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DNA Barcode Authentication and Improvement of Andrographolide Yield in *Andrographis paniculata* Plant

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A – research concept and design; B – collection and/or assembly of data; C – data analysis and interpretation; D – writing the article; E – critical revision of the article; F – final approval of article.

Abstract

Background: Andrographolide, the major bioactive compound responsible for most pharmacological activities such as anticancer, antimicrobial activity exhibited by the *Andrographis paniculata* plant is present in small quantities. In addition, the genus *Andrographis* has about 28 species most of which possess no medicinal value. The deoxyribonucleic acid (DNA) barcode is utilized in species identification and plant authentication.
Objectives: This study aimed at authenticating *Andrographis paniculata* using DNA barcodes and improving the yield of andrographolide via enzymatic treatment.
Materials and Method: The DNA of *Andrographis* plant was obtained using the Qiagen kit. The psbA-trnH and rbcL DNA barcode regions were amplified using polymerase chain reaction (PCR). Presence of amplified regions was confirmed using gel electrophoresis and the amplicons were sequenced. A blast N search was performed on the sequenced DNA. The constituents of *A. paniculata* dried leaves was extracted using methanol, followed by treatment with and without β glucosidase. The extract obtained was dried and partitioned using ethyl acetate. The ethyl acetate fraction was concentrated and dissolved in methanol. Andrographolide content was determined using high performance liquid chromatography (HPLC).
Results: The psbA-trnH and rbcL DNA regions were successfully amplified having 358 and 604 bp respectively. The DNA barcode sequences obtained were identical to the psbA-trnH (97%) and rbcL (99%) genes of *A. paniculata* voucher MICET P00101. The mean andrographolide yield was 9.4±0.11mg/g and 8.9±0.13mg/g dry weight for the treatment and control groups respectively; statistical analysis at $p = 0.05$ shows a significant difference.
Conclusion: The *Andrographis* plant used in this study was confirmed to be *Andrographis paniculata*, enzymatic treatment increased andrographolide yield from the plant.
Keywords: *Andrographis paniculata*, andrographolide, authentication, DNA barcodes, β-glucosidase.

INTRODUCTION

Medicinal plants belong to a wide range of taxa. However, medicinal plants in the same genus often have numerous species which are closely related. For instance, in China, medicinal plants belong to 11,146 species of 2309 genera from 383 families and as such, there is a very high level of biodiversity (Chen *et al*., 2010; Zhou *et al*., 2014). A typical example is the *Andrographis* plant; the genus has at least 28 species, however, only few of the species possess medicinal value (Ukpanukpong *et al*., 2018).
The DNA barcoding is an inexpensive, rapid species identification process using short fragments (about 800 kilobase pairs) of gene sequences that evolve fast enough to differentiate species but have flanking regions that are sufficiently conserved to enable these regions to be amplified by universal primers (Selvaraj *et al*., 2013). Several plant specific barcodes candidates derived from the nuclear and plastid

genome such as the internal transcribed spacer (ITS) of the nuclear ribosomal DNA, the plastid psbA-trnH intergenic spacer, matK, rbcL, rpoC1, rpoB and ycf5 (Liu *et al*., 2011; Marcial-Quino *et al*., 2015) have demonstrated a high potential in discriminating between closely related species due to their unique properties ranging from high variation and identification potential to ease of PCR amplification.

The psbA-trnH spacer is a short DNA fragment found in the chloroplast genome with highly variable sequences amongst plant species and high amplification efficiency and as such, a potential DNA barcode candidate (Hao *et al*., 2010). The rbcL gene found in the plastid genome encoding the large subunit of ribulose- 1,5-bisphosphate carboxylase/oxygenase have been widely sequenced across numerous plant taxa with subsequent data used in phylogeny studies at the family and higher levels of classification (Patwardhan *et al*., 2014). This gene could discriminate amongst 85% of pairwise congeneric species with an increase to 88% discrimination efficiency when a combination of psbA-trnH and rbcL was used across 96 diverse species of 48 genera from 43 families (Guo *et al*., 2016). The DNA barcoding is being used by taxonomists, scientists in other fields as well as non-professionals such as custom officers for classification of plants into species, authentication of plant materials, determination of adulterants, which is of importance in quality control and detection of the presence of a specific herb in a commercial product (Sriramaa *et al*., 2010).

Andrographis paniculata (Burm.f.) Wall. ex Nees is an herbaceous medicinal plant that belongs to the Acanthaceae family. It is commonly known as king of bitters because of the extremely bitter taste found in all plant parts (Hossain *et al*., 2014). *Andrographis paniculata* is native to Southeast Asian countries (such as Thailand and Malaysia), India and China (Niranjan *et al*., 2010; Hossain *et al*., 2014). The plant represents an important constituent of many traditional formulations in ayurvedic medicines and Chinese herbal medicine being used to treat bacterial dysentery, carbuncles, colitis, tuberculosis, malaria, herpes, ulcer and venomous snake bites (Panossian and Georg, 2013). The genus *Andrographis* consists of at least 28 species, very few species have medicinal properties—the most common of which is *Andrographis paniculata* (Ukpanukpong *et al*., 2018). All *Andrographis* species are readily available in the countries of origin, it therefore becomes pertinent that the plant is verified to belong to the species of medicinal importance prior to use.

Andrographis paniculata has also been recognised in modern medicine to possess various pharmacological properties such as anticancer, antimicrobial, anti-inflammatory and anti-hyperglycaemic, hepatoprotective activity, amongst others (Hossain *et al*., 2014; Pandey *et al*., 2019). These activities have been attributed to various constituents such as the labdane diterpenoids, xanthones, stigmasterols and flavonoids present in the plant. The labdane diterpenoids form the major constituent of the *A. paniculata* plant, and andrographolide is the bioactive diterpenoid responsible for many pharmacological activities (Okhuarobo *et al*., 2014). Andrographolide however occur in small quantities—about 4%, 0.8-1.2% and 0.5-6%w/w dry weight andrographolide is present in the dried whole plant, stem and leaf (Tan *et al*., 2016).

The availability of several species of the *Andrographis* plant many of which have no medicinal value necessitated plant identification to ascertain that it belongs to the few species of medicinal value prior to further studies. Also, there is need to improve the yield of andrographolide compound obtained from the *A. paniculata* plant due to its numerous medicinal benefits thereby increasing its supply.

This study aimed at authenticating the *Andrographis* plant and increasing andrographolide yield in the leaves of the plant using enzymatic treatment.

METHODOLOGY

Materials and Method

Materials

Dried *Andrographis paniculata* leaves from the *A. paniculata* herb of Indian origin with voucher number: 80085 was purchased from Herbs in bottle Limited (Essendine, United Kingdom); *Aristolochia debilis* DNA, *Hypericum perforatum* DNA, psbA-trnH and rbcL primers were kindly provided by Maslinda Mahat; DNeasy plant mini kit, My Taq Red mix, agarose, SYBRsafe, Bioline DNA ladder, 1X TBE buffer were purchased from Qiagen (Manchester, United Kingdom); β-Glucosidase from almonds and pure andrographolide reference sample were purchased from Sigma Aldrich (Gillingham, United Kingdom); double distilled water (HPLC grade), methanol, hexane, ethyl acetate, dichloromethane, acetonitrile (all solvents of HPLC grade) were obtained from Fischer Scientific (Loughborough, United Kingdom).

Method

Extraction and purification of DNA from *A. paniculata* leaves

A 20 mg weight of *A. paniculata* dried young leaves (126 days old) obtained from a single collection was transferred into a microcentrifuge tube with beads and placed in a tissue lyser (Qiagen, Hilden, Germany) set to 1 min at 30 Hz frequency to obtain a fine powder. The total DNA was then extracted and purified using the DNeasy plant mini kit from Qiagen (Manchester, United Kingdom) according to the manufacturer's protocol (DNeasy plant handbook, 2012). The absorbance of the DNA solution was measured at 230, 260 and 280 nm using the UV spectrophotometer. The DNA concentration and purity were calculated from Equations 1 and 2, respectively. The DNA solution obtained was stored at 4°C for further analysis.

$$DNA\ concentration = Absorbance\ at\ 260\ nm\ x\ 50\ x\ Dilution\ Factor \quad ---1$$

$$DNA\ Purity = \frac{Absorbance\ at\ 260\ nm}{Absorbance\ at\ 280\ nm}\ and\ \frac{Absorbance\ at\ 260\ nm}{Absorbance\ at\ 230\ nm} \quad ---2$$

Amplification of DNA barcode regions

The primers specific to the psbA-trnH and rbcL DNA regions were utilized in the amplification of these candidate barcode regions in the obtained DNA using the polymerase chain reaction (PCR) technique. A mastermix for each primer set was prepared as indicated in Table 1.

Table 1: PCR master mix preparation for amplification of psbA-trnH and rbcL gene

Ingredients	Mastermix 1 (µL)	Mastermix 2 (µL)
My Taq Red mix	100	100
trnH	4	-
psbA	4	-
rbcLF	-	4
rbcLR	-	4
Double distilled water	84	84

All procedures were carried out on ice. A volume of 48 µL each of mastermix 1 was pipetted into three labelled 0.5 mL PCR tubes, 2 µL each of *A. paniculata* DNA, *Aristolochia debilis* DNA and double distilled water was added into the tubes representing the DNA sample, positive and negative control respectively. This procedure was repeated for mastermix 2 using *Hypericum perforatum* DNA as the positive control.

A PCR of the reaction tubes were run on the Applied Biosystems GeneAmp PCR System 9700 thermal cycler (Warrington, United Kingdom) using a different thermal cycler programme for each primer set. The programme for the trnH-psbA include: 5 mins at 95°C initial denaturation step, 35 cycles consisting of 1 min at 95°C, 30 s at annealing temperature (touchdown temperature begins at 58°C, reduced by 1°C per cycle until 48°C which is maintained for the remainder of the program) and 1 min at 72°C, final extension period of 7 mins at 72°C. For rbcL amplification, the cycling programme include: 5 mins 95°C initial denaturation step, 35 cycles consisting of 30 s at 95°C, 20 s at 52°C and 50 s at 72°C, with a final extension period of 5 mins at 72°C (Howard *et al*., 2012).

Gel electrophoresis of amplified DNA, sequencing and analysis

Agarose gel of 1% in 1X TBE buffer was prepared, allowed to cool at room temperature till hand hot and 1 µL of SYBRsafe DNA stain was added. This was poured into the gel tray fitted with a comb (placed such that the comb is close to the negative electrode) in a gel tank and allowed to set. The 1X TBE was poured into the tank until the gel was completely covered, the comb was removed and 5 µL each of the PCR products and Bioline Easy ladder was loaded into different wells. The lid was placed on the tank, a voltage of 90V was applied for 25 mins and the gel was subsequently observed under TransUV radiation and images were obtained using a BioRad illuminator (Watford, United Kingdom) with a ChemiDocXRS camera and Quantity One Software.

The psbA-trnH and rbcL regions of the *A. paniculata* plant successfully amplified were sequenced and viewed using the CLC DNA workbench 6.6.1. The similarity of the DNA sequenced obtained with existing sequence in the GenBank database was carried out using the nucleotide basic local alignment search tool (BLAST N) on National Center for Biotechnology Information (NCBI) database using default parameters.

Extraction and improving andrographolide yield from *Andrographis paniculata* leaves

Andrographis paniculata leaves sample of 500 mg was weighed and macerated in 10 mL of methanol:water (50:50) for 48 h. The sample was filtered and concentrated under vacuum, 50 mg of β-glucosidase enzyme was added to the concentrated

extracts and placed on the orbital shaker for 1 h. The extract was partitioned in 3 x 5 mL of ethyl acetate; 20 mg of sodium sulphate was added to the ethyl acetate fraction to remove excess water, the fraction was then filtered and concentrated under vacuum. The concentrate was dissolved in 2 mL of methanol for further analysis. The procedure was carried out in quadruplicate and repeated without β-glucosidase (control group) treatment.

Quantification of andrographolide in *A. paniculata* extract

The amount of andrographolide in the *A. paniculata* extract was determined by high performance liquid chromatography (HPLC). Solutions of 125–1000 µg/mL andrographolide reference standard in methanol was prepared, isocratic elution was carried out with acetonitrile:water (25:75) at a flow rate of 1 mL/min on a RP-18 5 µ column, temperature of 40°C and injection volume of 5 µL. Detection was at 235 nm using diode array detector, evaluation was via peak areas and a graph of peak area against andrographolide concentration was plotted. The extracts were subjected to HPLC analysis, the concentration of andrographolide in the extract was obtained from the calibration curve and the amount of andrographolide in the *A. paniculata* sample was subsequently calculated.

Statistical Analysis

Results are expressed as mean ± standard error of mean. Statistical difference in the mean values of andrographolide content was determined by T-test for independent sample using SPSS 20.0 statistical software. A p value < 0.05 was considered significant.

RESULTS AND DISCUSSION

Quantification of *A. paniculata* DNA The DNA absorbance at three wavelengths and the calculated DNA concentration and purity are presented in Table 2.

Table2: Absorbance of DNA solution at different wavelengths, DNA concentration and purity

Wavelength (nm)	Absorbance	DNA concentration (ng/µL)	DNA purity (260/230)	DNA purity (260/280)
260	0.204	102	-	-
230	0.130	-	1.57	-
280	0.112	-	-	1.82

DNA samples are usually contaminated with impurities mostly proteins, ribonucleic acid (RNA) and other substances used for purification which also absorb at the 260 nm DNA absorption wavelength. This can lead to over estimation of DNA concentration in the sample which will in turn affect subsequent assay or procedure to be performed (Berthold, 2020). For example, the use of low DNA concentration as starting material in the PCR will result in little or no amplification of genes such as the pbsbA-trnH and rbcL genes of interest in this study. Most of these impurities absorb between 230 and 320 nm wavelength, the DNA purity is usually estimated by determining the ratio of absorbance at 260:280 and 260:230. A 260:280 value of 1.7 to 2.0 and 260:230 value greater than 1.5 signifies a relatively pure DNA (BioSizeBio, 2016), therefore the DNA obtained contain less impurities and the estimated amount to be utilized for PCR will bring about successful amplification of the psbA-trnH and rbcL DNA regions.

Amplification and detection of psbA-trnH and rbcL DNA barcode regions in *A. paniculata*

The bands representing the psbA-trnH and rbcL regions of *A. paniculata* showing similar sizes (estimated using number of base pairs -bp) with the psbA-trnH region of *Aristolochia debilis* and rbcL region of *Hypericum perforatum,* respectively are represented in Plate 1.

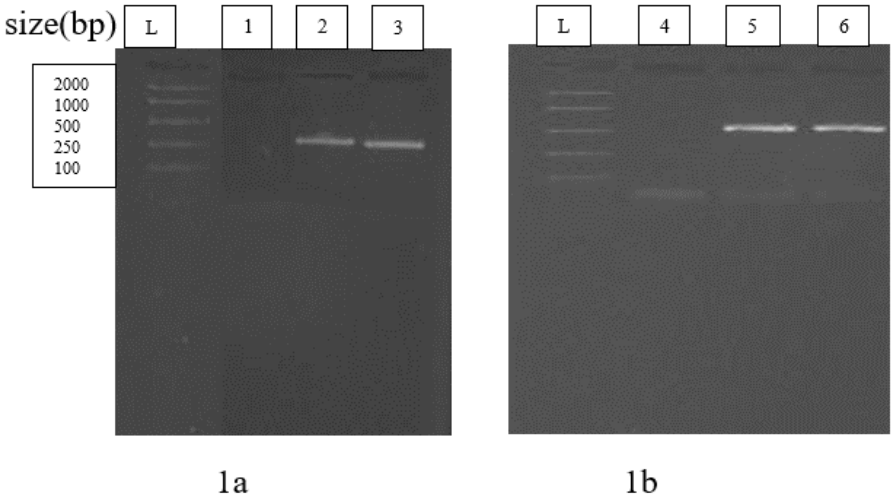


Plate 1: Gel image showing the psbA-trnH gene of *A. paniculata* and *Aristolochia debilis* (a) and rbcL region of *A. paniculata* and *Hypericum perforatum* (b).
L: Ladder, 1: distilled water (negative control), 2: psbA-trnH region of *A. paniculata* , 3: psbA-trnH region of *Aristolochia debilis* (positive control), 4: distilled water, 5: rbcL region of *A. paniculata*, 6: rbcL region of *Hypericum perforatum* (positive control).

The polymerase chain reaction is a molecular technique used to synthesize millions of specific regions in a target DNA. The successful amplification of the required psbA-trnH and rbcL DNA region was determined via gel electrophoresis- a technique which involves the separation of molecules such as DNA, RNA and proteins into different fragments based on their sizes and charge by the passage of an electric current (Houck and Siegel, 2015). The band sizes are determined using a standard DNA size marker consisting of bands of known sizes in base pairs (bp). A DNA band between 250 and 500bp (lane 2, Plate 1a) was obtained for the psbA-trnH gene of *A. paniculata*, a similar band was observed in the positive control-*A. debilis* (lane 3, Plate 1a) signifying the successful amplification of the psbA-trnH spacer region. The PCR product without DNA-negative control (lane 1, Plate 1a) showed no band as expected. Also, a band slightly above 500bp was observed in *A. paniculata* DNA (lane 5, Plate 1b); a band of similar size fragment was obtained in the *Hypericum perforatum* rbcL gene – positive control (lane 6, Plate 1b), the rbcL gene in the *A. paniculata* plant was therefore successful amplified. The band observed in the negative control sample (lane 4, Plate 1b) is the primer dimer band which is also present in the *A. paniculata* and *H. perforatum* PCR product (lanes 5 and 6 respectively), this can be circumvented by increasing annealing temperatures during PCR (Ruiz-Villalba *et al*., 2017).

***Andrographis paniculata* psbA-trnH and rbcL DNA sequencing and analysis**

The DNA sequences of the psbA-trnH and rbcL gene of *A. paniculata* is presented in Figures 1 and 2, respectively. Comparison of the obtained sequence with sequences in the GenBank database shows 97 and 99% identity with the psbA-trnH and rbcL gene of *A. paniculata* voucher MICET P00101, respectively as shown in Figures 3 and 4.

LOCUS 1-499_Apa_trnhpsbA_TrnH 358 bp DNA linear
1 CCGTCTTACG TTGGCTCTCC GCCCCTATAT ATAAAATATT AGATAGAAAA TTCATTGAGA
61 ATACTACTCA ATCATTAATC AATCCCTAAA ATTAGAAGGT CTTCCACTTC TTTATCTAAA
121 ATGAAAAGAA AAAAAGTTTA GGTAAGCAAA ATATTAATAA AAAACAATAC TAAATGAAAA
181 GAAAACCAAG GAGCAATAAA CTATTTCTTG TTCTATCAAG AAAATTTATT GCTCCTTATG
241 GGATTCTATG TTCAAAAACT CCTAGACACT AAAAGCAAGT CTTATCCATT TGTTGGAGCT
301 TCGACAGCAG CTAGATCTAA AGGAAAGTTA TGAGCATTAC TGCTGTGCAT AACACAGC

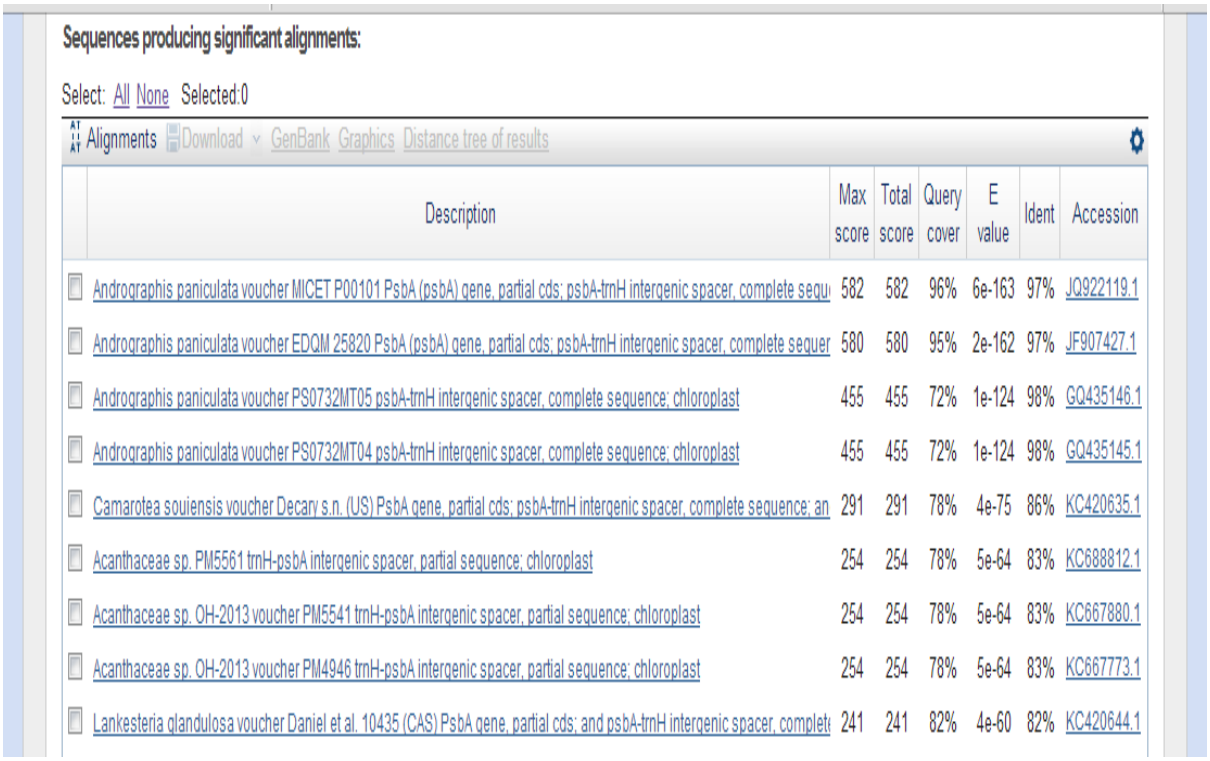
Figure 1: DNA sequence of psbA-trnH gene of *A. paniculata*
Key: A- Adenine nucleotide, G- Guanine nucleotide, T- Thymine nucleotide, C- cytosine nucleotide

LOCUS 2-499_Apa_rbcL_rbcLF 604 bp DNA linear

```
   1 GAGACGCCAT CATTCAGACG GGTGTTAAAG AGTACAAATT GACTTATTAT ACTCCTGAAT
  61 ACGAAACCAA AGATACTGAT ATCTTGGCAG CATTCCGAGT AACTCCTCAA CCCGGAGTTC
 121 CGCCTGAAGA AGCAGGGGCA GCGGTAGCTG CCGAATCTTC CACTGGTACA TGGACAACCG
 181 TGTGGACTGA TGGACTTACC AGCCTTGATC GTTACAAAGG GCGATGCTAC AACATCGAGC
 241 CCGTTCTTGG CGAAACAGAT CAATATATTT GTTATGTAGC TTACCCTTTA GACCTTTTTG
 301 AAGAAGGTTC TGTTACCAAC ATGTTTACTT CCATTGTAGG AAATGTATTT GGATTCAAAG
 361 CCCTGCGTGC TCTACGCCTG GAAGATCTGC GAATCCCTAC TGCTTATATT AAAACTTTCC
 421 AAGGTCCGCC TCATGGGATC CAAGTTGAGA GAGATAAATT GAACAAATAT GGTCGTCCTC
 481 TGCTGGGATG TACTATTAAA CCTAAATTGG GATTATCCGC TAAAAACTAC GGCAGAGCAT
 541 GTTATGAATG TCTTCGCGGG ATTGGGGATT TTTAAAAAAA ACATCCCCAT TTTTTTAATT
 601 TATC
```

Figure 2: DNA sequence of rbcL gene of *A. paniculata*

Key: A- Adenine nucleotide, G- Guanine nucleotide, T- Thymine nucleotide, C- cytosine nucleotide



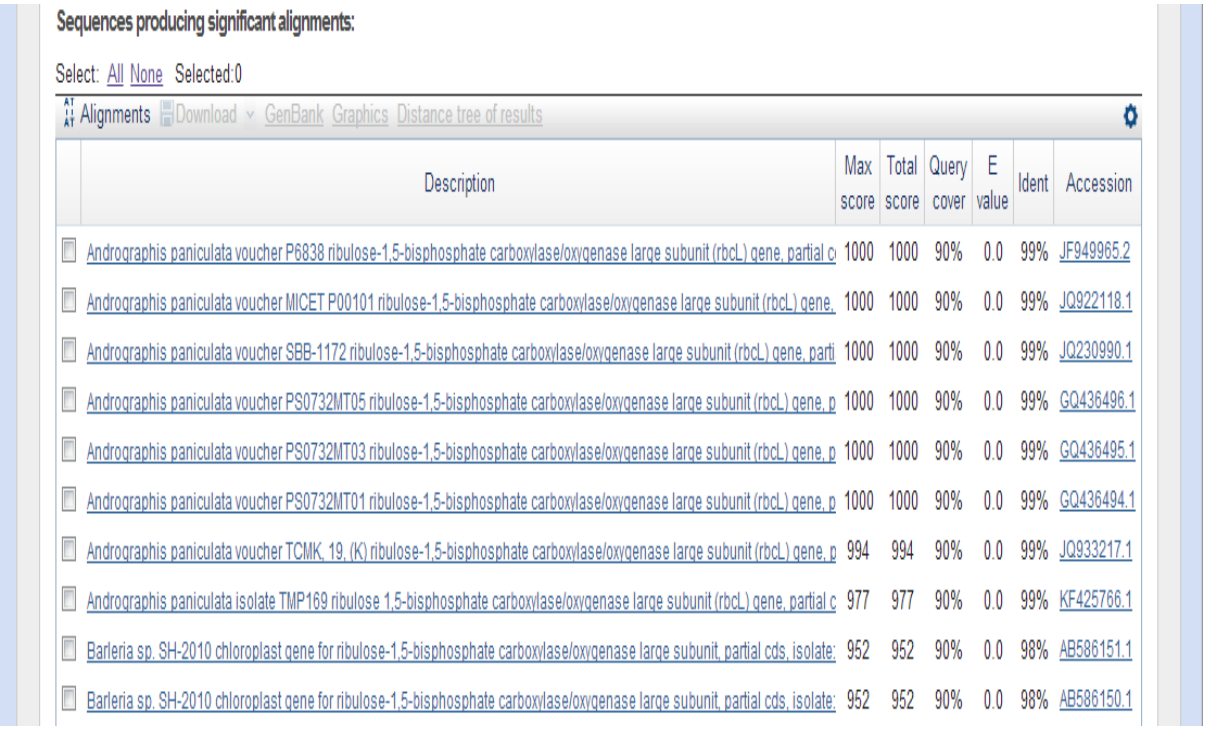
Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Andrographis paniculata voucher MICET P00101 PsbA (psbA) gene, partial cds; psbA-trnH intergenic spacer, complete sequ	582	582	96%	6e-163	97%	JQ922119.1
Andrographis paniculata voucher EDQM 25820 PsbA (psbA) gene, partial cds; psbA-trnH intergenic spacer, complete sequer	580	580	95%	2e-162	97%	JF907427.1
Andrographis paniculata voucher PS0732MT05 psbA-trnH intergenic spacer, complete sequence; chloroplast	455	455	72%	1e-124	98%	GQ435146.1
Andrographis paniculata voucher PS0732MT04 psbA-trnH intergenic spacer, complete sequence; chloroplast	455	455	72%	1e-124	98%	GQ435145.1
Camarotea souiensis voucher Decary s.n. (US) PsbA gene, partial cds; psbA-trnH intergenic spacer, complete sequence; an	291	291	78%	4e-75	86%	KC420635.1
Acanthaceae sp. PM5561 trnH-psbA intergenic spacer, partial sequence; chloroplast	254	254	78%	5e-64	83%	KC688812.1
Acanthaceae sp. OH-2013 voucher PM5541 trnH-psbA intergenic spacer, partial sequence; chloroplast	254	254	78%	5e-64	83%	KC667880.1
Acanthaceae sp. OH-2013 voucher PM4946 trnH-psbA intergenic spacer, partial sequence; chloroplast	254	254	78%	5e-64	83%	KC667773.1
Lankesteria glandulosa voucher Daniel et al. 10435 (CAS) PsbA gene, partial cds; and psbA-trnH intergenic spacer, complet	241	241	82%	4e-60	82%	KC420644.1

Figure 3: Blast N search result showing extent of similarity between the psbA-trnH sequence of *A. paniculata* obtained and the sequences present in the GenBank database.



Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Andrographis paniculata voucher P6838 ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (rbcL) gene, partial c	1000	1000	90%	0.0	99%	JF949965.2
Andrographis paniculata voucher MICET P00101 ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (rbcL) gene,	1000	1000	90%	0.0	99%	JQ922118.1
Andrographis paniculata voucher SBB-1172 ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (rbcL) gene, parti	1000	1000	90%	0.0	99%	JQ230990.1
Andrographis paniculata voucher PS0732MT05 ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (rbcL) gene, p	1000	1000	90%	0.0	99%	GQ436496.1
Andrographis paniculata voucher PS0732MT03 ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (rbcL) gene, p	1000	1000	90%	0.0	99%	GQ436495.1
Andrographis paniculata voucher PS0732MT01 ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (rbcL) gene, p	1000	1000	90%	0.0	99%	GQ436494.1
Andrographis paniculata voucher TCMK, 19, (K) ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (rbcL) gene, p	994	994	90%	0.0	99%	JQ933217.1
Andrographis paniculata isolate TMP169 ribulose 1,5-bisphosphate carboxylase/oxygenase large subunit (rbcL) gene, partial c	977	977	90%	0.0	99%	KF425766.1
Barleria sp. SH-2010 chloroplast gene for ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit, partial cds, isolate:	952	952	90%	0.0	98%	AB586151.1
Barleria sp. SH-2010 chloroplast gene for ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit, partial cds, isolate:	952	952	90%	0.0	98%	AB586150.1

Figure 4: Blast N search result showing extent of similarity between the rbcL sequence of *A. paniculata* obtained and sequences present in the GenBank database.

The psbA-trnH and rbcL regions of *A. paniculata* sequenced gave 358 bp and 604 bp, respectively. This is in line with the estimated size of bands observed on the gel.

The BLAST program is a bioinformatic tool used to compare unknown or query sequences (DNA, RNA, or protein) with the sequences present in the database. This program uses an algorithm to align, identify and score sequences similar or identical to the query sequence with corresponding statistical significance of alignment (NCBI, 2020). It is used to deduce evolutionary and functional relationships as well as identification of genes that belong to the same family. The BLAST search showed that the psbA-trnH query sequence had 97% identity with *two A. paniculata* psbA-trnH sequence with accession numbers JQ922119.1 and JF907427.1, respectively (Figure 3). However, the former had 96% query coverage, while the latter had 95% coverage, hence a lesser total score of 580 when compared to the former (a total score of 582). In addition, two *A. paniculata* psbA-trnH species with accession numbers GQ435146.1 and GQ435145.1 possess 98% identity with the query sequence. However, only 72% of the query sequence was used in determining the identity, thereby leading to a reduction in the total score (455) despite the high identity. Therefore, the amplified DNA region can be said to belong to the *A. paniculata* species and highly identical to the *A. paniculata* voucher MICET P00101 psbA-trnH intergenic spacer complete sequence with accession number JQ922119.1. Plants from other genera showed decreased identity with the query sequence.

The amplified rbcL region had 99% identity, 90% query coverage and total score of 1000 with six *A. paniculata* vouchers with accession numbers – JF949965.2, JQ922119.1, JQ230990.1, GQ436496.1, GQ436495.1 and GQ436494.1 (Figure 4). All accession numbers represent the ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (rbcL) gene, partial coding sequence of the chloroplast. Other *A. paniculata* vouchers and plants of different genera have lesser total score and %identity as the query sequence.

Comparison of the Blast N result obtained for both DNA barcode (Figures 3 and 4) gives a common *A. paniculata* voucher MICET P00101 with accession numbers JQ922119.1 and JQ922118.1 for its psbA-trnH and rbcL genes, respectively. It possesses the highest % identity, query coverage and total score for psbA-trnH and amongst the six *A. paniculata* vouchers with the highest %identity, query coverage and total score for rbcL gene. This shows that the sample provided is *A. paniculata* which likely belongs to the *A. paniculata* voucher MICET P00101.

Quantification of andrographolide in *A. paniculata*

The andrographolide content of *A. paniculata* showing an increase following treatment with β-glucosidase

enzyme is presented in Figure 5. Andrographolide yield with and without β-Glucosidase treatment is 8.9±0.13 and 9.4±0.11mg/g dry weight, respectively.

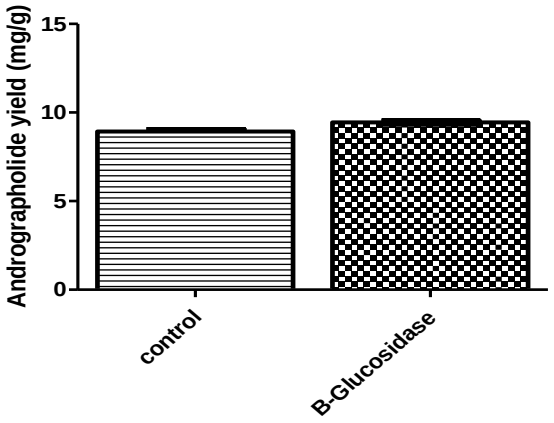


Figure 5: Andrographolide yield from *A. paniculata* with and without enzyme treatment

The *A. paniculata* leaves contain several compounds mainly diterpenoids, flavonoids, xanthones and stigmasterols. One of the diterpenoid constituent is andrographiside- a glycoside of andrographolide. A method involving the breakage of glycosidic bond to produce free andrographolide can increase the amount of andrographolide isolated from the *A. paniculata* plant. Enzyme treatment of methanolic extract of *A. paniculata* leaves with β- Glucosidase hydrolyses andrographiside producing andrographolide and glucose. The treatment of *A. paniculata* plant with the enzyme resulted in a 5.6% increase in andrographolide yield, the difference in the andrographolide content, although marginal is statistically significant ($p = 0.025$). The marginal difference may be because a dry sample of the *A. paniculata* leaves was used as the drying processes might have led to the loss of the andrographiside. In addition, age of plant at harvest and time of harvest can bring about variation in plant constituents (Tajidin *et al*., 2019).

CONCLUSION

The *A. paniculata* sample used in the study was successfully identified with close identity to the *A. paniculata* voucher MICET P00101. A simple and rapid enzymatic treatment of *A. paniculata* leaves extract increased its andrographolide yield
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