however could be revealed through extensive studies on the analyses of the chemical constituents of the rhizosphere soil. It allows the growth of some selected bacteria which have the ability to avert the existing unfavorable microenvironment. The study is compared with [14]. The diagrams are shown inPlate 1 and plate 2. Gentamycin (GEN) and Ofloxacin (OFL) were susceptible to the test isolates than other antibiotics present on the disc from rhizosphere of the plants shown in table (2 and 3) in this study.

The traditional identification of bacteria on the basis of phenotypic characteristics is generally not as accurate as identification based on genotypic methods. The present study has been carried out to identify the bacterial strain isolated from the rhizospheric part of the plants in Sabe community. Isolated colony from mixed populations, on nutrient agar plates were characterized and sub cultured to obtain pure cultures, and the isolated bacteria were identified based on colony characteristics, and were biochemically analyzed for the activities of Oxidase, Catalase, and Gram stained which was found to be *Serratia marcescens* and *Pseudomonas aeruginosa.* DNA was extracted and isolated using ZR Bashing bead. Prior to sequencing, the ribosomal rRNA gene. The extracted DNA was used as template for amplification of 16S rRNA gene. The optimum annealing temperature was found to be 55ºC [5,8]. The percentages of sequence matching were also analyzed. The accession number of the bacteria isolated from the rhizosphere of plants are KJ535328 for *Serratia marcescens* strain HA517 the E. value is equal to 0and for *Pseudomonas aeruginosa* Strain P1 is KF530797, the E. value is equal to 3.29, E-07.

5. Conclusion

The antibacterial activity of the plant showed that the plants leaves extract demonstrated a non-antimicrobial effect against the test isolates with lower activity on the leaf's extracts. Lowest antibacterial activity was recorded in ethanol, aqueous, N-Hexane, ethanol + aqueous. The antibacterial activity of the plant parts is due to the present of the phytochemicals which should be further studied. The minimum inhibitory concentration (MIC) ranges from 0.00-0.00 of the extracts. Findings from this work does not support the use of extracts from *Psidium guajavaAzadirachta indica* leaves as medicinal plant against gram positive bacterial such as *Salmonella species and Escherichia coli.*

Azadirachta indica extract has been reported because it is an important source of diverse compounds with ameliorative, bactericidal and bacteriostatic properties. Our results in this study that neem could not be recommended as anti-microbial against the two tested pathogenic organisms but its efficacy cannot be totally ruled out in near future [10].

We showed the use of 16S rRNA gene sequence to characterize the bacterial isolate from the rhizosp here of *Azadirachta indica* and *Psidium guajava* and were found to be *Serratia marcescens* Strain HA517 and *pseudomonas aeruginosa* Strain P1 (NCBI Gene Bank Accession No: KJ535328 and: KF530797). Thus, the genotyping method using 16S rRNA gene sequence is both simple and effective in strain identification. The bacteria isolates were resistant against the plants extracts and this is due to the presence of antimicrobial resistant genes present in the bacteria isolates which have been transferred through horizontal gene transfer (HGT) to the plants.

Conflicts of Interest

All the authors hereby declare that there is no conflict of interest before, during and after the conduct of this research.

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