

Antiproliferative Properties of *Embelia Ribes*

Beena Joy^{a,*} and S. Lakshmi^b

^aNational Institute for Interdisciplinary Science & Technology, Thiruvananthapuram- 695019, India

^bDivision of Cancer Research, Regional Cancer Centre, Thiruvananthapuram, 695011, India

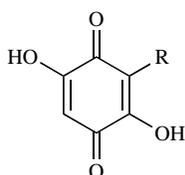
Abstract: *Embelia ribes* is a threatened woody shrub belonging to the family Myrsinaceae, which is sparsely distributed in the moist deciduous forests of the Western Ghats in India.

The previous reports showed the effect of Embelin in methylcholanthrene induced fibrosarcoma in albino rats besides enhancing their survival time. Embelin provided a good support for the chemopreventive effect against N-nitrosodiethylamine/ phenobarbital induced hepatocarcinogenesis in Wistar rats. The present studies on cancer cell lines revealed that the crude hexane extract of the fruits of *Embelia ribes* exhibited cytotoxicity against Human leukaemic cells (K562) and Dalton's Lymphoma ascites cells (DLA). Bioactivity directed fractionation and isolation yielded Embelin as the active principle. *In vitro* studies on Embelin suggests the potential of the compound on these two cell lines. However the compound did not exhibit toxicity on normal lymphocytes isolated from human blood preferentially attacking the tumour cells. This is the first report of *in vitro* cytotoxic activity of *Embelia ribes*.

Keywords: *Embelia ribes*, Embelin, Cytotoxicity, HPLC, HPTLC, MTT.

1. INTRODUCTION

Embelia ribes is a threatened woody shrub belonging to the family Myrsinaceae, which is sparsely distributed in the moist deciduous forests of the Western Ghats in India. In Indian system of medicine 'Ayurveda', the plant is popularly known as 'Vidanga'. Embelin, a naturally occurring quinonoid compound, is found to be the major constituent of *Embelia ribes* [1].



R= undecyl (**embelin**)

Chemical structure of embelin (2, 5-dihydroxy-3-undecyl-1, 4-benzoquinone).

Embelin has a long alkyl chain (undecyl) as a substituent, which confers solubility in the non-polar phase and cell permeability. The adjacent quinone and hydroquinone groups on embelin form intramolecular hydrogen bonds. Embelin shows a wide spectrum of biological activities. The antibacterial activity of embelin was reported [2]. The antitumor, antiinflammatory and analgesic activity of embelin has been reported [3]. Embelin shows antitumour activity in methylcholanthrene induced fibrosarcoma in

albino rats besides enhancing their survival time. The study of embelin suggests that, embelin can act as an antioxidant in physiological conditions [4].

2. MATERIALS AND METHODS

2.1. Isolation of Marker Compound Embelin and Purification

The hexane extract was subjected to column chromatography over silica gel (100-200 mesh). Elution of the column with benzene yielded an orange colour powder. The purification of embelin has been done by the crystallization using diethyl ether, yielded orange flakes of embelin. The spectral characterization of isolated compound was done, and was satisfied with the structure of embelin. The purity of embelin was confirmed by using HPLC and HPTLC analysis.

2.2. Cell Lines

Dalton's lymphoma ascites (DLA) cells, Human Leukaemic cells (K- 562) cells, Lymphocytes isolated from human blood. Daltons Lymphoma Ascites (DLA) cells (arose as a spontaneous carcinoma of Thymus) were obtained from Cancer Institute, Adayar.

DLA cells were propagated in the peritoneal cavity of mice by transplanting one million DLA cells per ml of Phosphate Buffered Saline (PBS). For experimental purposes, the tumour cells were aspirated from tumour bearing mice aseptically. K-562 (Human leukaemic cells) were obtained from National Centre for Cell Sciences, Pune, India and the cells were maintained in DMEM media supplemented with 10% FBS, 100 units/ml of Penicillin and 100 µg/ml Streptomycin in a humidified 5% CO₂ incubator.

*Address correspondence to this author at the National Institute for Interdisciplinary Science & Technology, Thiruvananthapuram- 695019, India; Tel: +91-471-2515354; Fax: +91-471-2491712; E-mail: bjoy39@yahoo.co.in

2.3. Experimental Animal maintenance

Balb/C mice (7-8 weeks old) weighing 20-25 gram were used for experiments. They were maintained on normal mouse chow (Lipton, India) and water *ad libitum* and housed in ventilated cages.

2.4. Reagents

Trypan blue, MTT (3-[4,5-dimethylthiazol-2-yl]2,5-diphenyltetrazolium bromide), Dulbecco's modified eagle's (DMEM) media, Fetal Bovine serum (FBS), Isopropanol, Dimethyl sulfoxide (DMSO), Sodium Dodecyl sulfate (SDS)

2.5. Short Term Cytotoxicity Studies

Short term cytotoxicity studies were done on DLA cells by Trypan blue exclusion method. Cells were aspirated from the peritoneal cavity of tumour bearing mice and washed in PBS twice and counted using a haemocytometer. 1 million cells were taken for cell cytotoxicity studies. Different concentrations of the compound was added to the cells and then made up to 1 ml with PBS. Cells were incubated for 3 hours at 37°C. After incubation, the cell death was evaluated using Trypan Blue exclusion method. To the cell suspension, 3 drops of Trypan Blue (0.5 % in PBS) was added and the cells were loaded immediately on to a haemocytometer. The number of Dead cells was counted and the percentage of dead cells was calculated. Viable cells exclude the dye while non viable cells take up the dye and appear blue in colour.

$$\% \text{ of Dead cells} = \frac{\text{Dead cells} \times 100}{\text{Total cells}}$$

2.6. Cell Viability Assay by MTT

MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay is based on the ability of viable cells to reduce MTT from a yellow water-soluble dye to a dark blue insoluble formazan product [5].

5x10³ cells were plated in 100 µl of the medium (DMEM with 10% FBS) in 96 well corning plates in the presence or absence of various concentrations of the compound for 48 and 72 hours [6]. Media treated cells was taken as negative control. At the end of the incubation, 25 µl of MTT solution (5 mg/ml in PBS) was added to each well. After a 2 hour incubation, at 37°C, 100µl of the extraction buffer was added (20% Sodium Dodecyl Sulfate) in 50% Dimethylformamide. After a 4 hour incubation, the optical densities at 570 nm was taken using a multiwell plate reader with the extraction buffer as blank [7]. The minimum concentration required for 50% cytotoxicity and Inhibitory concentration required for 50% cytotoxicity (IC₅₀) was calculated.

$$\% \text{ of cell cytotoxicity} = 100 - \frac{\text{Treated OD}}{\text{Control OD}} \times 100$$

2.7. Isolation of Lymphocytes from whole Blood

3ml of blood was taken in heparinised test tube. 5ml of PBS was added and mixed well by inversion. 3ml of ficoll hypaque solution was added in a conical centrifuge tube. Carefully layered 8ml of blood PBS mixture was added on to the ficoll hypaque. And then centrifuged at 2000 rpm for 30 minutes. The opaque interface containing mononuclear cells was aspirated and transferred into a clean conical centrifuge

tube with a Pasteur pipette and discarded the upper layer. 10ml of PBS solution was added and mixed by inversion. Centrifuged at 1500rpm for 10 minutes and supernatant was discarded. Resuspended pellet with 5ml PBS and again centrifuged at 1500rpm for 10 minutes. Repeated the step thrice and resuspended lymphocyte pellet in 500µl PBS.

2.8. Determination of Cytotoxicity Towards Normal Lymphocytes

The cytotoxicity of the compound towards normal lymphocytes was determined by MTT assay described above.

2.9. Statistical Analysis

The data were expressed as mean± S.E.M. Results were analyzed statistically by one-way analysis of variance (ANOVA). P-value <0.05 was regarded as statistically significant.

3. RESULTS

The fraction containing embelin, the lead molecule of *Embelia ribes* exhibited cytotoxicity against DLA and K-562 cells. Bioactivity directed fractionation led to the isolation of the lead molecule, Embelin. There is a graphical increase in the percentage of cytotoxicity with increasing time and concentration of the active compound. The compound exhibited 67% cytotoxicity on DLA cells at 800 µg/ml concentrations after 72 hours and 61.7% cytotoxicity on K-562 cells at the same time interval and concentration (Table 1, 2, 3 & 4). The Inhibitory concentration required for 50% cytotoxicity (IC₅₀) was determined graphically. The IC₅₀ of the active fraction against DLA cells and K-562 cells was 370 µg/ml and 114.5 µg/ml after 72 hours incubation (Graph 3 & 4). However the compound did not exhibit any cytotoxicity towards normal lymphocytes. The isolation, identification and quantification of embelin, the active principle of *Embelia ribes* had been investigated using HPLC, HPTLC, UV and different NMR techniques (Figs. 1,2,3 & 4).

Table 1. Short Term Cytotoxicity by Trypan Blue Exclusion Test

Concentration (µg/ml)	Percentage of Cytotoxicity
100	35.71
200	42.46
400	52.94
800	62.35

Table 2. DLA Cells (MTT Assay) 72 Hours

Concentration (µg/ml)	Percentage of Cytotoxicity
25	5.2
50	21.15
100	28.60
200	41.2
400	53.15
800	67

Table 3. K-562 (Leukemic Cells)-MTT Assay- 72 Hours

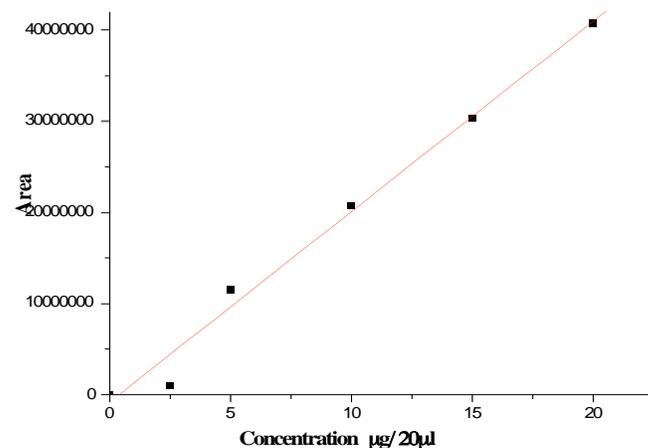
Concentration ($\mu\text{g/ml}$)	Percentage of Cytotoxicity
800	61.7
400	59.31
200	54.6
100	49.42
50	48.3
25	44.7

Table 4. Lymphocyte Proliferation Assay – To Determine Cytotoxicity Towards Lymphocytes Isolated from Normal Human Blood

Concentration ($\mu\text{g/ml}$)	Percentage of proliferation
25	99.1
50	98.7
100	98.2
200	98.1
400	98.1
800	97.6

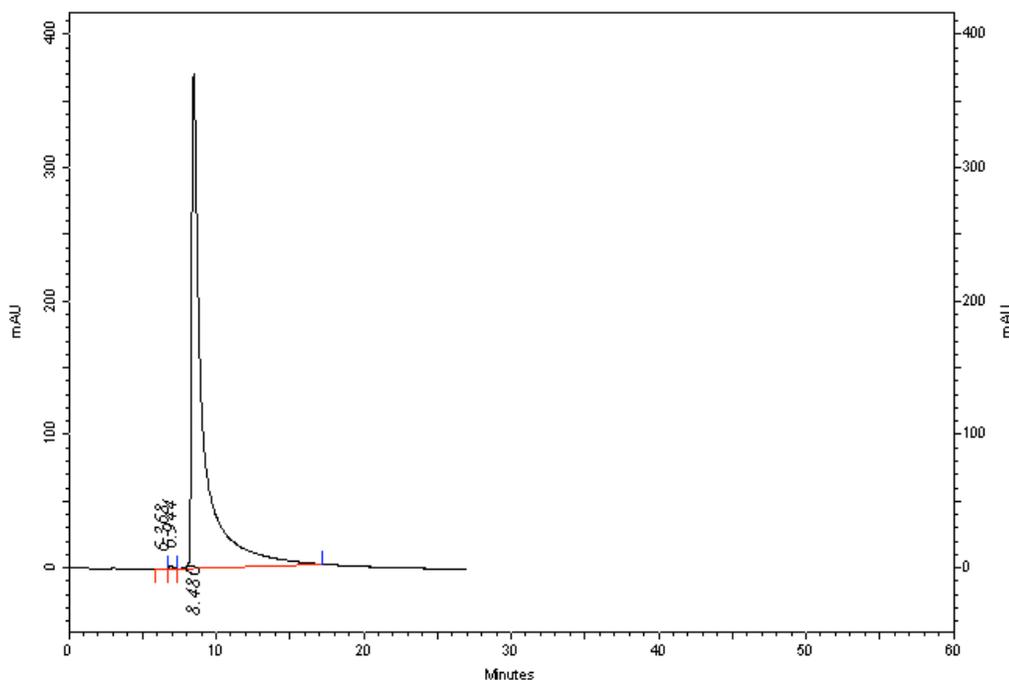
Any Cancer therapy is basically aimed at killing the cancer cells while sparing the normal cells. Plants are continuously being screened throughout globe, in search of novel compounds that are more specific, toward eliminating tumour cells with least side effects. The survey of literature pertaining to *Embelia ribes* showed that investigations were carried out regarding its medicinal properties but few at-

tempts had been made to investigate this plant, as a source of drug for cancer too. Further studies on the mechanism of action of the lead molecule on the cell cycle and apoptosis pathways are under progress.

**Fig. (2).** Calibration curve of embelin by HPLC.

4. DISCUSSION AND CONCLUSION

In vitro studies on Embelin suggest the potential of the compound on the suspension cell lines DLA (Graph 1, 2 & 3) and K 562 (Table 3 & Graph 4,5). However the compound did not exhibit significant toxicity on normal lymphocytes (Table 4, Graph 6) isolated from human blood preferentially attacking the tumour cells. The comparison of cytotoxicity (Graph 7) clearly indicated that this compound has no toxicity towards normal human cells. At the same time it is highly cytotoxic at $400\mu\text{g/ml}$ and $800\mu\text{g/ml}$ towards DLA and K-562 cell lines with IC 50 at 370 and $114.5\mu\text{g/ml}$ (Graph 4, 5, 6 & 7). The cytotoxicity for cyclophosphamide, the drug for the treatment of suspension type cancers is also

**Fig. (1).** HPLC Profile of isolated embelin at 298 nm.

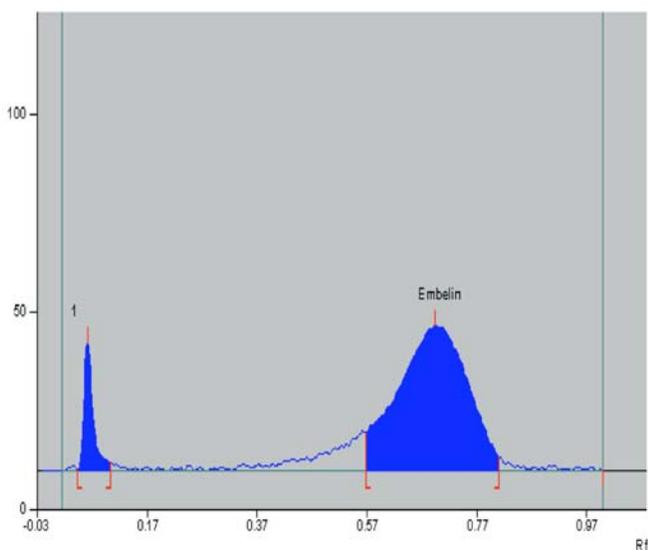


Fig. (3). HPTLC Profile of isolated embelin at 298 nm.

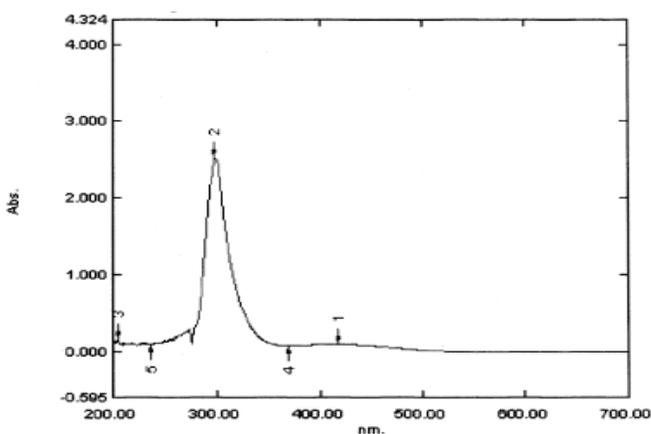
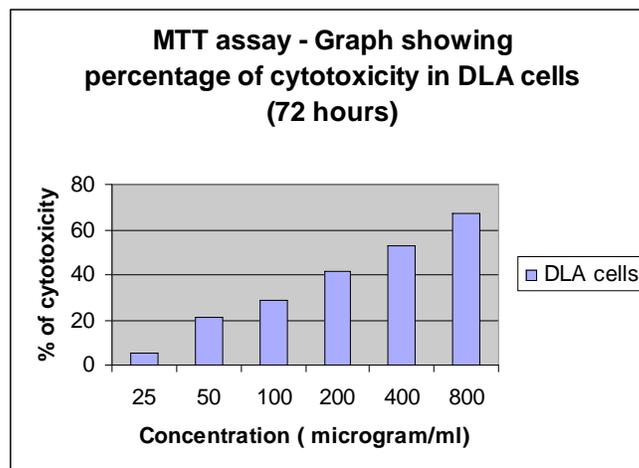
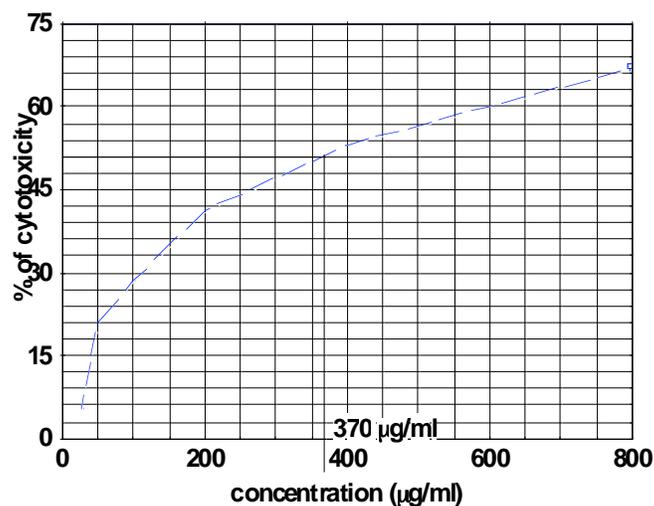


Fig. (4). UV absorption spectrogram of embelin.

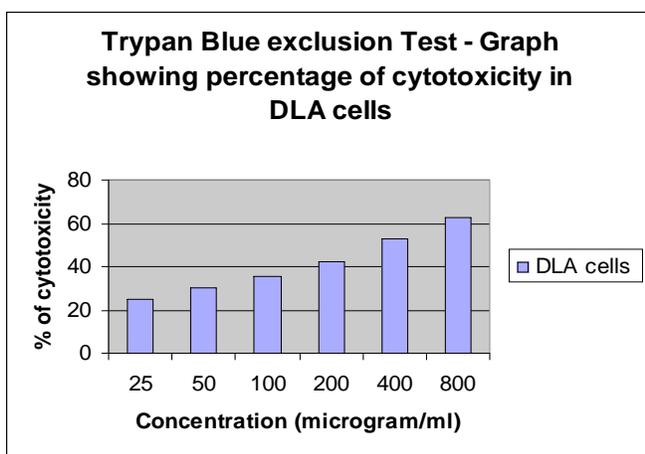
activity of *Embelia ribes* and its active principle embelin against DLA and K-562 cell lines.



Graph 2.

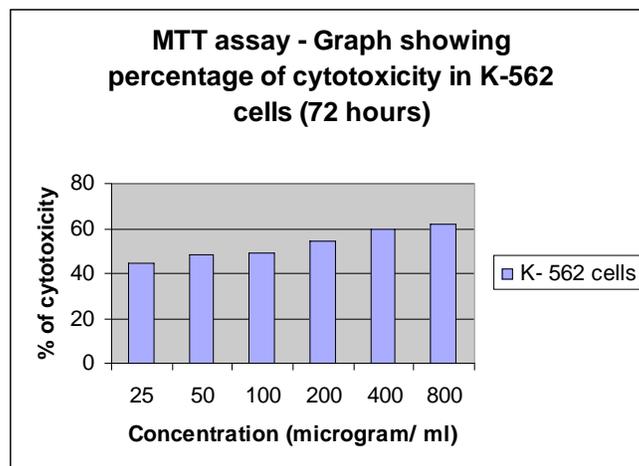


Graph 3. Determination of IC₅₀ on DLA cells by embelin after 72 hours

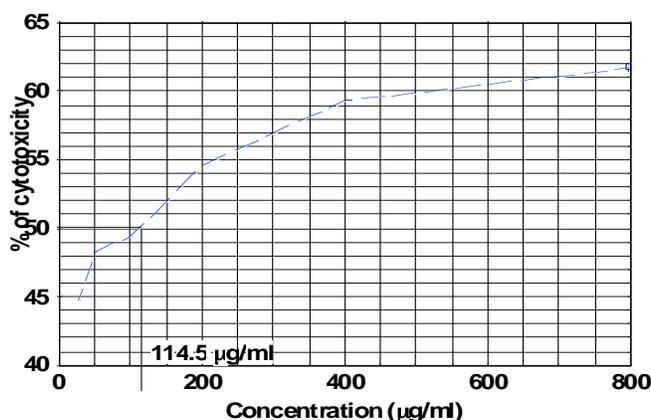


Graph 1.

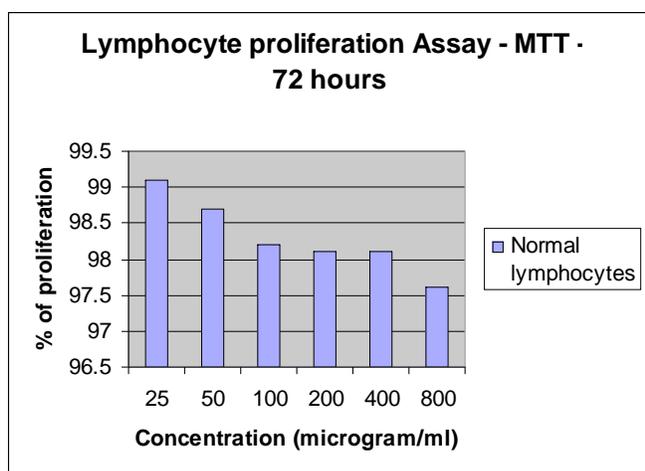
evaluated invitro and and it is found that its cytotoxicity is high with that of embelin. But the toxicity towards normal lymphocytes are also high for the drug. The study indicated that embelin preferentially attack the cancer cell lines than cyclophosphamide This is the first report of invitro cytotoxic



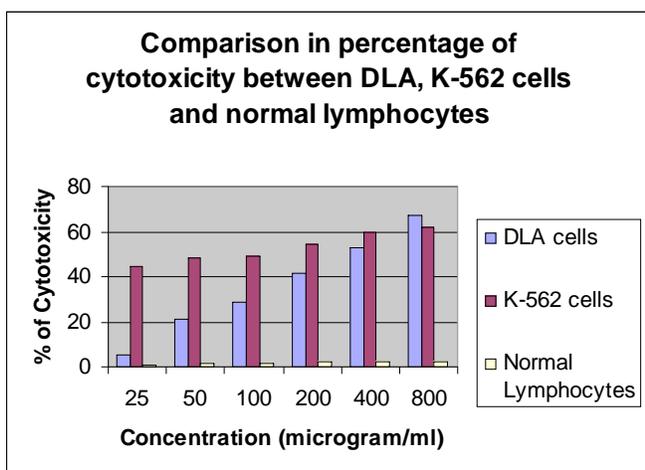
Graph 4.



Graph 5. Determination of IC 50 on K-562 cells by embelin after 72 hours.



Graph 6.



Graph 7. Percentage of cell cytotoxicity-comparison between DLA, K-562 and Lymphocytes.

5- O-ethylembelin and 5- O-methylembelin were reported to possess antiproliferative active against HL-60 leukemia cells and cervical carcinoma He La cells. Treat-

ment with 10 microM of 1 and 2 for 24 h induced apoptosis in HL-60 cells. These compounds arrested HL-60 cells in the G(0)/G(1) phase of the cell cycle in a dose- and time-dependent manner. In HeLa cells, exposure to 100 microM of 1 or 2 for 6 h induced a complete disassembly of the microtubule network and an increased number of cells blocked in mitotic stages. This evidence suggests that both 1 and 2 are promising novel antimetabolic and anticancer molecules targeting microtubular proteins [8]. Embelin induced caspase 3 and 9 activation in LNCaP and C4-2 cells by decreasing XIAP expression and was more potent than bicalutamide in killing prostate tumor cells irrespective of their androgen status [9]. Embelin is reported to be a fairly potent, nonpeptidic, cell-permeable, small-molecule inhibitor of XIAP and represents a promising lead compound for designing an entirely new class of anticancer agents that target the BIR3 domain of XIAP [10]. Embelin is reported to possess TRAIL induced apoptosis inducing activity in pancreatic cancer cells [11]. Our studies using colon cancer cells lacking p53 and Bax suggest that both lysosomes and mitochondria are prominent targets of embelin-induced cell death. Embelin induced cell-cycle arrest in G1 phase through p21, downstream of p53. In the absence of p21, the cells are sensitized to death in a Bax-dependent manner [12]. Our studies highlights the preliminary antiproliferative activity against Human Leukemic K-562 cells and Murine Lymphoma cells (DLA) for the first time and its relative non toxicity on normal human lymphocytes. More studies are being done in our lab in these Lymphoma as well as leukemic cells to evaluate its effects on the molecular mechanisms of apoptosis.

Our preliminary data of cytotoxic screening of different extracts of *Embelia ribes* showed hexane extract to be a promising one regarding its antitumour potential. The lead compound was also identified and it exhibited significant cytotoxicity towards tumour cells. As embelin is not soluble in water, we have made nanoembelin on biodegradable biopolymer to enhance the efficacy of the molecule in cancer therapy (Patent Submitted). And its antitumor activities in vivo as well as invitro are in progress.

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REFERENCES

- [1] Latha, C. Selective extraction of embelin from *Embelia ribes* by hydrotropes. *Sep. Sci. Technol.*, **2006**, *41*, 3721-3729.
- [2] Chitra, M.; Devi, S.C.S.; Sukumar, E. Antibacterial activity of embelin. *Fitoterapia*, **2003**, *74*, 401-403.
- [3] Chitra, M.; Sukumar, E.; Suja, V.; Devi, S.C.S. Antitumour, anti inflammatory and analgesic property of embelin, a plant product. *Chemotherapy*, **1994**, *40*, 109-113.
- [4] Joshi, R.; Kamat, J.P.; Mukherjee, T. Free radical scavenging reactions and antioxidant activity of embelin: Biochemical and pulse radiolytic studies. *Chem. Biol. Interact.*, **2007**, *167*(2), 125-134.
- [5] Mosmann, T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxic assays. *J. Immunol. Methods.*, **1983**, *65*, 55-63.
- [6] Scudiero, D.A.; Shoemaker, R.H.; Paull, K.D.; Monks, A.; Tierney, S.; Nofziger, H.; Currens, M.J.; Seniff, D.; Boyd, M.R. Evaluation of a soluble tetrazolium formazan assay for cell growth and drug sensitivity in culture using human and other tumour cell lines. *Cancer Res.*, **1988**, *48*, 4827-4833.

- [7] Anto, R.J.; Maliekal, T.T.; Karunakaran, D. L- 929 cells harbouring ectopically expressed Rel A resist Curcumin induced apoptosis. *J. Biol. Chem.*, **2000**, *275*, 15601-15604.
- [8] Xu, M.; Cui, J.; Fu, H.; Proksch, P.; Lin, W.; Li, M. Embelin derivatives and their anticancer activity through microtubule disassembly. *Planta Med.*, **2005**, *71*(10), 944-948.
- [9] Danquah, M.; Li, F.; Charles, B.D.; Duane, D.M.; Ram, I.M. Micellar Delivery of Bicalutamide and Embelin for Treating Prostate Cancer. *Pharm. Res.*, **2009**, *26*(9), 2081-2092.
- [10] Zaneta, N-C.; Liang, X.; Zengjian, H.; York, T.; Peng, L.; Peter, P. R.; Renxiao, W.; Xueliang, F.; Ribo, G.; Manchao, Z.; Marc, E. L.; Dajun, Y.; Shaomeng, W. Discovery of Embelin as a Cell-Permeable, Small-Molecular Weight Inhibitor of XIAP through Structure-Based Computational Screening of a Traditional Herbal Medicine Three-Dimensional Structure Database. *J. Med. Chem.*, **2004**, *47*(10), 2430-2440.
- [11] Tomohiko, M.; Ryuichiro, D.; Atsushi, K.; Kazuyuki, N.; Kazuhiro, K.; Daisuke, I.; Eiji, T.; Yoshiya, K.; Shinji, U. Effect of the XIAP Inhibitor Embelin on TRAIL-Induced Apoptosis of Pancreatic Cancer Cells. *J. Surg. Res.*, **2007**, *142*(2), 281-286.
- [12] Beena, J.; Rajeeve, S.; Abraham, T.E.; Mohan, J.; Praveen, K.S.; Mahendra, S.; Santhoshkumar, T.R. Lysosomal destabilization and cathepsin B contributes for cytochrome C release and caspase activation in embelin-induced apoptosis. *Mol. Carcinogenesis.*, **2010**, *49*(4), 324-336.

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