

Research Article**Isolation of embelin from *Embelia ribes* and evaluation of its anti-cancer potential in breast cancer**Veerpal Kaur,¹ Supandeep Singh Hallan,¹ Nidhi,¹ A.N. Kalia,² Neeraj Mishra^{1*}¹Department of Pharmaceutics, ISF College of Pharmacy, Moga, Punjab, India²Department of Pharmacognosy, ISF College of Pharmacy, Moga, Punjab, India

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Abstract

Objective: Aim of present study to evaluate anticancer activity of dried fruit of *Embelia ribes* containing embelin in various types of cancer. **Material and Method:** Embelin was extracted and isolated from *Embelia ribes* berries of Indian origin. Isolated embelin was characterized by melting point, TLC, partition coefficient, solubility, FT-IR, NMR and Mass spectroscopy. Assessment of anticancer activity was done by Cytotoxicity assay and Apoptosis assay by using MCF-7 breast cancer cells. Isolated Embelin was investigated on MCF-7 breast cancer cell line by MTT assay and apoptosis assay. Different concentration of embelin were used and observed that 80µg/ml was the IC₅₀ value of embelin in MCF-7 breast cancer cells and it was found that embelin was able to affect the viability of cells and also induce apoptosis of MCF-7 breast cancer cells in a dose dependent manner. **Results:** We have found that the isolated embelin have significant (*p < 0.0001) potency against the MCF-7 breast cancer cells in dose dependent manner and showed significant increase in apoptotic cells at IC₅₀ value. **Conclusion:** Isolated embelin have significant potency against the MCF-7 breast cancer cells in dose dependent manner.

Keywords: Embelin, Apoptosis, Cancer, Cytotoxicity, Breast Cancer, TLC

Introduction

The term “breast cancer” is a type of malignant tumor that originates from cells in the breast and is defined as the rapid and unlimited growth of abnormal cells in the mammalian glands which produces milk from breast or in the ducts that deliver milk to the nipples. The main site of breast cancer is stromal cell which include fibrous connective tissues and fatty. It is a leading disease affecting females in particular, and its occurrence is growing annually (Benson and Jatoo, 2012; Anderson et al., 2011). Widely, estimated number of cases of breast cancer reached 1.2 million. Worldwide, in every 3 minutes, a woman is identified with breast cancer and nearly about 1 million annually (Parkin, 2001). Hence, the crucial therapeutic methodology is required for the treatment of breast cancer.

However, market survey or literature review reveals that numerous pharmacological agents are used in the

treatment of breast cancer include anastrozole, arimidex, carboplatin, capecitabine, docetaxel, cisplatin, cyclophosphamide, doxorubicin, epirubicin hydrochloride, flurouracil, fulvestrant, gemcitabine, herceptin, letrozole, methotrexate, paclitaxel, pertuzumab, tamoxifen, zoladex, vincristine and vinblastine but these drugs shows many side-effects like bleeding, decrease in blood cell counts, diarrhoea, allergic reaction, loss of appetite, hair loss, joint pain (short term), change in taste, weight gain, numbness or tingling in the fingers or toes, nausea and vomiting. Hence, alternative therapy having fewer side effects with maximal therapeutic efficacy is required to combat with this dreadful disease.

Embelin, (2, 5-dihydroxy-3-undecyl-p-benzoquinone), a naturally occurring quinonoid compound, is found to be the major constituent of *Embelia ribes* (Myrsinaceae). *Embelia ribes* commonly known as Vidanga or Via-vidang is a large woody climbing shrub and is widely distributed throughout Pakistan and India. The fruit contains embelic acid, tannins, christembin and embelin as major constituents. Vilangin, 2, 5-isobutylamine salts, quercetol and volatile oil are also present. The dried fruit has been used in India from time immemorial as an anthelmintic.

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Embelia ribes has been shown to possess astringent, carminative, stimulant, antioxidant, anti-spermatogenic, antibacterial, anticancer, anti-inflammatory, antidiabetic, antidyslipidemic, analgesic and cardio-protective activity. The fruit cures tumors, ascites, bronchitis, jaundice and mental disorders. The plant is used as an anti-inflammatory drug to relieve rheumatism and fever. *Embelia ribes* is extensively used in Indian traditional medicine for treating various disease conditions including chronic inflammatory disorders, heart and urinary conditions, snake and insect bites, and tumor (Kapoor et al., 1983). Embelin is available commercially as tablets and powders in spite of its not absorbed at all in entire upper portions of gastrointestinal tract and remain as such in colon. The log P value of embelin about 4.8 is indicating its high lipophilicity. Chemical structure of embelin is having quite resemblance with the structure of natural Coenzyme Q10 (ubiquinones) and the role of this is well defined in various biochemical protective mechanism (Mozaina et al., 2008).

Embelin is a polyphenolic compound that binds to the Smac binding site in the BIR3 domain of XIAP protein molecules and inhibit XIAP (X-linked apoptosis protein) (Reuter et al., 2010; Hu et al., 2011; Nikolovska-Coleska et al., 2004). Various studies have confirmed that embelin has anti-oxidative biological and also has anti-inflammatory effects (Joshi et al., 2007; Sreepriya and Bali, 2006; Mahendran et al., 2011; Thippeswamy et al., 2011). It has been established that embelin has a wide-ranging antitumor nature and is competent to control the development of different tumor cells, like breast, prostate, hepatic pancreatic and colon cancer (Danquah et al., 2009; Dai and Wang, 2010; Aird et al., 2008; Mori et al., 2007). However, the exhaustive mechanism of embelin for its antitumor activity remains unknown.

Embelin releases cytochrome C and activates the caspase family to result in the induction of breast cancer apoptosis through regulation of the action of the Bcl-2/Bax family in the mitochondrial pathway (Li et al. 2013). It clearly inhibits NF- α B activation, which makes it a potentially effective suppressor of tumor cell survival, proliferation, invasion, angiogenesis, and inflammation (Ahn et al., 2007). PEG_{3.5K}-EB₂ conjugate and preliminary in-vitro study suggested that it functioned as a dual delivery system and showed synergistic activity with co-delivered PTX against several cancer cell lines (Huang et al., 2012). Similarly a conjugate of PEG_{5K} with two embelin molecules (PEG_{5K}-EB₂) forms small-sized micelles (20-30 nm) that effectively solubilize hydrophobic drugs such as PTX. PTX-loaded PEG_{5K}-EB₂ micelles show kinetics of sustained release and are effectively targeted to tumors in vivo with minimal nonspecific uptake by RES and exhibited potent

cytotoxicity to several cultured cancer cell lines (Lu et al., 2013). The aim of present study was to extract embelin from *Embelia ribes* and evaluate its anti-cancer activity on MCF-7 breast cancer cell line using MTT assay and apoptosis assay.

Materials and methods

Plant material

Embelia ribes fruits were purchased from Kharibabli, Delhi and authenticated by Dr. H.B Singh, Chief Scientist & Head, Raw Material Herbarium & Museum, NISCAIR, New Delhi, India (NISCAIR/RHMD/Consult/-2012-2013/2090/97).

Chemicals

Standard embelin was purchased from Indofine chemical company, Inc, USA. Taxol was obtained as gift sample from Mac-Chem Products (India) Pvt. Ltd., Mumbai. TLC aluminium sheets silica gel 60 was supplied by E. Merck, Mumbai, India. MCF-7 cancer cell line was purchased from NCCS, Pune. MTT (3-(4, 5-dimethylthazol-2-yl)-2, 5-diphenyl tetrazolium bromide) and Annexin V-FITC Apoptosis Detection Kit were purchased from Sigma Aldrich, USA. All solvents used in the experiment procedure were analytical grade.

Isolation of Embelin

The pure embelin was isolated from the *Embelia ribes* by using extraction procedure (Suthar et al., 2012). Berries of *Embelia ribes* were air dried, coarsely powdered and stored in closed container. Dried powder (250 gm) was extracted with n-Hexane by using Soxhlet apparatus for 6 hours. Residue was obtained by removal of solvent by distillation. Then the residue was washed with cold pet ether. After washing, the residue was dissolved in mixture of dichloromethane (DCM) and methanol. Then the solution was kept for crystallization for 24 hours. After crystallization the crystals were filtered out from the mother solution. Finally washed the crystals with n-hexane and followed by DCM. The golden coloured embelin crystals were obtained and characterized the embelin using organoleptic properties like appearance, colour and consistency, melting point, chemical test, TLC and solubility.

Physical Parameters of isolated embelin

Isolated embelin from *Embelia ribes* were identified according to standard procedures.

Colour and consistency

Physical appearance of embelia like colour and consistency was analyzed by visual observation.

Melting point

A capillary melting point apparatus was used to determine melting point of the drug. A small amount of drug was filled in the previously one sided sealed capillary and melting point analyzed in melting point apparatus to observe melting point range.

TLC profiling

1 mg isolated embelin was dissolved in 10 ml DCM. 10 μ l of test sample was applied on pre-coated silica gel G aluminum plate. The chromatogram was run in the mobile phase Ethyl acetate: Toluene: Methanol: Formic Acid (0.5: 0.4: 0.1: 0.05) in twin trough chamber. After the saturation of chamber, TLC plate was developed and observed under ultra violet light, R_f value was compared with the standard marker (Chauhan et al., 1999).

Chemical Test

Identification of embelin was confirmed by chemical test. Embelin was dissolved in pet ether and then diluted ammonia solution was added to it. Formation of bluish-violet precipitate indicates the presence of embelin.

FT-IR spectroscopy

FT-IR spectra were obtained using a FT-IR spectrophotometer (Thermo Nicolet, USA). The potassium bromide (KBr) disk method was employed, mixing small amount of drug powder with spectroscopic KBr and compressed in a vacuum press to obtain a disk. Infrared Spectrum was recorded by scanning over a wave number region of 400-4000 cm^{-1} using Nicolet Omnic software. The characteristic I.R. spectrum and peaks were observed and were compared with the spectrum and peaks of the reference spectrum of the embelin (Radhakrishnan et al., 2011).

¹H NMR Spectra

¹H-NMR was done by sophisticated multinuclear FT NMR Spectrometer model Avance-II (Bruker) in the Centre. The instrument is equipped with a cryomagnet of field strength 9.4 T. Its ¹H frequency is 400 Mhz, while for ¹³C the frequency is 100MHZ.

Partition coefficient

Partition coefficient is a measure of drug lipophilicity and an indication of its ability to cross bio-membrane factor. It is also useful in screening of biologic properties. The partition coefficient is defined as ratio of unionized drug distributed between organic and aqueous phase. The partition coefficient of embelin was determined by Shake Flask Method (De Bruijn et al., 1989). The partition coefficient of embelin was determined in octanol: water. The octanol: water mixture was kept to equilibrate for 24 hours in separating funnel. Then 1 mg of the embelin in 25 ml of water and 25 ml of n-octanol was added to it and after 5 hours both the phase was separated and organic phase was analyzed using UV spectrophotometer. The concentration in aqueous phase was determined by difference between initial drugs added to mixture with drug concentration in organic phase.

P_{ow} = Concentration in organic phase/concentration in aqueous phase

Saturation solubility

The solubility of embelin in various solvents was determined by using shake flask method (Ibrahim et al., 2011). Briefly, an excess amount of embelin was added to each vial containing 1 ml of the selected vehicle. After sealing, the mixture was vortex for 10 min in order to facilitate proper mixing of embelin with the vehicles. Mixtures were then stirred for 24h on magnetic stirrer maintained at room temperature. After stirring mixtures were centrifuged at 5000 rpm for 5 min and drug in the supernatant was analyzed for embelin by UV-visible spectrophotometric method at λ_{max} of 292.75 nm.

In-vitro Cytotoxicity Assay

Cell culture

MCF-7 breast cancer cells were purchased from the Culture Collection (NCCS, Pune, India). Following cell passage, cells were inoculated in MEM culture medium containing 10% fetal calf serum. The cells were then cultured in an incubator containing 5% CO₂ and 95% O₂, at 37°C (Chaudhari et al., 2012).

Cell viability

MCF-7 cells in the logarithmic growth phase were collected and cultured in 96-well culture plates inoculated for 24 h. When the cells had grown to adherence, different concentration of embelin (40, 60, 80, 100, 120 μ g/ml) were administered to various groups with triplicate wells for each concentration. A negative control group without embelin was also created. All cells were incubated in 5% CO₂ for further culture for 24 prior to the colour reaction. Subsequently, MTT was added to each well, and cells were cultured in CO₂ at 37°C in an incubator for 2 h. The optical density (OD) values (A570nm) of each well were measured with a microplate reader (Bio-Rad; Hercules, CA, USA) (Chaudhari et al., 2012).

Apoptosis Assay

Human breast cancer cell line (MCF-7) was treated with Annexin V-FITC Apoptosis detection kit. The cells were plated in 6 well plates and treated with different concentration of drugs and incubated. Media was removed and washed with PBS and re-suspended in annexin binding buffer. 1 μ l annexin V dye and PI was added and kept in dark for 30 min. Finally cells were washed and observed under fluorescent microscope using appropriate filters.

The cells should separate into three groups: live, apoptotic and dead. Live cells show only weak annexin V staining of the cellular membrane, while apoptotic cells show

asignificantly higher degree of surface labelling. Dead cells show both membrane staining by Annexin V and strong nuclear staining from the propidium iodide (Chaudhari et al., 2012).

Statistical analysis

All the results are expressed as mean \pm standard deviation. The treated groups were compared with control by applying analysis of variance (ANOVA). The statistical analysis was carried out Graph Pad Software Corp., San Diego, CA. The p value <0.0001 was considered to indicate a statistically significant difference between groups.

Results

The pure embelin was isolated from the *Embelia ribes* by using extraction procedure and conformer its purity by physical appearance like colour, consistency, solvent system, R_f value and chemical test. The results of identification tests are in Table 1.

Table 1. Summary of Identification tests for isolated Embelin

Test	Results
Colour	Golden Yellow
Consistency	Crystalline
% Yield	8.4%
Solvent System	Ethyl acetate : Toluene : Methanol : Formic Acid 0.5 : 0.4 : 0.1 : 0.05
R_f Value	0.752
Chemical Test	Pet ether solution of embelin gives bluish-violet precipitate with dil. ammonia solution, which indicates the presence of embelin.
Melting Point	Standard Embelin 143-146°C Isolated Embelin 142-145°C

FT-IR spectroscopy

Observed peaks in IR spectrum of standard embelin (Figure 1) and isolated embelin (Figure 2) were found to be concordant with functional groups present in structure of standard embelin and isolated embelin.

Table 2 shows frequency of observed bands and its interpretation. Purity of procured sample was confirmed from its IR spectrum.

Table 2. Interpretation of FTIR of Embelin

Standard embelin peaks (cm^{-1})	Isolated embelin peaks (cm^{-1})	Interpretation
1195	1194	C-O bond
1615	1615	Stretching vibration of C=O
2849	2848	Stretching vibration of methyl C-H
3309	3308	Stretching vibration of OH group).
2920	2920	Aromatic C-H stretching

NMR (Nuclear Magnetic Resonance)

(a) $^1\text{H NMR}$ Spectra of Standard Embelin

Molecular Formula $\text{C}_{17}\text{H}_{26}\text{O}_4$

Proton NMR ($^1\text{H NMR}$ - 300 MHz, DMSO) demonstrates chemical shifts at δ 7.8 (2H, board spectra, 2xOH), 5.8(1H, singlet, H-3), 1.5 (2H, triplet, H-7), 1.24 (18H, board spectra, H-8- H-16), 0.88 (3H, triplet, H-17). $^1\text{H NMR}$ Spectra of standard

embelin is shown in Figure 3.

(b) $^1\text{H NMR}$ Spectra of Isolated Embelin

Molecular Formula $\text{C}_{17}\text{H}_{26}\text{O}_4$

Proton NMR ($^1\text{H NMR}$ - 400 MHz, DMSO) demonstrates chemical shifts at δ 10.97 (2H, board spectra, 2xOH), 5.75(1H, singlet, H-3), 2.30 (2H, triplet, H-7), 1.36 (2H, board spectra, H-8), 1.24 (16H, board spectra, H-16), 0.86 (3H, triplet, H-17). $^1\text{H NMR}$ Spectra of Isolated Embelin is shown in Figure 4.

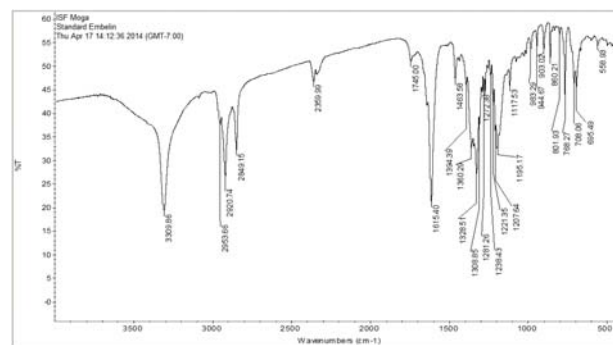


Figure 1. FT-IR spectrum of Standard Embelin

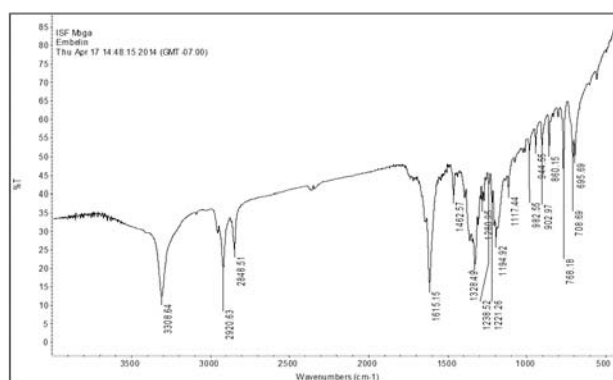


Figure 2. FT-IR spectrum of Isolated Embelin

Partition coefficient

Partition coefficient of isolated embelin was found to be 4.595, which is comparable to the standard drug *i.e.* 4.609 by using formula given below;

$$\text{Log } P_{o/w} = \log \left(\frac{\text{Solute}_{\text{Octanol}}}{\text{Solute}_{\text{water}}} \right)$$

Solubility of Embelin

Solubility studies were carried out by dissolving embelin in 1ml of different solvent and results were depicted Table 3.

Table 3 Solubility profile of Isolated Embelin

Solvents	Saturated solubility of Isolated Embelin (mg/ml)	Description
DMSO	120-126 mg/ml	Freely Soluble
Methanol	5-7 mg/ml	Slightly Soluble
Ethanol	4-6 mg/ml	Slightly Soluble
Water	0.2-0.3 mg/ml	Insoluble

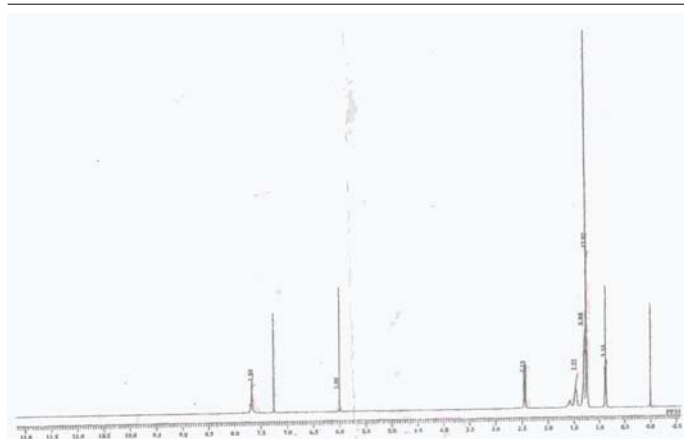


Figure 3. ^1H NMR Spectra of Standard Embelin

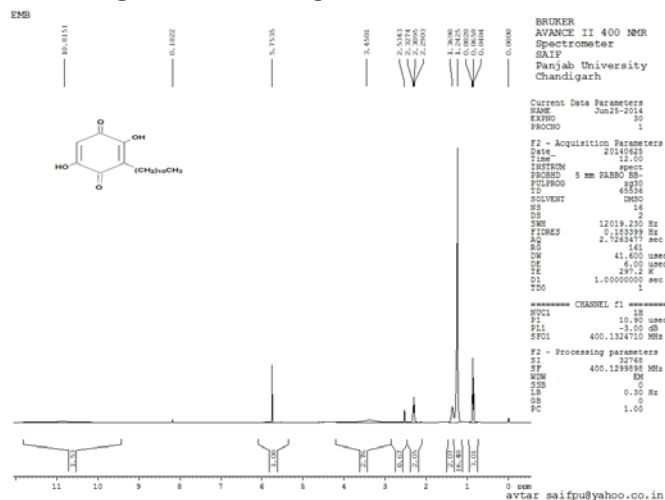


Figure 4. ^1H NMR Spectra of Isolated Embelin

In-vitro Cytotoxicity Assay

Embelin stimulates the inhibition of cell growth in MCFs-7 breast cancer cells. Various concentrations of isolated embelin i.e. 40, 60, 80, 100 and 120 $\mu\text{g/ml}$ were added to MCF-7 breast cancer cells for 24 h. Then MTT method was utilized to verify the cell activity which is different at different concentrations (Figure 5). The results demonstrated that cytotoxicity potential of breast cancer cells significantly decreased, when the concentration of isolated embelin was increased and isolated embelin has shown significant cytotoxic potential in MCF-7 breast cancer cells and its IC_{50} value 80 $\mu\text{g/ml}$. This result indicates that isolated embelin has an inhibitory effect on the growth of MCF-7 breast cancer cells.

Apoptosis Assay

Embelin also induces apoptosis of MCF-7 breast cancer cells. The cancerous cells were treated with different concentration of isolated embelin for 24 h and observed under fluorescent microscope to determine the effect on the rate of apoptosis (Figure 6). The observed results established that the rate of MCF-7 breast cancer cell apoptosis significantly increased with increase in concentration of embelin in dose-dependent manner. At IC_{50} value of isolated embelin 80 $\mu\text{g/ml}$ the rate of apoptotic

cells was significant.

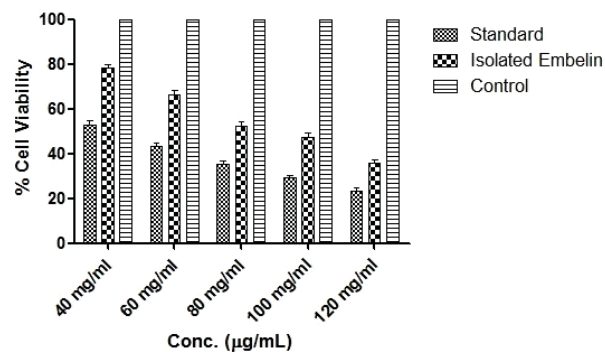


Figure 5. Cytotoxicity study of standard (Taxol) and Isolated Embelin in MCF-7 cell line by using MTT assay vs. control

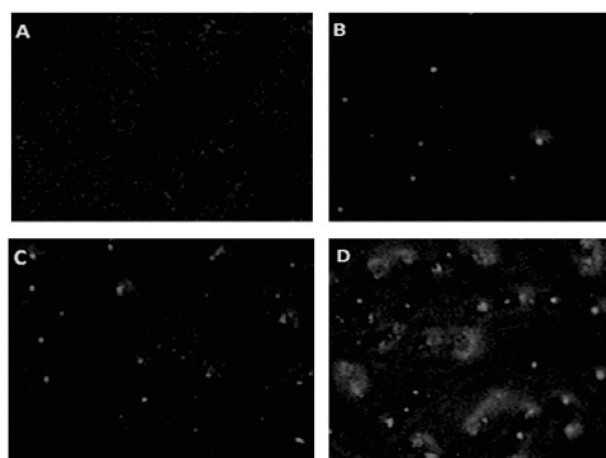


Figure 6. Apoptosis-inducing activity: (A) Plain MCF-7 cells, (B) Standard Embelin, (C) Standard Taxol (D) Isolated Embelin in MCF-7 cell line

Discussion

Cancer is a very fast developing, serious and life-threatening malignant tumor. It is important to study the disease in detail and find effective anticancer drugs, which are safe and efficient. Plants derived anticancer drugs have advantages of including a wide variety, low toxicity and few side-effects and adverse reactions. So, very effective anticancer drugs from plants are being explored. Embelin affects the explosion and apoptosis of a wide range of cancer cells because it acts as a small molecular inhibitor with specific inhibition of XIAP. Researchers have studied that embelin inhibits the cell multiplication of various abnormal cancerous cells, significant results have been seen in breast cancer and other solid cancerous cells (Allensworth *et al.*, 2012).

In the present study, isolation of embelin was carried out by using n-hexane as a solvent reported by Suthar *et al.* with little modification. Ganesan and

Madhavan demonstrated that isolation of embelin with other solvents like acetone, chloroform, carbon tetrachloride, diethyl ether, ethyl acetate, methanol and propanol (Ganesan et al., 2010; Madhavan et al., 2011). After isolation and purification, pure embelin was obtained in crystal form. The characterization studies of isolated embelin i.e. Colour, melting point, FTIR-Spectra, NMR-Spectra were compared with results of standard embelin, which were almost identical.

Based on our results cytotoxicity assay, we conclude that embelin is a potent inhibitor of XIAP, which make it potentially effective suppressor of tumor cell survival. We demonstrated that when breast cancer cells (MCF-7 cell line) had been treated with different concentration of embelin i.e. 40, 60, 80, 100, 120µg/ml for 24 hr, the rate of cell viability decrease with increase in concentration of embelin. This result indicates that the embelin showed inhibitory effect on the growth of breast cancer cells. The results were shown significant (*p < 0.0001) activity of the isolated embelin was compared with the Standard (Taxol). These results were found to be similar as the studies done by Yang et al., 2013 by using embelin (Li et al., 2013).

Embelin is capable to induce breast cancer cell apoptosis instead of directly causing cell death. In figure 5, control treatment group (0µg/ml) shows absence of apoptotic cells due to absence of embelin. The percentage of apoptotic cells was higher at 40µg/ml as compared to control group but less than the percentage of apoptotic cells at 60µg/ml. At IC₅₀ value (80µg/ml), high percentage of apoptotic cells was present. Our IC₅₀ were found to be similar with the studies done by Yang et al., 2013. However, embelin induce higher apoptosis at higher concentration in dose dependent manner.

Conclusion

In this study, embelin was isolated from *Embelia ribes* and confirmed its chemical structure by FTIR and ¹H NMR spectroscopy. We have found that the isolated embelin have significant (P < 0.0001) potency against the MCF-7 breast cancer cells in dose dependent manner and showed significant increase in apoptotic cells at IC₅₀ value. So from the above results we can conclude that isolated embelin can effectively used in the treatment of breast cancer in dose dependent manner.

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Declaration of Interest

The authors have declared no conflict of interest.

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