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DNA BARCODE ITS 2 REGION BASED MOLECULAR MARKER AUTHENTICATION OF MEDICINAL PLANTS, *NARDOSTACHYS JATAMANSI* AND ITS SUBSTITUTE *SELINUM VAGINATUM*

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Abstract— Commercial herbal products are available were either contaminated or substituted with other plant species with similar morphology which are not catalogued on the labels. Adulteration is a serious health threat to the consumer safety. Thus, the genetic level discrimination protocols could be safer to identify the exact and specific herbal products in the market. DNA bar-coding is safe, integrity and authenticated methodologies for species identification and taxon discrimination. The present study discriminated particular herbal plants such as *N.jatamansi* and *S.vaginatum* using the DNA bar-coding technology with ITS2 primers and authenticated the originality of the herbal plants.

Index Terms— DNA bar-Coding, Herbal products *N.jatamansi* and *S.vaginatum*

I. INTRODUCTION

DNA Barcoding is a novel molecular biology technique used to identify the taxa to which the biological samples or specimens belongs by using short standardized DNA sequences either nuclear or organellar genomic regions. [1] DNA Barcoding technique helps to generate universal DNA sequences that could be used for the genus and species identification and authentication at each taxon level. DNA Barcoding system in plants is a challenging process. [2] The Internal Transcribed Spacer 2 (ITS2) region of nuclear ribosomal DNA has been suggested as the most suitable region for DNA Barcoding application among the medicinal plants and their closely related species.[3] In this study, three medicinal herbs *Nardostachys jatamansi* (Jatamansi), *Selinum vaginatum* (Bhutakesi), *Valeriana wallachi* (Tagara)) were tested for the species identification and taxon discrimination using the ITS as DNA barcode region. [4]
The chief aim of the present work is to generate DNA Barcode data for *Nardostachys jatamansi* DC and its substitutes/ alternatives/ adulterants viz. *Selinum vaginatum* and *Valeriana wallachii*. *Nardostachys jatamansi DC* (Jatamansi) is an important Ayurvedic medicinal plant, incorporated in various Ayurvedic compound preparations. It has interesting pharmacological activities, immense trade value, prone to adulteration/substitution, vulnerable to illegal trafficking hence generating DNA Barcode is of utmost importance from both research and biodiversity monitoring perspective. [5]

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II. MATERIALS AND METHODS

A. Extraction of genomic DNA and amplification of target genes by Polymerase Chain Reaction (PCR) Plant Samples
Authenticated rhizome plant material of *Nardostachys jatamansi DC* (Jatamansi), *Selinum vaginatum (*Bhutakesi) *and Valeriana wallachii* (Tagara) were procured from Herbal Research and Development Institute, Uttarakhand.
DNA was extracted from the root samples of the three medicinal plant species, *Nardostachys jatamansi DC* (Jatamansi), *Selinum vaginatum* (Bhutakesi), *Valeriana wallachi* (Tagara)) by using Roche HIPURE PCR preparation template kit. Root tissues of the three medicinal plant species were taken in a homogenizer (Fast-Prep-24 instrument, MP Biomedicals, Ohio).

B. DNA Extraction
About 200 µl of sample material was added to a nuclease-free 1.5 ml micro centrifuge tube and 200 µl binding Buffer and 40 µl Proteinase K (reconstituted) were added, mixed and incubated at 70°C for 10 min after the incubation 100 µl isopropanol was added to the incubated mixture and again mixed well. The sample mixture was transferred into the high pure filter tube, it was connected to the collected tube. Then the tube was centrifuged for 1 min at 8,000 × g. After centrifugation removal of collected tube from filter tube then it was connected to the new collected tube. Same procedure repeated again with new collection tube. The whole high pure assembly was centrifuged for an additional 10s at full speed after the discarding the flow through. The removal of residual wash buffer was confirmed with the extra centrifugation time. The tubes were again centrifuged in the tube assembly for 1 min at 8,000 × g. The micro centrifuge tube containing the eluted, purified DNA, stored at +15 to +25°C for PCR amplification.

C. DNA quality check
The extracted DNA was quantified using spectrophotometer and by running in 0.8%agarose gel. Subsequently, DNA purity and quantity will be analyzed using the spectrophotometer, the ratios between the reading at 260nm

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and 280nm provide an estimate of DNA purity. Pure DNA samples will have a ratio of 1.8. If the sample was contaminated by protein, the ratio will be significantly less. The ratio of 2.0 or more indicated a high proportion of RNA in the sample. DNA has the maximum absorbance at 260nm. An absorbance of 1.0 corresponds to 10µg/ml for double-stranded DNA.

Table.1: First set of primers

S.No	Primer Name	Primer sequence 5' to 3'
1.	psbA3	GTTATGCATGAACGTAATGCTC
2.	trnH	CGCGCATGGTGGATTCACAATCC
3.	matK472F	CCCRTYCATCTGGAAATCTTGGTTC
4.	matK1248R	GCTRTRATAATGAGAAAGATTTCTGC
5.	matK21F	CCTATCCATCTGGAAATCTTAG
6.	matK5R	GTTCTAGCACAAGAAAGTCG
7.	ITS2-5.8S-BF	CGATGAAGAACGCAGCGAAATGCGAT
8.	ITS-2-25B R-2	TCCTCCGCTTAGTATATGCTTAA

F'-Forward region, R-Reverse region

Table.2: Second set of primers

S.No	Primer Name	Primer sequence 5' to 3'	Primer length	Category belongs to region	Universal/ Plant specific primers
1.	ITSU1	GGAAGKARAAGTCGTAACAAGG	22	18S F	Universal
2.	ITSU2	GCGTTCAAAGAYTCGATGRTTC	22	5.8S R	Universal
3.	ITSU4	RGTTTCTTTTCCTCCGCTTA	20	26S R	Universal
4.	ITSU3	CAWCGATGAAGAACGYAGC	19	5.8S F	Universal

Table.3: Third set of primers

S.No	Primer Name	Primer sequence 5' to 3'	Primer length	Category belongs to region	Universal/ Plant specific primers
1.	ITSP5	CCTTATCAYTTAGAGGAAGGAG	22	18S F'	Plant-specific
2.	ITSP4	CCGCTTAKTGATATGCTTAAA	21	26S R'	Plant-specific
3.	ITSP3	YGACTCTCGGCAACGGATA	19	5.8S F	Universal

D. Extraction of PCR primers

The reagents and DNA were removed from the freeze and they were allowed to thaw on ice. It can be thawed at room temperature, but it was put back on ice immediately after the thawing process.Using sterile pipette tips, add nuclease-free water to the master mix tube (125µl) as follows 18.3 µl of double distilled water, 2.5µl *Taq* DNA polymerase buffer (10x), dNTP mix (2mM) - 2.5µl, Forward Primers (10µM)-0.5µl, Reverse Primers (10µM) - 0.5µl,Template DNA -0.5µl and Taq DNA polymerase enzyme(5U/µl)-0.2µl.The total reaction volume was 25.0µl. It was taken in five 0.2ml or 0.5 ml PCR tubes and the master mix was vortexed or mixed thoroughly by tapping and aliquot 24.5 µl into each of the five reaction tubes. About 0.5 µl of each DNA sample was added to the respective PCR tubes. The point that negative control receives no DNA was kept in mind during the procedure.The tubes were capped and mixed by flicking with finger, and then briefly (~10 seconds) the tubes were centrifuged. The tubes were kept in PCR thermal cycler, the lid was closed, and the programme was started. After completion of thermal cycler programme PCR product was resolved onto 1% agarose gel and the results were observed.

E.Thermalcycler programme

i. Initial denaturation - 94ºC for 4 minutes
ii. Cycle denaturation - 94ºC for 30 Seconds
iii. Annealing - 55ºC for 40 Seconds
iv. Extension - 72ºC for 1 min
v. Go to step 2 for 34 cycles
vi. Final Extension - 72ºC for 10 minutes
vii. Hold 22ºC forever
viii. End programme

Gel Preparation: The required amount of agarose was added to the electrophoresis buffer (1X Tris-Borate EDTA-TBE) in a glass flask. Agarose was melted in the microwave and the flask was swirled to ensure even mixing. Melted agarose was cooled to a tolerable temperature (~55ºC). The flask was swirled after the addition of ethidium bromide (EtBr-5µg/mL) for even mixing. The casting apparatus with an inserted comb was filled with the melted gel.The gel was allowed to stand until it got solidified. The comb was removed gently from the gel plate after solidification.

Gel loading and running: The gel plate was placed in the electrophoresis tank. The wells were covered by pouring 1X TAE buffer.PCR products were mixed with 1 µl of loading buffer (6X) (MBI Fermentas) on parafilm and were loaded with a micropipette into the wells along with the marker (100-bp ladder) (MBI Fermentas) to determine their size. The gel was run for approximately half an hour at a voltage supply of 10V/cm till bromophenol migrated at least half the distance through the gel. Amplification was documented in a gel doc system after the gel was removed from the tank and placed on UV trans illuminator (UV TEC).

F. Elution of PCR amplified products

The PCR amplified products were eluted with the help of GenElute™ gel extraction kit from Sigma-Aldrich, USA.

Procedure:

Band excision: The DNA fragments of interest were excised from agarose gel with sharp, clean razor blade. The excess

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gel was trimmed away to minimize agarose amount. The gel slice was weighed in a tared colourless tube.

Gel solubilization: 3 volumes of gel solubilization solution were added to the gel slice. The gel mixture was incubated at 50-60 °C for 10 minutes, or until it was completely dissolved. The gel was dissolved with help of vortexing briefly for 2-3 min during incubation.

Preparation of binding column: The binding column was prepared while agarose was solubilized. The GenElute binding column G was placed into 2 ml collection tubes and centrifuged for 1 minute. The flow through liquid was discarded.

Checking the colour of the mixture: The color of the mixture was checked for color yellow after the gel slice was completely dissolved prior to the continuing the following step. As the color of the mixture was red, 10 mL of the 3 M Sodium acetate buffer, pH 5.2, was added and mixed well. The color was still yellow and hence, 3 M Sodium acetate buffer, pH 5.2, was added in 10 mL increments until the mixture turned yellow.

Addition of isopropanol: About 1 gel volume of 100% isopropanol was added and mixed until homogenous. For a gel with an agarose concentration greater than 2%, 2 gel volumes were used for 100% isopropanol.

Binding of DNA: The solubilized gel solution mixture was loaded from step 6a into the binding column that was assembled in a 2 ml collection tube. We were aware it was normal to see an occasional color change from yellow to red once the sample was applied to the binding column. After loading the column each time, it was centrifuged for 1 minute. The flow through liquid was discarded.

Column washing: The washing solution (700 mL) was added to the binding column and it was centrifuged for 1 minute. Flow –through liquid was discarded after the binding column was removed from the collection tube. The collection tube was fitted above the binding column and centrifuged again for 1 minute.

Elution of DNA: The binding column was transferred to a fresh collection tube. Nearly 50 µL of elution solution was added to the centre of the membrane and incubated for 1 minute. It was centrifuged for 1 minute. Prior addition to the membrane, the elution solution was pre-heated up to 65 °C for effective recovery of intact plasmid DNA. Preheating treatment recovers plasmid by 2 to 3-fold.

*Sequencing of eluted PCR products:*2 µl containing 10 picomol. of the same amplification reaction primers was used by the sequencing reaction. Sanger dideoxy bi-directional sequencing was performed in Centre for Cellular and Molecular Biology (CCMB, Hyderabad using an ABI automated sequencer Model 3730(Applied Biosystems, Foster City, CA) with fluorescently labeled DNA molecules.

G. Phylogenetic analysis

The phylogenetic analysis was done using the homology search tool i.e BLASTN (Basic Local Alignment Search Tool Nucleotide) from the NCBI (National Centre for Biotechnology Information). This bioinformatic tools compare our query DNA sequences with all available DNA the non-redundant databases. In this study, four results from the sequencing study were analysed with BLASTN online for homology and phylogenetic relationship among the query sequences. The blast tree (phylogenetic tree) was produced using BLAST pair wise alignment.

III. RESULTS AND DISCUSSION

A. Efficiency of DNA Barcoding

Three medicinal plants *Nardostachys jatamansi* DC (Jatamansi and its substitutes/adulterants viz. *Selinum vaginatum* (Bhutakesi) and *Valeriana wallachi* (Tagara) were selected for the study. The protocols for DNA extraction, PCR and sequencing worked for primers ITSp4, p3, u1 and u4. Genomic DNA was successfully extracted from the powdered root samples of the three medicinal plant species: *Nardostachys jatamansi DC* (Jatamansi), *Selinum vaginatum* (Bhutakesi), *Valeriana wallachi* (Tagara). Genomic DNA was found to be a mild smear in the agarose gel electrophoresis instead of a thick band (Figure 1).

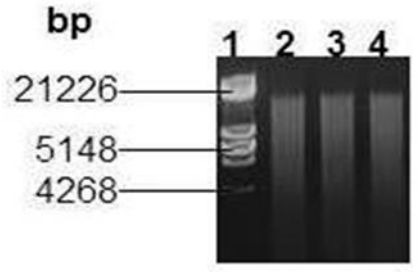

Figure.1. Agarose gel electrophoresis (1%) showing Lambda DNA/*Eco* R1 marker and genomic DNA extracted from the root samples of medicinal plant species.Lane 1 – Lambda DNA / *Eco* RI Marker, Lane 2 – Genomic DNA of S1J, Lane 3 – Genomic DNA of S2T, Lane 4 – Genomic DNA of SB3.

The PCR product proportions varied greatly among these three species, with *Nardostachys jatamansi*, *Selinum vaginatum* yielding the best results than the PCR amplification for *Valeriana wallachi*. First set of barcode genes such as psbA3, trnH, matK472, matK1248, matK21, matK5, ITS2-5.8BF-2 and ITS2-25BR-2 were randomly selected based on their universality. However, no amplification was observed for all the 4 set of primers enlisted in the Table. 1. Then the second set of primers for the ITS genes such as U1, U2, U3 and U4 were used for the PCR amplification. Three specifically selected primers for ITS marker regions such as P3, P4 and P5 were finally used to barcode the commercial herbals available in the market. This helps to the specific and significant identification of the commercially available herbs based on the DNA Barcoding techniques. In the second set of primers, U1 and U4primers amplified appropriately and generated the sharp and thick bands for all the three medicinal herbs used in the study. They showed PCR products ranged in molecular size around 700 base pairs.

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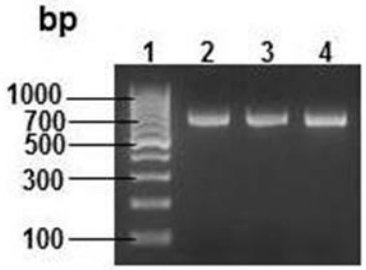

Figure.2. Agarose gel electrophoresis (2%) showing 100bp DNA ladder and PCR amplified products of genomic DNA from medicinal plant species using ITS P5 and P4 primers.Lane 1 – Lambda DNA / *Eco* RI Marker, Lane 2 – Genomic DNA of S1J, Lane 3 – Genomic DNA of S2T, Lane 4 – Genomic DNA of SB3.

The third set of primers ITS-P4 and P5 were the most commonly used one to amplify the plant-based identification in DNA barcoding procedures (Table 3). The PCR product sizes varied significantly among the different set of primers used in the study.ITS-P4 and P5 primers yielded the best results in PCR for all the three species, *N.jatamansi*, *S.vaginatum* and *V.wallachi* (Figure 3). These primers also had passed the first two steps of the barcoding technique, DNA extraction and PCR amplification. The PCR product found to be around 500 bp in molecular size for all the three commercially available medicinal herbs.

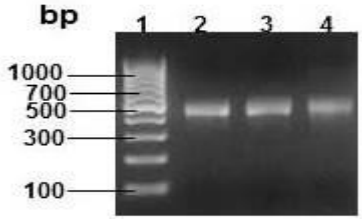

Figure.3. Agarose gel electrophoresis (2%) showing 100bp DNA ladder and PCR amplified products of genomic DNA from medicinal plant species using ITS P3 and P4 primers.Lane 1 – Lambda DNA / Eco RI Marker, Lane 2 – Genomic DNA of S1J, Lane 3 – Genomic DNA of S2T, Lane 4 – Genomic DNA of SB3.

The ITS-P3 and P4 were the third set of primers used in the experiment to randomly amplify the herbs utilizing the DNA Barcoding procedures. The PCR products were allowed to pass in the two percent agarose gel and revealed the significantly amplified products. ITS-P3 and P4 primers yielded the best results in PCR for all the three species: *N.jatamansi*, *S.vaginatum* and *V.wallachi* (Figure 4). These ITS-P3 and P4primers also had passed the preliminary steps of barcoding technique, DNA extraction and PCR amplification. In the 2% agarose gel, the one sharp and a thin band were found around 700 and 500bp in molecular size, respectively.The specificity of the plant-specific primer sets was validated with *in vitro* PCR experiments for three plant samples using ITS-P5/ITS-U4 for the whole ITS region, ITS-P5/ITS-U2 for ITS1 and ITS-P3/ITS-U4 for ITS2

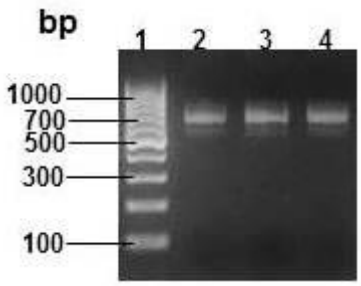

Figure.4.Agarose gel electrophoresis (2%) showing 100bp DNA ladder and PCR amplified products of genomic DNA from medicinal plant species using ITS U1 and U4 primers.Lane 1 – Lambda DNA / *Eco* RI Marker, Lane 2 – Genomic DNA of S1J, Lane 3 – Genomic DNA of S2T, Lane 4 – Genomic DNA of SB3.

B. Sequencing results of the plant ITS primers

Five universal and two plant-specific primers were designed for the entire ITS region, ITS1 and ITS2. The odd numbers in the primers were used for forward primers and even numbers for reverse primers. Among the seven different primers and combinations, ITS-P3 and P4 worked out well in the case of universal and plant-specific primers, respectively for the entire coverage of the ITS 2 region in the ribosomal subunit. Two fungal species were identified from the PCR amplified DNA isolated from the root samples of medicinal herbs, *N.jatamansi* and *S.vaginatum.* Those fungal sequences were Barcoded with ITS – U1 and U4 primer pairs (Figure 4a & 5& 6). The first and second sequences were identified as partial sequence of internal transcribed spacer (ITS 1) or 5.8S ribosomal RNA gene (small subunit) of *Trichoderma asperelloides* isolate SB3AL and S2T or else complete sequence of 5.8S ribosomal RNA gene, and internal transcribed spacer 2 (ITS 2), and also represented the partial sequence of large subunit ribosomal RNA gene and assigned with accession numbers MH371282 and MH371292 (Figure 5 & 6). It is a linear strand of DNA with 679bp in length. These sequences were amplified from the DNA isolated from the roots of *S.vaginatum* and *V.wallachi*.

IV. THE THIRD FUNGAL SEQUENCE WAS BARCODED AS PARTIAL SEQUENCE OF INTERNAL TRANSCRIBED SPACER (ITS 1) OR 5.8S RIBOSOMAL RNA GENE (SMALL SUBUNIT) OF *ASPERGILLUS FLAVUS* ISOLATE SJAL OR ELSE COMPLETE SEQUENCE OF 5.8S RIBOSOMAL RNA GENE, AND INTERNAL TRANSCRIBED SPACER 2 (ITS 2), AND ALSO REPRESENTED THE PARTIAL SEQUENCE OF LARGE SUBUNIT RIBOSOMAL RNA GENE AND ASSIGNED WITH ACCESSION NUMBER MH371291. IT IS A LINEAR STRAND OF DNA WITH 586BP IN LENGTH AND THIS SEQUENCE WAS AMPLIFIED FROM THE DNA ISOLATED FROM THE ROOTS OF *N.JATAMANSI.*

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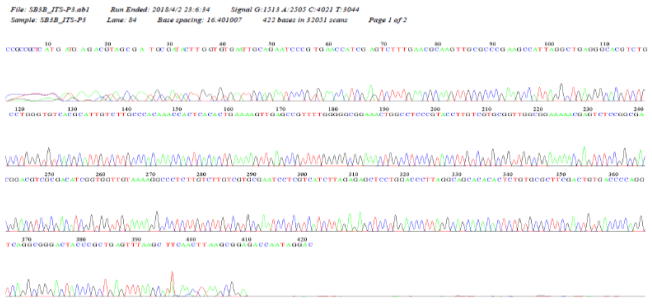

Figure.5.Sequencing result of commercial herb *Selinum vaginatum* using universal ITS-p3 primers.

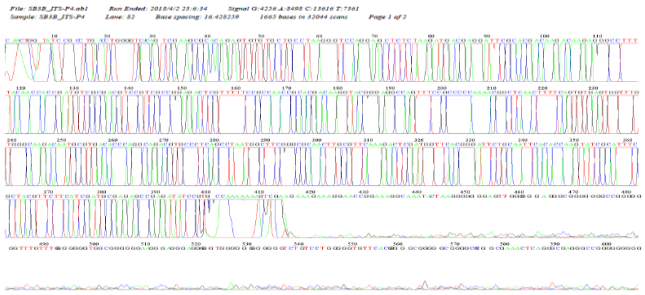

Figure.6.Sequencing result of commercial herb *Selinum vaginatum* using the plant-specific ITS-p4 primers.

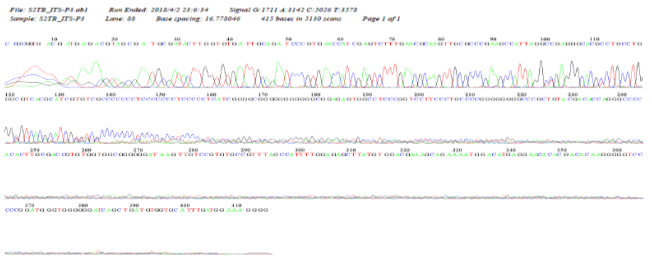

Figure.7. Sequencing result of commercial *S.vaginatum* using universal ITS primers

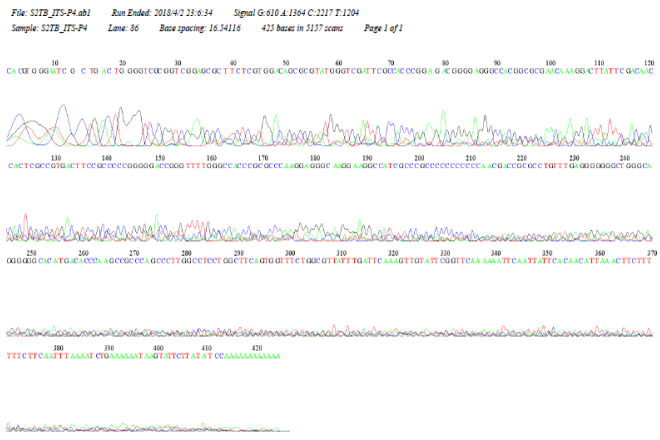

Figure.8. Sequencing result of commercial herb *N.jatamansi* using the plant-specific ITS primers.

The commercial available medicinal herb, *Nardostachys jatamansi*was identifiedusing the ITS - P3 and P4 primer and assembled sequence was submitted in the GenBank with the accession number, MH368047. It is a partial sequence of 5.8S ribosomal RNA gene and internal transcribed spacer 2 region with length of 368bp (Figure 9 & 10). This ITS - p3 and p4 primer pair had barcoded the exact taxon of the commercial herbals (*Nardostachys jatamansi)* available in the market.

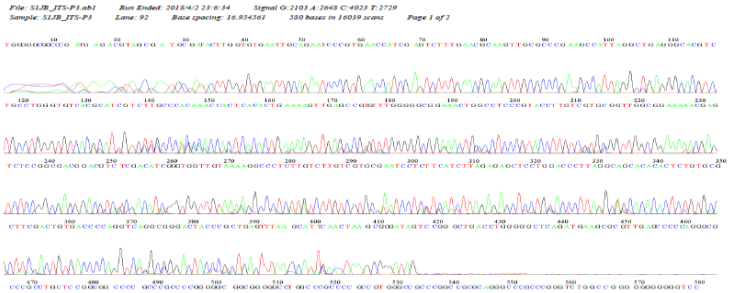

Figure.9. Sequencing result of commercial herb *Nardostachys jatamansi* using universal ITS-p3 primer

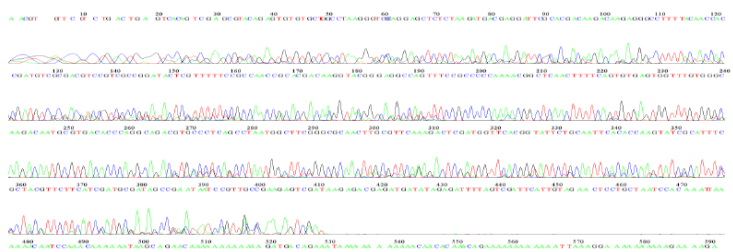

Figure.10.Sequencing result of commercial herb *N.jatamansi* using the plant-specific ITS-p4 primers

Consequently, the second important herb *Selinum vaginatum* also identified as the original sample from the market using the barcode technique with ITS - P3 and P4 primer through PCR amplification. It is a partial sequence of 5.8S ribosomal RNA gene and internal transcribed spacer 2 regions with the length of 203bp (Figure 5a & b). The respective assembled sequence was submitted to the GenBank with the accession number, MH368050. Both the primer combinations amplified for the specific and taxon of the commercial herbals, *Selinum vaginatum.*

C. GenBank Submission

The five DNA sequences isolated from the roots of two herbal plants and amplified using the two different set of ITS primers such as U1-U4 and P3-P4. Out of these two primer pairs, the ITS-p3 and p4 amplified the particular herbal plants, *N.jatamansi* and *S.vaginatum* and authenticated their originality among the commercial herbals. The assembled sequences of *N.jatamansi* and *S.vaginatum* were submitted to GenBank and assigned with accession numbers, MH368047 and MH368050, respectively. Two fungal sequences were amplified with ITS-P4 and P5 primers designed for whole ITS region including ITS1 and ITS2 nucleolar regions. These amplified DNA sequences were assembled and submitted in the GenBank and published with the databases. The source of the first and second fungal sequences were *S.vaginatum* and *V.wallachi* the amplified sequence was identified as the partial ITS2 region of *Trichoderma asperelloides* isolate SB3AL and S2T, which assigned with accession numbers, MH371282 and MH371292. Similarly, the source of the second fungal sequence was *N.jatamansi*and the amplified sequence was identified as the partial ITS2 region

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of*Aspergillus flavus* isolate SJAL, which assigned with accession number MH371291.

D. Phylogenetic analysis

TheITS-P3 and P4 primers were used for the amplification of ITS2 region in the three herbal samples for DNA barcoding purpose. The first sequence *Nardostachys jatamansi* submitted to the NCBI databases (MH38047) was nearly 97% homology *with Chaerophyllopsis huai, Ligusticopsis brachyloba*related DNA sequences among the Genbank submission (Figure 7). The second sequence was found to be highly related to Valerian family and about 100% homology with *Selnium vaginatum*. The same sequence was found 96% homology with *Valeriana lepidota, Valeriana urticifolia* and 94% homology with*Valeriana jatamansi* species in the blast tree (Figure 11).

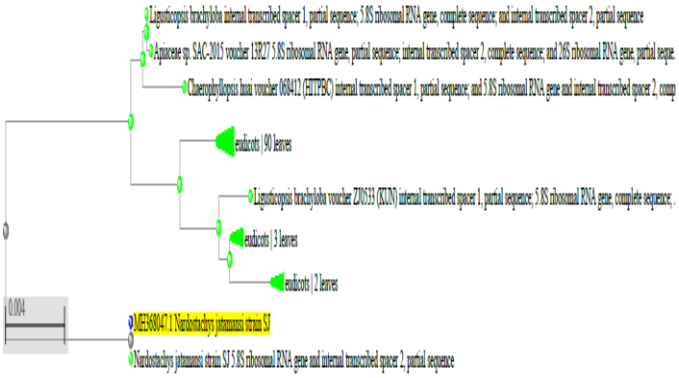

Figure.11. Phylogenetic relationship among the *Nardostachys jatamansi* and related species in the NCBI database using the ITS primers.

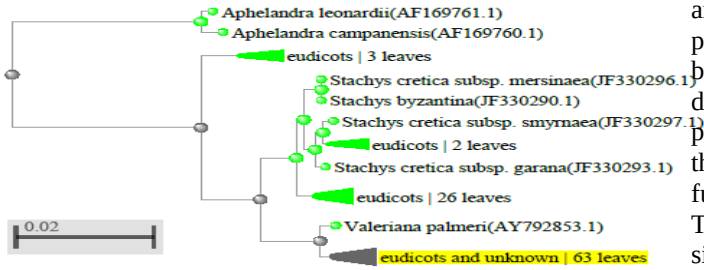

Figure.12. Phylogenetic relationship among the *Selenium vaginatum* and related species in the NCBI database using the ITS primers.

E. Discussion

Most of the plants are used as herbal medicines in traditional methods of treatments and they are available in the market as crude drugs. The quality of these crude herbals drugs is tested or verified by a set of rules and methods utilizing botanical, physicochemical and chemical analyses established by pharmacopoeias and experts of official compendia. However those methods are not absolutely reliable for exact species identification and former studies have revealed species substitutions. DNA-based molecular methods including the use of specific DNA sequences as markersfor the species identification are widely used now-a-days in various fields such as agriculture and food industry. There is a wide range of DNA-based methods available in the market such as RAPF, AFLP, PCR-DGGE, real-time PCR and sequencing-based systems, such as SSR. Choosing the correct methods among these techniques depends upon various factors including the emphasis of the study. Although several methods are available in hands, it may hinder the research and reduce the reproducibility of the study. Among these molecular methods, DNA barcoding has an aim to catalogue universal markers for all species in the world and fused the DNA-based procedures utilized for the species identification.

The DNA barcoding technology use the universal set of primers, databases and standardized methods help to file the species by researchers and groups all around the world with increasing the level of reliability among the number of species available for the study. Universal priming may not be possible due to the small disturbances in the different families, orders and species that are responsible for diverse levels of amplification. While in some cases, the usage of different primers might be the best ideology to keep way on the amplification and efficient sequencing. Processed samples such as root specimens used in this study are often meticulous, since the extraction of high-quality DNA may be difficult to achieve the purpose of the work. Similarly, in this study, the sample materials were root specimens and extraction of DNA from the root samples found very difficult and laborious. We obtained the medium-quality of DNA as a smear in the agarose gel electrophoresis.

In this study, we succeeded with good-quality PCR amplification and efficiency with the primers of ITS2 region to improve the accuracy rate of species identification in barcoding technology. Previous studies failed in the attempt to use ITS region as an accurate marker for the species identification. When compared to previous studies, ITS-P3 and P4 primers worked well for locating the ITS region in the plant-specific markers among DNA barcodes. There might be a wide range of species identification nuances among the different set of primers used for the barcoding technique. The presence of a fungus in the plant root samples might be due to the association of fungal pathogens or symbiotic nature of fungus in the root structures of commercial herbs.

The species discriminating power of ITS region was significantly increased when it was integrated with a single or combination of plastid markers such as *rbcL* and *matK*. The combination of *rbcL*+ ITS region was suggested to exhibit the maximum species identification and discriminatory power of all combinations for establishing potential DNA barcode for temperate woody bamboos. When compared to the other spacer from the chloroplast genome, ITS region was recommended as a possible and potential barcode that provided 100% species identification and discrimination in Dendrobium species. It remained as the best while considering for percent species resolution capabilities.

The ITS markers found to be the most efficient among the single-locus barcodes for the species discrimination of the genus by 52.27% in Venus slippers, whereas the combination of *matK* + *atpF-atpH* + ITS as a barcode in orchid species. The combination of ITS and rbcL loci offer a basic pipeline against the comparison of other genes and intergenic spacers which used in the system of plant DNA barcoding. Kress et al believed and recommended that the ITS and trnH-psbA could serve as better starting points for mass testing of DNA

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barcoding genes across a wide range of angiosperms. The same combination of DNA barcode loci has estimated the identification and discriminatory power over 8,000 flowering plants of Costa Rica.

When compared to all ribosomal DNA loci, ITS region is the most widely used and sequenced locus in the plant phylogeny studies. The overall advantage in using the ITS region, which can be broken and amplified into two smaller fragments (ITS 1 and ITS 2) with joining 5.8S locus. This 5.8S region might be quite conserved and possess enough phylogenetic signals for species-level discrimination and phyla. The ITS region is an authoritative phylogenetic marker at the taxon and species level revealed high levels of inter specific variations. The ITS region has the highest discriminating power than the plastid loci at the lower taxonomic levels, which was widely studied and can be used as the universal barcode.

There could be few limitations preventing it from being core barcode marker. Most of the times, fungal ITS sequences can be amplified and confused with plant sequences. Other than its limitations, the ITS region could be accepted as the universal barcode for a broader range of plant taxa identification. Mostly no sequences were found for 35 samples of species, *Valeriana officinalis*, where DNA barcoding protocol and efficiency of the genetic markers didn't work well in comparison with the identification of other species. The same issue was found in this study, where the ITS primers didn't amplify the exact species, *Valeriana wallachi.* The fungal sequences were found in the place of plant species, which might be due to the inefficiency of ITS primer sequences or else the limitation of the DNA barcoding technique. This study supported the results and facts of behind the ITS markers and barcoding procedure followed in the previous studies for *Valeriana officinalis.*

The combination of *rbcL* and ITS gene recovered DNA barcodes from most of the herbal products and identified the contaminated and substituted herbs. Thus the poor quality of the commercial herbs could be detected using the DNA barcoding technology for authenticating the herbal products through the raw material testing used in the product manufacturing. This method of authenticating commercial herbals would provide safe and high quality of products to the consumers.

IV. CONCLUSION

The present research study revealed another supportive evidence for the identification and authentication report of three commercial medicinal herbals from the market using the ITS region (biotechnological tools) that worked well for the purpose of species discrimination. These primers can be widely used as universal primer pairs for the species identification and help the consumer to stay safe and promising them for the authenticated herbal products from the market.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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