OPEN ACCESS Freely available online

Journal of Proteomics & Bioinformatics

Research Article

DNA Barcoding of *Cardiospermum halicacabum* using trnH- psbA and Analysis of its Anti-inflammatory Potential

Pavithra Srinivasan, Ivo Romauld*, Vivek Pazhamalai

Department of Bio-Engineering, School of Engineering, Vels Institute of Science Technology and Advanced Studies (VISTAS) Pallavaram, Tamil Nadu, India

ABSTRACT

Cardiospermum halicacabum is a highly medicinal value plant used in the traditional medicine for long time and well known for anti-inflammatory activity, widely known as weed in all over India and used to treat rheumatism, nervous diseases, demulcent in orchitis and in dropsy. It exhibits significant analgesic, anti-inflammatory and anti-vasodepressant activity, which is transient in nature. The present study was extracted the sample by polar solvent (ethanol), analyzed for phytochemical screening. The anti-inflammatory activity the sample was studied in HRBC (Human red blood cell stabilization) assay.The DNA of the *Cardiospermum halicacabum* was isolated and both quality and quantity of the DNA was checked. The isolated DNA was amplified in PCR by using the gene trnH-psbA and the DNA was sequenced. Phylogenetic tree for *Cardiospermum halicacabum* was constructed and the DNA barcode was generated.

Keywords: *Cardiospermum halicacabum*; Anti-inflammatory activity; trnH-psbA; Phylogenetic tree; Barcode generation

INTRODUCTION

DNA barcoding

The purpose of sequencing a DNA to know the base pair variations and deposited in the barcode database and it is termed as DNA barcodes. The DNA barcoding is a technique for identification of species without their morphological characters. The genetic codes are accessed in the library to identify the unknown species in the world. The barcoding of a DNA is normally a uniform short sequence of DNA the base pairs are up to 400-800 and it was generated to characterize the living organism [1]. The barcoding was first emerged to describe the microorganism. The information of the DNA barcoding takes the information of one or few regions to recognize the genome of the species in a genus [2]. The term DNA barcoding is defined as the distribution of inter-specific variation and intra-specific variation [3]. The barcoding of DNA is necessary for the identification of species [3] and discovery of new species [4].

Barcoding system

The molecular identification system was started during the year of 1990 by PCR method for identification of species. The barcoding system is a diagnostic tool based on their taxonomic data known as DNA barcode reference library. The data system resembles the information of achievement, storage, analysis and publication of barcode records. The DNA barcode of life data system was initiated in 2004 and established in 2007 [5]. The barcoded DNA used in the reference library for rapid identification of low taxonomy level taxa with specific short DNA sequence. By using the barcoding system, the pathogens and the species were identified in the fields of medicine and bio-industries.

Plant DNA barcoding

The Plant DNA barcoding plays a major role especially in identification of rare species. In the early stages of barcoding plant has not given much importance due to the inability of cytochrome oxidase (COX1) to work as barcode [6]. The universal barcode of plants are have not been found [7] due to lack of consensus region in the plant. The barcoded DNA data were available in the barcode libraries; it helps in knowing the complete database of DNA barcoding [8]. They require standard PCR primers per gene and it helps as a barcode marker in the taxa. The combination DNA regions is proposed and initiated to facilitate biodiversity studies [7,9,10]. The non-coding intergenic spacers like trnH-psbA [7,9,10] and plastidial coding sequence mat k gene [9]. And the chloroplast trnl (UAA) intron (10-143bp) relatively could be improved with highly conserved primers. The gene sequence of coding regions is matk, rbcl and rpoc and non-coding region ITS and psbA-trnH [11].

PsbA-trnH (D1 protein of photosystem)

The non-coding regions are coded by psbA-trnH intergenic spacer.

Correspondence to: Daniel Benharroch, Department of Bioinformatics, Science Block, Alagappa University, Karaikudi630 004, Tamil Nadu, India, E-mail: ivoromauld@gmail.com

Received: March 16, 2021; **Accepted:** March 30, 2021; **Published:** April 06, 2021

Citation: Srinivasan P (2021) DNA Barcoding of Cardiospermum halicacabum using trnH- psbA and Analysis of its Anti-inflammatory Potential. J Proteomics Bioinform. 14:534.

The function of the non-coding regions was unknown, it causes mutation and not restrained by selection [12,13]. The structure of psbA-trnH has a small spacer region consist of 200 bp-500 bp in angiosperms and gymnosperm species. They were amplified by the universal primers and developed [14]. The psbA-trnH intergenic region differs in their evolutionary conversation:

- The psbA 3'UTR is responsible for the regulation of gene expression.
- The psbA-trnH is the non-transcribed intergenic spacer has no function but shows variability across angiosperms.
- The psbA gene codes the D1 reaction center protein. The expression of the gene depends on light intensity, the development stage and physiological state of the plant [15].

The trnH-psbA is ubiquitous, high intra-specific sequence divergence and universal flanking primers allows easy amplification and sequence in both high molecular weight and effective in degrading the DNA [7,9,16,17]. But the trnH-psbA does not shows the continuous amplification or multiple bands [19] and irregular longer [17,18]. The trnH-psbA has shown less variation among closely related species [19] intra-specific variation is high [20].

Cardiospermum halicacabum

Plants are not only the source of food, fiber also rich in their medicinal value. The plant products are biologically important and less toxic than synthetic products. *Cardiospermum halicacabum* is an ornamental and medicinal plant. The name *Cardiospermum* is the combination of the latin words cardio- heart and sperma-white-heart shaped seed, commonly known as heart pea, love puff, cultivated in tropical and subtropical regions belongs to the family sapindaceae includes 142 genera and 1900 species. It is a deciduous climbing shrub grows about 3 m tall and branched fromthe base, climbs from the tendril in the length of 1.5 m-2 m in length. The stems are with 5-longitudinal ribs. The alternate, biternate leaves are of 4 cm-12 cm long and finely hairy to almost hairless young stem. The flowers are zygomorphic, in axillary racemiform thyrses, shorter than accompanying leaf. They are 3 mm-4 mm long and papery capsules (1 cm-3 cm long) are also relatively small, nectar glands 4, ovoid 0.4 mm long. The seeds of the plant contain 33% fatty acids there are 55% cyanolipids [31]. The secondary metabolite of *C.halicacabum* has the anti-filarial, anticancer, anti-diarrheal, anti-ulcer, anti-convulsant, anti-pyretic activities. The main objectives of the study: To extract the bio-constituents from the plant sample *Cardiospermum halicacabum* using the polar solvent, analyze both quantitative and qualitatively, anti-inflammatory activity in HRBC assay, isolated the DNA to generate a barcode constructed a phylogenetic tree.

MATERIALS AND METHODS

Powdered sample (*Cardiospermum halicacabum*), ethanol solvent, mortar and pestle, phytochemical reagents, sterile distilled water, agarose and trnH- PSBA primer.

Extraction of sample

The fresh *Cardiospermum halicacabum* was collected from southern region (Chennai) of Tamilnadu. With sterile distilled water; shade dried for 3 weeks to get rid of the moisture content and powdered. The powdered samples were extracted using polar solvent (ethanol). The solvent is added to the sample in the ratio of 1:3. The extracted sample was incubated at room temperature for twenty-four hours. The solvent was evaporated using hot plate at 90°C.

Phytochemical analysis

The test was done for carbohydrates, tannins, saponins, flavonoids, alkaloids, quinones, glycosides, cardiac glycosides, terpenoids, phenols, coumarins, steroids, phytosteroids, phlobatannins, anthraquinones [25-30].

Anti-Inflammatory potential (HRBC stabilization assay)

The in vitro anti-inflammatory analysis by HRBC membrane stabilization method [22] was studied in the extracted sample.

Hypotonicity induced human red blood cell (HRBC) membrane stabilization by HRBC assay

1mg of extract were taken at different concentrations and mixed with 0.2 M phosphate buffer, 0.5 ml of HRBC suspension, 0.5 ml of 0.25% hyposaline was added. The extract was incubated at 37°C for half-hour and centrifuged at 3000 rpm for 20 minutes. The presence of hemoglobin content is estimated at 560 nm in spectrophotometer [23]. The proportion of HRBC hemolysis and membrane stabilization was calculated by the formula.

$$\% \text{ of Hemolysis} = \frac{\text{Optical density of test sample}}{\text{Optical density of control}} \times 100$$

$$\% \text{ of Protection} = 100 - \frac{\text{Optical density of test sample}}{\text{Optical density of control}} \times 100$$

Isolation of DNA

The DNA of the sample was isolated by using CTAB buffer [24]. The isolated DNA were subjected to both qualitative and quantitatively.

Qualitative analysis of genomic DNA

Agarose gel electrophoresis: For the bulk of DNA samples, electrophoretic separation was dispensed in agarose gels. This is often DNA molecules and their fragments are considerably larger than proteins. Under an electrical field, any given fragment of DNA should move towards the anode with the identical mobility. Thus, the largest molecules will have difficulty in moving, whereas the smallest molecules are relatively unhindered. Consequently, the mobility of DNA molecules during gel electrophoresis will rely on size.

Quantitative determination of dna by spectrophotometric method

Proteins are usually the foremost contaminants in nucleic acids extract and these have absorption maximum at 280 nm. The ratio of absorbance at 260 nm and 280 nm hence provides a transparent idea about the extent of contamination within the preparation. 5 µl of the sample were taken in a quartz cuvette and made up to 3 ml with TE buffer. The TE buffer was taken as blank. The sample absorbance was measured at 260 nm and 280 nm. The concentration of the sample was calculated using the given formula.

Concentration of dsDNA A_{260} × 50 µg × dilution factor

Purity of the DNA

A_{260}: A_{28}0 ratio=A_{260}/A_{280}

=1.8: Pure DNA

=1.7–1.9 moderately pure DNA

=less than 1.8; presence of proteins.

=greater than 1.8; presence of organic solvent

Polymerase Chain Reaction (PCR)

PCR is an in vitro method of enzymatic synthesis of specific DNA sequence developed by Kary Muller in 1988. It is a very simple and inexpensive technology for characterizing, analysing, synthesizing, a selected DNA or RNA from virtually, any living organism, plant, animal, virus or bacteria. It exploits the natural function of polymerase present in all living things to copy genetic material or to perform molecular photocopy (Tables 1 and 2).

Table 1: Primer details.

Primer details	
trnH-psbA F	ACTGCCTTGATCCACTTGGC
trnH-psbA R	CGAAGCTCCATCTACAAATGG

Table 2: Concentration of sample in PCR.

Componets	Stock concentraton	Final concentration	Volume for 20 µl set up
Milli Q water	-	-	10.8 µl
dNTP mix	2 Mm	0.2 mM	2 µl
Taq buffer	10 X	1 X	2 µl
$Mgcl_2$	25 Mm	1 mM	0.8 µl
Forward primer	10 µM	10 µM	2 µl
Reverse primer	10 µM	1 µM	2 µl
DNA Template	Crude	-	1 µl
Taq DNA polymerase	10 U/µl	1 U	0.2 µl

The total volume of the reaction is 20 µl. Reaction condition was altered between 1.5 mM $MgCl_2$ and 1 mM $MgCl_2$. The entire reaction setup was taken out at 4°C. PCR products (20 µl) were mixed with 5 µl of gel loading dye (Bromophenol dye) in 1.5% Agarose gel containing ethidium bromide together with 5 µl of DNA ladder. The electrophoretic separation was performed at 100 V for half-hour. The resulting DNA fragments were visualized using an Ultraviolet transilluminator.

• Sequencing: It had been done by applied biosystem 3500 genetic analyzer using Sanger sequencing.

• Phylogenetic tree: Phylogenetic tree was constructed using maximum likelihood method in MEGAX. Phylogenetic tree is a graphical representation of the evolutionary relationship among three or more gene or organism.

• MEGAX: MegaX performs tree inference using the MegaX program and therefore the computer file should be 'meg' format. The file contains the sequence of DNA in mega format. Using MegaX it's possible to estimate a maximum likelihood tree and perform the bootstrap test in an automatic. The program will display the tree in a new window and superimpose bootstrap support values along each branch of the tree.

Barcode generation

The barcode is employed to identify the plant species and adulteration of gene. The invention of species till now depends on the morphological information provided by the taxonomists. The DNA barcode of life is to develop an even, rapid and inexpensive species identification method. It may be accessible to non-specialists around the world. The barcode of C.*halicacabum* is generated by uploading the FASTA sequence oftrnH-psbA within the Bioradar.

RESULTS AND DISCUSSION

The leaves of *Cardiospermum halicacabum* have a lot therapeutic potential like anti-inflammatory antioxidant anti-fungal etc., the leaf juice was used to treat earache, the vapor of the crushed leaves was inhaled to alleviate headache. The process of barcoding is to identify the adulteration and to analyze the anti-inflammatory potential of the sample in membrane stabilization assay.

Extraction and phytochemical analysis of C. halicacabum

C. halicacabum were extracted in the solvent ethanol. After extraction, the crude sample was analyzed for phytochemical screening of the bioactive compounds (Figure 1). The phytochemical analysis was tabulated in the following (Table 3).

Figure 1: Crude extract.

Table 3: Phytochemical screening of the C. halicacabum.

S.No.	Phytochemical test	Results
1	Carbohydrate	+
2	Tannin	+
3	Flavonoid	+
4	Alkaloid	+
5	Quinone	+
6	Terpenoids	+
7	Phenols	+
8	Coumarins	+
9	Steroids and Phytosteroids	+

The ethanolic extract showed the presence of carbohydrates, tannin, flavonoid, alkaloid, quinone, terpenoids, phenols, coumarins, steroidand Phyto steroids. The absence of Pholobotannins, anthraquinones, saponins, glycoside, and cardiac glycoside was observed (Figure 2).

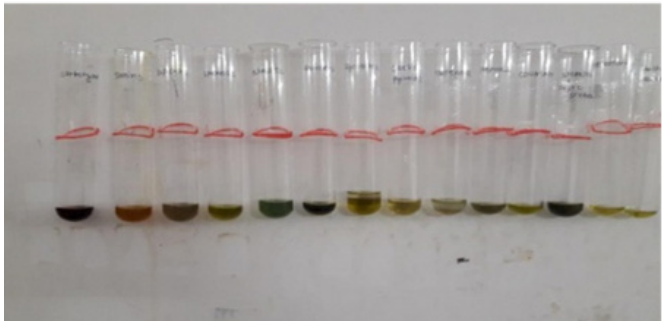

Figure 2: Qualitative analysis.

A) carbohydrate, B) tannin, C) saponin, D) flavonoid, E) alkaloid, F) quinone, F) glycosides, G) cardiac glycosides, H) terpenoids, I) phenol, J) coumarins test, K) steroids, L) phytosteroids, M) phlobotannins test and N) anthraquinones test

HRBC stabilization assay: The ethanolic extract of C. halicacabum is analyzed the property of anti- inflammatory property is analyzed by HRBC assay, the absorbance value was calculated at 560 nm and therefore the percentage of hemolysis were tabulated in Table 4. The property of the substance utilized in the treatment of swelling, analgesics, anti-inflammatory drugs (Table 5) (Figure 3).

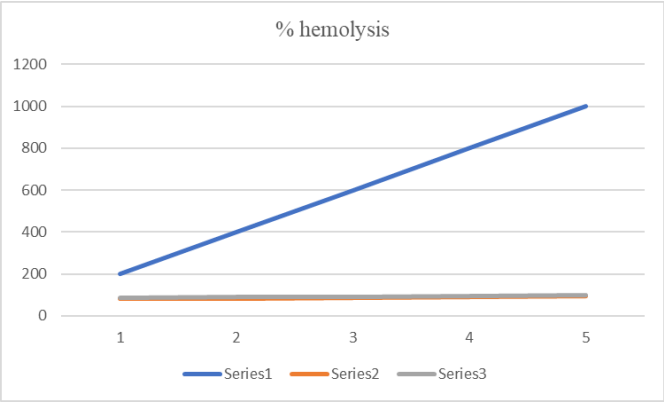

Figure 3: Plot of concentration of membrane stabilization of extracted sample.

Table 4: Absorbance of assorted concentration of sample.

S.No.	Concentration (µg/ml)	Volume to be taken (ml)	Volume of PO4 buffer (ml)	HRBC Suspension	Absorbance (560 nm)
1	200	0.1	1	0.5	0.0333
2	400	0.2	1	0.5	0.0310
3	600	0.3	1	0.5	0.0215
4	800	0.4	1	0.5	0.0213
5	1000	0.5	1	0.5	0.0129
	1 ml (ethanol)	-	1	0.5	0.0129

Table 5: Percentage of membrane stabilization.

S.No.	Concentration of sample (µg/ml)	% Membrane Stabilization	Positive control
1	200	82	85
2	400	83.25	90
3	600	88.3	92
4	800	88.5	96
5	1000	93.03	98

Calculation:

$$\% \text{ Membrane Stabilization} = \frac{100 - \text{OD of test}}{\text{OD of control}} \times 100$$

Agarose gel electrophoresis

The isolated DNA were checked qualitatively by agarose gel electrophoresis method the 1 kb ladder was employed as marker as shown in Figure 4. L-1 kb ladder genomic DNA, Sample (1-4) various concentration of plant DNA.

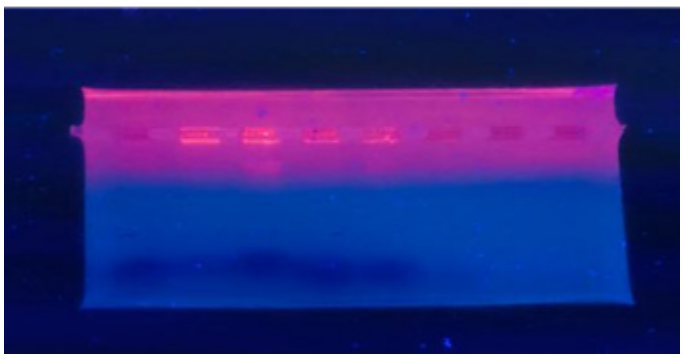

Figure 4: Agarose gel electrophoresis L-1kb ladder genomic DNA, Sample (1-4) various concentration of plant DNA.

Quantification of DNA by spectrophotometric method

The isolated DNA was quantified by spectrophotometer at two different wavelengths 260 nm and 280 nm. A_{260}/A_{280} ratio gives purity of the DNA as proteins absorb at 280 nm because of tyrosine and tryptophan residues. The ratios were in the range of 1.83 which indicated a pure DNA. DNA concentrations are given in Table 6.

Table 6: Absorbance of DNA at various concentrations.

S. No	Sample	Absorbance at 260 nm	Absorbance at 280 nm	Concentration (µg/ml)	Purity
1	Blank	0.000	S-	-	-
2	Sample	0.098	0.068	1056	1.83

Concentration of DNA

$$= A_{260} \times 50\ \mu g/ml \times \text{dilution factor}$$

Polymerase chain reaction: The thermocycler was used to obtain multiple copies of DNA for sequential analysis. The 500 kb of DNA was obtained (Figure 5).

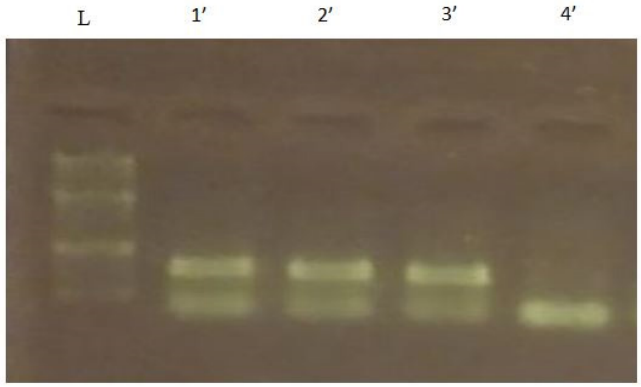

Figure 5: PCR with barcoding gene trnH-psbA.

L-1 kb ladder genomic DNA, Sample (1'-4') PCR product with 1.8% of agarose gel the samples 2', 3' were sequenced.

Sequencing

The obtained PCR product was sequenced by using trnH-psbA barcoding gene by sanger sequencing method. The sequenced DNA was analysed to study quality of the base pairs (Figure 6).

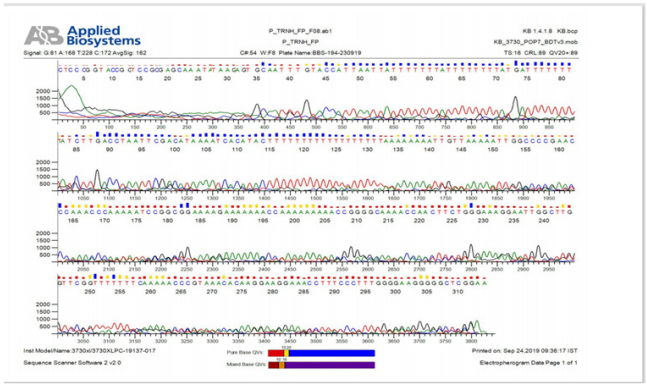

Figure 6: Sequence analysis using trnH-psbA gene.

>P_TRNH_FP_F08.ab1

CTCCCGGTACCGGTCCGCGAGCAAATATAAGAGTGCAA TTTGTACCATTAATTATTTTTTTATTTTTTTTATGATTTTTT

TTATCTTGACCTAATTCGACATAAAATCACATACTTTTTT TTTTTTTTTTTTAAAAAAAATTGTTAAAAATTGGCCCCGA

ACCCAAACCCAAAAATCCGGCGGAAAAGAAAAAAACC AAAAAAAAACCGGGGCAAAACCAACTTCTGGG AAAGGAATTGG

CTTGGTTCGGTTTTTTTCAAAAACCCGTAAACACAAGG AAGGAAACCTTTCCCTTTGGGGAAGGGGGCTCGGAA

Construction of phylogenetic tree

The evolutionary relationship of the species of *Cardiospermum halicacabum* was studied by constructing a phylogenetic tree (Figure 7).

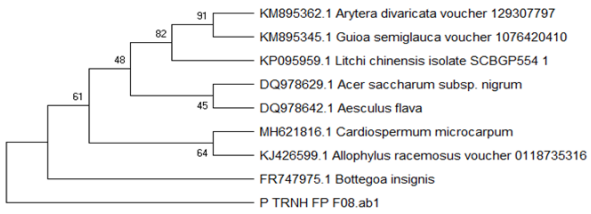

Figure 7: Phylogenetic tree.

Barcode generation: The DNA barcode were generated by using BIO-RAD (http://biorad-ads.com/DNABarcodeWeb/). By using the barcode, the species were identified easily by both the taxonomists and non- taxonomists. And the adulteration of the product is identified (Figure 8).

CONCLUSION

In this study, ethanolic extraction of C. halicacabum was carried in the dry powder. The bio active compounds of the sample were tested by phytochemical screening and quantitative analysis indicates the presence of tannins, saponins, steroids, alkaloids, carbohydrates, phytosteroids, glycosides, cardiac glycosides, anthraquinone and phlobotannins. The anti-inflammatory activity of C. halicacabum for identification of hemolysis was done by HRBC assay. The DNA was isolated from the fresh leaves of C. halicacabum. The quality and quantity of the DNA were checked by agarose gel electrophoresis and spectrophotometric method. The evolutionary relationship of C. halicacabum was studied by constructing a phylogenetic tree and the barcode for C. halicacabum were generated.

REFERENCES

1. Savolainen V, Cowan RS, Vogler AP, Roderick GK, Lane R. Towards writing the encyclopedia of life: An introduction to DNA barcoding. Philos Trans R Soc Lond B Biol Sci. 2005;360:1805-1811.
2. Lahaye R, van der Bank M, Bogarin D, Warner J, Pupulin F. DNA barcoding the floras of biodiversity hotspots. Proceedings of the National Academy of Sciences. 2008;105:2923-2928.
3. Hebert PDN, Ratnasingham S, DeWaard JR. Barcoding animal life: Cytochrome c oxidase subunit 1 divergences among closely related species. Proceedings of the Royal Society Biological Science. 2003;270:96-99.
4. Valentini AS, Mattiucci P, Bondanelli SC, Webb AA, Mignucci-Giannone MM, Llavina C, et al. Genetic relationships among anisakis species (Nematoda: Anisakidae) inferred from mitochondrial COX2 sequences, and comparison with allozyme data. J Parasitol. 2006;92:156-166.
5. Ratnasingham S, Hebert PDN. BOLD: The barcode of life data system (www.barcodinglife.org). Molecular ecology notes. 2007;7:355-364.
6. Cho Y, Mower JP, Qiu YL, Palmer JD. Mitochondrial substitution rates are extraordinarily elevated and variable in a genus of flowering plants. Proc Natl Acad Sci U S A. 2004;101:17741-17746.
7. Kress WJ, Wurdack KJ, Zimmer EA, Weigt LA, Janzen DH. Use of DNA barcodes to identify flowering plants. Proc Nat Academy Sci. 2005;102(23):8369-8374.
8. Ekrem T, Willassen E, Stur E. A comprehensive DNA sequence library is essential for identification with DNA barcodes. Mol Phylogenet Evol. 2007;43:530-542.
9. Chase MW, Cowan RS, Hollingsworth PM, van der Berg C, Madrinan S. A proposal for a standardised protocol to barcode all land plants. Taxon. 2007;56:295-299.
10. Kress WJ, Erickson DL. A two-locus global DNA barcode for land plants: The codingrbcL gene complements the non-coding trnH-psbA spacer region. PLoS One. 2007;2(6):e508.
11. Chen SL, Yao H, Han JP, Liu C, Song JY, Shi LC, et al. Validation of the ITS2 region as a novel DNA barcode for identifying medicinal plant species. PLoS One. 2010;5:e8613.
12. Olmstead RG, Michaels HJ, Scott KM, Palmer JD. Monophyly of the asteridae and identification of their major lineages inferred from DNA sequences of rbcL. Ann Missouri Botan Garden. 1992;79:249-265.
13. Hamilton MB, Braverman JM, Soria-Hernanz DF. Patterns and relative rates of nucleotide and insertion/deletion evolution at six chloroplast intergenic regions in new world species of the lecythidaceae. Mol Biol Evol. 2003;20:1710-1721.
14. Hamilton MB. Four primer pairs for the amplification of chloroplast intergenic regions with intra-specific variation. Mol Ecol. 2015;8:521-523.
15. He JX, Wen JQ, Chong K, Liang HG. Changes in transcript levels ofchloroplastpsbA and psbD genes during water stress in wheat leaves. Physiol Plant. 1998;102:49-54.
16. Erickson DL, Spouge J, Resch A, Weigt LA, Kress WJ. DNA barcoding in land plants: Developing standards to quantify and maximize success. Taxon. 2008;57:1304-1316.
17. Shaw J, Lickey EB, Beck JT, Farmer SB, Liu W, Miller J, et al. The tortoise and the hare II: Relative utility of 21-noncoding chloroplasts. American Journal of Botany. 2005;92:142-166.

18. Hollingsworth ML, Clark AA, Forrest LL, Richardson J, Pennington RT. Selecting barcoding loci for plants: Evaluation of seven candidate loci with species-level sampling in three divergent groups of land plants. Mol Ecol Res. 2009;9:439-457.

19. Sass C, Little DP, Stevenson DW, Specht CD. DNA barcoding in the Cycadales: Testing the potential of proposed barcoding markers for species identification of cycads. PLoS One. 2007;2:e1154.

20. Edwards D, Horn A, Taylor D, Savolainen V, Hawkins JA. DNA barcoding of a large genus, Aspalathus L. (Fabaceae). Taxon. 2003;57:1317-1327.

21. Viji M, Murugesan S. Phytochemical analysis and antibacterial activity of medicinal plant *cardiospermum halicacabum* linn. J Phytol. 2010;15:55-61.

22. Varghese CP, Ambrose C, Jin SC, Lim YJ, Keisaban T. Antioxidant and anti-inflammatory activity of Eurycoma longifolia Jack, a traditional medicinal plant in Malaysia. Int J Pharm Sci Nanotechnol. 2013;5(4):1875-1878.

23. Chippada SC, Volluri SS, Bammidi SR, Vangalapati M. In vitro anti-inflammatory activity of methanolic extract of Centella asiatica by HRBC membrane stabilisation. Rasayan J Chem. 2011;4(2):457-460.

24. Murray MG, Thompson WF. Rapid isolation of high molecular weight plant DNA. Nucleic acids Res. 1980;8(19):4321-4326.

25. Sofowora A. Medicinal plant and traditional medicine in Africa 2nd ed sunshine house, Ibadan.

26. Herborne JB. Phytochemical methods. A guide to modern techniques of plant analysis. 1973:5-11.

27. Jana S, Shekhawat GS. Phytochemical analysis and antibacterial screening of in vivo and in vitro extracts of Indian medicinal herb: Anethum graveolens. Res J Med Plant. 2010;4(4):206-212.

28. Sureshkumar CA, Varadharajan R, Muthumani P, Meera R, Devi P, Kameswari B, et al. Pharmacognostic and preliminary phytochemical investigations on the stem of Saccharum spontaneum. J Pharm Sci Res. 2009;1(3):129-136.

29. Manas B, Rajesh Y, Kumar VR, Praveen B, Mangamma K. Extraction, phytochemical screening and in-vitro evaluation of anti-oxidant properties of Commicarpus chinesis (aqueous leaf extract). Int J Pharm Bio Sci. 2010;1(4):361-366.

30. Kolawole OM. Studies on the efficacy of bridelia ferruginea benth. Bark extract in reducing the coliform load and BOD of domestic wastewater. Ethnobotanical Leaflets. 2006;2006(1):24.

31. Shareef H, Rizwani GH, Mahmood S, Khursheed R, Zahid H. In vitro antimicrobial and phytochemical analysis of *Cardiospermum halicacabum* L. L Pak J Bot. 2012;44(5):1677-1680.