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DNA BARCODING AND HPLC–DAD–ESI-MS/MS CHARACTERIZATION OF COMPOUNDS FROM *COMMIPHORA AFRICANA (*A. RICH.) ENGL. (BURSERACEAE)

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ABSTRACT

DNA barcoding is a novel method of species identification based on nucleotide diversity of conserved sequences. It provides authentic identification of plants on a molecular basis. *Commiphora africana* is a famous medicinal plant used traditionally in the treatment of various ailments such as inflammation, stomach disorders, diabetes, malaria, tumors, skin infections and as antidotes for venomous bites and stings in Nigeria and other parts of Africa. The present study aims to develop DNA barcodes and chemical profile for *C. africana.* The total genomic DNA was extracted from the young leaves of *C. africana,* the isolated total genomic DNA was PCR amplified using the *rbcl* and *matk* markers and the PCR products were resolved on agarose gel electrophoresis. The amplified band was sequenced and analyzed using Basic Local Alignment Search Tool (BLAST). The LC/MS was carried out on the *C. africana* stem bark ethanol extract through HPLC–DAD–ESI-MS/MS experiments. The primer (*rbcl*) gave good amplification and produced a barcode region with 550 bp in this plant species while the *matk* marker could not produce a visible band. About 10 chemical compounds were tentatively identified from the stem bark of *C. africana*. The results showed that the use of these conserved DNA sequences as barcodes and chemical profiles would be an accurate way for species identification and discrimination.

Keywords: DNA barcoding, *Commiphora africana*, BLAST*, rbcl.*

INTRODUCTION

Natural products, such as plants extract, either as pure compounds or as standardized extracts, provide infinite opportunities for new drug compounds or constituents because of the unmatched availability of chemical diversity (Chikezie *et al*., 2015). According to the World Health Organization (WHO), more than 80% of the world's population depends on traditional medicine for their primary healthcare needs (WHO, 2002). The traditional system of medicine employs medicinal plants to cure various illnesses but the herbal industry suffers from substitution and adulteration of medicinal herbs with closely related or unrelated species (Sofowara *et al*., 2013). The effectiveness of the drug may be altered if adulterated and, in some cases, harmful if substituted with toxic adulterants. Hence, the correct identification is important for the safety and efficacy of medicinal herb (Mishra *et al.,* 2016). The absence of some morphological features could make the identification difficult or sometimes impossible (Wu *et al*., 2021). DNA barcoding is a technique that is used to identify the species based on species-

specific differences in short regions of their DNA (Newmaster *et al.,* 2013). Following the assessment of several candidate *loci,* the Plant Working Group (PWG) of the Consortium for the Barcoding of Life (CBOL) recommended that sections of two plastid genes, *rbcL* and *matK*, be adopted as the standard plant DNA barcodes, with the acknowledgment that supplementary markers may be required (Lin *et al*., 2011). The medicinal values of the plants are due to the presence of chemical substances that produce a definite physiological action on human body and are called phytochemicals (Saxena *et al.,* 2013). In general, structures, distributions and percentage occurrence of secondary metabolites are valuable tools in providing taxonomic markers as well as defining evolutionary pathways (Xie *et al*., 2014). *Commiphora africana* is traditionally used for the treatment of a number of ailments including typhoid, wound healing, pain, dysentery, heart burn, snake-bites and as anti-malaria (Abubakar *et al*., 2016, Nuhu *et al*, 2016). In the present study, efforts have been made to develop a DNA barcode and chemical profile that could be useful in the identification and standardization of *C. africana*.

2. 0 MATERIAL AND METHODS

2.1 Plant Collection and Identification

The leaves of *C. africana* were collected around the vicinity of Ahmadu Bello University Dam, Samaru campus, Zaria, Kaduna state, Nigeria in the month of February 2021. The plant was identified and authenticated as *C. africana* by a Taxonomist at the Herbarium unit of the Department of Botany, Faculty of Life Sciences, Ahmadu Bello University, Zaria - Nigeria. The Herbarium specimen was cross matched with an existing specimen and thus allotted a reference specimen voucher number no. 2848. The sample was carefully packaged in Ziplock bag, transported to a laboratory facility and kept frozen until DNA extraction.

2.2 Genomic DNA Extraction

Exactly 3g of the frozen fresh plant material was homogenized using pestle and mortar, thereafter the Genomic DNA was extracted using the Easy Prep Plant Genomic DNA Miniprep Kit from Bioland Scientific (USA) according to the manufacturer's instructions.

2.3 DNA Quality and Quantification Assessment

The DNA quality was assessed by running the sample 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 and the quantity of DNA extracted was estimated via spectrophotometry (Gene Quant *pro*, Eppendorf AG, Germany) at 260 and 280 nm. An aliquot of the DNA was diluted in distilled water. The isolated DNA was stored at -20°C.

2.4 Primer Selection

The two most commonly used primers *rbcl* and *matk* were utilized in this study. The detail description is as listed in Table (1)

Table 1: Gene Region and Primer Sequence for DNA Barcoding of *C. africana*

Gene Region	Name of Primer	Primer Sequence 5′-3′
Rbcl	1F	ATGTCACCACAAACAGAAAC
	724R	TCGCATGTACCTGCAGTAGC
matK	KIM_3F	CGTACAGTACTTTTGTGTTTACGAG
	KIM_1R	ACCCAGTCCATCTGGAAATCTTGGTTC

2.5 PCR Optimization

A total volume of 20 µl of PCR reaction mixture prepared as follows: 8 µl of DTCS Quick start master Mix, 2 µL of genomic DNA, 1µL of each primer and adjusted with sterile distilled water (to 20 µl). The PCR amplification was performed with a PTC-100™ Programmable Thermal Controller (MJ Research INC.), the profile was adjusted to 35 cycles as follows: Initial denaturation cycle at 90℃ for 5 min, followed by 34 cycles consisting of 94℃ for 30 sec, annealing at 54℃ for 30 sec and extension at 72℃ for 1 min and a final extension cycle at 72℃ for 5 min.

2.6 Resolution of the PCR Product

The PCR amplification products were resolved on 1 % 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 amplicon was estimated from 100 bp DNA ladder.

2.7 Nucleotide Sequencing

The amplified PCR products were purified using a PCR Purification Kit (Thermo scientific) before being sequenced using the dideoxynucleotide chain termination method with a DNA sequencer (ABI 3130XL, Applied Biosystems) and a Big Dye Terminator version 3.1 Cycle Sequencing RR-100 Kit (Applied Biosystems).

2.8 Database search using BLAST

The obtained sequence was queried using the NCBI BLASTN 2.9.0+ online database web interface and the sequences were used to construct phylogenetic tree using Mega 6 (Tamura et al., 2013). The evolutionary history was inferred based on Neighbor-Joining method (Saitou and Nei 1987). The obtained sequence was submitted to the GenBank database.

2.9 Liquid Chromatography – Mass spectrometry of the Stem bark Extract of *C. africana*

2.9.1 *Extraction*

10.0 g of dried powdered plant material was extracted with 500 mL of a 70% of aqueous ethanol using the cold maceration technique for 24 hours. The mixture was filtered, the filtrate was dried using rotavapor, labeled and stored in the desiccator until required for use. The scum was discarded.

2.9.2 *Chromatography*

Separation of compounds in the *C. africana* stem bark extract was performed using Triple Quad LCT/MS / HPLC-Alliance 2695-watermaslynx LCT-MS equipped with an Agilent Zorbax C18 column (4.6 x 150 mm, 5 µm). Acidified water and methanol were used as mobile phases A and B, respectively. The gradient elution was programmed as follows: 30% B, 0-1 min; linear gradient from 30% B/70% A to 70% B/30% A, 1-3 min; linear gradient from 70% B/30% A to 100% B, 3-9 min; holding at 100% B, 9-13 min; linear gradient from 100% B to 30% B/70% A, 13-14 min; holding at 30% B/70% A, 14-15 min. The flow rate was set at 1mL/min throughout the gradient. The flow from the HPLC system into the ESI-Q-TOF-MS detector was 0.2 mL/min. The injection volume was 5 µL and the column temperature was maintained at 25^0C. The HPLC system was coupled to a Triple quadrupole-time-of-flight mass spectrometer (QToF-MS) and was operated with electrospray ionization (ESI) in positive mode at the mass resolution of 100 - 1000 and controlled by MassLynx 4.0 software. The source parameters were set as follows: capillary 3 kV, sampling cone 35 V, extraction cone 3 V, Source temperature 100^0C, desolvation temperature 250^0C, Desolvation gas flow 702 L/h, and cone gas flow 21 L/h. Detector: Waters 2487 UV Detector and Wavelength 254. The

following public databases were consulted: ChemSpider (http:// www.chemspider.com), SciFinder Scholar (https://scifinder. cas.org).

2.11 Data Analysis

The DNA fragments amplified by primers were analyzed by size and intensity from all scorable bands and comparing with a standard 100bp DNA ladder.

3.0 RESULTS

3.1 Quality of DNA Extracted

The DNA quality assessment through the A_{260}/A_{280} ratio gave a value of 1.8. The *rbcl* marker successfully generated amplified product of about 550 bp while the *matk* marker could not produce any band (Plate I).

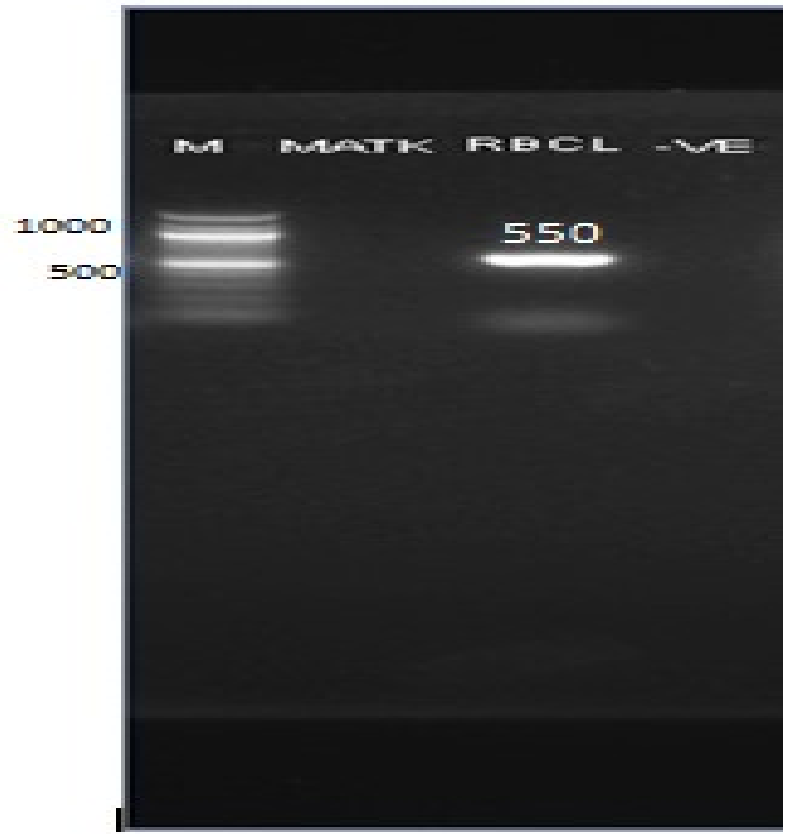

Figure I: PCR product for DNA Barcode Region of *C. africana*
M=*1kb DNA ladder, MatK = no band, RBCL =band of about 550bp, -ve = Negative control*

3.2 PCR product analysis

The rbcL nucleotide PCR-product generated a clear scorable band of about 550bp while the Matk marker could not produce any scorable band (Figure 1).

3.3 Sequence Analysis

The partial rbcL nucleotide sequence homology of *Commiphora africana* showed 99% similarity with other species of Commiphora and related taxa (Table 1). The sequence was assigned an accession number MN100136.

Table 1: The top 10 BLAST result of *rbcL* sequence of *C. africana* from the NCBI BLASTN 2.9.0+ online database

S/No.	Plant name	Family	Percentage similarity (%)	Accession Number
1	*Commiphora schimperi*	Burseraceae	99.81	JF265365.1
2	*Commiphora gileadensis*	Burseraceae	99.45	NC041104.1
3	*Commiphora wightii*	Burseraceae	99.45	MH042752.1
4	*Commiphora africana*	Burseraceae	99.43	JX572461.1
5	*Commiphora zanzibaria*	Burseraceae	99.24	JX572468.1
6	*Commiphora roxburghii*	Burseraceae	98.88	JN114798.1
7	*Commiphora foliaceae*	Burseraceae	98.71	NC041103.1
8	*Boswellia sacra*	Burseraceae	98.35	KY085915.1
9	*Canarium zeylanicum*	Frankincense	98.15	KF521882.1
10	*Protium heptaphyllum*	Burseraceae	97.98	MG833471.1

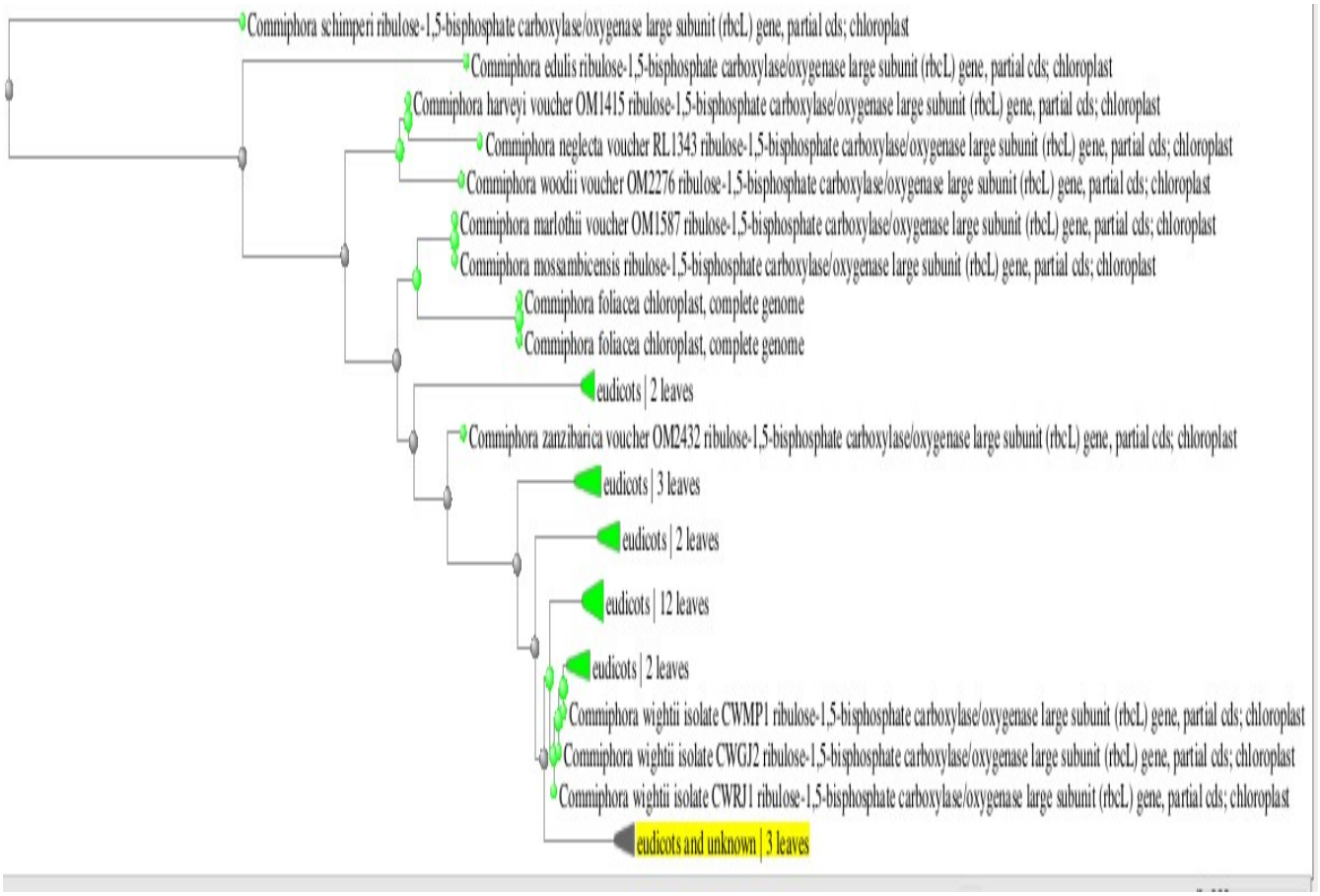

Figure 2: The Neighbor Joining tree showing the phylogenetic evolutionary relationship of *C. africana* with the related taxa.

3.3 Liquid Chromatographic Characters of the Stem bark Extract of C. *africana*

The chromatogram of the stem bark extract of *C. africana* reveals the presence of several recognizable peaks with their corresponding retention times (Figure 2).

3.4 Liquid Chromatography – Mass spectrometry of the Stem bark Extract of *C. africana*

About 10 chemical compounds were tentatively identified through HPLC–DAD–ESI-MS/MS experiments along with their retention times (Rt), detected accurate mass in positive ionization mode, molecular formula of each phytochemical, as well as the MS/MS fragment ions and the bibliographic references used in the characterization process (Table 2).

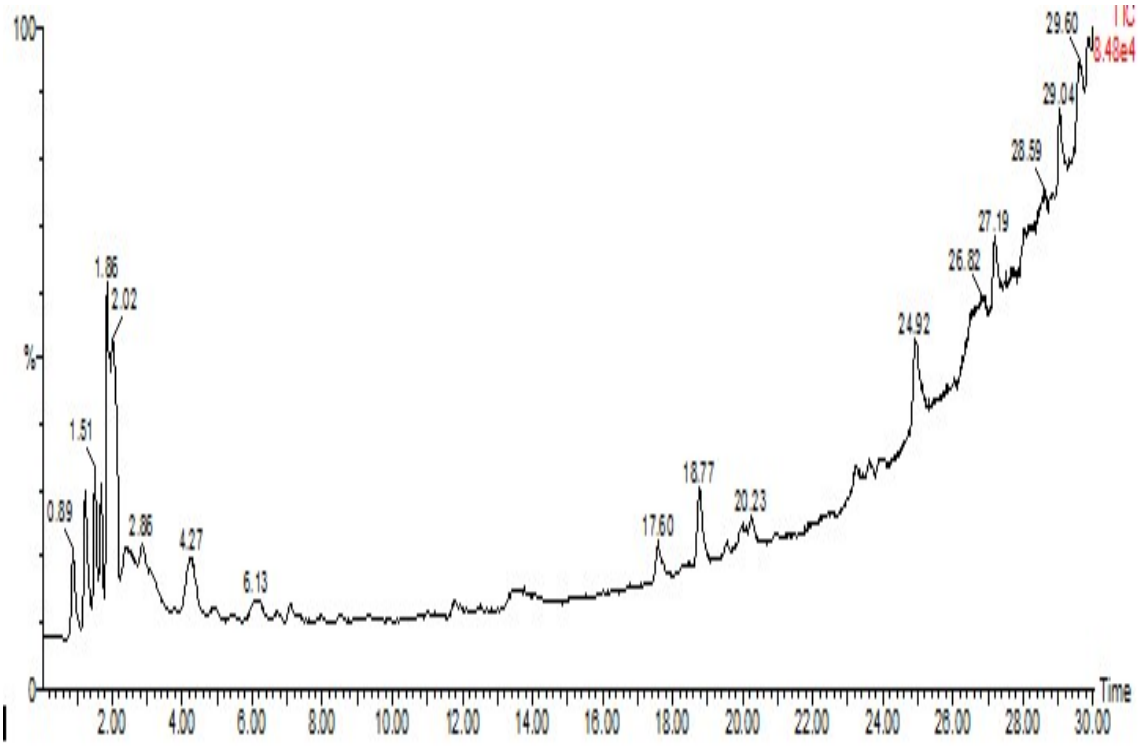

Figure 2. LCT Chromatogram of *C. africana* stem bark ethanol extract

Table 2: Phytochemical compounds detected and characterized in *C. africana* Stem bark using HPLC–DAD/QTOF-MS in positive ionization mode.

S/NO	Rt (min)	[M + H] (m/z)	Molecular formular	Tentative assignment	MS/MS fragment ions (%)	Reference
1	0.87	325.9301	$C_{15}H_{18}O_8$	Coumaryl hexoside	200 (100), 208(30), 316 (20)	Abu-Reidah *et al.,* 2015
2	1.84	885.2764	$C_{41}H_{41}O_{22}$	Kaempferol di deoxysyl hexoside	381(100), 365(60), 543(40)	Koley *et al.,* 2017
3	4.26	603.1922	$C_{32}H_{28}O_{12}$	Catechin ethyl dimmer	291(100), 313(20)	Rockenbach et al., 2012
4	7.07	613.3668	$C_{25}H_{24}O_{12}$	Quercetin rutinoside	453(100), 197(40)	Olchowik *et al*., 2012
5	17.58	827.3514	$C_{33}H_{41}O_{22}$	Tricaffeoyl-glucosyl-glucoside	425(100), 457(50), 347(40)	Abu-Reidah *et al*., 2015
6	20.95	877.5380	$C_{44}H_{30}O_{20}$	Kaempferol 7-*O*-(6"-galloyl)-glucosyl 6-*C*-(2'"pentosyl)-rhamnoside	239(100),271(40),185(40)	Tsang *et al.,* 2005
7	24.90	595.4684	$C_{27}H_{30}O_{15}$	Kaempferol rutenoside	309(100),527(20)	Abu-Reidah *et al.,* 2015
8	24.63	879.5389	$C_{44}H_{32}O_{20}$	Kaempferol galloyl glucoside	527(100), 393(70), 317(50)	Benayad *et al*., 2014
09	29.02	877.5240	$C_{44}H_{30}O_{20}$	Luteolin 7-*O*-(2"-galloyl)-glucosyl 6-*C*-(2'"pentosyl)-rhamnoside	301(100), 343(40),365(30)	Abu-Reidah *et al*., 2015
10	29.85	893.6373	$C_{47}H_{42}O_{18}$	Apigenin 7-*O*-(2"-dihydrogalloyl)-glucosyl-8-*C*-rhamnosyl-6-*C*-glucoside	353(100), 493(30)	Pollier *et al.,* 2011

4.0 DISCUSSION AND CONCLUSION

We investigated the efficacy of the two DNA barcode regions (*rbcL* and *matK*) for discriminating selected medicinal plant species belonging to the family Burseraceae. The DNA quantity of 46.0 ng and quality value of 1.9 indicates that the DNA extracted has a relatively low concentration and moderate purity. Ideally, a good quality DNA sample should have an A260/A280 ratio around 1.8 to indicate that there is minimal contamination by proteins or other molecules (Mishra *et al*., 2016). However, a value of 1.9 is still acceptable for most downstream applications, such as PCR and sequencing. To improve both the quantity and quality of the DNA extracted, it may be necessary to optimize the extraction protocols. The *rbcl*, sequence homology for the plant species was 99%, (Table 1). Many studies reported similar results when using *matK* and *rbcL* as barcodes for closely related taxa, such as Myristicaceae (Newmaster *et al.,* 2007), Rutaceae (Luo *et al*., 2010), Vitaceae (Fu *et al* 2011), Lamiaceae (De Mattia *et al.,* 2011). In this study, the matK marker gave a poor amplification as compared to rbcL, many other studies have shown similar patterns (Kress and Erickson 2007, Chen et al 2010, CBOL Plant Working Group 2009). The tropical flora seems more difficult to be amplified using matK as compared to temperate flora (Bruni *et al*., 2012). This might be due to a higher rate of evolution in the tropical flora compared to the temperate flora (Gillman *et al* 2010). The use of DNA

based markers as universal primers has significantly resulted in species identification as they exhibit good amplification pattern across different genomic regions among divergent species (Kumar *et al*., 2009). The qualitative analyses of the phenolic composition from the aqueous-ethanol extract of *C. africana* stem bark lead to the tentative characterization of ten compounds as follows;

A compound (Rt 4.26 min) at m/z 603 was presumably assigned to (epi) catechin ethyl dimers corresponding to an elemental composition of $C_{32}H_{30}O_{12}$, (epi) catechin ethyl dimers, condensed products of (epi) catechin with acetaldehyde, corresponding to two (epi) catechin units linked by an ethyl-bridge (He *et al.,* 2008). A compound (Rt 24.90 min) at m/z 595.16 ($C_{27}H_{31}O_{15}$), gave a fragment ion at m/z 287.05, corresponding to kaempferol aglycone in structure. This compound is likely a glycoside of kaempferol, meaning that kaempferol is attached to a sugar molecule (Abu-Reidah *et al.,* 2015). The molecular weight of the compound (595.16) is consistent with this, as 308.10 (the mass of the sugar) plus 287.05 (the mass of kaempferol) equals 595.16. The retention time of 24.90 minutes indicates that the compound has intermediate polarity, as it does not retain on the column for a long time, but also does not move through the column too quickly thus suggesting that the compound is likely a kaempferol glycoside with moderate polarity. The presence of kaempferol and its glycoside form in the sample could indicate potential health benefits, as kaempferol has been shown to have antioxidant, anti-inflammatory, and anti-cancer properties. Additionally, flavonoids such as kaempferol are commonly found in many fruits and vegetables, and their consumption has been associated with a reduced risk of chronic diseases such as cardiovascular disease and certain cancers (Abu-Reidah *et al.,* 2015). A compound (Rt 7.07 min) at m/z 611.16 ($C_{25}H_{24}O_{12}$) was suggested to be Quercetin rutinoside. The MS and MS/MS spectra showed a product ion [M+H]+ at m/z 303.05 quercetin (Olchowik *et al.,* 2012). A compound (Rt 11.19 min) at (m/z 863.18) corresponding to elemental compositions of $C_{45}H_{38}O_{18}$ was proposed to be a procyanidin trimer (Tsang *et al*., 2005). Two isomers (Rt 24.63 min) were to have A-type structures with two galloyl groups corresponding to elemental compositions of $C_{44}H_{32}O_{20}$ (m/z 879.14; and B-type structures with elemental compositions of $C_{44}H_{30}O_{20}$ (m/z 877.15) and were identified as Kaempferol galloyl glucoside (Pollier *et al*., 2011). The DNA barcode and chemical profile established in this study may be useful for the standardization and as well compilation of a suitable monograph on *C. africana.*

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