RESEARCH COMMUNICATION

Apoptosis Induction in MDA-MB-435S, Hep3B and PC-3 Cell Lines by *Rheum emodi* Rhizome Extracts

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Abstract

The study was aimed at evaluating apoptosis induction potentials of methanolic and aqueous extracts of *Rheum emodi* Wall. ex Meissn. rhizome. The ability of the extracts to induce apoptosis in MDA-MB-435S (human breast carcinoma), Hep3B (human hepatocellular carcinoma) and PC-3 (human prostate cancer) cell lines were tested by ELISA to detect cellular DNA fragmentation. Results obtained from the present study confirm that the extracts target the cancerous cells towards apoptosis. The study concludes that *R. emodi* possess anticancer metabolites that can be isolated and used as precursors in development of anticancer drugs. Suppression of apoptosis might contribute to tumor development by means of accumulation of continuously proliferating cells. The strategy employed in this study, to induce apoptosis in the tumor cells, could be a potential target of therapeutic intervention of cancers.

Keywords: Apoptosis induction - MDA-MB-435S - Hep3B - PC-3 - ELISA

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Introduction

Cancer imposes a major disease burden worldwide, with considerable variations in incidence, mortality, survival, occurrence and causative factors. It was estimated that the number of new cases of cancer will increase from an estimated 10 million cases in 2000 to 15 million in 2020 and by 2050, the cancer burden could reach 24 million cases per year worldwide (Parkin et al., 2001). While surgery, chemotherapy and radiation therapy are the main treatment options available to counteract all forms of cancers, at some stage resistance develops, leaving the current generation of cancer drugs ineffective after a particular period of time.

A great deal of research has been done all over the world to isolate the active component from natural resources. Countries are changing over to alternative medicines due to the more holistic approach and with substantially fewer side effects as opposed to a depleted immune system due to the prolonged use of allopathic medicine. Medicinal plants have received growing attention in recent years as potential chemotherapeutic agents.

Medicinal plants and their derivatives are widely used in the treatment of cancer. There are many classes of plant-derived natural products studied for development of drugs. Flavonoids from plants have been linked to reducing the risk of cancer and other major chronic diseases. They have also been reported to modulate P-glycoprotein (Pgp) and MDR (multidrug-resistance) protein (Walle et al., 2001). Flavanoids also inhibit many enzymes that are the targets in anticancer treatment, e.g. eukaryotic DNA topoisomerase I, Cox I and II and estrogen 2- and 4-hydroxylases. Flavonoids by interacting with P450 enzymes reduce the activation of procarcinogen substrates to carcinogens which makes them putative anticancer substances (Mukhtar et al., 1988). Anthraquinones, another class of plant compounds have been reported to inhibit cell growth in several tumor cells, including human lung carcinoma (Lee et al., 2001), hepatoma (Kuo et al., 2002; Yeh et al., 2003) and leukemia cell lines (Chen et al., 2004). Similarly, saponins from plants are believed to be useful for the human diet in controlling cholesterol (Mohamad et al., 2001) and in treatment of tuberculosis (Ong, 2004). Alkaloids can increase nutrient absorption and blood circulation, reduce pain and stimulate nerve system as they have narcotic effect (Ong, 2004). Plant resources hold many more of such potential compounds that are yet to be isolated and studied. The strategy of enhancing the efficacy of anticancer therapy employing such compounds, without significant side effects is a rather novel approach which warrants further investigation.

A growing number of plant resources are screened and reported for their capacities to induce apoptosis to cancer cells. *Euchresta formosana* radix extracts induce apoptosis to human hepatocellular carcinoma (Hep3B) cell line (Hsu et al., 2007). Li et al. (2009) reported apoptosis induction abilities of *Chrysanthemum indicum* extract in human HCC MHCC97H cells. Crude extracts of garlic are found to induce apoptosis via caspases-3 gene expression in human colon cancer cells (Su et al., 2006). Similarly, Dai et al. (2008) has reported that the extract of *Scutellaria*

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barbate induces apoptosis to hepatoma H22 cells via the mitochondrial pathway involving caspase-3.

Rheum emodi Wall. ex Meissn., is a leafy perennial herb distributed in altitudes ranging from 2800 to 3800 m in the temperate and subtropical regions of Himalayas from Kashmir to Sikkim in India (Nautiyal et al., 2003). It belongs to the family Polygonaceae. Roots of *R. emodi* are reported to have antibacterial and antifungal activities (Agarwal et al., 1976; Cyong et al., 1987; Harvey and Waring, 1987). In addition, several other biological activities such as laxative, diuretic, antioxidant, anti-cancer and *in vivo* inhibitory effects towards P388 leukemia in mice are also reported (Lu and Chen, 1989; Oshio and Kawamura, 1985; Zhou and Chen, 1988; Rajkumar et al., 2010).

However, reports on the abilities of the extracts to target cancerous cells towards apoptosis are still scarce and this question needs to be addressed. The aim of the present study was to evaluate the apoptosis induction efficacies of the methanolic and aqueous extracts of *R. emodi* on MDA-MB-435S, Hep3B and PC-3 cell lines.

Materials and Methods

Chemicals

L-15 (Leibovitz) cell culture medium (with L-glutamine), MEM (minimal essential medium) cell culture medium (with Earle's salt, NEAA and L-glutamine) and Nutrient Mixture F-12K, Kaighn's modification cell culture medium (with L-glutamine) were purchased from Himedia Laboratories Pvt. Ltd. (India). MDA-MB-435S (human breast carcinoma), Hep3B (human hepatocellular carcinoma) and PC3 (human prostate cancer) cell lines were obtained from National Centre for Cell Science (Pune, India). Cellular DNA fragmentation ELISA (Cat. No. 11 585 045 001) from Roche Diagnostics, Germany, was used to determine apoptosis.

Plant material

Rheum emodi rhizomes were collected from their natural habitat in the Garhwal Himalayas at Chamoli (30º24' N, 79º21' E), Uttaranchal, India in the month of June, 2007. The specimens were identified by C. Sathyanarayanan, Arya Vaidya Pharmacy (Coimbatore) Limited, Kanjikode, Palakkad, India. Collected specimen were shade-dried, powdered and used for solvent extraction. Voucher specimens were maintained at our laboratory for future reference (Accession no.: VIT/SBCBE/CCL/07/6/04; Dated: June 11, 2007).

Extraction

50 g of *R. emodi* rhizome powder was serially extracted using methanol and water as solvents in a Soxhlet apparatus. The powder:solvent ratio was maintained as 1:6. The extracts obtained were evaporated to dryness at 40 °C in reduced pressure (methanol: 337 mbar; aqueous: 72 mbar) in a rotary evaporator (BÜCHI, Switzerland). The dried extracts were then weighed to determine the yield of soluble constituents and subsequently stored in a vacuum desiccator (Pickett and Stephenson, 1980). 50 g of rhizome powder yielded 24.8 g of crude methanolic extract and 3.86 g of crude aqueous extract.

Cell culture

MDA-MB-435S, Hep3B and PC-3 cells were cultured in L-15, MEM Eagle medium (1 mM sodium pyruvate) and Ham F-12 culture media(supplemented with 10% fetal bovine serum) respectively, in a humidified atmosphere with 5% CO_2 (except for MDA-MB-435S cells) at 37 ºC. Cells were grown in polystyrene-coated T75 (75 cm^2) cell culture flasks, and were harvested in logarithmic phase of growth. Harvested cells were used for detection of cellular DNA fragmentation by ELISA.

Cellular DNA fragmentation ELISA

The ability of the extracts to induce apoptosis was studied using cellular DNA fragmentation ELISA kit as per supplier's instructions. The principle of the kit involves labeling of the cells in culture with non-radioactive thymidine analogue BrdU, which is incorporated into the genomic DNA and measuring apoptosis by capturing the BrdU labeled DNA fragments that are released in to the culture supernatant after treating with the apoptosis inducing agents.

Labeling the cells with BrdU

The cell number was adjusted to 2-4 x 10^5 cells/ml in the respective culture medium and BrdU labeling solution was added to a final concentration of 10μM. The tubes were incubated at 37 °C for 2 h after which they were centrifuged for 10 min at 250 x g. The supernatant containing BrdU was carefully and thoroughly removed and the cells were resuspended in BrdU-free culture medium.

Procedure for treating the cells with apoptosis inducing agents

The flat-bottom microtiter plates (MTP) were seeded with 100 µl of BrdU-labeled cells (1 x 10^5 cells/ml) and treated with 100 µl of apoptosis-inducing agent (varying concentration of the extracts) which were then incubated for 4 h at 37 °C in a humidified atmosphere (5% CO^2) or as per the cell requirements. After the incubation period, the supernating culture medium was removed and each well was added with 200 µl of 1x incubation solution. The plates were then incubated for 30 min at 15-25 °C to lyse the cells and followed by centrifugation at 1500 rpm for 10 min. The supernatant containing the apoptotic fragments was used for further analysis.

Coating of MTPs

Each well of a 96-well, flat-bottom microtiter plate (MTP) was added with 100 µl of anti-DNA antibody coating solution and incubated at 37 °C for 60 min. The coating solution was removed by aspiration.

Blocking of MTPs

The anti-DNA antibody coated MTP were blocked by addition of 200 µl of 1x incubation solution to prevent nonspecific binding sites, after which the MTP was covered with an adhesive cover foil and incubated at 15-25 °C for 30 min. The wells were then washed three

times with 250 µl of washing solution for 2-3 min each. The prepared MTP was used for ELISA procedure.

Procedure for ELISA

Each well of the pre-coated MTPs were added with 100 µl of the sample to be measured for apoptosis and incubated for 90 min at 15-25 °C after covering them tightly with an adhesive cover foil. The solution was removed and the plates were washed with 250 µl of washing solution. Washing step was repeated three times with 2-3 min interval. After the last wash, the washing solution was left in the well and the uncovered MTP was placed in the microwave oven along with 300 ml of water in a 500 ml beaker. The plates were irradiated for 5 min on medium power (500 W) to fix and denature DNA after which it was cooled for approximately 10 min at -20 °C. Removal of the existing solution was followed by the addition of 100 µl of anti-BrdU-POD conjugate solution. The plates were incubated at 15-25 °C for 90 min after covering them tightly with an adhesive cover foil.

Procedure for photometric measurement

After incubation, the wells were washed three times with 250 µl of washing solution for 2-3 min each and added with 100 µl of substrate solution. The plates were incubated in the dark at room temperature on a MTP shaker until the color development is sufficient. The reaction was stopped by the addition of 25 µl of stop solution followed by incubation for 1 min. Absorbance was measured at 450 nm within 5 min after the addition of stop solution.

Results and Discussion

Plant compounds are known to induce apoptosis in neoplastic cells (Chiao et al., 1995; Hirano et al., 1995; Jiang et al., 1996). It has become increasingly evident that apoptosis is an important mode of action for many anti-tumor agents, including ionizing radiation (Rodford et al., 1994), alkylating agents such as cisplatin and 1,3-bis(2-chloroethyl)-1-nitrosourea (D'Amico and McKenna, 1994), topoisomerase inhibitor etoposide (Kaufman et al., 1993), cytokine tumour necrosis factor (Shih and Stutman, 1996) and taxol (Gibb et al., 1997). Apoptosis induction has been a new target for innovative mechanism-based drug discovery (Workman, 1996). It is thus considered important to screen apoptosis inducers from plants, either in the form of crude extracts or as components isolated from them.

Both extracts of *R. emodi* were observed to be capable of inducing and targeting the cells towards apoptosis. This apoptosis inducing potential is vital for drug development targeted towards cancer. Figure 1 shows data for the ability of the aqueous and methanolic extracts to induce apoptosis in MDA-MB-435S, Hep3B and PC-3 cell lines. Both extracts showed considerable concentration dependence as evident from the apoptotic fragments detected via absorbance at 450 nm. The decrease in absorbance at high dose could be justified by the already reported cytotoxicity exhibited by the extracts (Rajkumat et al., 2010). The methanolic extract presented an interesting observation on its efficacy to target PC-3 cells towards apoptosis.

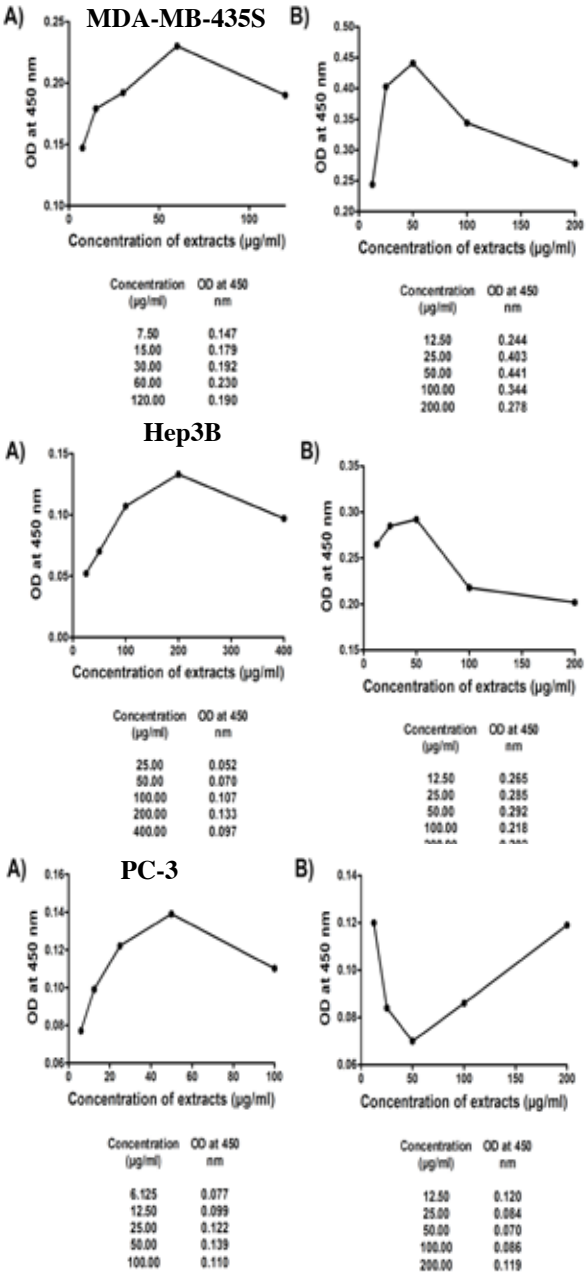

Figure 1. Apoptosis Induction by R. emodi Rhizome Aqueous (A) and Methanolic (B) Extracts as Determined by ELISA for DNA Fragmentation.

Maximum apoptotic fragments from PC-3 cells were detected at the lower treated dose of 12.50 µg/ml followed by a decrease of fragments at 50 µg/ml. Further increase in treatment dose lead to gradual increase of apoptotic fragments. This dichotomy observed might be due to the presence of a dual natured compound(s) present in the methanolic extract of *R. emodi*. The observed results of the study confirm the presence of anticancer metabolites in the extracts and warrant them as potential sources harboring anti-cancer metabolites that can be isolated and investigated. Overall, the methanolic extract *R. emodi* was found to be more effective in inducing apoptosis in comparison to its aqueous counterpart.

In conclusion, methanolic and aqueous *R. emodi* rhizome extracts were found to possess apoptosis induction potentials. The extracts possess anticancer

metabolites that may have prospective clinical use as precursor for preventive medicine. Further studies for isolation and identification of active components are in prospect.

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