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COMPARATIVE ANALYSIS OF ANTICANCER ACTIVITY OF *RUBIA CORDIFOLIA* L. & ADULTERANT ON MCF-7 CELLS AND SCAR MARKER DEVELOPMENT

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ABSTRACT: *Rubia cordifolia* Linn. (Rubiaceae) commonly known as Indian Madder, Majith, Manjistha etc. is widely dispersed throughout the lower hills of Indian. The plant is famous important drug in the Ayurvedic treatments. Extracts of this plant have shown many important medicinal properties. In this study we compared the therapeutic potential of methanolic extract of *R. cordifolia* Linn. and its adulterant on breast cancer cell line MCF-7. The adulterant exhibited significantly low anticancer activity in comparison to the authentic *R. cordifolia* as authentic plant showed IC_{50} value 400 µg/mL while the adulterant have not showed any significant cytotoxicity to the same. For the correct identification of the *R. cordifolia* we developed RAPD based easy and cheap SCAR markers (Rb-F and Rb-R). Developed SCAR markers were also validated with respect to the market samples.
Key words: *Rubia*; medicinal plant; RAPD; SCAR; MCF-7

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INTRODUCTION

Rubia cordifolia Linn. from the family Rubiaceae (commonly known as Indian Madder, Majith, Manjistha etc.) is widely dispersed throughout the lower hills of Indian Himalayas [1, 2]. The plant is well known and important drug in the Ayurvedic treatments. Extracts of this plant have shown hepatoprotective, antineoplastic properties and is also useful in the disintegration and elimination of urinary stones [3, 4]. Anti-inflammatory, anti-ulcer and anti-dysentric activities are also found in the roots of *Rubia cordifolia* Linn. [5]. It is also used in the treatment of diuretic, liver complaints, joint pains, uterine pains, in Rheumatoid arthritis [1].

The major compounds of this plant are anthraquinones alizarin and purpurin and their derivatives, ruberythric acid (alizarin-primeyeroside), pseudopurpurin and lucidinprimeveroside, rubiadin (1,3-Dihydroxy-2-methylanthracene-9,10-dione), munjistin, quinizarin, lucidin and 1,8-dihydroxyanthraquinone [6].

Apart from the above mentioned medicinal properties of this plant, its cytotoxic activity on cancer cell lines have also been studied previously. Because medicinal plants are generally replaced by an adulterant plant in the market due to their easy availability and low cost, the key question is how to identify a genuine plant? This is crucial, since adulterant plants possess low therapeutic potential. In the present study, we evaluated the cytotoxic property of the authentic plant on breast cancer cell line (MCF-7) and simultaneously compared the same with the extract of the market sample.

In this era, the authenticity of the medicinal plants is a key question where the genuine plants are replaced by an adulterant in the market due to their low cost and easy availability. The molecular identification achieved the highest interest and success due to its high specificity and sensitivity in identifying the genuine plant as other parameters like morphology or chemical constituents are governed by geographical area that may be found similar in plant species from the same area. In molecular level of identification of plants DNA-based molecular markers are routinely used. Beside the identification purpose DNA-Based molecular markers can also be used for the assessing genetic diversity in plants and for the up gradation of the plant species. Medicinal plants can be efficiently authenticated with the help of PCR based methods, including Randomly Amplified Polymorphic DNA (RAPD) [7, 8]. RAPD analysis has been applied in herbal medicine to discriminate between species in various genera [9]. In spite of several advantages like high speed, low cost, and the requirement of small amount of plant material RAPD also have a disadvantage that is its reproducibility which relies on PCR conditions.To prevail over this problem, it has to be converted into sequence characterized amplified region (SCAR) marker [10]. By the conversion of the RAPD marker into more specific, sensitive and reproducible markers like Sequence Characterized Amplified Region (SCAR) its industrial application can be increased tremendously. Because, the SCAR marker amplifies only specific RAPD marker fragment, it overcome the problems that have been commonly faced with RAPD markers, like amplification of a large number of non-specific fragments as well as overlapping fragments of similar sizes [11].

Briefly, in this study we compared the anti-breast cancer activity of *R. cordifolia* with its adulterant on MCF-7 cells simultaneously RAPD based SCAR marker was also developed for the correct identification of this plant.

MATERIAL AND METHODS

Plant material

Six different accessions of *Rubia cordifolia* L. were used in this study. Roots and aerial parts of plant were collected from six different geographical areas (Table. 1) of India. These samples were identified by Prof. M. P. Sharma, Head, Department of Botany, Jamia Hamdard. Voucher specimen is deposited at the herbarium of Department of Botany, Jamia Hamdard. New Delhi. Further identification of the plant was also done with the morphological examination of the T.S of mature root of the plant (Figure 1)

(A) Cytotoxic Study

Chemicals:

All the cell culture reagents were purchased from HiMedia Laboratories (USA). 3-(4, 5-dimethyl thiazol-2-yl)-5-diphenyl tetrazolium bromide (MTT) was purchased from Sigma (USA). MCF-7 cell line was procured from National Center for Cell Science (NCCS), Pune, India.

MTT Assay:

The extract of *R. cordifolia* was tested for in-vitro cytotoxicity on MCF-7 cells using MTT assay. The living cells reduces the yellow dye 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-terazolium bromide (MTT) to a blue formazan product which can be read calorimetrically after dissolving in DMSO. Briefly, the human breast cancer cell line MCF-7 cells were grown in DMEM media containing 10% Fetal Bovine Serum (FBS) at 37°C and 5% CO_2 in a humidified chamber**.** The cells were placed on 96 wells plate at a confluency of 10,000 cells per well and incubated for 24 hours before the testing of the extract. Then the cells were treated with different concentrations of the test compound (100, 200, 300, 400 and 500 µg/ml), ethanol (0.5%) for 24 hr. At the end of this period, 20 µl of MTT stock solution (5 mg/ml, Sigma, St. Louis, MO) was added to each well and the plates were further incubated for 4 hour at 37°C. The supernatant was removed and 100 µl of DMSO were added to each well to solubilize the water-insoluble purple formazan crystals. Then the absorbance was measured at 550 nm with Micro Plate Reader-spectra max 340 PC, Molecular devices, California, USA. All the measurements were performed in triplicate. Results are expressed as the percentage cell viability. The percentage cell viability was calculated by using the following formula:

Percentage cell viability = Abs (Sample) / Abs (Control) x 100.

(B) Molecular study

Genomic DNA isolation:

Sample preparation:

Dried root samples of *R. cordifolia* were chopped into small pieces and treated with ethanol in low to high concentration (10%, 30%, 50%, 70% & 90%) for removing excess dye content. After that these samples were kept overnight in water to make them soft than these roots sample were pressed to remove excess water, these soft roots were used for genomic DNA isolation.

DNA Extraction:

Genomic DNA of root sample of *R. cordifolia* was isolated by CTAB extraction method of Doyle and Doyle [12] with some modifications.

4.0 g of root sample was ground in liquid nitrogen to a fine powder. 15 mL of pre-warmed (65 ºC) 4 x CTAB buffer [4% CTAB, 1.4 M NaCl, 0.5 M EDTA(18.61 gm/100 mL) pH-8, 0.5 M TrisHCl (15.76 gm/100 mL) pH-8] and 0.2% β-mercaptoethanol was added just before use. The homogenate was incubated at 65ºC in a water bath for 45 min with intermittent mixing. Equal volume of Phenol: Chloroform: Isoamyl alcohol (25:24:1) was added and mixed well by inverting the tubes 3-4 times. The suspension was then centrifuged twice at 10000 rpm for 15 min at 25ºC. The aqueous phase was transferred to a fresh tube and 5 µL of RNase (50 mg/mL) was added to the aqueous phase and incubated at 37ºC for 1 hour. The aqueous phase was extracted again by adding equal volume of Chloroform/ Iso amyl alcohol (24:1). The suspension was mixed well and then centrifuged for 15 min at 4ºC at 10000 rpm. The aqueous phase was again transferred to a fresh tube. One volume of chilled Isopropanol was added to the aqueous phase and incubated at -20ºC for overnight for precipitation of DNA. DNA was pelleted by centrifugation at 10000 rpm for 15 min at 4ºC. DNA pellet was then washed with washing solution (70 % ethanol, 2M ammonium acetate) and air dried. The pellet was dissolved in appropriate volume (200-300 µL) of TE buffer or sterile MilliQ water. The DNA solution was stored at 4ºC for immediate use or at -20ºC for prolonged storage.

PCR Screening:

40 random primers of OPAA series (Table. 2) were used for the PCR screening of genomic DNA of all six accessions of *R. cordifolia*. Out of which 10 primers showing clear and reproducible polymorphic patterns in preliminary trials were selected to analyze the further RAPD.

PCR reactions were carried out in the 15 µl reaction volume containing (5U µl-1) Taq polymerase, 1X Taq buffer,(10 mM)$MgCl_2$, 10mM dNTPs (2.5 mM each), 10 µM of each primer and 50 ng/µl of DNA template.Amplification was performed in a thermal cycler ($T100^{TM}$ Thermal Cycler, Bio-Rad, USA).PCR conditions were set as: initial denaturation 95°C for 4 min, denaturation at 94°C for 30 sec, annealing at 34°C for 45 sec, extension at 72°C for 50 sec, and final extension at 72°C for 2 min and infinite hold on 4°C.Amplified PCR products (15µl) along with 2µl 6X bromophenol dye were loaded on 1-1.2% agarose gel containing ethidium bromide (0.5 µg ml-1) and electrophoresed on 100 volts for one and half hour. The amplified DNA was visualized on UV lamp and photographed under UV light using gel documentation system Alphalmager EC (USA).

Cloning and Sequencing of the Polymorphic Band:

The putative marker amplified by the random primer OPAA-04 was eluted from 1.2% agarose gel with sterile gel slicer and purified by using Gel Extraction Kit (Nucleopore – Genetix) was cloned using the pGEM-T easy vector I (Promega, U.S.A.).The ligated plasmid was introduced into *Escherichia coli* strain DH_{10B}, following the protocols for preparing competent cells and transformation using the calcium chloride method Saad et al. (2006) [13]. White colonies were picked from LB/ ampicillin/ X-gal/IPTG plates and grown overnight in LB medium. The plasmid DNA was isolated from the bacterial culture using plasmid isolation kit (RBC Real $Genomics^{TM}$, Taiwan). The inserted fragment was sequenced at Ist Base sequencing, Singapore with SP6 and T7 primer. Nucleotide sequence of 580 bp RAPD amplicon, specific for all the six accessions of *R. cordifolia*, was used for designing primers for development of SCAR marker.

Analysis of Sequence Data:

The DNA sequence was submitted to Gene Bank. Homology searches were performed within Gene Bank's non-redundant database using the BLAST 2.2.8 algorithm at http://www.ncbi.nlm.nih.gov/BLAST of the National Center for Biotechnology Information (NCBI), with the programme BLAST.

1 TAATCGGGCTGATGTATTCAAAGAACCCACCTAAACTACCTAAACTTTTTAGGCTTGTGT

61 TCTTATCAGGGCTCTTCTCTTTTTTCTTGGTAAGGGGTGTTTTTTAGGGGAGGGTTGAAT

121 GGACAAGAATGCAAGAATCATACCTCCTGATAAATCTCTAGCATGTTTGTTAAGTTGTCG

181 GCCTGGTCATAGTGGATGAGCCACGCCCCACAGGGATTATGCTTGGAAGACTCTCAACAG

241 AGTCATGTACTTCAACTTGTAATGAATCTGCAGAACATTAGGATAATGCCGTGACCAACT

301 AATCAAAAGAGTCTACAATACTTTTCATCATAGAATGTATACACCCCAAGATGCATAGTT

361 TCTAATAGCGAAGAATGAAGCAGTAAACTTTTTATATTTAAGATTTGATTATGTAGCTAA

421 ACCTAATTCTGAGGCCCAACAATTCAGAGCTATATGCAATTTATAAACCTCAATAGTCCC

481 AATTCTGAGAACTGATGTAGATTAGCAATCATATATCACCTGAAACTTCTTCTCCAGCCC

SCAR Primer Designing and Validation:

Based on the sequence of unique RAPD amplicon a pair of 21-20 bp oligonucleotide primers (Rb-F and Rb-R), defining a SCAR for each of the cloned fragments was designed and synthesized for specific amplification of the loci identified by the RAPD markers(Table. 3).

PCR reactions were carried out in the 15 µl reaction volume containing (5U µl-1) Taq polymerase, 1X Taq buffer,(10 mM)$MgCl_2$, 10mM dNTPs (2.5 mM each), 10 µM of each primer (SCAR-forward and SCAR-reverse) and 50 ng/µl of DNA template.Amplification was performed in a thermal cycler (T100^{TM} Thermal Cycler, Bio-Rad, USA). PCR conditions were set as: initial denaturation 95°C for 4 min, denaturation at 94°C for 30 sec, annealing at 58°C for 45 sec, extension at 72°C for 50 sec, and final extension at 72°C for 2 min and infinite hold on 4°C. Amplified SCAR product along with 2µl 6X bromophenol dye were loaded on 1-1.2% agarose gel containing ethidium bromide (0.5 µg ml-1) and electrophoresed on 100 volts for one and half hour. The amplified product was visualized on UV lamp and photographed under UV light using gel documentation system Alphalmager EC (USA).

Commercial crude drug sample of *R. cordifolia* was collected from the market, Delhi. DNA was isolated from the above sample using the above isolation method. The gene pool of the sample was then put to test with the above SCAR primers for validation.

RESULTS

(A) Cytotoxic study

In this study root extract of authentic *R. cordifolia* plant showed significant cytotoxic activity against the MCF-7 breast cancer cell line with IC_{50} value of 400 µg/mL while the root extract of market sample of the plant unable to show the substantial cytotoxicity to the MCF-7 cells (Figure 2).

(B) Molecular study

DNA extraction and RAPD:

High molecular weight genomic DNA was isolated from the dried plant samples. The DNA extraction procedure yielded 600–800 ng of DNA per 100 mg of tissue. An absorbance (A260/A280) ratio of 1.6–1.7 indicated contamination of proteins and polysaccharides which was overcome by the PCI (25:24:1) treatment after that the absorbance (A260/A280) ratio of DNA samples came to 1.7-1.8 which was highly purified.

40 RAPD 10-mer primers were used for screening of all the accessions of the genuine plant (*R. cordifolia*) and its adulterants. Primers (OPAA 01, OPAA 03, OPAA 04, OPAA 09, OPAA 11, OPAA 12, OPAA 13, OPAA16, OPAA 18 and OPAA 20) produced good quality, reproducible fingerprint patterns and showed a high level of consistency.

A total of 500 DNA fragments of 200-1500 bp were obtained. Confirmation of results was done using three different samples of each accession. Experiment was repeated three times to confirm reproducibility of RAPD pattern under the same conditions of composition of reaction volume, amplification profile and thermal cycler. Primer OPAA-04 consistently amplified an intense 580 bp band that was unique to all the accessions of *R. cordifolia* (Figure 3).

Table-1: List of Plant materials (*Rubia cordifolia* Linn.) used in this study

Plant name	Explant	Sample	Locality
Rubia cordifolia Linn.	Aerial part	Rb-1	IHBT(Palampur)
	Aerial part	Rb-2	Indus nursery (Bangalore)
	Root	Rb-3	Chopta forest (Himalyas)
	Root	Rb-4	University of Kashmir (Srinagar)
	Root	Rb-5	Chamba (Himachal Pradesh)
	Root	Rb-6	Hamdard laboratory (Ghaziabad)

Table-2: Nucleotide sequences of selected primers with the number of amplified products and fragment size range (bp).

Primer code	Primer Sequence (5′-3′)	No. of amplified products	Fragment size
OPAA-01	CCGGCCCTTC	32	200-800
OPAA-02	TGCCGAGCTG	30	220-900
OPAA-04	AATCGGGCTG	42	310-750
OPAA-06	GGTCCCTGAC	25	280-730
OPAA-07	GAAACGGGTG	20	400-600
OPAA-09	GGGTAACGCC	34	310-700
OPAA-11	CAATCGCCGT	20	400-1000
OPAA-13	CAGCACCCAC	38	420-1200
OPAA-16	AGCCAGCGAA	32	450-1500
OPAA-17	GACCGCTTGT	26	200-810
OPAA-19	CAAACGTCGG	24	425-1055
OPAA-20	GTTGCGATCC	29	300-850
BG-24	AAGCCTCGTC	38	280-800
BG-26	TGCGTGCTTG	22	440-1300
BG-28	GACGGATCAG	33	430-1400
BG-29	CACACTCCAG	20	290-950
BG-30	TGAGTGGGTG	24	435-1025

Table-3: Details of the *Rubia cordifolia* Linn. specific SCAR marker designed from the 560-bp polymorphic sequence.

Name of Random decamer primer used	Sequence of random decamer primer (5′-3′)	Name of the SCAR primer	Sequence of the SCAR primer (5′-3′)	G + C content (%)	Annealing temperature(°C)
OPAA-04	AATCGGGCTG	Rb-F	GGCTTGTGTTCTTATCAGGGC	52.3%	58°C
		Rb-R	GCTCTGAATTGTTGGGCCTC	55.0%	58°C

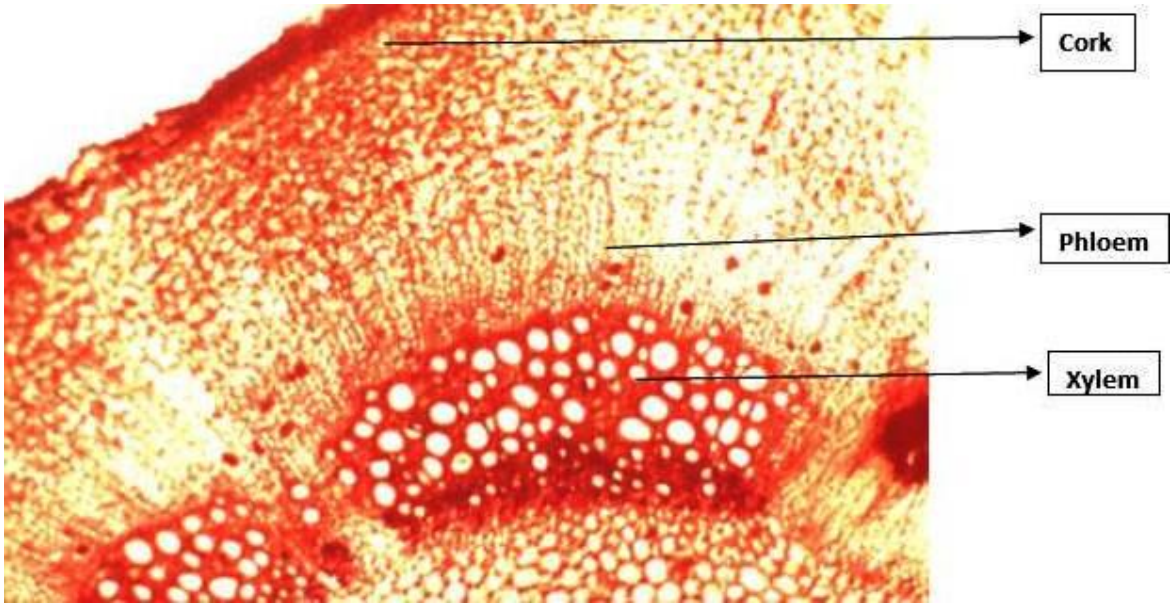

Figure-1: A portion T.S of mature root of *Rubia cordifolia* Linn.

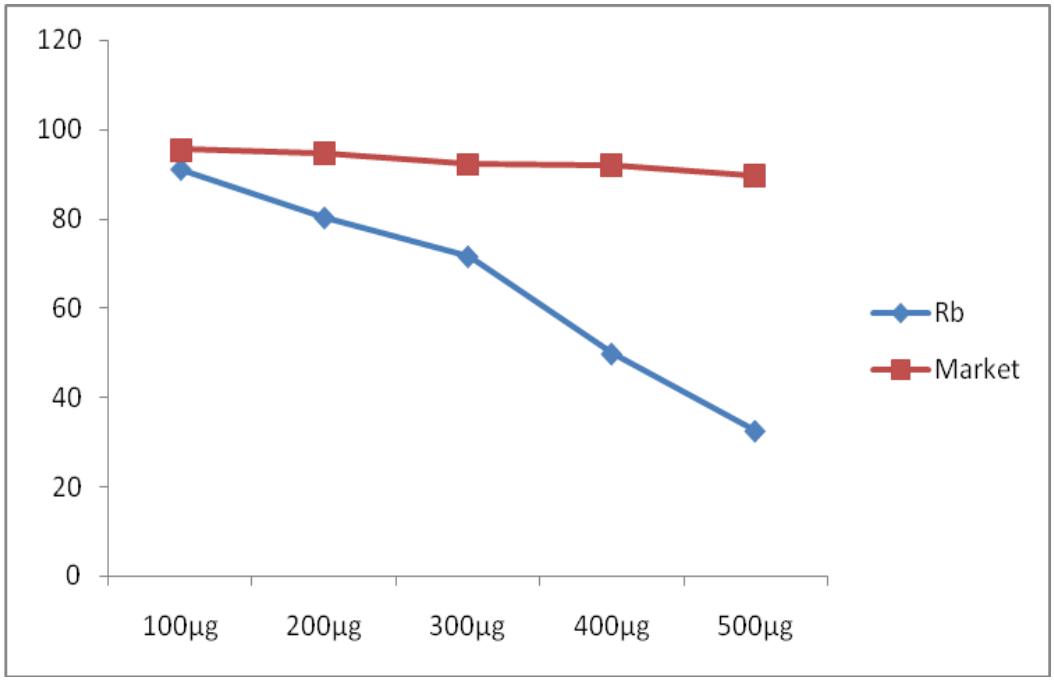

Figure-2: Comparative analysis of cytotoxicity of methanolic extract of *R. cordifolia* L. with respect to the market sample. Rb- *R.cordifilia* L. (authenticated) & Market- market sample of the plant

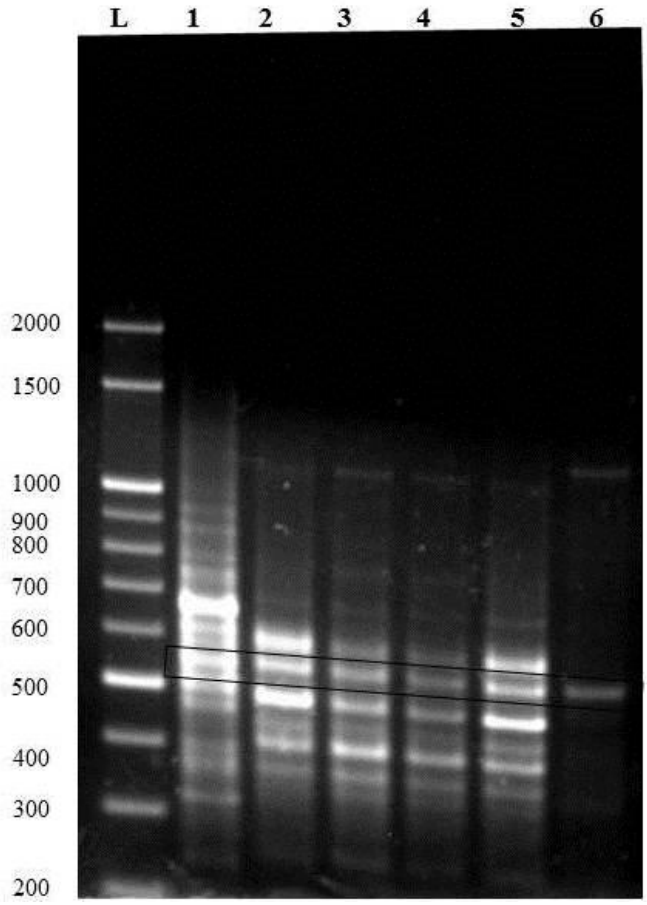

Figure-3: RAPD pattern with Primer-04
L-DNA Marker, 1- 6 DNA Samples of *Rubia cordifolia* L.

Cloning and Sequencing of Polymorphic Band:

A polymorphic band (580 bp) that was specific in all the accessions of *R. cordifolia* was selected. This specific band was eluted, cloned and sequenced. Restriction digestion from the *EcoR1* and *Spe1* enzymes revealed 580 bp band on 1.2% agarose gel that confirmed the presence of the insert of the desired gene in the vector. The sequencing of this recombinant construct was done by using SP6 and T7 primers.

Validation of SCAR Primers:

The genomic DNA of all the accessions of *R. cordifolia* and the market sample was amplified by the SCAR primers (Rb-F and Rb-R) for the applicability of the SCAR marker and for the molecular authentication of the plant. A single, bright and distinct band of 400 bp was obtained only from the genomic DNA of the authenticated accessions of the plant and no amplification product was obtained from the genomic DNA of the market sample of plant (Figure 4). This confirms the specificity and the sensitivity of the SCAR marker for the *R. cordifolia.* Further these results confirm that these SCAR markers can be used for the molecular identification of this plant.

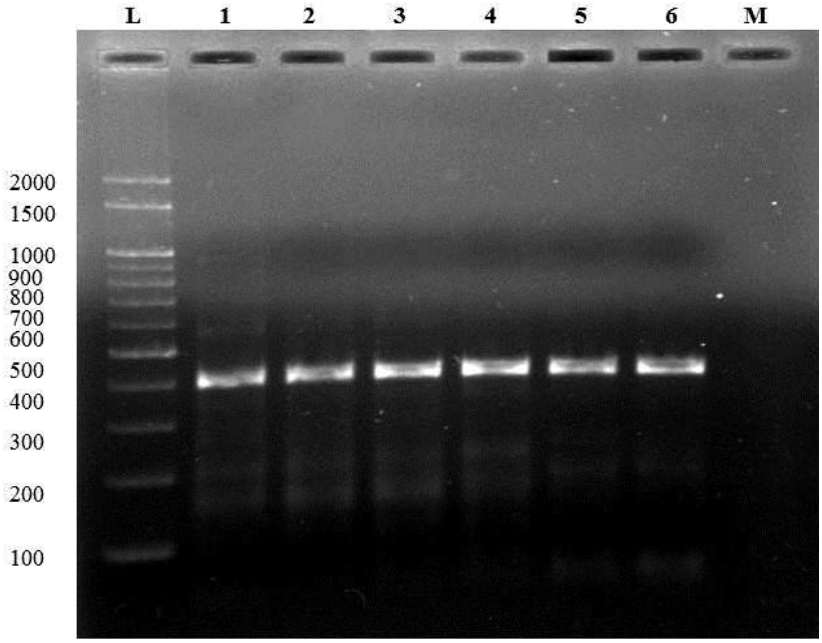

Figure-4: PCR amplification of *Rubia cordifolia* Linn. and market sample with SCAR (Rb-F & Rb-R) primers
L-DNA Marker, 1- 6 DNA Samples of *R. cordifolia,* M -Market sample

DISCUSSION

The methanolic extract of the authentic plant material has been successfully evaluated for its therapeutic potential on cancerous cells MCF-7 and showed the significant cytotoxicity, while the extract of the roots of market sample was unable to significantly kill the cancer cells. Therefore, our study successfully evaluated the comparison between the cytotoxic activities of the methanolic extract of authentic sample of *R.cordifolia* and market sample of *R.cordifolia*.

There are various studies that have already been reported showing the anticancer activities of different fractions of *R. cordifolia* roots extracts. The aqueous extract of *R. cordifolia* showed anti-proliferative activity on A549, LLC, Panc-1, Panc02, PC-3, LNCaP, MCF-7, MCNeuA as well as on Normal human mammary epithelial cells (HuMEC) [14].The extract of *R. cordifolia* showed cytotoxicity to the HEp-2 cell line and induced apoptosis through the elevation of reactive oxygen species generation [15]. The extract also showed anti-proliferative activity to human colon carcinoma (HT-29), human breast carcinoma (MCF-7) and human liver carcinoma (HepG 32) cell lines [16, 17]. The ethanolic fraction of the plant showed effective inhibition of human cervical cancer cell line [18]. The root extract of the plant through dichloromethane fraction displayed inhibition of human leukemia cell line and human histolytic lymphoma cell line. The ethanolic extract of plant showed cytotoxicity to human larynx carcinoma [17]and human cervical cancer whereas methanolic extract of the leaf of plant showed cytotoxicity to MCF-7 cell line [16].

For the correct identification of the genuine plant on molecular level we successfully developed the RAPD based SCAR marker for the *R. cordifolia*. For this, six accessions of the plant were used, as it is the high altitude plant and having very low availability, we were able to arrange only six accessions. As the roots of the *R.cordifolia* also content high amount of alizarin which is used as a red dye in textile industry and the plant samples we used are dried too. So, that isolation of good quality genomic DNA is a tedious job but we successfully isolate the ample amount of genomic DNA by doing some modifications in the CTAB method of Doyle and Doyle [12].Thus, establishing a consistent DNA fingerprinting for the dried root samples of the *R. cordifolia* is very significant for SCAR analysis. In our RAPD analysis significant genetic polymorphism was observed among *R. cordifolia* and its adulterants. The SCAR primers designed using this sequence variation was found to be specific for *R. cordifolia* making the technique more stringent and specific when compared with RAPD marker.

However at molecular level some works have already been reported on the genus *Rubia*. In *Rubia tinctorum* genetic diversity has been reported on the basis of agro-morphological traits, phytochemical content and RAPD markers [19]. But for the first time, our study involved molecular level of *R. cordifolia*, in which successful polymorphism has been developed in different accessions of *R. cordifolia*. By using the developed RAPD pattern SCAR marker has also been developed for the correct identification of this plant. So, our study makes the base for the further research on molecular level of *R. cordifolia* as this part of plant is still untouched.

The authentication and discrimination of genuine medicinal plants from their adulterants are essential for public health and for pharmaceutical company too. Chemical and morphological approaches limitations generated a necessity for the use of molecular methods for the authentication of herbal formulations. However, in the literature there are many molecular markers available for the identification of medicinal plants from their adulterants. There are many DNA based markers like AFLP, SSR, ISSR and RAPD that can be used for the molecular authentication of the plants but among these markers RAPD based SCAR markers are cheap, less time consuming and easy to develop. A PCR-based marker like SCAR signifies genetically defined single locus by amplification of genomic DNA with a pair of specific oligonucleotide primers. Further, RAPD technique can be performed without the preceding genetic information of the plant and it is a simple and rapid technique. The main disadvantage of RAPD that is about its low reproducibility but this disadvantage can be overcome mainly by the successful conversion of RAPD to the SCAR marker. So that SCAR marker seems to be the preeminent, easy and cheap technology for the identification and authentication of the important medicinal plants. By using SCAR marker one can easily differentiate between the morphologically similar species as well as genuine plant from their adulterants.

CONCLUSION

In our study, we successfully evaluated the anti-breast cancer potential of *R. cordifolia* which exhibits significantly high cytotoxicity against breast cancer cells MCF-7 as comparison to its adulterant. Simultaneously RAPD based SCAR markers were successfully produced for the identification of genuine plant.

CONFLICT OF INTEREST

The authors have no conflict of interest.

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Abbreviations

R. cordifolia	*Rubia cordifolia* Linn.
PCR	Polymerase Chain Reaction
RAPD	Randomly Amplified Polymorphic DNA
SCAR	Sequence Characterized Amplified Region
CTAB	Cetyl Trimethyl ammonium Bromide
EDTA	Ethylene Diamine Tetra Acetic acid
PCI	Phenol Chloroform Iso-amyl Alcohol
BLAST	Basic Local Alignment Search Tool
DMEM	Dulbecco's Modified Eagle's Medium
MCF-7	Michigan Cancer Foundation-7

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