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Research Article

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[1]Department of Science Laboratory Technology, Biological Sciences Unit, Microbiology Option, the Oke Ogun Polytechnic, Saki, Nigeria.
[2]Department of Pure and Applied Biology, P.M.B 4000, Ogbomosho, Ladoke Akintola University of Technology, Nigeria.
[3]Department of Zoology, Parasitology Unit, University of Ibadan, Ibadan, Nigeria.
[4]Genetics and Molecular Biology Unit, Department of Botany, University of Ibadan, Nigeria.
[5]Department of Zoology, Cellular Parasitology Unit, University of Ibadan, Ibadan, Nigeria

Contacts of Authors

*** To whom correspondence should be addressed: Adeoti O. Micheal**

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Antibiotic Effects of selected leaf extracts and molecular profile of *Klebsiella pneumoniae* and *Enterobacter bugandensis* strain (AdM2)

Adeoti O. Micheal[1, 2,5]*, Aboladale A. Olayemi[1], Adeoye K. Adenike[1, 3], Abiola A. Olabiyi[1], Olufemi S. Olufemi[1,4], Adedokun E. Olajumoke[1], Adesina D .Ademola[1] and Olaoye O. Joy[1]

Abstract

Only healthy surface tissues of healthy animals that are constantly in contact with the environment can easily be colonized by various microbial species, including pathogenic ones. However, these microorganisms are usually the cause of opportunistic infections in animals. Two plants; *Vernonia amygdalina* (taxonomic ID number 112605) and *Ocimum gratissimum* (taxonomic ID number 112606) were identified at the Nigerian Federal Research Institute in Ibadan and subsequently concentrated with methanol, water and n-hexane. Twelve pre-slaughter healthy cattle in the same ratio of male to female were selected for this study. Samples from the mouth, skin, nose, vaginal and gastrointestinal tract were collected. Susceptibility patterns for antimicrobial agents were performed using disk-diffusion agar method. All isolates were subjected to bacteriological and molecular identification (PCR and sequencing techniques). Preliminary identification was carried out based on standard bacteriological, microscopic identification, biochemical criteria, while PCR and DNA sequencing techniques were used for molecular identification and analysis. Basic local alignment sequence tools (BLAST) were used to compare retrieved bacterial sequences to a set of previously published strains in the database. The morphological and biochemical profiles of six out of eight normal flora from different sites were Gram-negative. All bacterial cultures were oxidase-positive, including 7 catalase-positive cultures. The majority of isolated bacteria were moderately sensitive to all the extracts tested but resistant to Amoxylin. Noticeable amplification of 16S rRNA genes from *Klebsiella pneumoniae* strain EMB and *Enterobacter bugandensis* strain AdM2 was obtained. The amplification of the resistant genes of the two isolates was approximately 1,500 base pairs; however, some of the suspected virulence and antibiotic genes were within this base pair range.

Keywords: *Vernonia amygdalina*, *Ocimum gratissimum*, Anti-bacterial, 16S rRNA, Cattle

Introduction

Antibacterial are kinds of antimicrobial agent used mainly against bacteria, for the treatment of bacterial infections [1]. The invention of antibiotics has assisted in the control of pathogenic bacteria until when most pathogens become resistant to the treatment. Increased alarming rate of Antibiotic resistance worldwide is causing difficulty and posing danger to humanity as various diseases and ailments are now difficult to treat and control [2].

In addition, majority of available synthetic antibacterial drugs are becoming less effective due to their side effects such as tendonitis, seizure, and Steven-Johnson syndrome [3]. The emergence of antibiotic resistance and the unbearable side effects (toxicity) of some of the commercially available antibiotics makes it imperative to search for newer, more effective and cheaper drugs that could serve as alternative therapy for the treatment of various infections and diseases [4]. Plants are reservoir of therapeutic substances which have a vital role in the sustenance of human health in the time past. World Health Organization (WHO) opines that plant parts or their potent make up are used in the productions of array of drugs in traditional therapies [5]. These plants have highly contributed to the development of human health and welfare. Simultaneously, there is an increase in data and huge patronage to herbal products round the world [6].

Medicinal plants such as *Ocimum gratissimum* have been affirmed to provide different culinary and medicinal properties which effect can be bacteriostatic and bacteriocidal on some bacteria. These effects have been attributed to the peptides, alkaloids, essential oils, phenols and flavonoids which are main constituents in these plants [7]. It is a therapeutic plant which has been used traditionally for the treatment of various disorder [8].

Cattle are reared for various reasons for consumption and economic purposes .they are used to pull farm implements in farmland and rural areas. Cattles are herbivores which mean they do not eat only plants, grass and cereals. Microbiota is the total of microorganisms that resides on or within any of a number of human tissues and bio-fluids. In new born, it is a powerful stimulus for the development of the immune system. Unlike humans which are also colonized by many microorganisms, some microbes are normal flora in human they are commensal, they co-exist without being detrimental to humans; others to humans host are mutualistic [9]. This study was to provide an insight into molecular identification of two pathogenic organisms and document their antimicrobial susceptibility profiles to locally available plants. Gene sequencing technology will be used for strain identification, and classification. In addition, we aim to use some nucleotide sequence analysis tools to perform a basic phylogenetic and diversity analysis in order to evaluate the genetic variability of the flora studied.

Materials and Methods

Plant collection and identification

Plants were selected based on ethno botanical information obtained from traditional medicine practitioners in Saki. All plants were collected around Challenge II area in Saki. Parts of the plants (leaves) were used for extraction. The identification was carried out in Forest Research Institute of Nigeria (FRIN), Ibadan with voucher specimen of 112605 and 112606 for *Vernonia amygdalina* and *Ocimum gratissimum* respectively.

Extraction and isolation of active agents

The materials of each was washed with distilled water and dried at room temperature by exposure to atmosphere for weeks. The dried materials were pulverized into fine powder using electric blender and packed into a plastic container with screw cap for further use. Four types of extracts (aqueous, methanol, methanol plus water, n-hexane) were done. 100 g of each bitter leaf powder were weighed into three different sterile bottles and was soaked with 500 ml of methanol; n-hexane and 250 ml of methanol plus 250 ml of distilled water. 50 g of each scent leaf powder were also weighed into three different sterile bottles and was soaked with 250 ml of methanol, n-hexane and 175 ml of methanol plus 175 ml of distilled water. The mixture was stirred carefully with sterile stirrer and was covered with foil paper. The bottles were kept at room temperature for 3 days.

The aqueous solution was prepared on the night of extraction by weighing 100g of bitter leaf powder in another bottle and was soaked with 500 ml of water and 50 g of scent leaf powder was also weighed in another sterile bottle and soaked with 250 ml of distilled water and covered with foil paper. The mixtures were extracted by sieving through muslin cloth, cotton wool and later filtered using Whatman filter paper into clean sterile bottles. The aqueous prepared was boiled for 15 minutes and it was allowed to cool and was extracted like it was done to the previous ones. The extracts filtrates were concentrated using rotary evaporator at 45°C at the Department of Pharmaceutical Chemistry Laboratory in University of Ibadan, Oyo State, Nigeria.

Determination of the antibacterial activity of the extract

Normal flora samples were collected from twelve cattle (6 males and 6 females) from different sites (nose, mouth, anus, vagina and skin) from the abattoir situated in Foofo, Saki, Ibadan, Oyo State, Nigeria. Swab sticks were used for the collection of specimen from different site from each cattle and were well labeled for recognition. Nutrient agar was prepared according to manufacturer's instructions. The specimens were directly streak on the prepared agar in different plates after solidifying and then incubated for 24 hours at room temperature. After 24 hours, the growth from the media incubated was transferred on MacConkey Agar and Eosin Methylene Blue for sub-culturing till pure culture is formed. Isolates were also tested on MRS and SSA agar but there was no growth.

Minimum inhibitory concentration

Each test organisms from serial dilution were streaked onto various plates and carried out in a septic condition and the plates were labeled accordingly. Disc diffusion method were introduced by using disc(sterilized perforated disc 6

mm in size seeded with different concentrations of the extract of 2 g, 4g and 6 g which is diluted into 10 ml of each methanol, n-Hexane, methanol + aqueous and aqueous and Amoxylin which is the control used was also diluted in 100 ml of distilled water. Then the seeded disc was placed on the surface of the inoculated agar medium. The inoculated plates were incubated at 37^0C the organism's susceptibility test with plant extracts were recorded after 24 hours by measuring the mean diameter of the clear zone of inhibition using Vernier caliper in millimeter (mm).

Biochemical tests for bacterial identification

Isolates were subjected to catalase and oxidase tests, which was identified using Bergey's determinative bacteriology manual [10]. In order to perform a catalase test, the production of gas bubbles has been used as an indication of a positive result. Isolate was placed on a glass slide with an inoculating loop 2 % of Hydrogen Peroxide was drop on the isolates and bubble isolates were recorded positive and vice versa. The oxidase test was used to detect the enzyme cytochrome oxidase activity. A purple coloration indicates positive results while no color change indicates oxidase negative results. Inoculums of each isolate were smeared on a clean filter paper and oxidase reagent (tetramethyl-phenylenediaminedihydrochloride) was drop on each isolate. Then colour changes were observed.

DNA extraction and 16S PCR

Bacterial cells broth was prepared and lysed through a ZR Bashing TM Lysis Tubes. 750µl lysis was pipetted in form of solution to the tubes. DNA extraction from bacterial samples was carried out using the ZYMO bacterial DNA extraction kit according to the protocol used by the manufacturer. The DNA quantity and quality was measured using gel electrophoresis techniques. Conventional PCR was applied to all the samples to amplify the 16S rRNA gene using previously reported protocol (**Figure 1**) [11].

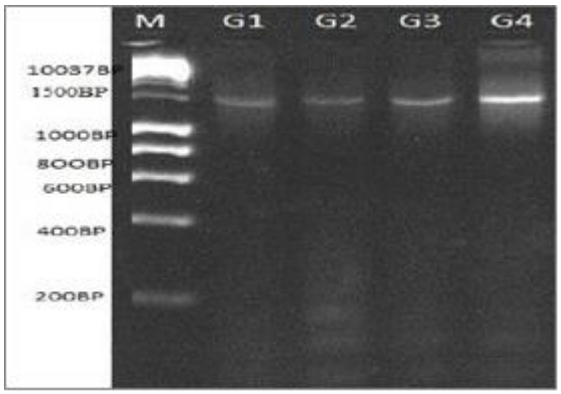

Figure 1: Agarose gel electrophoresis showing positive amplification of 1,500 bp fragment of 16S rRNA gene of *Klebsiella pneumoniae* performed with strain-specific primer. Lane M: 200-1037 bp DNA ladder. Strain lane G1:1500bp from cattle while G2: AdM2 (1000-1700) isolated from cattle. G3 and G4 (Isolates from other sources: rain and well water respectively).

The PCR results were sequenced by using Sanger sequencing technique and its products were subjected to analysis by Basic Local Alignment Search Tools [12] (BLAST) and similar sequences were retrieved from the National Centre for Biotechnology Information (NCBI) database [13]. The phylogenetic analysis was performed using MEGA 5.2 version [14].

Ethical considerations

Experiments on animals have been conducted, according the research protocol reviewed by animal ethics committees of Microbiology Option, the Oke Ogun Polytechnic, Saki, Nigeria.

Results and Discussion

The resultant nucleotides sequences of the 16S rRNA was matched to *Klebsiella pneumonia* and *Enterobacter bugandensis* (AdM2) with accession number MK719814 and MN213349 (**Figure 2**).

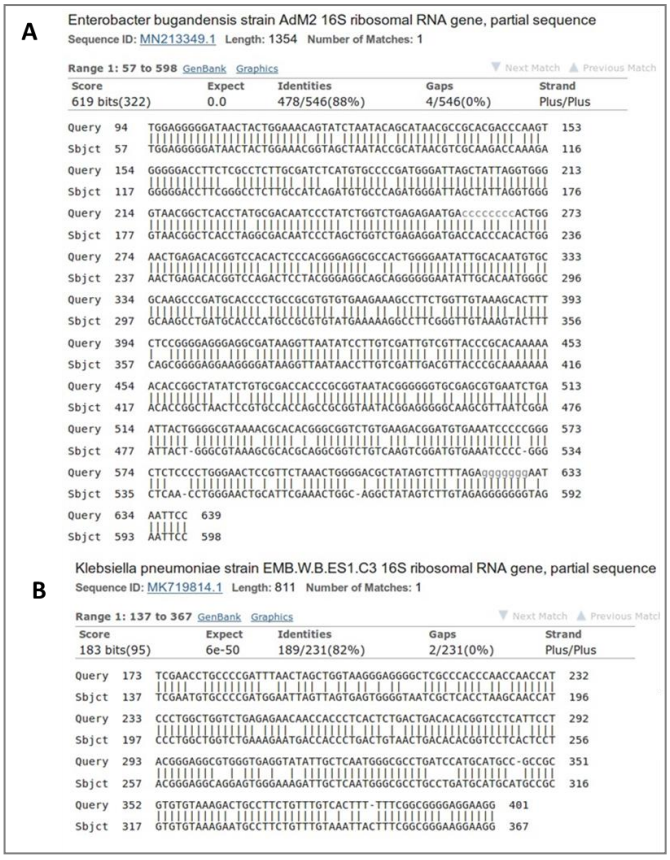

Figure 2: The BLAST results of 16S bacterial sequences obtained in the current study.

The antibacterial activity of both *V amygdalina* (bitter leaf) and *Ocimum gratissimum* (scent leaf) were found to be dosage and extraction solvent dependent. Methanolic extract of both plants at their highest tested doses possessed more antibacterial activities compared to the other extracts from different solvents when tested against the bacteria strain of *Klebsiella pneumoniae* and *E. bugandensis* (AdM2). This might attributed to the fact that methanol

extracted more of the bioactive constituents of the plant compared to other solvents. The methanolic extract of V amygdalina appeared more effective in the concentrations of 6g/ml when tested on *K. pneumonia* and showed moderate zone of inhibition on *Enterobacter bugandensis* compared to the other tested doses, though the trend revealed dose dependent activity, i.e the higher the concentration the higher the effectiveness of the plant extract. The aqueous extract showed little to no inhibitory activity on the tested organisms while ciprofloxacin used as control inhibited the growth than the extracts (**Table 1**). Zones of inhibition produced by methanolic extract of *Ocimum gratissimum* ranged from 7. 00 ± 0.00 at 2g/ml to 14.75±0.05 at 6g/ml against on *K. pneumonia*, followed by n-hexane extract ranged from 4.30±0.15 at 2g/ml to the highest dose of 6g/ml with value of 5.75±0.20, methanol + aqueous extract also showed moderate inhibitory activity while aqueous extract was found not to inhibit the growth of the test organisms at lower concentrations. However, at higher concentration, zones of inhibition were observed for the *Enterobacter bugandensis* ranges from 1.30±0.02 at 4g/ml and 2.85±0.03 at 6g/ml (**Table 2**).

Table 1: Antibacterial activities of bitter leaf extract on *K. pneumonia* and *E. bugandensis* (AdM2).

Leaf extract	**Concentration (g/ml)**	**Mean diameter of zone of inhibition of bacteria in mm (±SEM)**				
		Methanol	Methanol + Aqueous	n- Hexane	Aqueous only	Control (ciprofloxacin)
K. pneumonia	2.0g/ml	9.75 ± 0.03	4.75±0.02	3.30±0.15	1.00±0.00	22.00± 0.00
	4.0g/ml	11.35±0.02	5.00±0.00	3.10±0.22	1.40±0.08	24.00±1.00
	6.0g/ml	16.75±0.05	5.25±0.02	3.75±0.03	1.50±0.05	27.30±1.00
E. bugandensis	2.0mg/ml	4.50±0.05	2.50±0.08	2.00±0.00	0.00±0.00	10.00±0.00
	4.0g/ml	5.00±0.00	3.60±1.06	2.90±0.14	1.00±0.00	11.00±0.00
	6.0g/ml	6.30±0.10	4.30±0.14	3.30±0.00	1.00±0.02	15.00±1.00

Table 2: Antibacterial activities of scent leaf extract on *K. pneumonia* and *E. bugandensis* (AdM2).

Leaf extract	**Concentration (g/ml)**	**Mean diameter of zone of inhibition of bacteria in mm (±SEM)**				
		Methanol	Methanol + Aqueous	n- Hexane	Aqueous only	Control (ciprofloxacin)
K. pneumonia	2.0g/ml	9.75 ± 0.03	4.75±0.02	3.30±0.15	1.00±0.00	22.00± 0.00
	4.0g/ml	11.35±0.02	5.00±0.00	3.10±0.22	1.40±0.08	24.00±1.00
	6.0g/ml	16.75±0.05	5.25±0.02	3.75±0.03	1.50±0.05	27.30±1.00
E. bugandensis	2.0mg/ml	4.50±0.05	2.50±0.08	2.00±0.00	0.00±0.00	10.00±0.00
	4.0g/ml	5.00±0.00	3.60±1.06	2.90±0.14	1.00±0.00	11.00±0.00
	6.0g/ml	6.30±0.10	4.30±0.14	3.30±0.00	1.00±0.02	15.00±1.00

Veronia amygdalina produces a variety of flavonoids and bitter lactones which contribute to the bioactivities of this plant [15]. *V. amygdalina* serves well as a low cost are readily available source of important nutrients to human [16]. The result of the current study demonstrated that the six out of eight normal flora from different sites isolated from cattle were bacterial culture while others were positive according to the standard bacteriological identification and microscopic identification. The sensitivity tests show that there is high resistance of test organism against antibacterial activity of plant extracts. This means that scent leaf and bitter leaf extracts are more effective as antibacterial agent against test organism. Sequencing analysis was done to subject a Deoxyribonucleic Acid, Polymerase Chain Reaction to test organism so as to understand its features, structure, function and evolution [17,18].Some of the suspected antibiotic resistance genes and virulence genes were (hylA, tetA, tetE and blaTEM) respectively with base pairs of 1-1.5kb [19–21]. Phylogenetic analysis of retrieved bacterial isolates indicates a high level of identification of 16S rRNA sequences for bacterial genus and species (**Figure 3**). Such results could confirm the usefulness of using such genomic regions in the identification and characterization of bacterial isolates.

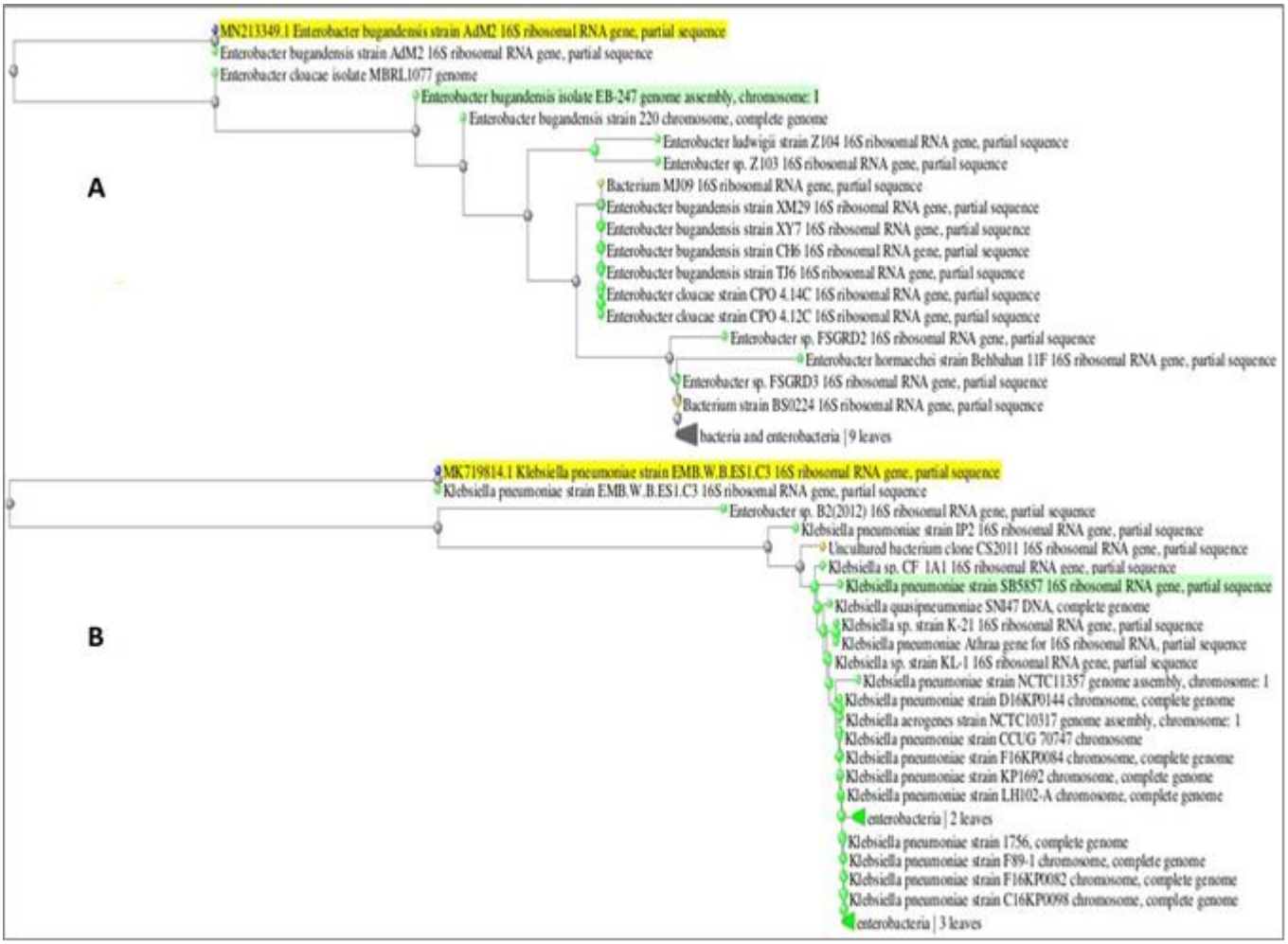

Figure 3: Phylogenetic trees of *Enterobacter bugandensis* (A) and *Klebsiella pneumoniae* (B) isolates from normal cattle flora compared to similar 16SrRNA gene sequences.

Conclusion

Most of the sensitive extracts were unable to inhibit the growth of the organisms and are presumably drug-resistant. Scent leaf and bitter leaf extract appeared more effective than their previously reported actions. Notwithstanding, *V. amygdalina possesses* various bioactivities with low or absence of side effects having great health promoting effects. It is however more advantageous to incorporate *V. amygdalina into* health supplement for both human and animal benefits.

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