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A STUDY ON DNA BARCODING OF FEW ANTI-ARTHRITIC PLANTS PRESENT IN AND AROUND DAKSHINA KANNADA, KARNATAKA.

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Abstract:

Plants being important source of medicine used time immemorial to cure various arthritic problems. Also the efficacy of drugs elucidated from plant depends on proper identification of plants at molecular level. This behaves as reliable method when compared to morphological characteristics alone and avoids usage of incorrect plant leading to poisoning, side effects etc. DNA barcoding methods have been shown to be an effective tool for species authentication targeting specific genes from genomic DNA which is widely used in molecular plant taxonomy. Thus, the goal of our study was to perform field survey to collect anti-arthritic plants present in and around Dakshina Kannada, Karnataka and subject them for DNA barcoding technique to authenticate those medicinal plants. DNA samples were extracted from shortlisted 6 plants such as *Cardiospermum helicacabum, Kirganelia reticulata, Pongamia pinnata, Scoparia dulcis, Urena lobata* and *Vitex negundo*, chosen based on their therapeutic efficacy and usage in traditional medicine. MatK, ITS2, Rbcl and PetB genes were selected to be best molecular markers for identification purposes. The mentioned genes were amplified using PCR and subjected to Sanger sequencing. The sequences were screened in NCBI BLAST for species identification and phylogenetice analysis was studies using Clustal Omega. Our findings authenticated the plant species as well as to determine their closely related species. Hence, DNA barcoding technique acted as a potential and simple method to authenticate selected plants which had ethnobotanical claim to treat arthritis.

Keywords: arthritis, genomic DNA, PCR, Sanger sequencing, NCBI, phylogeny

Introduction:

Plants are utilized as source of medicine from ancient times by many geographical cultures and many publications are available highlighting usage of plants to cure various diseases (Chao, 2010; Lin et al., 2008; Oh et al., 2011). Presently, herbal medicines have gained great popularity because of its demand, availability, cost effective and have fewer side effects. On an average, among the commercially available drugs in pharmaceutical industry one-third of them are either natural products or their derivatives. The conventional approach employed towards discovery of plant based drugs often involves large amount of time and expenditure. Also the traditional approaches available for identification of herbal product includes organoleptic methods like identification by sense such as taste, sight, smell, touch; macroscopic and microscopic methods including shape, colour and texture wherein professional taxonomists are needed (Techen et al., 2014). In addition, even the specialist may not be able to identify the specimen if it is damaged or at immature stage of development. And another identification method followed usually is by using chemical profiling wherein plant material may gets affected by physiological and storage conditions. To overcome all these, there lies a necessity of new approach to identify plant species in an efficient and accurate manner. Therefore, authentication using DNA barcoding helps in overcoming these problems and acts as a rapid and accurate identification method of plant species based on extracting genomic DNA from tiny sample of any organism. This allows taxonomists to recognize species quickly and accurately by retrieving information about them. Moreover, it allows non-experts to identify species from damaged or tiny material. There are several potential barcoding candidates reported so far such as matK, rbcL, psbK-psbI, trnH-psbA, ITS etc. (Luo et al., 2010; Pang et al., 2010).

In our current study we have done field survey in and around Dakshina Kannada, Karnataka for traditional usage of medicinal plants for treating arthritis. Based on the availability and effective treatment strategy employed by people, we have selected 6 plants such as *Cardiospermum helicacabum, Kirganelia reticulata, Pongamia pinnata, Scoparia dulcis, Urena lobata* and *Vitex negundo. Cardiospermum halicacabum* is a herb belonging to family Sapindaceae extensively found dispersed in tropical and subtropical areas of world, growing in plains of Africa, America, Bangladesh, India and Pakistan. The roots, leaves and seeds of this herb are employed as herbal medication due to the presence of triterpenoids, flavones, aglycones, glycosides along with a variety of fatty acids and volatile esters. Secondary metabolites present in this plant include alkaloids, proteins, carbohydrates, lignin, saponins, steroids, cardiac glycosides etc and phytochemicals such as β-arachidic acid, apigenin-7-O-glucuronide, apigenin, chrysoeriol-7-Oglucuronide, luteolin-7-O-glucuronide, beta-sitosterol and beta-D-glycoside are investigated (Syed et al., 2013). *Kirganelia reticulata* (Poir) belongs to family Euphorbiaceae which is a medicinal herb used widely in indigenous system of medicine. It is adored in ancient ayurvedic texts for its extraordinary medicinal properties and act as diuretic, astringent used in treating diarrhoea, small pox etc. Wide range of chemical compound like flavanoids, alkaloids, triterpenoids, glycosides, lignans, steroids,

coumerins, phenols are isolated from this species. The extracts and compounds isolated have shown a wide spectrum of biological properties including antibacterial, antidiabetic, antioxidant, antiplasmodial, hepatoprotective, analgesic, anti-inflammatory etc (Soni et al., 2013).

Pongamia pinnata belongs to family Fabacea being one of the many plants possessing diverse medicinal properties wherein all its parts are used in treating and preventing several kinds of ailments such as piles, skin diseases, wound healing etc. Phytoconstituents like different classes of flavonoid derivatives, flavones, flavans, chalcones, and compounds such as terpenes, fatty acids, steroids have been isolated from this plant. The pharmacological investigations revealed that various types of formulations, extracts, and individual compounds have exhibited broad spectrum biological activities such as antimicrobial, antioxidant, anti-inflammatory and anti-diabetic properties (Al Muqarrabuna et al., 2013). *Scoparia dulcis* belongs to Scrophulariaceae, commonly called as sweet broom weed found distributed throughout tropical and subtropical region around the world. This plant is used traditionally as remedy for treating diseases like kidney stones, stomach ailments, hypertension, inflammation, diabetes, haemorrhoids, bronchitis and urinary disorders. Studies have revealed the presence of various phytochemicals like terpenoids, steroids, flavonoids, glycosides, chemical constituents such as scoparic acid A-C, scopadulcic acid A and B, scopadulciol, scopadulin, ammelin etc. Aspects of several speculated pharmacological properties have been validated by scientific research including the presence of antimicrobial, antidiabetic, antioxidant, hypoglycaemic, antitumour promoting and antihyperlipidemic actions (Meera et al., 2017). *Urena lobata* is commonly known as Caesar weeds belonging to family Malvaceae. In India, it is widely seen in southern and some parts of northern regions, where in traditional medicine plant is used as febrifuge, for rheumatism, wound healing and an as antiseptic. Its stem, leaves, roots and bark have beneficiary activities such as inflammatory, antioxidant, antimicrobial, antidiabetic, antihyperlipidemic etc. (Babu et al., 2016). *Vitex negundo* belongs to Verbenaceae, its a hardy plant flourishing mainly in parts of Indian subcontinent. All parts of plant possess a multitude of phytochemicals and secondary metabolites which impart great variety of medicinal uses in traditional and folk medicine and few of them have been experimentally tested. This plant is component of many commercially available herbal formulations exibiting to be potent bio-control agent. Phytochemical analysis has shown bioactive secondary metabolites occurring in the plant like flavonoids, terpenoids, lignans, steroids and promising bioactivities such as antimicrobial, antiinflammatory, anti-nociceptive, anti-oxidant, anti-tumor, insecticidal, anti-osteoporotic, anti-androgenic, anti-cataract, anti-hyperglycemic and hepatoprotective activities (Vishwanathan and Basavaraju, 2010). In the present study, we have isolated genomic DNA from above plants and DNA barcoded them using nuclear, mitochondrial, chloroplast genes to authenticate the plant species.

Materials and methods

Collection of Plant material:

The fresh and not so tender leaves of selected medicinal plants *Cardiospermum helicacabum, Kirganelia reticulata, Pongamia pinnata, Scoparia dulcis, Urena lobata* and *Vitex negundo* were collected in and around Dakshina Kannada, Karnataka by discussing with people who use it in traditional medicine for treating arthritis. The leaves were washed with sterile water to remove the dirt and further processed for molecular studies.

Genomic DNA preparation:

Genomic DNA was extracted from fresh leaves using modified CTAB method (Cota et al., 2006). Approximately 200mg of sample was ground with 1ml of preheated CTAB buffer using pestle and mortar incubating in water bath at 60^0c for 30 mins. The content was allowed to cool down at room temperature and an equal volume of chloroform: isoamyl alcohol (24:1 ratio) was added and mixed gently. The mixture was centrifuged at 10,000 rpm for 10 min in a refrigerated centrifuge, the pellet was discarded and the supernatant was transferred to another fresh microtube to which equal volume of ice-cold isopropanol was added. This mixture was also centrifuged at 10,000 rpm for 10 min in a refrigerated centrifuge. Supernatant was discarded and 500µl of 70% ethanol was added to the pellet, followed by a quick spin at 10,000 rpm for 10 min in a refrigerated centrifuge. The supernatant was discarded and pellet was retained, which was allowed to dry for about 45 min, and then dissolved in 10µl TE buffer. This DNA was treated with RNAse and column purified to get rid of impurities. The isolated plant DNA was later subjected to electrophoresis for quantification and compared with standard1kb ladder.

PCR Amplification:

PCR amplification of the isolated plant DNA was done using primers: ITS2, matK, petB and rbcL. Amplification reaction was performed in 25µl reaction mixture, by adding 40ng DNA, 10pM of each primer, 10pM dNTPs, 2X buffer and 0.5µl Taq DNA polymerases. Gene amplification was performed using thermal cycler program of 35 cycles: initial denaturation step and final denaturation at 94^oc followed by annealing at 60^oc and extension at 72^oC. The quality of PCR products was checked on 1 % agarose gel electrophoresis stained with ethidium bromide.

Primer	Sequence	Annealing Temperature
Matk F	CCCRTYCATCTGGAAATCTTGGTT C	54^0C
MatkR	GCTRTRATAATGAGAAAGATTTCTGC	59^0C
ITS2-F	ATGCGATACTTGGTGTGAAT	53^0C
ITS2-R	GACGCTTCTCCAGACTACAAT	51^0C
Rbcl F	ATGTC CCACAAACAGAAAC	50^0C
Rbcl R	TCGCATGT CCTGCAGTAGC	56^0C
psbH–petB F	AACTACTCCTTTGATGGG	53^0C
psbH–petB R	CCTTTGATGGGAACTAC	55^0C

Table 1: Showing primers sequences used for gene amplification.

Sanger Sequencing:

Bidirectional sequencing of PCR products was carried in Sequencer- Applied Biosystems (Hitachi) 3130x1 Genetic Analyzer and processed in Finch TV. The electropherogram files in .AB1 format were converted to .pdf and fasta files using Sequence Scanner Software 2. The sequence data generated during this study were subjected to BLAST in nucleotide database GenBank (http://blast.ncbi.nlm.nih.gov/) to determine their most probably closely related taxa.

Construction of phylogenetic tree:

Phylogenetic inference requires computational approaches implementing the optimality criteria and methods of parsimony, and maximum likelihood (ML) based on Bayesian inference. All these depend upon an implicit or explicit model describing the evolution of characters observed. All the sequences obtained were aligned using Clustal omega to understand the relationship of unknown sequences with other related species.

Results and discussion:

DNA was isolated by CTAB method and the quality and quantity of the DNA was checked in gel electrophoresis as shown in **figure 1**. Using the genomic DNA PCR was performed using ITS2, MatK, PetB and RbCl primers and obtained PCR product was gel purified to get rid of salt contamination and other impurities as shown in **figure 2**. Sequencing files obtained were in .AB1 format which were viewed using software FinchTV as electropherogram peaks of individual nucleotide in its position. Quality of the obtained sequence can be observed through Electropherogram peaks as seen in **figure 3** and **figure 4**. FASTA sequences for further analysis were obtained by converting .AB1 file in Seq Scanner 2.0. Using BLAST server unknown sequences were identified based on query coverage, percentage identity and e-value. **Table 2** depicts the percentage similarity between the predicted species as per BLAST analysis. Further phylogenetic analysis was done to find out the evolutionary relationship of our query sequence. The Phylogenetic tree obtained for *Cardiospermum helicacabum* sequences from all four genes are shown

in **figure 5**. Similarly, the Phylogenetic trees obtained using Clustal Omega for *Kirganelia reticulata, Pongamia pinnata, Scoparia dulcis, Urena lobata* and *Vitex negundo* are as shown in **figure 6, figure 7, figure 8, figure 9 and figure 10**.

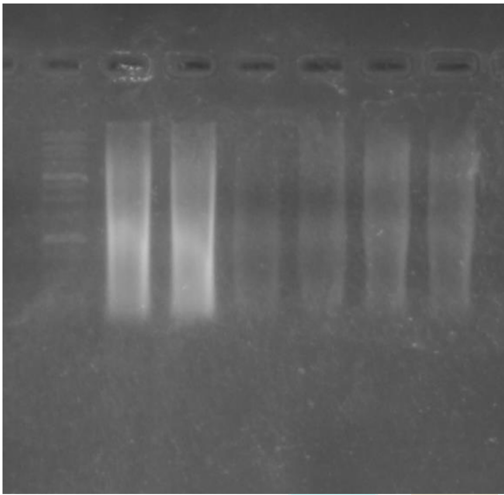

Figure 1: Genomic DNA of 6 plants compared with 1kb ladder.

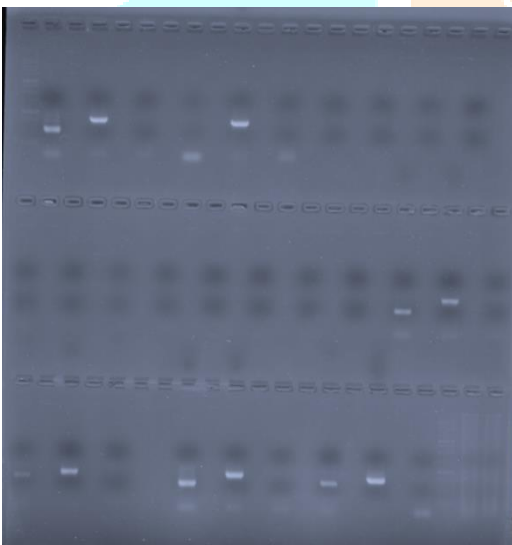

Figure 2: Amplified ITS2, MAtk, RbCl, PetB genes (~700bp) from plant DNA and compared with 100bp ladder

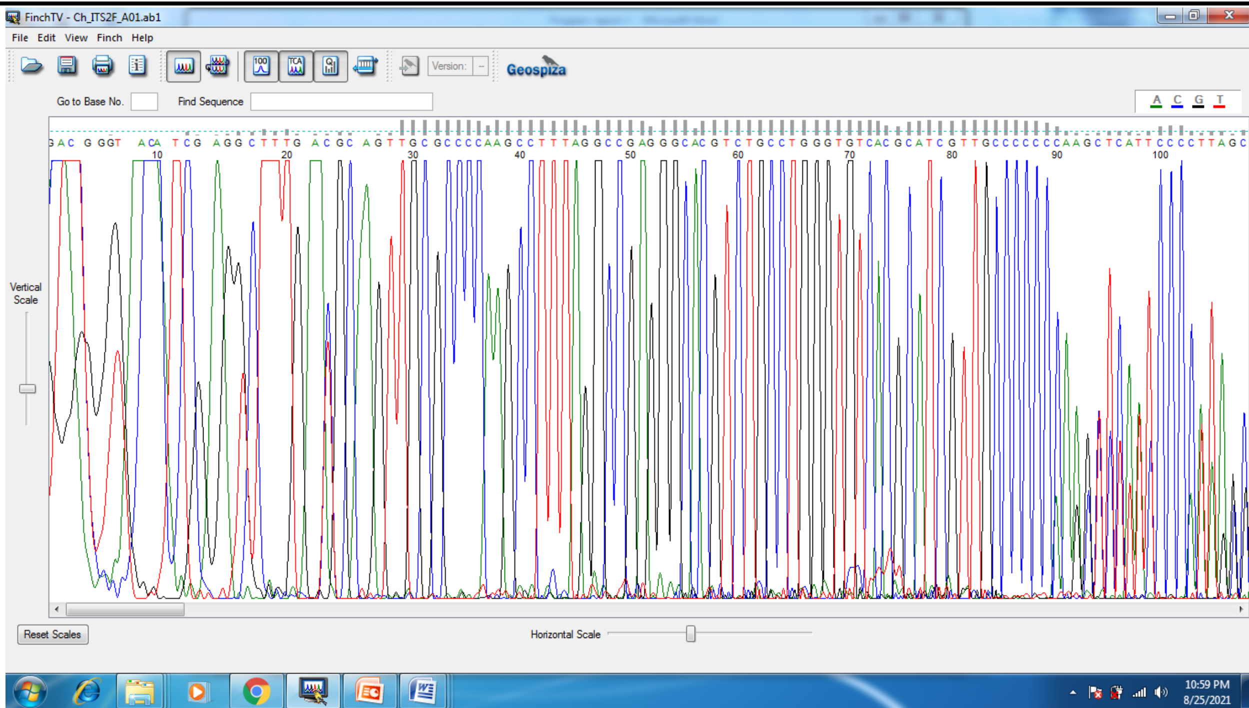

Figure 3: Representative Electropherogram peaks showing the nucleotide bases obtained from Sanger sequencing.

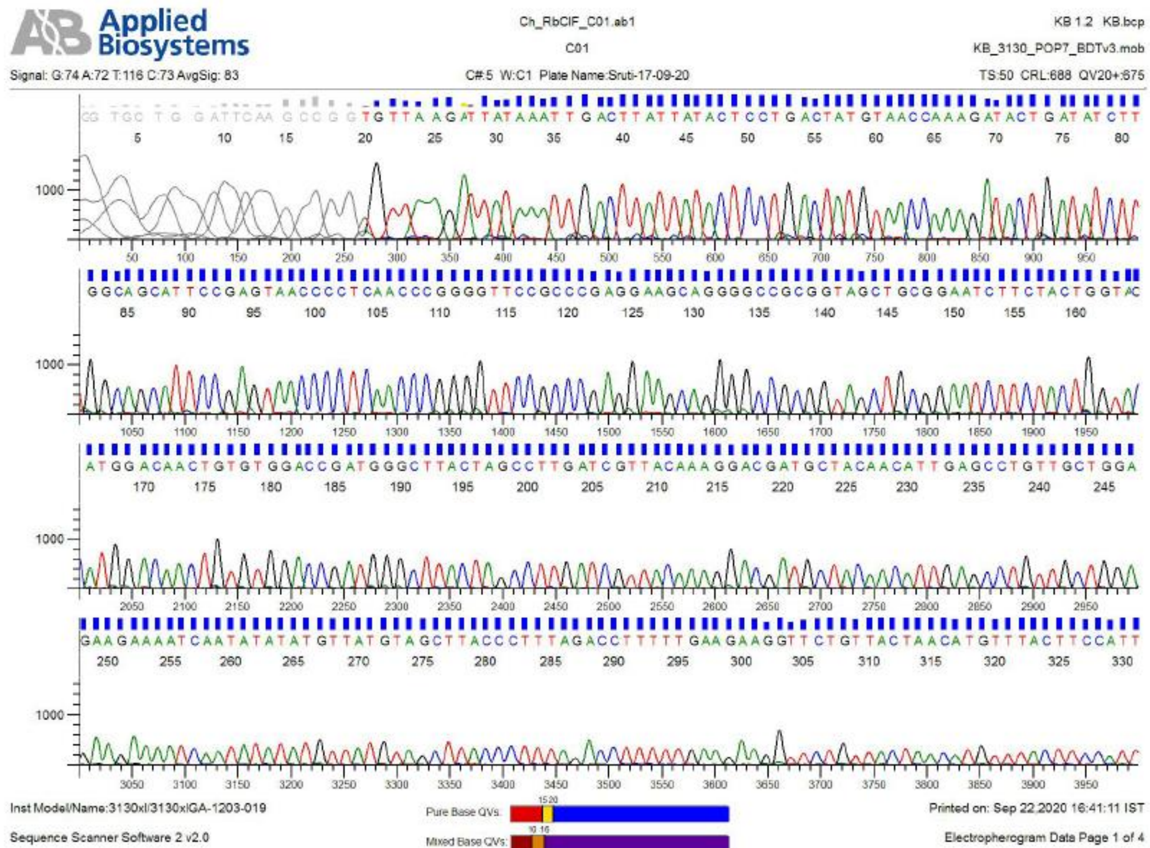

Figure 4: Representative pdf file showing overall view of the sequence as obtained from genetic analyzer.

Sample name	Description	Query coverage (%)	E Value	% Identity	Accession No.	Gene
1	*Cardiospermum halicacabum* 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S	62	3e-76	95.40	KJ845654.1	ITS2
1	*Cardiospermum halicacabum* voucher QRI 549 ribulose-1,5-biphosphate carboxylase/oxygenase large subunit	98	0.0	98.36	MK348962.1	RBCL2
1	*Cardiospermum halicacabum* voucher VCW25 ribulose-1,5-bisphosphate carboxylase/oxygenase large	98	0.0	98.36	MN989853.1	PETB
1	*Cardiospermum microcarpum* voucher Trotta950526 maturase K (matK) gene, partial cds; chloroplast	98	0.0	99.02	MH621643.1	MATK
2	*Phyllanthus reticulatus* voucher 1035 5.8S ribosomal RNA gene, partial sequence; internal transcribed spacer	92	0.0	99.98	MG731035.1	ITS2
2	*Phyllanthus reticulatus* isolate SCBGP039_1 ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit	99	0.0	97.47	KP094227.1	RBCL2
2	*Phyllanthus reticulatus* 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	99	0.0	97.08	AY765290.1	PETB
2	*Phyllanthus reticulatus* voucher OM224 maturase K (matK) gene, partial cds	98	0.0	95.08	JF270893.1	MATK
3	*Pongamia pinnata* var. pinnata isolate G06484 small subunit ribosomal RNA gene, partial sequence; internal transcribed	66	4e-150	99.34	MN076231.1	ITS2
3	*Pongamia pinnata* small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence	91	0.0	98.33	KF848293.1	RBCL2
3	*Pongamia pinnata* var. minor isolate G08002 maturase K (matK) gene, partial cds; chloroplast	96	0.0	96.96	MN076302.1	PETB
3	*Pongamia pinnata* var. minor isolate G06456 maturase K (matK) gene, partial cds; chloroplast	99	3e-157	95.26	MN076304.1	MATK
4	*Scoparia dulcis* SD01 genes for 18S rRNA, ITS1, 5.8S rRNA, ITS2, 26S rRNA, partial and complete sequence	76	4e-175	96.57	LC376943.1	ITS2
4	*Scoparia dulcis* ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (rbcL) gene, partial cds; chloroplast	99	0.0	94.03	LC376946.1	RBCL2
4	*Scoparia dulcis* SdTPS18 mRNA for putative delta-cadinene synthase, complete	98	0.0	96.97	LC505627.1	PETB

	cds					
4	*Scoparia dulcis* SD01 chloroplast gene for maturase K, complete cds	98	0.0	96.49	LC376944.1	MAT K
5	*Urena lobata* isolate URLO_G1_P.B_PM_2of3 5.8S ribosomal RNA gene, partial sequence; internal transcribed region	95	0.0	98.66	KY700529.1	ITS2
5	*Urena lobata* voucher Trotta950138 ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit	96	0.0	97.42	MH550034.1	RBCL 2
5	*Urena lobata* voucher Trotta950138 psbA-trnH intergenic spacer, partial sequence; chloroplast	93	3e-135	94.80	MH622137.1	PETB
5	*Urena lobata* isolate OSBAR 000938 maturase K (matK) gene, partial cds; chloroplast	92	0.0	95.76	MH552391.1	MAT K
6	*Vitex negundo* genomic DNA sequence contains ITS1, 5.8S rRNA gene, ITS2, specimen voucher USTH:012824	99	0.0	95.58	LT219443.1	ITS2
6	*Vitex negundo* chloroplast, complete genome	98	0.0	93.42	MW366787.1	RBCL 2
6	*Vitex negundo* haplotype 2 tRNA-Gly (trnG) gene, intron; chloroplast	99	0.0	95.99	DQ304785.1	PETB
6	*Vitex negundo* var. incisa isolate AD3HJ30 maturase K (matK) gene, partial cds; chloroplast	98	0.0	95.16	MF350269.1	MAT K

Table 2: Showing the predicted hits from individual gene sequences from plant species using NCBI BLAST

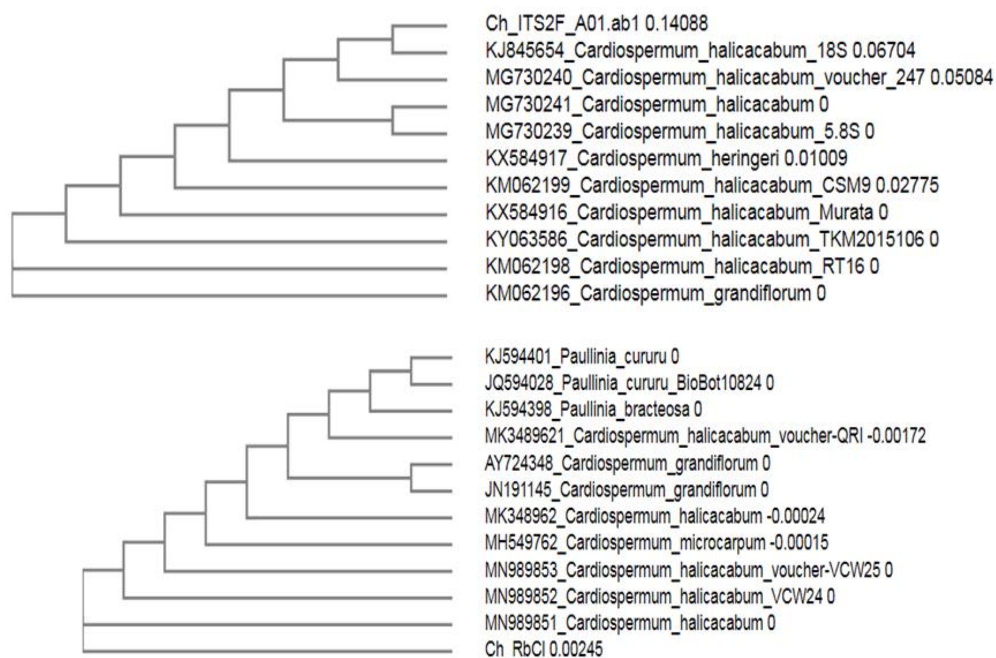

Figure 5: Phylogenetic tree showing the evolutionary relationship of plant Cardiospermum halicacabum.

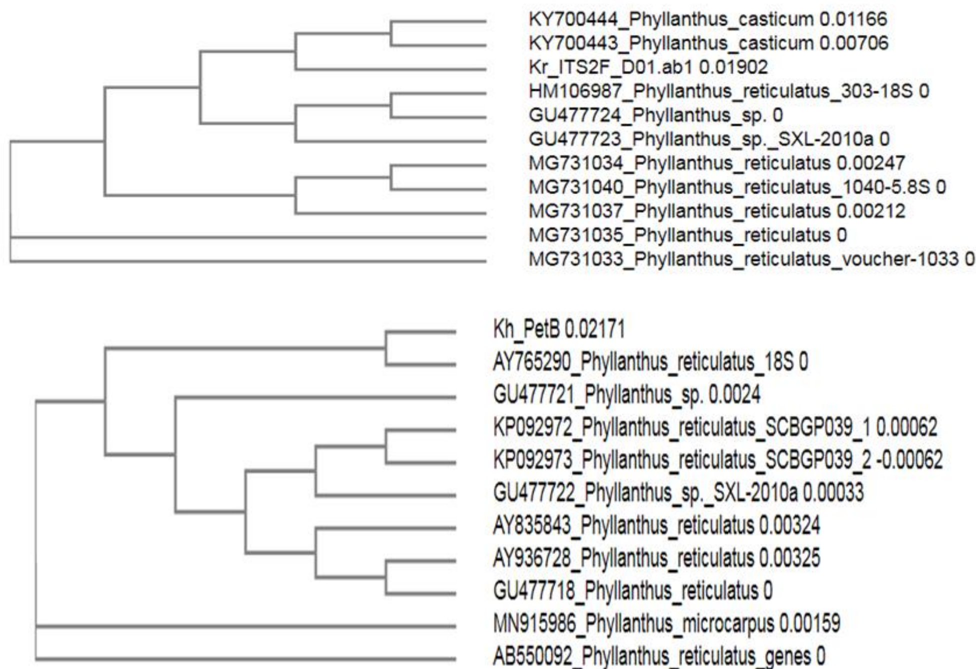

Figure 6: Phylogenetic tree showing the evolutionary relationship of plant *Kirganelia reticulata.*

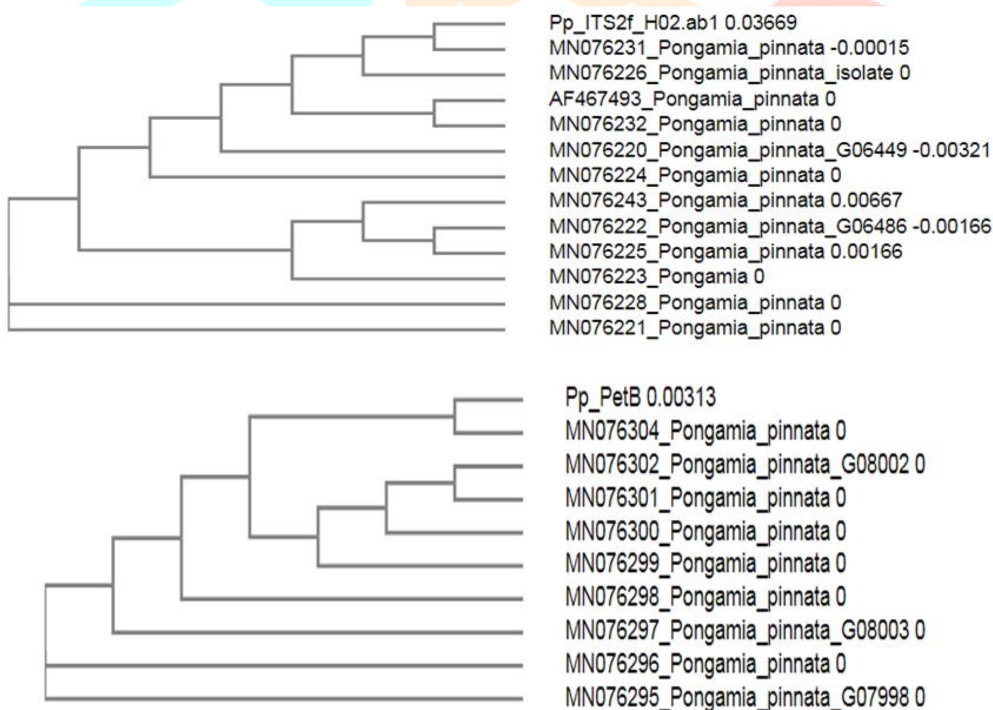

Figure 7: Phylogenetic tree showing the evolutionary relationship of plant *Pongamia pinnata.*

Figure 8: Phylogenetic tree showing the evolutionary relationship of plant *Scoparia dulcis*.

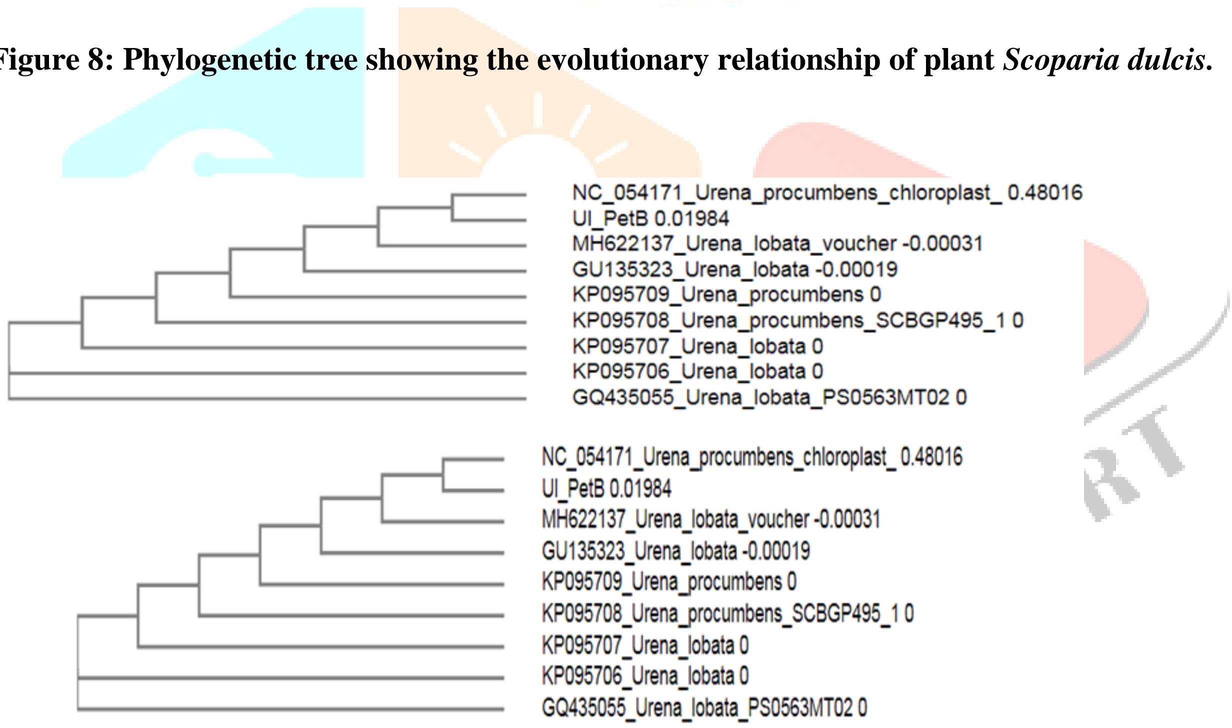

Figure 9: Phylogenetic tree showing the evolutionary relationship of plant *Urena lobata*.

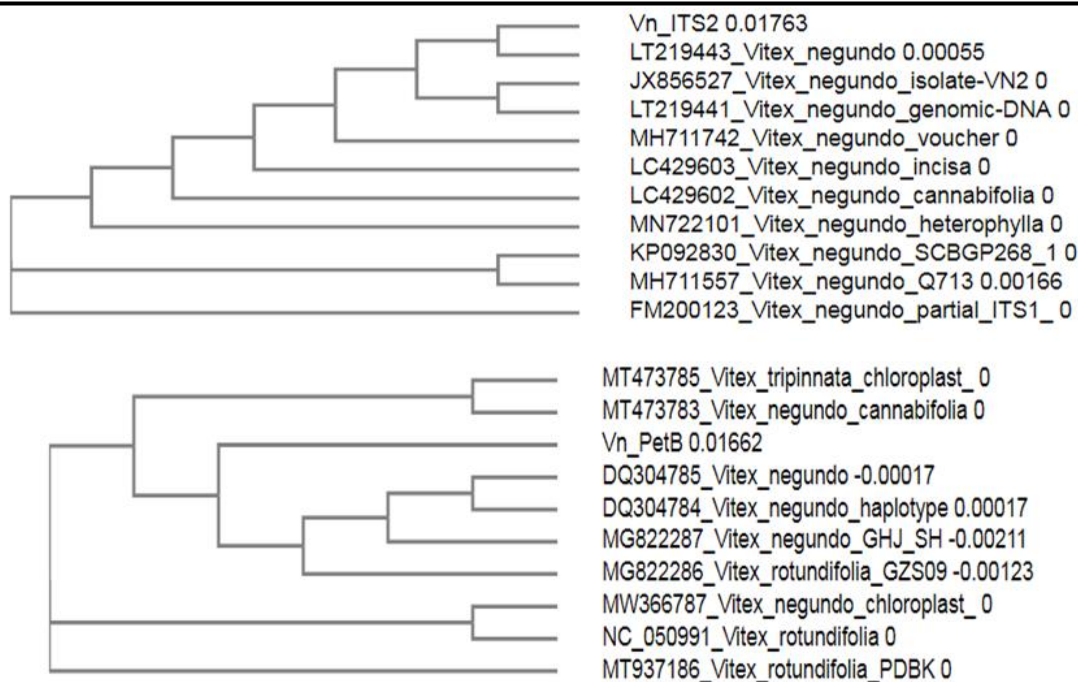

Figure 10: Phylogenetic tree showing the evolutionary relationship of plant *Vitex negundo*.

DNA barcoding is applicable in the field of taxonomy and has become very popular in past few years (Luo et al., 2010). This technique is useful tool for taxonomists because of its rapid and accurate method of species identification and acts as an important parameter to ensure that the products made from medicinal herbs are pure and safe. Also, there is a need to explain this rapid and simple DNA barcoding identification system to all the biologists to avoid the burden on taxonomists (Chase et al., 2005). Currently the candidate barcodes available are ITS2 of nuclear ribosomal DNA, as well as matK, psbA-trnH, ycf5, rbcL, rpoC1, petB etc in the chloroplast. The previous observations has shown the potential of ITS2 being suitable marker for taxonomic classification when compared to other markers. This ability of conventional amplification using universal primer pairs and generation of unambiguous bidirectional DNA sequences with minimal editing acts as major criteria of selecting an ideal DNA barcode. ITS2 has good primer sites in the gene as its region is short and easy to amplify and sequence them (Kress et al., 2005). Results obtained from our study demonstrated that the primers used provide promising universal DNA barcode to recognize 6 selected medicinal plants by providing high percentage of maximum identity for each sample. However, few plants have showed less percentage of maximum identity around 95% and below than 95% was rejected because of chances of ambiguous species identification (Kool et al., 2012). Here, maximum identity indicates measure of likeliness of identified species and every successful species identification will be influenced by factors like quality of DNA, a suitable primer used and the sequence quality (Mahadani and Ghosh, 2013). In most of the cases the successful rates of DNA sequencing can be relatively low due to the presence of polymorphic DNA in multiple copies (Alvarez and Wendel, 2003) and also may be because of contamination by fungal or other parasitic species (Hollingsworth et al., 2011, Che et al., 2018).

Thus, DNA barcoding has been a standardized technique to identify and differentiate between species in recent years (Dhananjaiah et al., 2020). In addition, the phylogenetic analysis highlighted the diversity between our plants and other known species, which helped us to understand the evolutionary relationship of our plant species.

Conclusion:

DNA barcoding acts as a reliable tool for authentication of the selected medicinal plants with the minimal help of core taxonomists. Our present study was to authenticate the anti-arthritic plants at molecular level using DNA barcoding technique. It is highly satisfactory to get species level identity with maximum percentage and identify plants as *Cardiospermum helicacabum, Kirganelia reticulata, Pongamia pinnata, Scoparia dulcis, Urena lobata* and *Vitex negundo.* Therefore, our findings provided strong base for usage of proper plant species for traditional medicine, and help misguiding regional people in collecting suitable species. The usage of many potential markers like ITS2, matK, petB and rbcL can be implemented for studying and identifying medicinal plants. Further studies towards proving these plants to be anti-arthritic through scientific methods are in the process.

References:

1. Chao WW, Lin BF, 2010, Chinese Medicine 5(1).
2. Lin SY, Wang CC, Lu YL, Wu WC, Hou WC, 2008, Food and Chemical Toxicology, 46(7), 2485-2492.
3. Oh MJ, Hamid MA, Ngadiran S, Seo YK, Sarmidi MR, Park CS, 2011, Archives of dermatological research, 303(3), 161-170.
4. Techen N, Parveen I, Pan Z, Khan IA, 2014, Current Opinion in Biotechnology, 25, 103-110.
5. Luo K, Chen S, Chen K, Song J, Yao H, Ma X, Li X, 2010, Science China Life Sciences, 53(6), 701-708.
6. Pang X, Song J, Zhu Y, Xie C, Chen S, 2010, Planta medica, 76(15), 1784-1786.
7. Syed Atif Raza, Shahzad Hussain, Humayun Riaz and Sidra Mahmood, 2013, Review of beneficial and remedial aspects of Cardiospermum halicacabum L, African Journal of Pharmacy and Pharmacology 7(48):3026-3033.
8. Soni, Rajesh and Dixit, Vihangesh and Chandra, Akanksha and Irchhaiya, Raghuveer and Singh, Nandlal and Singh, Harsh. (2013). KIRGANELIA RETICULATA (POIR) BAILL.-A REVIEW ON ITS BOTANY, ETHNOBOTANY, PHYTOCHEMISTRY AND PHARMACOLOGY. Journal of Drug Delivery and Therapeutics. 3. 10.22270/jddt.v3i6.688.

9. Al Muqarrabuna LMR, Ahmata N, Ruzainaa SAS, Ismaila Sahidinb NH, 2013, Medicinal uses, phytochemistry and pharmacology of Pongamia pinnata (L.) Pierre: A review, Journal of Ethnopharmacology 150(2).
10. Meera Paul, Kavitha Vasudevan, Krishnaja KR, 2017, Scoparia Dulcis: A review on its phytochemical and pharmacological profile, Innoriginal International Journal of Sciences 4(4) :17-21.
11. Babu SS, Madhuri DB, Ali SL, 2016, A pharmacological review of Urena lobata plant, Asian Journal of Pharmaceutical and Clinical Research, 9(2) pp. 20-22.
12. Vishwanathan AS, Basavaraju R, 2010, A Review on Vitex negundo L. – A Medicinally Important Plant, EJBS 3 (1): 30-42.
13. Cota-Sánchez JH, Remarchuk K, Ubayasena K, 2006, Plant Molecular Biology Reporter, 24(2), 161-167.
14. Luo K, Chen S, Chen K, Song J, Yao H, Ma X, Li X, 2010, Science China Life Sciences, 53(6), 701-708.
15. Chase MW, Salamin N, Wilkinson M, Dunwell JM, Kesanakurthi RP, 2005, Philosophil Transactions of The Royal Society B, 360, 1889-1895.
16. Kress WJ, Wurdack KJ, Zimmer EA, Weight LA, Janzen DH, 2005, The National Academy of Sciences, 102, 8369.
17. Kool A, De Boer HJ, Kruger A, Rydberg A, Abbad AA, Bjork L, Martin G, 2012, PLos One, 7, e39459.
18. Mahadani P, Ghosh SK, 2013, DNA Barcodes, 1, 35-38.
19. Alvarez I, Wendel JF, 2003, Molecular Phylogenetics and Evolution, 29(3), 417-434.
20. Hollingsworth PM, Graham SW, Little DP, 2011, PLoS One, 6(5), e19254.
21. Che Nasriyyah, Che Husin, Darlina Md. Naim , Mardiana Idayu Ahmad, 2018, Authentication of a selected medicinal plants using DNA barcoding technique, SHS Web of Conferences 45, 05004.
22. Dhananjaiah TM, Shalmali Chatterjee, Chandrika S Tanthry, Shruthi SD, 2020, DNA Barcoding of Cynadon Dactylon and Molecular Characterization of Its Endophytic Fungi, International Journal of Science, Engineering and Management (IJSEM) 5(7).