Evaluation of anthraquinones from Himalayan rhubarb (*Rheum emodi* Wall. ex Meissn.) as antiproliferative agents

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ABSTRACT

Rheum emodi (Polygonaceae), a multipurpose medicinal herb is a rich repository of pharmacologically active secondary metabolites known as anthraquinones. The present study entails HPLC quantitation and *in vitro* activity of four major constituents and the extracts of *R. emodi*. The anthraquinone glycosides were more abundant than their aglycone constituents *viz.* emodin and chrysophanol. MTT assay was used to assess the *in vitro* antiproliferative activity of anthraquinones and extracts on four cancer cell lines namely MIAPaCa-2, HCT-116, MCF-7 and T47D. The cytotoxicity was more obvious on MIAPaCa-2. Further, the study of mechanism of action involving cell cycle analysis and determination of mitochondrial membrane potential (MMP) loss was also investigated. The extracts significantly reduced cell viability by inducing apoptosis/necrosis and cell cycle arrest with concurrent loss of MMP ($\Delta\psi$m) in a concentration dependent manner. The methanolic extract of rhizome (SPL5) with the least IC_{50} value (25 µg/ml) showed a significant increase in the percentage of apoptotic/necrotic cells (42.3% at 100 µg/ml) compared to that of vehicle treated cells (11.6%). These cellular manifestations corresponded remarkably with a decrease in the integrity of the mitochondrial membrane. In conclusion, SPL5 with emodin and chrysophanol as the preponderant constituents exhibited considerable antiproliferative activity possibly by reducing cell viability and stirring up $\Delta\psi$m loss.

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1. Introduction

The clonal disorder, cancer, is a diverse class of complex diseases which differ widely in their cause and biology. It is a pathological condition that involves upregulation of certain oncogenic signaling pathways that prop up proliferation, hold back apoptosis, and allow the malignancy to spread and trigger angiogenesis. Cancer, identified by the WHO (World Health Organization) as one of the four main threats to human health and development, is the leading cause of deaths across the world. In the United States alone, one in four deaths is due to cancer (Siegel et al., 2013). As per the new version of IARC's (International Agency for Research on Cancer) database GLOBOCAN about 12.7 million new cancer cases occurred worldwide in 2008 that accounted for 14% of all deaths, with breast and colorectal cancers sharing the major proportion (Jemal et al., 2011). Moreover, pancreatic cancer is one of the most malignant and aggressive forms of cancer (Li et al., 2004). It is the fourth most common cause of cancer-related deaths in the United States and the eighth worldwide (Hariharan et al., 2008).

Plants have been used as medicine since antiquity probably from middle Paleolithic age some 60,000 years ago (Solecki, 1975). As per the estimation of the WHO about 75% of the population of Asian and African countries still relies on conventional and traditional methods for the treatment of various diseases (Wani et al., 2013). These preventive measures mostly involve the use of herbal extracts which are cheap and comparatively offer high safety and lesser side effects. The traditionally observed gamut of pharmacological efficacy in competent herbal medicine may only arise because of the synergistic action of multiple

ingredients. In the last few decades, the plant derived natural products have found their use as efficient chemotherapeutic and/or chemopreventive agents for the effective treatment of vast diversity of cancers. Despite topical advances in our understanding of the biological processes leading to the development of cancer, there is still a need for new and effective agents to bring cancer under control. Phytomedicine offers a great deal to the health system as presently about 25% of the pharmaceutical prescriptions in the United States contain at least one plant-derived ingredient. Based on the traditional knowledge around 121 pharmaceutical products were formulated in the last century (Pandey et al., 2011). The metabolomes of medicinal plants serve as a valuable natural resource for pharmaceuticals and provide a room for renewed attention from both practical and scientific viewpoints for the evidence based development of new phytotherapeutics and nutraceuticals.

Rheum (*Rheum emodi* Wall. Ex. Meissn. Syn: *Rheum australe* D. Don., Polygonaceae) is an endemic, robust, perennial, diploid ($n = 11; 2\times$) medicinal and vegetable herb distributed in the temperate and subtropical regions of the Himalayas from Kashmir to Sikkim, at elevations ranging from 2000 to 4500 m. The plant has dark reddish-purple flowers, in dense branched clusters in a long inflorescence which can be 1 ft long. The stems are stout, streaked green and brown, and 1.5–2 m high. Plants propagate either by seeds or by rootstock. *R. australe* is commonly known as Himalayan rhubarb or red-veined pie plant in English and pumbachalan in Kashmiri. It is native to India, Myanmar, Nepal, Pakistan and Sikkim. *R. emodi* finds an extensive use in Ayurvedic and other traditional medical systems, like homeopathic, Tibetan, Unani and Chinese systems (Bhatia et al., 2011). Extracts from the roots, bark and leaves of rhubarb have been used as a laxative since ancient times and presently are widely used in various herbal preparations (Wang et al., 2007). The major phytoconstituents reported from the plant include anthraquinones (emodin, aloe-emodin, chrysophanol, rhein, physcion) and stilbenes, and their respective glycoside derivatives. These frequently occurring key constituents are reportedly known for various biological activities including antioxidant, cytotoxic, antimicrobial, antifungal, antitumor, antidiabetic, antiproliferative and immunoenhancing (Zargar et al., 2011). Emodin is a selective casein kinase II inhibitor which competitively binds to the ATP binding pocket of the kinase (Fullbeck et al., 2005). In human cancer cells emodin inhibits TPA-induced cell invasiveness and MMP-9 expression through suppression of both AP-1 and NF-κB signaling pathways (Huang et al., 2004). Increasing focus is being directed towards alternative types of cell death like autophagy, mitotic catastrophe and necrosis. The clear understanding about the molecular events leading to cell death can facilitate towards a more rational approach for anticancer treatments (de Bruin and Medema, 2008). The major anthraquinones with the exception of chrysophanol have been shown to induce apoptosis in various animal and human cancer cell lines. Chrysophanol stimulated ROS production, mitochondrial dysfunction, loss of ATP and DNA damage in J5 human liver cancer cells which finally lead to necrotic cell death (Lu et al., 2010).

About 60% of the approved drugs for treatment and prevention of cancer have their origin from natural sources (Gul et al., 2013). Nature has provided various potent antitumor agents like microbial derived drugs such as bleomycin, doxorubicin and dactinomycin. There are also plant based drugs like vincristine, vinblastine, irinotrecan, topotecan, etoposide and paclitaxel with wide pharmacological significance. In the last few decades robust evidences from *in vivo* and *in vitro* studies by using human normal and malignant cells indicate that plant extracts protect against various cancers. Presently natural products continue to offer a fabulous range of lead structures, which act as a source for the development of new drugs (Abdullaev, 2001). Furthermore, plant natural products and scaffolds hold promise to design new and potent multi-target drugs and drug combinations that are efficacious for the treatment of various human ailments. In the present study we attempted to explore the *in vitro* antiproliferative activities of four major anthraquinones and of the methanolic and ethyl-acetate herbal extracts from the rhizome and leaves of *R. emodi*. The diversity in concentration of key chemical/bioactive constituents of plant samples collected from four different geographic locations of North Western Himalayas with an altitudinal range of 1600 m to 4500 m was also determined by a slightly modified standard HPLC method.

2. Materials and methods

2.1. Plant material

The plant samples of *R. emodi* were collected from four different locations of Kashmir Himalayas (Jammu and Kashmir State, India) in the months of June–July 2012. The located geographic zones are spread over an altitudinal range of 1600 to 4500 m (Table 1). The plant material was well identified and further authenticated by Dr. S. N. Sharma, Senior Technical Officer, Janaki Ammal Herbarium, Indian Institute of Integrative Medicine (CSIR), Jammu, India. A voucher specimen (RRL-22175) has been preserved for future reference in the Herbarium.

2.2. Chemicals

DMSO, RPMI-1640, DMEM, penicillin G and streptomycin were purchased from Sigma Aldrich, St. Louis, USA. Fetal bovine serum (FBS), phosphate buffered saline (PBS), propidium iodide and rhodamine-123 were obtained from Merck, Darmstadt, Germany. All other chemicals and solvents used were of analytical and HPLC grade. Fresh ultra-pure distilled water with resistivity greater than 18 MΩ was used.

2.3. Pure compounds

Pure standards of two major anthraquinones namely emodin and chrysophanol were purchased from Sigma Aldrich (St. Louis, USA) and their respective glycosides; emodin glycoside and chrysophanol glycoside (Fig. 1) were obtained from the Institute of Himalayan Bioresource Technology, Palampur, India (kindly provided by A. K. Sinha). Their purities were above 99% as determined by HPLC analysis.

2.4. Extraction procedure

Plant samples of *R. emodi* collected from four different Himalayan locations were chopped, properly cleaned and completely dried under gentle air stream (temperature 25 ± 2 °C and relative humidity 65 ± 5%). The leaves as well as the rhizomes of all these plant samples were ground separately into fine powder with a mortar and pestle. The powdered rhizomes (50 g of each sample) and leaves (20 g of each sample) of different samples were serially extracted each with methanol (3 $\times$ 250 ml) and ethyl-acetate (3 $\times$ 200 ml) using Soxhlet apparatus. The extracts were filtered through a Whatman No. 1 paper filter and solvents were removed under reduced pressure using a rotary evaporator, and then kept at 4 °C until use.

Table 1
Places of collection of different samples of *R. emodi*.

Location code	Location	Geographic co-ordinate
1	Bonera Farm, Pulwama, Kashmir	33° 52′ 59″ N, 74° 55′ 00″ E; 1630 m asl
2	Yarikhah Farm, Gulmarg, Kashmir	34° 04′ 797″ N, 74° 26′ 448″ E; 2119 m asl
3	Pense La Top, Ladakh	33° 51′ 08″ N, 76° 21′ 57″ E; 4287 m asl
4	Nyoma Valley, Ladakh	33° 08′ 661″ N, 78° 34′ 742″ E; 4415 m asl

A   B
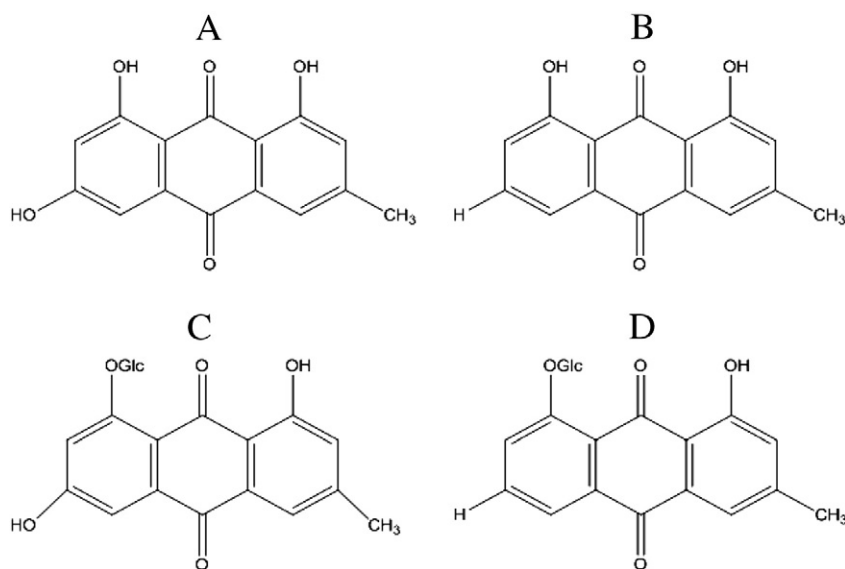
C   D

Fig. 1. Chemical structures of emodin (A), chrysophanol (B), emodin glycoside (C) and chrysophanol glycoside (D).

2.5. Sample preparation and HPLC conditions

The stock solutions (1 mg/ml) of each of the four anthraquinones were freshly prepared in a mixture of methanol:acetonitrile (80:20, v/v). Similarly, the crude extracts of all the leaves and rhizomes of collected plant samples were also dissolved in the same solvent composition (20 mg/ml) and were filter sterilized with 0.25 μm membrane filters (Millipore, Bedford, USA). The HPLC (Shimadzu CLASS-VP V 6.14 SPI model) equipped with RP-18e column (E-Merck, 5 μm, 4.6 × 250 nm), a photo-diode array detector (SPD-M10A VP model) and a pump (LC-10AT VP model) was used for the analysis of the anthraquinones. Acetonitrile:methanol (95:5, v/v) (solvent B) and water:acetic acid (99.9:0.1, v/v, pH 3.5) (solvent A) were used as a mobile phase with a linear gradient elution as follows: 0–15 min, 20% B; 15–25 min, 20% B; 25–30 min, 50% B; 30–40 min, 70% B; 40–45 min, 100% B; 45–50 min, 20% B; and 50–55 min, 20% B; at a flow rate of 0.8 ml/min. The detection wavelength was set at 254 nm. Injection volume of the sample was 10 μL and the column temperature was 30 °C.

2.6. Detection and quantification of anthraquinones

Identification and quantification of anthraquinones were accomplished using a reverse-phase HPLC at a set wavelength of 254 nm. The detection was made on the basis of retention time of reference compounds under a specific set of column operating conditions. Elution positions were established with authentic samples and by comparison with literature data. Relative contents of different anthraquinone constituents were determined and expressed as percentage peak area. The HPLC chromatograms of all the screened samples are depicted in Fig. S1–S7.

2.7. Cell lines and culture conditions

MCF-7 and T47D (breast), HCT-116 (colon), and MIAPaCa-2 (pancreas) cells were routinely maintained in Dulbecco's Modified Eagle's Medium (DMEM) while fR2 (normal epithelial) cells were maintained in Roswell Park Memorial Institute-1640 medium (RPMI-1640) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin G and streptomycin at 37 °C in a humidified incubator with 5% CO_2. All stock solutions of the samples were prepared in cell culture grade dimethyl sulfoxide (DMSO) and stored at −20 °C. The samples were diluted in culture media prior to their use in experiments.

2.8. MTT cell viability assay

The different crude extracts of plant samples and four of the secondary metabolite constituents namely emodin (SPL1), chrysophanol (SPL2), emodin glycoside (SPL3) and chrysophanol glycoside (SPL4) were subjected to antiproliferative assay against four cancer cell lines viz. MCF-7, T47D, HCT-116, and MIAPaCa-2 and a normal epithelial cell line, fR2. All the selected samples were dissolved in cell culture grade DMSO before screening. Cell viability of the treated cells was measured by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. Briefly, cells (10^4 cells/well) were cultured in 96 well tissue culture plates and treated with different concentrations of compounds for 48 h. At the end of incubation, 20 μL of MTT (2.5 mg/ml) was added to the wells and incubated for 4 h. Absorbance was recorded at 570 nm using Eliza Plate Reader.

2.9. Cell cycle analysis by propidium iodide (PI) staining

After 24 h of incubation, to exponentially growing MIAPaCa-2 cells (2×10^5 cells/ml/well) 10, 50 and 100 μg/ml of extracts SPL-5, 6, 7 and 8 were added and incubated further for 48 h. Control cells were treated only with vehicle DMSO (<0.5%). Both control and extract treated cells were harvested by trypsin treatment, washed in cold PBS (phosphate buffer saline), fixed with 70% ethanol for about 48 h at −20 °C and again washed with cold PBS. In order to remove RNA, the cells were treated with 40 μg of RNase for 1 h and 30 min at 37 °C followed by staining with 5 μg of PI (propidium iodide) at RT in the dark for 20 min before analyzing by FACS Aria II flow cytometer (BD Biosciences, San Jose, USA).

2.10. Mitochondrial membrane potential (MMP) assay

The human pancreatic cancer cell line, MIAPaCa-2, was seeded into 6-well culture plate (2×10^5 cells/ml/well) and incubated overnight. Different concentrations of extracts SPL5, SPL6, SPL7 and SPL8 (10, 50 and 100 μg/ml) were added to the cells and incubated for 48 h. 1 h before the completion of 48 h treatment, rhodamine-123 (Rh-123) was added at a concentration of 1 μM to each well. The cells were trypsinized and washed in PBS once. The cell pellets were resuspended in 500 μL of PBS and analyzed using a FACS Aria II flow cytometer for detecting the mitochondrial depolarization patterns.

3. Results and discussion

The scope of the present investigation extends towards the evaluation of in vitro cytotoxic potential of herbal extracts of *R. emodi* along with their known key chemical constituents, namely anthraquinones. It entailed the evaluation of the cytotoxic response of MIAPaCa-2 upon exposure to crude extracts to elucidate the mechanism of action by employing cell cycle analysis and MMP assay. Another important aim of this study was to identify elite chemotypes with desirable chemical profiles for pharmaceutical and commercial purposes. The metabolite accumulation is an efficient strategy mainly adopted by plants to thrive across diverse habitats under the umbrella of different kinds of biotic and abiotic conditions of stress. The interesting differences in the concentration of major bioactive phytoconstituents within the same species across an altitudinal gradient with varying temperature ranges present a differential and significant perspective for their potential use in modern medicine. This study also provides an outlook to explore desirable populations of *R. emodi* with higher contents of chemical constituents for pharmacological applications.

3.1. Determination of major anthraquinone constituents

Various methods for the identification and quantification of anthraquinone derivatives have been reported previously. However, so far there has been no such comprehensive study undertaken that involves locational comparison in anthraquinone concentrations in rhizome as well as leaf samples of *R. emodi* across the altitudinal gradient of North Western Himalayas ranging from 1600 to 4500 m (Table 1). The standard HPLC method with slight modifications was employed to determine the concentrations of major anthraquinones (Verma et al., 2005). The results showed interesting differences in the content of anthraquinone constituents. Tissue-specific chemoprofiling revealed preponderance of anthraquinones and their glycosides in rhizomes in comparison to leaves.

The methanolic extracts of the rhizomes showed the highest concentration out of the four chemical constituents at the highest altitude in Nyoma Valley (Ladakh). Here, the concentration of emodin and chrysophanol averaged 24.569 ± 0.91 mg/g and 54.512 ± 1.95 mg/g respectively. The mean values for their respective glycosides were 128.328 ± 4.83 mg/g and 76.305 ± 3.07 mg/g (Fig. 2a; Table 2). The increased levels of anthraquinones at higher altitudes are in conformity with the previous reports wherein it has been shown that an increase in secondary metabolite content is positively correlated with altitude (Farooq et al., 2013). However, ethyl-acetate extracts of rhizomes showed the reverse trend, wherein anthraquinone constituents showed maximum accumulation except for chrysophanol at Bonera location. Here, the maximum recorded concentrations for emodin and chrysophanol were 14.535 ± 0.86 mg/g and 39.939 ± 1.40 mg/g and for their respective glycosides they averaged 211.952 ± 8.39 mg/g and 74.427 ± 1.61 mg/g, respectively (Fig. 2b; Table 2). A similar decreasing trend, with increasing altitude, in concentrations of major anthraquinones was observed in both methanolic and ethyl-acetate extracts of leaf samples (Fig. 2c; Table 2). A similar trend in the pattern of secondary metabolite accumulation has previously been reported in *Lupinus argenteus* also (Carey and Wink, 1994).

The enormous diversity of bioactive constituents in plants are primarily derivatives of few biochemical motifs for undertaking various functions in the operating metabolic circuits. One of the important decorations is through glycosylation which is related to specific plant functions like xenobiotic detoxification, regulation of hormone homeostasis, and biosynthesis and storage of important phytoconstituents and shows its widespread occurrence in plants. Among the four anthraquinones quantified, the overall concentration of glycosides was found to be higher than those of their respective aglycone forms in all the extracts across the altitudinal gradients (Fig. 2; Table 2). This shuffle of anthraquinones in the direction of glycosylation, like
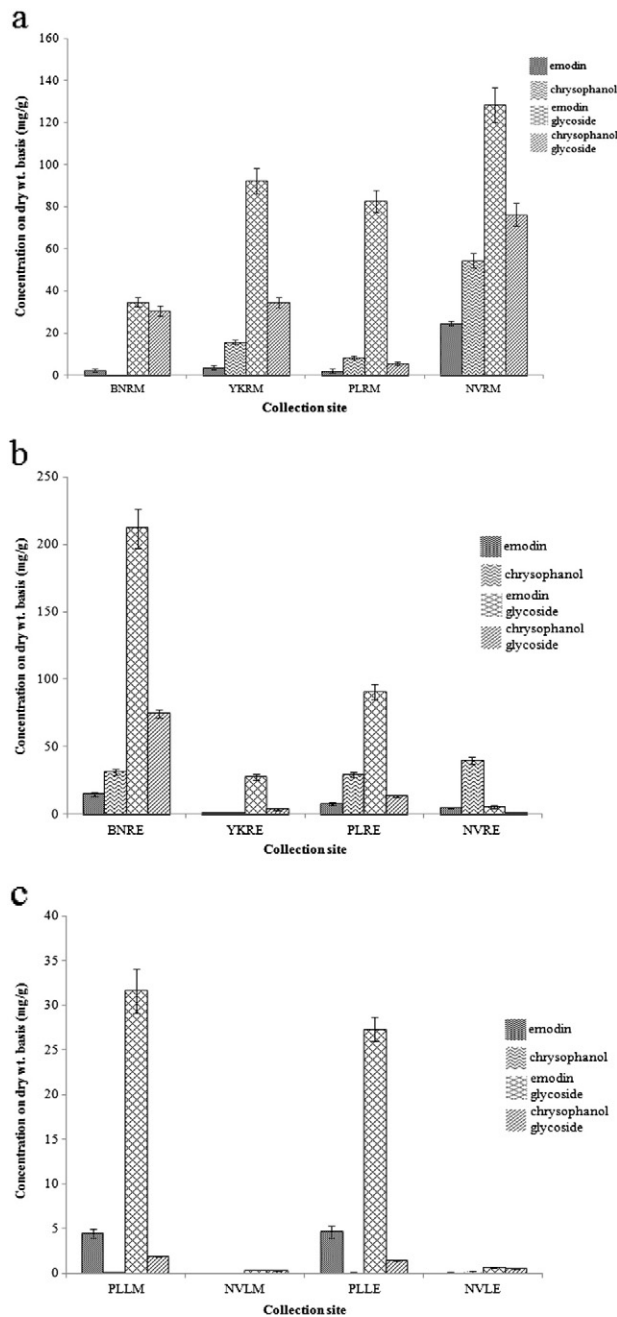
Fig. 2. Anthraquinone concentrations in extracts. a) graph depicting concentration of major anthraquinones in methanolic extracts of rhizomes; b) in ethyl-acetate extracts of rhizomes; and c) in methanolic and ethyl-acetate leaf extracts. BNRM, YKRM, PLRM and NVRM are the methanolic rhizome extracts from locations 1, 2, 3, and 4 respectively; BNRE, YKRE, PLRE and NVRE are ethyl-acetate rhizome extracts from locations 1, 2, 3 and 4 respectively; PLLM, NVLM and PLLE, NVLE are methanolic and ethyl-acetate leaf extracts from locations 3 and 4 respectively. All values obtained were means of triplicate with standard error.

many other plant natural products may be towards increasing solubility and stability for facilitating their easy storage and accumulation in plant cells.

3.2. Antiproliferative activity

The standards as well as the rhizome and leaf-specific extracts were evaluated for their in vitro anticancer activity against four cell lines at three different concentrations viz. 100, 50, 10 μM for standard molecules

Table 2
Concentrations of major anthraquinone constituents from plant samples collected from four different geographical locations of North Western Himalayas.

| Location code	Emodin (mg/g DWB[a])	Chrysophanol (mg/g DWB)	Emodin glycoside (mg/g DWB)	Chrysophanol glycoside (mg/g DWB)
BNRM	2.273 ± 0.056	0.0098 ± 0.0021	34.617 ± 1.278	30.579 ± 1.469
YKRM	3.543 ± 0.318	15.896 ± 0.556	92.229 ± 3.428	34.681 ± 1.435
PLRM	2.224 ± 0.329	8.33 ± 0.439	82.61 ± 3.005	5.578 ± 0.399
NVRM	24.569 ± 0.909	54.512 ± 1.952	128.328 ± 4.835	76.305 ± 3.074
BNRE	14.535 ± 0.867	31.33 ± 1.265	211.952 ± 8.399	74.427 ± 1.613
YKRE	0.416 ± 0.019	0.957 ± 0.0216	27.674 ± 1.261	3.611 ± 0.357
PLRE	7.651 ± 0.480	29.403 ± 0.995	90.683 ± 3.363	13.256 ± 0.571
NVRE	4.552 ± 0.331	39.939 ± 1.402	5.396 ± 0.467	0.305 ± 0.0106
PLLM	4.42 ± 0.296	0.146 ± 0.0087	31.613 ± 1.416	1.877 ± 0.054
NVLM	0.013 ± 0.00037	0.059 ± 0.00139	0.325 ± 0.0118	0.295 ± 0.014
PLLE	4.617 ± 0.4019	0.166 ± 0.0093	27.279 ± 0.774	1.444 ± 0.049
NVLE	0.111 ± 0.0074	0.214 ± 0.0079	0.594 ± 0.0158	0.479 ± 0.012

[a] Dry weight basis.

and 100, 50, 10 μg/ml for extracts. BEZ-235, a dual PI3K–mTOR inhibitor, was used as positive control (0.01 μM) because most of the cancer cell lines used have PI3K mutations. The half maximal inhibitory concentration (IC_{50}), a measure of the effectiveness of a compound in inhibiting some specific biological or biochemical function was used as a parameter of cytotoxicity. The percentage growth inhibition for standard molecules and extracts including their IC_{50} values against various cell lines are depicted in Table 3. The extracts SPL5 (methanolic extract of rhizome) and SPL8 (ethyl-acetate extract of leaf) were found to be most active with an IC_{50} value range of 40–41 μg/ml in the case of HCT-116 and 25–34 μg/ml in MIAPaCa-2 compared to control, fR2 (43–45 μg/ml). Further, none of the extracts were found to be effective against any of the breast cancer cell lines, with IC_{50} values of 77 μg/ml or more (Fig. 3a, b; Table 3). The cytotoxicity ratio of normal (fR2) to

Table 3
Percentage growth inhibition at 100, 50, 10 μM for standard molecules (SPL1, SPL2, SPL3 and SPL4) and 100, 50, 10 μg/ml for extracts (SPL5, SPL6, SPL7 and SPL8) and their IC_{50} values against breast cancer cell lines (MCF-7, T47D), colon cancer cell line (HCT-116), pancreatic cancer cell line (MIAPaCa-2) and normal epithelial cell line (fR2) with MTT assay. BEZ-235 (0.01 μM) was used as positive control. Values are means of three replicates ± SD.

Tissue type			Breast		Colon	Pancreatic	Normal epithelial
Cell type			MCF-7	T47D	HCT-116	MIAPaCa-2	fR-2
S. no	Code	Conc.	% growth inhibition				
1	SPL1	100 μM	26 ± 1.98	15 ± 0.87	38 ± 2.10	56 ± 1.93	65 ± 2.85
		50 μM	18 ± 0.97	10 ± 0.86	17 ± 2.02	40 ± 1.02	38 ± 2.05
		10 μM	13 ± 1.85	10 ± 1.89	4 ± 0.01	4 ± 1.01	3 ± 2.03
IC_{50}			>100 μM	>100 μM	>100 μM	82 μM	72 μM
2	SPL2	100 μM	32 ± 0.02	21 ± 1.01	22 ± 0.08	59 ± 3.02	65 ± 0.02
		50 μM	27 ± 1.05	16 ± 2.79	10 ± 1.87	44 ± 1.88	43 ± 1.03
		10 μM	26 ± 2.04	3 ± 0.01	5 ± 2.05	35 ± 2.11	14 ± 0.94
IC_{50}			>100 μM	>100 μM	>100 μM	70 μM	70 μM
3	SPL3	100 μM	15 ± 1.02	25 ± 1.86	26 ± 3.02	11 ± 1.02	11 ± 1.01
		50 μM	14 ± 0.01	19 ± 0.95	17 ± 0.85	9 ± 1.04	6 ± 0.02
		10 μM	11 ± 0.03	18 ± 1.03	10 ± 0.92	7 ± 1.01	4 ± 0.11
IC_{50}			>100 μM	>100 μM	>100 μM	>100 μM	>100 μM
4	SPL4	100 μM	26 ± 0.01	42 ± 2.04	10 ± 1.09	37 ± 1.96	20 ± 0.89
		50 μM	19 ± 0.01	25 ± 1.03	8 ± 2.06	28 ± 1.01	13 ± 0.87
		10 μM	9 ± 0.87	15 ± 3.01	1 ± 0.01	10 ± 0.99	6 ± 0.01
IC_{50}			>100 μM	>100 μM	>100 μM	>100 μM	>100 μM
5	SPL5	100 μg/ml	40 ± 1.02	38 ± 1.02	77 ± 2.03	83 ± 1.93	65 ± 1.01
		50 μg/ml	32 ± 0.01	22 ± 1.04	63 ± 0.91	80 ± 1.02	61 ± 0.94
		10 μg/ml	1 ± 0.04	3 ± 0.86	6 ± 0.94	32 ± 0.89	1 ± 0.96
IC_{50}			>100 μg/ml	>100 μg/ml	41 μg/ml	25 μg/ml	43 μg/ml
6	SPL6	100 μg/ml	50 ± 1.01	50 ± 1.79	79 ± 1.01	78 ± 2.04	71 ± 0.97
		50 μg/ml	33 ± 2.88	31 ± 1.93	55 ± 1.99	73 ± 3.01	66 ± 1.03
		10 μg/ml	2 ± 0.01	6 ± 1.01	1 ± 0.01	27 ± 2.02	2 ± 1.01
IC_{50}			>100 μg/ml	>100 μg/ml	46 μg/ml	37 μg/ml	40 μg/ml
7	SPL7	100 μg/ml	50 ± 1.02	37 ± 1.05	77 ± 2.02	80 ± 0.88	73 ± 1.03
		50 μg/ml	35 ± 1.01	22 ± 0.02	58 ± 0.01	77 ± 1.03	63 ± 0.94
		10 μg/ml	2 ± 0.01	13 ± 0.88	7 ± 0.94	25 ± 1.06	10 ± 0.01
IC_{50}			100 μg/ml	>100 μg/ml	44 μg/ml	29 μg/ml	40 μg/ml
8	SPL8	100 μg/ml	57 ± 2.01	13 ± 0.98	73 ± 1.93	74 ± 2.11	65 ± 0.89
		50 μg/ml	42 ± 3.04	28 ± 1.86	64 ± 2.78	70 ± 1.01	55 ± 1.86
		10 μg/ml	10 ± 0.02	3 ± 1.01	4 ± 1.03	20 ± 1.02	11 ± 2.04
IC_{50}			77 μg/ml	>100 μg/ml	40 μg/ml	34 μg/ml	45 μg/ml
Control	BEZ-235	10 nM	37 ± 2.94	36 ± 4.94	34 ± 3.77	43 ± 2.03	58 ± 0.01
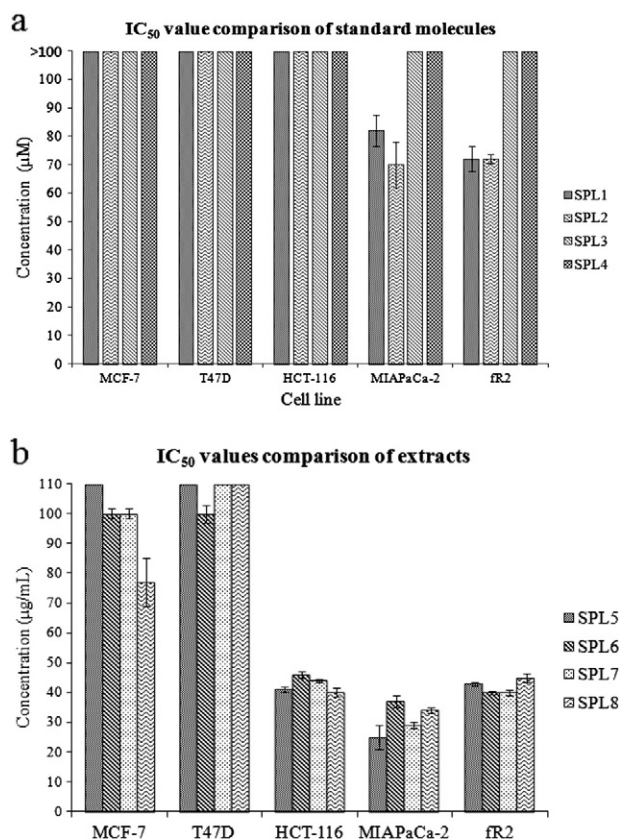
Fig. 3. MTT assay. a) Comparison of IC$_{50}$ values of standard molecules (SPL1, SPL2, SPL3 and SPL4); and b) extracts (SPL5, SPL6, SPL7 and SPL8) against breast cancer cell lines (MCF-7, T47D), colon cancer cell line (HCT-116), pancreatic cancer cell line (MIAPaCa-2) and normal epithelial cell line (fR2). SPL1, emodin; SPL2, chrysophanol; SPL3, emodin glycoside; SPL4, chrysophanol glycoside; SPL5, methanolic rhizome extract; SPL6, rhizome ethyl-acetate extract; SPL7, methanolic leaf extract; SPL8, leaf ethyl-acetate extract. Values are means of thee replicates ± SD.

cancerous cell line (MIAPaCa-2) was more evident in SPL5 (1.72) compared to that of SPL6 (1.08), SPL7 (1.38) and SPL8 (1.32). The higher cytotoxic ratio of SPL5 may confer it with a better possible role in antiproliferative activity.

Since MIAPaCa-2, the human pancreatic cancer cell line was chiefly sensitive to the crude extracts used for elucidating the cytotoxic effects, further studies were carried out with this cell line. To the best of our knowledge, anthraquinones from *R. emodi* have not been tested on pancreatic tumors, especially on MIAPaCa-2, a solid pancreatic tumor cell line. The results possibly indicate towards the existence of a synergistic effect of crude extracts as they were more potent in inducing cell death than the individual chemical compounds *viz.* emodin and chrysophanol.

3.3. Mechanisms involved in cytotoxicity

The desirable cytotoxic response of MIAPaCa-2 upon exposure to crude extracts prompted us to elucidate the mechanism of action by employing cell cycle analysis and MMP assay. The cytotoxic potential of the extracts against MIAPaCa-2 was evaluated using BEZ-235 (0.01 μM) as a positive control and the untreated control cultures received only the vehicle (DMSO < 0.5%).

3.3.1. Cell cycle phase distribution
The cell cycle machinery interprets oncogenic signals and reflects the biology of cancers.

Cell cycle arrest is a common cause of growth inhibition. To determine whether the effect of extracts involve alterations in cell cycle progression, cell cycle distribution analysis was carried out using flow cytometry. The binding of water soluble, DNA intercalating propidium iodide correlates with the amount of DNA within a given cell and the relative content of DNA indicates the distribution of a population of cells throughout the cell cycle. The effects of varying concentrations of different crude extracts on cell cycle progression of PI stained MIAPaCa-2 seeded cell line was analyzed by flow cytometry. All four tissue-specific extracts showed a concentration dependent increase in the percentage of apoptotic cells. Moreover, SPL8 caused both apoptotic cell death and S-phase arrest in increasing order at all the concentrations used (Fig. 4L–N). The extract SPL5 (100 μg/ml) showed a significant effect (58.9%) on viability reduction compared to that of SPL6, SPL7 and SPL8 which induced a considerable proportion of S-phase arrest with a corresponding decrease in G1 and G2/M cell population (Fig. 4C–N). The positive control BEZ-235 increased the apoptotic cell population to 13.6% with S phase arrest of 20% (Fig. 4B). The lowest IC$_{50}$ against MIAPaCa-2 as well as the major reduction in cell viability against 9.3% of vehicle treated cells (Fig. 4A) of the methanolic extract of rhizome (SPL5) may be attributed towards the highest concentration of its major chemical constituents *viz.* emodi and chrysophanol. The hang-up of cell cycle succession might be one of the molecular events connected with the selective anticancer efficacy of the crude extract as is shown by MIAPaCa-2 cell line.

3.3.2. Analysis of mitochondrial membrane potential (Δψm)
The mitochondrion plays an important role in the propagation of apoptosis and is responsible for 90% of the energy needed by the cell. The Δψm loss is an early event in mitochondrial mediated apoptosis. To assess whether the extracts affect mitochondrial integrity, the potential changes in mitochondrial membrane were analyzed by using rhodamine-123, a dye indicating Δψm loss (Pieme et al., 2013). Flow cytometric analysis of MIAPaCa-2 cells after treatment with different concentrations of extracts for 48 h revealed a reduction in Δψm in a concentration dependent manner. The SPL5, SPL6, and SPL7 caused MMP loss from 11.6% in the case of untreated control (Fig. 5A) to a maximum of 42.3%, 37.1% and 26.6% respectively at a higher concentration of 100 μg/ml (Fig. 5E, H, K). The maximum MMP loss of 24.4% in SPL8 was observed at a concentration of 50 μg/ml (Fig. 5M). The positive control BEZ-235 showed a 16.4% loss of MMP (Fig. 5B). This loss is primarily due to the activation of mitochondrial permeability transition (PT) pore which leads to the consequent release of cytochrome C from mitochondria and accordingly elicits other apoptotic factors to induce cell death. In earlier studies it has been shown that permeability of inner mitochondrial membrane increases upon interaction of various anticancer agents with the mitochondria (Wani et al., 2013). The results indicate that extracts may be directly affecting the mitochondria to increase membrane permeability and decrease Δψm which may further lead to cell death (apoptosis/necrosis).

The plant parts and the extraction solvents greatly influence the antiproliferative activity of the same plant species (Gul et al., 2013). As described above, the concentrations of bioactive constituents varied among the extracts screened for antiproliferative activity, and this quantitative divergence is in correspondence with the percentage growth inhibition of different cancer cell lines used. The extract SPL5 with a higher concentration of emodin and chrysophanol was found to be highly active. This inference provides a way for the identification of elite chemotypes with better pharmacological significance to ensure their possible use as potential chemotherapeutic agents. Moreover, as per the reports in the literature and the results we obtained, a possibility of occurrence of synergism is plausible in the herbal extracts.

In conclusion, the overall results are indicative of the possible existence of a synergistic effect of crude extracts as they were found to be more active than the individual chemical agents which may in all probability bring-out the role of aglycone forms of major
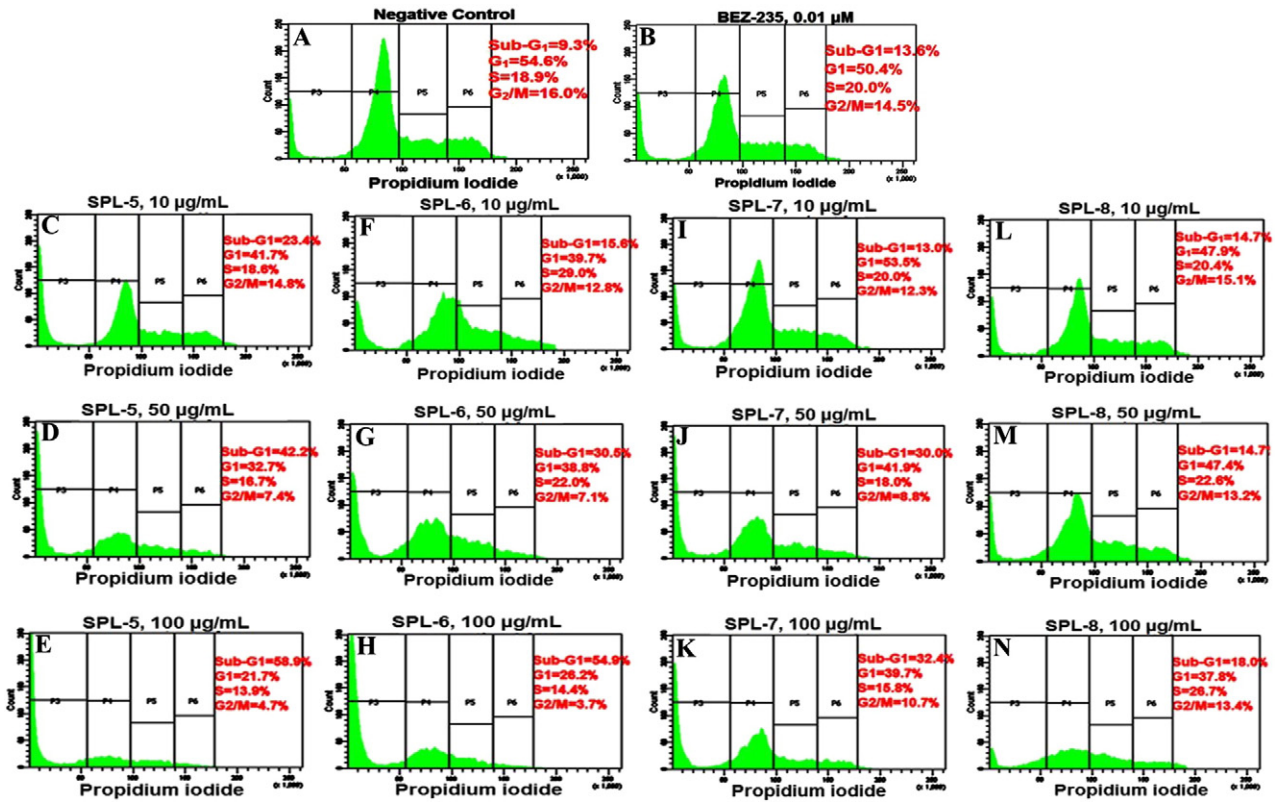
Fig. 4. Flow cytometric analysis of human pancreatic cancer cell line (MIAPaCa-2). Cells were exposed to different concentrations (10, 50 and 100 μg/ml) of the extracts (SPL5, SPL6, SPL7 and SPL8) for 48 h and stained with PI (5 μg) to determine DNA fluorescence and cell cycle phase distribution using FACS Aria II flow cytometer. A) Negative control < 0.5%; B) positive control (0.01 μM). The data are representative of three independent experiments.
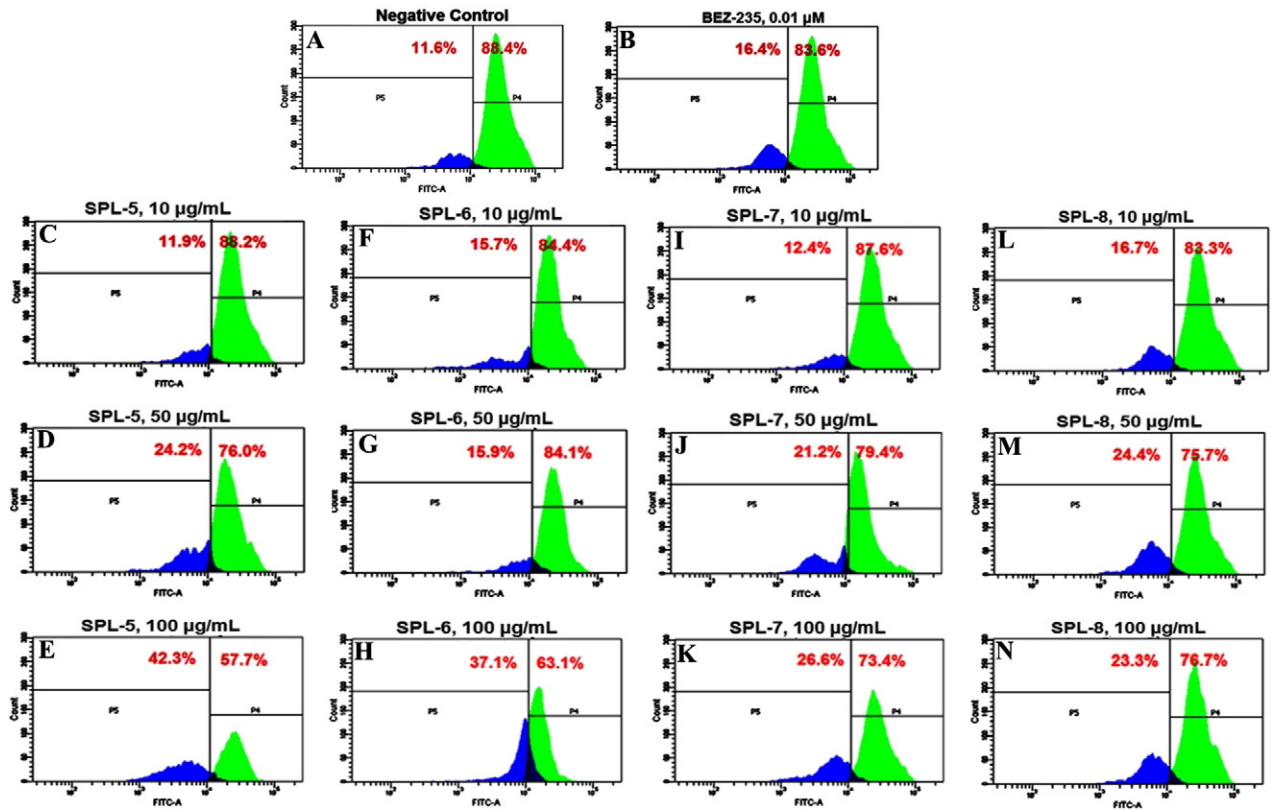

Fig. 5. Effect of extracts on the integrity of mitochondrial membrane. The MIAPaCa-2 cells were incubated with extracts (SPL5, SPL6, SPL7 and SPL8) at varying concentrations (10, 50 and 100 μg/ml) for 48 h. Thereafter, they were stained with rhodamine-123 (1 μM) for 1 h and analyzed in FACS Aria II flow cytometer to determine the loss of mitochondrial membrane potential (Δψm). A) Negative control (<0.5%); B) positive control (0.01 μM). The data are representative of three independent experiments.

anthraquinones, emodin and chrysophanol as probable chemo-agents in inducing the death of cancer cells. Additionally, different plants from varying altitudes presented significant differences in the anthraquinone accumulation. It has a prospect of providing high yielding populations of *R. emodi* for pharmacological and commercial utilization.

Conflict of interest

The authors declare that there is no conflict of interest.

Acknowledgments

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.sajb.2014.07.012.

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