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Geographical variation in Chemo profile, Genetic profile and *In-vitro* Anticancer activity of *Chitraka* (*Plumbago zeylanica* Linn.) collected from Himalayas and Western Ghats

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ABSTRACT

Therapeutic actions of medicinal plants are due to the chemical components present in them. These chemical constituents often vary with change in geographical and climatic conditions. Climate, altitude, and temperature play an important role to make a herb therapeutically potent. Ancient Ayurvedic physicians mentioned that medicinal plants which grow in Himalayan region are more potent as compared to the plants growing in other regions. A modest study was therefore undertaken to compare the in vitro anticancer activity of *Plumbago zeylanica* Linn. collected from Himalayan region and Western Ghats with respect to their chemo profile and genetic patterns. Variation in physio-chemical, phyto-chemical, the genetic profiles were observed in plants collected from these two regions and corresponding variation was also seen in their anticancer activities.

Key words: *Plumbago zeylanica Linn., Geographical Variation, Chemo Profile, Genetic Profile, Invitro Anticancer Activity.*

INTRODUCTION

Climate is one of the major determinants of plant species distribution and, therefore, changes in climate and geographical location result in distributional shifts. Same plants grown in the different climates, different altitudes, are known to differ in the concentrations of their chemical constituents.[1] Like variation in morphology, chemo profile and genetic make up of plants growing in different geoclimatic locations may also differ. The chemical composition of genetically similar plants has been observed to vary ultimately affecting the pharmacological action.[2]

Ayurveda had long back considered these variations and described different factors to be considered before collection of the plant/crude drug for therapeutic use. Acknowledging these factors, Acharya Charaka had claimed that Himalayan region is the best habitat for collection of medicinal plants.[3],[4] Also it has been mentioned that plants growing in Northerly direction should be collected.[5],[6] However, unfortunately many of these variations and corresponding changes in the biological activities have not been scientifically validated using modern technologies.

A modest attempt has been made to understand the changes in chemical profiles, genetic characteristics and corresponding biological activity of *Chitraka* (*Plumbago zeylanica* Linn.), which is easily and abundantly available in Himalayan as well as Western Ghat and is used as a common ingredient in many Ayurvedic formulations for its anticancer activity.

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Materials and Methods

Chemo profile

Roots of Chitraka were collected from natural populations growing in Jammu (Himalayan region) and Belgaum (Western Ghats region) in the month of June and March as suggested in classics and were identified and authenticated by qualified plant taxonomist at KLEU'S Shri. B.M.K Ayurvedic College, Central Research Facility (AYUSH approved drug testing laboratory for ASU drugs). Voucher specimens (accession no.crf/214,crl/215) has been deposited to the herbarium of Central Research Facility of KLEU'S Shri.B.M.K Ayurvedic College. After collection the roots were washed, dried and powdered into course particles and preserved in the air tight containers for further studies.

The pharmacognostic and phytochemical studies of *Chitraka* were conducted in K.L.EU'S Shri. B.M.K Ayurvedic College, Central Research Facility. The sample was coded as A (Jammu) and B (Belgaum).

Macroscopical characterization

Macroscopical studies of root were carried out by naked eye examination for shape, color, taste and odor of roots were determined and reported.

Physico-chemical evaluations

Physico-chemical parameters of both the samples were determined and reported as total ash, and acid-insoluble ash values. Alcohol, water-soluble and hydro alcoholic extractive values were determined to find out the amount of water and alcohol soluble components. The moisture content was also determined by standard methods.

Preliminary phyto-chemical screening

Aqueous, alcoholic and hydro alcoholic extracts were prepared under reflux for 8 hours, and filtered. The filtrate was dried on water bath by taking both the samples (25g each), and solvents were taken in the ratio of 1:4. The total of six samples were screened for preliminary phytochemicals.

Thin layer chromatography (TLC)

Methanolic extracts of samples A and B along with standard marker Plumbagin (Sigma, USA) were subjected to thin layer chromatography (TLC) and solvent system was toluene (3) : chloroform (7) and was run upto 8 cm.

Genetic profile

Collection of plant material

A total of 10 samples were collected from five different places from Himalayan region (Jammu) and Western Ghats (Belgaum) and the samples were identified and authenticated by qualified taxonomist at RMRC Belgaum (Table 1). The voucher specimens have been deposited to the herbarium of Regional Medical Research Centre, Belgaum. Genetic profiling of *Plumbago zeylanica* samples was conducted at RMRC Belgaum.

Table 1: Plant samples and place of collection.

SN	Plant samples	Places of collection
1	Sample 1	Natural habitat Yellapur (Karnataka)
2	Sample 2	Natural habitat Kanbargi (Karnataka)
3	Sample 3	Natural Habitat Kankumbi (Karnataka)
4	Sample 4	Natural Habitat Sirshi (Karnataka)
5	Sample 5	Natural Habitat Dandeli (Karnataka)
6	Sample 6	IIIM Herbal garden (Jammu)
7	Sample 7	Natural habitat Samba (Jammu)
8	Sample 8	Natural Habitat Suchani (Jammu)
9	Sample 9	Natural habitat Basoli (Jammu)
10	Sample 10	Natural habitat Udhampur(Jammu)

DNA Isolation

This was done as per modified methodology of Doyle et.al. 1991.[7]

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Quantification of DNA

The quantity of extracted DNA was determined using Nanodrop 1000 spectrophotometer (JH Bio Sciences) at 260/280 nm. The quality of DNA was determined visually by carrying out horizontal gel electrophoresis with 2 µl of each sample loaded on 1% agarose gels stained with Gel Red (Biotium). The agarose gels were visualized and documented under gel documentation system (Syngene, UK).

DNA Finger printing

Random Amplified Polymorphic DNA (RAPD) and Inter-Simple-Sequence Repeat (ISSR) finger printing techniques were used for assessment of genetic differences among individual plants and to characterize individual plants.

RAPD Finger printing Assay[8]

RAPD finger printing assay was performed in 25 µl reaction volumes containing 50 ng genomic DNA, 0.5 µL of 10 mM primer (RPI Series, Bangalore Genie, India) 0.5 µL of 2.5 mM each of dNTP, 0.5µL of 3U/ µL of Taq DNA polymerase in 10 X Taq buffer containing Tris with 15 mM MgCl2 (Merck, India). The amplification reaction consisted of aninitial denaturation at 95°C for 6min followed by 28 cycles of denaturation at 94°C for 40 sec, annealing at 37°C for 60 sec, and extension at 72°C for 1 min with final extension at 72°C for 5 min.

ISSR Finger printing Assay

ISSR fingerprinting assay was performed in 25 µl volumes containing 50 ng genomic DNA, 0.5 µL of 10 mM of primer (UBC, Canada) 0.5 µL of 2.5 mM each of dNTP, 0.5µL of 3U/ µL of Taq DNA polymerase in 10 X Taq buffer containing Tris with 15 mM MgCl2 (Merck, India). Amplification was carried out in a BioRadiCycler Thermal cycler with aninitial denaturation at 94°C for 4 min followed by 45 cycles of denaturation at 94°C for 30 sec, annealing at 50°C for 45 sec, and extension at 72°C for 2 min. with final extension at 72°C for 4 min.

Documentation and Data Interpretation

The amplified products were electrophoressed on a 2% agarose gel, stained with Gel Red (Biotium, USA) and photographed using a gel documentation system (Syngene, UK). The presence and absence of each band was recorded and this exercise was repeated at least thrice for selection of the reproducible bands only. Reproducible bands were scored as 0 (absence) and 1 (presence) for analysis. Fig 2,3,4,5.

Anticancer activity

Anticancer activities of above mentioned extracts were studied at Indian Institute of Integrative Medicine (IIIM), Jammu where 5 cell lines, Colo – 205 (colon), M C F -7 (breast), HCT – 15 (colon), THP-1, PC–3 (prostate) were maintained and used in ideal laboratory conditions. The cell lines were grown in RPMI 1640 medium containing 10% fetal bovine serum and 2mM L-glutamine. Cells were inoculated into 96 well micro-titer plates. With 100 µL/well at appropriate plating densities, depending on the doubling time of individual cell lines. After cell inoculation, the microtiter plates were incubated at 37°C, in 5% CO_2, 95% air and 100% relative humidity for 24 hours prior to addition of experimental drugs.

After 24h, cells from one plate of each cell line were fixed *in situ* with TCA, to represent a measurement of the cell population for each cell line at the time of drug addition (Tz). Experimental extracts were solubilized in appropriate solvent (DMSO) 400-fold of the desired final maximum test concentration and stored frozen prior to use. At the time of drug addition, an aliquot of frozen concentrate was thawed and diluted 10 times the desired final maximum test concentration with complete medium. Aliquots of 10 µl of these different dilutions were added to the appropriate micro-titer wells already containing 90 µl of cell suspension, resulting in the required final drug concentrations of 10,30,50,70, and 100 µg/ml.

For each of the experiments a known anticancer drug was used as a positive control.

End point measurement

After addition of test extracts plates were incubated at standard conditions for 48 hours and assay was terminated by the addition of cold TCA. Cells were fixed *in situ* by gentle addition of 50 µl of cold 30%

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(w/v) TCA (final concentration, 10% TCA) and incubated for 60 minutes at 4°C. The supernatant was discarded; the plates were washed five times with tap water and air dried.

Sulphorodamine B (SRB) solution (50 µl) at 0.4 % (w/v) in 1% acetic acid was added to each of the wells, and plates were incubated for 20 minutes at room temperature. After staining, unbound dye was recovered and the residual dye was removed by washing five times with 1% acetic acid. The plates were air dried. Bound stain was subsequently eluted with 10 mM Trizma base, and the absorbance was read on an Elisa Plate Reader at a wave length of 540nm with 690 nm reference wavelength.[9]

$$\% \text{ Growth Inhibition} = 100 - \frac{\text{Mean OD of individual Test Group}}{\text{Mean OD of Control Group}} \text{ x } 100$$

Table 2: Samples used for cytotoxicity evaluation

SN	Institute Codes	Samples (Root of *Plumbago Zeylanica*)
1.	M-4529	Aqueous extract (Bgm)
2.	M-4530	Aqueous extract (Jmu)
3.	M-4531	Alcoholic extract (Bgm)
4.	M-4532	Alcoholic extract (Jmu)
5.	M-4533	Hydro Alcoholic extract (Bgm)
6.	M-4534	Hydro Alcoholic extract (Jmu)

RESULTS

Macroscopical study

The roots of both the samples were long and thin and 20-30 cm in length and 5-6 cm in breadth, surface was smooth, colour of sample A (Jmu) was brown from outside and yellow from inside but the colour of sample B was reddish brown from outside and light brown from inside, the odour of both the samples was characteristic and taste was acrid.

Table 3: Physico Chemical Analysis

Particulars	Sample A	Sample B	API Standard
Loss on Drying	7.05%	4.2%	-
Total Ash value	1.5%	1.523%	Not more than 3 %
Acid insoluble ash	0.5%	0.963%	Not more than 1 %
Foreign Matter	nil	nil	Not more than 3 %

Table 4: Extractive Values

Extractive Values	Sample A	Sample B	API Standard
Ethanol	13.456	13.125%	Not less than 12
Water	14.176	15.256%	Not less than 12
Hydroalcohol	13%	12.5%	--------

Table 5: Showing photo-chemicals present in *Chitraka Moola*

Chemicals present	Aq. (Jammu) (A)	Aq. (bgm) (B)	Al. (jammu) (C)	Al. (bgm) (D)	H.A (Jmu) (E)	H.A (Bgm) (F)
Carbohydrates Molisch's Test	+ +	+ +	+	+	+	+
Reducing sugar Benedict's test	+ (1%) green	+ (1.5 %) brown	+ (2%) brown	+ (2%) brown	+(2%) brown	+(2%) brown

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Monosaccrides Barfoed's Test	-ve	- ve	-ve	-ve	-ve	-ve
Amino acids Ninhydrin Test	+ ve	+ ve	-ve	-ve	-ve	-ve
Steroids Salkowski reaction	+ ve	+ ve	+ + ve	+ + ve	++	++
Glycosides Foam Test	-ve	-ve	-ve	-ve	-ve	-ve
Alkaloids Wagner's Test	++++	+++	+++	+ + +	++++	++++
Tannins $FeCl_3$ solution	+++	+++	+ + + +	+ + + +	+ + +	+ + +
Flavonoids Lead acetate solution	++	++	+ +	+ +	+ +	+ +
Proteins Million's Test	+ve brick red ppt	+ve brick red ppt	-ve	-ve	-ve	-ve

Inorganic elements

Iron, Sulphate and Carbon was present in both the samples

Table 6: Showing the Rf values of methanolic extracts of *plumbago zeylanica* Linn. roots.

SN	Samples	Short wave (Rf values)	Long wave (Rf values)
1.	Sample A	O.12, 0.97	0.62, 0.78, 0.9, 0.97
2.	Sample B	0.15, 0.9	0.06, 0.56, 0.62, 0.9
3.	Standard (Plumbagin)	-----	0.9

Genetic profile

In RAPD there was not proper amplification of the DNA, but ISSR showed the genomic amplification in which the Himalayan samples showed unique finger printing as compared to the samples of Western Ghats.

Three (1,4,5) out of four (1,2,4,5) individual plants of *Plumbago zeylanica* from belgaum region were genetically identical.

All four 6,7,8,10 individual plants of *Plumbago zeylanica* collected from Himalayas are diverse and showed unique finger printing patterns.

It shows that genetic diversity of *Plumbago zeylanica* in the Northern India (Himalayas) is more than that those in the southern region (Western Ghats)

Interestingly the genetic finger printing of plant #10 collected from Himalayan region was identical to those of the most common genotype collected from Belgaum (1,4,5)

The study also revealed that ISSR-Finger printing with primers 813 and 814 worked better than Rapd with primers RPI -2 and RPI-3 for finger printing of individual plants of *Plumbago zeylanica* (Fig. 2,3,4,5).

Fig. 1: Genetic Profile Results

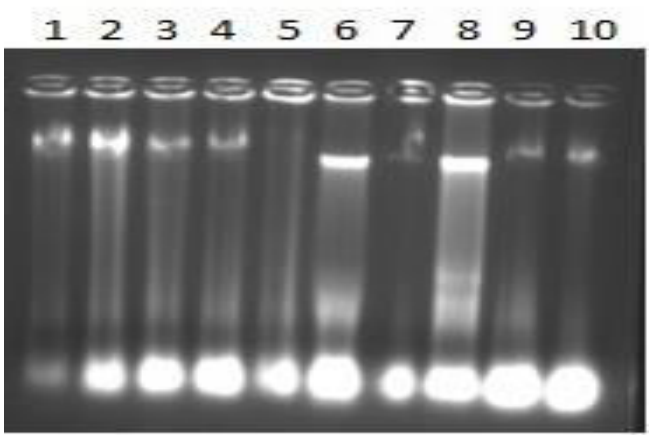

Fig. 1: Isolated DNA from the samples visualized under horizontal gel electrophoresis

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Table 7: Showing DNA quantification of *Plumbago zeylanica* by using Nanodrop Spectrophotometer

Samples	DNA Quantity ng/µl
1.	886.9ng/µl
2.	346.9 ng/µl
3.	1452.6 ng/µl
4.	759.4 ng/µl
5.	928.0 ng/µl
6.	193.8 ng/µl
7.	190.4 ng/µl
8.	320.3 ng/µl
9.	311.3 ng/µl
10.	229.2 ng/µl

All the samples showed satisfactory concentration of DNA by Spectrophotometeric method which ranged from 190.4 ng//µl to 928.0 ng//µl.

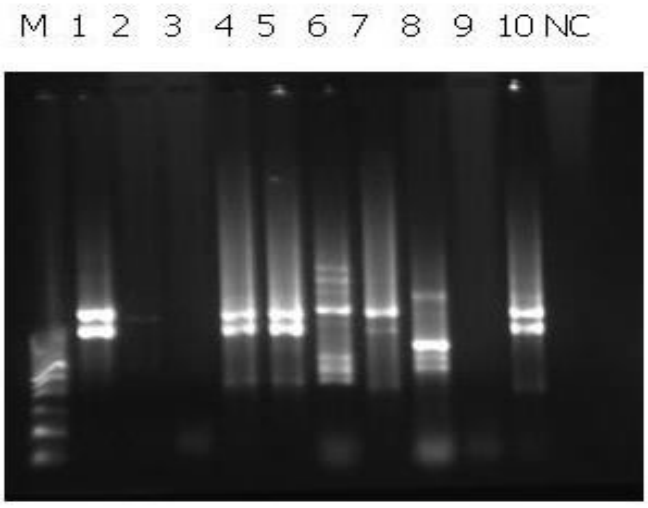

Fig. 2: ISSR fingerprints of *Plumbagozeylanica* samples using Primer 813

(M: Mol. wt markers, NC: Negative control, Lane 1-11 of *Plumbago zeylanica* samples)

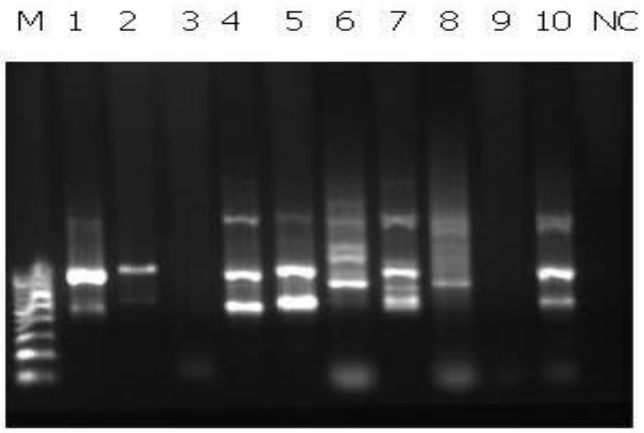

Fig. 3: ISSR fingerprints of *Plumbago zeylanica* samples using Primer 814

(M: Mol. wt markers, NC: Negative control, Lane 1-11 of *Plumbago zeylanica* samples)

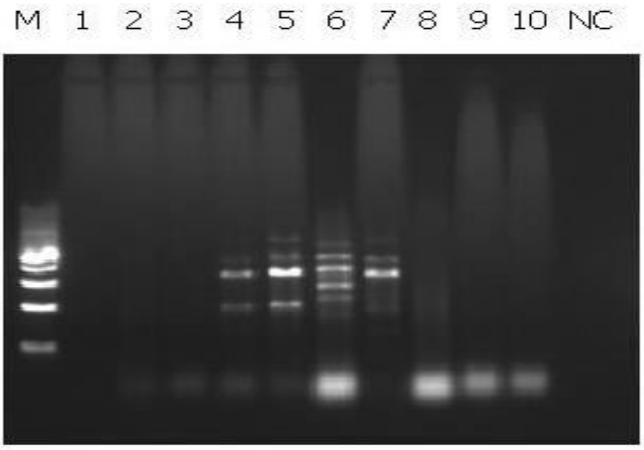

Fig. 4: RAPD Fingerprints of *Plumbagozeylanica* (RPI 2)

(M: Mol. wt markers, NC: Negative control, Lane 1-10 of Plumbago*zeylanica* samples)

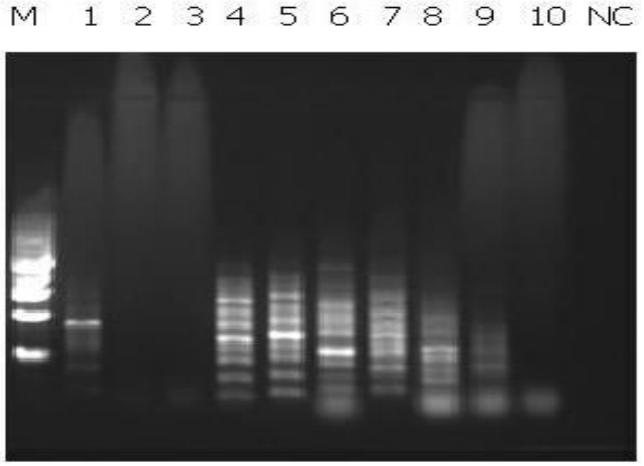

Fig. 5: RAPD Fingerprints of *Plumbago zeylanica* (RPI 3)

(M: Mol. wt markers, NC: Negative control, Lane 1-10 of Plumbago *zeylanica* samples)

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(a) Sample A (b) Plumbagin (Sigma) (c) Sample B

Fig. 6: Showing Thin layer chromatography of methanolic extracts of sample A and B

Table 8: Genetic profile based on ISSR Fingerprinting using primers 813 ana 814

Samples	ISSR (813)	ISSR (814)	Genetic profile
1	1	1	1 1
2	0	0	0 0
3	0	0	0 0
4	1	1	1 1
5	1	1	1 1
6	2	2	2 2
7	3	3	3 3
8	4	4	4 4
9	0	0	0 0
10	1	1	1 1

Table 9: *In vitro* cytotoxicity against human cancer cell lines.

Cell Line Type			HCT-15	Colo-205	MCF-7	PC-3	THP-1
Tissue			Colon	Colon	Breast	Prostate	Leukemia
Instt. Code	Code	Conc.	% Growth Inhibition				
M-4529	Aq. BGM	10	0	0	28	8	0
		30	3	0	43	15	0
		50	7	0	69	28	2
		70	71	0	78	46	2
		100	92	74	79	49	5
IC_{50} (µg/ml)			85	>100	33	>100	>100
M-4530	Aq. JMU	10	52	0	10	0	0
		30	56	0	30	0	0
		50	76	0	51	30	0
		70	90	0	74	65	9
		100	96	0	80	68	17
IC_{50} (µg/ml)			11	>100	52	>100	>100
M-4531	Alc. BGM	10	60	0	0	1	28
		30	89	15	0	4	35
		50	95	35	0	9	50
		70	99	50	5	15	67
		100	100	70	30	31	73
IC_{50} (µg/ml)			3	69	>100	>100	45
M-4532	Alc. JMU	10	49	10	0	25	0
		30	68	20	10	30	12
		50	74	51	12	50	58
		70	100	65	52	69	61
		100	100	77	70	80	69
IC_{50} (µg/ml)			11	58	81	46	64
M-4533	Hyd. Al.BGM	10	35	0	0	0	0
		30	74	0	0	0	0
		50	100	0	0	3	0

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		70	**100**	5	17	36	9
		100	**100**	13	20	36	42
IC_{50} (µg/ml)			**16**	**>100**	**>100**	**>100**	**>100**
M-4534	**Hyd. Al.JMU**	**10**	**70**	0	0	0	0
		30	**80**	0	0	0	0
		50	**100**	0	0	0	4
		70	**100**	**54**	20	0	10
		100	**100**	**90**	30	15	30
IC_{50} (µg/ml)			**1**	**>100**	**>100**	**>100**	**>100**
	5-FU		**54**	**57**	-	-	**50**
	Doxorubicin				**60**		
	Mitomycin					**61**	

The test material showing > 50% activity at 100ug/ml is considered to be active .

Table 10: Showing the comparison of IC_{50} values of both the samples.

Samples	HCT-15 colon	Colo-205	MCF-Breast	PC-3 Prostate	THP Leukemia
Aq. Bgm	85 µg/ml	>100	33 µg/ml	>100	>100
Aq. Jmu	11 µg/ml	>100	52 µg/ml	>100	>100
Al. Bgm	3 µg/ml	69 µg/ml	>100	>100	45 µg/ml
Al. Jmu	11 µg/ml	58 µg/ml	81 µg/ml	46 µg/ml	40 µg/ml
H.Al. Bgm	16 µg/ml	>100	>100	>100	>100
H.Al. Jmu	1 µg/ml	>100	>100	>100	>100

Discussion

To achieve adequate therapeutic results primarily collection of *Dravya* (drug) plays an important role. More over ecological factors are to be considered during collection as it influences on the medicinal value of the drug, The study was thus carried out to understand the Geographical variation phytochemically, genetically and pharmacologically in the Plant *Plumbago zeylanica* Linn. Collected from the Himalayan region and Western Ghats. No much difference was observed in both the samples but during collection it was observed that the Sample A was yellow colored from inside and this may be due to presence of high concentration of plumbagin in it as plumbagin is a yellow colored molecule also preliminary phytochemical evaluation revealed that the concentration of Tannins and alkaloids was high in sample A as compared to sample B (Table no. 5) and this may be because of the climatic conditions. While performing Thin layer chromatography the standard molecule of *Plumbago zeylanica* i.e. Plumbagin (Sigma) was also made to run along with both the samples, the Rf value for plumbagin is 0.910, different Rf values of different bands were recorded but in both the samples a band of light green colour having Rf value 0.97 and 0.9 was observed which indicates the presence of plumbagin in both the samples (fig. no. 6).

Among the molecular markers used, DNA markers are omnipresent as they are very stable and specific and help in easy and reliable identification of plant species 11. So genetic profile of both the samples was carried. The ISSR finger printing using primer 813 showed sample numbers 1,4,5 (Western Ghats) had similar amplification pattern as of 10^{th} sample (Himalayan). It implies that the samples 1, 4, 5, and 10 might be genetically identical. The ISSR fingerprinting using primer 814 showed same results as that of 813 This may be attributed to genetic uniformity of these plants in both the regions. (Fig.no.2,3). All four (6,7,8,10) individual plants of *Plumbago zeylanica* collected from Himalayas are diverse and showed

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unique finger printing patterns. It shows that genetic diversity of *Plumbago zeylanica* in the Northern India (Himalayas) is more than that those in the Southern region (Western Ghats). There was no genomic amplification observed in RAPD using primers RPI 1 and RPI 2, among the 10 plant samples. (Fig.no.4, 5)The present study detected genetic identity among the 3 plant samples collected from Karnataka with that of 1 sample of Himalayan region. However further studies based on chemo profile of these plants is required for definite conclusion.

The studies also revealed that the ISSR based DNA fingerprinting using primers 813 and 814 worked better and are much more reliable than RAPD finger printing to study the genetic diversity of individual plants of *Plumbago zeylanica.* The study showed that analysis of ISSR data can be used to detect genetic differentiation of *P. zeylanica*.

In vitro anticancer activity of both the samples was done by performing SRB Assay. In this pharmacological study the three extracts showed the maximum activity against the HCT -15 Colon, and the extracts of sample A possessed better anticancer activity as compared to the extracts of sample B. The above observation suggests that roots of *Plumbago* collected from Jammu have better cytotoxic activity than those from Belgaum. Samples from Jammu region had shown better Anticancer activity as compared to the sample of the Bgm region and it may be due to the presence of high concentration of alkaloids, and Tannins present in the sample A, as compared to sample B as the Alkaloids and Tannins 12 are reported for their antitumor activity and plumbagin is the Alkaloid present in *P.zeylanica.*

As per literary survey this is the first report of *Plumbago zeylanicas* anticancer activity reported against HCT-15 colon cancer cell lines.

CONCLUSION

Depending upon the above parameters it is confirmed that Geographical variation was seen in both the plants of both the area with slight changes in Chemo profile, Genetic profile and in its *Invitro* Anticancer activity. Also we can say that the samples collected from the Himalayan regions worked better on the parameters which were selected for the study as compared to the samples of the western ghat regions sample. To ensure the best quality of medicinal plant the plants should therefore be collected during the appropriate season and place.

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