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USE OF GENETIC AND PROTEIN MARKERS FOR CHARACTERIZATION OF MEDICINAL AYURVEDIC PLANTS

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ABSTRACT

Randomly amplified polymorphisms DNA (RAPD) has been widely used for authentication of plant species of medicinal importance. It is particularly useful in case of plants that are frequently substituted or adulterated with other species or varieties that are morphologically and/or phytochemically indistinguishable. In this study *Hemidesmus indicus* and its substitute plants were selected to develop gene and protein based coding for medicinal plants. DNA and protein was isolated from all the plants, i.e. the medicinal plant as well as the substitute plants. A modified DNA and protein extraction procedure was used for dry roots and powder of *Hemidesmus indicus*. Further a RAPD technique was used to develop DNA fingerprint pattern for all plants using self designed random primers. Sequence Characteristic Amplified Region (SCAR) marker was developed for *Hemidesmus indicus*. MALDI-TOF technique was used to differentiate between plant parts. Unique markers were developed for *Hemidesmus indicus,* to identify whether main plant is used or substitute plant, which part is added in formulation.

INTRODUCTION: Gene based coding of medicinal plants has become an important international intellectual property rights issue along with tracking biodiversity. As compared to other medicinal therapies, herbal drug therapies are easy availability and have fewer side effects; hence herbal drug therapies are getting importance. Development and enforcement of quality control standards have become crucial for regulatory authorities and industries due to global interest in botanicals as drug and dietary supplements. In assuring quality of botanical drugs correct identification and characterization of plant species and plant part is important. Authentication of raw materials is done by conventional techniques like macroscopy, microscopy and chemical profiling. Identifying closely related species and adulterants that may resemble the genuine botanical material may not be possible due to limitation of these methods. As macroscopy and microscopy are prone to subjective bias while chemo-profiles are sensitive to environmental conditions, physiology, plant part used and processing practices [1].

Considering the above mentioned limitations, we have made attempts to develop genetic and protein markers as complementary tools in confirming the botanical identity. DNA fingerprinting refers to the use of techniques based on polymerase chain reaction (PCR), to reveal the specific DNA profile for a particular organism which is as unique as a fingerprint [2]. A DNA fingerprint is generally independent of environment, and is consistent throughout different parts and developmental stages of the organism. Similarity of DNA fingerprints depends on genetic closeness of tested samples. DNA fingerprinting can distinguish plants from different families, genera, species, cultivars (cultivated variety), and even sibling plants [3]. DNA- based molecular markers have proved their utility in fields like taxonomy, physiology, embryology, genetics, etc. This is especially useful in case of those that are frequently substituted or adulterated with other species or varieties that are morphologically and/or phyto-chemically in-distinguishable. Protein fingerprint using Matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF) technique are used to identify the plant part used. This work is an attempt to apply genetic and protein markers for authentication of Ayurvedic based medicinal plants by taking *Hemidesmus indicus* [4] as a case example. This plant is found mainly in Southern India [5, 6] and the roots, especially, have been found to have very high medicinal properties.

However, roots of these plants are extremely deep below the ground and hence there is tendency of plant collectors or traders to adulterate plant material with easily available substitute plants [7, 8]. *Hemidesmus indicus* (locally called *Anantmool*) is an important plant [9, 10, 11], particularly due to its use to make beverages [12] and also in traditional medicine [11]. The plant enjoys a status as tonic, alterative, demulcent, diaphoretic, diuretic and blood purifier. It is employed in nutritional disorders [13], syphilis, chronic rheumatism [14], gravel and other urinary diseases [9] and skin affections [15, 16, 17]. It is administered in the form of powder, infusion or decoction as syrup [12]. It is also a component of several medicinal preparations [6, 18].

The current work involves DNA and protein fingerprinting of *Hemidesmus indicus* and its substitute plants [4, 8, 10] viz, *Hemidesmus indicus variety pubescens, Decalepis hamiltonii, Cryptolepis buchanii, Ichanocarpus frutescens, Vallaris solanaceae* using RAPD (Randomly Amplified Polymorphic DNA) [19, 20] analysis followed by development of SCAR (Sequence Characteristic Amplified Regions) markers for DNA fingerprinting and MALDI-TOF for Protein fingerprint. Thus, DNA/Protein -analytics can be effectively used as tool for correct identification of plant species,

plant part used and thereby support standardizing quality control of Ayurvedic medicinal plants and other botanicals, used as drugs or dietary supplements [1, 21].

MATERIAL AND METHODS:

- **For Genomic (DNA) study:**

Plant material: Fresh leaves and roots of *Hemidesmus indicus* (main plant) were collected from Thane district, Maharashtra, India. These were washed with sterilize distilled water followed by 80% alcohol. Fresh roots samples were kept at 4˚C before use and another representative of root samples of main plant was kept for drying at room temperature. These fresh and dried samples were subsequently processed for DNA extraction [22], also commercially available powder of *Hemidesmus indicus* available in market was used [1]. Similarly variety and substitute plants viz., *Hemidesmus indicus variety pubescens*, *Decalepis hamiltonii, Vallaris solanaceae, Ichanocarpus frutoscen, Cryptolepis buchanani* were processed for DNA extraction [23] and for subsequent RAPD analysis.

- **Reagent and chemicals for DNA study:**

  - CTAB (20% [w/v]), 1M Tris- HCl (pH 8), 5 M NaCl, 5M EDTA (pH 8), ethanol, chloroform: IAA (24:1 [w/v]), polyvinylpyrrolidone (PVP) (Sigma), β-mercaptoethanol
  - Extraction buffer pH 8 - 3% (w/v) CTAB, 100 mM Tris-HCl (pH 8), 2M NaCl, 20 mM EDTA (pH 8), 1% PVP (w/v) and 0.3% β-mercaptoethanol (v/v) (added to buffer just before use)
  - TE buffer - 10 mM Tris-HCl (pH 8), 1 mM EDTA (pH 8).
  - *Taq* DNA polymerase enzyme, dNTP mix (A, T, G, C), *Taq* DNA polymerase buffer (Fermentas, Life sciences, Canada), Primers (Sigma).
  - Plants genomic DNA Isolation- Two grams of fresh/dried root sample was taken in pre-chilled mortar and pestle and crushed in the presence of liquid nitrogen.
  - To each tube 5cm^3 of 0.5M EDTA solution was added [24] and kept at room temperature for overnight and after incubation centrifuge at 10000rpm for 10mins and the supernatant was discard.
  - To this tube 10 cm^3 of extraction buffer pH 8 was added and transferred into 30ml centrifuge tube [25]. It was incubated at 70°C for 2 hr.
  - The tube was intermittently mixed; pH was monitored and adjusted to 8.
  - To this one third volume of 5M potassium acetate was added [26] and kept in ice for 1hr.

Equal volume of Phenol: Chloroform: Isoamylalcohol (25:24:1) was added, mixed and centrifuged at 10,000rpm for 15min. The aqueous layer was taken in another tube. To this aqueous solution equal volume of Chloroform: Isoamylalcohol (24:1) was added, mixed and centrifuged at 10,000rpm for 15 minutes.

The aqueous layer was transferred in another tube. To this equal volume of 30% PEG 6000 [26] was added and the tube was kept in ice for 1 hour (fresh sample) to overnight (dried sample). Centrifugation was carried out at 10,000rpm for 30minutes. The supernatant was discarded and the pellet was washed with 70% ethanol. After 70% ethanol wash supernatant was discarded and the pellet was dried at room temperature. The pellet was reconstituted in 100µl of 1X TE Buffer [27].

The crude DNA sample was treated with 1 ml RNase (10 mg/ml stock) for 30 min at 37°C and equal volume of Chloroform: Isoamylalcohol (24:1) was added. It was thereafter centrifuged at 8000 rpm for 10min at 4°C. The aqueous phase was taken and 0.6 volumes of isopropanol were added.

It was then kept at 20°C for 10 min. The mixture was centrifuged at 8000 rpm for 5 min at 4°C and the supernatant was decanted carefully. The pellet was washed with 70% ethanol twice and dried at 37°C for 10 min. Finally DNA pellet was dissolved in 50 ml of 1x TE buffer [22, 28].

Quantification of DNA: The DNA yields per gram of fresh and per gram of dry root tissues were determined using a UV-VIS spectrophotometer (*DU-600, Beckman coulter*) at 260 and 280nm. The purity of extracted DNA was determined by calculating the ratio of absorbance at 260 and 280nm.

RAPD analysis to obtain fingerprint pattern for all selected plants: The following random primers were designed and used for PCR amplification for RAPD analysis [29, 30].

F1 5'-CGGTAGACTCATACTTGC-3',

R1 5'-GGTAGGTGTACGTGTTGA-3',

F2 5'-CATATCTCTCTCTCGGTC-3',

R2 5'-CATCACCTCAATCACATGGC-3',

F3 5'-ATCACGCTTACCGT-3',

R3 5'-TCTCCGTATCGAGT-3',

F4 5'-ATGGACTTACCAGCCTT-3',

R4 5'-CAAGGGTGTCCTAAAGTT-3',

F5 5'-CCACTTATCTTTCAGG-3',

R5 5'-GACTTCTTCTTCCG-3',

F6 5'-GCATTTTATATGCAAAG-3',

R6 5'-CGTTTTATCTAGGTACTGG-3'

Amplification was performed in 50µl reaction with 25ng genomic DNA, 1XPCR buffer, 2.5mM $MgCl_2$, 200µM dNTPs, 20 picomole primer and 2.5 units of Taq polymerase using thermal cycler (BioRad) [31, 32, 33]. The thermal cycler was programmed at initial denaturation temperature of 96°C for 5 min, amplification reaction were cycled 35 times at 96°C for 1 min, 35°C for 1 min and 72°C for 1.30 min. A final amplification was allowed for 5min at 72°C.

RAPD analysis for all selected plants to make SCAR marker: The following random primer were used for PCR amplification for RAPD analysis [29, 30].

FWD 3H 5'-CGGAAGGATCATTGTCGAAT-3'

REV 3I 5'-ATCACCTCAATCACATGGCA-3'

Amplification was performed in 50µl reaction with 25ng genomic DNA, 1X PCR buffer, 2.5mM $MgCl_2$, 200µM dNTPs, 20 picomole primer and 2.5 units of Taq polymerase using thermal cycler (BioRad) [31, 32, 33]. The thermal cycler was programmed at initial denaturation temperature of 96°C for 5 min, amplification reaction were cycled 35 times at 96°C for 1 min, 35°C for 1 min and 72°C for 1.30 min. A final amplification was allowed for 5min at 72°C. A bulk PCR reaction was carried out and loaded onto a 2% agarose gel. The required PCR amplified band was eluted using the QIAquick gel extraction kit. The purified PCR amplified DNA was collected in 1.7cm^3 eppendoff tube and stored at -20°C until further analysis.

Sequencing of DNA marker: Sequencing of the purified PCR amplified DNA was carried out on Beckman coulter CEQ 8000 DNA sequencer. The nucleotide sequence data was analyzed and BLAST search on NCBI[34] was carried out.

Primer designing: The sequence obtained from sequencer was analyzed to design primer using the Primer BLAST tool from NCBI site. The designed primer FWD 3G 5'-GGTAGGCAGTTGGGTTCAAA-3' and REV 3J 5'-GCAACACTCGTCCTGCATAA-3' was used to obtain SCAR marker for *Hemidesmus indicus*.

PCR of SCAR marker: Amplification for marker was performed in 50µl reaction with 25ng genomic DNA, 1X PCR buffer, 2.5mM $MgCl_2$, 200µM dNTPs, 20 picomole primer (FWD 3G & REV 3J) and 2.5U Taq polymerase using thermal cycler (BioRad). The thermal cycler was programmed at initial denaturation temperature of 96°C for 5 min, amplification reaction were cycled 35 times at 96°C for 1 min, 60°C for 1 min and 72°C for 1.30 min. A final amplification was allowed for 5min at 72°C.

- **For Proteomic (Protein) study:**

Plant material: Plant parts viz. leaves, stem and root of *Hemidesmus indicus* was used for protein extraction. Roots of variety and substitute plants viz., *Hemidesmus indicus variety pubescens*, *Decalepis hamiltonii, Vallaris solanaceae, Ichanocarpus frutoscen, Cryptolepis buchanani* were processed for Protein extraction

Reagent and chemicals for Protein study:

- Liquid nitrogen
- 1M Tris HCl pH 7.5
- Sucrose
- Triton X-100
- 100mM Phenyl Methli Sulfonyl Fluoride
- 0.5M EDTA
- Distilled water
- Tri Chloro Acetic acid
- 15% Non Denaturing Polyacrylamide gel for Native PAGE gel electrophoresis
- 10mM ammonium hydroxide
- 10mM acetic acid
- cyano-4-hydroxy-cinnamic acid (CHCA)
- 3, 5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid, SA)

MALDI- TOF (Matrix- assisted laser desorption/ionization- time of flight mass spectrometry) instrument (Bruker Daltonics-Microflex, Germany).

Protein extraction: Plant part (root/stem/leaves) 2g was crushed using liquid nitrogen in mortar and pestle. The crushed powder was transferred to beaker containing 30cm^3 Protein extraction buffer (0.6 cm^3 of 1M Tris HCl pH 7.5, 2.55gm of Sucrose, 300 µl of Triton X-100, 300 µl of 100mM Phenyl Methyl Sulfonyl Fluoride (PMSF), 600 µl of 0.5M EDTA and the volume was made up to 30 cm^3 using distilled water) and sonicated for 30min.The above solution was centrifuged at 10,000rpm for 15min. The supernatant was transferred into clean test tube (50 cm^3). 50% Tri Chloro Acetic acid (TCA) was added to precipitate the protein. Mixed and centrifuge at 10,000 rpm for 15min. The supernatant was discarded. Protein pellet was stored at 20°C until further analysis.

Non Denaturing Poly Acrylamide Gel Electrophoresis (Native gel electrophoresis): The protein pellet was dissolved in 10mM ammonium hydroxide/ 10mM acetic acid and loaded in 15% non- denaturing PAGE. The gel was stained using coomasie brilliant blue stain.

MALDI-TOF analysis of protein: Protein pellet was dissolved in 10mM ammonium hydroxide or 10mM acetic acid. Dissolved Protein (1µl) + 1µl of matrixes α- cyano- 4- hydroxy- cinnamic acid (CHCA) or 3, 5- dimethoxy- 4- hydroxycinnamic acid (sinapinic acid, SA) spotted on MALDI ground steel target plate, dried and analyzed in MALDI-TOF instrument.

RESULTS:

- **DNA marker results:** The samples were run on 0.7% agarose gel and stained with ethidium bromide. Lane 1 shows 1kb ladder; Lane 2 shows genomic DNA isolated from leaf of Hemidesmus indicus; Lane 3 shows genomic DNA isolated from root of *Hemidesmus indicus*; Lane 4 shows genomic DNA isolated from leaf of *Hemidesmus pubescens*; Lane 5 shows genomic DNA isolated from leaf of *Decalepis halmintonii*; Lane 6 shows

genomic DNA isolated from leaf of *Vallaris solanaciae*; Lane 7 shows genomic DNA isolated from leaf of *Cryptolepis buchanni*; Lane 8 shows genomic DNA isolated from leaf of *Ichanocarpus fructosen*; Lane 9 shows genomic DNA isolated from root of *Ichanocarpus fructosen* (**Fig. 1**).

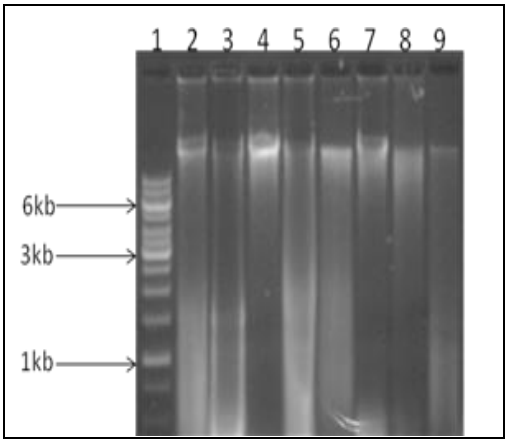

FIG. 1: AGAROSE GEL ELECTROPHORESIS

The samples were run on 0.7% agarose gel and stained with ethidium bromide. Lane 1 shows 1kb ladder; Lane 2 shows genomic DNA isolated from commercial powder of *Hemidesmus indicus* root, Lane 3 shows genomic DNA isolated from one month dried root of *Hemidesmus indicus* (**Fig. 2**).

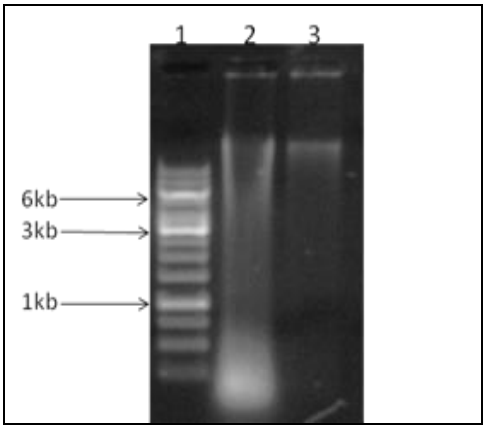

FIG. 2: AGAROSE GEL ELECTROPHORESIS

The samples were run on 0.7% agarose gel and stained with ethidium bromide. Lane 1 shows 1kb ladder; Lane 2 shows genomic DNA isolated from roots of *Hemidesmus indicus* (main plant); Lane 3 shows genomic DNA isolated from roots of *Hemidesmus indicus variety pubescens*; Lane 4 shows genomic DNA isolated from roots of *Decalepis hamiltoni*; Lane 5 shows genomic DNA isolated from roots of *Vallaris solanaceae*; Lane 6 shows genomic DNA isolated from roots of *Cryptolepis buchanani*; Lane 7 shows genomic DNA isolated from Ichanocarpus frutescens (**Fig. 3**).

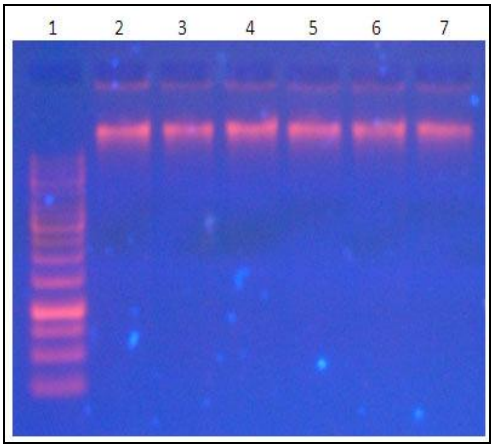

FIG. 3: AGAROSE GEL ELECTROPHORESIS OF GENOMIC DNA TREATED WITH RNASE ENZYME

The samples were run on 0.7% agaorse gel and stained with ethidium bromide. Lane 1 shows 1kb ladder; Lane 2 shows genomic DNA isolated from dry roots of *Hemidesmus indicus* (main plant)*;* Lane 3 shows genomic DNA isolated from commercial powder of *Hemidesmus indicus* (main plant) (**Fig. 4**).

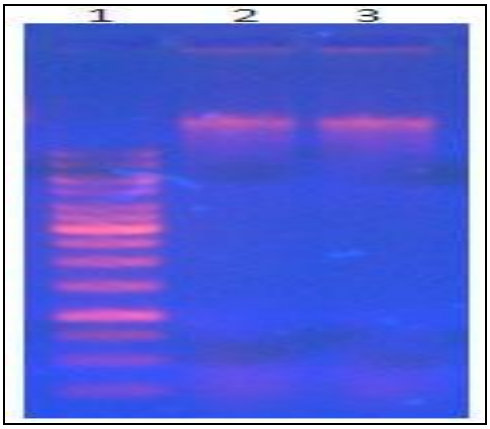

FIG. 4: AGAROSE GEL ELECTROPHORESIS

Genomic DNA isolation and quantitation: This procedure yields 40-70µg of DNA per gram of fresh leaves/root tissue and 25-50µg of DNA per gram of dried roots of *Hemidesmus indicus*. For substitute plants the above procedure yielded 40-50 µg of DNA per gram of fresh leaves/ root tissue. An A_{260}/A_{280} ratio 1.91 indicates insignificant levels of contaminating RNA. Total DNA isolated from *Hemidesmus indicus* and its substituted was checked by means of agarose gel electrophoresis. Figure 1 and 2 shows genomic DNA isolated from fresh leaf tissue, fresh and dried root. RNase treatment was given to samples as shown in figure 3 and 4.

The samples were run on 2% agarose gel and stained with ethidium bromide. Lane 1 shows 100bp ladder; Lane 2 shows amplification at ~150bp, ~400bp, ~1kb & ~1.5kb for *Decalepis hamiltoni* (~60ng/µl); Lane 3 shows amplification at ~450bp, ~1kb, ~1.5kb & ~2kb for *Vallaris solanaceae* (~60ng/µl); Lane 4 shows amplification at ~150bp, ~1kb & ~1.5kb for *Hemidesmus indicus variety pubescens* (~60ng/µl);Lane 5 shows amplification at ~1kb for *Ichanocarpus fructescens* (~60ng/µl); Lane 6 shows amplification at ~1kb & ~1.5kb for *Cryptolepis buchanani* (~60.5ng/µl); Lane 7 shows amplification at ~150bp, ~400bp, ~500bp & ~1kb for *Hemidesmus indicus* (main plant) (59.8ng/µl) (**Fig. 5**).

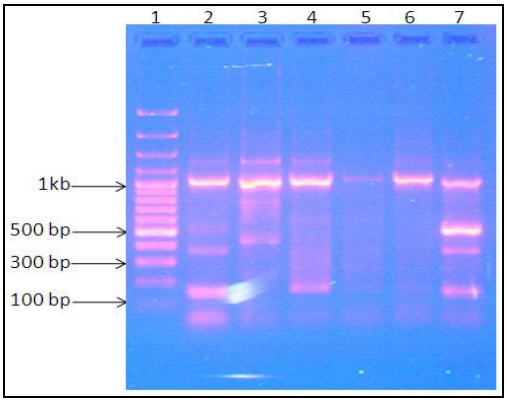

FIG.5: AGAROSE GEL ELECTROPHORESIS OF RANDOM AMPLIFIED POLYMORPHIC DNA (RAPD) USING MULTIPLE PRIMERS

RAPD analysis to obtain fingerprint pattern for all selected plants - The DNA isolated by this modified protocol was consistently amplified with random primers used for PCR amplification [34, 35, 36]. The RAPD analysis with random primer resulted in amplification of fragments of different sizes, thus when samples were run on 2% agarose gel band pattern was observed (figure 5). This pattern was different for all the plants selected for the study. Fingerprint pattern was observed in *Hemidesmus indicus* (main plant). Amplification at ~500bp band was unique for Hemidesmus indicus (main plant). Hence RAPD finger print pattern and present or absence of marker will help to distinguish main plant from its substitutes and fingerprint pattern will help to know which plant is used for adulteration.

The samples were run on 2% agarose gel and stained with ethidium bromide. Lane 1 shows 100bp ladder; Lane 2 shows amplification at ~600bp for *Hemidesmus indicus,* ; Lane 3 shows amplification at ~350bp for *Hemidesmus variety pubescens*; Lane 4 shows no amplification for *Decalepis hamiltoni*; Lane 5 shows amplification at ~350bp for *Vallaris solanaceae*; Lane 6 shows amplification at ~350bp for *Cryptolepis buchanani*; Lane 7 shows no amplification for *Ichanocarpus frutescens* (**Fig. 6**).

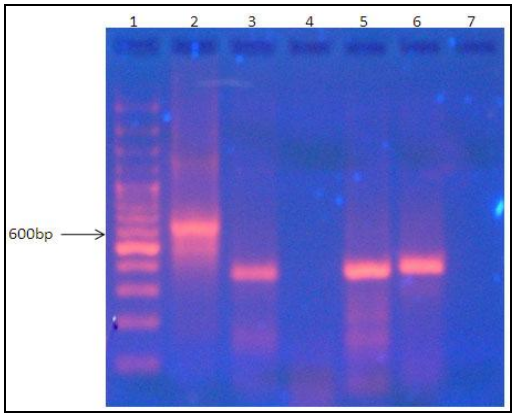

FIG.6. AGAROSE GEL ELECTROPHORESIS OF RANDOM AMPLIFIED POLYMORPHIC DNA (RAPD) USING SINGLE PRIMER SET

RAPD analysis for all selected plants to make SCAR marker: A pair of random primer was used to obtain amplification at ~600bp which was present only in *Hemidesmus indicus* and not in other plants (figure 6). A bulk PCR reaction was setup and the samples were resolved on 2% agarose gel. For further analysis, amplicon of ~600bp was purified using Qiagen gel extraction kit.

Sequencing of ~600bp band to make SCAR marker: The purified amplicon was sequenced using DNA sequencer (CEQ 8000, Beckman coulter). The nucleotide sequence obtained was analyzed using bioinformatics tool like BLAST search [31]. The first BLAST (NCBI) result (**Table 1**) showed that sequence matches 94% with the available nucleotide sequences of *Hemidesmus indicus* (Accession no.: DQ916851.1).

TABLE 1: GENE BANK BLAST SEARCH RESULT (FIRST TWENTY FIVE)

Accession	Description	Max score	Total score	Query coverage	E value	Max indent	Links
DQ916851.1	*Hemidesmus indicus* voucher Civeyrel 1008 (TL) 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 26S ribosomal RNA gene, partial sequence	632	632	75%	5e-178	94%	
AJ581679.1	*Mondia whitei* 18S rRNA gene (partial), ITS1, 5.8S rRNA gene, ITS2, and 26S rRNA gene (partial)	494	494	77%	3e-136	87%	
DQ916863.1	*Sacleuxia newii* voucher Bruyns 8653 (K) 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 26S ribosomal RNA gene, partial sequence	492	492	73%	9e-136	88%	
DQ916862.1	*Raphionacme lobulata* voucher Dold 4461 (GRA) 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 26S ribosomal RNA gene, partial sequence	483	483	74%	5e-133	87%	
AJ581691.1	*Schlechterella abyssinica* 18S rRNA gene (partial), ITS1, 5.8S rRNA gene, ITS2 and 26S rRNA gene (partial)	479	479	75%	7e-132	87%	
DQ916861.1	*Raphionacme galpinii* voucher Abbott s.n. (Z) 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 26S ribosomal RNA gene, partial sequence	477	477	77%	3e-131	87%	
DQ916855.1	*Pentopetia longipetala* voucher Allorge 2422 (P) 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 26S ribosomal RNA gene, partial sequence	477	477	75%	3e-131	87%	
AJ581687.1	*Raphionacme elata* 18S rRNA gene (partial), ITS1, 5.8S rRNA gene, ITS2 and 26S rRNA gene (partial)	477	477	77%	3e-131	87%	
AJ581685.1	*Raphionacme angolensis* 18S rRNA gene (partial), ITS1, 5.8S rRNA gene, ITS2 and 26S rRNA gene (partial)	477	477	77%	3e-131	87%	
DQ916852.1	*Mondia whitei* voucher ex hort. Ollerton s.n, Bot. Gard. Univ. Natal Pietermaritzberg 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 26S ribosomal RNA gene, partial sequence	473	473	77%	3e-130	86%	
DQ916866.1	*Tacazzea apiculata* voucher Renvoize 5760 (MO) 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 26S ribosomal RNA gene, partial sequence	472	472	77%	1e-129	86%	
AJ581690.1	*Raphionacme madiensis* 18S rRNA gene (partial), ITS1, 5.8S rRNA gene, ITS2 and 26S rRNA gene (partial)	472	472	77%	1e-129	86%	

Accession	Description	Max score	Total score	Query coverage	E value	Max indent	Links
DQ916848.1	*Finlaysonia insularum* voucher Middleton 1164 (A) 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 26S ribosomal RNA gene, partial sequence	466	466	77%	6e-128	86%	
AJ581688.1	*Raphionacme flanaganii* 18S rRNA gene (partial), ITS1, 5.8S rRNA gene, ITS2 and 26S rRNA gene (partial)	466	466	77%	6e-128	86%	
AJ581681.1	*Pentopetia grevei* 18S rRNA gene (partial), ITS1, 5.8S rRNA gene, ITS2 and 26S rRNA gene (partial)	460	460	75%	3e-126	87%	
DQ916836.1	*Batesanthus purpureus* voucher Hart 620 (MO) 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 26S ribosomal RNA gene, partial sequence	459	459	77%	9e-126	86%	
DQ916849.1	*Finlaysonia lanuginosa* voucher Livshultz 03-41 (BH) 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 26S ribosomal RNA gene, partial sequence	457	457	74%	3e-125	86%	
DQ916867.1	*Zygostelma benthami* voucher Middleton 849 (A) 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 26S ribosomal RNA gene, partial sequence	455	455	77%	1e-124	86%	
DQ916850.1	*Gymnanthera oblonga* voucher Forster 6133 (K) 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 26S ribosomal RNA gene, partial sequence	455	455	77%	1e-124	86%	
DQ916835.1	*Baseonema gregorii* voucher Cox & Abdullah 2028 (K) 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 26S ribosomal RNA gene, partial sequence	455	455	70%	1e-124	87%	
AJ581694.1	*Stomatostemma monteiroae* 18S rRNA gene (partial), ITS1, 5.8S rRNA gene, ITS2 and 26S rRNA gene (partial)	453	453	74%	4e-124	86%	
DQ916847.1	*Epistemma rupestre* voucher deWilde 7462 (MO) 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 26S ribosomal RNA gene, partial sequence	451	451	77%	2e-123	85%	
AJ581689.1	*Raphionacme hirsuta* 18S rRNA gene (partial), ITS1, 5.8S rRNA gene, ITS2 and 26S rRNA gene (partial)	449	449	77%	6e-123	85%	
AJ581693.1	*Stomatostemma monteiroae* 18S rRNA gene (partial), ITS1, 5.8S rRNA gene, ITS2 and 26S rRNA gene (partial)	448	448	74%	2e-122	86%	
AJ581686.1	*Raphionacme dyeri* 18S rRNA gene (partial), ITS1, 5.8S rRNA gene, ITS2 and 26S rRNA gene (partial)	444	444	77%	3e-121	85%	

The samples were run on 2% agarose gel and stained with ethidium bromide. Lane 1 shows 100bp ladder; Lane 2 shows amplification at ~400bp for *Hemidesmus indicus,* Lane 3 shows no amplification at ~400bp for *Hemidesmus indicus variety pubescens,* Lane 4 shows no amplification at ~400bp for *Decalepis hamiltoni,* Lane 5 shows no amplification at ~400bp for *Vallaris solanaceae,* Lane 6 shows no amplification at ~400bp for *Cryptolepis buchanani,* Lane 7 shows no amplification at ~400bp for *Ichanocarpus frutescens* (**Fig. 7**).

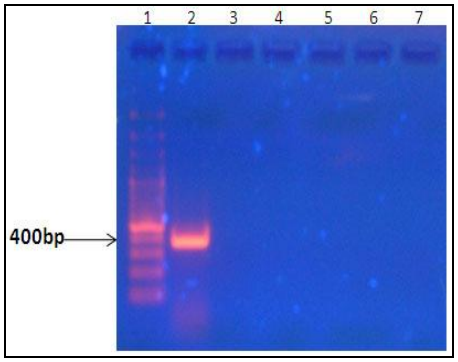

FIG. 7: AGAROSE GEL ELECTROPHORESIS OF SEQUENCE CHARACTERISTIC AMPLIFIED REGION (SCAR) MARKER

The samples were run on 2% agarose gel and stained with ethidium bromide. Lane 1 shows 100bp ladder; Lane 2 shows amplification at ~400bp for dry roots of *Hemidesmus indicus,* Lane 3 shows amplification at ~400bp for commercial powder of *Hemidesmus indicus* (**Fig. 8**).

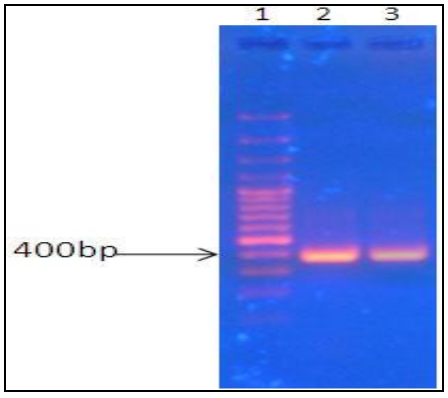

FIG. 8: AGAROSE GEL ELECTROPHORESIS OF SEQUENCE CHARACTERISTIC AMPLIFIED REGION (SCAR) MARKER

PCR of SCAR marker: The primers for SCAR marker were designed from the sequence obtained using primer BLAST tool. The PCR reaction was optimized to obtain amplification of marker band at ~400bp in *Hemidesmus indicus* (main plant) in such a way that no amplification is observed at that size under similar experimental condition with selected primers for substituted and adulterant plants (figure 7 & 8). Hence annealing temperature was optimized to 60°C for amplification of ~400bp marker. Thus presence or absence of marker band will help in identification of *Hemidesmus indicus* from variety and substitute plants.

Protein marker results: Lane 1 shows medium molecular weight marker. Lane 2 shows protein band pattern for *Hemidesmus indicus* dry root, Lane 3 shows protein band pattern for *Hemidesmus indicus* dry root, Lane 4 shows protein band pattern for *Hemidesmus indicus variety pubescens* root, Lane 5 shows protein band pattern for *Vallaris solanaceae* root, Lane 6 shows protein band pattern for *Ichanocarpus frutescens* root, Lane 7 shows protein band pattern for *Cryptolepis buchanani* root, Lane 8 shows medium molecular weight marker, Lane 9 shows protein band pattern for *Hemidesmus indicus* fresh root, Lane 10 shows protein band pattern for *Hemidesmus indicus* leaves, Lane 11 shows protein band pattern for *Hemidesmus indicus* stem (**Fig. 9**).

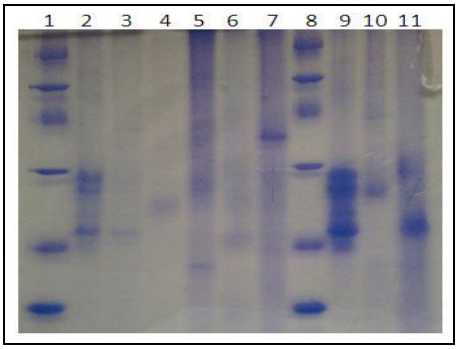

FIG. 9: NON DENATURING POLYACRYLAMIDE GEL ELECTROPHORESIS (NATIVE-PAGE) FOR PLANT PROTEIN SAMPLES

Protein isolation and non denaturing PAGE analysis: Total protein was isolated from plant parts (root/stem/leaves) of *Hemidesmus indicus,* its variety and substitute plants[37,38,39]. The extracted protein was analyze on 15% native PAGE gel[40,41] as shown in **figure 10**.

m/z	SN	Quality Fac.	Res.	Intens.
4338.128	46	1880	79	300
4426.943	193.4	86381	290	1261
4563.93	138.4	43154	240	902
4718.814	42.2	4386	18	275
5372.799	79	10021	196	515
5786.304	48	8011	244	313
8515.248	17.8	1230	128	116

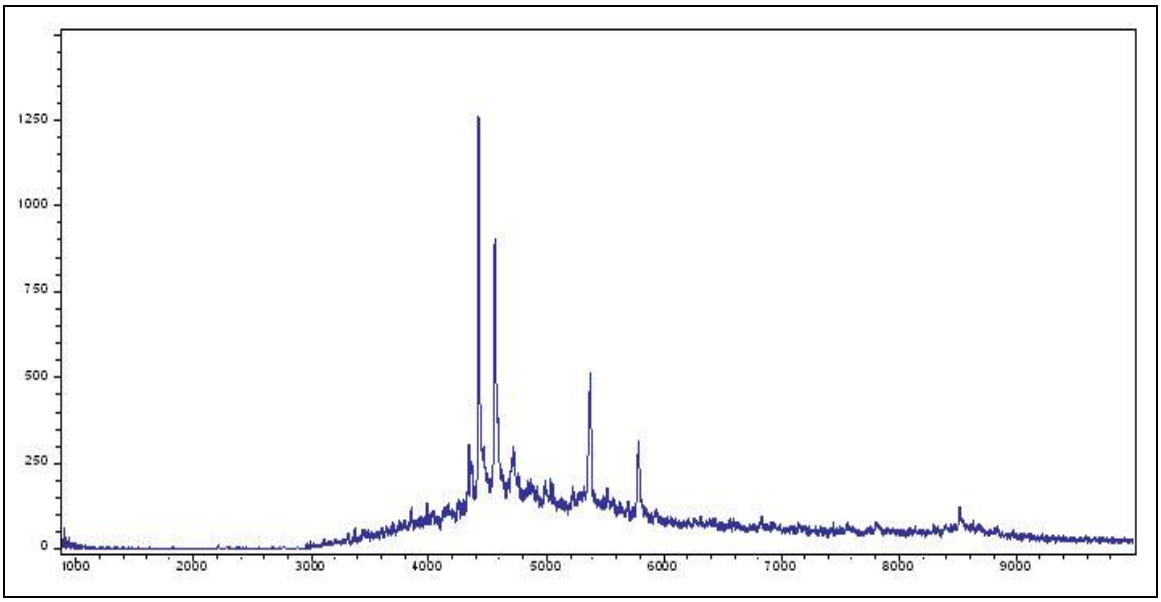

FIG. 10: SHOWS MALDI-TOF SPECTRA OF *HEMIDESMUS INDICUS* FRESH ROOT

Protein fingerprinting using MALDI-TOF Mass Spectrophotometer: Extracted protein was dissolved in 10mM ammonium hydroxide/10mM acetic acid. Dissolved protein (1µl) plus matrix i.e. CHCA/ SA (1µl) was spotted on to MALDI plate and scanned [42, 43]. The mass/charge region 1000 to 10000 was selected to obtain protein fingerprint pattern for all selected plants. Unique fingerprint pattern for root, stem, leaves of *Hemidesmus indicus,* its variety and substitute plants was observed as shown in figure 10, 11, 12 & 13. The protein marker peak of mass/charge at 4426.943 and 5372.799 is consider the protein marker for *Hemidesmus indicus* which is not present in stem, leaves of *Hemidesmus indicus* and in roots of variety as well as substitute plants.

DISCUSSION and CONCLUSION: The yield of DNA from dry powdered samples of *Hemidesmus indicus* using the DNA extraction protocols by Doyle *et al*., [23] was poor, and hence a modified DNA extraction method was used. It was found that overnight incubation with EDTA [24] leaches out the water soluble components and mineral moieties. Precipitation of DNA with PEG [25] at 20°C for overnight improves the yield as compared to isopropanol. This DNA was found to be consistently suitable for RAPD analysis.

RAPD analysis using multiplex PCR was carried out with six pairs of different primers on *Hemidesmus indicus* as well as other substituted plants. Results showed a unique fingerprint pattern for *Hemidesmus indicus* and other plants. After analyzing this pattern, it was found that amplification at ~500bp is unique band for *Hemidesmus indicus* which was not found in all other substitute or contaminating plants.

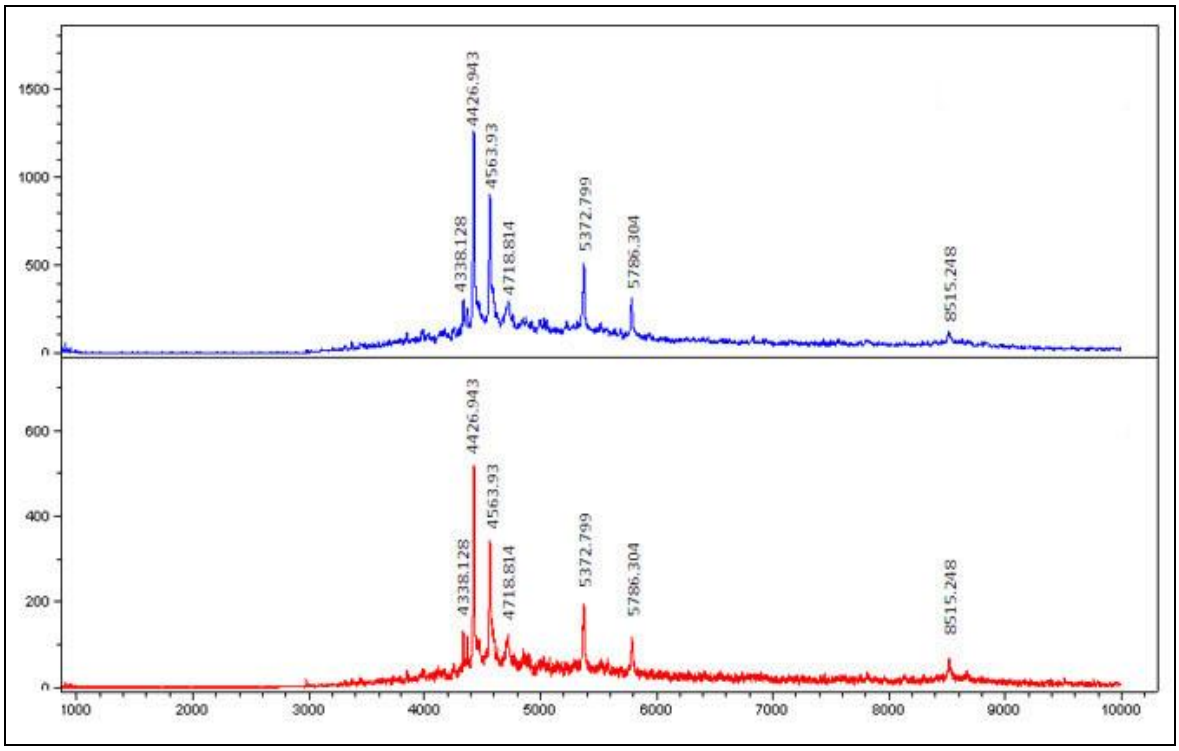

FIG. 12: SHOWS MALDI-TOFF SPECTRA OF *HEMIDESMUS INDICUS* FRESH ROOT AND DRY ROOT

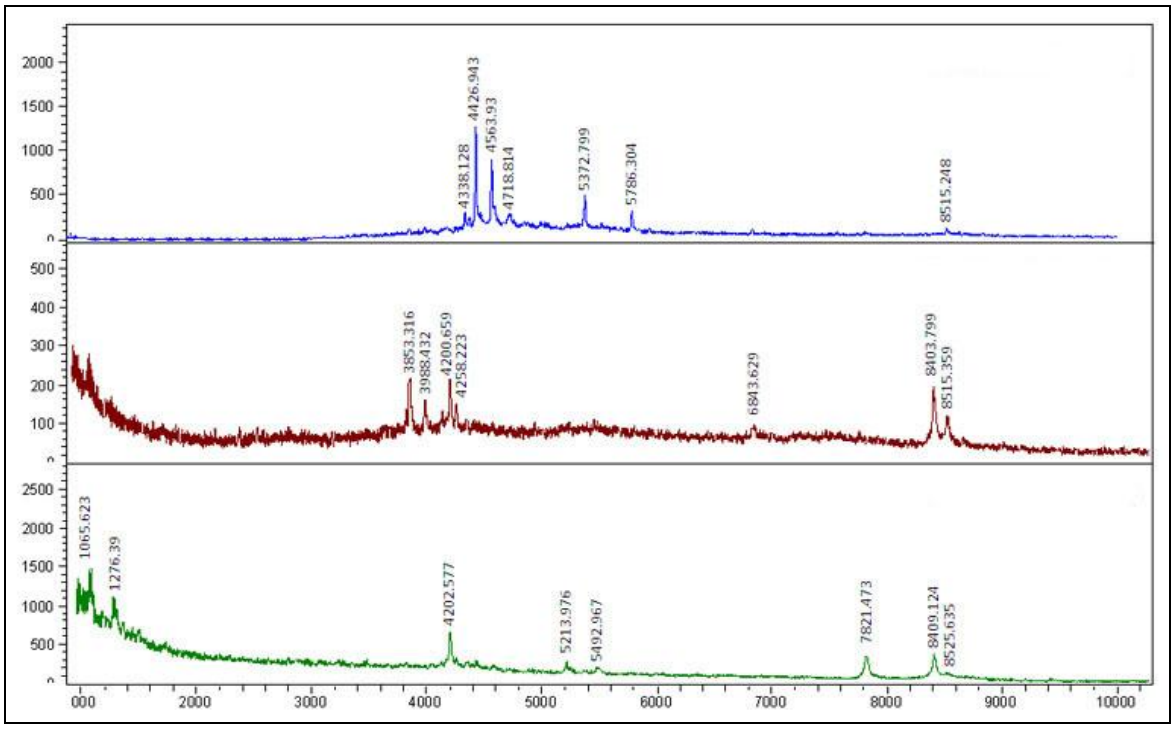

FIG. 12: SHOWS MALDI-TOFF SPECTRA OF *HEMIDESMUS INDICUS* ROOT, STEM AND LEAVES

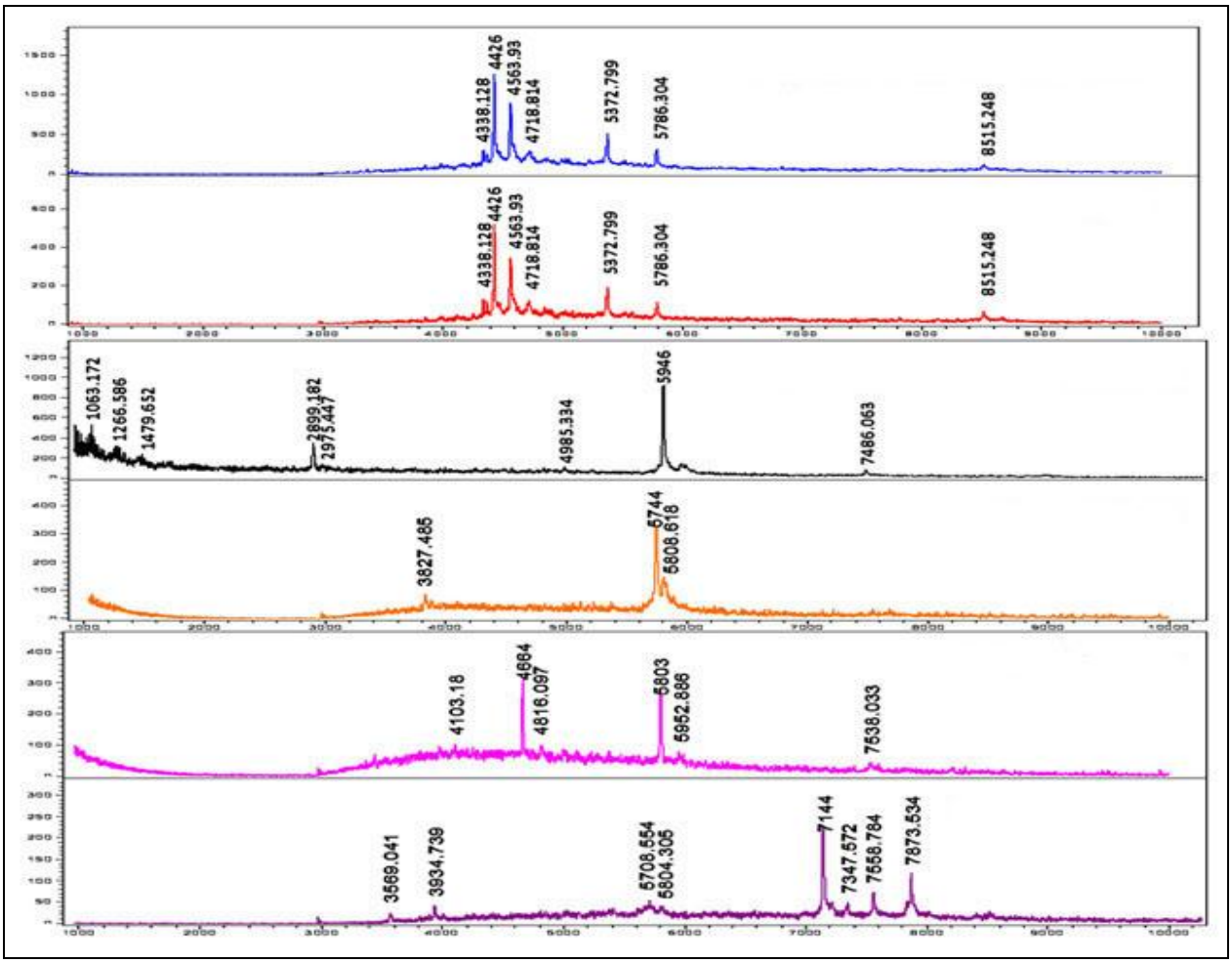

FIG. 13: SHOWS MALDI-TOFF SPECTRA OF *HEMIDESMUS INDICUS* FRESH ROOT, *HEMIDESMUS INDICUS* DRY ROOT, *HEMIDESMUS INDICUS VARIETY PUBESCENS* ROOT, *ICHANOCARPUS FRUTESCENS* ROOT, *VALLARIS SOLANACEAE* ROOT AND *CRYPTOLEPIS BUCHANANI* ROOT

Another RAPD analysis was carried out using single pair of primer. Results showed a unique amplification at ~600bp for *Hemidesmus indicus* and not for other plants. This amplified band was cut and gel elution was carried out. This gel eluted pure PCR amplified DNA was further taken for sequencing and the data was analyzed using BLAST (NCBI). BLAST search results showed 94% homology with *Hemidesmus indicus* voucher Civeyrel 1008 (TL) 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 26S ribosomal RNA gene, partial sequence, accession no. DQ916851.1. With the help of sequence primer were design using Primer BLAST tool from NCBI site. These primer were further used for development of specific Sequence Characteristic Amplified Region (SCAR) marker for *Hemidesmus indicus.* Results showed amplification at ~400bp for *Hemidesmus indicus* only and not for other plants. This band is the marker band for identification of *Hemidesmus indicus* from variety and substitute plant.

The new modified protocol consistently produced a good yield of high-quality DNA. This method of DNA isolation is suitable for plants with acidic tissue extracts [1, 35]. It can be successfully used for isolating amplifiable DNA from dry tissue

powders. Distinguishing profiles were produced for all plants selected for study using random primers. If there is adulteration or substitution of *Hemidesmus indicus* with other plants, RAPD analysis could help to find out whether the plant used is genuine or substituted. The presence of the characteristic marker band would also help in identification of *Hemidesmus indicus* from other plants.

Such RAPD markers could also be used for typing the flora and fauna or biodiversity of a region. Biodiversity in plants refers to total variation in plant life, including the number of species, the degree of genetic variation within species, the different types of ecosystems, and its functions. Local communities tend to collect the highest value or most popular plant species, leading to over-harvesting or species extinction [44], as these medicinal plants provide important development options for communities [45, 46]. When supply of medicinal plants like *Hemidesmus indicus* is insufficient, there is a tendency of the collector to adulterate the genuine plant material. Thus, leads to expansion of unregulated trade and commercial use of medicinal plants which poses a major threat to biodiversity in the region [47]. Hence molecular biology techniques such as RAPD analysis helps to develop a fingerprint pattern for genuine medicinal plants. This fingerprint pattern or presence of DNA marker helps to identify genuine plant from substituted plant material.

DNA fingerprinting/DNA marker will not be able to distinguish between the plant parts (root/leaves/stem) used in adulteration of *Hemidesmus indicus*. Thus, there is need for complementary technique like Protein fingerprint t*o* differentiate plant parts used as protein expression in each part viz root, stem, leaves is different [48, 49]. Hence MALDI-TOF analysis was carried out *for Hemidesmus indicus roots* (fresh, dry, commercial powder), stem and leaves. Also MALDI-TOF analysis of roots of variety i.e. Hemidesmus indicus variety pubescens, and substitute plants Vallaris solanaceae, Ichanocarpus frutoscen, Cryptolepis buchanani was also carried out. The Non denaturing and MALDI-TOF analysis showed unique protein fingerprint patter for each plant part of *Hemidesmus indicus*. Also unique protein fingerprint pattern was observed for variety and substitute plants. Peak of mass/charge at 4426.943 and 5372.799 is consider the protein marker for *Hemidesmus indicus* which is not present in stem, leaves of *Hemidesmus indicus* and variety as well as substitute plants [50, 51].

Thus, the advantages of fingerprinting based on molecular markers over morphological character is;

- High degree of non-tissue specific polymorphism
- Minimal influence of environment
- Simple inheritance pattern

DNA/Protein fingerprint will provide proof or defense against allegations of breach of intellectual property right. Such infringements would occur when;

i) a registered variety is cultivated/marketed unauthorisidely under its own or a different name,
ii) plant material comprising seeds, flowers, fruits or other plant products are falsely sold under the name of a registered variety,
iii) plant material is collected from wild and commercially exploited without the authorization of biodiversity authority [52].

The demand for medicinal plants has increased world wide and there is need for proper documentation regarding collection of plant materials, its analysis using various scientific

techniques and way it is formulated to final product [53].

Hence DNA/Protein-analytics can be effectively used as tool for correct identification of plant species and thereby support standardizing quality control of Ayurvedic medicinal plants and other botanicals, used as drugs or dietary supplements.

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