Antiproliferative effects of *Plumbago rosea* and its purified constituent plumbagin on SK-MEL 28 melanoma cell lines

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A B S T R A C T

Background: *Plumbago rosea* is used in traditional systems of medicine for the preparation of formulations used for treating inflammations, cough, bronchitis, and gastrointestinal disorders, and also in conjunction with cancer chemotherapy. In the present study, the cytotoxic and anti-proliferative effects of plumbagin, and the ethanolic root extract of *P. rosea* (ETPR) was evaluated on SK-MEL 28 melanoma cell lines and human lymphocytes. **Materials and Methods:** MTT and apoptotic assays were used for the evaluation of cytotoxic and anti-proliferative effects, respectively. In addition, the effect of Plumbagin and ETPR in down regulation of BCL-2 expression is investigated using RT-PCR analysis. **Results:** Both plumbagin and ETPR dose-dependently decreased the cell viability more potently in melanoma cell lines. *P. rosea* extract demonstrated significant synergy in inhibiting BCL-2 expression than plumbagin. Moreover plumbagin showed more toxicity in human lymphocytes. **Conclusion:** Plumbagin has anti-cancer potential, but the side effects limits its use; yet plumbagin, in combination with other ingredients in *Plumbago rosea* extract, displays significant synergy leading to a stronger anticancer effect with significantly less toxicity.

Key words: BCL-2, ethanolic root extract of *P. rosea*, high performance liquid chromatography, *Plumbago rosea*, plumbagin, SK-MEL 28

INTRODUCTION

Cancer is one of the leading causes of death in both developed and developing countries and continues to be a major public health problem in many parts of the world.[1] In spite of various efforts to reduce the threat caused by cancer, only modest progress has been made in reducing the morbidity and mortality of this dreadful disease.[2]

Cancer treatment involves various treatment strategies including surgery, radiation therapy, immunotherapy, hormone therapy, or chemotherapy. These treatment regimes are often unsatisfactory and proved unsuccessful with the majority including serious side effects such as cardiac and other toxicities.[3] Tumor cells were found to

acquire resistance to apoptosis by various mechanisms that interfere at different levels of apoptosis, such as over-expression of anti-apoptotic genes. Studies have shown a correlation between high levels of BCL-2 expression and the severity of malignancy of human tumors.[4-6] Moreover, it has been shown in *in vitro* and *in vivo* models that BCL-2 expression confers resistance to many kinds of chemotherapeutic drugs and irradiation.[4,7] In some types of tumors, a high level of BCL-2 expression is associated with a poor response to chemotherapy and seems to be predictive of shorter, disease-free survival.[5,6,8] As a result, research is currently shifting towards traditional herbal drugs. These herbal drugs contain complex mixtures of chemical components[9] that can act individually or synergistically by activating multiple pathways, thus suppressing cancer with minimal toxicity to normal cells.

Plumbago rosea Linn. (syn. *Plumbago indica* Linn.), commonly known as red chitrak of the family Plumbaginaceae, is a highly reputed Indian medicinal plant mentioned

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in Ayurvedic literature. It is a perennial shrub widely distributed in the tropics, more specifically in Southern India.[10] The tuberous roots of the plant have been used in traditional medicine for the treatment of rheumatism, edema, piles, common wart, secondary syphilis, and leprosy. It has also been used to treat skin diseases and intestinal worms[10-12] these properties are believed to be attributed due to the presence of plumbagin, a natural quinoid constituent. Plumbagin possess various pharmacological activities such as antibacterial antifungal, anticancer,[13,14] and anti-inflammatory activity.[15] In addition to the quinoid constituent plumbagin, several other potential bioactive components such as, sitosterol, stigmasterol, campesterol, napthaquinone, and 5, 6-dihydroxy-2-methyl-1, 4-naphthoquinone (6-hydroxyplumbagin) α-amyrin, β-sitosterol[15-18] were also reported. However, only a few scientific studies have been conducted to evaluate the efficacy of these constituents. Hence, the present work was intended to compare the effect of purified plumbagin and *P. rosea* extracts on SK-MEL 28 melanoma cell lines.

MATERIALS AND METHODS

Chemicals and reagents
Plumbagin (CID 10205), MTT (3-(4, 5-dimethyl thiazol-2-yl)-2, 5-diphenyltetrazolium bromide, Fetal bovine serum (FBS), and penicillin were purchased from Sigma Aldrich, USA. Primers for the study were purchased from Synergy Scientific, India. All analytical grade reagents and solvents were purchased from Merck (Mumbai, India).

Collection and extraction of root powder
Whole plants of *P. rosea* were collected from different parts of Kanyakumari district, Tamil Nadu. The plant was authenticated at Botanical Survey of India, Coimbatore (BSI/SRC/5/23/2010-11/Tech-1282). A voucher specimen was deposited at the Department of Biotechnology, Bharathidasan Institute of Technology, Anna University, Tiruchirappalli for future reference. Roots of the plant were washed thoroughly, air dried, and ground into a powder. Powdered *P. rosea* roots (75 g) were then subjected to soxhlet extraction with ethanol at 72°C for 24 h. The extracts were then evaporated to dryness in rotary evaporator (Yamato, Japan).

Analysis of *P. rosea* extract by HPLC
Analysis of ethanolic extract of *P. rosea* roots (ETPR) was carried out with high performance liquid chromatography (Gilson, France) using C18-Kromasil column (250 mm × 4.6 mm) attached to Gilson 321 binary gradient pump. Aqueous 0.2 M acetic acid (pH adjusted to 3.5 with trimethylamine) and methanol was used as the mobile phase. The flow rate was 0.8 ml/min. Injection volume was 15 μl and HPLC chromatogram was monitored at 254 nm. Plumbagin in crude root extract was quantified

by comparison with the standard curve obtained using the authentic plumbagin (Sigma Aldrich, USA).

Cell culture
SK-MEL 28, human melanoma cell lines were procured from National Centre for Cell Science (NCCS), Pune, India. Cells were cultured in DMEM medium supplemented with 10% heat inactivated FBS, 1% glutamine, and 1% penicillin. The cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2.

Human lymphocytes culture was used to evaluate the cytotoxicity of *P. rosea*. Lymphocytes were isolated using HiSep (HiMedia, India) as per the manufacturer's instruction. Briefly, Hisep media was added to the blood in the ratio 1:3 (media: blood), and centrifuged at 160 g for 20 min. The lymphocytes were then transferred into fresh tube and washed with sterile PBS by centrifuging at 140 g for 15 min. The pellet were cultured in RPMI medium (HiMedia) supplemented with 10% heat inactivated bovine serum, antibiotics (streptomycin, penicillin), $NaHCO_3$. Phytoheamagglutinins were used as a mitogen for the proliferation of lymphocytes. The culture was filtered by using sterilized 0.2-μm pore size cellulose acetate membrane filter (Sartorius). Isolated human peripheral lymphocytes were incubated for 72 h at 37°C.

3-(4, 5dimethythiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay
MTT is a colorimetric assay that measures the reduction of yellow 3-(4, 5dimethythiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) by mitochondrial succinate dehydrogenase. The reduction of MTT by mitochondrial succinate dehydrogenase in to formazan crystals was read spectrophotometrically at 540 nm.

Cell viability was calculated using the formula

% of viability = (Mean absorbance of the sample/Mean absorbance of the control) × 100

Cytotoxicity of ETPR and plumbagin on human lymphocytes
The cytotoxic effects of plumbagin and ETPR were determined in human lymphocytes with MTT assay. Different concentrations of plumbagin (50-100 μg/mL) and ETPR (100-800 μg/mL) were added to the cultured human lymphocytes after 24 h of pre-incubation. The toxicity of cells was determined after 48 h.

Antiproliferative effect of ETPR and plumbagin on SK-MEL 28 melanoma cell lines
SK MEL 28 cells were seeded in tissue culture plates at a concentration of 1×10^5 cells/ml and exposed to increasing concentration of plumbagin (25-200 μg/mL) and ETPR

(50-400 μg/mL). Cell viability values were determined after 24 h using MTT cell viability assay as descried before.

Colony formation assay

Cells at the initial density of 1×10^5 in 2 ml medium were seeded in plates containing 2 ml of 0.5% agar with medium. The cultures were incubated with ETPR and plumbagin at 37°C in a humidified 5% CO_2 atmosphere for 10 days. The number of colonies were determined by counting them under an inverted phase-contrast microscope at ×40 magnification Colonies consisting of more than 50 cells were fixed with formaldehyde (4%) for 2 h and then stained with trypan blue for 30 min followed by crystal violet and counted.

Acridine orange–ethidium bromide double staining

Acridine orange and ethidium bromide staining confirms the nuclear integrity of treated cells. SK-MEL 28 cells were previously incubated at 37°C in a humidified 5% CO_2 atmosphere. ETPR (400 μg/mL) and plumbagin (10 μg/mL) were then added into the wells and further incubated for 24 h. The cells were treated with acridine orange and ethidium bromide (1:1). and viewed under fluorescence microscope (Olympus CKK41) at ×20 magnification.

Percentage viability was determined by using the following formula,

% of viability = (A/B) ×100

Where,
A represents the total number of live cells
B represents the total number of cells.

BCL-2 gene expression in plumbagin- and ETPR-treated cells by RT-PCR method

Expression of anti apoptotic gene, BCL-2 was studied using reverse transcriptase-PCR (RT-PCR). SK-MEL 28 cell lines were previously incubated with ETPR (400 μg/mL) and Plumbagin (10 μg/mL).

Isolation of RNA

For preparation of total RNA, the phenol–guanidinium thiocyanate-based RNA Sol™ was used. To the cells, 600 μl of RNA sol reagent was added and lysed by repetitive pipetting and allowed to stand for 5 min followed by addition of 200 ml of chloroform: isoamyl alcohol (24:1) for phase separation. The mixture was then vigorously vortexed for 15 sec and allowed to stand for 15 min followed by centrifugation at 12000 g for 15 min at 4°C. The superficial layer containing RNA was transferred to a fresh sterile DEPC-treated microfuge tube. To this, 500 ml of ice cold isopropanol was added, gently mixed, and allowed to stand for 10 min and centrifuged at 12000 g

for 15 min at 4°C. The supernatant was discarded and the RNA pellet was washed with 1 ml of 70% ethanol in DEPC-treated water and again centrifuged at 14000 g for 10 min at 4°C to obtain total RNA. This pellet was dissolved in 25 μl of sterile RNase free water by heating at 55°C for 20 min and stored at −20°C until use.

Reverse transcriptase polymerase chain reaction (RT-PCR)

Isolated mRNA was converted to cDNA and amplified using specific primers (BCL-2) as per the manufacturer's instruction (RT PCR kit, Synergy scientific, India). To amplify the cDNA, PCR ready mix was used according to manufacturer's instruction. cDNA synthesis was carried out at 42°C followed by amplification (94°C, 10 min; 94°C, 30 s; 55°C, 30 s; 72°C, 30 s, and final hold at 4°C) for 35 cycles. The final extension was performed at 72°C for 5 min.

PCR products were separated on a 1% agarose gel and stained with fluorescent dye Green view™. Further, the expression of BCL-2 was analyzed semiquantitatively using IMAGE J software.

RESULTS

Analysis of *P. rosea* extract by HPLC

Ethanolic extraction yielded 0.17g plumbagin/g dry wt. Plumbagin was detected at the retention time of 5.3 min and the total calculated content was 2.53% [Figure 1]. Thus amount of plumbagin extracted from roots of *P. rosea* corresponds to 4.3 mg/g. dry wt.

Cytotoxic effect of ETPR and Plumbagin on human lymphocytes

The cytotoxic effect of plumbagin and ETPR on human lymphocytes was evaluated by MTT assay. Cell lines were sensitive to plumbagin when compared to ETPR, indicating that plumbagin could well induce cytotoxicity to the lymphocyte cell lines. In addition, the LD_{50} for plumbagin was 46.24 μg/mL when compared to 420.45 μg/mL for ETPR suggesting that very low concentration of
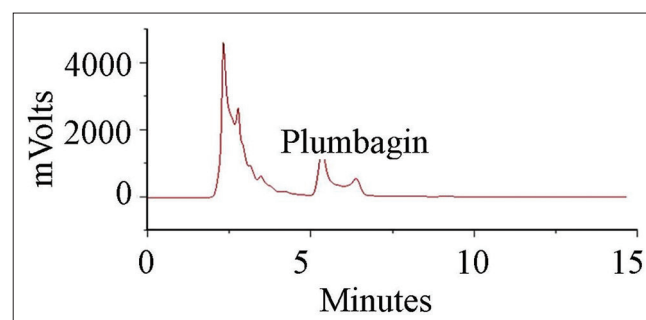
**Figure 1:** Qunatification of plumbagin in ethanolic extracts of *Plumbago rosea* roots using HPLC

plumbagin is sufficient to induce cytotoxic effect on normal cells [Figure 2].

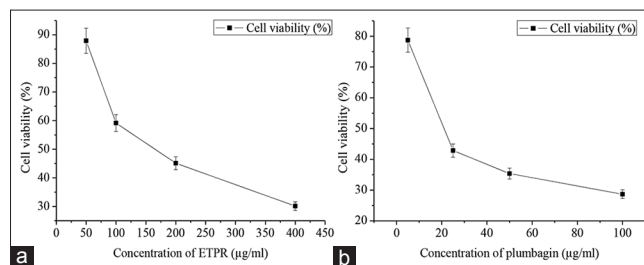## Antiproliferative effect of ETPR and plumbagin on SK-MEL cells

To investigate the effect of ETPR and plumbagin on SK-MEL 28 cell proliferation, the cells were treated with various concentrations of ETPR and plumbagin for 24 h. ETPR showed 50% inhibition of cell proliferation only at 84.74 µg/mL whereas with plumbagin, it was observed at a very low concentration of 30.48 µg/mL. In either case, the antiproliferative activity was dose-dependent [Figure 3].
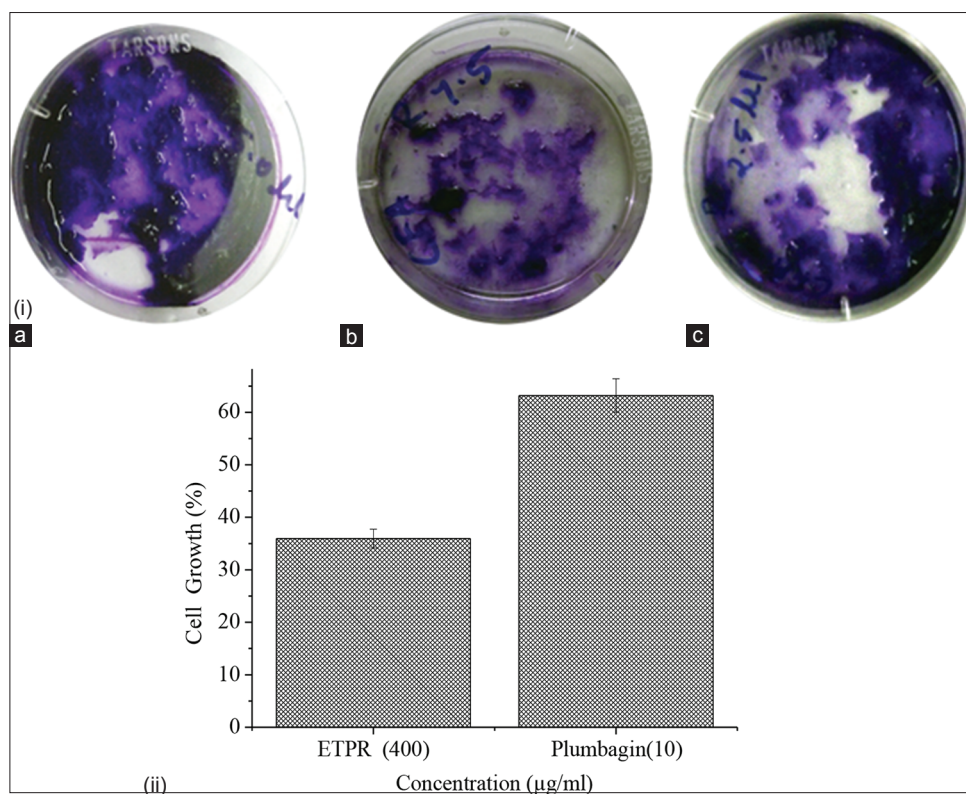
Colony formation assay

The assay for colony formation assay was carried out to compare the long term effect of ETPR and plumbagin on SK MEL 28 cell growth. The cells were incubated individually with ETPR and plumbagin for 10 days, the colonies were counted and photographed. After 10 days incubation of the indicated concentration of plumbagin and ETPR, the colony numbers were counted and the colony photos were taken. The results showed that ETPR-treated cells resulted in significantly lesser cell growth and colony formation when compared with plumbagin-treated cells.
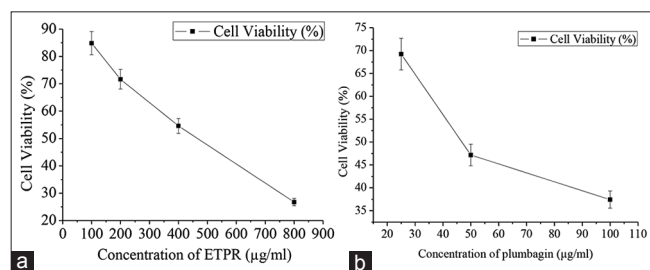
Figure 2: Cytotoxic effect of (a) ETPR, (b) Plumbagin on human lymphocytes cells after 48-h treatment. Each data point represents the mean from three independent experiments (mean ± SE)

Figure 3: Antiproliferative effect of (a) ETPR, (b) Plumbagin on SK-MEL 28 cells after 24-h treatment. Data are expressed as a percentage of control (statistics data). Each data point represents the mean of four wells from three independent experiments (mean ± SE)

Figure 4: (i) Colony formation and cell growth in SK-MEL28 melanoma cell lines treated with plumbagin & ETPR, (a) control, (b) 400 µg/mL of ETPR, (c) 10 µg/mL of plumbagin in DMEM containing 10% FBS, (ii) Cell numbers determined by hemocytometer. Values are expressed as mean ± SE from three independent experiments

Although reduced cell growth (35.23%) was observed with ETPR, the concentration measured was 400 μg/mL, whereas with plumbagin 10 μg/mL was sufficient to reduce the cell growth to 61.17% [Figure 4].

Double staining of SK-MEL 28 cell lines with acridine orange and ethidium orange

In order to determine whether the decrease in cell viability was due to apoptosis, a morphological change at cellular and nuclear levels was assessed with ETPR and plumbagin-treated cells. Cellular and nuclear morphological changes of treated cells were assessed to determine whether observed decrease in cell viability were consequence of an apoptosis process. Acridine orange selectively stains the living cells as green whereas ethidium bromide stains dead cell DNA as red [Figure 5]. The untreated control SK-MEL 28 cell lines were characterized by bright green nucleus with uniform intensity and the absence of ethidium bromide uptake. From the results it can be observed that ETPR-treated cells showed higher percentage of apoptotic cells compared to plumbagin, which confirms the synergistic anticancer potential of crude extract [Figure 5].

Analysis of BCL-2 mRNA expression

BCL-2 mRNA expression was analyzed in both plumbagin- and ETPR-treated SK-MEL 28 cells.

Plumbagin- and ETPR-treated SK MEL 28 cells showed increased amounts of RNA transcripts of the BCL-2 gene compared to the untreated cells. Levels of RNA transcript of BCL-2 was decreased markedly in ETPR-treated cells (400 μg/ml) when compared to plumbagin-treated cells (10 μg/ml) [Figure 6]. GAPDH was used as internal control.

To validate the synergistic effect of phytochemicals present in ETPR by inhibiting the expression of BCl-2, mRNA isolated from SK-MEL cell lines exposed to ETPR was transcribed to cDNA using primers supplied by sigma RT-PCR amplification mix (Synergy scientific, India). The cDNA presence was confirmed by agarose gel electrophoresis. The band intensities were visualized using UV transilluminator. From the results it can be observed that *P. rosea* extract has shown significant inhibition of BCL-2 when compared with plumbagin alone. The intensity of expression was analyzed using IMAGE J VIEW software.

DISCUSSION

Skin cancer is the third most common human malignancy. Increase of the incidence of malignant skin cancer is rising at an alarming rate.[19,20] However, the main problem to treat
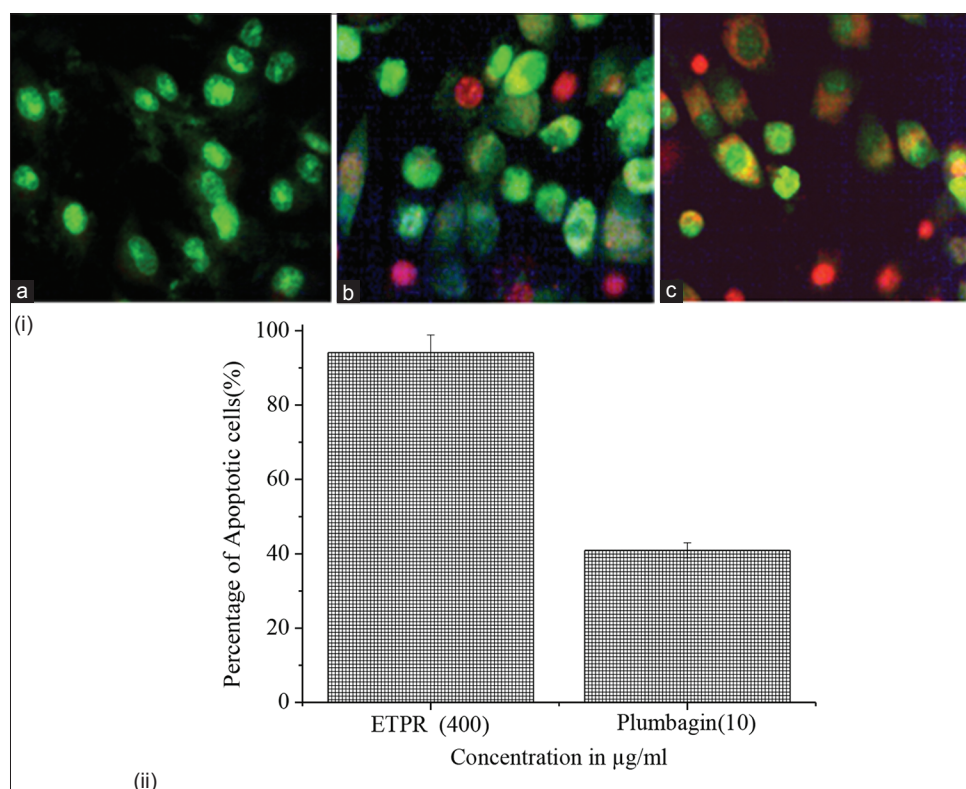
Figure 5: (i) Fluorescence photomicrographs of SK-MEL 28 melanoma cell lines stained with acridine orange and ethidium bromide, after being treated with ETPR and plumbagin. (a) Control cells with bright red nucleus, (b) cells treated with 10 μg/ml plumbagin, (c) cells treated with 400 μg/ml ETPR. ii) The bar graph shows the percentage of apoptotic cells. The baseline apoptosis in the untreated group was normalized with data from the treated group. The data shown are representative of the combined means from three independent experiments
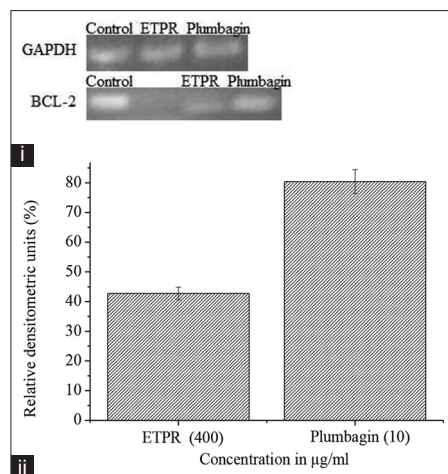
Figure 6: (i) Levels of RNA transcript of BCL-2 was decreased markedly in 400 µg/ml ETPR-treated cells when compared to 10 µg/ml plumbagin-treated cells. GAPDH was used as internal control, (ii) Densitometric analysis of RNA transcript using IMAGE VIEW™ software. Represented data values were obtained from triplicate analysis and expressed as the mean ± SD

melanoma is its resistance to radiation and conventional chemotherapeutic agents.[20,21] Hence, novel therapies are needed to reduce the effects of the increasing incidence in human melanoma.

Plumbago rosea Linn a shrubby perennial herb, commonly known as rakta chitrak, grows in wild and in abundance in India.[22] It is heavily used in traditional systems of medicine for the preparation of formulations used to treat a variety of disease conditions. The juice of the leaves and roots mixed with oil is employed as an application for rheumatism paralysis and leprosy.[22] The roots are used in dyspepsia, colic, inflammations, cough, bronchitis, hemorrhoids, elephantiasis chronic intermittent fever, ring worm, skin diseases diarrhea, and piles.[23] The anticancer potential of the plant *P. rosea* has been previously explored in animal models.[24]

Plumbagin, is a natural napthaquinone, possessing various pharmacological activities namely antimalarial, antimicrobial, anticancer, cardiotonic, antibiotic, and antineoplastic activities.[11,13,25] Potential role of plumbagin, as an anti-cancer agent has been recognize[24,26-28] and its anti-cancer effects have been reported in diverse cancer models such as prostate[29], lung [30,31], cervical[32,33], ovarian[34] as well as melanoma.[34]

SK MEL 28 cells and lymphocytes showed growth inhibition in a dose-dependent manner when treated with plumbagin. Plumbagin was found to be slightly more toxic to normal lymphocytes. Recently, much focus has been attributed to multi-component phyto or natural compounds as anticancer agents because of their prominent pharmacological activity at low concentrations and minimal toxicity on normal cells.[35] In addition these phyto extracts would increase the efficacy of drugs by modulating several molecular targets. Extracts of the medicinal plants are believed to contain a wide array of polyphenolic compounds, which might possess cancer preventive and/or therapeutic properties.[36]

Crude extracts exert the antitumor activities through regulation of different cell signaling pathways. SK MEL cells treated with ETPR for 24 h showed growth inhibition in a dose-dependent manner. The LD_{50} value of ETPR on SK MEL cells was found to be 124 µg/ml. It is well-established that herbal extracts have lesser degree of toxicity compared to purified constituents. In the light of this fact, we assessed the effect of ETPR on lymphocytes (as normal). Our results are consistent with previous studies, wherein *Angelica sinensis* extracts showed better responses compared with that of the single purified phthalides.[37]

The nuclear morphology analyzed quantitatively by staining suggested that an apoptotic cell death mechanism was more potentially involved in the antiproliferative effect of ETPR compared to plumbagin. As apoptosis is controlled by complex interplay between regulatory proteins of bcl-2 family. Therefore, Bcl-2 proteins have emerged as attractive targets for the development of novel anticancer agents as down regulation of Bcl-2 by plumbagin has been previously reported. An attempt has been taken to explore the synergistic potential of crude extracts by comparing the extent of down regulation of Bcl-2 by plumbagin and crude extracts. The extent of inhibition of Bcl-2 was found to be higher in crude extracts when compared to plumbagin alone. This might be due to the potentiating of activity of plumbagin by the other bioactive constituents present in crude extracts.

Double staining revealed the percentage of apoptotic cells being increased in crude extract-treated cell lines when compared with the purified constituent-treated cells. The presence of phenolic compounds in plant extracts might be responsible for chemopreventive properties such as antioxidant, anticarcinogenic, or antimutagenic and anti-inflammatory effects. Phenolic compounds also contribute to their apoptosis property by arresting cell cycle, regulating carcinogen metabolism and ontogenesis expression, inhibiting DNA binding and cell adhesion, migration, proliferation or differentiation, and blocking signaling pathways.[38-41]

The *in vitro* and *in vivo* colorectal anticancer study of Tian-Xian liquid (crude herbal medicine) showed significant multi-targeted to down regulation of metastatic markers than its bioactive fractions.[42] Tomoxifen showed drug resistance in breast cancer patients; therefore, MCF-7 cells were treated with tomoxifen combined with coptis extracts.

The result showed that tomoxifen possesses significant promising potential anticancer effect and it triggers many anticancer pathways due to the synergistic effect.[43] Similarly, *P. rosea* extract also demonstrated a synergistic effect with a significantly stronger antiproliferative activity on skin cancer cells than plumbagin. The results suggested that *P. rosea* might have a wider pharmacological application because in addition to the active compound plumbagin, other components in *P. rosea* might also contribute to the herbal anti-tumor effect and/or enhance the action in a synergistic manner. These results are also in line with the long-established principle of traditional Ayurvedic practice, which recommends, instead of using purified ingredient, a single or mixture of herbs containing various active compounds in specific proportions can be used to treat and prevent diseases by operating synergistically.[44,45] Every country has its own traditional medicinal system and it should be studied by the recent scientific techniques[46] because sometimes herbal preparation may meet with problems such as identifying plants, variable growing conditions, and batch-to-batch variations.[47]

CONCLUSION

The present study concluded that the *P. rosea* extracts exhibited selective cytotoxic and anti-proliferative effects in human skin cancer SK MEL-28 cells. Furthermore, for the first time, a significant synergy of cytotoxicity towards cancer cells was demonstrated in the use of *P. rosea* extract due to interactions among plumbagin and other components present in the herb. Further mechanistic studies and *in vivo* anti-cancer models need to be done to fully assess the anticancer potential of plumbagin and herbal extracts. Therefore, *P. rosea is* worthy to be further investigated and developed as herbal medicine for potential use in cancer therapy.

ACKNOWLEDGMENT

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