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Table of Content

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Full Length Research Paper

Improved SSRs-based genetic diversity assessment of coconuts (*Cocos nucifera* L) along the coast of Kenya

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Coconut is the most important cash crop along the Coast of Kenya, yet its genetic diversity has not been fully established. Genetic diversity study of 48 coconut genotypes, collected at the Coastal Kenya was conducted with 13 polymorphic short sequence repeats (SSRs) markers. SSR analysis was performed using GeneMapper while data analysis was done with PowerMarker and DARwin softwares. Analysis revealed a total of 68 alleles ranging from 2 to 11 per locus with a mean of 5.23 per marker. Gene diversity and polymorphic information content (PIC) ranged between 0.41 to 0.83 and 0.33 to 0.79, respectively. Neighbour - joining dendrogram grouped the genotypes into three major clusters with distinct sub-clusters. This study underscored that capillary electrophoresis is a more accurate and informative technique for SSRs allele scoring as opposed to agarose gels. The clusters observed forms the basis to isolate conservation blocks, which is key to establishing a genebank, since there is no documented coconut genebank for *ex-situ* conservation in Kenya.

Key words: Coconut genetic diversity, capillary electrophoresis, polymorphic information content.

INTRODUCTION

Coconut (*Cocos nucifera* L.), commonly known as the "tree of life" in the world (Huang et al., 2013**;** McKeon et al., 2016), is an export commodity for many countries, such as Indonesia (largest coconut-producing country, with 30% of the total world production (Burton, 2021)) and several other Southeast Asian countries (McKeon et al., 2016). Coconut (*Cocos nucifera* L.) is among the estimated 2600 living palm species (Christenhusz and Byng, 2016). Indonesia is the largest coconut-producing country, with 30% of the total world production (Burton, 2021).

It is also the main tropical cash crop and provides a source of income to the people of the coastal lowlands of Kenya (Wekesa et al., 2017). Coconut is proposed to have originated from south Asia and disseminated initially to the Pacific and East African shores, floating on sea waves and later to the Atlantic and American shores by humans after cultivation (Harries, 1978). Consequently, in most studies, African and South Pacific coconut germplasm are closely related to those in the South Asian subcontinent (Lebrun et al., 1998; Teulat et al., 2000; Perera et al., 2003). There are three varieties; tall

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(up to 60 feet), dwarf (up to 25 feet) coconut types as well as their intermediates, which are thought to be their hybrids (16 feet), at the coast of Kenya (Oyoo et al., 2015). Diversity is higher in tall varieties (out-crossing) than in the (in-breeding) dwarf varieties (Teulat et al., 2000; Meerow et al., 2003; Perera et al., 1999, 2000; Rivera et al., 1999). These varieties contribute significantly towards their social, economic and environmental wellbeing of the coastal people in the following counties where they are grown; Kwale, Kilifi, Mombasa, Tana River, Lamu and Taita Taveta. The coconut industry plays an important role in the protection of fragile environments such as small islands and coastal zones as well as providing a good tropical canopy for the tourism industry (Bourdeix and Prades, 2018).

Despite these diverse ecological services, the genetic diversity of coconut germplasm is under threat from climate change, pests and diseases, poor conservation strategies and urbanization, logging for timber and land fragmentation for housing, especially in coconut growing areas (Batugal et al., 2009; Martinez et al., 2009) to mention a few.

Assessment of the genetic diversity is an essential component of coconut genetic resource characterization, genetic improvement and adoption of conservation strategies (George and Angels, 2008). For coconut, germplasm from different geographical areas might look similar but may be genetically different. They might also show morphological differences but may be genetically the same. Molecular marker technologies reduce the inclusion of duplicates in breeding programmes and conservation blocks (Batugal et al., 2009). Once the genetic diversity has been assessed using reliable DNA markers such as short sequence repeats (SSRs), distinct coconut representatives can be identified, collected and conserved in genebanks. Coconut diversity hotspots can be documented with precision and the richness of the genepool can be determined and regeneration of conserved accessions can be enhanced (Rao, 2004; Yao et al., 2013).

Molecular marker technology is an ideal tool for assessing coconut genetic diversity within and between coconut populations (Batugal et al., 2009). Genomic markers such as Random Amplified Polymorphic DNA (RAPD) (Masumbuko et al., 2014), Restriction Fragment Length Polymorphism (RFLP) (Lebrun et al., 1998), Amplified Fragment Length Polymorphism (AFLP) (Perera et al., 2000; Teulat et al., 2000), Short Sequence Repeats (SSRs) or microsatellites (Amiteye, 2021; Dasanayaka et al., 2009; Martinez et al., 2009; Xiao et al., 2013; Wu et al., 2019) and inter-simple sequence repeats (ISSRs) (Manimekalai and Nagarajan, 2006) have been used to characterise coconut populations. Diversity in worldwide coconut germplasm show grouping patterns primarily according to dissemination routes from their source of origin (Rivera et al., 1999; Perera et al., 2003). SSRs have been shown to be the most efficient and valuable molecular marker technology for genetic diversity studies (Caro et al., 2022) and variety identification as are rich in polymorphism, high stability, and good repeatability (Dong et al., 2017).

Effective coconut genebanking strategies rely on analysis of genetic diversity at the DNA level in two ways: (1) to ensure that distinct coconut varieties from different geographical areas are represented in genebanks and (2) the DNA profiles serve as reference in regeneration of old coconut accessions for the maintenance of important agronomic traits (Rao and Tobby, 2002; Dasanayaka et al., 2009; Martial et al., 2013)

The binary scoring method based on the presence or absence of bands (Wang et al., 2009) was deployed in the previous study (Oyoo et al., 2016). The genetic diversity of the same set of coconut germplasm using 13 SSRs markers failed to provide accurate genetic distances of the germplasm. Presently, the famous method for gene detection and isolation is polyacrylamide gel electrophoresis (PAGE) or agarose gel electrophoresis. However, these cannot reveal the accurate size of amplified target DNA fragments and has low detection efficiency. Furthermore, it is difficult to effectively integrate and accurately compare DNA fingerprinting data from large-scale samples and different batches of samples. Capillary electrophoresis based on DNA band and accurately determine the size of detected fragments thereby identifying subtle length differences This study reported here employed the more informative and accurate capillary electrophoresis approach to resolve the multiple alleles that could be generated for a single marker across the 48 coconut genotypes and precisely sized them. In SSRs analyses, a missing amplification band does not indicate an absent SSRs allele. Furthermore, a visible band that appears in several different individuals are often several alleles with slightly different sizes (Mueller and Wolfenbarger, 1999). Some samples may present two different alleles for a single marker, indicating a heterozygous locus where the two diploid chromosomes each carry a different allele. Therefore, when SSRs markers are scored as presence or absence of alleles, such as when using agarose gels, their co-dominance and multi-allelic features are not considered, which can lead to misinterpretations (Jones et al., 1997; Wang et al., 2003).

The objective of this study was to assess the diversity of 48 coconut palms growing along the coast of Kenya using improved SSRs markers resolution by capillary electrophoresis.

MATERIALS AND METHODS

Area of study

The study was confined in the coastal region of Kenya which covers approximately 82,383 km^2 with a population of 4,329,474 (KNBS, 2019). The coastal region is comprised of 6 counties namely: Lamu, Kwale, Kilifi, Tana River, Mombasa and Taita Taveta. These counties are known for their coconut diversity as reported in the

Table 1. Summary of concentration and volumes for individual PCR reagents per reaction.

Master mix components	Stock	Final concentration	Volume (µL)for 1 reaction
PCR Buffer without $MgCl_2$	10 ×	1 ×	1.0
$MgCl_2$	50 Mm	2 Mm	0.4
DNTPs	2Mm	0.16 Mm	0.8
M13 fluorescent Forward prime	2 µM	0.16 µM	0.8
Forward primer	2 µM	0.04 µM	0.2
Reverse primer	2 µM	0.2 µM	1.0
Taq polymerase	5 U	0.2 U	0.2
Template DNA		20 ng/ µL	1.0
Sterile water			4.6
Total volume			**10**

Source: Author

previous study (Oyoo et al., 2015). The climate of this area is tropical humid with an annual mean rainfall of about 1200 mm mainly confined to the long rains between April to July and short rains between October and December (Mwachiro and Gakure, 2011).

Sampling

Coconut leaf samples were obtained from Kwale, Kilifi, Tana River and Lamu counties. A total of 48 individual coconut genotypes previously collected by Oyoo et al. (2015) comprising of 37 tall, 8 semi-tall (suspected hybrids) and 3 dwarf types. They were from different agro-ecological zones of the coastal lowlands of Kenya. Leaf samples were collected and coded according to Oyoo et al. (2015).

DNA extraction and evaluation

DNA was extracted from dry, frozen coconut leaves (preserved in silica gel at -20°C of the 48 varieties (tall, dwarf and hybrids) using a modified CTAB protocol described by Doyle and Doyle (1987). Two steel balls were placed in 2 mL labelled eppendorf tubes for each sample. Approximately 80 mg of dry leaves were weighed from each sample, cut into small pieces and ground into a fine powder to increase surface area for detergent activity, using a Tissue Lyser II (Qiagen®). This was followed by incubation with 800 µL of preheated CTAB extraction buffer (3% CTAB w/v, 100 mM Tris-HCl (pH 8.0), 1.4 M NaCl, 20 mM EDTA, 3% β-mercaptoethanol (BME), 2% Polyvinylpyrollidone (PVP)(w/v) in a water bath at 65°C for 30 min with occasional mixing. BME 3% was used in a replica experiment to determine if it reduced the degree of degradation. Solvent extraction was done by adding 800 µl of chloroform:isoamyl alcohol (24:1) followed by thorough mixing. They were then centrifuged for 10 min at 13000 rpm and approximately 500 µL of the supernatant was transferred into clean labelled tubes. The DNA was precipitated by addition of 350 µL of isopropanol (0.7 volume) stored at -20°C, left overnight to increase precipitation and then centrifuged for 20 min. The supernatant was decanted and the DNA pellet washed with 400 µL of 70% ethanol, air dried for 30 min and re-suspended in 200 µL of low salt TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA). RNase A (5 µL of 10 mg/mL) was added and the samples incubated at 37°C for 1 h to digest RNA. DNA was precipitated by the addition of 315 µL of ethanol sodium acetate (Ethanol: 3 M NaOAc 300 µL:15 µL) and incubated for 2 h at -20°C. The samples were then centrifuged at 14000 rpm for 25 min, the supernatant was decanted and the pellet was washed with 400 µL of 70% ethanol at 14000 rpm for 5 min. The DNA was air dried for 1 h in a laminar flow-hood, re-suspended in 50 µL low salt 1×TE and stored at -20°C.

Evaluation of quality and quantity of the genomic DNA

The quality of DNA was evaluated by electrophoresis using 0.8% (w/v) agarose gels stained with 5 µL/100 ml Gel Red® (Biotium Inc., USA) to enable visualization. A mixture of 4 µl of DNA and 2 µl of loading buffer (25 mg bromophenol blue (0.25%), 25 mg xylene xyanol (0.25%), 4 g sucrose (40%), was loaded onto the gel and run for 1 h at 80 volts in a 0.5 × TBE buffer (0.1 M Tris base, 0.1 M boric acid and 0.02 M EDTA; pH 8.0). The fragments were visualized under UV light and photographed using a transilluminator. The amount and purity of the DNA quantity was determined by spectrophotometry using a Nanodrop© 1000 (Thermo Scientific, USA) which was programmed to measure absorbance (A) from 220 to 350 nm and display the DNA concentration according to Wilfinger et al. (1997).

PCR amplification

PCR reactions were conducted in 10 µl final volume containing 1 × PCR buffer (20 mM Tris-HCl (pH 7.6); 100 mM KCl; 0.1 mM EDTA; 1 mM DTT; 0.5% (v/v) Triton X - 100; 50% (v/v) glycerol), 2 mM $MgCl_2$, 0.16 mM dNTPs, 0.16 µM of a labelled M13-primer, 0.04 µM M13 - forward primer (InqabaBiotec, South Africa), 0.2 µM reverse primer (InqabaBiotec™), 0.2 units of Taq DNA polymerase (SibEnzyme Ltd, Russia), and 20 ng of template DNA as shown in Table 1

A total of 30 pairs of SSRs primers (Supplementary Table 1) developed by Perera et al. (2000) were used in this study. For detection of PCR fragments during capillary electrophoresis, each forward primer was labeled with one of the three 6-Carboxyfluorescein fluorescent dyes: 6-FAM®, 6-VIC ® or 6-PET® (Life Technologies Corporation, Carlsbad, USA). During capillary electrophoresis, the amplification products passed through a detection window and a light excited the fluorescent dye. The fluorescence was then visualized using a computer programme as relative fluorescent units (RFU) against fragment length in base pairs. An allele was scored for each data point as length in base pairs at the highest RFU peak.

Reactions were performed on a thermocycler (GeneAmp PCR system 9700®, Applied Biosystems, USA). Initially, the thermocycler

Table 2. Touch down PCR conditions.

PCR touchdown programme			
	Step	**Temperature (°C)**	**Time (s)**
16 Cycles	1st denaturation	94	180
	2nd denaturation	94	30
	Annealing	62 – 1 for each cycle	15
	Elongation	72	15
25 Cycles	Denaturation	95	15
	Annealing	58	15
	Elongation	72	30
1 Cycle	Final elongation	72	420
	Holding temp	15	∞

Source: Author

was progammed with an initial denaturation of 94°C for 5 min, followed by 35 cycles of 95°C for 30 s, 57°C for 1 min, and elongation at 72ºC for 2 min. Final elongation was done at 72ºC for 20 min, and PCR products held at 15°C. Since the markers had a range of annealing temperatures from 56 to 46°C, this protocol did not work well for all primer sets and a gradient PCR protocol was introduced. The thermocycler was programmed with an initial denaturation of 94ºC for 3 min followed by 16 touch down cycles of 62ºC annealing for 15 s and 72ºC for 15 s in every cycle, with the annealing temperature decreased by 1ºC in each subsequent cycle, generating a range of annealing temperatures from 62 to 46ºC. This was followed by 21 cycles of 95ºC for 15 s, 58ºC for 15 s and 72ºC for 30 s. Final chain elongation was done at 72ºC for 7 min and reaction products held at 15ºC. To amplify markers that did not succeed with this protocol, the touch down cycles were increased from 10 to 15 cycles and the annealing time increased by 15 s. The protocol is summarised in Table 2 and was adopted after optimization of the annealing temperatures.

Success of PCR was determined by electrophoresis using a 2% (w/v) agarose gel stained with GelRed® (Biotium, USA) and visualized under UV light. For SSR fragment size analysis, 2.0 to 3.0 µl of 2 different markers amplification products were co-loaded along with the internal size standard, GeneScan™ –500 LIZ® (Applied Biosystems, USA) and Hi - Di™ Formamide (Applied Biosystems, USA). The DNA (PCR products) in this mixture was denatured for 3 min at 95ºC and chilled on ice for a few minutes.

The products were then separated by capillary electrophoresis using an ABI Prism® 3730 Genetic analyzer (Applied Biosystems, USA) (Koumi et al., 2004). This provided automated and accurate estimates of allele sizes, which is better than using traditional gels because of the differences that can occur in migration between lanes in a gel (Life Technologies Corporation user guide, Carlsbad USA).

Fragment analysis

Fragment analysis was performed using Gene Mapper 4.0 (Applied Biosystems, USA) and allelic data for every marker was further analyzed by PowerMarker V3.25 (Liu and Muse, 2005) and DARwinV.6 (Dissimilarity Analysis and Representation for Windows®) software (Perrier and Jacquemound-Collet, 2006). Analysis of the molecular variance (AMOVA) was performed using Arlequin V.3.5.2.2. PowerMarker was used to calculate diversity parameters including: Inbreeding co-efficient, gene diversity and polymorphic information content (PIC), allele number; estimation of allelic and genotypic frequency, Hardy-Weinberg disequilibrium and linkage disequilibrium. PIC, which is a measure of diversity, was calculated using the formula:

$$\text{PIC} = 1 - \sum_{u=1}^{k} \tilde{p}2\text{lu} - \sum_{u-1}^{k-1} \sum_{v=u+1}^{k} 2\tilde{p}2\text{lu}\ 2\tilde{p}2\text{lv}$$

Where P_{lu} is the allele population frequency at the l^{th} locus and P_{lv} is the genotype population frequency at the l^{th} locus.

Dissimilarity was calculated by Darwin software using the formula:

$$\text{dij} = 1 - \frac{1}{L} \sum_{l=1}^{L} \frac{ml}{\pi}$$

Where d_{ij} is the dissimilarity between units i and j, L is the number of loci, π is the ploidy and ml is the number of matching alleles for locus l.

DARwin software was used to generate dendograms based on coconut evolutionary relationships using the dissimilarity matrix (Perrier and Jacquemound-Collet, 2006) as well as Principle Coordinate Analysis (PCoA) graphs.

RESULTS AND DISCUSSION

Genotyping the coconut genotypes using capillary electrophoresis

Some common markers as those of Oyoo et al. (2016) were used in the current study. The common markers used were CAC02, CAC03, CAC04, CAC06, CAC56, CAC72 and CN1C6, while CAC13, CAC20, CAC65, CN11E10, CN1G4, CN2A4 were specific to this study. Markers used by Oyoo et al. (2016) were CAC10, CAC21, CAC23, CAC71, CAC84, CN11E6 and CN1H2 (Table 3). In this study, 14 of the 30 markers failed to amplify DNA while three (3) were monomorphic, these were eliminated from the analysis. The markers that amplified well and produced bands across the 48 coconut genotypes are as follows: CN1C6, CAC56, CAC03,

Table 3. Summary statistics of allelic data analysis for 13 markers used to amplify the 48 coconut DNA samples in coastal Kenya.

Marker	Allele No	Genotype No	Major allele frequency	Gene Diversity	Expected heterozygosity	PIC
CAC02	7.0	7.0	0.208	0.821	0.000	0.790
CAC03	2.0	2.0	0.625	0.473	0.000	0.359
CAC04	4.0	4.0	0.458	0.676	0.000	0.623
CAC06	7.0	7.0	0.437	0.682	0.000	0.635
CAC13	2.0	2.0	0.521	0.503	0.000	0.375
CAC20	9.0	9.0	0.521	0.704	0.000	0.676
CAC56	4.0	4.0	0.500	0.632	0.000	0.570
CAC65	6.0	7.0	0.385	0.749	0.021	0.713
CAC72	11.0	12.0	0.385	0.792	0.021	0.769
CN11E10	2.0	2.0	0.708	0.413	0.000	0.328
CN1C6	3.0	3.0	0.500	0.624	0.000	0.549
CN1G4	4.0	4.0	0.667	0.513	0.000	0.469
CNZA4	7.0	7.0	0.229	0.832	0.000	0.803
Mean	**5.231**	**5.384**	**0.472**	**0.647**	**0.003**	**0.589**

PIC = Polymorphic information content.
Source: Author

CA08, CAC11, CAC20, CAC13, CAC39, CAC10, CAC23, CAC72, CN11A10, CN11E10, CN11E6, and CN1G4 as shown in Figure 1. Seven markers visible bands though faint are as follow: CN1C6, CAC56, CAC03, CAC13, CAC20, CAC72 and CN1G4 produced as shown in Figure 1.

The 7 polymorphic markers used to amplify three random coconut DNA samples using 2% agarose gel electrophoresis as shown in Figure 2 using similar procedure as used by Oyoo et al. (2016). From this, it was not possible to tell by visual assessment alone whether the PCR products generated for each marker were similar or of different sizes. For example, for marker CAC20, the allele sizes indicated on the gel look the same (Figure 1), but capillary electrophoresis determined that they were actually different band scores; 161, 163 and 165 bp respectively. It should also be noted that CAC56, CAC03, CAC13, CAC20 and CAC72 displayed the co-dominance of SSRs markers, presenting two heterozygous alleles for some of the genotypes; this was not distinguishable in the previous work by Oyoo et al. (2016).

Oyoo et al. (2016) assessed markers on the basis of presence or absence of the expected band. In contrast, in the current study, differences in allele sizes amplified for a single marker for the 48 coconut DNA samples were precisely assessed with capillary electrophoresis. For example, for CAC56, six possible genotypes were distinguished. Furthermore, none of the 13 polymorphic markers used in this study presented any absent alleles. Such absent PCR products should be considered carefully as they could have occurred as a result of failed PCR amplification or failure of primers to bind to the allele locus, and not necessarily due to an absent allele.

Genomic diversity studies

The allelic data for the 13 SSRs markers was analyzed by PowerMarker® version 3.25 and diversity summary statistics of each marker presented in Table 3.

Polymorphism of the SSRs and genetic diversity of cape goose berry accessions

An average of five genotypes was detected by each marker. Marker CAC72 was the most sensitive because it differentiated the coconut population into twelve genotypes while markers CAC03, CAC13 and CN11E01 were the least sensitive differentiating only two genotypes. Overall, six SSR markers were able to differentiate the population into more than five genotypes and were considered to be sensitive (Table 3). The other six SSRs differentiated the population into either two, three or four genotypes only and were considered to be less sensitive.

In this study, a total of 68 observed alleles were detected, ranging from 2 for markers CN11E10, CAC13 and CAC03 to 11 for marker CAC72 with a mean of 5.231 alleles per marker. The numbers of alleles reported in the study are comparatively higher than those reported by Oyoo et al. (2016) using agarose gel electrophoresis. This finding informs that capillary electrophoresis has a higher resolution of detecting different alleles at a given locus than gel electrophoresis.

The highest major allele frequency was 0.708 (CN11E10) and the least was 0.208 (CAC02) with a mean 0.472 (maximum possible value is 1). This means that, using capillary electrophoresis at any given locus

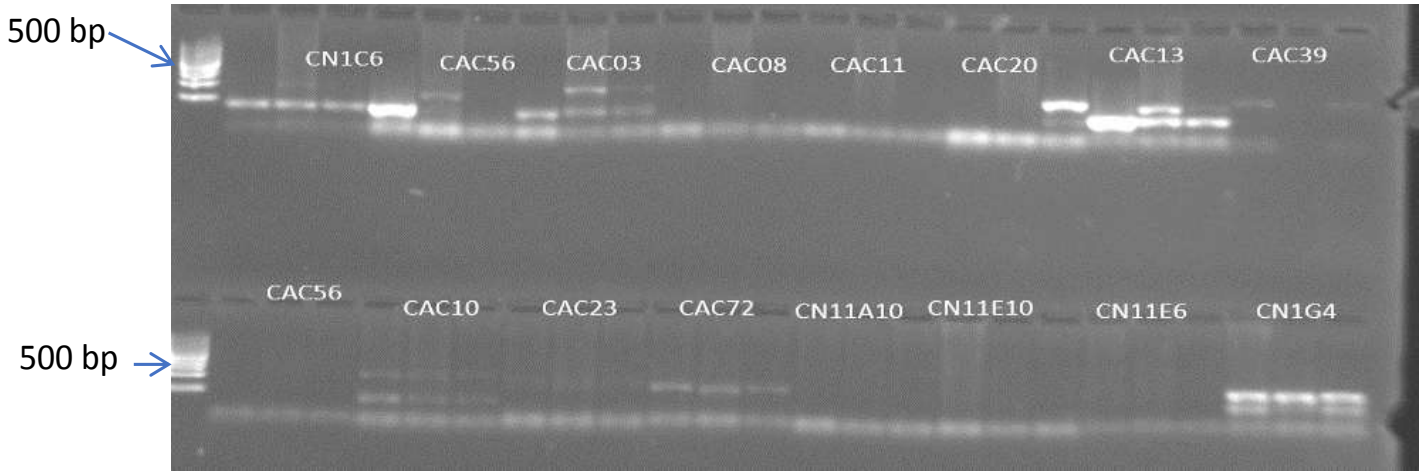

Figure 1. Agarose gel image (2 % w/v) of PCR products for SSRs markers.
Source: Author

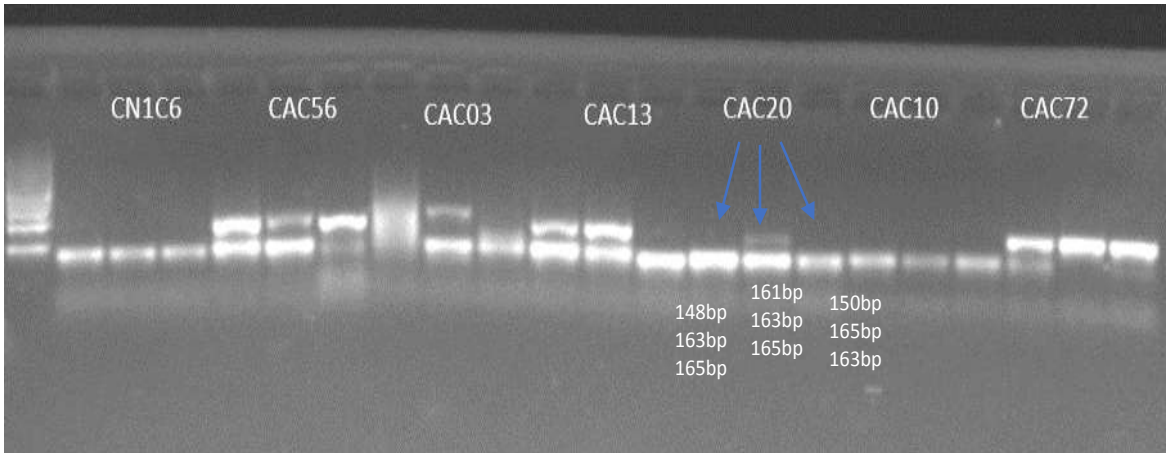

Figure 2. Agarose gel image (2.0 % w/v) of PCR products from 7 markers to confirm amplification of SSRs alleles prior to capillary electrophoresis.
Source: Author

the mean chance of any of the alleles being detected is about 0.5 showing that this method has higher resolution for band size separation and no bias towards dominant alleles (Njung'e et al., 2013). This finding is in contrast with results of Oyoo et al. (2016), who reported a higher major allele values with a mean of 0.807 showing that gel electrophoresis has a lower resolution for band size separation and higher bias (80%) for detecting the dominant allele. Polymorphic information content (PIC), a measure of how well the marker distinguished the samples tested, ranged from 0.33 for marker CAC03 to 0.80 for marker CNA4 with a mean of 0.59. This was in contrast to Oyoo et al. (2016) who reported who reported the highest PIC of 0.364 with a mean of 0.235. Six primers CNZA4 (0.8026), CAC02 (0.7904), CAC72 (0.7697), CAC04 (0.6228), CAC06 (0.6346), CAC20 (O.6763) and CAC65 (0.7134) showed higher PIC values. These primers should therefore be given priority in genotyping coconut because they have higher segregation capacity. The PIC values showed in this study were higher compared to Oyoo et al. (2016). This shows that capillary electrophoresis increases the efficacy of SSR markers and therefore increases their resolution for detecting diversity in coconut genotypes. This suggests that these markers are suitable for detecting the genetic diversity of coconut accessions from the Coast of Kenya.

Expected heterozygosity for the selected markers was generally low with a mean of 0.003 (minimum possible value is 0) indicating that the materials tested were genetically pure, that is, the loci were stable and not prone to high outcrossing frequencies. These markers

Table 4. AMOVA results of four coconut populations from the coastal lowlands of Kenya.

Source of variation	Degrees of freedom	Sum of squares	Variation components	Percentage of variation
Among populations	3	21.265	-0.06779	-1.59 (p = 0.001)
Among individuals within populations	44	380.402	4.31233	101.10 (p = 0.999)
Within individuals	48	1.000	0.02083	0.49 (p = 0.001)
Total	95	402.667	4.26538	

Significance level = 0.05.
Source: Author

were good for diversity studies especially in the tall coconut varieties, due to their outcrossing nature (Liu et al., 2011; Oyoo et al., 2016).

The study reaffirms that SSRs are powerful tools in genetic diversity studies and their usefulness could be enhanced if capillary electrophoresis was used than the standard procedure. In DNA markers diversity studies, allelic data analysis is the key towards attaining conclusive and reliable results (Vemireddy et al., 2007). The choice of allele sizing and separation platforms determines the accuracy in allele sizing, reproducibility and interpretation of the data obtained (Wang et al., 2009). A comparative analysis across 8 microsatellite loci in 12 rice varieties (Vemireddy et al., 2007) demonstrated that capillary electrophoresis is the most accurate and preferred method for allele size estimation, with errors of less than 0.73 bp compared to slab gels such as polyacrylamide, which produce error rates of up to 1.59 bp and agarose gels, which give error rates of up to 8.03 bp. Capillary electrophoresis has also been proven to have greater reproducibility (3 bp). Oyoo et al. (2016) who used 2% (w/v) agarose gels could not separate accurately alleles with size differences of as little as 2 bp where the average allele length is 150 to 500 bp long. Such alleles will migrate the same distance in an agarose gel and will therefore be assumed to be of the same size leading to limited interpretation. SSRs results should not be scored as present or absent as they are co-dominant markers and missing alleles can be as a result of low quality DNA or due to a marker that could not bind specifically to the allele locus. In this study, capillary electrophoresis was used to separate alleles and hence added value to the previous study by Oyoo et al. (2016).

Population structure of coconuts along the coast of Kenya

Genetic distances of coconut genotypes among counties

The results of partitioning of genetic variance within and among coconut populations sampled are presented in Table 4. The AMOVA was performed using Arlequin software. Samples obtained from four counties; were considered as constituting 4 different populations.

Results of the study showed a significant negative variation -1.59% (p = 0.001), in coconut genotypes in different counties indicating lack of population structuring according to county of collection. While the population molecular variance value reported in this study was lower than that reported by Oyoo et al. (2016) (2%) who used the same markers and gel electrophoresis detection platform, both studies reported lack of population differentiation. The lack of population differentiation may be attributed to a number of factors; the nature of markers used may lack enough resolution to group the genotypes according to the county of collection (Excoffier et al., 2005). There are chances that similar genotypes were sampled between counties or there is exchange of germplasm by farmers between counties. The negative variance can also be attributed to the highly outcrossing nature of coconut (Gunn et al., 2011) resulting in exchange of genes between populations/counties and reducing inter-population diversity compared to within same population (Excoffier et al., 2005).

Overall, no geographical divergence was observed in coconuts evaluated in the study across the selected counties. This result shows that coconut populations in the select counties at the Kenyan coast are similar.

Genetic variations among coconut genotypes within counties

The majority of the variation displayed in the within populations rather than among populations. The percentile variation among individuals within counties was not statistically significant 101.10 (p = 0.999) indicating that the differences observed in the coconuts within counties could be due to the fact that outcrossing species had less genetic differentiation among their populations (Table 4). The percentage variation within individuals in the same county was significant 0.4927 (p = 0.001) indicating existence of different distinct genotypes in the counties (Table 4). The molecular individual variance was significantly higher than the population variance due to breeding system and seed dispersal

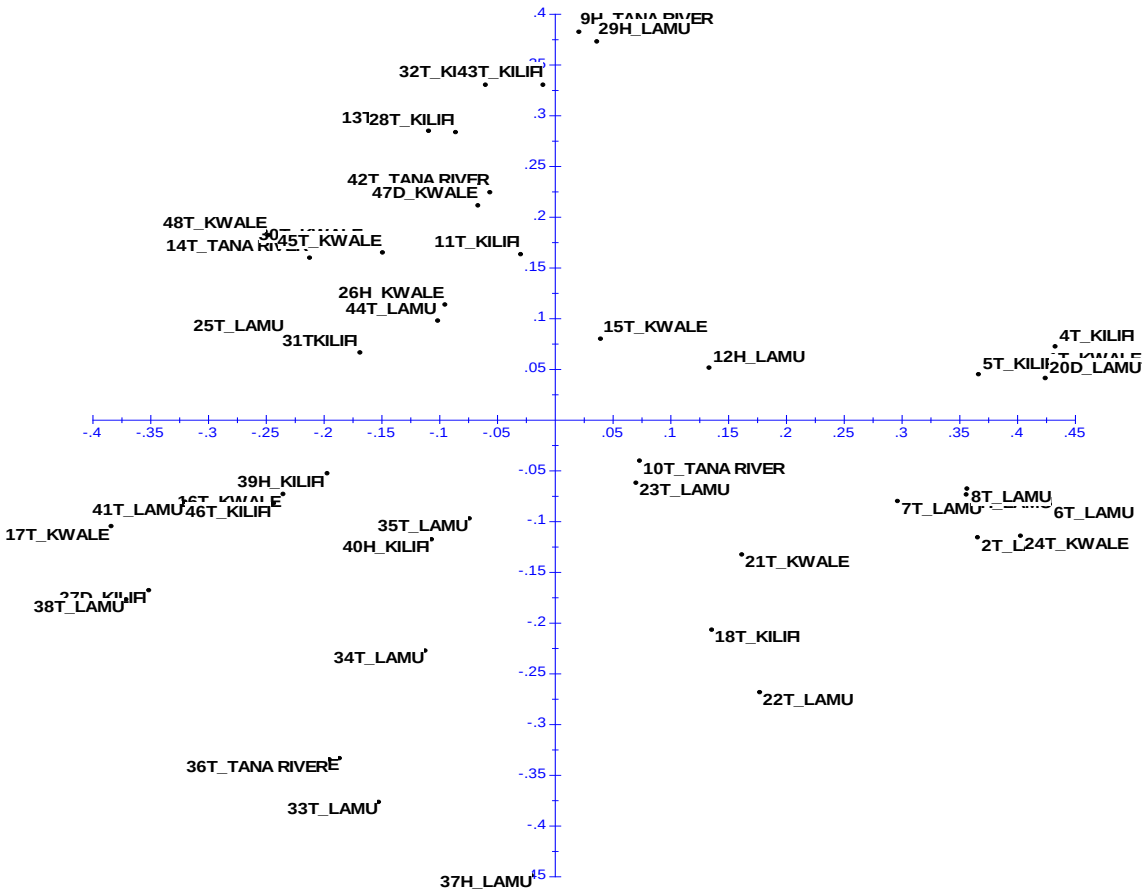

Figure 3. Factorial analysis of the coconut genotypes from select Kenyan coast counties.
Source: Author

mechanism. Life form and breeding system had been reported to have had highly significant influences on genetic diversity and its distribution in woody plants (Hamrick and Godt, 1996)

The genetic differentiation among regions was not significant. This finding is as expected for long-lived, woody plants which inherently display greater variations within populations (Hamrick et al., 1992). Additional studies revealed that low amount of the variation in genetic structure among species can be explained by life history traits alone. Thus, in this study, the high genetic homogeneity and intra-population variability recorded across populations could have resulted from high levels of gene flow, and long-life history of coconut plants as earlier suggested. The coconut from different counties may also have not been geographically separated for an evolutionary significant time to accumulate detectable genetic variation. This also explains the PCA cluster analysis in this work.

Overall, the coconut genotypes within the selected Kenyan coastal counties are genetically diverse. It can therefore be inferred that diversity of the coconut in the select counties is as a result of genetics and not geography.

Factorial analysis

Factorial analysis was performed to evaluate the genetic relationship of the genotypes within and between the counties. The scatter plot was derived from the dissimilarity matrix calculated using raw binary data using Darwin 6.0.21 software. Factorial analysis failed to group the genotypes into any discernible pattern either based on county of collection or genetic makeup (Figure 3), hence dispersal.

This implied that these coastal counties were not specialized habitats for the coconut found at the coastal Kenya. This observation further confirms the genetic relationships of coconut in the area of study in as much as capillarity electrophoresis was able to detect more DNA bands. Cluster analysis failed to group samples as per their origin; the SSR studies of Perera et al. (2000) and Teulat et al. (2000), and the RFLP of Lebrun et al. (1998), both of which were heavily sampled African and south Asian genotypes, support this dispersal theory.

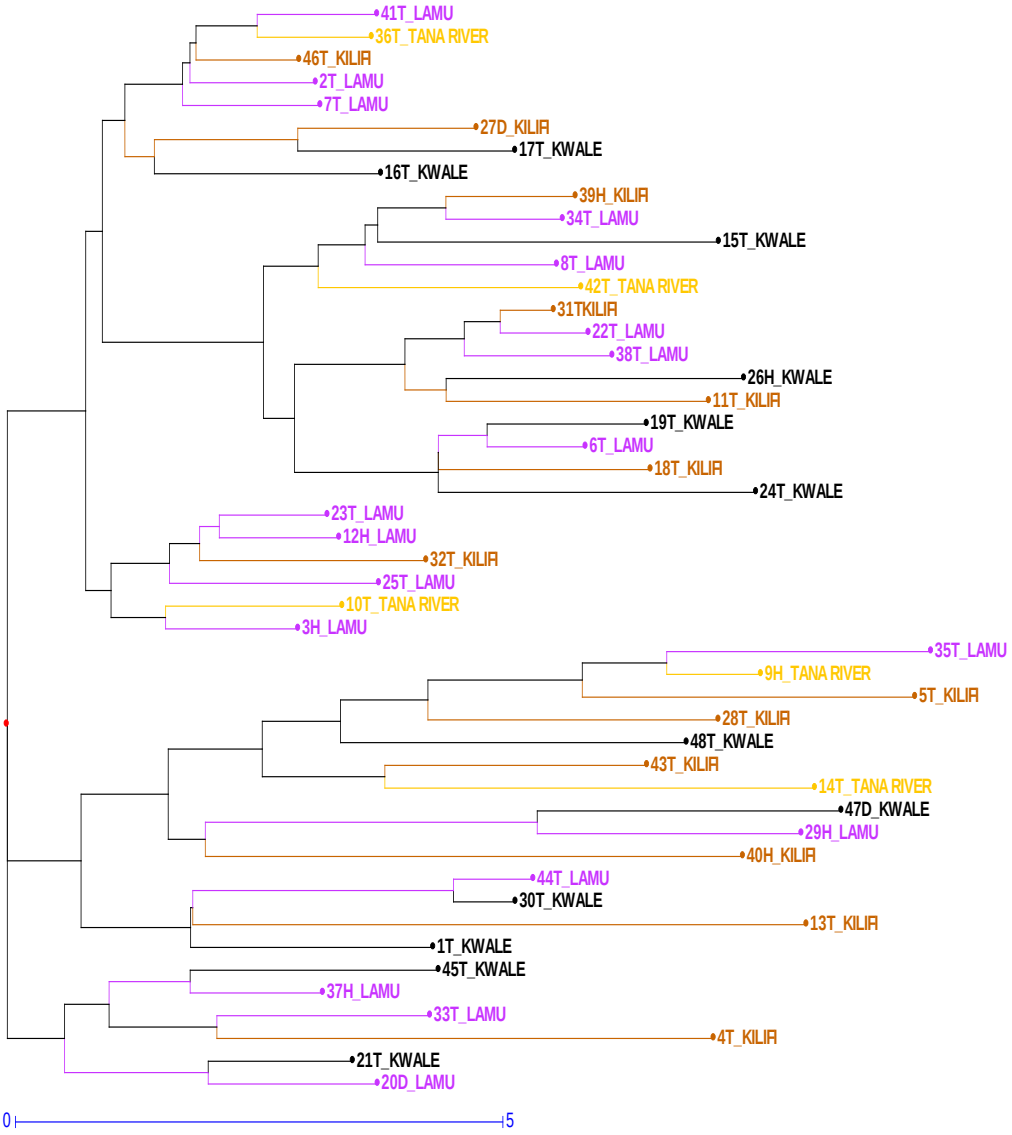

Figure 4. Neighbour-joining DENDOGRAM showing patterns of clustering of the coconut genotypes in the four counties of coastal Kenya. Genotypes are represented by numerical identities followed by a letter designating the coconut variety. T=Tall, D=Dwarf, H=Hybrid.
Source: Author

This could be due to farmers to farmer exchange of planting material, accidental material movements within the counties or ocean currents, inbreeding and outbreeding the life form of coconut.

Phylogenetic analysis

Relationship of the genotypes was presented in a Neighbor Joining dendrogram derived in Darwin 6.0 based on Sokal - Michener modalities after one thousand bootstraps (Figure 4). Phylogenetic analysis grouped the genotypes into three main clusters and eleven sub clusters randomly without any specific pattern. Analysis of markers did not segregate the genotypes based on either genetic makeup or county of collection. This observation further highlights the inefficiency of the markers used in detecting diversity within the coconut genotypes in the four select counties (Kwale, Lamu, Kilifi and Tana River) of the Kenyan coast (Figure 4). The lack of population partitioning is common in free mating populations where alleles are freely shared within and between populations. This postulation is further confirmed by the low expected heterozygosity values reported in the study (Table 3). The lack of population differentiation can also be attributed to high cultivation and domestication

coconut leading farmers transferring germplasm within and across the counties.

The lack of genotype structuring may be due to genetic closeness of the coconut genotypes and or low resolution of the markers used in the study. This finding is similar to results of Oyoo et al. (2016) who also failed to achieve segregation of the genotypes using the same markers.

CONCLUSION AND RECOMMENDATIONS

This study demonstrates an improved allele scoring technique to that of the study of Oyoo et al. (2016) with similar SSRs markers as shown with marker efficacy indices, genetic diversity indices and AMOVA as capillarity electrophoresis revealed more variation. This new information was used to enhance the existing description of the diversity of these genotypes and guide establishment of a coconut genebank for ex-situ conservation.

This study revealed the power of capillary electrophoresis in gene diversity studies in coconut as more variation was detected in this work than reported by Oyoo et al. (2016). Coconut germplasm however failed to cluster according to the county of collection and coconut type indicating low resolution of the SSR markers and genetic redundancy in Coastal Lowlands of Kenya even with the use of this novel technique. To expand coconut genetic base in Kenya, there is need to introduce germplasm and initiate a deliberate breeding programs that involve making controlled crosses. DNA based genetic characterization will also help avoid duplications in conservation blocks and breeding programs, maximising the use of genetic diversity between coconut populations to breed superior varieties and to identify coconut populations with narrow genetic bases and adoption of appropriate conservation strategies (Martial et al., 2013; Rao and Tobby, 2002).

The study also highlighted on the need to apply platforms with higher resolving power such as genotyping by sequencing (GBS) and genome wide sequencing (GWAS) in coconut diversity studies to expand coconut genetic base in Kenya, through introductions and controlled crosses of elite parents

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Supplementary Material

Table 1. List of 30 SSR primers used in the study.

S/N	Primer	Repeat type	F	R	Size range	Tm
1	CAC02	$(CA)_{15}(AG)_7$	AGCTTTTTCATTGCTGGAAT	CCCCTCCAATACATTTTTCC	225-240	49
2	CAC03	$(CA)_{12}(AG)_{14}$	GGCTCTCCAGCAGAGGCTTAC	GGGACACCAGAAAAAGCC	176-183	55
3	CAC04	$(CA)_{19}(AG)_{17}$	CCCCTATGCATCAAAACAAG	CTCAGTGTCCGTCTTTGTCC	185-207	53
4	CAC06	$(AG)_{14}(CA)_9$	TGTACATGTTTTTTGCCCAA	CGATGTAGCTACCTTCCCC	146-164	49
5	CAC08	$(AG)_{10}(CA)_9$	ATCACCCCAATACAAGGACA	AATTCTATGGTCCACCCACA	198-290	53
6	CAC10	(AG)13(CA)9	GATGGAAGGTGGTAATGCTG	GGAACCTCTTTTGGGTCATT	156-163	53
7	CAC11	(CA)n(TA)n	GATCTTCGGCGTTCCTCA	TCTCCTCAACAATCTGAAGC	144-147	53
8	CAC13	$(CA)_9(TA)_5A\ (TA)_4(CA)_6$	GGGTTTTTTAGATCTTCGGC	CTCAACAATCTGAAGCATCG	151-153	53
9	CAC20	$(CA)_{19}$	CTCATGAACCAAACGTTATA	CATCATATACATACATGCAACA	124-133	52
10	CAC21	$(CA)_{11}$	AATTGTGTGACACGTAGCC	GCATAACTCTTTCATAAGGGA	149-151	53.7
11	CAC23	$(CA)_8$	TGAAAACAAAAGATAGATGTCAG	GAAGATGCTTTGATATGGAAC	170-179	53.9
12	CAC39	$(CA)_{15}$	AATTGAGATAAGCAGATCAGT	GTCGGTCTTTATTCAGAAGG	142-166	53.8
13	CAC52	$(CA)_{19}$	TTATTTTCTCCACTTCTGTGG	ATATTACCCATGCACAGTACG	142-160	53.5
14	CAC56	$(CA)_{14}$	ATTCTTTTGGCTTAAAACATG	TGATTTTACAGTTACAAGTTTGG	138-162	53.9
15	CAC65	$(CA)_{15}$	GAAAAGGATGTAATAAGlCTGG	TTTGTCCCCAAATATAGGTAG	150-173	53.4
16	CAC68	$(CA)_{13}$	AATTATTTTCTTGTTACATGCATC	AACAGCCTCTAGCAATCATAG3	130-146	54
17	CAC71	$(CA)_{17}$	ATAGCTCAAGTTGTTGCTAGG3	ATATTGTCATGATTGAGCCTC3	172-283	54
18	CAC72	$(CA)_{18}$	TCACATTATCAAATAAGTCTCACA	GCTCTCTTTCTCATGCACA	124-132	54
19	CAC84	$(CA)_{13}$	TTGGTTTTTGTATGGAACTCT	AAATGCTAACATCTCAACAGC	150-163	54
20	CN11A10	$(CT)_{30}$	GTTGGAGATTTAATTTTCTTG	CCCAATAATATTTTATAACAG	81-119	46
21	CN11E10	$(GT)_{22}(GA)_{14}$	AGAGAGAGTAAATGGGTAAGT	CCCTTTCATTTTTCCTTATTC	99-151	50
22	CN11E6	$(CT)_{21}$	TACTTAGGCAACGTTCCATTC	TAACCAGAAAGCAAAAAGATT	85-128	50
23	CN1C6	$(CT)_1$·$TT(GT)_5$	AGTATGTGAGTAGGATTATGG	TTCCTTGGACCCTTATCTCTT	175-184	52
24	CN1G4	$(CT)_{15}$	GTCGTCCTATACTCATCATCA	GATGCGTATGAGATGTGAGAG	112-132	54
25	CN1H2	$(GA)_{18}$	TTGATAGGAGAGCTTCATAAC	ATCTTCTTTAATGCTCGGAGT	230-321	52
26	CN2A4	$(CT)_{15}TT(CT)_3$	CAGGATGGTTCAAGCCCTTAA	GGTGGAAGAGGGAGAGATTGA	87-111	56
27	CN2A5	$(CT)_{12}TT(CT)_3$	AAGGTGAAATCTATGAACACA	GGCAGTAACACATTACACATG	88-121	50
28	CNZ01	$(CT)_{15}(CA)_9$	ATGATGATCTCTGGTTAGGCT	AAATGAGGGTTTGGAAGGATT	109-131	52
29	CNZ02	$(GA)_{15}$	CTCTTCCCATCATATACCAGC	ACTGGGGGGATCTTATCTCTG	143-161	56
30	CNZ03	$(GA)_7$	CATCTTTCATCATTTAGCTCT	AAACCAAAAGCAAGGAGAAGT	91-97	50

Source: Author

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Full Length Research Paper

Evaluation of *Fagara zanthoxyloïdes* and *Mucuna pruriens* extracts phenolic profile and their *in vitro* antioxidant activity

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In this study, the antioxidant activity and phytochemical analysis of extracts from the bark of *Fagara zanthoxyloides* and seeds of *Mucuna pruriens* were performed. Phytochemical analysis quantified total polyphenols, flavonoids, tannins and anthocyanins and the antioxidant activity was evaluated by DPPH method. The high levels of polyphenols are obtained from the aqueous extract of *F. zanthoxyloïdes* (488.6 ± 1.1 GAE/g) and the methanolic extract of *Mucuna pruriens* (142.9±12.9 GAE/g). The acetone extracts of *F. zanthoxyloïdes* and ethyl acetate extract of *M. pruriens* showed better flavonoid levels with 9.80±0.72 and 15.6±0.02 QE/g, respectively. For tannins, the aqueous extracts of *F. zanthoxyloides* and methanolic from *M. pruriens* showed a level of 19.2±1.41 and 5.7±0.74 CE/g. The antioxidant activity of the different extracts of the plants proved the highest antioxidant activity for the acetone extract of *F. zanthoxyloïdes* (IC_{50}=3.44 mg/L). The methanolic extract of *M. pruriens* showed weak antioxidant activity IC_{50}=3176.22 mg/L, the highest antioxidant activity of *F. zanthoxyloïdes*.

Key words: *Mucuna pruriens*, *Fagara zanthoxyloïdes,* polyphenols, antioxidant, *in vitro* activity, DPPH.

INTRODUCTION

Traditional medicine remains the main remedy for a large majority of African populations to solve their health problem, as it constitutes an important part of the cultural heritage. According to the World Health Organization, nearly 80% of populations depend on traditional medicine for primary health care (WHO, 2002). Medicinal plants are plants used in traditional medicine of which at least a part has medicinal properties, their actions come from their chemical compounds (primary or secondary metabolites) or from the synergy between the different compounds present. The universal role of plants in the treatment of disease is illustrated by their use in all major systems of medicine. Oxidative stress is involved in a wide spectrum of diseases which have a huge impact on the health of populations. These diseases causing oxidative stress are generally due to the excessive production of reactive oxygen species (ROS) and reactive nitrogen species (ERN) which could become

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toxic to major components of the cell: lipids, proteins, and nucleic acids; this would cause cellular dysfunction and would be involved in various pathologies such as: cardiovascular diseases, cancer, diabetes, neuro-degenerative diseases, and the aging process. A large number of scientific studies have been carried out to discover the functional properties of compounds of plant origin, antioxidant and others, which could be effective for health.

Fagara zanthoxyloïdes Lam (Rutaceae) is a plant that grows spontaneously in tropical Africa and preferably on cool, moist soils. Their roots are used and sold in African markets; they have a very popular spicy flavor and are used to calm dental pain. Its use as a toothpick is particularly recommended. This plant, well known to traditional therapists, is traditionally used to treat sickle cell crises by preventing hemolysis of red blood cells (Sofowara et al., 1979).

Mucuna pruriens (Linn.) belongs to Fabaceae family, commonly known as common cowith, cowhage, kavach, velvet bean, kapikachhu and naikaranam. It is an indigenous leguminous plant, is well known for producing itch. It is one of the most popular drugs in the Ayurvedic system of medicine. All parts of *M. pruriens* have valuable medicinal properties (Caius, 1989). The roots are bitter, sweet thermogenic, emollient, stimulant, purgative and diuretic. The seeds are astringent, laxative, antihelmentic, alexipharmic and tonic (Taylor, 2005). The leaves are broadly ovate, elliptic or rhomboid ovate, unequal at base. Leaves used as aphrodisiac, antihelmentic, tonic and are useful in stomach ulcers, inflammation, helminthiasis, cephalalgia and general debility (Sathyanarayanan and Arulmozhi, 2007). *M. pruriens* posses a wide range of pharmacological activities such as anti inflammatory (Hishikar et al., 1989), neuro-protective activity (Manyam et al., 2004), antioxidant activity (Tripathi and Upadhyay, 2001), antidiabetic (Davies, 1994; Dawan et al., 1980), anti protozoal activity (Rathi et al., 2002), and antimicrobial activity (Ekanem et al., 2004). Therefore, in the present investigation, efforts have been made to study the antioxidant activity of hexane, chloroform and methanolic extracts from *M. pruriens* leaves against various bacterial and fungal species *in vitro*.

The literature does not mention a phytochemical study and antioxidant activity of these two in the Central African Republic.

MATERIALS AND METHODS

Reagents and products

Products and reagents used for the various analyses were provided by Sigma-Aldrich-Fluka (Saint Quentin France).

Collection of plant material and preparation of extract

The plant materia l was collected in October 2020 in M'baïki (Boukoko) in the south of the Central African Republic. The specimen (PJK102020) was identified by botanist (Dr. Olivia Semboli) in the Department of Life Sciences and the Center for Studies in Pharmacopoeia and Traditional Medicine (CERPHAMETRA) from the University of Bangui. Extraction was done by macerating 200 g of sample in 2 L of each solvent for 4 h. The extracts were evaporated in a rotavapor at 35°C.

Determination of total phenolics

Total polyphenols were quantified using the method of Namkona et al. (2017), and then slightly modified. We mixed 20 µL of extract plus 100 µl of sodium carbonate (75 g/L in 20 ml H_2O), the whole was mixed with 100 µl of 0.2 N Folin reagent. The whole was stirred for 30 min and then incubated for 15 min. We then read the absorbance at 765 nm. The blank was measured under the same conditions as the Folin reagent is replaced by water. Gallic acid (GA) was used as standard. The results are expressed as mg of gallic acid equivalent by gram of dry mass (dw).

Determination of flavonoids

The rate of flavonoids was obtained using the method described by Namkona et al. (2017). In 96-well microplates, 100 µL of different plant extracts were mixed with 100 µL of aluminum trichloride ($AC1_3$) in methanol (2%) and the absorbance was read at 415 nm after 15 min incubation at room temperature. The blank was measured under the same conditions by mixing 100 µL extract and 100 µL of methanol. Quercetin is used as a standard and is expressed as a quercetin equivalent by gram of dry mass.

Determination of tannins

The condensed tannins were quantified by mixing 50 µL with 150 µL of vanillin (1% H_2SO_4 in 7 M), according to the method of Namkona et al. (2017) and then slightly modified. The whole was incubated at 25°C for 15 min. The absorbance of the solution was measured at 500 nm. The measurement was made of for a blank when the vanillin was replaced with water. Catechin was used as standard. The results were expressed as mg of catechin equivalent by gram of dry mass.

Determination of total anthocyanins

Total anthocyanins were quantified in this study using the Namkona et al. (2017) pH difference method and then lightly modified. 20 µL of each extract was added to 180 µL of the pH 1.0 and 4.5 solutions of hydrochloric acid, potassium chloride (pH 1.0, 0.2 M) and acetic acid, sodium acetate. The corresponding absorbance was calculated using the following formula:

A = [(A510-A700) pH1.0- (A510-A700). pH4.5].

Molar extinction coefficient of 29600 was used. The results were expressed as mg of cyanidin-3-glucoside equivalent of dry mass.

Antioxidant activity by DPPH

The anti-radical activity of the extracts was determined by the method of Bekir et al. (2013) and then slightly modified. In a 96-well microplate, 20 µL of each extract and 180 µL of the DPPH solution were introduced. The mixture was stirred for 30 s and then incubated for 30 min in the dark. The reading was made at 524 nm.

Table 1. Extraction yield chemical composition (families) of *F. zanthoxyloïdes* extracts.

Solvents	Yields (%)	Phenolics (GAE)[a]	Tannins (CE)[a]	Flavonoids (QE)[a]	Anthocyanins (C3GE)[a]
Ether petrolum	22.3	nd	2.02±0.55	7.39 ±0.53	nd
Chloroform	24.7	nd	9.21±2.79	5.14±0.35	nd
Acetone	25.2	133.3±1.7	4.29±1.34	9.80±0.72	nd
Water	27.4	488.6±1.1	19.2±1.41	6.31±0.21	nd

[a]mg/g dw; nd: no detected.
Source: Author

Table 2. Extraction yield chemical composition (families) of *M. pruriens* extracts.

Solvents	Yields (%)	Phenolics (GAE)[a]	Tannins (CE)[a]	Flavonoids (QE)[a]	Anthocyanins (C3GE)[a]
Cyclohexane	0.67	88.9±10.4	5.5±0.14	15.6±0.03	1.06±0.63
Dichloromethane	0.50	93.6±7.5	4.5±0.28	15.5±0.05	5.06±0.58
Ethyl acetate	0.65	108.0±15.7	4.4±7.49	15.6±0.02	0.65±0.08
Methanol	6.10	142.9±12.9	5.7±0.74	15.5±0.01	0.6±0.34

[a]mg/g dw; nd: no detected.
Source: Author

The yields inhibition of the extract was calculated by the following equation:

$$\%\text{Inhibition} = \frac{\text{A(blanc)} - \text{A(ext)x100}}{\text{A(blanc)}}$$

The antioxidant activity of the extract is expressed as IC_{50} which defines the concentration of the extract that reduces by 50% the free radical (DPPH). Ascorbic acid was used as standard.

Data analysis

Data were processed by the software Excel and slopes, variances, standard deviations, and IC_{50} were determined. The threshold of 5% margin of error was used as a criterion for significance in all cases.

RESULTS AND DISCUSSION

Chemical composition of extracts

Table 1 shows the results of the phytochemical screening of *F. zanthoxyloides*. The best extraction yield is obtained with water and the low yield with petroleum ether with 27.4 and 22.3%, respectively. Bossokpi (2003) resulted in extraction yields of 4% (petroleum ether), 6% (dichloromethane) and 8% (methanol) and 1.8 (water) with the bark of the roots. Our yields are better compared to that of the latter results. This difference could be due to the organs used, since we worked on the bark of the trunk, on the other hand they used the bark of the roots. Four chemical families (polyphenols, flavonoids, tannins and anthocyanins) were quantified in this study. For petroleum ether extract, the polyphenols, flavonoids and tannins levels are in the order of 488.6 ± 1.1; 39 ± 0.53 and 2.028 mg/g ± 0.554, respectively. The chloroform extract gave the following results, 5.14 mg/g ± 0.35 for flavonoids and, 211 mg/g ± 2.795 for condensed tannins. The acetone extract contains 133.3 mg/g ± 13.7 polyphenols, 9.80 mg/g ± 0.72 flavonoids and 4.290 mg/g ± 1.347 condensed tannins. Finally, the aqueous extract quantified 6.31 mg/g ± 0.21 of flavonoids and 19.2 mg/g ± 1.412 of tannins. Adefisoye et al. (2012) demonstrated the presence of flavonoids and tannins in the roots of this plant in Nigeria. This confirms the flavonoids and tannins quantified in this study.

Table 2 shows the results of the phytochemical study of extracts from *M. pruriens*. For total polyphenols, the results are respectively 88.9 mg/g ± 10.4 (cyclohexane extract), 93.6 mg/g ± 7.5 (dichloromethane extract), 108.0 mg/g ± 15.7 (ethyl acetate extract) and 142.9 mg/g ± 12.9 for the methanol extract.

The methanolic extract contains more tannins than the other extracts with 5.7 mg/g ± 0.74. On the other hand, the highest rate of flavonoids is obtained with the acetone extract 9.80 mg/g ± 0.72 (Figure 4). Muhali et al. (2019) made it possible to find 3130.1 mg/g ± 15.5 for polyphenols, 63.3 mg/g ± 1.9 for flavonoids in aqueous and ethanolic extracts of mature fruits.

The levels of polyphenols and flavonoids of the latter are higher than those obtained in the present study. These differences could be due to the difference in part of the plant used. Also, the difference between the solvents used would affect the values of the quantified chemical compounds.

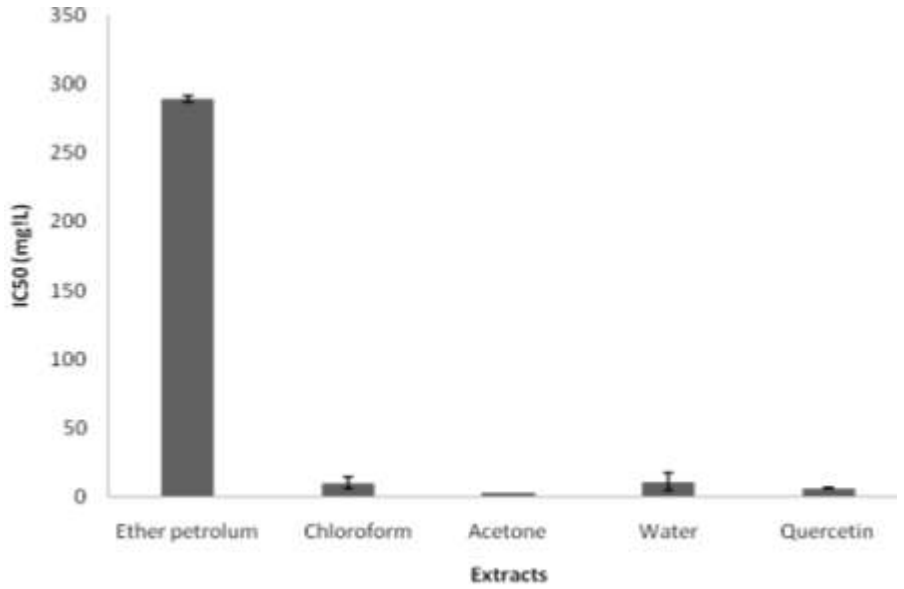

Figure 1. Results of antioxidant activity of *F. zanthoxyloides* extracts.
Source: Author

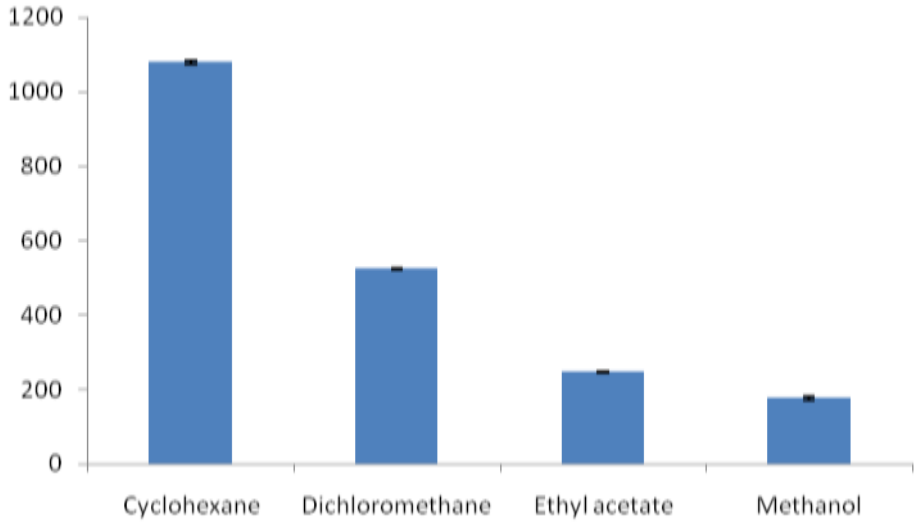

Figure 2. Results of antioxidant activity of *M. pruriens* extracts.
Source: Author

Antioxidant activity by DPPH

The histogram (Figure 1) shows the results of the antioxidant activity of the extracts and of the quercetin used as a reference molecule (Figure 3). The acetone extract showed strong antioxidant activity with IC_{50} equal to 3.44 mg/L, chloroform and aqueous extracts have IC_{50} values of 10.13 and 11.06 mg/L, respectively. The petroleum ether extract gave an IC_{50}> 250 mg/L.

The IC_{50} value of acetone extract proves that this plant has a very high antioxidant activity than the reference molecule which has an IC50 of 6.3. Antioxidant activity was demonstrated by the work of Bossokpi (2012). Imaga et al. (2011) found an IC_{50} of 500 mg/L with the aqueous extract of the stems of *F. zanthoxyloïdes*. The difference between our results and that of the latter could be in the different parts of the plant used. Climatic and soil variations can influence chemical composition.

Figure 2 shows the IC_{50} values for the antioxidant activity of *M. pruriens* extracts. The best antioxidant activity was obtained with the methanol extract with 176.22 mg/L followed by the ethyl acetate extract 246.22

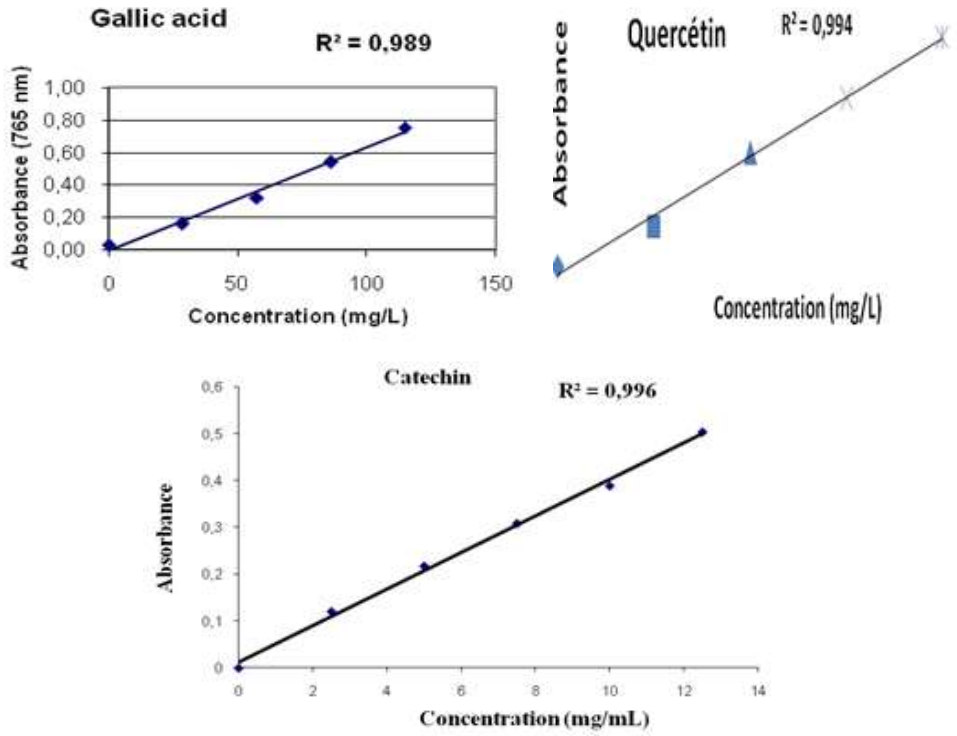

Figure 3. Calibration curves of reference molecules
Source: Author

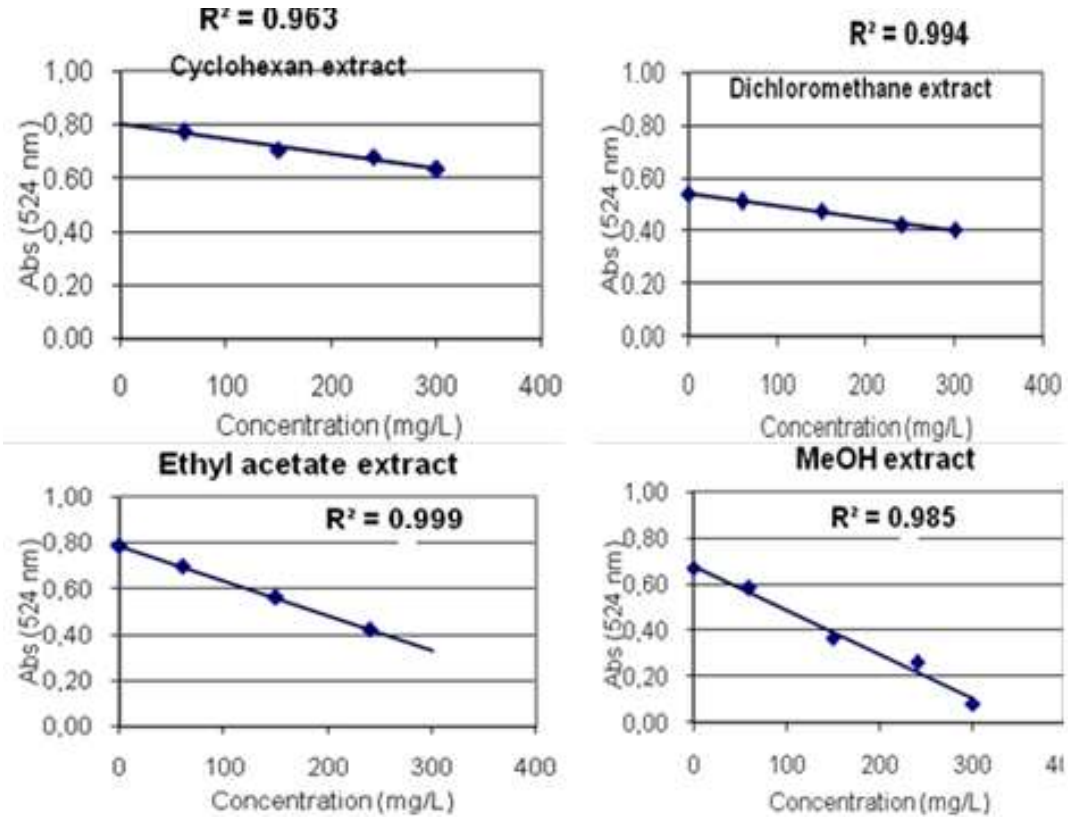

Figure 4. Determination curves of inhibitory concentrations of extracts
Source: Author

mg/L. The cyclohexane extract exhibited a lower IC_{50} with 1078.46 mg/L. The work of Ruhi et al. (2020) proved the antioxidant activity of *M. pruriens*. Kumar et al. (2010) demonstrated that extracts of *M. pruriens* were effective against free radicals. The antioxidant activity proven by the study is in line with this previous work.

Conclusion

This study allowed us to assess the antioxidant activity and quantify some chemical families (total polyphenols, tannins, flavonoids and anthocyanins) of *F. zanthoxyloïdes* and *M. pruriens*, used by different populations to treat themselves against various diseases.

It emerges from this study that these plants are rich in phenolic compounds which are very important in the fight against cardiovascular diseases. The antioxidant activity of extracts from these plants proven in this study is in accordance with their total polyphenol compositions. These plants could be used in the treatment of diseases linked to oxidative stress which presently poses a serious public health problem.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Antibacterial Activities of selected Medicinal Plants Against *Salmonella typhi*, *Salmonella paratyphi* A, B and C, Clinical Isolates in North Central, Nigeria.

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The aim of the research was to investigate the antibacterial activities and the phytochemical properties of aqueous and ethanolic extracts of Mango (*Mangifera indica)* and Neem (*Azadirachta indica*) leaves, on clinical isolates of *Salmonella* typhi*, Salmonella* paratyphi A, B and C, in Lafia, Nigeria. The agar well diffusion technique was used and the analyses were done in triplicates. At the highest aqueous and ethanolic extract concentration of 80 mg/ml, *S. typhi,* showed inhibition zones of 29.4±0.1mm and 30.0±0.01 mm respectively, while *S.* paratyphi A, B and C, exhibited inhibition zones of 14.4±0.2, 21.2±0.4, 13.4±0.1 mm and 18.0±0.03, 20.0±0.04, 21.0±0.04 mm, respectively. At aqueous and ethanolic lower extract concentration of 10 mg/ml, S. typhi, exhibited inhibition zones of 8.4±0.01 and 9.0±0.1 mm, respectively, while *S. paratyphi* A, B and C, showed inhibition zones of 9.5±0.01, 9.1±0.01, 6.2±0.01 mm and 9.0±0.01, 9.0±0.01, 9.0±0.01 mm, respectively of Neem leaf extract. The MIC and MBC, of aqueous and ethanolic extracts against organisms were 2.5 and 5.0 mg/ml, respectively. The qualitative phytochemical results showed the presence of alkaloids, anthraquinones, carbohydrates, cardiac glycosides, flavonoids, tannins, terpenoids and saponins. Conclusively, the aqueous and ethanolic extracts of the studied medicinal plant exhibited bactericidal activities against all tested organisms, and this supports the claim by traditional medical practitioners and vendors of the use of the plants for the cure of typhoid fever. Further studies are recommended on the toxicity and safe dosage regimen of the plants since the infusion of the plant is taken orally by people for cure of typhoid fever.

Key words: *Salmonella* organisms, typhoid fever, phytochemical constituents, bacteria, medicinal plants.

INTRODUCTION

There is a great concern over the emergence and the spread of bacteria resistant to drug all over the world (Boucher et al., 2009). Therefore, there is the need to find an efficient and safe alternative to microbial resistance to drugs, and the alternative resides in phytomedicine.

Although, various brands of new antibiotics have been manufactured by pharmacological companies every year, the global emergency of multidrug resistant (MDR)

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bacteria is limiting the potency of these drugs (Hancock, 2005). For example, multidrug resistant *Salmonella* Typhi, showed resistance to all three first line of drugs: Ampicillin, Chloramphenicol and Trimethioprim-sulfamethoxazole (Ackers et al., 2000). This poses challenges to researchers which prompted studies to explore the phytotherapeutic potential of medicinal plants including Mango (*Mangifera indica*) and Neem (*Azadirachta indica*), as a source for alternative medicine to microbial resistant drugs which are cheaper and safer than conventional drugs.

Medicinal plants such as Mango (*M. indica*) and Neem (*A. indica*), have been used to treat human ailments including malaria, anemia, diabetes and cancer, for many years because they possess organic compounds including alkaloids, flavonoids, phenols, tannins, saponins, terpenoids, and cardiac glycosides, that are antibacterial and have definite physiological action on the human body (Byarygaba, 2004). For instance, the use of plant extracts for antimicrobial therapy has proven to be a promising remedy in Chinese phytomedicine, Indian Ayurvedic, Arabic and Unani medicines (WHO, 2002 - 2005). In Africa, the use of medicinal plants for the treatment of diseases has been since the introduction of modern medicine (Kabir et al., 2005). In Southern Nigeria, for example, *Acalypha wilkesiana*, a member of the Euphorbiaceae family, is used to treat malaria, dermatological and gastrointestinal disorders and has antimicrobial properties (Kabir et al., 2005; Oladunmoye, 2006; Erute and Oyibo, 2008). The interest in plants is because they are available, easily accessible, cheap for both the rich and the poor populace, and safer and more cost-effective sources for alternative medicine (Doughari et al., 2007; Osuagwu et al., 2015).

At least 25% of drugs in modern pharmacopoeias are derived from compounds isolated from medicinal plants, such as Mango (*M. indica*) and Neem (*A. indica*) (De Silva, 2005). Accordingly, extracts from medicinal plants have a wide range of bioactive and pharmacological activities which essentially include antibacterial, antifungal and anti-inflammatory properties (Okwu, 2005). Infusion and concoctions prepared from different parts of medicinal plants are used in the treatment of various human diseases, including typhoid fever, a systemic disease which is caused by the *Salmonella* organism, which has been reportedly resistant to both Trimethioprim sulfamexazole and Chloramphenicol (WHO, 2007). Fabricant and Farnsworth (2001) and Bussmann et al. (2006), have reported that plant extracts are traditionally used in most nonindustrialized nations for the treatment of human diseases, including typhoid fever. Gatsin et al. (2007), Agada et al. (2010), Uhuo et al. (2015) and Rachuonyo et al. (2016), have demonstrated the bactericidal effect of plant extracts on *S typhi, Salmonella paratyphi* A, B and C, in the West Province of Cameroon, Jos Plateau State, Ebonyi State and Kenya, respectively.

Rachuonyo et al. (2016) studied the *in-vitro* antimicrobial activity of methanolic leaf extracts from four plants (*A. secundiflora, Bulbine frutescens, Vernonia lasiopus* and *Tagetes minuta)* against *S. typhi* in Kenya, using a disc diffusion method. They reported *A. secundiflora* bactericidal against tested organisms at low concentration of 5.5 mg/ml; MIC of plant extracts on targeted organism varies from 5 to 9 mg/ml; MBC against organism ranged from 7 to 11 mg/ml. Reported qualitative phytochemicals include alkaloids, flavonoids, tannins and saponins. Uhuo et al. (2015), reported herb extracts of *Vernonia amygdalina, Allium sativum* and *Allium cepa,* had a strong inhibitory effect against *S. typhi,* in their study on antibacterial activities of some medicinal plants on *S. typhi* isolates in Abakiliki, Ebonyi State, Nigeria, using an agar diffusion method. It records the presence of secondary metabolites, including alkaloids, glucose, tannins, glycosides, flavonoids, steroids and phenolic compounds. Gatsing et al. (2007), recorded MIC values of 6 mg/ml against *S. typhi and S. paratyphi* B, and MBC value of 300 µg/ml against *S. typhi and S.paratyphi* B*,* in their study of antibacterial agents from methylene chloride and methanol leaf extract of *Crinum purpurascens* herb collected from the West Province of Cameroon, using both agar diffusion and broth dilution methods.

The mango (*M. indica L*) plant belongs to the family Anacardiaceae, which consists of about sixty genera and 600 species (Akinpelu and Onakoya, 2006). Mango is one of the tropical fruit bearing trees in the world (Kabuki et al., 2000). Mango is a medicinal herb that is used traditionally in the treatment of diseases including mouth infection in children, diarrhea, dysentery, gastrointestinal tract disorders, typhoid fever, sore throat and scurvy (Campbell et al., 2002; Fowler, 2006). Its ground seeds and leaves have been used to treat diabetes, colic and irritation from scorpions and bee stings (Doughari and Manzara, 2008). The leaves of *M. indica* have been reported to contain glycoside and mangiferin, which is an antimicrobial agent and mangiferin has been demonstrated to possess antiviral activity against the herpes simplex type 2 virus (Zakaria et al., 2006). The Neem (*A. indica*) plant, on the other hand, belongs to the family of Melioceae. Neem is a tropical plant that has adapted to a wide range of climatic, topographic and environmental factors and has immense potentials. In Indian traditional Ayurvedic medicine, different parts of Neem tree have been used for the treatment of various ailments. The Neem oil, bark and leaf extracts have been used to control leprosy, intestinal helminthiasis and respiratory disorders.

Mango and Neem plants are medicinal herbs that have been used traditionally in Chinese phytomedicine, Indian Ayurvedic, Arabic, Unani, and African medicines particularly in Nigeria, to treat various human ailments, including typhoid fever, but their antibacterial activity against clinical isolates of *Salmonella* organisms in Lafia, Nigeria, has not been tested. Therefore, the aim of the

research is to study the antibacterial activity and phytochemical properties of mango and Neem leaf, aqueous and ethanolic extracts against clinical isolates of *S. typhi, S. paratyphi* A, B and C, in Lafia, North Central, Nigeria, as an alternative to medicare for typhoid fever.

MATERIALS AND METHODS

Collection of plant materials

The procedures described by Osuagwu et al. (2015), good agricultural collection practice and Field collection practice (GACP and FCP, 2004), were the sampling techniques adopted for the collection of plant materials. Fresh leaves of mango (*M. indica*) and Neem (*A. indica*), were randomly collected from different location sites (Figure 1): Gandu and Akunza, in Lafia local government area, of Nasarawa State, in the morning before sunrise (to avoid degradation of plants biocomponents by ultra violet rays). The leaves were identified by a trained taxonomist and Botanist in the Department of Botany, Federal University of Lafia, Nasarawa State. A voucher specimen was assigned to the samples for record purposes. The leaves were then washed 5 to 6 times with clean water to remove dust and dirt; thereafter, dried in shade at room temperature 27°C till crisp and brittle to touch; separately ground to powder using a laboratory mortar and pestle; separately sieved through a sieve 0.5 to 10 mm to obtain fine particle size (GACP and FCP, 2004). The powdered plant samples were stored in a labeled airtight clean, dried opaque polythene container, until ready for analysis.

Preparation of plant extracts (aqueous and ethanolic)

Aqueous extraction

One hundred grams each of dried finely powdered plant samples were weighed separately into different glass beakers using analytical weighing balance (aeAdam, Model PW 124, UK). Then, 500 ml of sterile distilled water were added into each beaker. The mixtures were heated on a hot plate (Model SB 160, UK), at 30 to 40°C with intermittent stirring for 20 min. Thereafter, the aqueous extracts were filtered through Whatman filter paper, number one. The filtrates were evaporated separately in a water bath at 65°C, and the crudes were labeled and stored in a refrigerator until ready for analysis (Asowata et al., 2013).

Ethanolic extraction

Using analytical weighing balance (aeAdam, Model PW 124), 100 g of dried finely powdered mango and neem leaves were weighed into separate glass beaker, and 70% ethanol added to each beaker. The mixture was soaked and agitated intermittently for 72 h. After that the contents were filtered through Whatman filter paper number one. Thereafter, the filtrates were evaporated separately in a water bath at 60°C. The yield was weighed and recorded. The dried crude was stored in a refrigerator till ready for analysis (Asowata et al., 2013).

Phytochemical evaluation of the plant extract

Qualitative analysis of phytochemical constituents in plant extracts

Test for alkaloids: The alkaloids content of plant samples was determined by the method described by Aiyelaagbe and Osamudiamen (2009). 0.5 g of the crude extract was mixed with 3 ml of 1% HCL, and boiled for 5 min. The mixture was cooled and filtered. Thereafter, the filtrate was treated with Mayer's, Wagner's and Dragendroff's reagents. The turbidity of the resulting precipitate was an indication of the presence of alkaloids.

Test for reducing sugars: Reducing the sugars content of plant extracts was determined by the method described by Aiyelaagbe and Osamudiamen (2009). One milliliter of plant extract was treated with a mixture of 2 ml of Fehling's solution (A and B) in a test tube. The setup was gently boiled. The color changes from deep blue to brick red, which indicates the presence of reduced sugars.

Test for glycosides: The method described by Aiyelaagbe and Osamudiamen (2009), was adopted for glycoside determination in plant crude extracts. One millimeter of plant crude extract was mixed with 2 ml of chloroform and 2 ml of acetic acid in a test tube. The setup was cooled in an ice bath, followed by the careful addition of concentrated H_2SO_4. A color change from violet to blue to green indicates the presence of steroidal nucleus, that is, glycone portion of the glycosides.

Test for flavonoids: The method described by Aiyelaagbe and Osamudiamen (2009) was adopted for the presence of flavonoids in the plant extract. 2 g of plant extract in a glass test tube was detanned using acetone. After that the setup was placed in a hot water bath for traces of acetone to evaporate. Thereafter, boiling water was added to the detanned sample and the mixture was filtered while hot and allowed to cool. Then 5 ml of 20% NaOH solution was added to an equal volume of the filtrate. A yellow solution was evidence of the presence of flavonoids.

Test for tannins/phenol: The method described by Aiyelaagbe and Osamudiamen (2009) was adopted for the presence of tannins in plant crude extract. 0.5 g of plant crude extract was dissolved in 1 ml of distilled water in a glass test tube, and filtered through Whatman filter paper number one. 3 ml of the filtrate in a test tube was added 2 mL of 2% solution of $FeCl_3$. A blue-green or black coloration was an evidence of the presence of tannins.

Test for terpenoids: The method described by Aiyelaagbe and Osamudiamen (2009) was adopted for analysis of terpenoids in plant crude extract. 0.1 g of plant crude extract was dissolved in 2 ml of chloroform in a test tube, then evaporated on a water bath to dryness. Thereafter 2 ml of concentrated H_2SO_4 was carefully added to the residue, then heated for 2 min. The grayish coloration was evidence of the presence of terpenoids.

Test for anthraquinones: Procedures described by Aiyelaagbe and Osamudiamen (2009), were the method used to determine anthraquinones in plants crude extract. 0.5 g of plant crude extract was dissolved in 5 ml of chloroform in a test tube, and the setup was shaken for 5 min. The resulting mixture was filtered, and 3 ml of the filtrate was shaken with an equal volume of 100% ammonia solution in a test tube. A pink, violet/red color in the ammoniacal layer indicates the presence of free anthraquinones.

Test for saponins: The method described by Aiyelaagbe and Osamudiamen (2009), was used to determine the presence of saponins in plant crude extracts. 0.5 g of the extract in a test tube was shaken with distilled water. The presence of saponins in the sample was indicated by persistence of front during warming.

Quantitative analysis of phytochemicals in plant crude extract

Determination of alkaloids

Harborne (1973) was the method used for the analysis of alkaloids

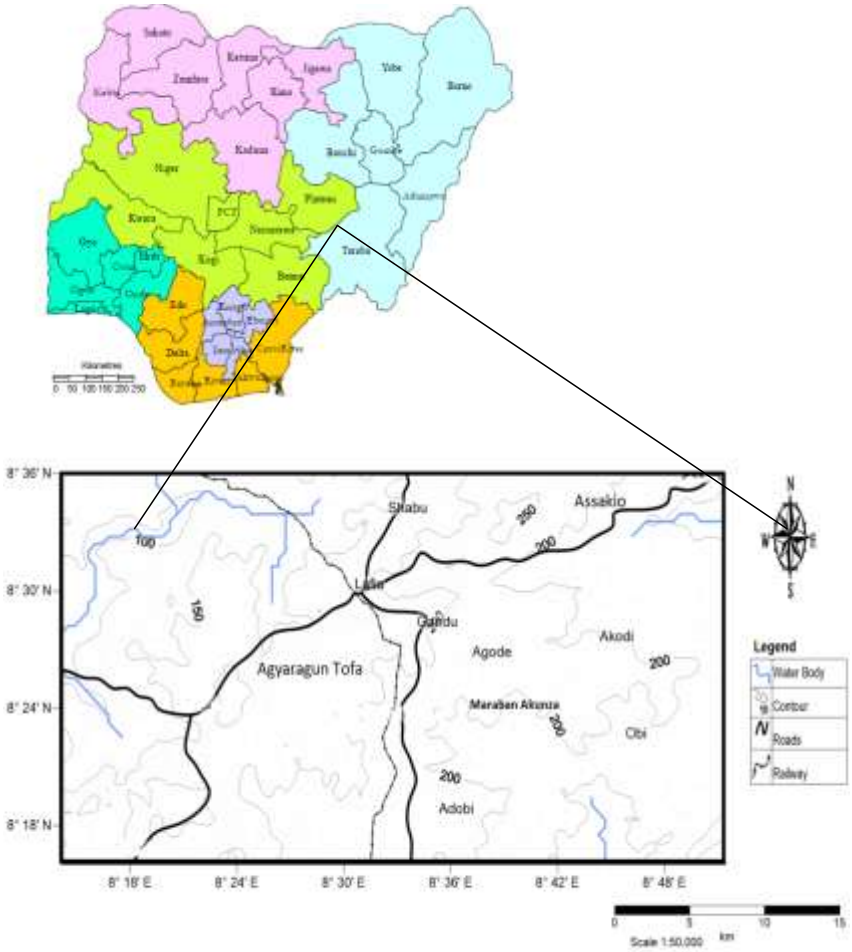

Figure 1. Topographic map of Lafia showing the study area.
Source: Umar et al. (2019).

in the crude extract. The test was done in triplicate. 5 g of the sample in a 250 ml beaker and 200 ml of 10% acetic acid in ethanol was added, then covered with a watch glass and allowed to stand for 4 h. The mixture was then filtered, and the filtrate was concentrated in a water bath to one quarter of its original volume. Then concentrated ammonium hydroxide was added to the concentrated filtrate drop wise till the precipitate was completed. The precipitate was then collected on a weighed filter paper and washed in diluted ammonium hydroxide (2M). The residue was dried in an oven at 80°C and the weight was calculated and expressed as a percentage of the weight of the sample.

Assay of flavonoids

Procedures adopted by Boham and Kocipai-Abyazan (1994), were used for the determination. Analysis was done in triplicates. 10 g of the plant sample was extracted repeatedly with 100 ml of 80% aqueous ethanol at room temperature until the residue becomes colorless. After that, the whole mixture was filtered through Whatman filter paper number one. Thereafter, the filtrate was transferred into a weighed crucible and was evaporated to dryness in a water bath. The difference in the constant weight of the crucible gave the value of the flavonoids of the assayed sample and the result was expressed as a percentage of the original sample.

Determination of saponins

The method described by Obadoni and Ochuko (2001), was used for the assay and analysis was carried out in triplicates. 20 g of the plant sample was placed in a 250 ml conical flask and 200 ml of 20% ethanol was added. The setup was heated in a water bath at 55°C for 4 h with continuous stirring. After that, the mixture was filtered and the residue was re-extracted with another 200 ml of 20% ethanol. The combined extracts were reduced to 40 ml in a water bath at 90°C. Then, the concentrate was transferred into a glass separatory funnel and 20 ml of diethyl ether was added and the setup was shaken vigorously. After that, the aqueous layer was recovered while the ether layer was discarded. The purification process was carried out once more. Then, 60 ml normal butanol was added and was washed twice with 10 ml 5% aqueous sodium chloride. After that, the solution was evaporated in a water bath and the residue was dried in an oven at 80°C to a constant weight.

Saponin content was calculated and the result expressed as percentage of the original sample.

Assay of tannins

For the analysis of tannins in the plants crude extract, Van Burden and Robinson (1981), procedures were used for analysis of tannins

in plant crude extract. The analysis was done in triplicates. 5 g of plant sample was in a 100 ml plastic bottle and 50 ml distilled water was added and the setup was shaken for 1 h on a mechanical shaker. After that, the mixture was filtered into a 50 ml volumetric flask and diluted to the mark with distilled water. 5 ml of the filtrate were pipetted into a test tube and mixed with 2 ml of 0.1M $FeCl_3$ and 0.1M HCL and 0.008M $K_4[Fe(CN)_6]$. A blank sample was also prepared. The absorbance of the sample was read at 395 nm against the blank within 10 min of preparation using the T60 UV-Visible spectrophotometer (PG Instrument, UK). A standard curve was prepared using tannic acid to get the 100 ppm measurement limit.

Assay of total phenol

The analysis was carried out in triplicates. 10 g of powdered plant sample was defatted with 100 ml diethyl ether for 2 h using a Soxhlet apparatus. The free fat sample was then boiled with 50 ml of ether to extract the phenolic component before being filtered. Then 5 ml, of the filtrate was pipetted into a 50 ml volumetric flask and 10 ml of distilled water, 2 ml NH_4OH solution and 5 ml amyl alcohol were added and diluted to mark with distilled water. The setup was allowed to react for 30 min for color development. Thereafter, the absorbance of the solution was read at 505 nm using the T60 UV-VIS Spectrophotometer (PG Instrument, UK). A standard curve was prepared using 0, 25, 50, 75, and 100 mg/l solutions of gallic acid in methanol: water (50:50 v/v). The total phenol value was expressed in g gallic acid equivalent per 100 g dry weight (g GAE/100 g dry mass) (Ebrahimzaded et al., 2008).

Determination of cardiac glycosides

The method described by El-Olemy et al. (1994), was used for the analysis of cardiac glycosides of plant extracts. The experiment was done in triplicates with 1 g of fine powdered plant sample used in 250 ml beaker. Ten milliliters of 70% alcohol were added to the setup and allowed to soak for 2 h. After that, the mixture was filtered and 8 ml of the filtrate was diluted to 100 ml with distilled water in a 100 ml standard flask. Then 8 ml of the diluted filtrate was transferred into a 100 ml standard flask and 8 ml of 12.5% lead acetate solution (to precipitate resins, tannins and pigments) was added and the setup was mixed by shaking and was diluted to the mark with distilled water and later filtered. After that, 50 ml of the filtrate was transferred into a 100 ml standard flask and 8 ml of 4.7% disodium hydrogen phosphate solution was added (precipitate excess lead ions) and the content was diluted to the mark with distilled water and the mixture was filtered twice through Whatman filter paper number one (obtain purified filtrate). Then 10 ml of purified filtrate was transferred into a 50 mL beaker and 10 ml of freshly prepared Baljet's reagent was added into the beaker and the content was mixed and the setup was allowed to stand at room temperature for 1h for color development. A blank sample was treated similarly. The sample was read at 495 nm against a blank using the T60 UV-Vis Spec (PG Instrument). Differences between the intensity of sample and blank, gave the absorbance which is proportional to the concentration of cardiac glycoside in the analyzed sample and the result was expressed in percentage from the relation:

% glycoside = A × 100/17;

where A = absorbance of sample at 495 nm.

Preparation of media

The media used include Muller-Hinton agar, nutrient broth, nutrient agar, urea broth, triple sugar iron agar, Simmon's citrate agar, and motility agar. The media was prepared in accordance with the Manufacturer's instructions. Each batch of prepared media was tested for sterility before being used.

Bacterial stains

Clinical isolates of *S. typhi, S. paratyphi* A, B and C, were collected from the Department of Microbiology, Dalhatu Araf Specialist Hospital, Lafia, Nasarawa State, Nigeria. All of the bacterial strains were preserved on Bijou agar slants and stored at 4°C until they were ready for analysis.

Bacterial strain confirmation

Bacterial strains were confirmed by biochemical screening including Gram stain, triple sugar iron (TSI), urease, citrate utilization, motility, methyl red and Voges-Proskauer tests, as described by Collins et al. (2004): ISO 6579: 2002 (Sharma, 2009) and the serological test as described by Cheesbrough (2000): ISO 6579: 2002 (Collins et al., 2004; Andrews et al., 2005; Sharma, 2009).

Bacterial strain confirmation by commercial kit

The procedures adopted by Cheesbrough (2000): ISO 6579: 2002 (Andrews et al., 2005), were the methods used to confirm the bacterial strains. Identification of *S. typhi, S. paratyphi* A, B and C, was performed by slide agglutination tests. Commercial kit was used to confirm serogroup *S. typhi, S. paratyphi* A, B and C, by their somatic (O) and flagella (H) antigens (A, B, C and D). A single pure colony of individual test organism was picked and placed separately on a ceramic tile and was rocked with the corresponding antisera. The reaction was observed for 2 min. The agglutination reaction confirmed the *Salmonella* subgroup and was evidenced by a positive organism under test.

Standardization of inoculum

Asowata, et al. (2013), method was used for the standardization of bacterial inoculum. Five colonies of each test organisms were picked aseptically with a wire loop and transferred into separate glass test tubes containing 5 ml of nutrient broth and mixed. The setup was then incubated at 37°C for 24 h. The turbidity that resulted was adjusted to match 0.5 McFarland standard which yielded approximately 1×10^7 ml^{-1} bacteria.

Preparation of various concentrations of plant extracts

Double dilution procedures were used to obtain various concentrations of plant extracts of 80, 40, 20, 10, 5, and 2.5 mg/ml, for antibacterial activity, MIC and MBC, using sterile distilled water. Thus, 8 g crude plant extract was reconstituted in 100 ml of sterile distilled water to obtain 80 mg/ml solution (that is, 8 g crude plant extract = 8000 mg/100 ml distilled water = 80 mg/ml). A known volume of 80 mg/ml solution was diluted with equal volume of sterile distilled water to obtain 40 mg/ml solution. Double dilution continues until lower concentrations were obtained (Asowata, et al., 2013).

Determination of antibacterial activity

The antibacterial screening of aqueous and ethanolic plant crude extracts were carried out using the agar well diffusion method.

Several dilutions of crude extracts of mango and Neem were separately made in separate glass test tubes as described by (Asowata, et al., 2013). The dilutions were 80, 40, 20, 10, 5 and 2.5 mg/ml. A suspension of *S. typhi*, *S. Paratyphi* A, B and C, compared to the 0.5 McFarland standard was each seeded on separate nutrient agar plates and spread with a glass rod and the excess was drained off. A sterile cork borer of 6 mm diameter was used to bore 8 wells on each plate. 0.1 ml of reconstituted extracts were introduced into six labeled well using automatic variable micropipette, and into the remaining 2 wells, one for Ciprofloxacin (250 mg/100 ml), positive control and the other one distilled water, negative control. The setup was allowed to stay on laboratory bench for 1 h for the extracts to diffuse into the agar. Then, the setup was incubated aerobically at 37°C for 24 h. The diameters of inhibition zones were measured with a 120 mm graduated ruler and the results were reported in millimeters (Asowata, et al., 2013).

Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

MIC of the plant extract was determined by the method of Asowata, et al., 2013). 0.1 ml of suspension of each standardized *S. typhi*, *S. Paratyphi* A, B and C, was inoculated into different series of sterile labeled test tubes of nutrient broth which contained various concentrations (80, 40, 20, 10, 5, and 2.5 mg/ml) of two-fold dilution of plant extract and was incubated at 37°C for 24 h. The minimum inhibition concentration was recorded as the least concentration (highest dilution) that inhibited the growth of tested organisms.

MBC of the plant extract was determined by the method described by Asowata et al. (2013). 1 ml of plant extract was pipetted from tubes which showed no visible growth of MIC, and was sub cultured on freshly prepared nutrient agar plates and incubated at 37°C for 24 h. The MBC was read and recorded as the highest dilution (lowest concentration) of the extract that did not show any colony growth on a new agar plate.

Statistical analysis

Quantitative phytochemical parameters and the minimum inhibitory diameter zone of plant crude extracts analyzed were carried out in triplicates. The data was subjected to statistical analysis to evaluate the differences between the phytochemical constituents and inhibitory diameter zone of the studied plants crude extracts. Data were expressed as mean standard error. Comparison of means was analyzed using one-way analysis of variance (ANOVA) on a statistical programme: Statistical Package for Social Science (SPSS) version 16.0 windows. The difference was significant at $P < 0.05$.

RESULTS AND DISCUSSION

The qualitative phytochemical constituents' present in the studied medicinal plants are shown in Table 1. Carbohydrates, tannins, cardiac glycosides, terpenoids, alkaloids, anthraquinones, flavonoids and saponins were the secondary metabolites present in the medicinal plants analyzed. This result agrees with the report by Paulsamy and Jeeshua (2011) who documented that plant secondary metabolites exhibit bioactive and physiological activities. These secondary metabolites are pharmacologically important and could account for their antibacterial activity.

Compared to aqueous solvent, ethanolic leaf extracts had higher quantity of secondary metabolites with a high degree of precipitation (+++) of alkaloids, tannins and terpenoids in the Mango leaves than in the Neem leaves and in the aqueous solvent (Table 1). Moderate degree of precipitation (++) of carbohydrates, cardiac glycosides, flavonoids, tannins and saponins were found in both aqueous and ethanolic extracts of Mango and Neem leaves (Table 1). Lesser/Slightly degree of precipitation (+) of alkaloids, anthraquinones, carbohydrates, cardiac glycosides, flavonoids and saponins, were found in both aqueous and ethanolic extracts of mango and Neem leaves (Table 1).

The ethanolic solvent extracts recorded a higher degree of precipitation than the aqueous solvent. This could be ascribed to the ethanolic solvents higher polarity, which contain a greater variety of plant constituents than aqueous solvent (Paulsamy and Jeeshua, 2011). Also, Doughari and Manzara (2008), reported that different solvents have different capacities for different phytoconstituents. Thus, the differences in the observed activities of various extracts may be due to varying degrees of solubility of the active constituents in the solvent used. However, aqueous solvent could not extract saponins from the Neem leaves in the study. This does not mean saponins are lacking in the plant. Rather, the analytical technique employed was not sensitive enough to extract the substance. In both aqueous and ethanolic leaf extracts of mango and Neem leaves, mango leaf extracts contained higher constituents of phytochemicals than neem leaf (Table 1). The differences in phytochemical constituents in plants could be due to geographical location, genetic constituents and the extraction method employed. However, the obtained secondary metabolite results agreed with the findings reported by Gatsing et al. (2007), Uhuo et al. (2015) and Rachuonye et al. (2016).

Table 2 shows the quantitative phytochemical profile of the studied medicinal herbs. Alkaloids, flavonoids, saponins and tannins contents in mango leaves are higher than the content found in neem leaves. Also, the total phenol content in neem leaves is higher than the content in mango leaves. The quantitative phytochemical constituents ranged between 0.90±0.01% cardiac glycosides in mango leaves and 13.11±0.00% tannins in mango leaves (Table 2). This demonstrates that the studied plants contain compounds which exhibited biological and physiological activities. As well as pharmacological significance that is directly related to secondary metabolites and accounts for their antibacterial activities (Gatsing et al., 2007; Uhuo et al., 2015; Rachuonye et al., 2016). Also, Petti and Scully (2009), documented that plants with a higher amount of phenolic content have the ability to be used to treat inflammatory diseases and can be implicated in wound healing. For instance, in the plants under study, the presence of phytochemical compounds probably justifies the use of

Table 1. Qualitative analysis of phytochemical screening of aqueous and ethanolic crude extracts of medicinal plants investigated.

Plant part	Solvents	Alkaloids	Anthraquinones	Carbohydrates	Cardiac glycosides	Flavonoids	Tannins	Terpenoids	Saponins
Mango leaves	Aqueous	+	+	+++	+++	+++	++	++	+
	80% ethanol	+++	+	++	++	++	+++	+++	++
Neem leaves	Aqueous	+	+	+	+	+	++	++	ND
	80% ethanol	++	+	++	++	++	++	++	++

+ = Slightly present; ++ = Moderately present; +++ = Heavy present; ND = Not detectable.
Source: Author

Table 2. Quantitative assays of phytochemical crude extracts of medicinal herbs studied.

Plant part	% Alkaloids	% Cardiac glycosides	% Flavonoids	% Total phenol	% Saponins	% Tannins
Mango leaves	1.62±0.13^{d}	0.90±0.01^{a}	11.37±0.20^{b}	9.68±0.03^{c}	13.27±0.04^{c}	13.11±0.00^{c}
Neem leaves	1.42±0.11^{d}	0.91±0.01^{a}	10.79±0.20^{b}	10.52±0.13^{b}	12.62±0.02^{d}	11.32±0.01^{e}
Overall mean	0.30	0.18	2.21	2.02	2.59	2.44
±SEM	0.05	0.01	0.16	0.16	0.06	0.10

$^{a-e}$Mean in the same column with different superscript differ significantly (P<0.05). •±SEM = Standard Error Mean. •Values are means of three determinations.
Source: Author

the selected plants for the treatment of typhoid fever. The differences in the phytochemical profile of the studied samples could be due to geographical location, genetic constituents, soil condition, variation in the season cycle, the age of plants and extraction method employed. However, results of the study, agree with the findings reported by Gatsing et al. (2007), Petti and Scully (2009), Uhuo et al. (2015) and Rachuonye et al. (2016). Notably, there are significant differences between the phytochemical constituents of the medicinal herbs studied (P < 0.05) (Table 2).

Antibacterial activities of aqueous and ethanolic extracts of mango and neem leaves against *S. typhi* and *S. paratyphi* A, B and C, at different extract concentrations are shown in Tables 3 and 4, respectively.

The extracts inhibited the growth of tested organisms at varying degrees of extract concentrations as shown by their diameter (mm) inhibition zones in Tables 3 and 4, respectively. *S. typhi* showed inhibition zones of 29.4±0.1^{b} mm at the highest aqueous extract concentration of 80 mg/ml, while *S. paratyphi* A, B and C, exhibited inhibition zones of 14.4±0.02, 21.2±0.04 and 13.4±0.01 mm, respectively (Table 3). Also, *S. typhi* exhibited inhibition zones of 30.0±0.1 mm at the highest ethanolic extract concentration of 80 mg/ml, while *S. paratyphi* A, B and C, showed inhibition growth of 18.0±0.03, 20.0±0.04 and 21.0±0.04 mm, respectively (Table 4).

At aqueous lower extract concentration of 10 mg/ml, *S. typhi* showed inhibition zone of 8.4±0.01 mm, while *S. paratyphi* A, B and C exhibited inhibition growth of 9.5±0.01, 9.1±0.01 and 6.2±0.01 mm, respectively as exhibited by neem leaf extracts (Table 3). Similarly, ethanolic extract at a lower concentration of 10 mg/ml, *S. typhi* showed an inhibition zone of 9.0±0.01 mm, while *S. paratyphi* A, B and C exhibited inhibition zones of 9.0±0.01, 9.0±0.01 and 9.0±0.01 mm, respectively in neem leaf extracts (Table 4).

Essentially, the neem leaves exhibited the highest diameter (mm) inhibition zones compared to mango leaves at all extract concentrations. This could be attributed to the genetic heterogeneity of plant species. In general, this study suggests that the plant extracts possess antibacterial potential

Table 3. Antibacterial activity of aqueous extracts of medicinal plant studied showing diameters (mm) inhibition zones at different extract concentrations against tested organisms.

Sample	Diameters (mm) Inhibition Zones											
	Salmonella Typhi [Concentration (mg/ml)]						*Salmonella Paratyphi A* [Concentration (mg/ml)]					
	80	40	20	10	+C	-C	80	40	20	10	+C	-C
Mango leaves	29.4±0.1^{b}	25.5±0.05^{b}	7.6±0.01^{b}	7.5±0.01^{b}	31.0±0.3^{b}	0.0±0.0^{b}	14.4±0.02^{b}	13.3±0.02^{b}	13.2±0.02^{b}	8.4±0.02^{b}	31.0±0.3^{b}	0.0±0.0^{b}
Neem leaves	29.1±0.1^{a}	27.7±0.05^{a}	9.7±0.01^{a}	8.4±0.01^{a}	31.0±0.3^{a}	0.0±0.0^{a}	15.2±0.03^{a}	13.5±0.02^{a}	3.5±0.01^{a}	9.5±0.01^{a}	31.0±0.3^{a}	0.0±0.0^{a}
Overall mean	5.9	5.3	1.7	1.8	6.2	0.0	3.0	2.7	1.9	2.0	6.2	0.0
	Salmonella Paratyphi B [Concentration (mg/ml)]						*Salmonella Paratyphi C* [Concentration (mg/ml)]					
	80	40	20	10	+C	-C	80	40	20	10	+C	-C
Mango leaves	19.2±0.03^{c}	12.2±0.02^{b}	8.3±0.01^{c}	31.0±0.3^{b}	0.0±0.0^{b}	19.2±0.03^{c}	13.4±0.01^{g}	10.5±0.01^{g}	10.6±0.01^{g}	3.4±0.01^{g}	31.0±0.3^{a}	0.0±0.0
Neem leaves	20.1±0.03^{a}	19.1±0.03^{a}	9.1±0.01^{a}	31.0±0.3^{a}	0.0±0.0^{a}	20.1±0.03^{a}	14.1±0.02^{b}	15.2±0.02^{b}	11.2±0.01^{b}	6.2±0.01^{b}	31.0±0.3^{a}	0.0±0.0
Overall mean	3.9	3.1	1.9	6.2	0.0	3.9	2.6	2.6	2.2	1.0	6.2	0.0

•a-g Mean in the same column with different superscript differ significantly ($p < 0.05$). • Values are means of three determinations. • ±= Standard Error. + = Positive control (2.5mg/ml, Ciprofloxacin). - = Negative control (Sterile distilled water).
Source: Author

Table 4. Antibacterial activity of ethanolic extracts of medicinal plant studied showing diameters (mm) inhibition zones at different extract concentrations against tested organisms.

Sample	*Salmonella* Typhi [Concentration (mg/ml)]						*Salmonella paratyphi A* [Concentration (mg/ml)]					
	80	40	20	10	+C	-C	80	40	20	10	+C	-C
Mango leaves	30.0±0.1^{b}	28.0±0.05^{b}	16.0±0.02^{b}	8.0±0.01^{b}	31.0±0.3^{b}	0.0±0.0^{b}	17.0±0.03^{b}	19.0±0.03^{b}	16.0±0.03^{c}	6.0±0.02^{b}	31.0±0.3^{b}	0.0±0.0^{b}
Neem leaves	30.0±0.1^{a}	26.0±0.05^{a}	18.0±0.02^{a}	9.0±0.01^{a}	31.0±0.3^{a}	0.0±0.0^{a}	18.0±0.03^{a}	20.0±0.03^{a}	18.0±0.03^{a}	9.0±0.01^{a}	31.0±0.3^{a}	0.0±0.0^{a}
Overall mean	6.0	5.4	3.4	1.7	6.2	0.0	3.5	3.9	3.4	1.5	6.2	0.0
	Salmonella paratyphi B [Concentration (mg/ml)]						*Salmonella paratyphi C* [Concentrations (mg/ml)]					
	80	40	20	10	+C	-C	80	40	20	10	+C	-C
Mango leaves	16.0±0.04^{c}	19.0±0.03^{c}	18.0±0.03^{b}	8.0±0.01^{c}	31.0±0.3^{b}	0.0±0.0^{b}	19.0±0.04^{g}	20.0±0.04^{g}	18.0±0.01^{g}	8.0±0.01^{g}	31.0±0.3^{b}	0.0±0.0^{b}
Neem leaves	20.0±0.04^{a}	20.0±0.03^{a}	19.0±0.03^{a}	9.0±0.01^{a}	31.0±0.3^{a}	0.0±0.0^{a}	21.0±0.04^{a}	21.0±0.04^{a}	16.0±0.03^{a}	9.0±0.01^{a}	31.0±0.3^{a}	0.0±0.0^{a}
Overall mean	3.6	3.9	3.7	1.7	6.2	0.0	3.0	4.1	3.4	1.7	6.2	0.0

•a-g Mean in the same column with different superscript differ significantly ($p < 0.05$). • Values are means of three determinations. •±= Standard Error. + = Positive control (2.5mg/ml, Ciprofloxacin). - = Negative control (Sterile distilled water).
Source: Author

for the tested organisms and demonstrates that inhibition zones increase with increasing extract concentrations, indicating that concentration influences the activities against the test organisms. The extracts of these plants could be alternative medicare for typhoid fever. The positive control,

Table 5. Minimum inhibition concentration (MIC, mg/mL) and minimum bactericidal concentration (MBC, mg/mL) of aqueous extract of studied medicinal plants against test organisms.

Sample	*Salmonella typhi*						*Salmonella Paratyphi A*						*Salmonella Paratyphi B*						*Salmonella Paratyphi C*					
	MIC			MBC			MIC			MBC			MIC			MBC			MIC			MBC		
	Extract Conc. (mg/mL)			Extract Conc. (mg/mL)			Extract Conc. (mg/mL)			Extract Conc. (mg/mL)			Extract Conc. (mg/mL)			Extract Conc. (mg/mL)			Extract Conc. (mg/mL)			Extract Conc. (mg/mL)		
	10	5	2.5	10	5	2.5	10	5	2.5	10	5	2.5	10	5	2.5	10	5	2.5	10	5	2.5	10	5	2.5
ML	+	+	-	+	-	+	+	+	-	+	-	+	+	+	-	+	-	+	+	+	-	+	-	+
NL	+	+	-	+	-	+	+	+	-	+	-	+	+	+	-	+	-	+	+	+	-	+	-	+

ML = Mango Leaf, NL = Neem Leaf, MIC = Minimum Inhibition Concentration, MBC = Minimum Bactericidal Concentration, Conc = Concentration(mg/ml), + = Growth/No Inhibition, - = No growth/ Inhibition of growth.
Source: Author

Table 6. Minimum inhibition concentration (MIC, mg/mL) and minimum bactericidal concentration (MBC, mg/mL) of ethanolic extracts of studied plants against tested organisms.

Sample	*Salmonella* Typhi						*Salmonella paratyphi A*						*Salmonella paratyphi B*						*Salmonella paratyphi C*					
	MIC			MBC			MIC			MBC			MIC			MBC			MIC			MBC		
	Extract Conc. (mg/mL)			Extract Conc. (mg/mL)			Extract Conc. (mg/mL)			Extract Conc. (mg/mL)			Extract Conc. (mg/mL)			Extract Conc. (mg/mL)			Extract Conc. (mg/mL)			Extract Conc. (mg/mL)		
	10	5	2.5	10	5	2.5	10	5	2.5	10	5	2.5	10	5	2.5	10	5	2.5	10	5	2.5	10	5	2.5
ML	+	+	-	+	-	+	+	+	-	+	-	+	+	+	-	+	-	+	+	+	-	+	-	+
NL	+	+	-	+	-	+	+	+	-	+	-	+	+	+	-	+	-	+	+	+	-	+	-	+

ML = Mango Leaf, NL = Neem Leaf, MIC = Minimum Inhibition Concentration, MBC = Minimum Bactericidal Concentration, Conc. = Concentration (mg/ml), + = Growth/ No Inhibition, - = No Growth/Growth Inhibition.
Source: Author

Ciprofloxacin (2.5mg/ml), showed an inhibition zone of 31.0±0.3 mm, while the negative control, sterile distilled water, recorded no inhibition zone. However, the zone of inhibition exhibited by the standard drug Ciprofloxacin, is higher than the plants extracts. This could be due to the crude nature of plant extracts which contained other constituents that do not possess antibacterial properties. Also, the ability of plant extracts to diffuse through the gel agar may be hindered because of the large molecules. Even at a higher extract concentration, the inhibition zones are not comparable with the zones of standard drugs.

However, the results of the study are in agreement with the findings documented by Gatsing et al. (2007), Tambekar and Dahikar (2011), Pankaj et al. (2015), Uhuo et al. (2015) and Rachuonyo et al. (2016). At different extract concentrations, there are significant differences between the mean diameter (mm) inhibition zones of aqueous and ethanolic plant extracts against tested organisms ($p < 0.05$).

The minimum inhibition concentration (MIC, mg/ml) and minimum bactericidal concentration (MBC, mg/ml) of aqueous and ethanolic extracts of studied medicinal plants against tested organisms are shown in Tables 5 and 6 respectively.

At different plant extract concentrations tested organisms were inhibited by both aqueous and ethanolic extracts. Aqueous extracts showed MIC against *S. typhi*, *S. paratyphi* A, B and C, at 2.5 mg/ml extract concentration, while at 5 mg/ml concentration, the aqueous extract showed MBC against the tested organisms (Table 5). Similarly, at 2.5 mg/ml ethanolic extract concentration, the extracts exhibited MIC against tested organisms, while at 5 mg/ml ethanolic extract concentration the extracts showed MBC against tested organisms (Table 6).

The lowest MIC of 2.5 mg/ml was exhibited by all tested organisms, and shows that the

organisms are more sensitive to the extracts (Tables 5 and 6). This supports the claim by traditional medical practitioners and vendors that the crude extracts of the investigated medicinal plants are a remedy for the cure of typhoid fever. All tested organisms showed a higher MBC than the MIC (Tables 5 and 6). This demonstrates that higher concentrations of extracts were needed to kill the bacteria than to inhibit their growth.

However, the results of the study differ with the findings by Gatsing et al. (2007), Agada et al. (2010) and Rachuonye et al. (2016). This could be due to genetic heterogeneity of plant species, soil factor, variation in season cycle, the age of plants, climatic influences and different geographical locations where plants were collected. Notably, Rao and Rout (2003), reported that there is a relationship between chemical composition of plants and their geographical location.

Conclusion

The aqueous and ethanolic extracts of investigated medicinal plants showed activities against all tested organisms at different extract concentrations. The aqueous and ethanolic plant extracts exhibited MIC and MBC against all tested organisms at 2.5 and 5 mg/ml concentrations, respectively. The qualitative and quantitative profile of studied medicinal plants is rich in phytochemical compounds which exhibit biological and physiological activities. This demonstrates that the plants have pharmacological significance that is directly related to the secondary metabolites that account for their antibacterial properties. The diameter (mm) inhibition zones of the medicinal plants studied indicate that they are good candidate for typhoid medicare alternative. However, there are significant differences between the quantitative phytochemical content and diameter (mm) inhibition zones of aqueous and ethanolic extract concentrations against tested organisms ($p < 0.05$).

Recommendations

Further studies are recommended on the toxicity and safe dosage regimen of the plants since the infusion of the plants are taken orally by local people for the treatment of typhoid fever. Traditional medical practitioners and vendors should be educated about modern and traditional medicine through the use of plants compounds. This will eliminate the challenges to phytomedicine, such as the lack of reproducibility of biological activity of individual herbal extracts after the success of the initial screening process, toxicity, contamination and adulteration, standardization and drug interaction issues. The loss of medical plant species due to risk of extinction as a result of high harvest and destruction of habitats, decrease in wildlife reservoir due to growing human population and excessive conservation of plants should be avoided and should be backed up by legislation.

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CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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