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Antibacterial kinetics and phylogenetic analysis of *Aloe vera* plants

Paul Akinduti[1]*, Yemisi D. Obafemi[1], Patrick O. Isibor[2], Rapheal Ishola[3], Frank E. Ahuekwe[1], O. A. Ayodele[4], O. S. Oduleye[5], Olubukola Oziegbe[1], O. M. Onagbesan[6]

[1]Department of Biological Sciences, Microbiology Unit, Covenant University, Ota, Ogun State, Nigeria; [2]Department of Biological Sciences, Applied Biology and Biotechnology Unit, Covenant University, Ota, Ogun State, Nigeria; [3]Department of Medical Microbiology and Parasitology, Olabisi Onabanjo University, Sagamu Campus, Ogun State, Nigeria; [4]Department of Medical Microbiology, University College Hospital, Ibadan, Nigeria; [5]Department of Science Laboratory Technology, Microbiology Unit, D.S Adegbenro ICT Polytechnic, Itori, Ewekoro, Nigeria; [6]Department of Animal Physiology, Federal University of Agriculture, Abeokuta, Nigeria

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***Correspondence:** Paul Akinduti, Microbiology Unit, Department of Biological Sciences, Covenant University, PMB 1023, Ota, Ogun State, Nigeria.
E-mail: paul.akinduti@covenantuniversity.edu.ng
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Abstract

BACKGROUND: Uncontrolled use of antibiotics has resulted in the emergence of resistant bacteria. It has necessitated the evaluation of antibacterial activities and phylo-diversity of Aloe vera (also called Aloe barbadensis) plants as antimicrobial agent in Nigeria.

METHODS: Biotyped enteric bacilli of 251 strains obtained from fecal samples of patients with various gastro-intestinal complications are profiled for antibiogram. Resistant biotypes were assayed for susceptibility to A. vera latex and further evaluated for time-kill kinetics and phylo-diversity.

RESULTS: More than 30% of enteric bacilli, including Citrobacter freundii, Escherichia coli, and Proteus mirabilis, were resistant to cotrimoxazole, ciprofloxacin, and tetracycline, respectively, at minimum inhibitory concentration (MIC) >16 µg/ml ($p = 0.004$). A. vera latex significantly inhibited 39.5% resistant enteric biotypes with a significant average reduction of the viable count at 1 × MIC and 2 × MIC to <3.0 Log10CFU/mL after 24 h. Flavonoids, alkaloids, terpenoids, and anthraquinine in anti-enteric sap significantly correlated and regressed with antibacterial activity ($p < 0.05$), while two of the antimicrobial A. vera plants showed phylogenetic relatedness with other homologous.

CONCLUSION: Antibacteria efficacy of some Nigerian A. vera latex could provide alternative therapy, while its phylo-diversity and genomic profiling would offer a promising avenue for identification and development of antimicrobial agents as drug candidates for natural antibiotics.

Significance of the Study

- Significant level of bioactive compounds (phytochemicals) in antibacterial of *A. vera* sap provide enhanced synergistic inhibitory activity against resistant enteric bacilli
- Phylo-diversity and genomic profiling offer a novel method for characterization of *A. vera* plant
- Application of phylogenetic analysis and genotyping aid characterization and development of natural antibiotics that are accessible and available with therapeutic benefit as drug candidates

Introduction

Over the years, inadvertent and uncontrolled use of antibiotics had resulted in the emergence of resistant bacteria strains to commonly used antibiotics; this necessitates developing newer antibiotics to check the prevailing infection [1], [2]. Highly diverse resistance to different antibiotic classes presents a very worrisome situation [3], [4]. This alarming trend necessitates an urgent search for an alternative source of antimicrobial agents, mostly from plant and herbal sources [5].

Various plants and herbs are now being harvested, processed and analyzed using biotechnology for human use, making the plants much more effective,

fast, economical, and easily available. One such plant is Aloe vera. *This* plant belongs to the Lily Family having thick, green leaf-like structures that grow from a central point. It has been reported to have been used for the treatment of different aliments, mostly as a laxative and cosmetics [6]. Since centuries, the leaves and gel were been used as medicinal, beauty, and skincare [7]. It is now recognized in some developing countries that plants are the main therapeutic source to treat various infectious diseases, but its leaf latex extract has not been explored for its efficacy against prevailing communicable diseases and several enteric bacterial pathogens in Southwest Nigeria [8].

This latex was documented to contain phytochemical (bioactive) compounds such as anthraquinones and their derivatives such as Barbaloin-IO-aloe, emodin-9-anthrone, Isobarbaloin, Anthrone-C-glycosides, and chromones which possess purgative, antimicrobial, analgesic, anti-inflammatory, and antibacterial properties [9]. Nevertheless, the phylogenetic analysis could suggest the probable antimicrobial active species of Aloe plants based on the geographical location and relatedness to other species [10].

Prevalent enteric infectious diseases (particularly diarrhea) characterized by severe loss of electrolytes through frequent stooling, abdominal pain and cramps, caused by pathogenic Gram-negative bacteria (such as *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Escherichia coli,* and *Salmonella typhimurium*) are becoming severe, with high intestinal morbidity mostly among children [11]. Therefore, this study focused on the anti-enteric bacteria activities and diversity of *A. vera* plant producing antibacterial latex in Nigeria.

Methods

Biotype sampling

Enteric bacilli of 251 were obtained from patients attending Federal Medical Centre which serve as one of the largest referral center for internal medicine in southwest Nigeria with ethical permission. Each isolate was characterized by cultural and cellular morphological examination and further biotyped using Analytic profile index kit (API 24E test KIT).

Antibiotic assay

Antibiogram of the biotypes was determined using Kirby–Bauer disc diffusion method [12] on Mueller Hinton for the panel of these antibiotics; Tetracycline (30 μg), Cefuroxime (30 μg), Clavulanic acid (10 μg) + Amoxicillin (20 μg), Ceftazidime (30 μg), Gentamycin (10 μg), Trimethoprim (5 μg) + Sulfamethaxazole (25 μg), Ofloxacin (10 μg), Ampicillin (10 μg), and Ciprofloxacin (10 μg). Inhibition zones produced were interpreted according to CLSI guidelines (2016) [13]. Minimum inhibitory concentration (MIC) of each antibiotic was evaluated with the use of standard broth micro-dilution assay [14], and isolates showing antibiotic MIC of >16 μg/mL were selected as resistant.

A. vera plant collection and examination

More than 16 *A. vera* plants commonly used for skin treatment and other nutraceuticals in southwest Nigeria were selected for this study and were examined for their morphological parameters [15], [16]. Based on the leaf length, which was measured from the base of attachment of the mature leaf to the tip of the leaf, the presence of white spots on leaves and arrangement and a number of spines present on each plant leaves were observed and recorded. The medial striation or lateral line was also examined in each plant leaves.

Preparation of leaf latex

The leaf latex was prepared as described with minor modifications [16]. Several healthy matured thick leaves of the *Aloe* plant were plucked from the stem and allowed to drain its fresh yellow latex for a period of 30 min into a sterile container. The latex was sterilized by steaming at 65°C for 15 min [17].

Bioactive compound analysis

Latex from 5 leaf strands of the same plant was pooled, and 1 ml was diluted with 19 ml of sterile water to make 1:20 dilution and estimated for alkaloids [18], tannins [19], anthraquinones, saponins [20], phenol [21], flavonoids, glycoside, phlobatannins [22], and terpenoids [23].

Spectrophotometric assay for antibacterial activity

Adjusted 0.5 McFarland turbid broths of resistant biotypes were tested against varying dilutions of the latex in a standard micro-tube dilution bio-assay for determination of the minimum inhibitory dilution (MID) with sterile 1% peptone to serve as a control. The micro-plate was incubated at 37°C in ambient air for 24 h. The absorbance of each well before and after incubation was measured using Jenway 6405UV/Vis spectrophotometer microplate reader at 590 nm wavelength to determine the concentration of the bacterial growth (turbidity), which is proportional to the absorbance. Therefore, the MID is defined as the lowest concentration of the latex that shows no growth (showing similar absorbance as blank). The respective

MIDs shown by the latex against the isolates were interpreted [24].

Time kill kinetics

This assay was carried out according to the modified method of Sopirala *et al.* [25] and Hedges [26]. Each overnight broth of 23 prepared 10^5 CFU/ml antibiotic resistant biotypes was added to 1 ml of different dilutions of latex and incubated on a shaker at 37°C in ambient air. Aliquots of 100 µl were removed at 0, 6, 12, 18, and 24 h post-inoculation. At each 6-h interval, the 100 µl broth culture challenged with latex was serially diluted in sterile 0.85% standard saline solution to estimate the total viable colony count for a period of 24 h in Log_{10} CFU/ml.

Polymerase chain reaction (PCR) amplification of internal transcribed spacer (ITS) gene marker

Ribosomal DNA (rDNA) was extracted from *A. vera leaf from* using the Cetyl trimetyl ammonium bromide (CTAB) method as described by Doyle and Doyle [27] using newly emerging succulent healthy small young leaf shoot of the plant from which the latex was extracted for an antibacterial test. Harvested leaf shoot was brought in an ice pack to the Laboratory and thoroughly washed with tap water and rinsed with distilled water, blot dried, and weighed. Leaf tissue of 0.5 g was thoroughly homogenized in 2 ml CTAB buffer in a sterile mortar to form slurry, which was used to extract the DNA [28]. Precipitated rDNA was spooled and washed with 70% ethanol, dried at room temperature, and dissolved in 50 µl of 1 × TE buffer. The quality and quantity of extracted rDNA were estimated on Thermoscientific Nanodrop Spectrophotometer at the absorbance of 260 nm filter, and an appropriate quantity of PCR Mastermix was prepared. Amplification of conserved ITS region for genomic taxonomy of Asphodelaceae plants was performed using ITS4 and ITS5 primers (Table 1) [10].

Table 1: PCR Amplification protocol for *ITS*4 and *ITS*5 genes

Primer	Primer Sequence (5' – 3')	Target gene	Amplicon size (bp)	Reference
ITS 4	TCC TCC GCT TAT TGA TAT GC	ITS 4	165	[10]
ITS5	GGA AGT AAA AGT CGT AAC AAG G	ITS 5	614	

PCR assay was carried out in a volume of 20 µl 2 × PCR buffer, 1.5 µl 25 mM $MgCl_2$, 0.5 µl of 10 mM dNTPs (dATP, dGTP, dCTP, and dTTP), 0.2 µl ITS 4 primer of 2500 pmole, 0.2 µl of ITS 5 primer of 2500 pmole, 0.2 µl *Taq* polymerase enzymes, 15.4 µl distilled water, and 2.0 µl of each extracted chromosomal DNA template to make final reaction volume of 20.0 µl. The amplification reaction was carried out in 26 cycles of denaturation at 97°C for 60 s, annealing at 50°C for 60 s, and elongation at 72°C for 3 min.

ITS amplicon sequencing

Amplicon products were cleaned and purified using QIAquick PCR purification kit (Qiagen, USA) and quantified with Thermoscientific Nanodrop Spectrophotometer at an absorbance of 260 nm to obtain the required dilution of amplicon for sequencing. Six microliters of diluted DNA was added to 4 µl of ITS primer at a concentration of 0.8 pmol/µl to make final reaction mixtures 10 µl, which was added to Taqcycle sequencing kit (containing tag-oligonucleotide *Taq* polymerase) according to manufacturer's instruction (Applied Biosystem Company, USA) and sequenced using ABI Prism Big Dye Terminator version 3.0 cycle and sequenced products subsequently analyzed on an ABI PRISM 3700 DNA Analyzer. Homological analyses were performed by aligning the raw nucleotide sequence product into BLAST (Basic Local Alignment Search Tool) Web-based tool of the National Institutes of Health (NIH, USA) software to search similar submitted nucleotide sequences and homologous sequences of more than 98% were selected with their respective accession number, nucleotides sequences, and location.

Phylogenetic analysis

Diversity of the *A. vera* sequences was analyzed using MEGA software version 6 [29], where nucleotide sequences were manually aligned to make a phylogenetic tree with maximum parsimony assessing the sequences as independent, unordered, and equally weighted, according to Fitch parsimony [29]. Fitch bootstrap percentages were calculated for each node (bootstrap option in PAUP 3.1.1., with 1000 replicates of heuristic search).

Data analysis

The significance of the resistant bacteria susceptibility was determined using Chi-square and bioactive compound from latex by t-test ($p < 0.05$). Analysis of variance (ANOVA) for the significance of the antimicrobial activity of each of the species of the Aloe barbadensis miller against different multi-antibiotic resistant enteric biotypes and level of the bioactive compound was correlated with antibacterial activity ($p < 0.05$).

Results

Resistance profile of enteric biotypes

Among the 251 collected enteric biotypes, *Citrobacter freundii* (14.3%), *E. coli* (13.4%) and *Proteus mirabilis* (9.1%) showed resistance to more

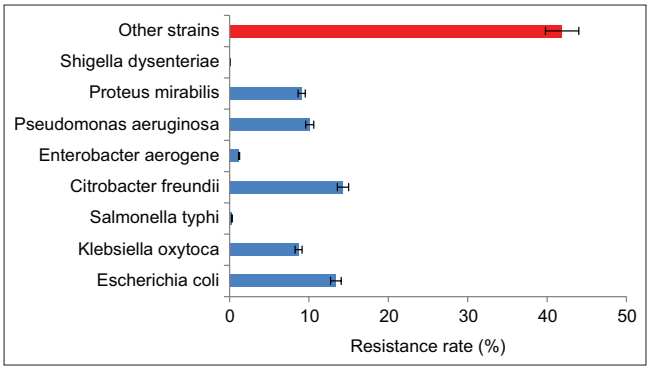

Figure 1: Antibiotic resistant rates of collected enteric biotypes from fecal samples (Other strains indicate collections of several identified bacteria isolates)

than two classes of antibiotics by disc diffusion assay (Figure 1). Only 44.7%, 38.9%, and 33.9% were significantly resistant to cotrimoxazole, ciprofloxacin, and tetracycline, respectively, with MIC >16 μg/ml ($p = 0.004$) (Figure 2).

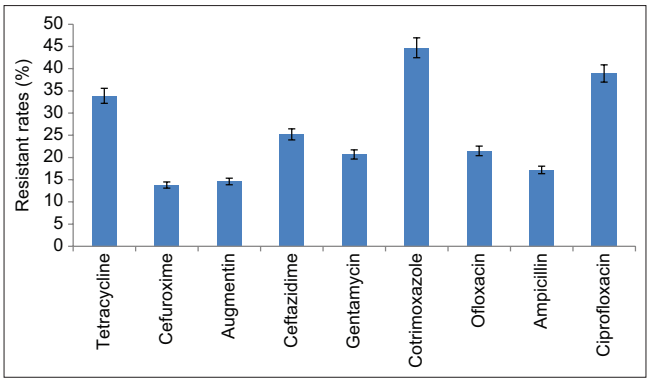

Figure 2: Resistance pattern of the recovered enteric bacilli strains (n = 251) to various antibiotics at minimum inhibitory concentration >16 μg/ml

Antimicrobial activity and time-kill kinetics of latex against multi-antibiotic resistant biotypes

From the *A. vera* plant latex of 16 variants tested against the resistant isolates, only variants A, B, and C showed significant MID_{50} and MID_{90} (Table 2). Among the *A. vera* with significant MID, the overall activity *A. vera latex from designated variant A* showed a significant inhibitory rate (39.5%) against resistant enteric biotypes at MID 1:16 compared to 5.3% and 2.6% recorded from other two variants (Figure 3). More than 50% inhibitory rate was recorded against *Klebsiella oxytoca, Salmonella* species*, Enterobacter cloaca,* and *P. mirabilis*. Latex from variant A showed a significant average reduction rate in viable colony count for all the enteric bacterial isolates at 1 × MIC and 2 × MIC to <3.0 Log_{10} CFU/mL after 24 h, which is an indication of bacteriocidal activity except resistant *P. aeruginosa*, which recorded more than 3.0 Log_{10} CFU/mL at 1/2 × MIC after 24 h incubation (Table 3). The levels of flavonoids, alkaloids, terpenoids, and anthraquinone in *A. vera* variant A significantly correlated and regressed

Table 2: Minimum inhibitory dilution (MID) of variants *Aloe vera* latex against multi-antibiotic resistant isolates

Aloe vera variants	MID_{50}	MID_{90}	p value
A	1	8	0.001
B	2	8	0.001
C	2	16	0.001
D	8	16	0.053
E	8	32	0.064
F	8	32	0.055
G	8	32	0.051
H	8	64	0.059
I	16	64	0.061
J	16	64	0.054
K	32	128	0.050
L	32	128	0.058
M	64	256	0.082
N	64	512	0.075
O	64	512	0.071
P	128	512	0.052

$p < 0.05$ significant.

with antibacterial activity ($p < 0.05$), as shown in Table 4.

Phylogenetic diversity

Only 16 A. barbadensis miller plants with more than 98% homologous sequences were selected from GenBank and were analyzed for phylogenetic diversity with nucleotide sequences of *A. barbadensis* miller from Nigeria using Maximum parsimony at <50% bootstrapping reliability (Figure 4).

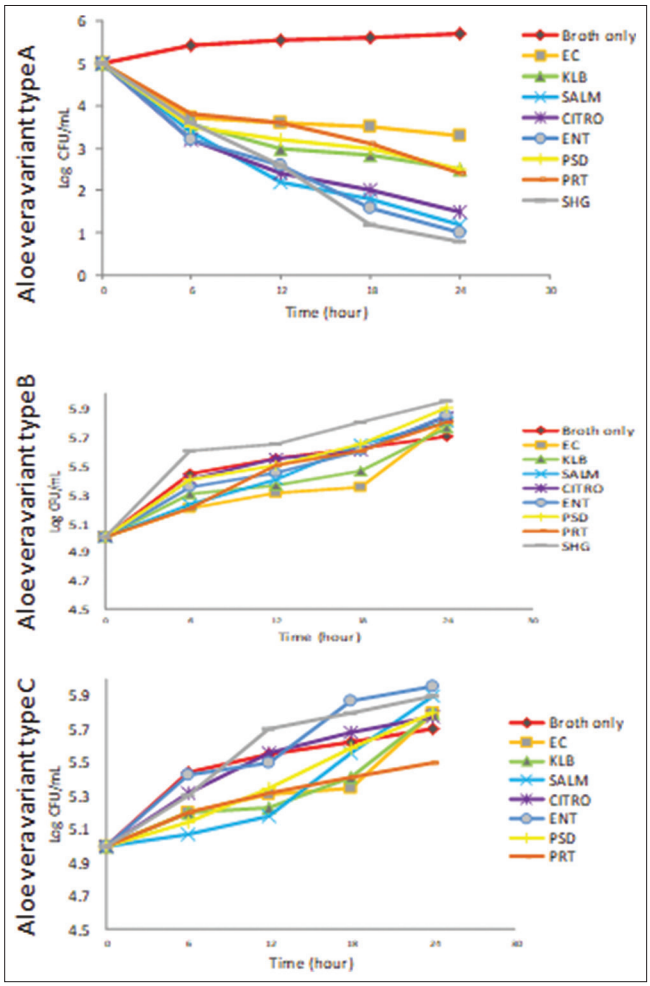

Figure 3: Kinetic of latex inhibitory activity at optimum minimum inhibitory dilution (1:16) of Aloe barbadensis miller variants against resistant enteric strains

Table 3: Evaluation of Time kill rate of *Aloe barbadensis* miller latex from variant A

Enteric isolates	Log_{10} CFU/mL								
	2×MIC			1×MIC			1/2×MIC		
	0 h	12 h	24 h	0 h	12 h	24 h	0 h	12 h	24 h
Escherichia coli	5.01	3.32	2.50	5.02	3.38	2.79	5.70	3.52	3.12
Salmonella species	5.03	3.12	2.56	5.05	3.27	2.78	5.02	3.44	2.94
Citrobacter freundii	5.00	3.01	2.50	5.23	3.21	2.95	5.32	3.50	3.21
Pseudomonas aeruginosa	4.98	2.85	2.51	5.12	3.20	3.00	5.21	3.50	3.23
Proteus mirabilis	4.87	3.01	2.74	5.10	3.28	2.90	5.10	3.40	3.03
Shigella species	5.32	2.85	2.24	5.06	3.22	2.54	5.15	3.46	3.00

A. barbadensis miller *variant A (*Nigeria) clustered with *A. vera* species HE995579 Canaria, Spain; *A. vera* GQ435495 Haidian, China; and *A. vera variant* B (Nigeria) to clade C. Only *two A. vera* AJ512309 Heidelberg, Germany; *A. vera* AJ290298 Surrey, UK; *A. vera* AY323649 Heidelberg, Germany; *A. vera* AY323649.1 Heidelberg, Germany; *A. vera* JQ273907 Missouri, USA and *A. vera* JQ273907.1 Missouri, USA clustered into clade A. Clade D is distinct, clustering only the Nigerian *A. vera variant* C with *A. vera* L05029 Missouri, USA.

Discussion

High rate of enteric biotypes including *C. freundii*, *E. coli*, and *P. mirabilis* showing resistance to Cotrimoxazole, Ciprofloxacin, and Tetracycline, respectively, at MIC>16 μg/ml suggest consistent resistance to most commonly used antibiotics in Nigeria, and this would continue to be a growing problem with increase morbidity especially in children [30]. The increasing antimicrobial resistance observed in this study is primarily linked to excessive and unnecessary antibiotics use in humans and food animals coupled with low health orientation and guidelines [31], [32]. Surveillance of multi-resistant enteric isolates in healthy populations has demonstrated commensals also constituting a rich reservoir of genetic material from which pathogens can readily transfer resistance on mobile genetic elements [33].

Table 4: Analysis of latex susceptibility with its phytochemical compounds

Physicochemical properties	Correlation analysis		Regression analysis			
	r-value	p-value	Coefficient (β)	SE	t-value	p-value
Tannins	0.326	0.405	-109.647	111.219	-1.524	0.378
Phenol	0.040	0.691	10.201	29.427	0.340	0.846
Flavonoids	0.714	0.017	21.245	19.136	2.145	0.028*
Glycosides	0.217	0.755	-135.090	42.605	-0.584	0.871
Steroids	0.139	0.636	194.930	105.567	1.053	0.323
Phlobatannins	0.041	0.889	192.545	256.97	0.479	0.599
Alkaloids	0.621	0.023*	65.321	61.23	1.872	0.020*
Anthraquinone	0.607	0.034*	11.763	53.983	3.740	0.039*
Terpenoids	0.502	0.043*	30.265	18.402	3.976	0.046*

*p < 0.05 is significant.

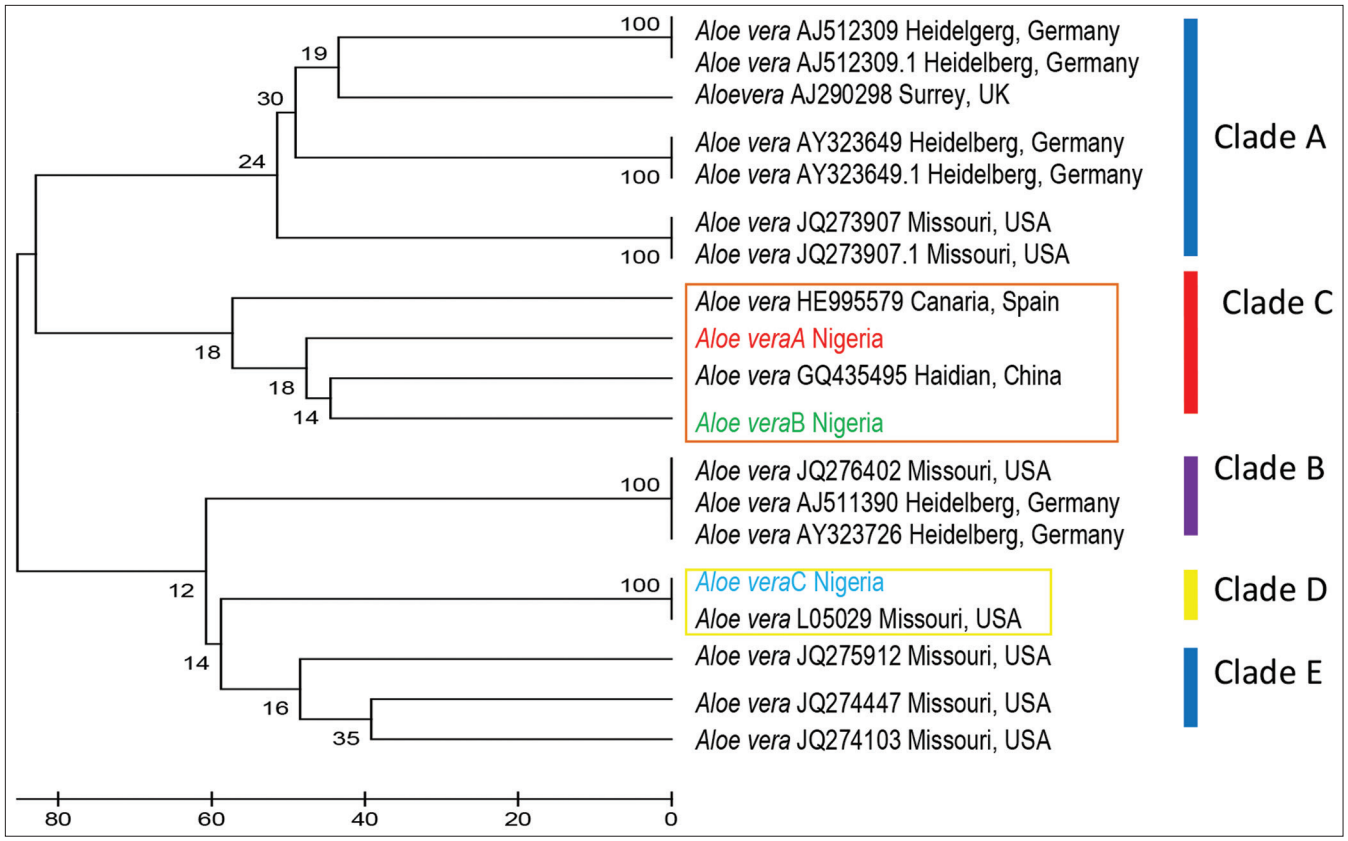

Figure 4: Phylogenetic diversity of Nigerian Aloe vera variants with repository A. vera metadata deposited in GenBank (colored black with GenBank accession number, location and country, bootstrapping values at node indicating level of tree reliability and scale bar indicating nucleotide sequence homology)

Despite the continued emergence of resistant enteric bacteria strains, *A. vera* has been identified as an alternative remedy from natural products that can circumvent resistance mechanisms, improve the containment, treatment, and eradication of these strains [9]. Preliminary examination of various *Aloe* plants indicated only three variants from this location to have antibacterial activities. The morphological and floral features reveal distinct structures in spine arrangement, white spot distribution, and greenish coloration that are not descriptive enough for the taxonomy of the medicinal *Aloe species* [34].

However, there are discrepancies between classical taxonomy [35] within the *Asphodelaceae*, the sub-family *Alooideae*, which is now recognized as difficult to classify of its succulent leaf morphology [36]. The uncertainty eroded biosynthetic investigations of the leaf latex phytochemical studies to presumably differentiate various species [34], [37]. Phytochemicals, defined as non-nutrient chemical compounds or bioactive components responsible for protecting the plant against microbial infections or infestations by pests [38], [39], are of significant proportion in latex from Nigerian *A. vera*.

There is a paucity of reports of anti-enteric bacilli activities of latex solution of Nigerian *Aloe* plants with variation in flavonoids, alkaloids, terpenoids, anthraquinone, and glycosides level. This study observed the effect of environmental changes and geographical location as attributable factors for its antimicrobial efficacy. However, these latexes' activity on resistant biotypes is a promising development of antibacterial agents with potent inhibitory action [40]. Antibacterial activity of tested latex showing significant inhibitory action at low dilutions (MID) suggests effect of high flavonoids, alkaloids, and tannin as antioxidants that makes effective scavenging for reactive oxygen species (including superoxide anions and hydroxyl radicals) as well as other free radicals causing damage to bacteria cellular functions. These activities result in oxidative stress leading to cytotoxic damage by removing "free-oxygen radicals," thereby giving rise to metastable radicals, which tend to trap electrons from the molecules in immediate surroundings [41], [42].

Evaluation of time-kill kinetics over 24 h caused a high reduction rate in viable colony count for all the bacterial strains at 2 × MIC, which is defined as bacteriocidal [43]. Nonetheless, <2.42 Log_{10}CFU/mL count recorded at 1:16 dilution further confirmed this natural product's potency with dependable efficacy as a growth inhibitory agent with dose and time-dependent to produce an effective time-kill rate. The effectiveness of time-kill kinetics may be accounted for by the synergistic activity of individual phytochemical compounds that possibly enhance the antibacterial action in sequential antioxidation of saponin, flavonoids, anthraquinine, and alkaloids. Of which saponin disrupts the outer phospholipidic membrane carrying the structural lipopolysaccharide components of the cell wall of bacteria cell leading to its permeability for other phytochemical compounds into bacteria cytosol [44], [45] and alkaloids causing inhibition of outer membrane bacteria cell wall synthesis, thereby damaging the cell membrane barrier function allowing other compound(s) to diffuse into the bacterial cells [46], [47].

Similarly, alkaloids act as DNA intercalating agents that adversely affect DNA replication [48], while flavonoids and phenol deprive hydrogen bonding, protein, and some microbial enzymes metabolism resulting in inhibition of bacteria cell replication and cytosol metabolic activities [49]. Culminated synergistic antibacterial activities of these phytochemical compounds through different mechanisms would not be circumvented by resistant bacteria. This was further confirmed by correlation and regression analysis, providing evidence of some bioactive compounds effectively producing antibacterial activity.

In spite of this antibacterial activity, hundreds of *A. vera* plant species had been identified in different parts of the World, but only a few had been characterized to be of medicinal importance. The Nigerian *A. vera* variants clustered with *A. vera* from Spain and China confirm the plants distinct phylogenetic diversity. Similar diversity was observed in Nigerian Aloe plant (*A. vera* A type) with other species from Germany, UK, and USA that clustered into clade A while clade D is distinctly clustering with other Nigerian variant species of *A. vera* from Missouri, USA. To a greater extent, the diversity of these plants reveals genomic unrelatedness with high polymorphism despite the geographical origin [50]. This could infer other *A. vera* in these clades possess high-level antimicrobial properties as they shared related genetic makeup [51]. Thus, showing the usefulness of genetic diversity as a reliable tool for improved taxonomy of *A. vera* of medicinal importance. Genetic diversity clearly predicts classification and identification of antimicrobial *A. vera* species sharing a typical genomic nucleotide profile [52], phylogenetic variation, and signature on nuclear DNA regions as reliable markers for characterization of homogeneity and detection of adulterant variants [50]. This would assure quality control in medicinal plant research and the production, clinical use, and forensic examination of *A. vera* to develop antimicrobial agents [51].

However, an important qualitative aspect of genomic architecture is nucleotide compositions, which express GC content. This would be needed to further identify various *Aloe* species and differentiate by the genomic impact of DNA composition phenotypically expressed in the absence of spot patches and lateral, medial line in some plants [53], [54]. In the recent past, considerable attention has been given to the biological relevance of GC ratio [55], having known only for the amount of the total phylogenetic diversity [56], [57] and high thermal stability that confers stability to many *Aloe* plants adaptability [58].

Conclusion

The efficacy of *A. vera latex* as an antibacterial agent on resistant enteric bacilli could provide alternative therapy, and the synergistic activity of various phytochemical compounds would make this latex a novel, natural and antibacterial product. Phylodiversity analysis would further offer a promising avenue for medicinal variant identification and development of antimicrobial agents accessible, available, and therapeutic benefit as drug candidates in research for natural antibiotics.

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Data Availability

The data supporting the results of this study are available on request to the corresponding author.

Authors' Contributions

Akinduti Paul A - conceptualization; methodology; data collection; sample analysis; data analysis; validation; data curation; writing – the initial draft; writing – revisions; student supervision; project leadership; project management; and funding acquisition.

Isibor Patrick O - data collection; sample analysis; data analysis; validation; data curation; writing – the initial draft; writing – revisions; and funding acquisition.

Obafemi Yemisi D - conceptualization; methodology; data collection; sample analysis; student supervision; project leadership; project management; and funding acquisition.

Ishola R - methodology; data collection; validation; data curation; project management.

Ahuekwe E.F - data analysis; validation; data curation; writing – revisions; student supervision; project leadership; project management; and funding acquisition.

Ayodele Olufunmilayo A - conceptualization; methodology; data collection; sample analysis; data analysis; validation; writing – revisions; project management.

Oduleye O.S - data collection; sample analysis; data analysis; validation; data curation; writing – revisions; student supervision; and funding acquisition.

Oziegbe O - sample analysis; data analysis; validation; data curation; writing – revisions; student supervision; project leadership; and funding acquisition.

Onagbesan K.O - conceptualization; methodology; data collection; sample analysis; data analysis; validation; data curation; writing – the initial draft; writing – revisions; student supervision; project leadership; project management.

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