ASSESSMENT (RAPD) OF AFRICAN MAHOGANY (*Khaya senegalensis* (Desr.) A. Juss) AND NEEM (*Azadirachta indica* A.Juss) AND THEIR HERBAL PRODUCTS MANAGEMENT OF DIABETES IN NASARAWA STATE AND THE F.C.T., ABUJA

BY

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DECLARATION

I declare that this dissertation entitled "Assessment (RAPD) of African Mahogany (Khaya *senegalensis* (Desr.) A. Juss) and Neem (*Azadirachta indica* A. Juss) and their Herbal Products Management of Diabetes in Nasarawa State and the F.C.T., Abuja" was written by me and it is a report of my research work. It has never been presented in any previous application for Masters' degree. All quotation is indicated and sources of information specifically acknowledged by means of references.

_____

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CERTIFICATION

This dissertation entitled "Assessment (RAPD) of African Mahogany (Khaya *senegalensis* (Desr.) A. Juss) and Neem (*Azadirachta indica* A. Juss) and their Herbal Products Management of Diabetes in Nasarawa State and the F.C.T., Abuja" meet the regulation governing the Degree of Masters of Science in Plant Genetics and Breeding of Nasarawa State University, Keffi for its contribution to scientific knowledge and literary presentation.

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DEDICATION

This work is dedicated to God Almighty. I also wish to dedicate it to my late sister, Miss Mowobi, Gbolahan Oluwatoyin, an icon of peaceful approach to everything in life. RIP Sister.

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TABLE OF CONTENT

Tittle - - - - - - - - - i

Declaration - - - - - - - - - ii

Certification - - - - - - - - - iii

Dedication - - - - - - - - - iv

Acknowledgments - - - - - - - - v

Table of content - - - - - - - - vi

List of Tables - - - - - - - - - x

List of Figures - - - - - - - - - xi

List of Plates - - - - - - - - - xii

Abstract - - - - - - - - - xiii

CHAPTER ONE

INTRODUCTION

1.1 Background of the study - - - - - - 1

1.2 Statement of the problem - - - - - - 6

1.3 Justification - - - - - - - - - 6

1.4 Aim - - - - - - - - - 7

1.5 Objectives - - - - - - - - - 7

CHAPTER TWO

LITERATURE REVIEEW

2.1 Botanical description of *Khaya senegalensis* - - - 8

2.2 Botanical description of *Azadirachta indica* - - - 9

2.3 Molecular basis of genome-based authentication of plant - - 12

2.4 Molecular techniques used for medicinal plant authentication- - 13

2.5 Molecular markers - - - - - - - 14

2.6 Molecular biological methods used for the authentication of medicinal Plants - - - - - - - - - 16

2.7 Nuclear and chloroplast gene used for authentication of medicinal plant- 19

2.8 RAPD (Random Amplified Polymorphic DNA) - - - 21

2.9 Diabetes Prevalence- - - - - - - - 23

2.10 Adulterants, substitutes, and confused species - - - 25

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study area - - - - - - - - 26

3.2 Collection and storage of herbs/plant - - - - 26

3.3 Genomic DNA extraction - - - - - - 27

3.3.1 Modified SDS method - - - - - - 27

3.3.2 Modified CTAB method - - - - - - 28

3.3.3 Modified CTAB method 2 - - - - - - 28

3.4 Estimation of quality and quantity of isolated genomic DNA - 29

3.4.1 Agarose gel electrophoresis - - - - - - 29

3.4.2 Estimation of the quality and quantity of the extracted genomic DNA by UV spectrophotometry - - - - - - - 30

3.5 RAPD-PCR - - - - - - - - 30

3.6 Preparation of stock solutions for primers - - - - 32

3.7 Preparation of T.E. buffer - - - - - - 32

3.8 Constitution of primers - - - - - - 32

3.9 Preparation of working solutions for primers - - - - 32

3.10 Procedure for RAPD-PCR - - - - - - 33
3.11 RAPD-PCR reaction mixture - - - - - - - 33
3.12 PCR Agarose gel electrophoresis - - - - - - 36
3.13 Analysis of Data - - - - - - - - 36

CHAPTER 4

RESULTS

4.1. DNA extraction result - - - - - - - 38

4.2. Quantity and quality analysis result of genomic DNA extracted - 41

4.3. PCR Picture of the different primer on both the herbal and plant sample 44

4.4. Scoring of the different primers for both the herbal and plant sample - 56

4.5. Percentage polymorphism of primers - - - - - 59

4.6. Dendogram - - - - - - - - 62

CHAPTER FIVE

DISCUSSION, CONCLUSION AND RECOMMENDATION

5.1. Discussion - - - - - - - - 64

5.1 Conclusion - - - - - - - - - 67

5.2. Recommendations - - - - - - - - 68

References - - - - - - - - - 69

LIST OF TABLES

CHAPTER THREE

MATERIALS AND METHODS

3.6 Primer designation and sequence - - - - - 31

3.11 Cycling conditions for RAPD – PCR - - - - 34

3.12 Masyer mix for RAPD-PCR amplification - - - - 35

CHAPTER 4

RESULTS

4.2 Quantitative and qualitative analysis result of genomic DNA extracted from Plant sample of Both *Khaya senegalensis* and *Azadirachta indica* - 42

4.3 Quantitative and qualitative analysis result of genomic DNA extracted from herbal sample - - - - - - - - - 43

4.4 Scoring of the primer for herbal sample - - - - 57

4.4 Scoring of the primer for plant sample - - - - 58

4.5 Percentage polymorphism of primers for plant sample - - 60

4.5 Percentage polymorphism of primers for herbal sample - - 61

LIST OF FIGURE

CHAPTER 4

RESULTS

Dendogram indicating similarity matrix among the herbal and plant sample- 63

LIST OF PLATES

CHAPTER TWO

LITERATURE REVIEEW

2.2 *Khaya senegalensis* - - - - - - - 10

2.2 *Azadirachta indica* - - - - - - - 11

CHAPTER 4

RESULTS

4.1 Plant sample DNA - - - - - - - 39

4.1 Herbal sample DNA - - - - - - - 40

4.3 PCR picture of primer OPL 01 plant sample - - - - 45

4.3 PCR picture of primer OPL 01 herbal sample - - - 46

4.3 PCR picture of primer OPA 01 herbal sample - - - 47

4.3 PCR picture of primer OPA 01 plant sample - - - - 48

4.3 PCR picture of Primer OPJ 01 plant sample - - - - 49

4.3 PCR picture of primer OPJ 01 herbal sample - - - - 51

4.3 PCR picture of primer OPA 02 herbal sample - - - 52

4.3 PCR picture of primer OPA 02 plant sample - - - - 53

4.3 PCR picture of primer OPB 01 herbal sample - - - 54

4.3 PCR picture of primer OPB 01 plant sample - - - - 55

ABSTRACT

Khaya senegalensis and *Azadirachta indica* are common plants that have been used in the management of diabetes. In the present investigation, RAPD Marker Assessment was carried out on Herbal Products from *Khaya senegalensis* and *Azadirachta indica being* used in the Management of Diabetes in Nasarawa State and Federal Capital Territory (FCT). Eleven decamer oligonucleotide primers were screened in the RAPD analysis for authentication of claim for genuine and adulterant samples. The Deoxyribonuclic Acid (DNA) isolated from the dried herbal samples was used as templates in polymerase chain reactions with the eleven primers. Five of the eleven primers gave species specific reproducible unique amplicons. The unique amplicons obtained in PCR amplification and the data generated from the amplification of the DNA were subjected to Numerical Taxonomy System of Statistic (NTSYS). From the phylogenetic tree generated among the 24 samples based on PCR product, it was observed that the relatedness was 37.5%. The analysis also showed that there was no strong interrelationship between herbal samples and plant sample at that index. At the end of the investigation, the result showed that the herbs contained both *Khaya senegalensis* and *Azardirachta indica* as they claim though; the quantity is low (37.5%).

CHAPTER ONE

INTRODUCTION

1.1 Background of the Study

Many plants have been used for herbal and medicinal purposes not only by humans since prehistoric times (Hart, 2005 and Halberstein, 2005), but are also used to treat various ailments by our closest relatives, the African great apes (Huffman, 2001; Krief*et al.*, 2005). Up on till now, medicinal plants constitute the source of a large number of chemical compounds used as drugs in Western medicine and serve as the primary therapeutic resource for most of the world's population living in developing countries. The use of herbal preparations for health care purposes is also gaining popularity in developed countries at the same time (Ernst, 2005). The increased demand for botanical products is met by an expanding industry and accompanied by calls for assurance of quality, efficacy and safety (Cardellinaj, 2002). Many ethnobotanical surveys on medicinal plants used by the local population have been performed in different parts of the world including Nigeria, China, Morroco, Saudi Arabia, Taiwan, and Trinidad and Tobago etc (Ivorra*et al.,* 1989; Bnouham*et al.*, 2002 and Al-Rowais, 2002).

Herbal medicines are often used as therapeutic remedies in combination with allopathic drugs. Most of the doctors did not report any complication, but nausea, vomiting, gastric problems were the common adverse effects reported with Polyherbal formulations (PHF). Usually ayurvedic drugs are being used due to their minimum toxicity. The basis prescription of ayurvedic drugs was mainly found to be past experience of the patients. Practicing physicians expected that controlled clinical trials of the herbal

antidiabetesshould be conducted in humans at different hospitals to substantiate the efficacy claim. Herbal medicines can be relevant today only if they are applied and tested within the framework of modern sciences and subjected to the rigorous criteria for quality, safety and efficacy. Only then, herbal products can be comparable with modern medicines and can bring necessary confidence in prescribing doctors. Major hindrance in amalgamation of herbal medicine in modern medical practices is lack of scientific and clinical data proving their efficacy and safety. There is a need for conducting clinical research in herbal drugs, developing simple bioassays for biological standardization, pharmacological and toxicological evaluation, and developing various animal models for toxicity and safety evaluation. It is also important to establish the active components from these plant extracts (Dey*et al.*, 2002).

Diabetes mellitus is a complex metabolic disorder resulting from either insulin insufficiency or insulin dysfunction. Type I diabetes (insulin dependent) is caused due to insulin insufficiency because of lack of functional beta cells. Patients suffering from this are therefore totally dependent on exogenous source of insulin while patients suffering from Type II diabetes (insulin independent) are unable to respond to insulin and can be treated with dietary changes, exercise and medication (Matteucci and Giampietro, 2000). Type II diabetes is the more common form of diabetes constituting 90% of the diabetic population. Symptoms for both diabetic conditions may include:

(i) high levels of sugar in the blood;

(ii) unusual thirst;

(iii) frequent urination;

(iv) extreme hunger and loss of weight;

(v) blurred vision;

(vi) nausea and vomiting;

(vii) extreme weakness and tiredness;

(viii) irritability, mood changes etc. (Matteucci and Giampietro, 2000).

Though pathophysiology of diabetes remains to be fully understood, experimental evidences suggest the involvement of free radicals in the pathogenesis of diabetes (Matteucci and Giampietro, 2000), and more importantly, in the development of diabetic complications (Lipinski, 2001: Baynes and Thorpe, 1997). Free radicals are capable of damaging cellular molecules, DNA, proteins and lipids leading to altered cellular functions. Many recent studies reveal that antioxidants capable of neutralizing free radicals are effective in preventing experimentally induced diabetes in animal models (Naziroglu and Cay, 2001) as well as reducing the severity of diabetic complications (Lipinski, 2001).

Medicinal plants are being looked up once again for the treatment of diabetes. Many conventional drugs have been derived from prototypic molecules in medicinal plants. Metformin exemplifies an efficacious oral glucose-lowering agent. Its development was based on the use of *Galegaofficinalis* to treat diabetes. *Galegaofficinalis* is rich in guanidine, the hypoglycemic component. Because guanidine is too toxic for clinical use, the alkyl biguanidessynthalin A and synthalin B were introduced as oral anti-diabetic agents in Europe in the 1920s but were discontinued after insulin became more widely available (Dey*et al.*, 2002). However, experience with guanidine and biguanides

prompted the development of metformin. To date, over 400 traditional plant treatments for diabetes have been reported, although only a small number of these have received scientific and medical evaluation to assess their efficacy. The hypoglycemic effect of some herbal extracts has been confirmed in human and animal models of type II diabetes (Dey*et al.,* 2002). The World Health Organization Expert Committee on diabetes has recommended that traditional medicinal herbs be further investigated.

*Azadirachtaindica*hasbeen used for many natural chemist products such as antiviral, antibacterial, antifungal and in the management of diabetes. Neem tree is used since time immemorial in the treatment of large number of diseases. It uses has been mentioned in the early test as naturally occurring wonder drugs curing diseases like blood pressure and diabetes. In the modern times, diabetes is more of a life style diseases rather than anything else; neem is being widely used by herbal medicine manufacturers and pharmaceutical companies as more and more people all across the globe fall back to it (Singh *et al.*, 1999).

Neem leaf extracts and seeds are used as active ingredient as an effective cure for diabetes. The popularity of Neem based anti diabetes drugs is due to the fact that not only are they more effective in curing the diseases but also have no side effects. There are no synthetic material used in the production of the drugs. Neem is considered to be one of the safest medicinal herbs available (Kundu, 2000).

Khayasenegalensis is a medicinal plant that is widely used in the treatment of various diseases in Nigeria and other West African countries. The stem back extract is used in the treatment of malaria and jaundice. The seed and the leaves are also used to treat diabetes,

fever and headache while the root extract is used to treat mental illness, leprosy and syphilis. (De bie*et al.,* 1998). Although *Khayasenegalensis*is of such great medicinal value, information on its effects on diabetes is very scanty. People taking diabetes treatment also use *Khayasenegalensis*to further relief their pain or to benefit from its other medicinal properties. The efficacy and safety of the co-administered drugs as it is practice, may not be objectively isolated and assessed (De bie*et al.,* 1998).

Molecular approach has proved itself an increasingly valuable tool in the identification of plant varieties (Morell*et al*., 1995). Some of the commercially desirable plant is reproduced by micro-propagation; therefore, all individuals belonging to a given variety share an identical genome. Lack of intra-variety genetic variability greatly simplifies the task of identification because any difference between two given individuals unambiguously indicates that they belong to different varieties. One of the most successful techniques in this field is random amplified polymorphic DNA (RAPD) (Welsh and McClelland, 1990; Williams *et al.,* 1990). It has two main advantages: it allows random sampling of markers over whole genomic DNA and does not require any previous information on the genome of the organism under investigation.

PCR-based markers involve amplification of particular DNA loci, with the help of specific or arbitrary oligonucleotide primers and a thermostable DNA polymerase enzyme (Gunther, 2010). The major advantages of PCR techniques are that mainly only a small amount of DNA is required, no prior sequence information is needed, and many genetic markers can be generated within a short time. Hybridization-based methods (RFLP) involves DNA hybridization a process in which two single stranded DNA fragments anneal into a double-stranded nucleic acid. Restriction fragment length

polymorphism (RFLP) implies that a single restriction enzyme produces fragments of different lengths from the DNA marker of different strains of a species or from different related species (Gunther, 2010).

1.2 Statement of the Problem

Diabetes is an ailment popular among the old and even in recent time, it is becoming alarming among the children who inherited it through genetic transfer. The frequency among Nigerian population is also on the increase, this has prompted the interest in the choice of research. Also, Poor identification methods of medicinal plant and their extract has been the major factor that has affecteddevelopment of herbal medicine and practices. The concoction of Khaya and Neem is among othersemphasizedas treatment for diabetes and the use of (RAPD) molecular method of identification is among other method important for the verification of this claim.

1.3 Justification

The use of molecular maker to identify and authenticate this medicinal plant has reduced the dangerous effect of some of this concoction; it has also help in quantifying the active ingredient in this plant. Random Amplified Polymorphic DNA marker will use to identify and quantify the presence or absence of the so called active ingredient (Khaya and Neem). The research work will help to confirm or disprove the claim by this herbal practitioners in and aroundNasarawa State and the FCT and there Environs.

1.4 Aim

This project is aimed at using RAPD marker to assess *Khayasenegalensis* and *Azadirachtaindica* herbal products used in the management of diabetes sold in Nasasrawa State and Abuja F.C.T.

1.5 Objectives

The specific objectives of the project are as follows:

i. To design and develop best RAPD marker that are able to amplify *Khayasenegalensis* and *Azadirachtaindica* medicinal plant
ii. To use amplifiable RAPD markers from K*hayasenegalensis* and *Azadirachtaindica* to charracterise the herbal products from Nasarawa State and F.C.T Nigeria.

CHAPTER TWO

LITERATURE REVIEW

2.1 Botanical Description of *Khayasenegalensis*

Khayasenegalensis (Plate 1) also known as African mahogany or dry-zone mahogany belongs to the family Meliaceae that is native to Africa. *Khayasenegalensis* is a deciduous tree, 15-20 m tall, reaching up to 35 m on fertile soils; diameter can get up to 1.5 m and with 8-16 m clean bole; buttresses not prominent or absent. The bark is often dark grey, with slash dark pink with red latex. Leaves are compound, up to 20 cm long, with 3-7 pairs of usually opposite leaflets and each leaflet is 7-12 cm long, with a width of 3-5 cm, underside grey (Nikel*et al.*, 2008). The flowers are small about 5 mm with white petals that are unisexual, but with well-developed vestiges of the opposite sex, making it difficult to distinguish between male and female flowers. Flowers are borne on up to 20 cm long, much branched inflorescences. Flowering occurs shortly before or in the beginning of the rainy season and the flowers are pollinated by insects (White and Powell, 1997).

The timber of *Khayasenegalensis* is rated as one of the best African mahogany woods. It is moderately hard and of medium density (0.6-0.8). The heartwood is usually deep red-brown with a purplish tinge and the attractive appearance that makes it one of the most favoured timbers for furniture. Besides timber, the species is valued for medicinal purposes, especially the bark which is used to treat a number of diseases. The leaves are used for fodder at the end of the dry season but the forage value is low. In West Africa,

oil from the seed is used for cooking. The seeds have an oil content of 67% and are rich in oleic acid. The wood ashes are used for storing millet seed (Nikel*et al.,* 2008).

2.2Botanical Description of*Azadirachtaindica*

Azadirachtaindica(Plate 2)also known *as* neem or margosa, has original natural distribution that is by widespread cultivation, it also belongs to the family Meliaceae. Neem*(Azadirachtaindica)* tree is a hard fast growing evergreen tree with a straight trunk, long spreading branches and moderately thick, rough, longitudinally bark. Matured trees attain a height of 7-15m (23-50 feet) (Koul*et al.,* 2006). The tree starts producing the yellowish ellipsoidal drupes (fruits) in about four years, and becomes fully productive in ten years and may live for more than 200 years. The leaves are compound imparipinnate, comprising up to 15 leaflets arranged in alternate pairs with terminal leaflets (Koul*et al.,* 2006). The leaflets are narrow, lancelet and up to 6cm long. The flowers are abundant with sweet smelling white panicles in the leaf axils. The seed of Neem*(Azadirachtaindica)* fruit is yellow when ripe and is about one inch long (Koul*et al.,* 2006). Neem roots have its applications in medicine (Udeinya*et al.,* 2008).

Wood from Neem tree is very strong and valued for furniture, carts, boards, panels, etc. Small twigs are used as toothbrushes. Neem Fruits are rich in azadirachtin and other pesticide compounds. Leaves are good fodder and also have medicinal and insect repellent properties. The pulp of the ripe fruits is used as tonic, purgative, emollient and anthelmintic. Seed kernel used as an insecticide effective ag-ainst a large number of insects. The seed kernel also yields margossa oil, which is mainly used in the making of soap and toothpaste. Bark is used for various medicinal purposes, primarily anti-

protozoal, anti-allergic, anti-dermetitic and anti-fungal. Anti-spermicidal properties have also been identified in neem plant (Mohd*et al.*, 2012).



Plate 1: *Khayasenegalensis*(Field survey)



Plate 2: *Azadirachtaindica*(Field survey)

2.3 Molecular Basis of Genome-Based Authentication of Plant

Plant DNA comprises three independently replicated genomes. In addition to the nuclear genome that is organized in chromosomes, plants contain circular chloroplast and mitochondrial genomes (Sucher and Carles, 2008). The nuclear DNA content (C-value) varies approximately 1000-fold across the angiosperms but exact C-values based on genome sequencing have not been obtained for any angiosperm to date (Bennett and Leitch, 2005). The chloroplast genome in angiosperms ranges in size between 120 and 220 kb (Lilly *et al.*, 2001) and the plant mitochondrial genome vary in size from 200 kb in Brassica to over 2.5 Mb in watermelon and are substantially larger than that in animals, which is only between 15–18 kb (Levings and Brown, 1989). Interestingly, "whole" genome size determined by sequencing is generally smaller than the C-values indicate, as considerable amounts of genomic DNA cannot be cloned and sequenced with currently available techniques (Bennett and Leitch, 2005). For example, the *Arabidopsis* Genome Initiative estimated the "genome" size of *Arabidopsis thaliana* at ~ 125 Mb (115.4 Mb in the sequenced regions plus an estimated 10 Mb in unsequenced regions) but recent data indicate that it may be considerably larger at 157 Mb (Bennett and Leitch, 2005). The use of genome-based methods for the authentication of medicinal plants should be seen in the context of plant phylogenetic studies and a general effort aimed at barcoding of all plants (Kress *et al.*, 2005; Newmaster*et al.*, 2006; Small *et al.*, 2004).

Genetic loci commonly used for the authentication of medicinal plants have included the internal transcribed spacers (ITS) that separate the coding regions of the nuclear 5.8S, 18S and 26S rRNA genes (Baldwin *et al.*, 1995; Alvarez and Wendel, 2003) and the intergenic spacers that separate multiple repeated copies of the nuclear 5S rRNA gene

(Hillis and Dixon, 1991). On the other hand, genetic loci used in phylogenetic studies include several chloroplast-based genes (Olmstead and Palmer, 1994; Palmer *et al.,* 1998) such as intron and intergenic spacers between the genes. It is noteworthy that the ITS and 5S spacers have been found to lack sufficient discriminatory power in some phylogenetic studies. In fact, sequence data from a single gene have proved to be insufficient for barcoding purposes in plants because multiple closely related species have been found to possess identical sequences at some loci. Consequently, the consensus view has developed that the unequivocal identification and barcoding of all plant species will require consideration of sequence data from more than one locus (Kress *et al.,* 2005; Newmaster*et al.,* 2006; Pennisi, 2007).

The generation of molecular "barcodes" of medicinal plants and deposition of sequence data in publicly accessible databases will be worth the concerted effort of the medicinal plant research community and contribute to the on-going effort of defining barcodes for every (plant) species on earth. Along these lines, future studies aimed at the authentication of medicinal plants using genomic methods should focus on genetic loci that have been found useful for barcoding of plants in general, in addition to those previously described in the literature (Sucher and Carles, 2008).

2.4 Molecular Techniques used for Medicinal Plant Authentication

These procedures can be broadly divided into two general approaches. In one approach, investigators determine the nucleotide sequence of one or more genetic loci ("genes") in the plants of interest and identify nucleotide sequences that are characteristic (i.e., inherited by all members) of a given species(Sucher and Carles, 2008). Examples of

techniques that are based on this approach include allele-specific diagnostic PCR, amplified refractory mutation system (ARMS), multiplex amplification refractory mutation system (MARMS), DNA microarray and DNA sequencing. Nuclear chloroplast gene used for authentication of medicinal plant is selected from a particular region for the phylogenetic studies in plant.

In a second approach, rather than focusing on specific genetic loci, researchers make use of species-specific variations (polymorphisms) of the nucleotide sequence that are spread randomly over the entire genome resulting in characteristic "fingerprints" of genomic DNA. Examples of techniques that are based on this approach include amplified fragmented length polymorphism (AFLP), arbitrarily primed PCR (AP-PCR), direct amplification of length polymorphism (DALP), randomly amplified polymorphic DNA (RAPD), restriction length polymorphism (RFLP), inter simple sequence repeat anchored PCR and simple sequence repeat polymorphism (SSR). The PCR and its numerous variations are central to both approaches and virtually all of the published genome-based authentication work employs this technique. PCR was originally developed for the directed amplification of predetermined regions of genomic DNA using primers with a specific sequence and is used in this way for the cloning and sequencing of specific genetic loci (Sucher and Carles, 2008).

2.5 Molecular Markers

Molecular markers are commonly used to characterize genetic diversity within or between populations or groups of individuals because they typically detect high levels of polymorphism. Furthermore, RAPDs and AFLPs are efficient in allowing multiple loci to

be analysed for each individual in a single gel run (Dalirsefat*et al.,* 2009). In analysing banding patterns of molecular markers, the data typically are coded as (0,1)-vectors, 1 indicating the presence and 0 indicating the absence of a band at a specific position in the gel. With diploid organisms and codominant markers, such as allozymes, RFLPs or SSRs, the banding patterns may be translated to homozygous or heterozygous genotypes at each locus, and the allelic structure derived is utilized for comparison between individuals (Peakall*et al.,* 1995; Maguire *et al.,* 2002). More often, however, the binary patterns obtained are used directly in comparisons of similarity of individuals (Kosman and Leonard, 2005).

A genetic marker may be defined as gene or a nucleotide sequence on a chromosome that has the potential to differentiate cells, individuals or species. As the DNA sequences are highly specific, they can be identified with the help of the known molecular markers which can find out a particular sequence of DNA from a group of unknown (Showkat*et al.,* 2005)

Molecular tools facilitate in the identification of genomic locations linked to traits of interest and help in indirect selection of such complex traits beside the phenotypic measurements (Awaad*et al.*, 2010).

DNA- based molecular markers that are based on analysis of the unique genetic structure are undoubtedly higher level markers and have the upper hand over other marker systems because these are not affected by environmental factors, age and physiological conditions (Showkat*et al.,* 2005). However, these molecular markers are not tissue specific and so can be detected at any stage of plant development. Compared with phenotypic and

chemical markers, DNA- based technology can provide an efficient, accurate and cheaper means of testing the authenticity of hundreds of samples simultaneously as these can be amenable to automation. DNA based authentication of medicinal plants can be useful as a tool for quality control and safety monitoring of herbal pharmaceuticals and neutraceuticals and will significantly add to the medical potential and commercial profitability of herbal products (Showkat*et al.,* 2005).

2.6 Molecular Biological Methods Used for the Authentication of Medicinal Plants

Polymerase chain reaction (PCR): This provides an *in vitro* method for the rapid enzymatic amplification of fragments of deoxyribonucleic acid (DNA) (Saiki *et al.,* 1988 and Saiki *et al.,* 1885). In the PCR procedure, two oligonucleotide primers (often referred to as "upstream" and" downstream" or "forward" and "reverse" primers) that are complementary to the 5 ′ and 3 ′ flanking sequences of the DNA to be amplified are used to prime a heat-stable DNA polymerase that performs the copying of each strand of DNA. The denaturation of the DNA double helix, the annealing of the oligonucleotide primers to each complementary strand, and the synthesis of new strands by DNA polymerase are performed at their optimal temperature resulting in a three-step reaction. PCR is conducted in fully programmable thermocyclers that change the reaction temperatures at each step automatically (Dieffenbach and Dveksler, 2003).

Allele-specific diagnostic PCR: Primers with allele specific 3 ′ Ends and labelled with different fluorochromes at their 5 ′ end are used together with a common primer in PCR (Gupta *et al.,* 2001). The resulting amplicons can be analyzed by gel electrophoresis or capillary electrophoresis using an automated DNA sequencer.

Amplification refractory mutation system (ARMS): This variation of the PCR is based on the fact that the primers only bind to their target sequence when their 3′ -ends are complementary. Oligonucleotides with mismatched ("mutated") 3 ′ end residues will not bind to the "normal" target sequence and no amplification will take place (Newton *et al.*, 1998).

Amplified fragmented length Polymorphism (AFLP): In this technique, genomic DNA is digested with restriction enzymes. In a ligation reaction specific oligonucleotide adapters are added to the ends of the fragments, which can then be selectively amplified by PCR using primers that are complementary to the adapter and restriction site sequence (Vos*et al.*, 1995).

Arbitrarily primed PCR (AP-PCR): Similar to RAPD but AP-PCR is performed using sets of two longer primers (>18nucleotides) of arbitrary sequence (Vos*et al.*, 1995).

Direct amplification of length polymorphism (DALP): This is conducted with variable forward primers that contain a universal core sequence at their 5 ′ end and a constant reverse primer resulting in multiple amplicons that can be separated by gel electrophoresis, isolated and directly sequenced (Desmarais*et al.*, 1998).

Multiplex PCR: Multiple sets of forward and reverse primers in the same reaction resul in parallel amplification (Dieffenbach and Dveksler, 2003).

PCR-selective restriction (PCR-SR): The PCR amplicons obtained with gene specific primers are cut with restriction enzymes and analysed by gel electrophoresis (Lin *et al.*, 2006).

Randomly amplified polymorphic DNA (RAPD): Genomic DNA (gDNA) is amplified by PCR using a single, short (10 nucleotides) primer with arbitrary sequence result in gin multiple amplicons of different lengths ("fingerprint" pattern) that are analysed by gel electrophoresis (Williams *et al.,* 1990).

Sequence characterized amplified region (SCAR): Genomic DNA is cut with sequence specific DNA restriction endonucleases resulting in the generation of a number of small fragments of various lengths, which are separated according to their molecular size by gel electrophoresis. The band pattern obtained with a specific DNA source and a specific restriction enzyme is called a DNA finger print of that source (Sucher and Carles, 2008).

DNA microarray: A DNA microarrays, also often referred to as gene chip, DNA chip, or gene array, consists of a solid support matrix (e.g. a glass slide, silicon chip or synthetic membrane) to which DNA has been covalently bound in the form of a collection of microscopic spots (Schena*et al.,* 1998). Each spot contains DNA of a defined sequence that is referred to as the probe. Fluorescently labelled target DNA is hybridized to the chip, which is washed and then analysed using a microarray reader(Schena*et al.,* 1998).

DNA sequencing: DNA sequencing is now almost exclusively performed using cycle sequencing, which is conducted using a heat stable DNA polymerase and fluorescently labelled dideoxynucleotides in a thermocyler. The resulting polymerase products are separated according to length using capillary electrophoresis, detected by laser-induced fluorescence and automatically analysed by computer software (Kretz*et al.*, 1994). Older methods making use of radio actively labelled nucleotides and gelelectrophoresis are still

in use and may be the only option, when access to automated sequencers is not available(Kretz*et al.,* 1994).

Inter simple sequence repeat-anchored PCR (ISSR-PCR): In ISSR-PCR, primers anchored at simple sequence repeat (SSR) sequences (e.g., CACACACA; see below) are used to amplify the DNA regions between the flanking SSR (Zietkiewicz*et al.*, 1994).

Multiplex amplification refractory mutation (MARMS): Multiplex PCR using a common primer and multiple mutation specific primers as used in ARMS (Ziet-kiewicz*et al.*, 1994).

Simple sequence repeat polymorphism (SSR): Simple sequence repeats (SSRs) or micro satellites are short sequence motifs consisting of 2 or more nucleotides (e.g., CA and ATG), which repeat in tandem (e.g., CACACA and ATGATGATG). The repeats vary in length (e.g., CACACA vs. CACACACACACA) and are ubiquitously and randomly distributed in all eukaryotic genomes. The length polymorphisms can be easily detected by gel electrophoresis of amplicons generated by PCR using unique pairs of primers flanking the repeat (Weber and May, 1989).

2.7 Nuclear and Chloroplast Gene Used for Authentication of Medicinal Plant

18SrRNA (Nuclear): The 18S ribosomal ribonucleic acid (rRNA) sequences have been widely used for phylogenetic studies in plants (Kuzoff*et al.*, 1998).

Internal transcribed spacers (ITS) of 18S, 5.8S and 26S rRNA (Nuclear): In land plants, the 18S, 5.8S and 26S rRNA genes form a linearly arrayed unit (acistron) in which the individual coding regions separated by 2 internal transcribed spacers (ITS; ITS1

between the 18S and 5.8S genes and ITS2 between 5.8S and 26S genes). The cistron itself is tandemly arrayed separated by external transcribed spacers (ETS) on one or more chromosomes (Small *et al.,* 2004, Hillis and Dixon 1991 and Wendel and Álvarez 2003). The ITS region has been used in many phylogenetic studies (Baldwin *et al.,* 1995).

Intergenic spacer of the 5S rRNA [(5S gene spacer) Nuclear]: In land plants, the genes for the 5S ribosomal RNA (rRNA) are arrayed as tandem repeats separated by intergenic spacers on one or more chromosomes (Small *et al.,* 2004). The 5S rRNA sequence has been used for construction of the phylogenetic tree of major organisms (Hori and Osawa, 1987).

26SrRNA (Nuclear): The entire coding region of the 26S rRNA gene can be amplified by DNA and was reported to provide -3 times more phylogenetically informative characters than the 18S rRNA. The 26S rRNA sequence consists of conserved core and highly variable expansion regions (Kuzoff*et al.*, 1998).

atpA, atpB, atpF, atpH (Chloroplast): Single copy chloroplast genes coding for the ATP synthase subunits α (atpA), β (atpB), I (atpF), and δ (atpH)), res (Sugiura, 1992).

chlB (Chloroplast): A chloroplast gene coding for a subunit of the light-independent protochlorophyllidereductase that catalyzes the reduction of protochlorophyllide to chlorophyllide in photosynthetic bacteria, algae, and gymnosperms but is not present in angiosperms (Liu *et al.*, 1993).

matK (Chloroplast): The matK gene, which is located within the trnK intron and comprises -1.6kbp. It is assumed to be involved in the splicing of group II introns (Heubl*et al.,* 2006).

psbA, psbK, psbI (Chloroplast): The pbs genes code for proteins of photosystem II.

rbcL (Chloroplast): Large subunit of the enzyme ribulose-1,5-biphosphate carboxylase (rbcL) is one of the largest (-1.4kbp) genes in the chloroplast genome. It has been sequenced in a large number of plants beginning in the mid-1980s (Olmstead and Palmer, 1994 and Palmer, 1988).

rp14, rpl16 (Chloroplast): Chloroplast genes coding for the ribosomal proteins L14 and L16, constituents of the large subunit (50S) of the chloroplast ribosome. The chloroplast (70S) and nuclear (80S) ribosomes are of different size (Sugiura, 1992).

rpoB, rpoC1(Chloroplast): Chloroplast gene coding for DNA-directed RNA polymerase beta and gamma chains, respectively (Sucher and Carles, 2008).

rps16 (Chloroplast): Chloroplast gene coding for the ribosomal proteins S16, a constituent of the small subunit of the chloroplast ribosome(Sucher and Carles, 2008).

trnC, trnD, trnF, trnK, trnL (Chloroplast): Genes coding for the transfer RNA (tRNA) for cystein, aspartate, phenylalanine, lysine, and leucine, respectively. Chloroplast genomes code for 20 to 40 different tRNAs (Sugiura, 1992). Regions used in molecular taxonomy include the trnL intron and various tRNAintergenic spacer regions (Chase *et al.*, 2005).

2.8 RAPD (Random Amplified Polymorphic DNA)

The RAPD technology utilizes low-stringency polymerase chain reaction (PCR) amplification with single, short, and arbitrary synthetic oligonucleotide primers (usually 10 bp lengths) to generate a high number of anonymous DNA fragments (Gunther, 2010).

DNA polymorphism can be due to mismatches at the primer site, appearance of a new primer site, and the length of the amplified region between primer sites. Amplification products are generally separated on agarose gels and stained with ethidium bromide. Decamer primer kits are commercially available from various sources. RAPDs are inherited as dominant-recessive, a character which means that homozygotes and heterozygotes cannot be distinguished. Low expense, efficiency in developing large number of DNA markers in a short time and requirement of basic molecular equipment has made the RAPD technique valuable although band reproducibility, problems of comigration, and scoring errors are a major problem (Gunther, 2010).The technique has been applied in many plant groups such as *Glycyrrhiza* (Yamasaki *et al.,* 1994), *Atractylodes* (Kohjyouma*et al.,* 1997), *Astragalus*(Cheng *et al.,* 2000), *Amomun* (Wang *et al.,* 2000), *Scutellaria* (Hosokawa *et al.,* 2000) etc.

Several molecular techniques are available for the assessment of plant genetic diversity. These markers are mostly dominant and detect variation in both coding as well as non-coding regions of the genome. RAPD analysis is technically simple and suitable for large scale germplasm characterization and can be performed even in a moderately equipped laboratory (Rafalski and Tingey 1993). Yadav*et al.* (2014), worked on Assessment of genetic diversity using RAPD marker among different accessions of *SalvadoraOleiodes* of North-West India. The genetic diversity of 19 accessions of Salvadoraoleoides collected from different regions of North-West India was reported using 16 RAPD primers. Congiu*et al.* (2000) also use random amplified polymorphic DNA (RAPD) markers to identify strawberry varieties: a forensic application in which RAPD technique

was applied to settle a lawsuit involving unauthorized commercialization of a patented strawberry variety of high economical relevance (‘Marmolada’).

A comparative study of seed morphometric traits and isozymes has been carried out in Neem (Kundu, 1999). However, environmental factors, as well as the developmental stage of the plants, influence such traits. DNA-based markers provide useful information regarding genetic diversity and relationships among accessions, as these remain unaffected by environmental factors. Polymerase chain reaction (PCR)-based markers including random amplified polymorphic DNA (RAPD) (Williams *et al.,* 1990) have been extensively used as genetic markers for the assessment of genetic diversity and relationship measures in various plant species (Barker *et al.,*1999; Degani*et al.,* 2001; Bekessy*et al.,* 2002). RAPD markers are generated by using short, 10-mer oligonucleotides of arbitrary sequence.

2.9 Diabetes Prevalence

Diabetes is a chronic disorder of carbohydrate, fat and protein metabolism characterized by increased fasting and post prandial blood sugar levels. The global prevalence of diabetes is estimated to increase, from 4% in 1995 to 5.4% by the year 2025 (Ramachandran *et al.,* 2002), WHO has predicted that the major burden will occur in developing countries. Studies conducted in India in the last decade have highlighted that not only is the prevalence of diabetes high but also that it is increasing rapidly in the urban population (Ramachandran *et al.,* 2002). It is estimated that there are approximately 33 million adults with diabetes in India. This number is likely to increase to 57.2 million by the year 2025.

For the development of diabetic complications, the abnormalities produced in lipids and proteins are the major etiologic factors. In diabetic patients, extra-cellular and long lived proteins, such as elastin, laminin, and collagen are the major targets of free radicals. These proteins are modified to form glycoproteins due to hyperglycemia. The modification of these proteins present in tissues such as lens, vascular wall and basement membranes are associated with the development of complications of diabetes such as cataracts, microangiopathy, atherosclerosis and nephropathy (Gluglianoet *al.,* 1996). During diabetes, lipoproteins are oxidized by free radicals. There are also multiple abnormalities of lipoprotein metabolism in very low density lipoprotein (VLDL), low density lipoprotein (LDL), and high density lipoprotein (HDL) in diabetes. Lipid peroxidation is enhanced due to increased oxidative stress in diabetic condition. Apart from this, advanced glycation end products (AGEs) are formed by non-enzymatic glycosylation of proteins. AGEs tend to accumulate on long-lived molecules in tissues and generate abnormalities in cell and tissue functions (Elgawish*et al.,* 1999). In addition, AGEs also contribute to increased vascular permeability in both micro and macrovascular structures by binding to specific macrophage receptors. This results in formation of free radicals and endothelial dysfunction. AGEs are also formed on nucleic acids and histones and may cause mutations and altered gene expression.

More than 400 plants have been incorporated in approximately 700 recipes which are used to treat diabetes mellitus in almost two thirds of the world population. A large number of in vivo studies have been conducted on animals to test the claimed activity have demonstrated the hypoglycemic property of many plants, already reported in various literatures (Li *et al.,* 2004). The plant families, including the species most studied for

their confirmed hypoglycemic effects include, Leguminoseae, Lamiaceae, Liliaceae, Cucurbitaceae, Asteraceae, Moraceae, Rosaceae, Euphorbiaceae and Araliaceae (Li *et al.,* 2004).

Many studies have confirmed the benefits of medicinal plants with hypoglycemic effects in the management of diabetes mellitus. The effects of these plants may delay the development of diabetic complications and even assist in correcting the metabolic abnormalities. Moreover, during the past decade and especially in last few years some of the new bioactive drugs isolated from hypoglycemic plants showed antidiabetic activity with more efficacy than synthetic oral hypoglycemic agents. Therefore, plants, as folk remedies, are widely used to treat diabetes mellitus(Li *et al.,* 2004).

2.10 Adulterants, Substitutes, and Confused Species

Medicinal plants have been used successfully in Nigeria over a long period of time they have never been subjected to the stringent International quality standards and regulations for pharmaceutical products in Europe or USA. In other to make traditional and innovative plant products acceptable for modern medicine, it is important to have reliable phytochemical, molecular and botanical tools for the identification of medicinal plant species (Liang *et al.,* 2004). Increasing interest in herbal medicines is one of the reasons for the belief that, for been natural and traditionally used over a long period of time, they are safe and hence do not possess the potential for negative side effects. The cost low compared to conventional medications which is the major attraction of these treatments. Quality of an herbal drug may be negatively affected by the use of inherent toxic herbs,

by substitution or adulteration, inappropriate labelling, misidentification, confusion of species or contamination (Ng *et al.,* 2009 and Mazzanti*et al.*, 2008).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study Area

The research work covers Nasarawa State and the Federal Capital Territory (FCT) Abuja Nigeria both of which are located in the middle belt of Nigeria. Nasarawa State is geographically situated on latitude of $8^{o}5^{'}$ N and longitude $7^{o}5^{'}$ E. The temperature range is between $25^{o}C$ – $31^{o}C$ and about 602mm of precipitation falls annually (Akwe*et al.*, 2007). Abuja is located in the Guinea savannah. Raining season begins from April and ends in October in Abuja and the average day time temperature reach $28^{0}C$ to $30^{0}C$. In dry season, average daily temperature can soar as high as $40^{o}C$ while Annual rainfall ranges from 1100 mm to 1600 mm. Gwagwalada is geographically located on latitude $8^{0}56'59''N$ of the Equator and longitude $7^{0}5'59''$ E. The experiment was conducted at Biotechnology Advance Research Center, Sheda Science and Technology Complex (SHESTCO) Abuja, Nigeria **(**Ochuko, 2013**).**

3.2 Collection and Storage of Herbs/Plant

A total of 12 samples (from Nasarawa State and from Federal Capital Territory) each of both plants (*Khayasenegalensis* and *Azadirachtaindica*) and herbs made from them were assessed. Young leaves of both plants were harvested, appropriately labelled and Stored in the freezer at $-20^{0}C$. The leaves were used for DNA extraction, while excess leaf materials were stored for future DNA extraction.

Six (6) of the samples were collected from the FCT (Zuba, Giri, Kubwa, Orozo, Nyanyan and Apo Village) and six (6) from Nasarawa State (Garaku, Keffi, Uke, Kaduna Road,

Auta and Nasarawa) to allow for a good representation of Nasarawa State and the FCT. Both plant and herbal sample where collected in the same location. Collected herbal samples were stored in a cool and dry place at room temperature (28 ± 2^0C) and also for further DNA extraction.

3.3 Genomic DNA Extraction

3.3.1 Modified SDS Method

0.2g of young fresh leaves/herbal sample collected,the leaves were crushed in liquid nitrogen with mortar and pestle and 0.2 g of both samples was put in 2 ml eppendorf tube, 700 µl of the extraction buffer (CTAB 2%, 1.4 M NaCl, 100 mMTrisph 8.0, 20mM EDTA PH 8.0, 2% PVP, 20 ul β-mercaptoethanol) pH 8.0was added, it was incubated at 65°C in heating block for 2 hours; it was cooled for 5 - 10minutes at room temperature, Chloroform Isoamylalchohol (24:1) 600 µl was added in the fume hood, it was mixed gently by inversion for 5 minutes. It was centrifuged for 5 minutes at 14000 rpm. The supernatant was then transferred into fresh tubes, 500 µl of ice cold isopropanol was added and mixed well gently by inversion for DNA precipitation. It was then incubated at -20°C for 30 overnight, it was centrifuged at 2,000 rpm for 15 minutes at 4°C.The supernatant was decanted in the fume hood, the residue was washed with 100 µl 70% ethanol,procedure 12 above was repeated,the pellet was dried at room temperature.The dry pellet was redesolve in 50 µl TE buffer and 2 µl of RNase was added and stored at -4^0C (Vengadesh*et al.,* 2016).

3.3.2 Modified CTAB Method

In 2x CTAB buffer (CTAB 2%, 1.4M NaCl, 100mM Trisph 8.0, 20mM EDTA PH 8.0, 1% PVP, 0.1% β-mercaptoethanol), about 1.3 µl Mercaptoethanol and a pinch of PVP was added (CTAB buffer was pre heated at 65^{o}C), 1g of herbal powder was weighed into 2 ml extraction tubes. 700 µl of CTAB buffer was added in number 2 above and incubated at 65^{o}C for 1 hour. Between times, vigorous vortexing was done for a good mix. 500 µl of chloroform/isoamylalchohol (24:1) was added and mix for 5mins, sample was Centrifuge at 14,000 rpm for 5mins.liquid phase was removed into a new 1.5 ml tube, 500 µl of ice cold isopropanol was added then it was incubated at – 20^{o}C for about 30 min to 1 hour. It was then centrifuge for 2min at 14,000 rpm, liquid was removed carefully so as not to throw away DNA rings at the bottom of the tube. 100 µl of 70% ethanol was added to wash DNA, and then it was spin for 2mins at 14,000 rpm, ethanol was removed and number 10 above was repeated. DNA pellet was dry at room temperature for the ethanol to evaporate, 100 µl of 1x TE buffer and 2 µl RNase was added (Dellaporta*et al*., 1983).

Modified CTAB Method 2

In 2x CTAB buffer (CTAB 2%, 1.4M NaCl, 100mM Trisph 8.0, 20mM EDTA PH 8.0, 1% PVP, 0.1% β-mercaptoethanol), about 1.3 µl Mercaptoethanol and a pinch of PVP was added (CTAB buffer was pre heated at 65^{o}C). 1g of herbal powder was weighed into 2 ml extraction tubes, 700 µl of CTAB buffer was added in number 2 above and incubated at 65^{o}C for 1 hour. Between times, vigorous vortexing will be done for a good mix.500 µl of chloroform/isoamylalchohol (24:1) was added and mix for 5mins, sample

was Centrifuge at 14,000 rpm for 5min, and liquid phase was remove into a new 1.5 ml tube.500 µl of ice cold isopropanol was added then it was incubated at – 20^{o}C for about 30 min to 1 hour.It was then centrifuge for 2min at 14,000 rpm. The liquid was removed carefully so as not to throw away DNA rings at the bottom of the tube. 100 µl of 70% ethanol was added to wash DNA, then it was spin for 2mins at 14,000 rpm. Ethanol was removed and number 10 above was repeated, DNA pellet was dry at room temperature for the ethanol to evaporate.

1. l of 5M NaClwas added to the dry DNA pellet, 73 µl of absolute (100%) ethanol was also added and then votexed, the sample was incubated on ice for 10 minute. It was then centrifuge at 10 000 Rpm for 15 minute, the solution was then removed in a new tube. 300 µl of Isopropanol was added for precipitation.It was then incubated at – 20^{0}C for 1 hour. It was centrifuge at 10 000 Rpm for 15 minute to remove the supernatant. It was then washed with 70% ethanol, DNA pellet was dry at room temperature for the ethanol to evaporate. 50 µl of 1x TE buffer and 2 µl, RNase was added (Dellaporta*et al*., 1983).

3.4 Estimation of Quality and Quantity of Isolated Genomic DNA

3.4.1 Agarose gel electrophoresis

Fifty millilitres (50ml) of 1% (w/v) of agarose gel were prepared by weighing accurately 0.5g, of agarose powder (BDH Biochemical England) and made up to 50mls with X1 TBE (Trisma base- Boric acid- EDTA) as a solvent. To obtain a homogenous solution, it was heated in a microwave oven for about 1minute. In the fume hood, 10,000X (5µl) of GR Green fluorescent dye (Inqaba biotech South Africa) were added with vigorous

shaking and was allowed to cool. It was poured in gel casting tray after cooling down to about 40^0C.

The prepared gel was placed in the electrophoresis tank and x1 TBE buffer was added to submerge the gel. The extracted genomic DNA samples (5µl) mixed with 1µl of 6x DNA loading dye from Thermo Scientific was loaded in the wells of the gel accordingly as well as the step-ladder DNA (Thermo Scientific O' Gene Ruler, 1kb plus). The set up was covered and connected to the power pack and was run at 55volts for 45 minutes, after which it was visualized using Alpha Innotech Gel documentation system model 201103 (Taiwan). This process was carried out for each of the extraction methods optimized (Charles *et al.,* 2015).

3.4.2 Estimation of the quality and quantity of the extracted genomic DNA by UV Spectrophotometry

For spectrophotometric analysis, 2µl of DNA samples was diluted to 100µl with T.E. buffer and 100µl of the blank solution were prepared. The spectrophotometer readings was recorded, the blank sample was read in the machine first, the DNA samples diluted in T. E. buffer was recorded at 260 nm/280 nm using Spectophotometry (UV absorbance) at 260nm and 280nm, using U.V Spectophotometer (Lab Kit model ST-UV 755B). DNA concentration was calculated using absorbance values at 260 nm using the following formula.

$$\text{Concentration of DNA (µl/ml)} = \frac{\text{Absorbance at 260 nm x dilution factor x 50µg/ml}}{1000}$$

(Charles *et al.,* 2015).

3.5 RAPD-PCR

A set of eleven (11) primers obtained from Inqaba Biotech (South Africa) was used for RAPD-PCR. The primer designation and sequence are presented in Table 1.

Table 1: Primer Designation and Sequence

RAPD primer designation	Sequence 5´-3´
OPB-1	GTTTCGCTCC
OPB-2	TGATCCCTGG
OPD-1	ACCGCGAAGG
OPF-1	ACGGATCCTG
OPF-2	GAGGATCCCT
OPJ-1	CCCGGCATAA
OPJ-2	CCCGTTGGGA
OPA-1	CAGGCCCTTC
OPA-2	TGCCGAGCTG
OPL-1	GGCATGACCT
OPL-2	TGGGCGTCAA

(Inqababiotecch)

3.6 Preparation of stock solutions for primers

This was prepared according to manufacturer's instruction. Stock solutions of primers were prepared in a sterile T. E. buffer solution (10mM TrisHCl, pH 7.5 to 8.0, 1mM EDTA).

3.7 Preparation of T.E. Buffer

One times (1X) TE buffer solution consists of 10 mMTris, adjusted to pH 7.5 with HCl and one (1) mM EDTA. 1 ml of TrisHCl was measured from an already prepared 1 M stock solution of TrisHCl and 0.2mls of EDTA was measured from 0.5M stock solution of EDTA. This solution was made up to 100mls with distilled water, sterilised and stored at room temperature.

3.8 Constitution of primers

Lypholisedoligos were centrifuged to obtain pellets that might be displaced from the bottom of the tube during shipment. Primer stock solution was prepared (100 µM) with sterile T.E. buffer solution. Working solutions were diluted from the stock solution with sterile nuclease free water to prevent inhibition of enzymatic reactions and degradation of the nucleic acids by nucleases. From the stock solution, 25µl (15 µM concentration), was divided into smaller aliquots for long term storage to avoid frequent freeze thaw cycles and accidental contamination.

3.9 Preparation of working solutions for primers

Fifteen (15) µM concentrations of primers for working solution was prepared from the stock solution. Microcentifuge tubes were labelled for working solutions appropriately. The tubes were disinfected with UV light in equipped laminar flow hood for 15 min.

Eighty –five (85) µl of T.E. buffer were added to each tube. Twentyfive (25) µl of the 100µM primer stock solution were added to each tube. It was vortexed for 30seconds at 3, 000 rpm. Tubes were sealed with parafilm and placed in -20°C freezer. Eleven (11) tubes to be used within the month were kept in -20°C, while the rest was stored in a -80°C freezer.

3.10 Procedure for RAPD-PCR

All reagents needed for RAPD-PCR were arranged in a freshly filled ice bucket. They were allowed to thaw completely before setting up the reaction. The RAPD-PCR Program/cycling conditions inputted into the PCR machine (Peltier Based ThermacyclerLabkits PCR-MGL-96+/Y) are presented in Table 2.

3.11 RAPD-PCR reaction mixture

PCR tubes were labelled accordingly with an ethanol resistant marker. A mastermix was prepared on ice in 6 eppendorf 1.5 ml tubes for the different primers (OPB 01, OPB 02, OPD 01, OPF 01, OPF 2, and OPJ 01). The PCR reagents were pippetted into the tube in the following order; sterile water, BSA, Primer, and Taq polymerase Master mix. It was mixed gently by pipetting up and down for at least 20 times. A 96 well plate was placed in the ice bucket as a holder for the 0.2ml thin walled PCR tubes. Three (3) ul of the DNA template was added into the PCR tubes. Twenty two (22) µl of the mastermix cocktail was gently added to the DNA template in the PCR tubes to make up the total volume of reaction to 25ul. The various components used for RAPD-PCR reaction are presented in Table 3.

Table 2: Cycling conditions for RAPD – PCR

Step	Cycle	Temperature	Time (min or secs)
Initial denaturation	1	94°C	3 min
Denaturation	40	94°C	1 min
Annealing	40	40°C	1 min
Extension	40	72°C	1 min
Final extension	1	72°C	10 min
Store/end		4°C	∞

Table 3: Master Mix for RAPD-PCR amplification

Reagent	1 sample (µl)	X 13 samples (µl)
Nuclease free water (SDW)	6.0	78.0
Taq polymerase Master mix	12.5	162.0
Primer	2.5	32.5
BSA	1.0	13.0
DNA template (50ng)	3.0	
Total	**25.0**	**285.5**

The tubes were placed in the PCR machine and covered. The RAPD-PCR program was run using the cycling conditions (Table 2). After the PCR programme was finished the tubes were removed from the thermocycler and stored at 4°C in the refrigerator. This procedure was repeated for primers OPJ 02, OPA 01, OPA 02, 0PL 01 and OPL 02.

3.12 PCR Agarose gel electrophoresis

One percent (1.5%) agarose gel was prepared by weighing accurately 0.75g of agarose powder, which was added to 50 mls of 1X TBE (Tris boric EDTA) buffer; it was dissolved in a microwave oven for 2 min to obtain a clear solution. GR Green (fluorescent dye) was added to the solution with vigorous shaking and allowed to cool for 40^0C. The gel was poured into the gel casting tray and was allowed to set for about 20 min. The prepared gel was placed in the electrophoresis tank and 1X TBE buffer was added to cover the gel. Five (5) µl of the RAPD - PCR product was loaded into wells on the agarose gel. Two (2) µl of the 10, 000bp and 1000bp molecular weight marker (Fermentas) were also loaded in the first and last well. Agarose gel electrophoresis was run for 45 min at 55volts. It was then visualised with a gel documentation system and photographed (Charles *et al.*, 2015).

3.13 Analysis of Data

The RAPD profile was scored for the presence, (represented as 1) or absence (represented as 0) of bands of various molecular weight sizes in the form of binary matrix. The molecular data collected were subjected to Numerical Taxonomy System of Statistic (NTSYS) (Jaccard, 1908). In NTSYS analysis, the similarity indices between pairs of genotypes were calculated for the combination of the data from the molecular studies by

selecting for Qualitative Analysis (SimQual) using method of Jaccard (1908). The similarity matrix is used for clustering of the genotypes based on Unweighted Pair Clustering Group Arithmetic Average (UPGMA) of Jaccard (1908), which does not consider the joint absence of the character as an indication of similarity by selecting Sequential Hierarchical Numeric option (SHAN) in clustering and the clustering was used to generate dendrogram by selecting tree plot option.

The similarity index of Jaccard between plant i and j is given by

$$S_{ij} = a / (a + b + c)$$

$$D_{ij} = 1 - S_{ij}$$

Where a, is the number of characters present in the plants i and j, b is the number of characters present in i not in j and c is the number of characters present in j and not in i and D is the distant coefficient.

CHAPTER FOUR

RESULTS

4.1 DNA Extraction

The DNA picture of the plant sample as observed in Plate 3 showed that most of the DNA band is close to the neck of the well. The DNA band is heavy; they remain close to the neck of the well. The Ladder used here is a 10 kb.

The herbal DNA band as observed in Plate 4 is also heavy and close to the neck of the well. There is some smearing and sheared DNA band. There is the presence of some other constituent of the herbal sample (phenol).

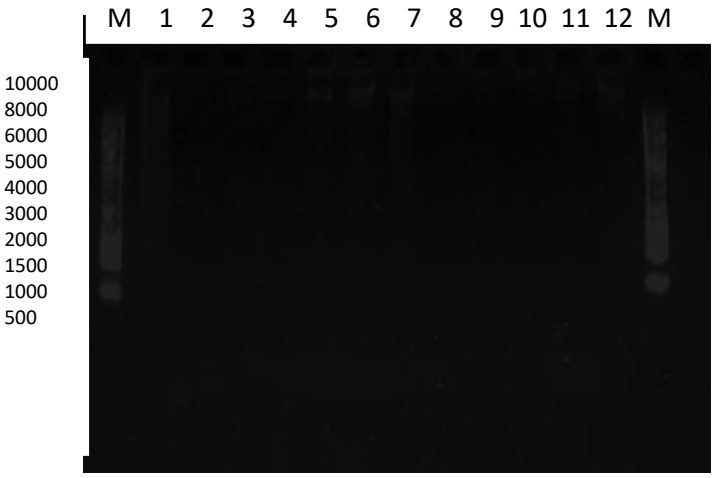


Plate 3: Plant Sample DNA

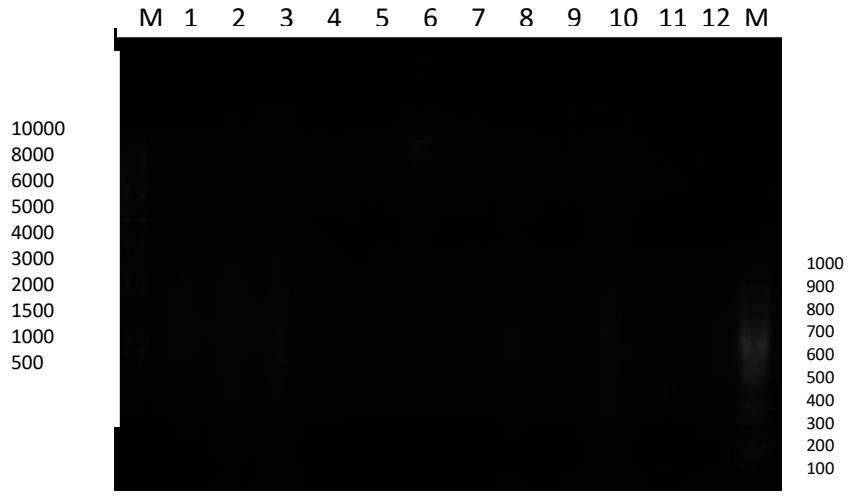


Plate 4: Herbal Sample DNA

4.2. Quantity and Quality Analysis Result of Genomic DNA Extracted

The quantity and quality of genomic DNA extracted from plant sample showed that the absorbance is better as presented in Table 4.

In Table 5, the quantity and quality of genomic DNA extracted from herbal sample was not so good and the addsorbance is a little low, it also showed poor result.

Table 4: Quantitative and Qualitative Result of Genomic DNA Extracted from Plant Sample of Both *Khayasenegalensis* and *Azadirachtaindica*

Sample No	Concentration (µg/µl)	Absorbance (260nm)	Absorbance (280nm)	Ratio (260/280nm)
1	0.55	0.219	0.133	1.65
2	0.46	0.182	0.113	1.61
3	0.56	0.225	0.131	1.72
4	0.55	0.221	0.130	1.69
5	0.61	0.246	0.146	1.67
6	0.63	0.251	0.147	1.70
7	0.50	0.201	0.122	1.67
8	0.45	0.180	0.106	1.70
9	0.48	0.191	0.116	1.65
10	0.47	0.187	0.111	1.68
11	0.49	0.197	0.117	1.68
12	0.57	0.226	0.132	1.71

Table 5: Quantitative and QualitativeAnalysis Result of Genomic DNA Extracted from Herbal Sample

Sample No	Concentration (µg/µl)	Absorbance (260nm)	Absorbance (280nm)	Ratio (260/280nm)
1	0.23	0.092	0.080	1.50
2	0.35	0.139	1.120	1.60
3	0.44	0.176	0.115	1.53
4	0.36	0.144	0.095	1.52
5	0.61	0.245	0.157	1.56
6	0.53	0.210	0.136	1.54
7	0.33	0.133	0.084	1.58
8	0.28	0.112	0.074	1.51
9	0.23	0.092	0.059	1.56
10	0.39	0.157	0.100	1.57
11	0.23	0.092	0.060	1.53
12	0.22	0.089	0.057	1.56

4.3. PCR Picture of the Different Primer on both the Herbal and Plant Sample

The PCR Picture of Primer OPL 01 Plant Sample with sequence GGCATGACCT (Plate 5), Primer OPL 01 herbal Sample with sequence GGCATGACCT (Plate 6) and Primer OPA 01 herbal Sample with sequence CAGGCCCTTC (Plate 7), showed that there was polymorphism across the different plant sample and there location. They were quite different from those of the plant sample. The band observed were scattered as such two different ladders were employed 10kb and 1kb.

The PCR Picture of Primer OPA 01 Plant Sample with sequence CAGGCCCTTC as presented in plate 8, showed that there was polymorphism across the different plant sample and there location. The band observed in sample 2 to 5 had some smear.

PCR Picture of Primer OPJ 01 Sample with sequence CCCGGCATAA as presented in Plate 9, showed polymorphism across the sample and there location but they were different from those of the herbal sample of the same primer.

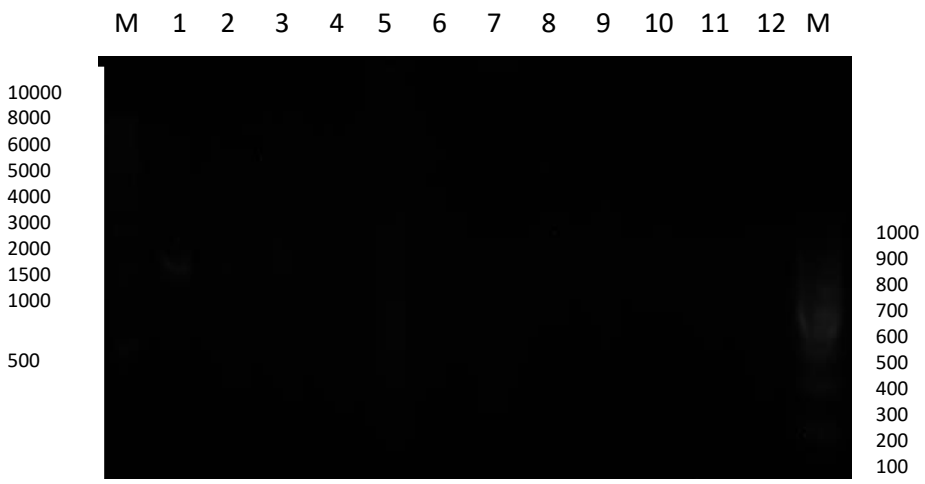


Plate 5: PCR of Primer OPL 01 Plant Sample

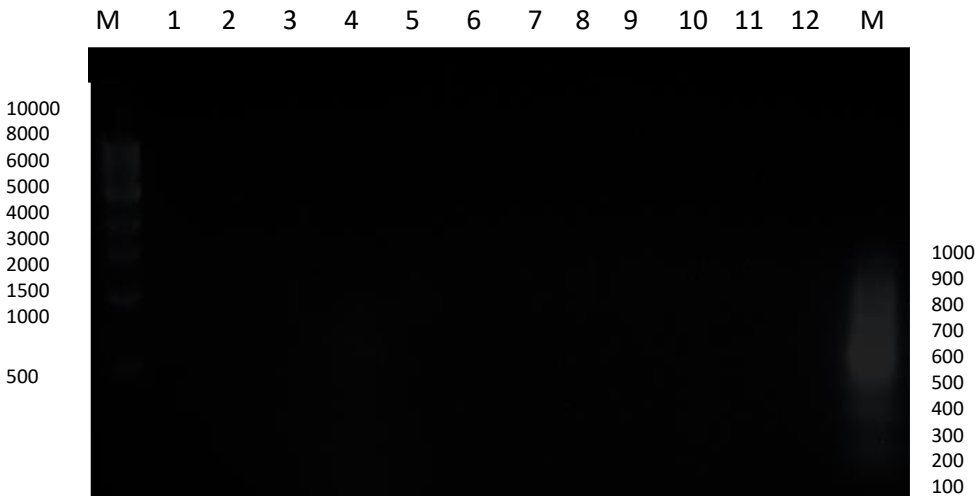


Plate 6: PCR of Primer OPL 01 Herbal Sample

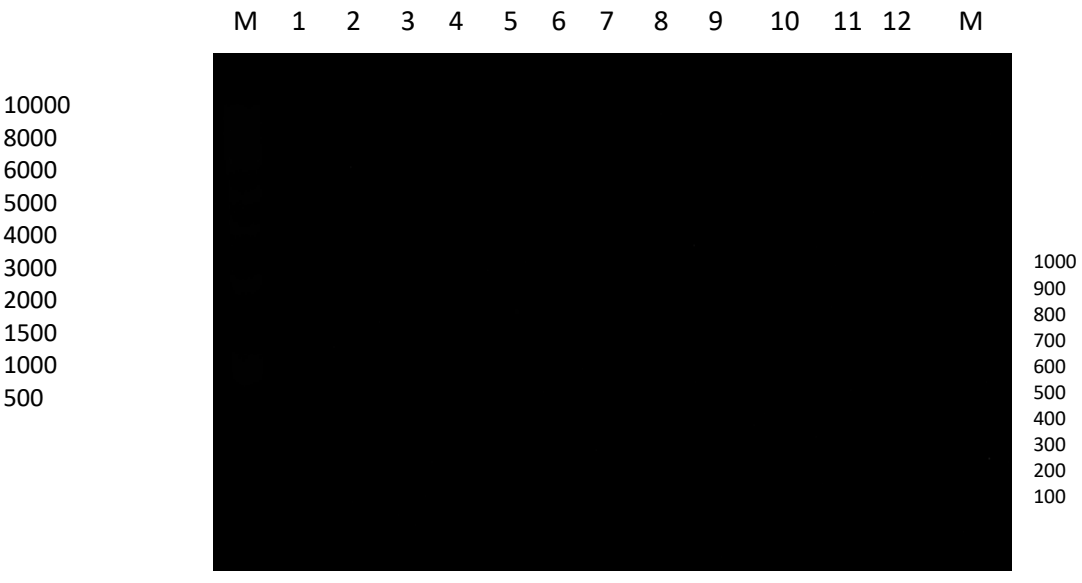


Plate 7: PCR of Primer OPA 01 Herbal Sample

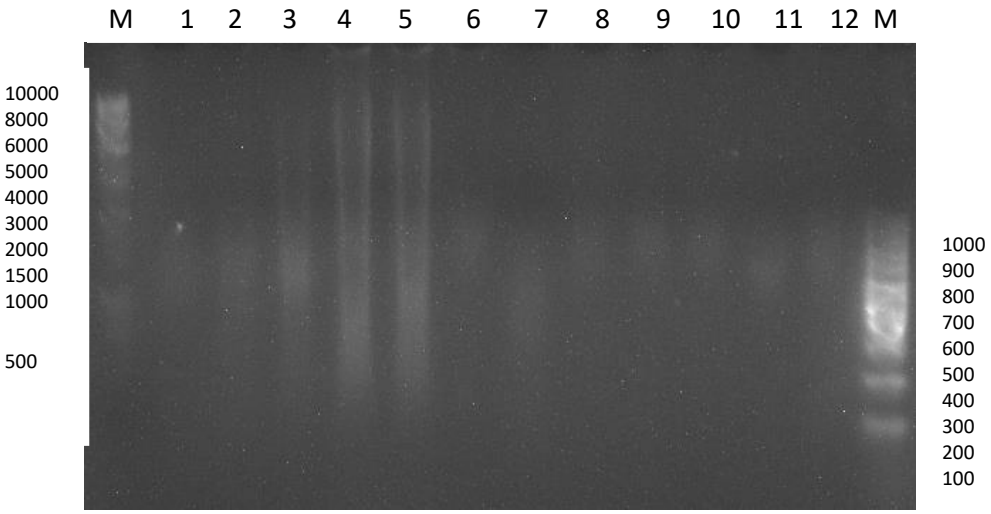


Plate 8: PCR of Primer OPA 01 Plant Sample

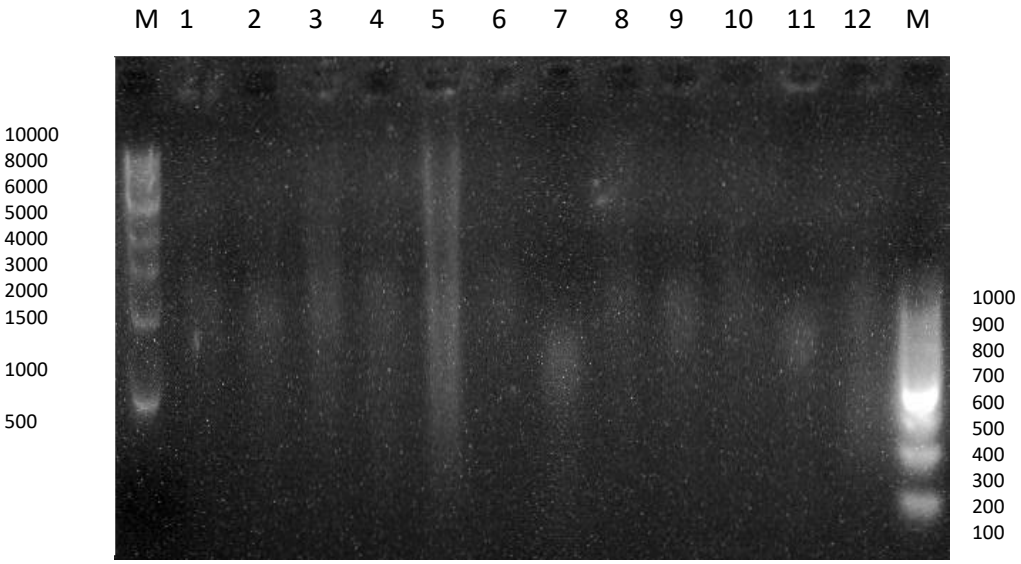


Plate 9: PCR of Primer OPJ 01 Plant Sample

PCR Picture of Primer OPJ 01 herbal Sample with sequence CCCGGCATAA (Plate 10), Primer OPB 01 plant Sample with sequence GTTTCGCTCC (Plate 14) and Primer OPB 01 herbal Sample with sequence GTTTCGCTCC (plate13) showed that there was polymorphism across the different herbal sample and there location but they were quite different from those of the herbal sample. The band observed were also scattered but closely related.

PCR Picture of Primer OPA 02 herbal Sample with sequence CAGGCCCTTC as presented in Plate 11 showed that there was polymorphism across the different herbal sample. The band observed was almost similar in their pattern.

PCR Picture of Primer OPA 02 plant Sample with sequence CAGGCCCTTC (Plate 12), showed that there was polymorphism across the different plant sample. The band observed was scattered and though sample 5 had a smeared pattern.

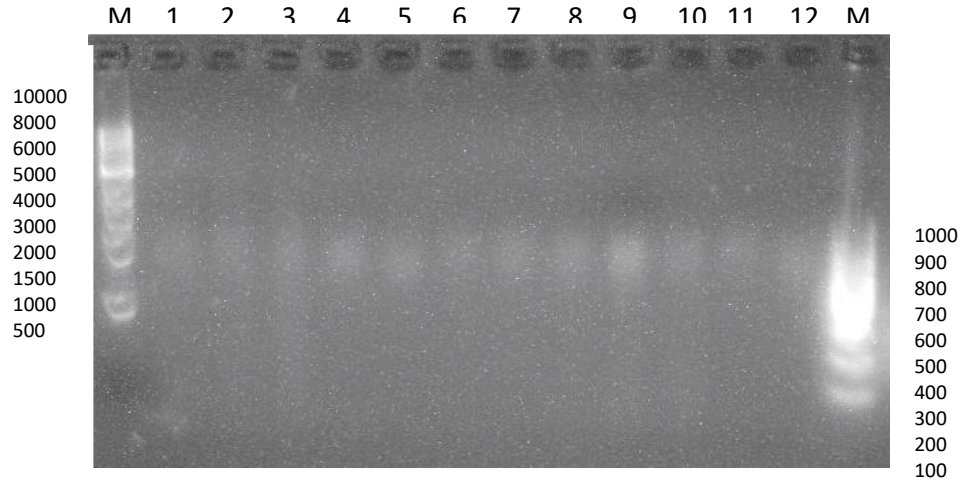


Plate 10: PCR Picture of Primer OPJ 01 Herbal Sample

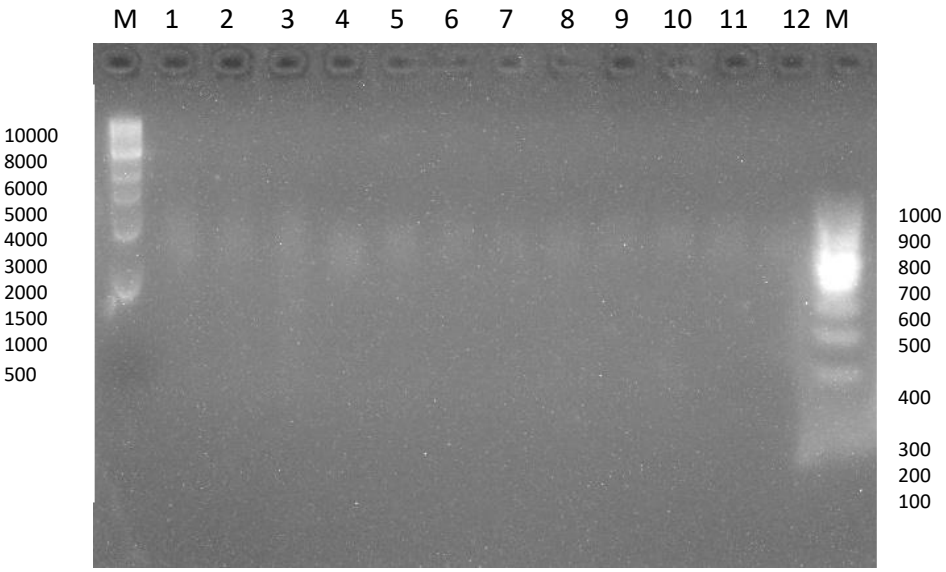


Plate 11: PCR Picture of Primer OPA 02 Herbal sample

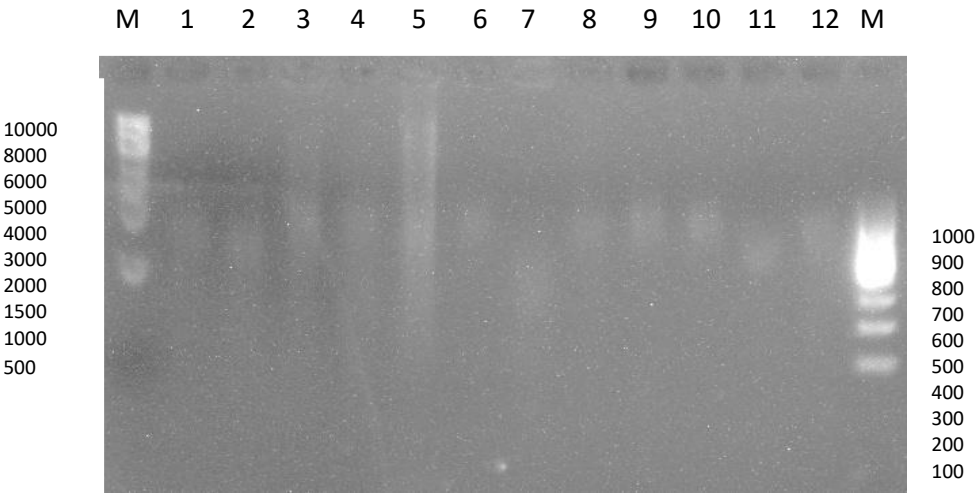


Plate 12: PCR Picture of Primer OPA 02 Plant Sample

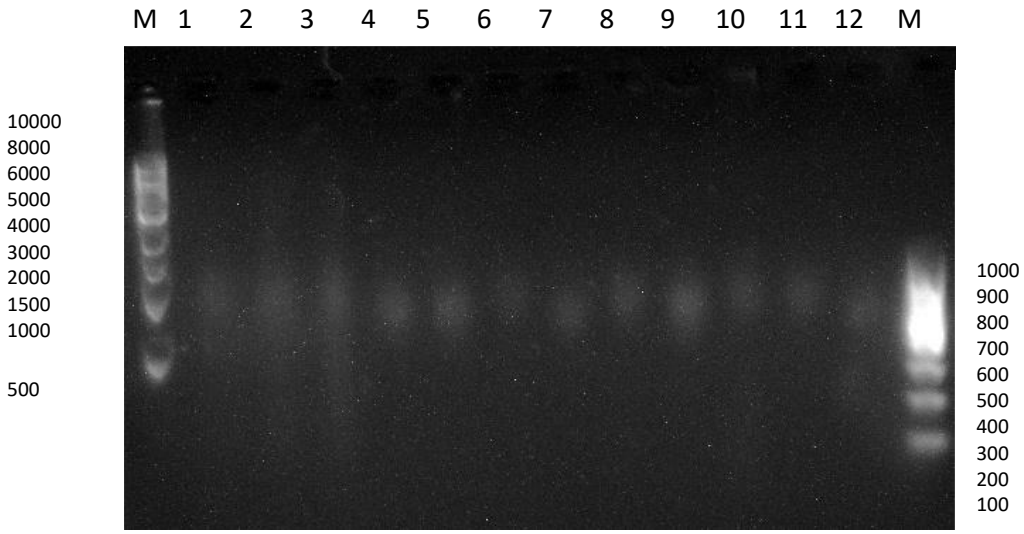


Plate 13: PCR Picture of Primer OPB 01 Herbal Sample

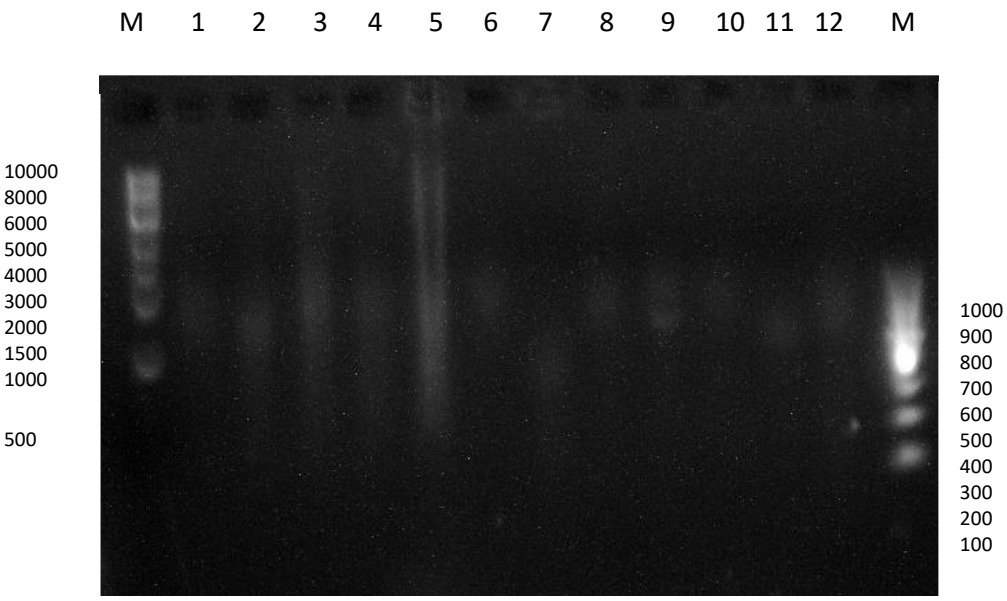


Plate 14: PCR Picture of Primer OPB 01 Plant Sample

4.4. Scoringof the Different Primers for both the Herbal and Plant Sample

Table 6 showed the scoring of the herbal sample primer OPL 01 with sequence (1) GGCATGACCT, OPA 01 with sequence (2) CAGGCCCTTC, OPJ 01 with sequence (3) CCCGGCATAA, OPA 02 with sequence (4) TGCCGAGCTG and OPB 01 with sequence (5) GTTTCGCTCC. OPL 01 has two polymorphic band at 1000 and 900 across the different location. While at band weight 800 and 700 the sample was unique. OPA 01 also had two polymorphic bands but at 900 and 800 and it was monomophic at band weight 1000. Primer OPJ 01 had two monomophic band weight at 1000 and 900 and polymorphic 800. OPA 02 had three polymorphic band weight at 1500, 900 and 800 but monomophic at 1000 band weight. OPB 01 showed three polymorphic bands and at 1000, 800 and 700 while it was unique at 600 and 500 but monomophic 900.

Table 7, showed scoring of primers OPL 01 plant sample (1) GGCATGACCT, OPA 01 plant sample (2) CAGGCCCTTC, OPJ 01 plant sample (3) CCCGGCATAA, OPA 02 plant sample (4) TGCCGAGCTG, OPB 01 plant sample (5) GTTTCGCTCC. OPL 01 was polymorphic at 1500 and 1000, but unique at 700. OPA 01 result also shows polymorphism at 1000 and 700 band weight, it also had one monomophic band weight at 900. OPJ 01 has two polymorphic (1500 and 700) and unique (800 and 600) band weights and only one monomophic band weight at 1000. OPA 02 had three polymorphic band weights at 1500, 1000, 900 and unique at 800. OPB 01 was polymorphic at 1000, 900, 800 and 600. Though, it was unique at 700 and 500 band weight.

Table 6: Scoring of the Primers for herbal samples OPL 01, OPA 01, OPJ 01, OPA 02 and OPB 01.

Ladder	ZubaGiriKubwaOrozoNyanya Apo						GarakuKeffiUke			Kaduna road AutaNewKaru			Status
	FCT	FCTFCTFCTFCTFCT				NAS	NASNASNASNASNAS						
(1). 1000	1	0	1	0	1	0	1	1	1	1	1	1	Polymophic
900	0	0	0	0	1	1	1	1	1	1	1	1	Polymophic
800	0	0	0	0	1	0	0	0	0	0	0	0	Unique
700	0	0	0	0	1	0	0	0	0	0	0	0	Unique
(2). 1000	1	1	1	1	1	1	1	1	1	1	1	1	Monomophic
900	0	1	0	1	1	1	1	1	1	1	1	1	Polymophic
800	0	0	0	1	0	0	1	1	1	1	0	0	Polymophic
(3). 1000	1	1	1	1	1	1	1	1	1	1	1	1	Monomophic
900	1	1	1	1	1	1	1	1	1	1	1	1	Monomophic
800	1	0	1	1	1	1	1	0	0	0	0	0	Polymophic
(4). 1500	1	1	1	0	0	0	0	0	0	0	1	1	Polymophic
1000	1	1	1	1	1	1	1	1	1	1	1	1	Monomophic
900	1	1	1	0	1	1	1	1	1	1	1	1	Polymophic
800	0	0	1	1	1	1	1	1	1	1	0	0	Polymophic
(5). 1000	1	1	1	0	0	0	0	1	0	1	1	0	Polymophic
900	1	1	1	1	1	1	1	1	1	1	1	1	Monomophic
800	1	1	1	1	1	0	1	1	1	0	0	1	Polymophic
700	1	0	0	0	0	0	1	0	1	0	0	0	Polymophic
600	0	0	0	0	0	0	0	0	0	0	0	1	Unique
500	0	0	0	0	0	0	0	0	0	0	0	1	Unique

Table 7: Scoring of the Primers for Plant Sample OPL 01, OPA 01, OPJ 01, OPA 2 and OPB 01

Ladder	Zuba Khaya FCT	Giri Khaya FCT	Kubwa Khaya FCT	Orozo Neem FCT	Nyanya Neem FCT	Apo Neem FCT	Garaku Khaya NAS	Keffi Khaya NAS	Uke Neem NAS	Kaduna road Neem NAS	Auta New NAS	Karu Neem NAS	Status
(1). 1500	0	0	1	1	0	1	0	1	1	1	0	1	Polymophic
1000	1	0	0	0	0	0	0	0	0	0	1	0	Polymophic
700	0	0	0	0	0	0	1	0	0	0	0	0	Unique
(2). 1000	0	1	1	1	1	1	0	1	1	1	0	1	Polymophic
900	1	1	1	1	1	1	1	1	1	1	1	1	Monomophic
700	0	0	0	1	1	1	0	0	0	0	0	0	Polymophic
(3). 1500	0	0	1	1	0	1	0	1	1	0	0	0	Polymophic
1000	1	1	1	1	1	1	1	1	1	1	1	1	Monomophic
800	0	0	0	0	0	0	1	0	0	0	0	0	Unique
700	0	0	0	0	0	0	1	0	0	0	1	0	Polymophic
600	0	0	0	0	0	0	1	0	0	0	0	0	Unique
(4). 1500	1	0	1	0	0	1	0	1	1	1	0	1	Polymophic
1000	1	1	1	1	0	1	0	1	1	1	0	1	Polymophic
900	1	1	1	0	0	0	1	0	0	0	0	0	Polymophic
800	0	0	0	0	0	0	1	0	0	0	0	0	Unique
(5). 1000	1	0	0	0	0	1	0	1	1	1	0	1	Polymophic
900	1	1	1	0	0	1	0	1	1	1	0	1	Polymophic
800	0	1	1	1	1	0	0	0	0	0	1	0	Polymophic
700	0	0	0	0	0	0	1	0	0	0	0	0	Unique
600	0	0	0	0	1	0	1	0	0	0	0	0	Polymophic
500	0	0	0	0	0	0	0	0	0	0	0	1	Unique

4.5. Percentage Polymorphism of Primers

Table 8 showed the percentage polymorphism of the different primer across the plant sample. It was observed to 66.6% for OPA 01 and 80% for OPJ 01 wish implies that the polymorphism did not cut across the plant sample completely. While, OPL 01, OPA 02 and OPB 01 had 100% polymorphism.

Table 9 also showed the percentage polymorphism of primers for herbal sample. It was 100% in OPL 01 primer only. While OPA 01, OPJ 0, OPA 02 and OPB 01 had 66.6%, 33.3 %, 75% and 83.5% respectively.

Table 8: Percentage Polymorphism of Primers for Plant Sample

Primer	Fragment Size Range	Total No of Fragment	No of Polymophic Band	Percentage Polymorphism
1.(OPL 01)	1500-700	3	3	100%
2.(OPA 01)	1000-800	3	2	66.6%
3.(OPJ 01)	1500-600	5	4	80%
4.(OPA 02)	1500-800	4	4	100%
5.(OPB 01)	1000-500	6	6	100%

Table 9: Percentage Polymorphism of Primers for Herbal Sample

Primer	Fragment Size Range	Total No of Fragment	No of Polymophic Band	Percentage Polymorphism
1. (OPL 01)	1000-700	4	4	100%
2.(OPA 01)	1000-800	3	2	66.6%
3.(OPJ 01)	1000-800	3	1	33.3%
4.(OPA 02)	1500-800	4	3	75%
5.(OPB 01)	1000-500	6	5	83.5%

4.6. Dendogram

Figure 1 is a dendrogram generated which showed the output of the data from the Numerical Taxonomy System of Statistic (NTSYS). The generated data showed the genetic diversity among the samples under study. The cluster generated from the dendrogram contained two distinct clusters. Group 1 contained only one sample while the rest accessions were clustered in group 2. Group 1 contained GarakuNasarawa plant sample at 0.63 similarity index. Group 2 has two separate clusters that grouped the remaining twenty-three both herbal samples and plant samples.

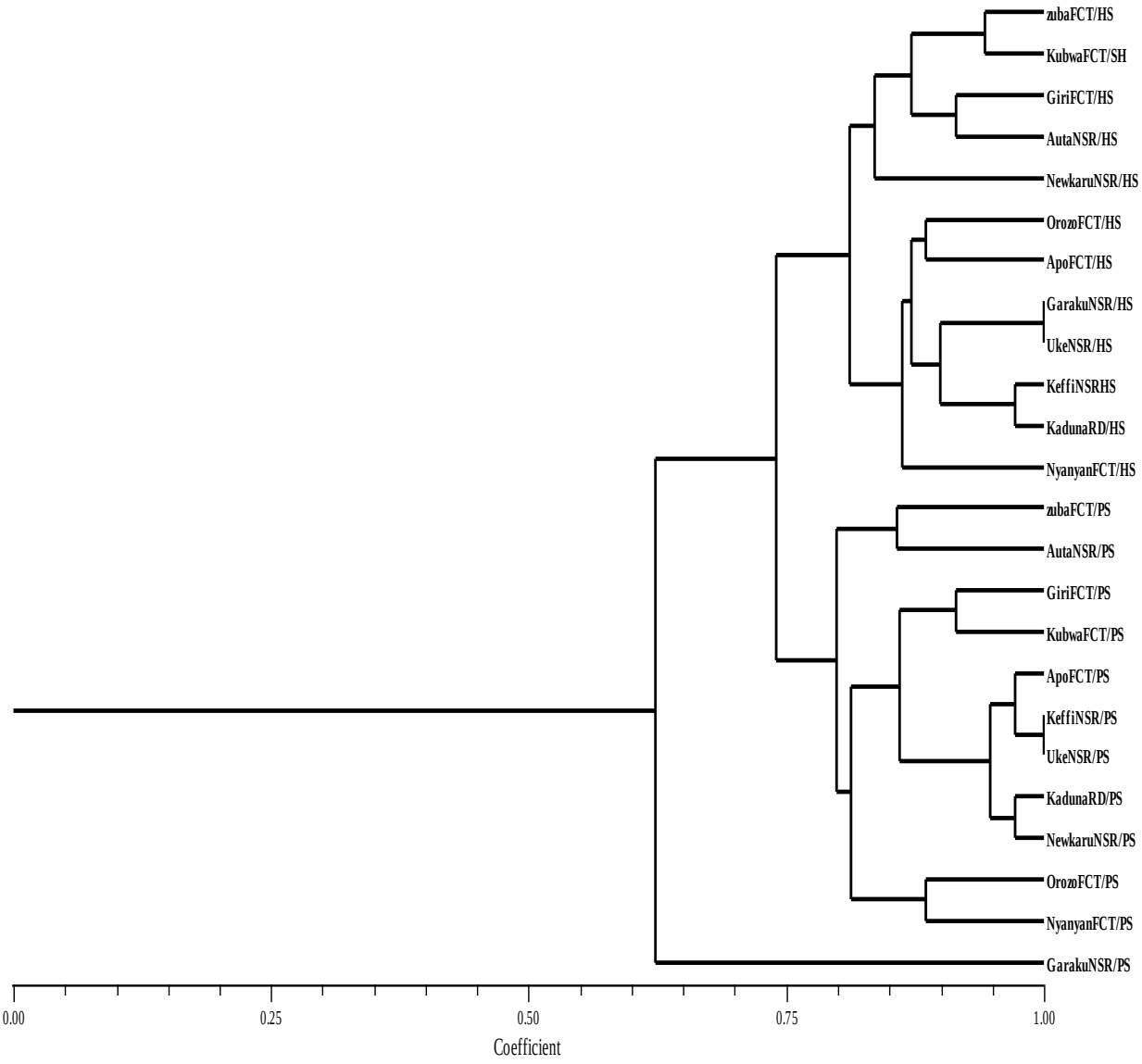


Figure 1:DendogramIndicating Similarity Matrix among the Herbal and Plant Sample

CHAPTER FIVE

DISCUSSION, CONCLUSION AND RECOMMENDATIONS

5.1. Discussion

The data generated from the amplification of the DNA were subjected to Numerical Taxonomy System of Statistic (NTSYS). The output of the data from the NTSYS generated a dendrogram showing the genetic diversity among the samples under study as shown in figure 1. The cluster generated from the dendogram contained two distinct clusters. Group 1 contained only one sample while the rest accessions were clustered in group 2. Group 1 contained GarakuNasarawa plant sample at 0.63 similarity index. Group 2 has two separate clusters that grouped the remaining twenty-three both herbal samples and plant samples. The first cluster of group 2 contains 3 subgroups that had 11 herbal samples with Zuba FCT plant sample and AutaNasarawa plant sample in one sub group, while Giri FCT plant sample and kubwa FCT plant sample clustered together, the implication is that they have similar genetic traits at that coefficient. Kaduna road plant sample and New KaruNasarawa sample clustered from the other accessions leaving Apo FCT plant sample, KeffiNasarawa plant sample clustered with UkeNasarawa Plant sample, it means that at 0.63 similarity index both of them have the same genetic composition hence there is no difference among them.

The other clusters in group 2 consist of three major clusters that made up 100% of the herbal samples under study. The first subgroup had four herbal samples obtained from Zuba, Kubwa, Giri all from FCT with Auta from Nasarawa herbal sample. This showed they have the similar composition. Although New KaruNasarawa herbal sample clustered

with them but with little degree of genetic differences, Nyanyan FCT herbal sample stands separately from Kaduna Road herbal sample and KeffiNasarawa herbal sample. At 0.63 similarity index herbal samples from GarakuNasarawa has the same content in terms of ingredient. Orozo FCT herbal sample and Apo FCT herbal sample made up the remaining accessions in the third sub-cluster.

As adopted in this study, the PCR protocol resulted in reproducible pattern of amplicons using specific combination of accession and primer. Only the primers which displayed reproducible, scorable and clear bands were considered for analysis as presented Tables 6 and 7. Image profiles of the banding patterns as obtained in the PCR gell picture (Plates 5 - 14) were recorded and molecular weight of each band was determined by NTSYS Analyst software. The banding pattern was also scored based on the presence or absence of clear, visible and reproducible bands (Tables 6 - 7). A band is considered to be 'polymorphic' if it is present in some individuals and absent in others, and 'monomorphic' if present in all the individuals or accessions (Plates 5 - 14). The most intense monomorphic band from each accession with each primer was used as reference to calibrate different lanes for the amounts of DNA present (Table 6). When the band was not monomorphic, the band with maximum frequency in each accession was used for calibration. In each lane, bands were scored present if their intensity was at least 10% of the monomorphic reference band within the same lane (Anjum*et al.*, 2003).

Traditional medicinal systems use whole plants or their parts for combating a great number of disorders. Hence maintaining the purity and quality of herbal drugs are necessary to identify the substitutes and adulterants. Medicinal plants constitute an effective source of traditional and modern medicine. Adulterations and substitutions are

common in herbal material trade and medicinal plants as observed in the result. Adulteration in market samples is one of the greatest drawbacks in promotion of herbal products which could be the reason for the 37.5% relatedness obtained in the phylogenetic tree. Due to this adulteration and altered efficacy, the faith in crude drug promotion has declined (Dubey*et al.,* 2004). The RAPD, PCR based assay described here is fast, reliable, and easy to conduct in any laboratory. It can be carried out in very short period using unknown genomic DNA from any developmental stages and body parts of herbs (Kethidi*et al.,* 2003). The high quality and purity of genomic DNA free from secondary metabolites was isolated from dried root powder of genuine and adulterant samples by modified CTAB method for RAPD-PCR (Salim*et al.,* 2007). When DNA was isolated using methods described by (Doyle and Doyle, 1987) and (Murray and Thompson, 1980), DNA of desired quality and quantity could not be isolated. For RAPD reaction, it was necessary to standardize the following variables for successful amplification using PCR. RAPD amplification is not reproducible below a certain concentration of genomic DNA and produces 'smears' or results in poor resolution if DNA concentration is high.

Despite close identity as observed in the PCR analysis and Picture, a great deal of polymorphism was observed among the accessions. *Khayasenegalensis* and *Azadirachtaindica*also showed significant genetic variations with each other. Numerical Taxonomy System of Statistic (NTSYS) analysis showed only 37.5% relatedness in the 12 herbal sample and 12 plant sample collections, which is deemed to be useful in authentication of herbal product and identification of medicinal plant material.

5.1 Conclusion

From the phylogenetic generated among the 24 samples based on PCR product, it was observed that the relatedness was 37.5% that shows an appreciable relationship. It was interesting to know that there was no inter relationship between herbal samples and Plant sample at that index.

This showed that they contain both *Khayasenegalensis* and *Azardirachtaindica*as they claim though; the quantity is what is now in contempt. This is simply because 37.5% of a result is not good enough for the loud claim of the ability of both *Khayasenegalensis* and *Azardirachtaindica*to treat Diabetes. Form the DNA extraction process, a lot of phenolic substance was observed as the DNA was cloudy in some case. The quality of the DNA was also not good as observed under the Gell Documentation System.Development of RAPD markers that can correlate DNA fingerprinting data with quantity of selected phytochemical markers associated with that specific medicinal herb, would have extensive applications in quality control of herbal product and also on their raw materials.

Based on this study, the large range of similarity and dissimilarity values for the medicinal plants and herbal sample using RAPD provides the greater confidence for assessment of genetic authentication and relationships. The practical approach developed in the study will also be useful in DNA fingerprinting and identification of plant species from the adulterants and substitutes. This will also make identification and characterization of genotype very easy.

5.2. Recommendations

From the RAPD marker assessment conducted it was observed that the herbal sample contain very little percentage (37.5%) of the acclaimed plant sample (*Khayasenegalensis* and *Azardirachtaindica*). The percentage of the herbal sample is very low and as such cannot be said to have such herbal potency to cure diabetes as claimed. Though this is not to say other content of the sample do not have such potency as such statement is subject to research. The herbal sample also contains a lot of mixture that made it difficult to conclude its potency.

Sequencing in this research is further recommended using a better and advance molecular biology technique to ascertain this claim by herbal practitioner in and around Nasarawa State and the FCT. Other herbal claims by this local herbs seller should also be research in and around Africa because there are a lot of yet to be discovered genuine herbal combinations that treat many ailments. Molecular markers should always be employed as a qualitative/quantitative diagnostic tool for identification of medicinal herbs from harvesting to finished product.

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APPEDIX

4.6. Similarity Coefficient and Dendogram

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