Annals of Phytomedicine 3(2): 87-92, 2014

Journal homepage: www.ukaazpublications.com

ANNALS OF PHYTOMEDICINE
An International Journal

ISSN 2393-9885

Molecular diversities among *Cardiospermum halicacabum* Linn. populations in Kerala assessed using RAPD markers

M.S. Sheeba*, K.H. Muneeb Hamza, N. Krishna Radhika*** and V.V Asha****

**Government College, Pattambi-679303, Palakkad, Kerala, India*

***Plant Based Bioactives and Disease Biology Laboratory, Rajiv Gandhi Centre for Biotechnology, Thiruvananthapuram-695014, Kerala, India*

****Central Tuber Crops Research Institute, Thiruvananthapuram-695017, Kerala, India*

Received November 5, 2014: Revised November 25, 2014: Accepted November 30, 2014: Published online December 30, 2014

Abstract

A collection of *Cardiospermum halicacabum* Linn. plants was made from five different regions of Kerala to determine the extent of genetic variability using analysis at DNA level. As germplasm diversities are not influenced by the environment, RAPD (Random Amplified Polymorphic DNA) provides different approach towards the genotype based classification of plants. The present study was aimed to reveal the genetic polymorphism among five different populations of *C. halicacabum*. For this, the plants were collected from five different locations in Kerala. DNA was isolated from the leaves by CTAB (Cetyltrimethyl Ammonium Bromide) method and quantified spectrophotometrically. The isolated DNA was subjected to RAPD analysis by Polymerase chain reaction (PCR) using 120 different primers. Among the 120 primers, 25 primers yielded the best product for RAPD analysis. Unweighted Pair Group Method and Arithmetic Mean (UPGMA) dendrogram analysis resulted in two clusters such as the plants collected from Trivandrum, Kanyakumari and Kollam were grouped in one cluster and the remaining populations from Alappuzha and Palakkad grouped in the other cluster. From the results, it has been observed that the plants collected from five different locations showed a low polymorphism. The total polymorphism among the plants was 38% and the the number of polymorphic fragments ranged from 125 bp to 3.5 Kb.

Key words: Genetic diversity, polymerase chain reaction, germplasm, polymorphism, arbitrary primers, UPGMA.

1. Introduction

Cardiospermum halicacabum Linn. (Synonym: *Cardiospermum microcarpum* Kunth.) is a herbaceous climber of the family Sapindaceae. Cardiospermum is an annual or perennial climber, widely distributed in tropical and subtropical Africa and Asia. *C. halicacabum* has been used in Ayurveda and folk medicine since ancient days (Mona *et al*., 2014). DNA based markers have the obvious advantage of sampling the genome directly (Helentjaris *et al*., 1985). As a medicinal plant, well adapted to the soil conditions of India, especially Kerala, revelation of the genetic polymorphism among populations of C. *halicacabum* is very crucial in order to identify the plant populations, having better medicinal properties. Genetic diversity within a plant species may not result in morphological changes to a great extent but creates a variation in the phytochemical composition of plants (Annamalai *et al*., 2012). Investigation on the species based on genetic variation shown by accessions would obviously be advantageous; such information can be obtained using molecular markers which afford many benefits for identifying variations and estimating biological diversity.

Author for correspondence: Dr. V.V. Asha
Scientist E2, Rajiv Gandhi Centre for Biotechnology, Thiruvananthapuram - 695014, Kerala State, India.
E-mail: ashavv@rediffmail.com; vvsha@rgcb.res.in
Tel.: + 09447428598

RAPD is a DNA fingerprinting technique that uses a single oligonucleotide primer in a PCR with low stringency. The technique requires no sequence information prior to analysis and only a minute amount of DNA is sufficient (Welsh *et al*., 1990a; Welsh and McClelland, 1990b). In RAPD, short oligonucleotides of arbitrary sequence are used singly to support the amplification of regions of the test plant genome and the amplification products are separated by gel electrophoresis; differences between genotypes are reflected as different banding patterns. Molecular techniques have been found to be more useful and accurate for determination of both interspecies and intraspecies genetic variations in plants. RAPD markers, in particular, have been successfully employed for determination of intraspecies genetic diversity in several plants (Demeke *et al*., 1992). However, the principal disadvantage of RAPD is that they are usually dominant markers, and reproducibility of DNA banding patterns can be affected by different concentrations of reaction components and cycle conditions (dos Santos *et al*., 1994). The detection of polymorphism by RAPD method is based on the presence or absence of particular bands in electrophoresis. RAPD markers have been used for identifying component species in Chinese medicine materials (Cheng *et al*., 1997; Shaw and Butt, 1995) and differentiating between genuine and counterfeit materials (Cheng *et al*., 1998). DNA fingerprinting patterns could be useful in identifying the species and the sources of various medicinal plants and, thus, serve as an aid to quality control.

C. halicacabum is considered as a beneficial plant for digestive and pulmonary disorders. The entire plant is diaphoretic, diuretic, emetic, laxative, stomachic and sudorific. The juice of the herb is used to cure ear ache and to reduce hardened tumors. Different active extracts of this plant possess many pharmacological activities like anti-inflammatory activity (Sadique *et al*,. 1987; Sheeba and Asha, 2009), analgesic, antipyretic, gastoprotective, antidiarrheal and antibacterial (Asha *et al*.,1999; Sheeba and Asha, 2006; Rao *et al*., 2006; Annadurai *et al*., 2013; Raman *et al*., 1998). The whole plant contains saponins, flavanoids, phytosterols and traces of alkaloids, which collectively might contribute to the diverse pharmaceutical actions of the plant (Ahmed and Ahmed, 1993; Ferrara and Montesano, 1996). As the plant *C. halicacabum* has so much traditional uses, and some of which have been scientifically validated through studies in our lab, we intended to do the genetic analysis of the plant by RAPD. Genetic variation within and between five populations of *C. halicacabum* from different geographical regions of Kerala, India was investigated using RAPD.

2. Materials and Methods

2.1 Plant material

C. halicacabum Linn. samples were collected from five different locations in or around Kerala, namely; Trivandrum, Kollam, Kanyakumari, Alappuzha and Palakkad (Figure A).

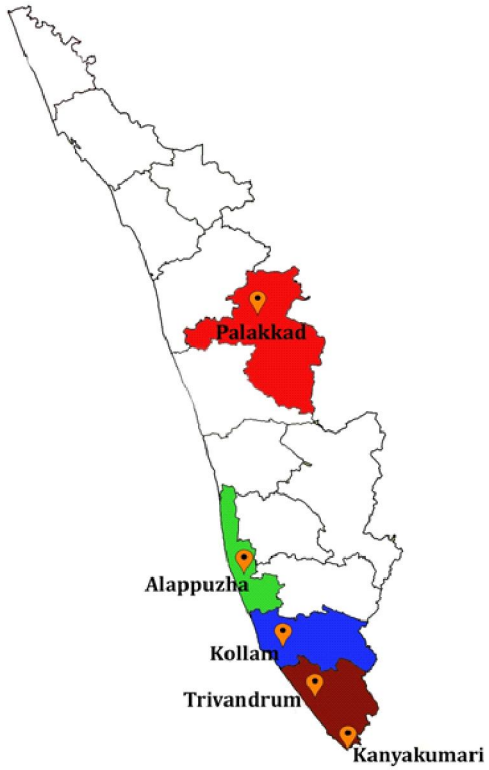

Figure A: Showing the places from where the plant samples were collected

2.2 DNA extraction and quantification

DNA was extracted from fresh tender leaves of *C. halicacabum* by the CTAB method (Bousquet *et al*., 1990). Approximately, 200 mg of fresh leaf was ground to a powder in liquid nitrogen, using mortar and pestle. The powder was transferred to a 25 mL falcon tube with 3 mL CTAB buffer. The extraction buffer consisted of 2% (w/v) CTAB, 1.4 M NaCl, 20 mM EDTA, 10 mM Tris-HCl and 0.2% b-mercaptoethanol. The homogenate was incubated at 60^0C for 1h with intermittent shaking. After incubation, chloroform-isoamyl alcohol (24:1) was added, shaken well and centrifuged at 10,000 rpm for 30 min. DNA was precipitated from the aqueous phase by mixing with ice cold absolute ethanol. The DNA pellet was washed in 70% ethanol, air dried and resuspended in TE (Tris-EDTA) buffer. DNA quantity was estimated spectrophotometrically by measuring absorbance at 260 nm and 280 nm. Agarose gel 1% was used to determine DNA concentration by comparing the band intensities using 1Kb DNA ladder marker.

2.3 RAPD analysis

A total of 120 random decamers (OPA1-20, OPC1-20, OPD1-20, OPH1-20, OPF1-20, OPG1-20; M/S Operon technologies, USA) were screened for their efficiency in generating random fragments from this plant. The PCR reaction conditions (Williams *et al*., 1990) included 45 cycles of denaturation at 94^0C for 1min, annealing at 36^0C for 1 min and extension at 72^0C for 2 min. The final cycle included extension at 72^0C for 7 min duration. All reactions were carried out in 25 µLvolume and contained 50ng genomic DNA (quantification was done by spectrophotometrically), 5 picomole primer, 125 mM dNTP, 25 mM Mg^{2+} in assay buffers (10X reaction buffer) supplied along with the enzyme 0.5 to 1 unit of the thermo stable *Taq* DNA polymerase. After overlaying the reaction mixture with one drop of mineral oil, the tubes were placed on the thermocycler (iCycler Bio-rad, USA) for PCR. The entire PCR product was resolved on a 1.4% agarose gel with 1X TBE buffer (0.009 M Tris borate, 0.0002 M EDTA), visualized after Ethidium bromide staining under UV illumination, and photographed with multi-imager (Molecular imager FX Biorad).

2.4 Data collection and statistical analysis

Amplification with each primer was repeated thrice and clearly resolved reproducible fragments were considered in data collection. RAPD profiles were manually scored as 1 for presence and 0 for absence of a band for each individual. Bands amplified from 125 bp to 3500 bp were considered for analysis. 1Kb ladder was used as marker and the data obtained was used to construct a dendrogram using Phylipversion 3.6b. The dendrogram analysis was done utilizing the Unweighted Pair Group Method and Arithmetic Mean (UPGMA) programme.

3. Results

Among the 120 primers screened, 25 primers yielded the best product for RAPD analysis (Table I). A total of 174 fragments with an average of 6.96 bands per primer were amplified; of that 62 were found to be polymorphic and the remaining were monomorphic. The total percentage polymorphism was 38%. The approximate size of the largest fragment produced was 32 Kb and the smallest easily recognizable fragment produced was approximately 50 bp. Out of the 25 different decamers tested, two (OPD-7 and OPD-13) produced amplification products that were monomorphic across all populations (Figures D and E). The size of the monomorphic DNA fragments produced by these primers ranged from 125 bp to 2.5 Kb for OPD-7 and 0.2 Kb to 2.5 Kb for OPD-13. The representative RAPD profiles are shown in (Figures B and E).

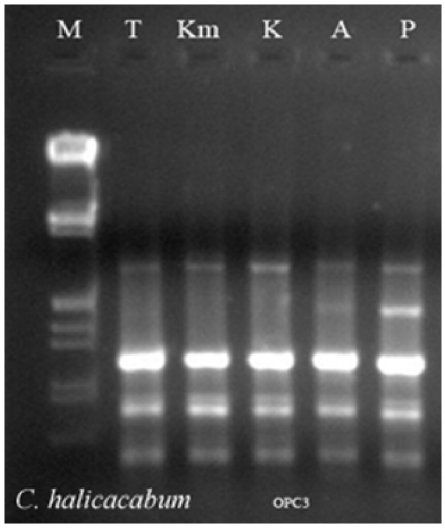

Figure: A OPC-3

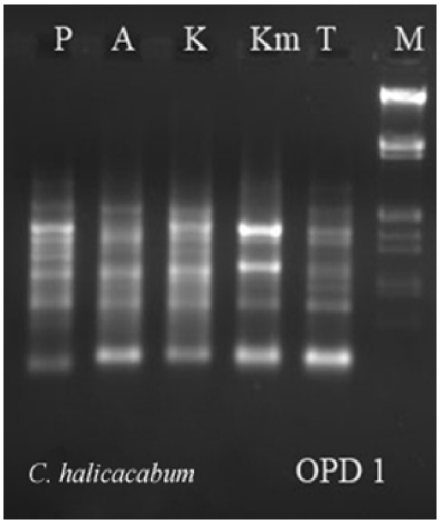

Figure: B OPD-1

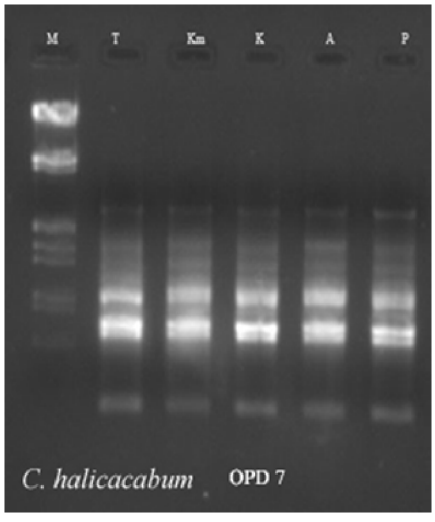

Figure: C OPD-7

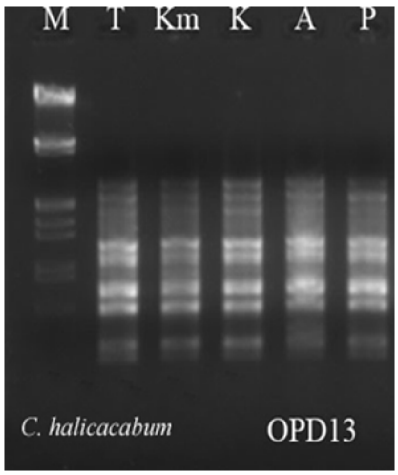

Figure: D OPD-13

RAPD profile of *C. halicacabum* selected populations with Operon primers. Figures A and B showing polymorphic bands and Figures C and D showing no polymorphism. (M- marker ; T-Trivandrum; Km- Kanyakumari; K-Kollam; A- Alappuzha; P- Palakkad)

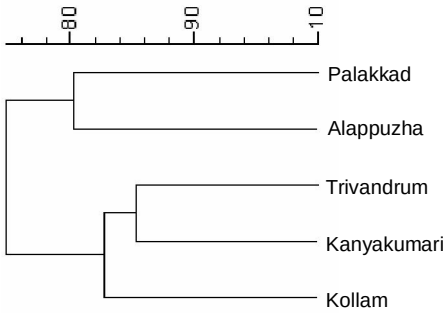

Figure E: Showing dendrogram generated from the RAPD profile of *C. halicacabum* populations with operon primers using UPGMA cluster analysis

Each DNA band was treated as a unit character and each population was scored for the presence or absence of band. Genetic similarity (GS) was analyzed using the equation

$$GS = \frac{Nab}{(Na + Nb - Nab)}$$

Where N_{ab} is the number of shared fragments between populations 'a' and 'b'. 'N_a' is the number of scored fragments of population 'a' and N_b, the number of scored fragments of population 'b'. Genetic distance (GD) was then calculated as

$$GD = 1\text{-}GS$$

Genetic distance among different populations of *C. halicacabum* were determined based on unweighted method (Jaccard similarity coefficient). The similarity indices were calculated between pairs of genotypes among the five accessions from the RAPD band data for all the primers cumulatively. Based on the unweighted method, the genetic distance among different populations of *C. halicacabum* varied from 0.261 to 0.161. The genetic similarity and genetic distance is represented in Tables 2 and 3. The results of both UPGMA cluster based on Jaccard similarity coefficient and pairwise distance analysis agree with each other.

Table 1: Showing nucleotide sequence, number of polymorphic and monomorphic bands and percentage of polymorphism for each primer used.

Primer	Sequence	Polymorphic Bands	Monomorphic Bands	Percentage polymorphism
OPD-7	5' TTGGCACGGG 3'	0	7	0
OPD-13	5' GGGGTGACGA 3'	0	9	0
OPH-13	5' GACGCCACAC 3'	1	11	8.3
OPF-6	5' GGGAATTCGG 3'	1	10	9.09
OPG-13	5' CTCTCCGCCA 3'	1	8	11.11
OPD-2	5' GGACCCAACC 3'	1	5	16.6
OPC-3	5' GGGGGTCTTT 3'	1	5	16.66
OPG-18	5' GGCTCATGTG 3'	1	3	25
OPA-12	5' TCGGCGATAG 3'	2	5	28.57
OPA-1	5'CAGGCCCTTC 3'	1	2	33
OPF-19	5'CCTCTAGACC 3'	3	6	33.33
OPG-8	5' TCAGGTCCAC 3'	3	5	37.5
OPH-2	5' TCGGACGTGA 3'	3	4	42.85
OPH-20	5' GGGAGACATC 3'	4	5	44.4
OPC-8	5' TGGACCGGTG 3'	1	1	50
OPD-1	5' ACCGCGAAGG 3'	4	4	50
OPH-4	5' GGAAGTCGCC 3'	3	3	50
OPH-5	5' AGTCGTCCCC 3'	3	3	50
OPH-12	5' ACGCGCATGT 3'	4	4	50
OPA-3	5'AGTCAGCCAC 3'	4	3	57.14
OPH-3	5' AGACGTCCAC 3'	4	3	57.14
OPF-12	5' ACGGTACCAG 3'	4	3	57.14
OPF-4	5' GGTCATCAGG 3'	5	2	71.4
OPA-8	5'GTGACGTCGG 3'	4	1	80
OPH-14	5' ACCAGCGTGG 3'	5	1	83.3

Table 2: Showing genetic similarities (GS) among populations of *C. halicacabum* based on unweighted method

	Trivandrum	Kanyakumari	Kollam	Alappuzha	Palakkad
Trivandrum	0.000				
Kanyakumari	0.839	0.000			
Kollam	0.878	0.838	0.000		
Alappuzha	0.878	0.793	0.865	0.000	
Palakkad	0.742	0.754	0.739	0.817	0.000

Table 3: Showing genetic distance (GD) among populations of *C. halicacabum* based on unweighted method

	Trivandrum	Kanyakumari	Kollam	Alappuzha	Palakkad
Trivandrum	0.000				
Kanyakumari	0.161	0.000			
Kollam	0.122	0.162	0.000		
Alappuzha	0.122	0.207	0.135	0.000	
Palakkad	0.258	0.246	0.261	0.183	0.000

4. Discussion

Medicinal plants contain active compounds as secondary metabolites (Subramoniam, 2014). The availability of these compounds varies according to the environment and genotype. *C. halicacabum* is distributed throughout India as "DNA based markers are increasingly being recognized as useful tools for assessing genetic diversity among germplasm". The genotypic variation between the populations can be figured out by molecular fingerprinting techniques like RAPD and AFLP (Tanksley *et al.*, 1989). As AFLP method is proven to be time consuming and costly, RAPD analysis could help in tracing the affinities of different accessions within a species. The profile of amplified DNA primarily depends on nucleotide sequence homology between the template DNA and oligonucleotide primer at the end of each amplified product. Nucleotide variation between different sets of template DNAs will result in the presence or absence of bands because of changes in the priming sites. Sequence Characterized Amplified Regions (SCARs) analysis of RAPD polymorphisms (Paran and Michelmore, 1993) showed that one cause of RAPD polymorphism is chromosomal rearrangements such as insertions/deletions. Therefore, amplification products from the same alleles in a heterozygote differ in length and will be detected as presence and absence of bands in the RAPD profile (Skroch and Nienhuis, 1995). In addition, the polymorphisms of RAPD markers were observed as different-sized DNA fragments from amplification. The profile of RAPD bands is similar to that of low stringency minisatellite DNA fingerprinting patterns and is, therefore, also termed RAPD fingerprinting. On average, each primer directs amplification of several discrete loci in the genome so that allelism is not distinguishable in RAPD patterns. In other words, it is not possible to distinguish whether a DNA segment is amplified from a locus that is heterozygous or homozygous. RAPD markers are therefore dominant. The UPGMA dendrogram for five selected populations of *C. halicacabum* showed two clusters. Accessions from Trivandrum, Kanyakumari and Kollam were grouped in one cluster whereas populations from Alappuzha and Palakkad in the other cluster. Our RAPD studies on these populations indicated only 38% of polymorphism. It has been found that the genetic distance among the populations was ranging from 0.261-0.161, indicating high similarity between populations. The polymorphic patterns among the samples subjected to RAPD analysis revealed low genetic variations present in *C. halicacabum* samples selected. We presume that habitat fragmentation under human disturbance might be a factor leading to the low genetic variation observed.

5. Conclusions

DNA based markers are increasingly being recognized as useful tools for assessing genetic diversity among germplasm. Being one of the plants in Dasapushpa, the detection of adulteration of the plant *C. halicacabum,* in Ayurveda drug industry is critical. RAPD analysis provided a molecular method for analyzing the genetic polymorphism among populations of *C. halicacabum*. This is the first report of partitioning of genetic variability within and among wild populations of *C. halicacabum* in Kerala, at the DNA level.

Acknowledgement

The authors express their gratitude to Dr.M.Radhakrishna Pillai, Director, Rajiv Gandhi Centre for Biotechnology for the facilities provided for the research. We thank Mr. Sreejith, Greeshma, and Sheena for their careful editing and reviewing of the manuscript. We also thank Miss. Ciji Varghese for her technical assistance throughout this work. The fund provided by Department of Biotechnology, Govt.of India, is gratefully acknowledged. We thank, Dr. M.N. Sheela, Principal Scientist, CTCRI, Sreekaryam, TVM for the critical evalution of the manuscript and suggestions.

Conflict of Interest

We declare that we have no conflict of Interest.

References

Ahmed, I. A. M. and Ahmed, A. (1993). Chemical investigation of the genus *Cardiospermum halicacabum* of the family sapindaceae. Lahore: Sci. Int.

Annadurai, A.; Elangovan, V.; Velmurugan, S. and Ravikumar R. (2013). Preliminary phytochemical screening and antibacterial of *Cardiospermum halicacabum* L. Adv. Appl. Sci. Res., **4**: 302-308.

Annamalai, A.; Christina, V.L. and Lakshmi, P.T.V. (2012). Evaluation of genetic diversity in *Cardiospermum halicacabum* based on Geographical variation using RAPD markers. Phcogcommun., **2**: 62-65.

Asha, V. V. and Pushpangadan, P. (1999). Antipyretic study of *Cardiospermum halicacabumLinn*. Indian J. Exp. Bio., **37**: 411-414.

Cheng, K. T.; Chang, H. C.; Su, C. H. and Hsu, F. L. (1997). Identification of dried rhizomes of *Coptis* species using random amplified polymorphic DNA. Bot. Bull. of Acad. Sinica., **38**: 241-244.

Cheng, K. T.; Su, C. H.; Chang, H. C. and Huang, J. Y. (1998). Differentiation of genuine and counterfeits of *Cordyceps* species using random amplified polymorphic DNA. Planta Med., **64**: 451-453.

Bousquet, J.; Simon, L. and Lalonde, M. (1990). DNA amplification from vegetative and sexual tissues of trees using polymerase chain reaction. Can. J. Forest Res., **20**: 254-257.

Demeke, T.; Adams, R. P. and Chibbar, R. (1992). Potential taxonomic use of random amplified polymorphic DNA (RAPD): A case study in *Brassica*. Theor. Appl. Genet., **84**: 990-994.

dos Santos, J. B.; Nienhuis, J.; Skroch, P.; Tivang, J. and Slocum, M. K. (1994). Comparison of RAPD and RFLP genetic markers in determining genetic similarity among *Brassica oleracea* L. genotypes.Theor. Appl. Genet., **87**: 909-915.

Ferrara, I. S. O. and Montesano, D. (1996). Triterpanoids from *Cardiospermum halicacabum* L. Phytother. Res., **10**: 192-194.

Helentjaris, T.; King, G.; Slocum, M.; Siedenstrang, C. and Wegman, S. (1985). Restriction fragment length polymorphisms as probes for plant diversity and their development as tools for applied plant breeding. Plant Mol. Biol., **5**: 109-118.

Lee, M. (1995). DNA markers and plant breeding programmes. Adv. Agron., **55:** 265-344.

Mona, R.; Ajay, K.; Punam, D. and Rajiv, R. (2014). *In vivo* investigation of the neuro-protective projection of the *Cardiospermum halicacabum* Linn. Int. J. Pharm. Sci., **6**: 64-66.

Paran, I. and Michelmore, R. W. (1993). Development of reliable PCR-based markers linked to downy mildew resistance gene in lettuce. Theor. Appl. Genet., **85**: 985-993.

Raman, N.; Radha, A. and Ravi, M, (1998). Antibacterial activity of Cardiospermum *halicacabum* against human and plant pathogens .Indian drugs, **35**;29-31.

Rao, N.V.; Prakash, K.C. and Shantakumar, S.M. (2006). Pharmacological investigation of *Cardiospermum halicacabum* Linn. in different animal models of diarrhea. Indian J. Pharmacol., **38**: 346-349

Sadique, J.; Chandra, T.; Thenmozhi, V. and Elango, V. (1987). Biochemical modes of action of *Cassia occidentalis* and *Cardiospermum halicacabum* in inflammation. J. Ethnopharmacol., **19**: 201-212.

Shaw, P. C. and Butt, P. P. H. (1995). Authentication of *Panax* species and their adulterants by random primed polymerase chain reaction. Planta Med., **61**: 466-469.

Sheeba, M.S. and Asha, V. V. (2009) *Cardiospermum halicacabum* ethanol extract inhibits LPS induced COX-2, TNFα and iNOS expression which is mediated by NF-Kb regulation in RAW 264.7 cells. J. Ethnopharmacol., **124**: 39-44.

Sheeba, M.S. and Asha, V. V. (2006). Effect of *Cardiospermum halicacabum* on Ethanol-induced gastric ulcers in rats. J. Ethnopharmacol., **106**: 105-110.

Subramoniam, A. (2014) Present scenario, challenges and future perspectives in plant based medicine development. Ann. Phytomed. **3**(1):31-36.

Skroch, P. and Nienhuis, J. (1995). Impact of scoring error and reproducibility of RAPD data on RAPD based estimates of genetic distances. Theor. Appl. Genet., **91**:1086-1091.

Tanksley, S. D.; Young, N. D.; Paterson, A. H. and Bonierbale, M. W. (1989). RFLP mapping in plant breeding: New tools for an old science. Biotechnol., **7**:258-264.

Welsh, J. and McClelland, M. (1990b). Fingerprinting genomes using PCR with arbitrary primers. Nucleic Acids Res., **18**: 7213-7218.

Welsh, J.; Liu, J. P. and Efstratiadis, A. (1990a). Cloning of PCR-amplified total cDNA: Construction of a mouse oocyte cDNA library. Genet. Anal. Tech. Appl., **7**:05-17.

William, J. G.; Kubelik, A. R.; Livak, K.J.; Rafalski, J. A. and Tingey, S.V. (1990). DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Res., **18**: 6531-6535.