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DNA and HPLC Fingerprint of *Commiphora africana* (Burseraceae)

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ABSTRACT

Commiphora africana is a famous medicinal plant used traditionally in the treatment of various ailments such as inflammation, stomach disorders, diabetes, malaria, tumours, skin infections and as antidotes for venomous bites and stings in Nigeria and other parts of Africa. The present study aims to evaluate the molecular and chemical properties that could be of value in the standardization and quality assessment of the medicinal values of *C. africana*. The randomly amplified polymorphic deoxyribonucleic acid – polymerase chain reaction (RAPD - PCR) analysis was carried out on the leaf sample. The high-performance liquid chromatography (HPLC) was carried out on the leaf and barks of stem and root extracts. The RAPD-PCR amplified products generated fingerprint patterns ranging from 250 to 1600 base pairs. The oligoprimers (A, B and E) produced distinct banding patterns for *C. africana*, the number of well-defined and major bands for a single primer ranged from 2 to 7 with maximum number of well-defined bands observed with primer 'a' (7 bands) and the minimum number with primer 'e' (2 bands). The HPLC chromatograms of the extracts of leaf and barks of stem and root of *C. africana* showed some similarities and differences in the type, number and concentration of the compounds in the various morphological parts of the plant. The DNA and HPLC fingerprints obtained from this research can be useful in the identification and authentication of *C. Africana* and thus may be useful in the standardization of this plant.

Keywords: Medicinal plant, *C. africana*, HPLC, RAPD.

Introduction

All through the ages, humans have relied on nature for their basic needs for the production of food, shelters and medicines. Plants are the main ingredient in traditional medicine systems that have been in existence for thousands of years and continue to provide mankind with new remedies.[1] According to World Health Organization, use of traditional medicine around the globe is increasing and in the developing countries nearly 80% of the population relies on these drugs for their primary health care needs.[2] Due to renewed interest and popularity in the use of herbal medicines, adulteration of herbal materials is frequently becoming a common occurrence.[3] Proper identification and authentication of plant species is necessary to improve the use of novel medicinal plants.[4] Traditionally, plants are being identified with the help of flora of different regions based on visual assessment of morphological and phenological traits in the field.[5] To protect the patent and quality standards of plant varieties for industries, it is necessary to develop authentic and unquestionable plant identification methods such as DNA fingerprinting. DNA based molecular fingerprints are based on the polymorphism at molecular level instead of morphological characteristics.[6] DNA-based techniques have been widely used for authentication of plant species of medicinal importance. DNA markers are more reliable because the genetic information is unique for each species and is independent of age, physiological conditions and environmental factors.[7,8] Among the polymerase chain reaction (PCR)-based molecular techniques, random amplified polymorphic DNA (RAPD) is convenient in performance and does not require any information about the DNA sequence to be amplified.[9] The Random Amplified Polymorphic DNA (RAPD) assay based technology has been widely used by several research groups as an efficient tool for identification of markers linked to important traits and cultivars.[4] In the last two decades, efforts have been made to develop an effective tool to resolve problems in standardization of herbal drugs. Using chromatographic techniques like thin-layer chromatography (TLC), high performance liquid chromatography (HPLC) and liquid chromatography mass spectrometry LC/MS, a profile of their various chemical constituents is obtained which is referred to as chemical fingerprints. Using chemical fingerprinting, plants can be demarcated on the basis of their species, strain and geographical origin.[3] *Commiphora Africana* is a shrub or small tree, sometimes reaching 10 m but usually not more than 5 m high with short bole and sprawling branches usually spined from the family Burseraceae.[10] It is traditionally used for the treatment of a number of ailments including typhoid, wound healing, pain, dysentery, heartburn, snake-bites and as anti-malaria.[11,12] Other uses include management of cold, fever and stomach ache and also as arbortifacients, cancer and inflammatory diseases, measles, insecticides, antilipidaemic, antiobesity and management of cardiovascular disorders.[13-15] In the present study, efforts have been made to identify, authenticate and standardize *C. africana* by using the RAPD-PCR and HPLC techniques.

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Materials and Methods

Plant sample

The young shoots (with intact leaves) from *C. africana*plant as authenticated by the taxonomist were collected around the vicinity of Ahmadu Bello University Dam, Samaru campus, Zaria, Kaduna state, Nigeria. The sample was carefully placed in a sealable polythene bag, transported to a laboratory facility (DNA Labs Kaduna) and then kept frozen until DNA extraction.

Development of DNA fingerprints for C. africana by RAPD-PCR
DNA Extraction

The young leaves of the plant were properly grounded with the aid of a mortar and pestle and thereafter the total genomic DNA was extracted by using the Plant Genomic DNA Miniprep Kit from Bioland Scientific (USA). The DNA isolated was used for the randomly amplified polymorphic DNA (RAPD) analysis.

Determination of DNA quality and quantity

The DNA quality was assessed by running the samples on 1% agarose gel by electrophoresis at 80 V for 40 min. The gel was stained in ethidium bromide followed by analysis on Gel Documentation and Analysis System (Gel Doc 2000, Biorad, UK) and the quantity of DNA extract was estimated via spectrophotometry (GeneQuant*pro*, Eppendorf AG, Germany) at 260 nm. The isolated DNA was stored at -20^{o}C.

RAPD-PCR Primers

For using PCR based markers, different random decamer primers were obtained for the study from Inqaba Biotech South Africa (Table 1) labeled A - E. The key important feature of RAPD is the use of a single 10-oligonucleotide arbitrary primer to amplify template DNA without prior knowledge of the target loci. Two basic criteria as suggested by Williams must be met for the base pair sequences of RAPD primers i.e. minimum of 40% GC content (50% - 80% GC content is generally used) and the absence of palindromic sequence[8].After primary screening, only those primers giving polymorphic bands i.e primer a, b and e were selected for further use.

Table 1: Decamer primers obtained from Inqaba Biotech for the RAPD study.

Primer name	Sequence (5' – 3')	GC content (%)	Bases	Tm (^{o}C)
A	GGTGCGGGAA	70	1010	43.6
B	GTTTCGCTCC	60	1010	39.5
C	GTAGACCCGT	60	10	39.539.5
D	AACGCGCAAC	60		43.6
E	CCCGTCAGCA	70		

RAPD-PCR Optimization

For PCR reaction mixture, PCR reaction kit (Bioneer) was used and 18ul volume of PCR reaction was prepared using IX Taq buffer, 2.5mM $MgCl_2$, 2mM dNTPs, 10pmol primer, 0.5UTaq enzyme and 50ng of the isolated genomic DNA. Amplification was carried out in PTC-100 Programmable thermal controller (MJ-Research Inc. USA) with initial denaturation cycle at 94℃ for 5 min, followed by 45 cycles consisting of 94℃ for 30 sec, annealing at 34^{o}C for 30 sec and extension at 72^{o}C for 1 min and a final extension cycle at 72℃ for 7 min. (Table 2). Every PCR reaction was repeated twice to get reproducible results.

Resolving of PCR Product for Scoring and Data Processing

The PCR amplification products were resolved on 1.5% agarose gel electrophoresis (AGE) with 1X Tris Acetate-EDTA buffer (pH 8.3), stained with ethidium bromide and visualized under UV light (Dolphin Gel Documentation system). The size of the amplicons was estimated from 100bp to 10,200bp with DNA ladder mix (Bioneer).

HPL fingerprints of the morphological parts of C. africana

Extracts for HPLC analysis were prepared by extraction of 10 g each of the leaf, stem and root bark powders with 200 mL 80% aqueous methanol using maceration technique for 48 hrs after which the filtrates were concentrated using a rotatory evaporator and a fraction of the residue was subsequently diluted with methanol to a final concentration of 50 mg/mL. Analysis of 10 µL extract aliquots was conducted on a Phenomenex Aqua C18 column (250 mm x 2.1 mm), using an HPLC1260 system equipped with a 996 PDA detector, at a constant mobile phase flow rate of 2 mL/min at a wavelength of 250 nm. The mobile phase consisted of methanol: acetonitrile (75:25) in acetic acid.

Data Analysis

The DNA fragment amplified by RAPD primers were analyzed by size and intensity of all the scorable bands. Statistical differences between samples were evaluated using Student's t-test and noted to be significantly different where $p < 0.05$.

Results and Discussion

Quantity and Quality of the Extracted DNA

The quantity of DNA extracted was estimated to be 46.0 ng at A_{260} nm and DNA quality assessment was done through the A_{260}/A_{280} ratio that gave a value of 1.9 which shows very high purity. The quality of DNA was assessed by its physical appearance, spectrophotometry, gel electrophoresis and PCR amplification. The A_{260}/A_{280} ratio is an indication of protein contamination. For high-quality DNA, A_{260}/A_{280} ratio should range between 1.8 and 2.2 and A_{260}/A_{230} ratio between 1.5 and 1.8.[16,18]

Table 2: Optimized PCR Conditions Using *Taq*DNA Polymerase.

Pipetting Volume (µL)		Cycling Protocol		
Component	25 µLReaction	Cycle Step	3 Step Protocol	Cycles
			Temp. Time	
H_2O	14.4	Initial Denat.	94˚C 5 min	
1X PCR Buffer	2.5	Denaturation	94˚C 30 sec	
2 mM dNTPs Mix	2.5	Annealing	34˚C 30 sec	
25 mM $MgCl_2$	2.5	Extension	72˚C 1 min	45
Primer A (15 µm)	0.8	Final Extension	72˚C 7 min	
Primer B (15 µm)	0.8			
Primer E (15 µm)	0.8			
DNA Polymerase	0.5			
Genomic DNA (50 ng)	0.5			

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RAPD-PCR Analysis

The three decaprimers observed to produce at least a scorable band during the initial screening were chosen for further study. The selected three (3) primers A, B and E generated 11 scorable amplification products against genomic DNA sample of *C. africana.* The number of well-defined and major bands/loci for a single primer ranged from 2 to 7with the approximate size ranges from 250 to 1500 bp. The maximum number of well-defined or major bands was observed with primer 'a' 7bandsand the minimum number with primer 'e' 2 bands (Figure 1, Table 3). The most important application of DNA marker is polymorphism, which can be used to categorize the different plant accessions/genotypes. The specificity of RAPD loci indicated that markers could be used to identify genotypes of important plants. DNA based markers are not tissue specific and thus can be detected at any stage of plant development. They thus offer advantages over the traditional phenotypic and chemical markers which can be affected by age, environmental factors and physiological conditions. Among different molecular markers; RAPD, SSR and AFLP are extensively used in germplasm characterization and DNA fingerprinting. RAPD has been successfully utilized for the identification of medicinal plants and herbal medicinal components.[16,17] Our study showed that the protocol used worked well for the plant species studied. DNA based authentication of medicinal plants can be useful as a tool for quality control and safety monitoring of herbal pharmaceuticals and nutraceuticals and can significantly add to the medical potential and commercial profitability of herbal products.[18] RAPD has been successfully utilized for the identification of medicinal plants,[16]and herbal medicinal components.[17]RAPD has frequently been used for the detection of genetic variability in plants. The advantages of RAPD method are its rapidity, simplicity and lack of need for any prior genetic information about the plant and RAPD patterns are consistent irrespective of the plant source or age.

HPLC Fingerprints of the leaf, stem and root barks of C. africana

The HPLC chromatograms of the leaf and barks of stem and root extracts (Figure 2) from *C. africana*differed significantly from each other. The presence of five compounds that were common to the leaf and barks of stem and root extracts of *C. africana*were observed at retention times ranging between 1.00 – 2.33 mins. Two compounds at retention times of 1.00 – 1.03 and 1.96 – 2.33 min were observed to be common to all the three morphological parts at a similar concentration respectively, though the compound at Rt 1.03 min was the most exceptionally predominant of all with a peak area concentration greater than 90% (Table 4). This compound when properly characterized may serve as a chemotaxonomic marker for this plant species. A compound at a retention time range of (2.33 – 2.35 mins) with a percentage integration area of greater than 1% (Table 4) was found to be common to both the stem and root barks though, at a lesser percentage integration area in the root, this compound was absent in the leaves. Three compounds at retention time ranges of (2.62 – 2.70, 2.88 – 2.95 and 3.23 – 3.33 mins) with a percentage integration area of less than 1% (Table 4) were found to be common to both the stem and root barks but were absent in the leaves.

Fingerprinting is a quality control model that builds upon spectroscopic and chromatographic technology. It is different from the traditional quality control model in the sense that fingerprinting looks at the complete information or comprehensiveness of the chromatograph and displays integrated quality information. Fingerprint analysis focuses on accurate identification (of similar peaks), and not on precise calculation. The comparison of fingerprints emphasizes similarity and the fingerprints compared do not need to be exactly the same. When it is impossible to find out all the complex components of a herbal medicine, fingerprints can be used to check the stability of the intrinsic quality of the medicine.[8] The HPLC analysis results could form the basis of a more detailed comparative study of the morphological parts of the plant as regards secondary metabolites and any marked similarity in their pattern may draw reasonable conclusions of the relationships of the chemical constituents within a given plant species.[19] Groups of secondary metabolites are used as markers for chemotaxonomical classification, which is based on the assumption that systematically related plants will show similar chemical characteristics.[18]

It is thus evident that HPLC-UV may be used as a tool in identifying species that belong to the same chemotaxonomic group. Similarities and differences were noted in the chromatograms of the leaf and barks of stem and root of a *C. africana*. These differences may account for the variation in the biological activities within the various parts and explain why a particular morphological part may be preferred over other for the treatment of a particular ailment in traditional medicine.

Table 3: The detail of bands produced by 3 RAPD primers selected for the study in *C. africana.*

Primer name	Sequence (5' – 3')	Total number c bands	Band range (bp)
A	GGTGCGGGAA	6	300 - 1500
B	GTTTCGCTCC	3	250 - 700
E	CCCGTCAGCA	2	250 - 550

Table 4: The retention times and the percentage integration areas for detected peaks from the morpholgical parts of *C. africana.*

S/No	Rt (min)	CAL	CAS	CAR
1	1.00 – 1.03	***	***	***
2	1.42 – 1.44	*	**	*
3	1.53 - 1.65	*	*	*
4	1.75 – 1.87	*	*	**
5	1.96 – 2.33	**	**	**
6	2.33 – 2.35	-	**	*
7	2.62 – 2.70	-	*	*
8	2.88 – 2.95	-	*	*
9	3.13 – 3.16	*	-	*
10	3.23 – 3.33	-	*	*
11	3.56	-	-	*
12	3.94	-	-	*
13	4.01	-	*	-
14	4.45	-	-	*
15	4.53	-	*	-
16	4.69	-	-	*
17	5.22	*	-	-
18	5.51	*	-	-
19	5.68	-	-	*
20	5.82	-	*	-
21	6.41	-	-	*
22	6.58	-	*	-
23	8.42	*	-	-
24	12.64	*	-	-
25	13.13	-	*	-

Where: Rt = represents the retention time in minutes,
* = presence of peak, - = absence of peak.
*** Peak area ≥ 10% ** peak area ≥ 1% , *peak area < 1%
CAL = *C. africana* (leaf), CAS = *C. africana* (stem), CAR = *C. africana* (root)

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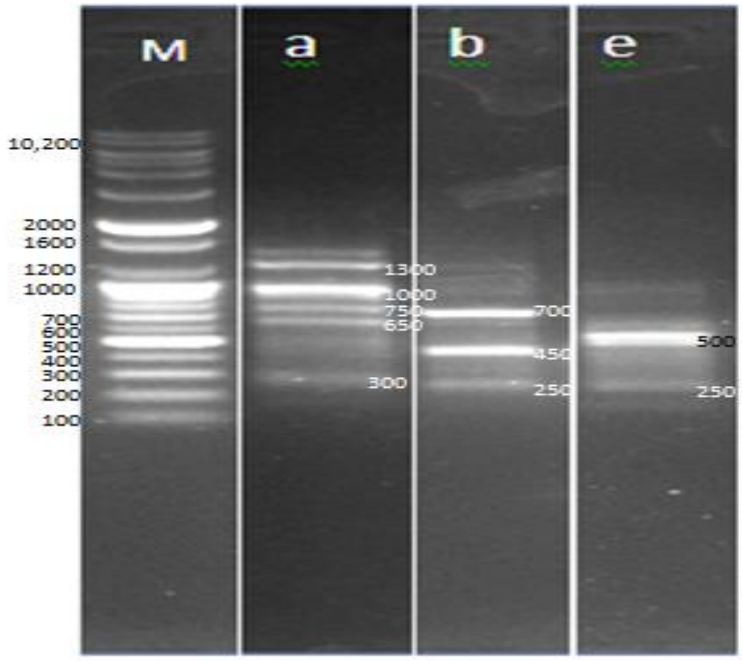

Figure 1:RAPD-PCR product profiles of *C. africana* plant. Lane M = 10,200-bp molecular weight marker; lanes a, b and e = primers A, B and E, respectively.

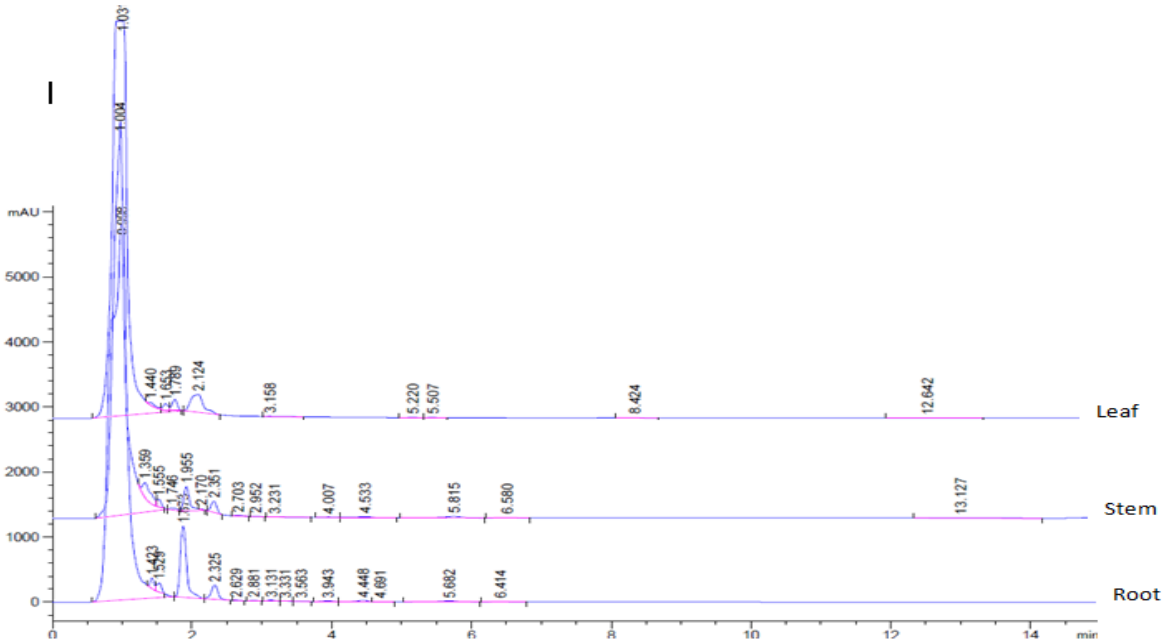

Figure 2: HPLC Chromatograms of the Leaf, Stem and Root bark extracts of *C. africana.*

Conclusion

The study demonstrates that out of the five oligoprimers (A-E) used, primers (A, B and E) produced distinct banding patterns for *C. africana* which can be useful as a characteristic DNA fingerprint for this plant species. The HPLC chromatograms showed some similarities and differences in the type, number and concentration of the compounds in the various morphological parts of the plant and thus also give a characteristic pattern. The DNA and HPLC fingerprints obtained from this research can be useful in the identification and authentication of *C. Africana* and thus may be useful in the standardization of *C. africana* commonly used in traditional medicine in Northern Nigeria.

Conflict of interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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