



NIRMA
UNIVERSITY

INSTITUTE OF PHARMACY

**Anticancer potential of
Dillenia indica and *Dillenia pentagyna*
plants and its correlation with
presence of active phytoconstituent**

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INTRODUCTION

- ❖ A vast knowledge on medicinal plants exists as oral among folklore and primitive societies of India, where a large number of potent medicinal herbs are found growing wild.
- ❖ Plants belonging to Dilleniaceae family are amongst many plants used by tribal communities of Dang forest and also even in many other forests of India, like Mizoram district, Vindhya region in M.P.
- ❖ *Dillenia indica* Linn. and *Dillenia pentagyna* Roxb. are two plants found to grow in Dang forest, Gujarat, India.

INTRODUCTION...

- ❖ *D. indica* and *D. pentagyna* known as 'Karmal' and 'Mota Karmal' in Dang forest of Gujarat.
- ❖ Traditionally the different parts of these plants have curing properties like cancer, wound healing, diabetes, diarrhea, bone fracture, in cut and burns, abdominal pains etc.
- ❖ As the folklore medicines are evolved by the individual and ethnic experiences, it needs further investigations in stipulations of diverse branches of medical science to endeavor the issues like that of standardization, identification, pharmacology etc.

INTRODUCTION...

- ❖ Based on phytochemical investigations these plants are reported to contain active constituents like betulin, betulinic acid, dillenetin, dipoloic acid, myricetin, quercetin derivatives etc.
- ❖ Betulinic acid (BA) is found to be present as an active phytoconstituent in both plants, which is chemically ((3 β)-3-Hydroxylup-20(29)-en-28-oic acid).
- ❖ Because of selective cytotoxicity against tumor cell lines compared to normal cells, BA is a promising experimental anticancer agent for the treatment of human cancers.

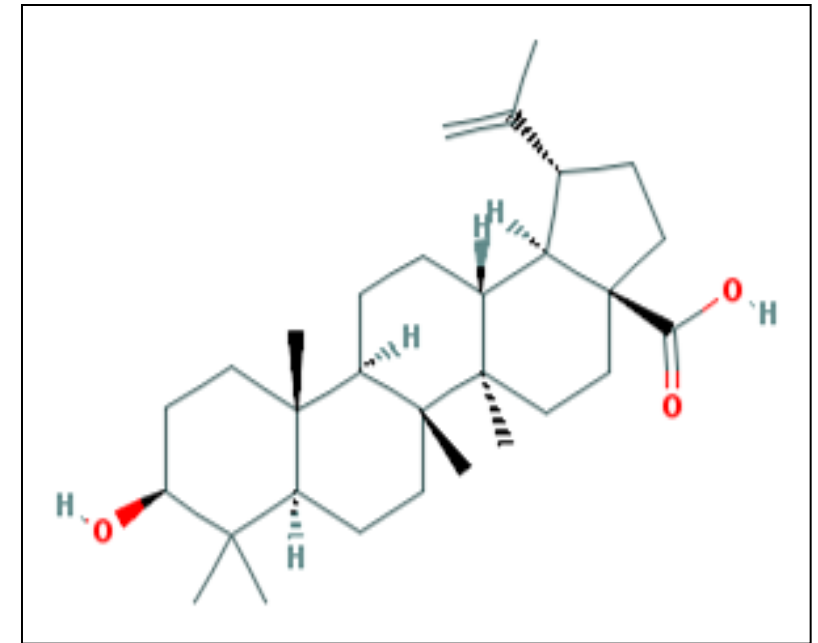
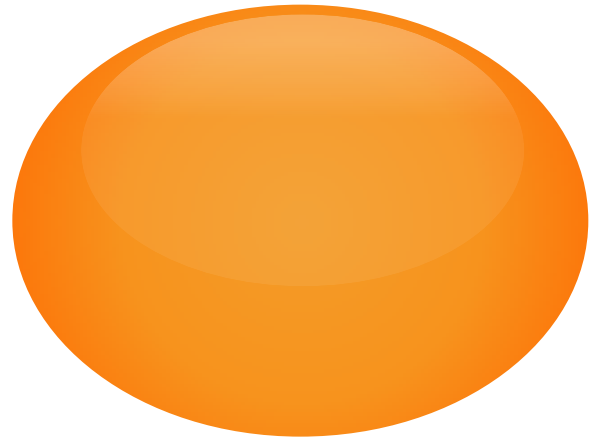


Fig. 1 Structure of Betulinic acid

OBJECTIVE OF THE STUDY

- ❖ Bark and leaves of these two plants has been selected to check for presence of active constituent.
- ❖ Keeping in mind the importance of betulinic acid which may be responsible for anticancer potential of both plants; development of RP-HPLC method for estimation of betulinic acid using fractions prepared for cell line.
- ❖ In order to prove anticancer potential of two ethno medicinal plants from India, *Dillenia indica* and *Dillenia pentagyna* in vitro cell line studies has been performed taking bark and leaves of both plants
- ❖ Correlation of the amount of betulinic acid with effect observed using cell line studies.



**RP-HPLC METHOD FOR ESTIMATION OF
BETULINIC ACID FROM
BARK AND LEAVES OF
D. PENTAGYNA AND *D. INDICA***



EXPERIMENTAL WORK

Plant material and Chemicals

- ✓ Bark and leaves of *D. indica* and *D. pentagyna* were collected from Waghai botanical garden of Dang district, Gujarat, India.
- ✓ Collected bark and leaf samples were authenticated by botanist Dr. Jasrai, School of Botany, Gujarat University, Gujarat, India.
- ✓ Standard BA (99 % purity) was purchased from Sigma-Aldrich (India).
- ✓ All other reagents were of HPLC grade or AR grade as per requirement.

Bark and Leaf of Both Plant Species

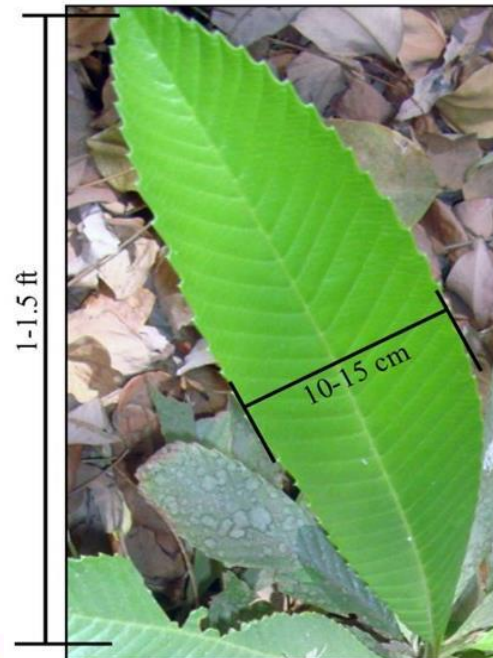
Fig. 2: Morphology of Bark (a) and leaf (b) of *D. indica* and Bark (c) and leaf (d) of *D. pentagyna*



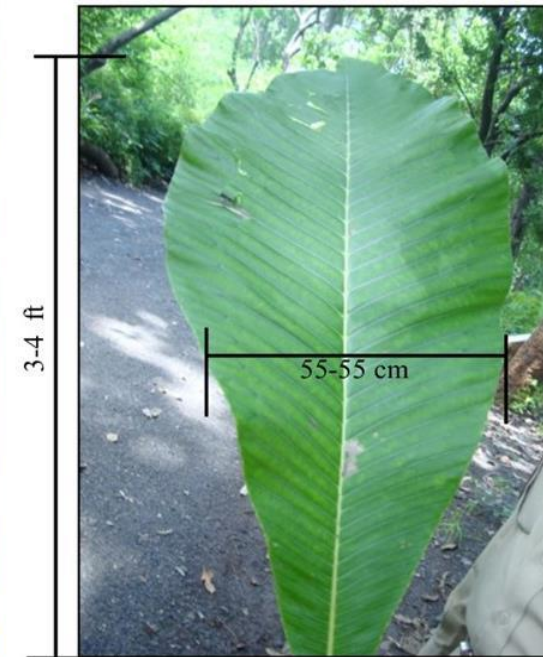
[A]



[C]



[B]



[D]

EXPERIMENTAL WORK

RP-HPLC method development and optimization

Chromatographic conditions for RP-HPLC method

Following chromatographic conditions were optimized and were kept constant throughout the analysis.

- Column: C18 PUROSPHERE STAR Hyber 250 × 4.5 mm i.d., with 5 μm particle size
- Mobile phase: Acetonitrile : Water (93:7 v/v)
- Flow Rate: 1.2 mL/min
- Detection wavelength: 207 nm;
- Injection volume: 20 μL,
- Run time :20 min.

Preparation of standard solutions for RP-HPLC analysis

Betulinic acid (100 mg) was weighed and dissolved in 100 ml methanol; 1000ppm stock solution prepared

From this solution, working standard solution of 50, 100, 200, 400, 600, 800 $\mu\text{g}/\text{mL}$ of Betulinic acid was prepared.

These freshly prepared solutions were used further for preparation of calibration curve and further chromatographic analysis.

50 g of bark and leaf of both plants (Individually)

MeOH combined and evaporated to dryness to get Residue.
Fractionated with...

ETHYL ACETATE

(30x3 mL) - Solvent was
evaporated to dryness

Residue dissolved in ACN and transferred in 50
mL vol. flask and make up the vol ACN.

Bark fraction further
diluted taking 1 ml to
10 mL with ACN

HPLC analysis

Leaf fraction taken
for HPLC analysis

BENZENE

(30x3 mL) - Solvent was
evaporated to dryness

Residue dissolved in ACN and
transferred in 50 mL vol flask and
make up the vol ACN .

Bark fraction further
diluted taking 1 ml to
10 mL with ACN

HPLC analysis

Leaf fraction taken for HPLC
analysis

Scheme: showing
preparation of sample
solutions for HPLC analysis

RESULTS OF RP-HPLC ANALYSIS

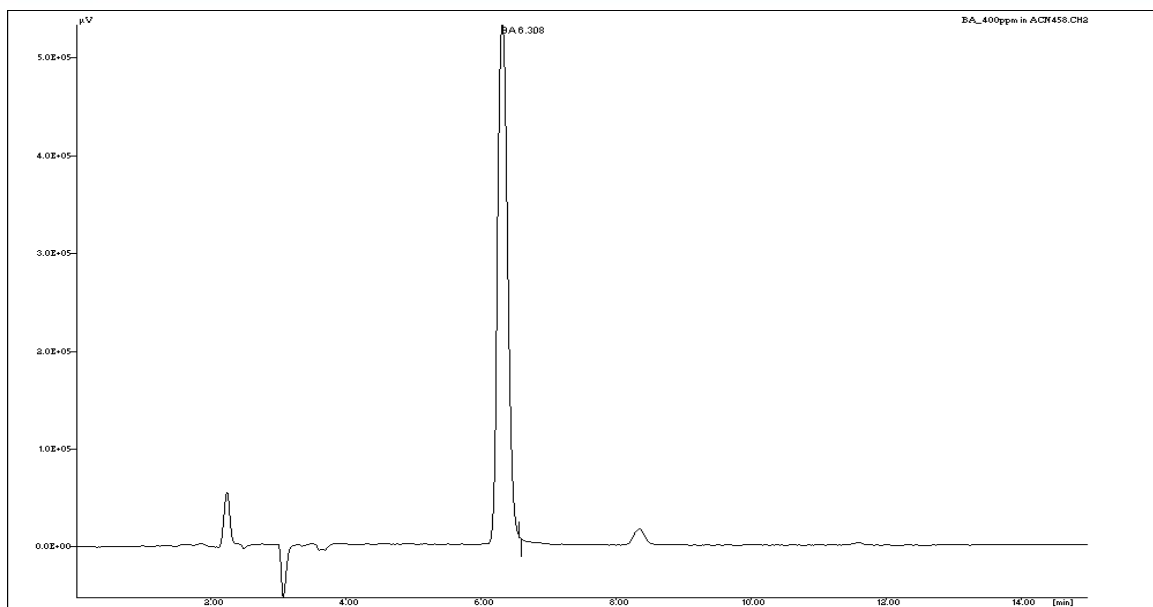


Fig. 3 RP-HPLC chromatogram of BA (400ppm)

TABLE 1 System suitability parameters for BA (400 ppm)

Parameters	Observation
Rt (min)	6.308
Peak Area	5775214
% Area	100
Theoretical plates	10111
Asymmetry	1.11

Preparation of calibration curve for betulinic acid

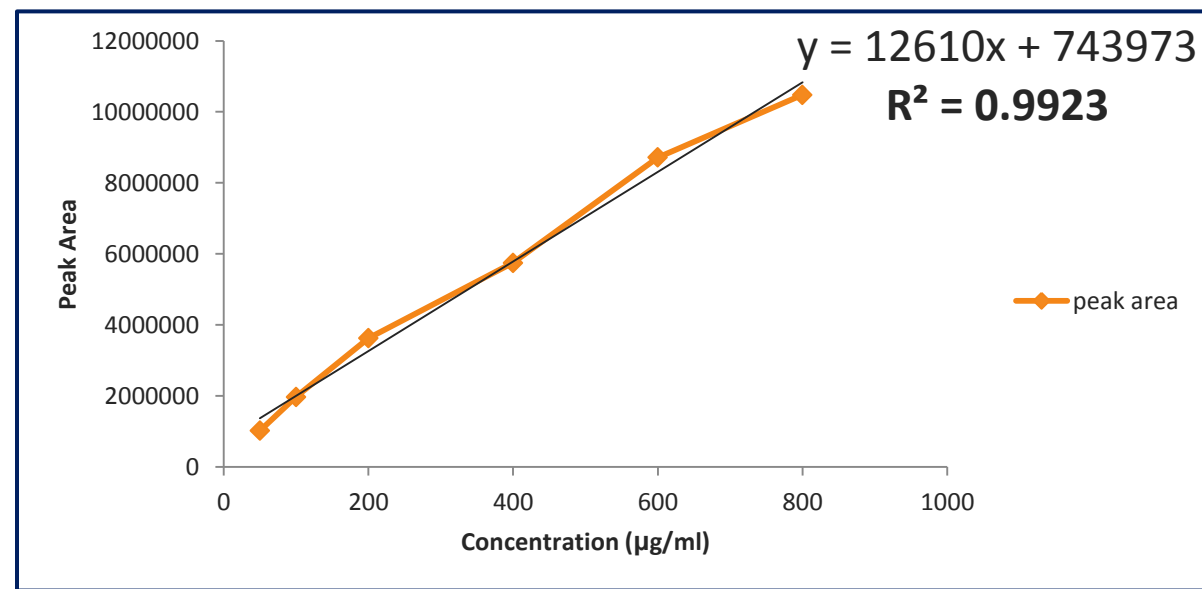


Fig. 4 Calibration curve of developed RP-HPLC method for BA

- Freshly prepared **50-800 ppm** working standard solutions of was taken for calibration curve.

Peak purity spectra of prepared fraction

Table 2 Peak purity of BA in prepared benzene fraction of DIB using RP-HPLC

Parameters	Observation
Rt (min)	6.301 (Peak Top @ 207 nm)
Purity (Tail) [1..1000]	997.129
Purity (Front) [1..1000]	998.679
Theoretical plates	9984.9
Peak purity	Pass
Specificity	Specific

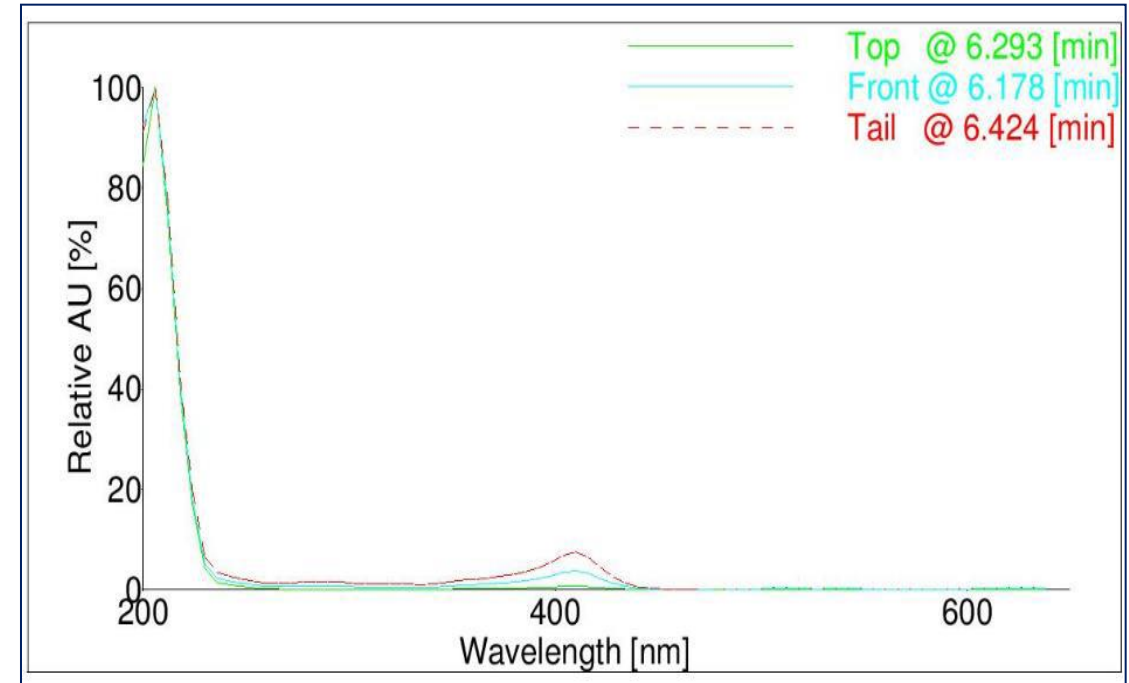


Fig. 5 Peak purity spectra BA in benzene fraction of DIB using RP-HPLC

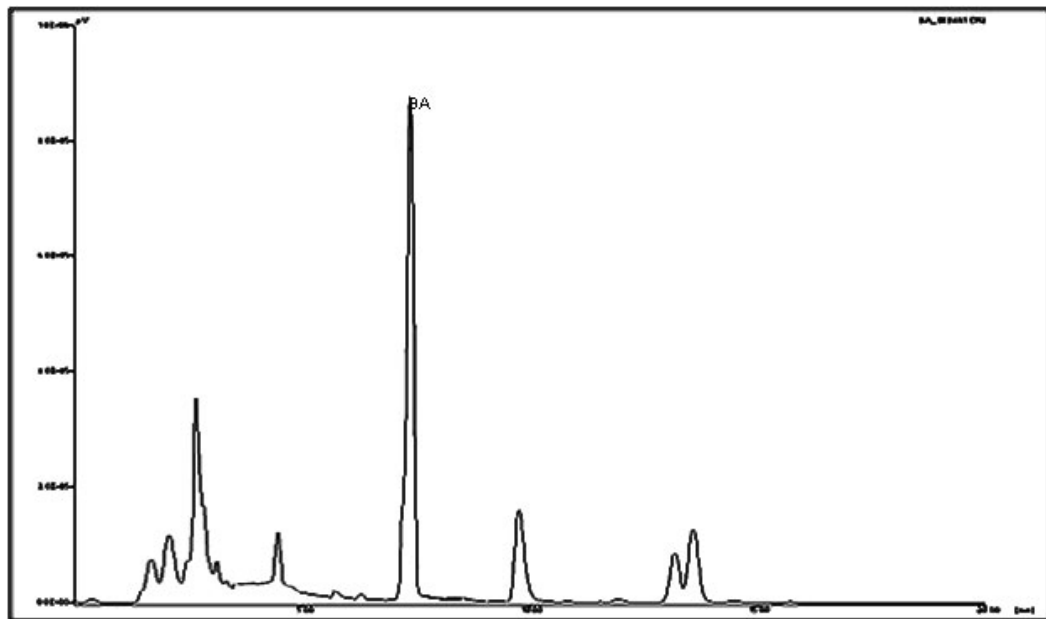


Fig.6 HPLC Chromatogram of DIB (Benzene fraction)

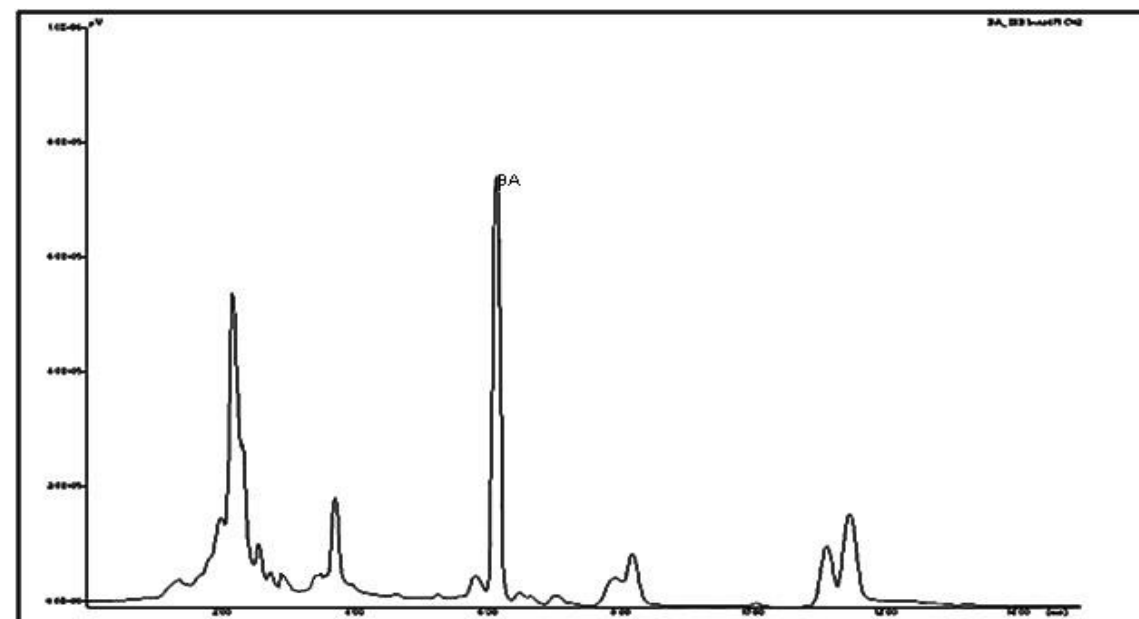


Fig. 7 HPLC Chromatogram of DIB (EtOAc fraction)

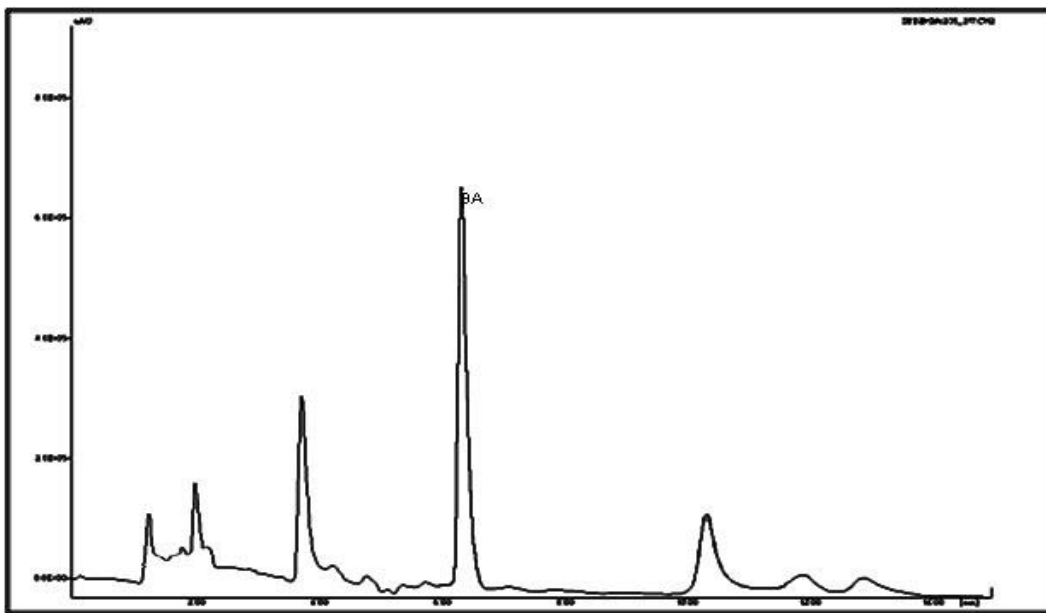


Fig. 8 HPLC Chromatogram of DPB (Benzene fraction)

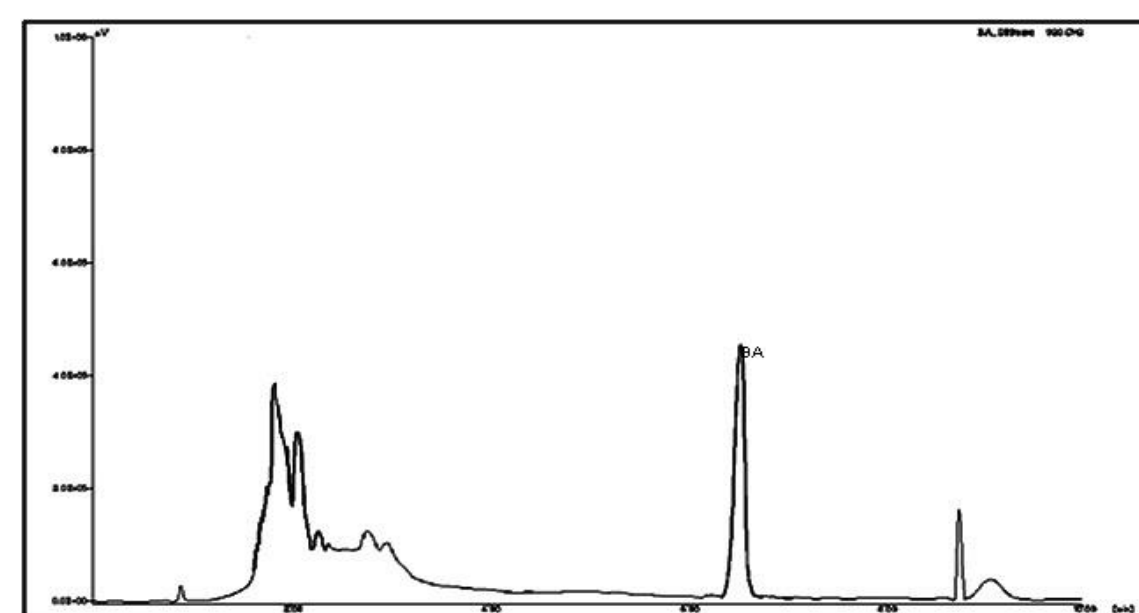


Fig. 9 HPLC Chromatogram of DPB (EtOAc fraction)

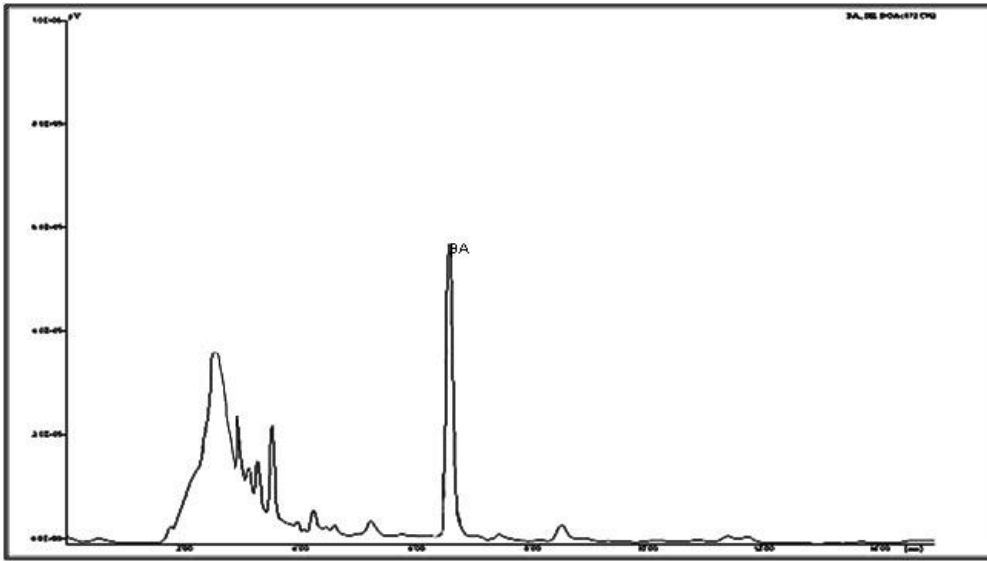


Fig. 10 HPLC Chromatogram of DIL (Benzene fraction)

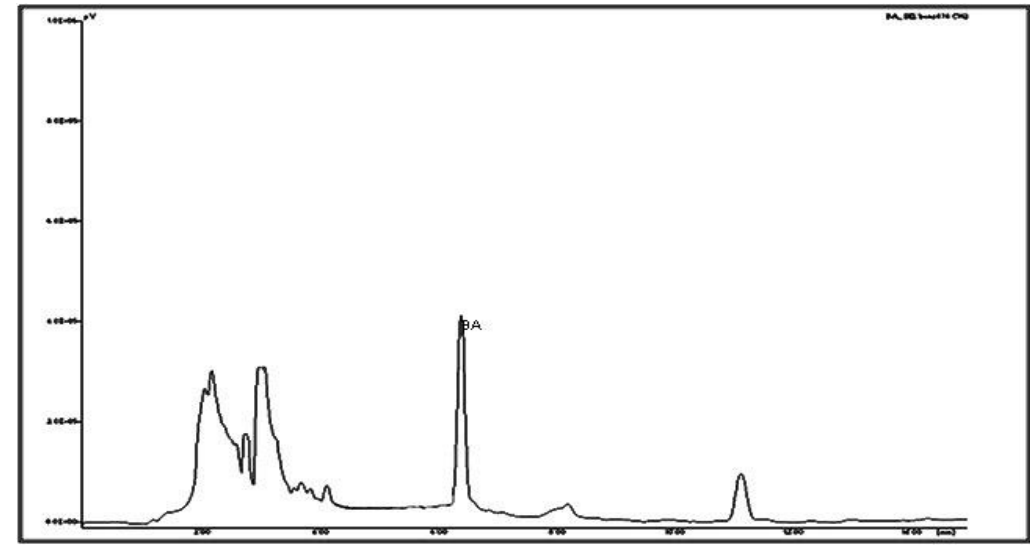


Fig. 11 HPLC Chromatogram of DIL (EtOAc fraction)

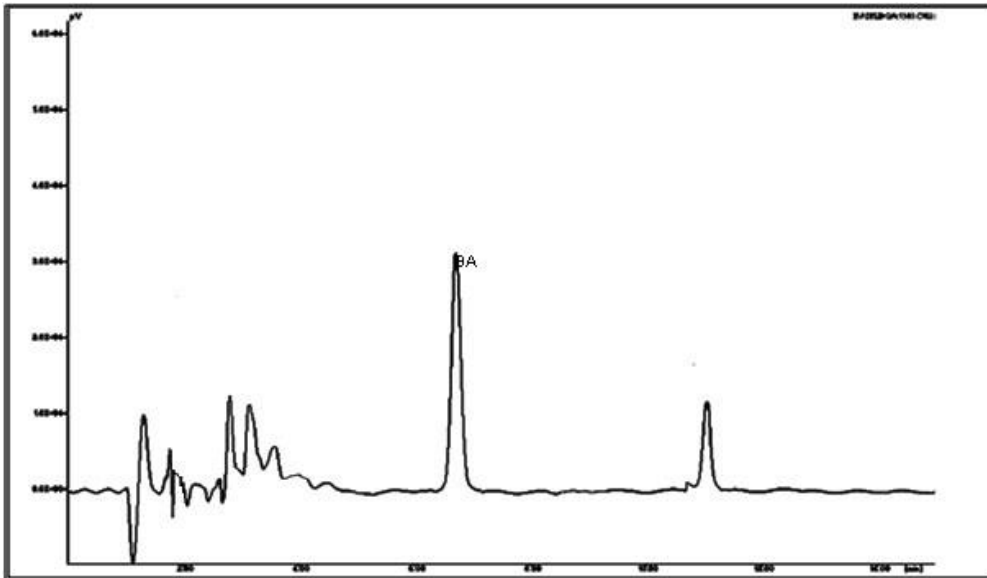


Fig. 12 HPLC Chromatogram of DPL (Benzene fraction)

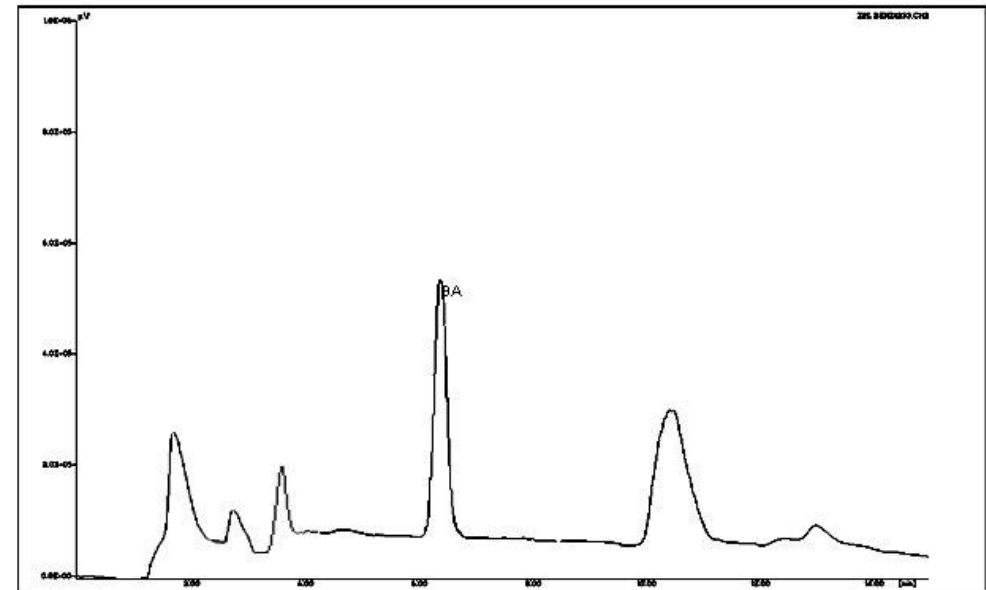
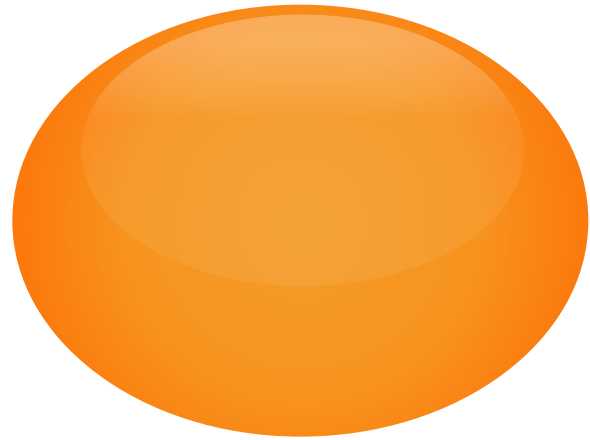


Fig. 13 HPLC Chromatogram of DPL (EtOAc fraction)

RESULTS: Quantity of Betulinic acid in both plants

TABLE 3 Amount of betulinic acid in different prepared fractions by RP- HPLC method

Part of Plant	Prepared Extract	%w/w in fraction	%w/w in Sample
DPB	Benzene Fraction	14.30	0.315
DIB		19.11	0.421
DPL		6.82	0.151
DIL		8.97	0.196
DPB	Ethyl Acetate Fraction	8.50	0.287
DIB		10.21	0.325
DPL		6.65	0.147
DIL		7.60	0.167



CELL LINE STUDIES OF BETULINIC ACID AND PREPARED PLANT EXTRACTS

In-vitro cytotoxicity screening method of BA and different prepared extracts

Sample and standard solutions:

- Fractions prepared for HPLC analysis taken further for cell line studies.
- Standard BA (99 % purity) was purchased from Sigma-Aldrich (India).

MTT Assay of betulinic acid and prepared fractions

- MTT assay has been performed on three different cell lines such as HCT-15, DU-145 and A-375 cell lines to check the potential of prepared plant extracts.
- IC_{50} values were calculated using the nonlinear regression program origin. The average of two (duplicates manner) were taken in determination.

Results of A-375 cell line

TABLE 4 IC50 values of BA and prepared fractions of plants

Sr. No.	Compound	IC50 values
1	Doxorubicin	9.71
2	Colchicine	11.92
3	BA Std	10.91
4	DIB- Benz	15.88
5	DPB- Benz	17.18
6	DIB- EtOAc	31.48
7	DPB- EtOAc	29.13
8	DIL- Benz	13.36
9	DPL- Benz	26.56
10	DIL- EtOAc	33.53
11	DPL- EtOAc	28.97

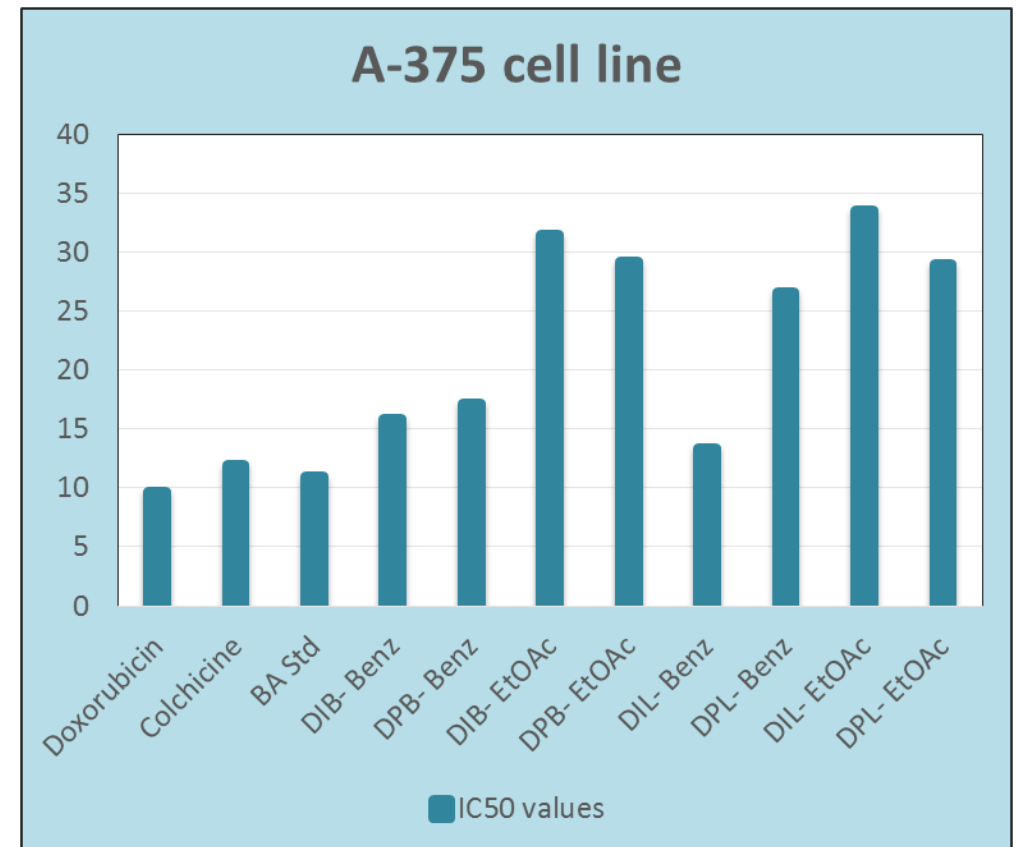


Fig. 14 Graph plotted IC50 values of different fraction on A-375

Results of DU-145 cell line

TABLE 5 IC50 values of BA and prepared fractions of plants on DU-145 cell line

Sr. No.	Compounds	IC50 value
1	Doxorubicin	6.80
2	Colchicine	10.17
3	BA Std	7.37
4	DIB- Benz	14.58
5	DPB- Benz	20.19
6	DIB- EtOAc	16.03
7	DPB- EtOAc	18.87
8	DIL- Benz	17.72
9	DPL- Benz	20.76
10	DIL- EtOAc	35.04
11	DPL- EtOAc	24.87

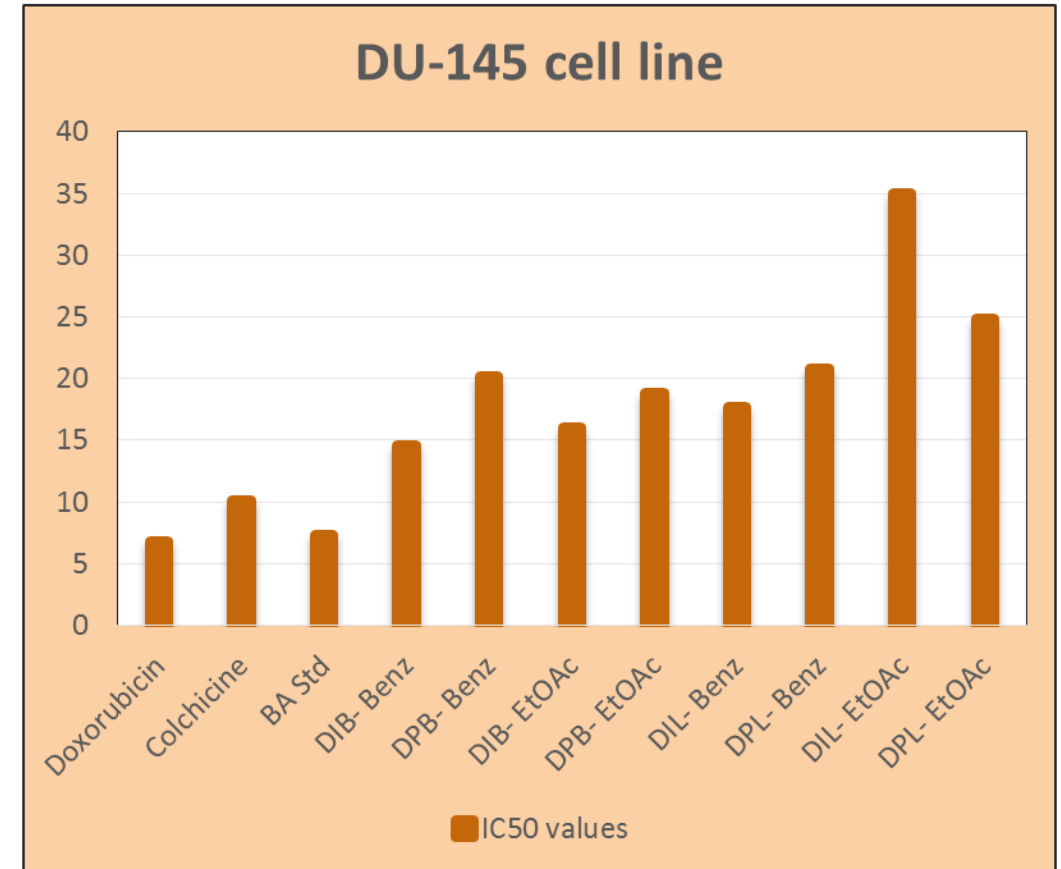


Fig. 15 Graph plotted IC50 values of different fraction on DU-145

Results of HCT-15 cell line

TABLE 6 IC50 values of BA and prepared fractions of plants

Sr. No.	Compound	IC50 values
1	Doxorubicin	10.29
2	Colchicine	12.17
3	BA Std	16.76
4	DIB- Benz	17.42
5	DPB- Benz	33.16
6	DIB- EtOAc	40.29
7	DPB- EtOAc	34.57
8	DIL- Benz	23.42
9	DPL- Benz	32.45
10	DIL- EtOAc	22.89
11	DPL- EtOAc	48.65

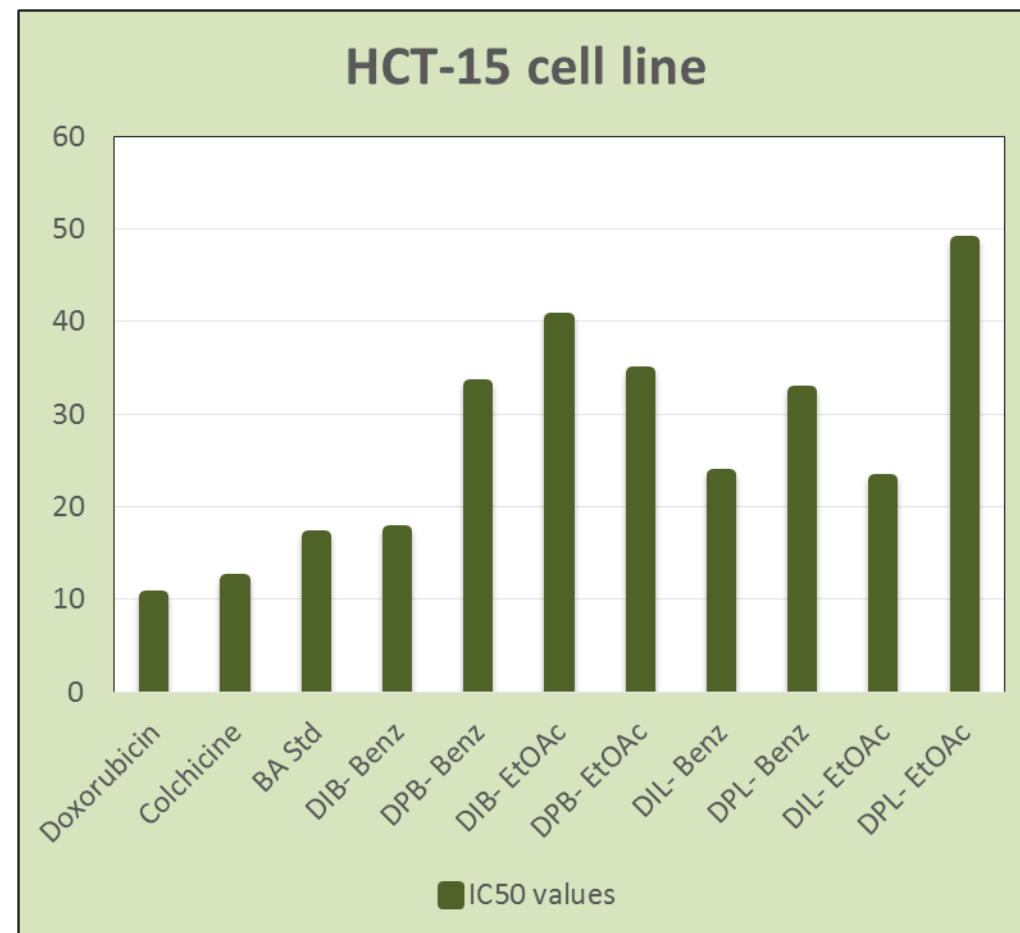


Fig. 16 Graph plotted IC50 values of different fraction on HCT-15

- **Activity order:** As compare to std. anticancer drugs, IC50 value of betulinic acid also showed as effective as standard, the highest activity order of plant fractions in A-375 cell line term of IC50 (10.91 µg/ml) values can be given as:

DIB-Benz> DIL-Benz> DIB-EtOAc > DIL-EtOAc (Less activity)

DPB-Benz> DPL-Benz > DPB-EtOAc > DPL-EtOAc(Less activity)

- **Activity order:** As compare to std. anticancer drugs, IC50 value of BA found equally potent, the highest activity order in DU-145 cell line term of IC50 (07.37 µg/ml) values can be given as

DIB-Benz>DIB-EtoAc> DIL-Benz > DIL-EtoAc(Less activity)

DPL-EtoAc> DIB-Benz> DIB-EtoAc > DIL-Benz (Less activity)

- **Activity order:** As compare to std. anticancer drugs, IC50 value of betulinic acid also found to be equally effective but as compared to other two cell line less, the highest activity order in HCT-15 cell line term of IC50 (16.76 µg/ml) values can be given as:

DIB-Benz > DIB-EttoAc> DIL-EtoAc> DIL-Benz (Less activity)

DPB- Benz > DPL-EtoAc DIB- Benz DPL-EtoAc (Less activity)

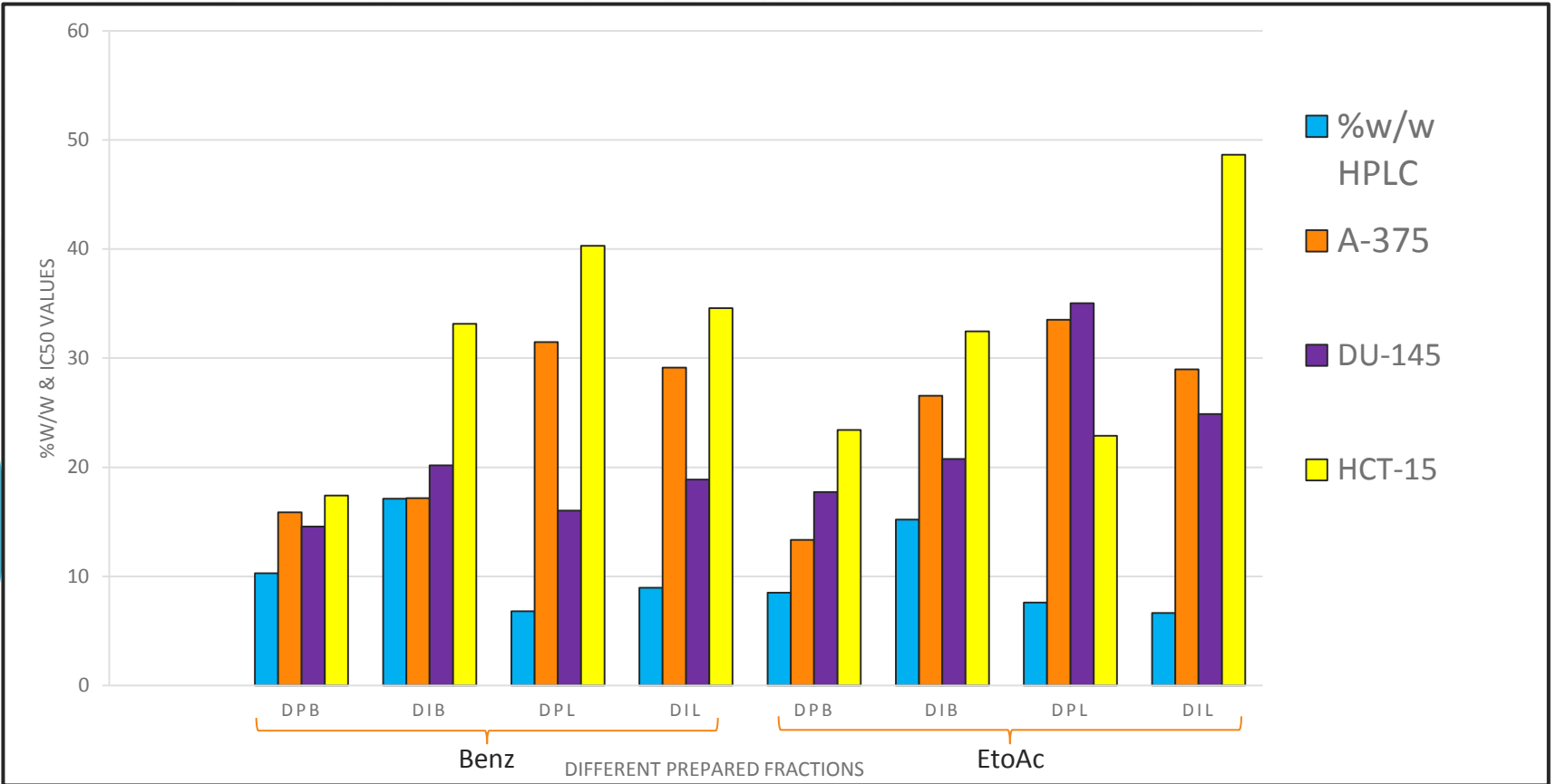
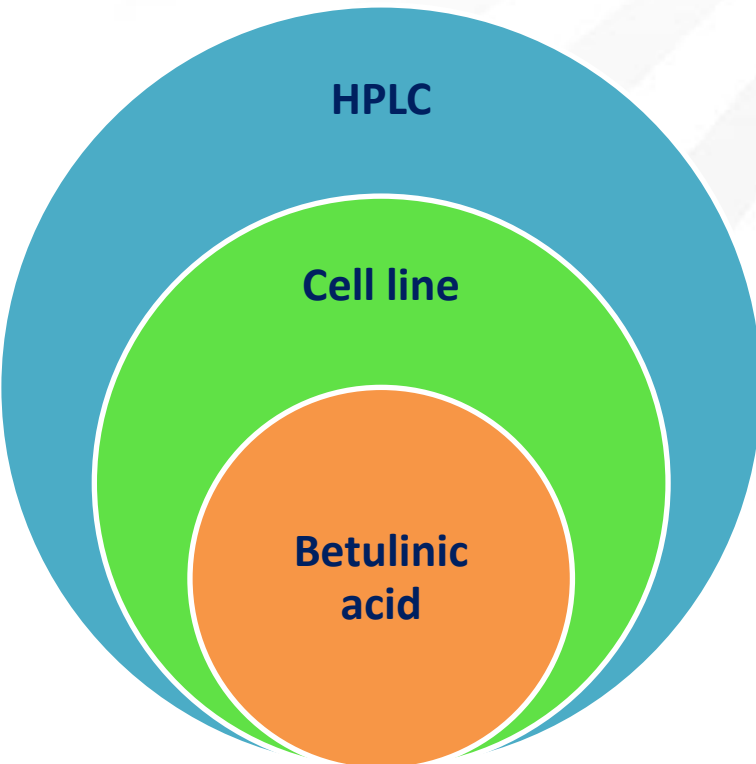


Fig. 17 Comparison Amount of BA Vs IC50 values on various cell lines in prepared fraction

CONCLUSION

- The proposed HPLC method is simple, rapid, specific and accurate technique developed and validated as per ICH guideline.
- The developed reversed-phase HPLC method using C18 column provides sufficient retention and baseline separation for analyzing betulinic acid in two different fractions of bark and leaves of *D. indica* and *D. pentagyna*.
- The developed method can be successfully used for routine quality control of plants. The proposed RP-HPLC method can be successfully applied for estimation of BA from other plants.

CONCLUSION...

- From the reported data of cell line studies it can be concluded that Betulinic acid is found to be as effective as standard anticancer drugs Doxorubicin and Colchicine on various cell lines.
- It can be also be observed that extracts benzene fraction of *Dillenia indica* bark showed highest potential than other fractions on all cell lines.
- In contrast to bark, benzene fraction of *Dillenia pentagyna* leaves showed least IC50 value.
- Overall all prepared fractions showed significant effect on various cell lines which can prove anticancer potential of these plants.

CONCLUSION...

- It has been observed that content of betulinic acid and IC_{50} values are positively related to each other as IC_{50} is decreasing with increase in amount of BA in prepared fractions It is known that lesser IC_{50} value, more potent is compound against cell line.
- This is indicating that the fractions and plants are possessing good anticancer potential and also confirmed scientifically the usefulness of its applications in folklore medicine for the treatment of cancer.

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THANK YOU!

Any questions?

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