The Journal of Phytopharmacology 2017; 6(5): 288-292
Online at:www.phytopharmajournal.com

Research Article

ISSN 2320-480X
JPHYTO 2017; 6(5): 288-292
September- October
Received: 31-09-2017
Accepted: 07-11-2017

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Emodin, isolated and characterized from an endophytic fungus *Polyporales sp*., induces apoptotic cell death in human lung cancer cells through the loss of mitochondrial membrane potential

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ABSTRACT

Emodin (1,3,8-trihydroxy-6-methylanthraquinone) is a Chinese herbal anthraquinone that exhibits numerous biological activities, such as antitumor, antibacterial, antiinflammatory, and immunosuppressive. From an endophytic fungus, a close relative of *Polyporales sp*., found in association with *Rheum emodi*, Wall. ex Meissn a compound (Rz) was isolated and characterizedby different spectroscopic techniques (^{1}H-NMR, ^{13}C-NMR, ^{2}D-NMR, and HRMS). The compound (Rz) displayed a range of cytotoxicities against different human cancer cell lines like THP-1(Leukemia), A549 (Lung), NCI-H322 (lung) and Colo-205(colon) at a concentration of 70 and 100 µM. The compound had strong anticancer activity by arresting the cell cycle at *G1* and *G2/M* phase and loss of mitochondrial membrane potential in A-549 lung cancer cells in concentration dependent manner. The study suggests that emodin induced anticancer effects may have novel therapeutic applications for the treatment of lung cancer.

Keywords: NMR, Cytotoxic, *Rheum emodi*, Cancer cell lines.

INTRODUCTION

Cancer is the major threat 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 to15 million in 2020 and by 2050, the cancer burden could reach 24 million cases per year worldwide [1]). Lung cancer accounts for 13% (1.6 million) of the total cases and 18% (1.4 million) of the deaths in year 2008 [2]. Lung cancer is the leading cause of cancer death worldwide and can be classified into small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). NSCLC accounts for 85%-87% of all lung cancer worldwide [3].

Emodin (1,3,8-trihydroxy-6-methyl-anthraquinone) is a natural anthraquinone derivative found in the roots and rhizomes of numerous plants [4], has been reported to exhibit anticancer effect on several human cancers such as lung cancer [5]. Emodin is an active component found in the roots and rhizomes of numerous plants including *Rheum emodi*. *Rheum emodi* Wall. ex Meissn. (Polygonaceae) 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 [6]. *Rheum emodi*, Pambchallan (Kashmiri) has been traditionally used to treat pathological ailments like fevers, ulcers, bacterial infections, fungal infections, jaundice and liver disorders [7-9]. Some workers have worked out antitumor activity of *Rheum emodi* [10], but very little is known about the mechanisms involved. This herb has been used in traditional Chinese medicine for the treatment of skin burns, infection, gallstones, hepatitis, inflammation, and osteomyelitis for years.

Ever since the discovery of the rich diversity of the endophytic fungi, their population dynamics, their role in improving plant growth, plant health [11], their distribution in the plant, the metabolites they secrete and their potency to produce novel compounds within the plants [12], have formed an important aspect of present day research. The discovery that an endophytic fungus (*Taxomyces andreanae*) also produced the anticancer drug paclitaxel (Taxol) derived from Pacific yew (*Taxus brevifolia*) was unexpected [13]. This background information led us to speculate that Kashmir valley that is endowed with a diversity of medical plant wealth might constitute another source of endophytic fungi with

biological activity. The isolation of endophytic fungi from the plant Rheum emodi is the first suchinitiative and has not been reported earlier. The present study was carried out todetermine the endophytic mycoflora in *Rheum emodi*, a widely used medicinal plant and the possible bioactive potential of the isolated endophytes.

MATERIALS AND METHODS

Fermentation of the fungal endophyte

The endophytic fungus was isolated from *Rheum emodi* Wall. Ex Meissn and identified as a close relative of *Polyporales sp.*, by molecular biology tools. The endophyte was transferred under sterile condition to the PDB medium for seed preparation. For chemical investigations the endophytic fungal strain was grown in potato dextrose broth at 28 °C for 14 days with rpm of 180 in an incubator shaker (New Brunswick, USA). The broth was initially inoculated with 1% of 7-day-old seed prepared. The 14th day grown culture of the endophyte was homogenized with 10% methanol under ultra sonicator.

Isolation and characterization of the compound

The fermentation broth (20 litres) of cultured endophyte was extracted with ethyl acetate and n-butanol respectively following the standard protocol of National Cancer Institute [41]. The ethyl acetate (5.7 g) and n-butanol (2.1 g) extracts thus obtained were combined and dissolved in minimum amount of methanol and adsorbed on silica gel to form slurry. The dried slurry was subjected to fractionation using silica gel column chromatography leading to various fractions. Repeated column chromatography of these fractions using different percentages of petroleum ether-ethyl acetate and ethyl acetate-methanol afforded the title compound as yellow solid. The molecular structure is identified by spectroscopic methods including ^{1}H- NMR, ^{13}C-NMR, 2D-NMR, LCMS.

Chemicals used in cell cytotoxity assay

The chemicals used for the *in vitro* cytotoxicity assays, Growth Medium RPMI-1640, Minimum Essential Medium (MEM), Fetal Calf Serum (GIBCO), Trypsin, Penicillin, Sulphorhodamine Dye (SRB), Streptomycin, DMSO and Phosphate Buffer Saline (PBS) and Human Cancer Cell Lines; THP-1(Leukemia), A549 (Lung), NCI-H322 (lung) and Colo-205(colon) were procured from European Collection of cell culture (ECACC), UK. Cells were grown in Roswell Park Memorial Institute medium (RPMI-1640) and Minimum Essential Medium (MEM) supplemented with 10% FCS and 1% penicillin. Penicillin was dissolved in PBS and sterilized by filtering through 0.2 µm filter in laminar air flow hood. Cells were cultured in CO2 incubator (New Brunswick, Galaxy 170R, eppendroff) with an internal atmosphere of 95% air and 5% CO_2 gas and the cell lines were maintained at 37°C. The media was stored at low temperature (2-8 °C). The medium for cryopreservation contained 20% FCS and 10% DMSO in growth medium.

Cell cytotoxicity assay

SRB assay was performed in which cell suspension of optimum cell density was seeded and exposed to 100 and 70 µm/ml concentrations of compound Rz and paclitaxel (1µm/ml) in the culture medium. Incubated the cells with concentrations of samples for 48 h. fixed the cells with ice-cold TCA for 1 h at 4°C. Plates were washed five times in distilled water and dried in the air. To each well of the dry 96-well plates 0.4% sulphorhodamine (SRB) solution was added and allowed the staining at room temperature for 30 min. The plates were washed quickly with 1% v/v acetic acid to remove the unbound SRB dye. The bound SRB dye was solubilised by adding 10 mM unbuffered Tris base (pH 10.5) to each 96 well plate on a shaker platform and read the plates at 540nm [15].

DNA content and cell cycle phase distribution

A-549 cells were seeded in 6 well plates at a concentration of (1×10^6 cells/well/ml) and incubated at 37ºC with 5% CO_2 to form the monolayer for 24 h, thereafter treated with 100, 70, 50 and 30 µg/ml concentrations of Rz. After 48 h of the treatment cells were collected, washed in PBS and fixed in 70% cold ethanol overnight. Cells were again washed with PBS, subjected to RNase digestion (400 µg/ml) at 37 °C for 90 mins. Finally, cells were incubated with propidium iodide (PI) (10 µg/ml) and analyzed immediately on flow cytometer FACS Diva (Becton Dickinson, USA) (16).

Measurement of mitochondrial membrane potential for cellular energy status

Changes in mitochondrial transmembrane potential ($\Delta\Psi_m$) as a result of mitochondrial perturbation were measured after staining with Rhodamine-123 (Rh-123). A-549 cells at a density of ($1x10^6$/ml/well) were grown in 6 well plates and treated with different concentration of Rz for 48 h. Rh-123 (1 µM) was added 30 mins before the termination of experiment. Cells were washed in PBS and centrifuged at 1500 rpm for 5 min and suspended in PBS. The decrease in intensity of fluorescence from 10,000 events because of mitochondrial membrane potential loss was analyzed in FITC channel on flow cytometer [16].

RESULTS

Identification of the fungal endophyte

The endophytic fungal strain IIIM2 was identified up to the genus level using ITS1-5.8S-ITS2 approach. The sequence was submitted to NCBI and accession number assigned to strain by NCBI is KC831589.

Extraction and isolation

The fermentation broth (20 litres) of cultured endophyte was extracted with ethyl acetate and n-butanol respectively following the standard protocol of National Cancer Institute [14]. The ethyl acetate (5.7 g), and n-butanol (2.1 g) extracts showed almost the similar secondary metabolite profiles on TLC plates and were therefore pooled and subjected to repeated column chromatography on silica gel (100-200 mesh) using hexane-ethyl acetate to afford compound **1** (15.5 mg).

Comparison of physical characteristics and spectral data of the compound, with that reported in literature [17], confirmed it to be Emodin as shown in Fig 1.

Yield: 15.5 mg; mp:258-260°C. UV (MeOH) λ_{max}: 218, 250, 262, 287, 436 nm. IR (KBr) ν_{max}cm^{-1}: 3450, 1660, 1630.^{1}H NMR ($CDCl_3$, 500 MHz) δ: 12.80 (1H, s, OH-3), 12.30 (1H, s, OH-1), 12.12 (1H, s, OH-8), 7.63 (1H, d, J = 1.3 Hz, H-4), 7.29 (1H, d, J = 2.4 Hz, H-7), 7.10 (1H, d, J = 1.3 Hz, H-2), 6.68 (1H, d, J = 2.4 Hz, H-5), 2.46 (3H, s, CH_3). ^{13}C NMR ($CDCl_3$, 125 MHz) δ: 189.9 (C-9), 181.5 (C-10),

165.6 (C-8), 164.4 (C-1), 161.4 (C-6), 148.3 (C-3), 135.2 (C-10a), 132.9 (C-4a), 124.2 (C-4), 120.5 (C-2), 113.4 (C-9a), 108.9 (C-5), 108.8 (C-8a), 107.9 (C-7), 21.8 (-CH3). HR-ESI-MS *m/z*: 271.0607 (calculated for $C_{15}H_{10}O_5$, 270.0528).

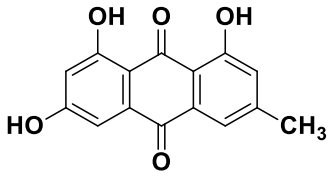

Figure 1: Structure of Compound 1 (Emodin)

Cell cytotoxicity assay

Sulpharhodamine B (SRB) assay is based on the measurement of cellular protein content. The assay relies on the uptake of the negatively charged pink aminoxanthene dye (SRB) by basic amino acid proteins under mild acidic conditions [18]. Treatment of cells with the compound, Rz (70 and 100 µm/ml) produced dose-dependent anti-proliferation effect on different human cancer cell lines THP-1, A549, NCI-H322, Colo-205 (Table 1). It was observed that the compound at higher concentration (100µm/ml) was the most potent with a high percentage cell growth inhibition of 87 and 92 against human lung (NCI-H322) and human colon (Colo-205) cancer cell lines respectively. At lower concentration (70 µm/ml), percent cell growth inhibition observed was 79 and 85 against the same cancer cell lines.

DNA content and cell cycle phase distribution

Incubation of A-549 cells with Rz induced alterations in cell cycle phase distribution. DNA content distribution in A-549 cells was observed to change after48 h treatment with various concentrations of Rz (0, 100, 70, 50 and 30 µg/ml) (Fig. 2). We could observe that Rz induced cell death by arrest the cells in G1 and G2/M phase of the cell cycle respectively. The percentage population of the cells in various phases of the cell cycle was found to be 2, 68, 11 and 16 in the untreated control cells where 68% of the cells are in the G1 phase and 16% of the cells represent the G2/M population. However treatment with increasing concentration of Rz increased the G2/M population from 14 to 21%. Camptothecin used as the positive control at 5 µM of for 6 h increased sub G0 phase cell population upto 39%. Overall as the concentration Rz increased, the percentage of cells in various phases of the cell cycle was changed.

Table 1: Anticancer activity of compound Rz

	Tissue Type		**Leukemia**	**Lung**		**Colon**
	Cell Type		THP-1	A549	NCI-H322	Colo-205
S. No	Code	Conc. (µm/ml)	%age Growth Inhibition	%age Growth Inhibition	%age Growth Inhibition	%age Growth Inhibition
1	Rz	100	12	**80**	**87**	**92**
		70	4	**75**	**79**	**85**
	Paclitaxel	1	**71**	**82**	**50**	**98**

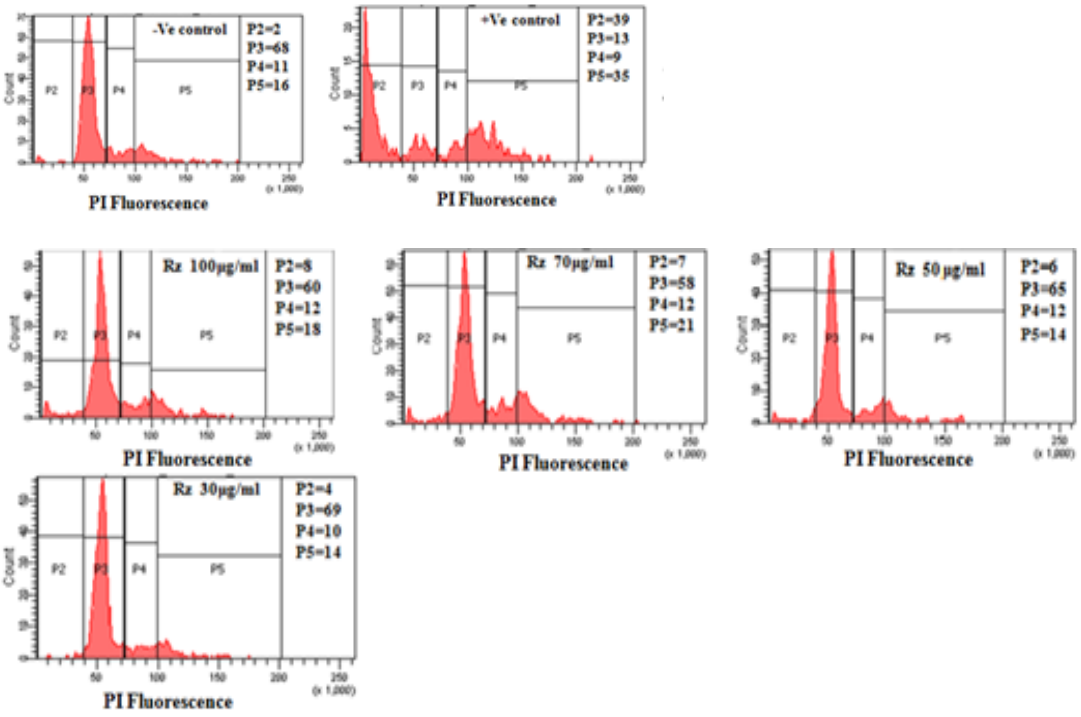

Figure 2: Showing the A-549 cells with Rz induced alterations in cell cycle phase distribution. P2 population showing cells in Sub G0/G1 phase, P3 showing G0/G1 phase population, P4 showing S phase population and P5 showing G2/M phase population.

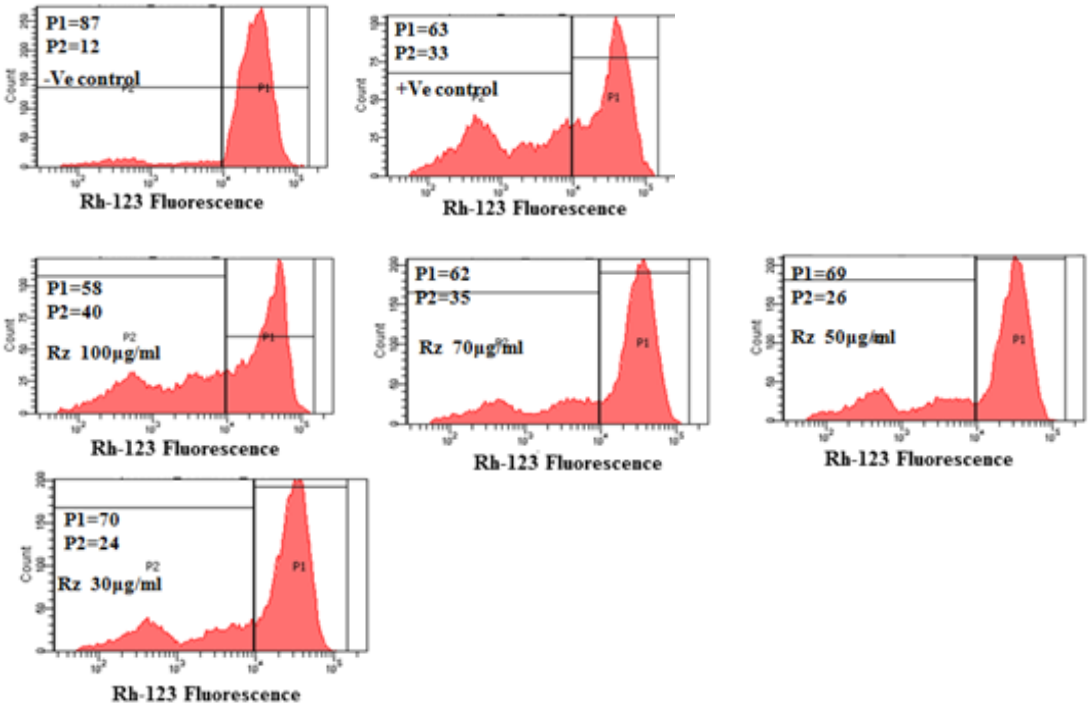

Figure 3: A-549 cells showing loss of mitochondrial membrane potential treated with different concentrations of Rz. P1 population showing cells with intact mitochondria and P2 population showing cells with disrupt mitochondria leading to loss in $\Delta\Psi_m$.

Measurement of mitochondrial membrane potential for cellular energy status

The loss of mitochondrial membrane potential ($\Delta\Psi_m$) is largely due to activation of mitochondrial permeability transition pore which leads to the subsequent release of Cytochrome C from mitochondria and consequently triggers other apoptotic factors. We observed decrease in ($\Delta\Psi_m$) in A-549 cell line treated with different concentration of Rzfor 48 h by flow cytometer after Rh-123 staining. Compared to the corresponding untreated control, incubation of A-549 cells with the compound caused an obvious decrease of ($\Delta\Psi_m$) after 48 h of incubation in a concentration dependent manner i.e. 24 to 40% at concentrations ranging from 30 to 100µg/ml. In case of untreated control upto 87% cells were functionally active with high Rh-123 signal represented by P1 population (Fig. 3). Moreover, A-549 cells treated with camptothecin used as a positive control showed a decrease in Rh-123 fluorescence up to 33% at only 5 µM camptothecin for 6 h.

DISCUSSION

Many novel and valuable bioactive compounds with antimicrobial, insecticidal, cytotoxic and anticancer activities have been successfully obtained from the endophytic fungi [19]. Keeping in view of these success stories of fungal endophytes, the present study was done. In this study an endophytic fungus a close relative of *Polyporales sp.*, IIIM2 was isolated from the medicinal plant*rheum emodi* and a compound (Rz) was isolated from this endophyte. Based on the spectroscopic data, ^{1}H-NMR, ^{13}C-NMR, HRMS and 2D- NMR the compound was identified as emodin. Emodin (1,3,8-trihydroxy-6-methyl-anthraquinone) is a natural anthraquinone derivative found in the roots and rhizomes oSf numerous plants [4]. It has been reported to exhibit anticancer effect on several human cancers such as lung cancer [5], chronic myelocytic leukemia [20], liver cancer [21] and gallbladder cancer [22].

Our study showed the cytotoxic potential of emodin against different human cancer cell lines THP-1, A549, NCI-H322, and Colo-205 at two different concentrations. It was observed that the compound at higher concentration (100 µm/ml) was the most potent with a high percentage cell growth inhibition of 87 and 92 against human lung (NCI-H322) and human colon (Colo-205) cancer cell lines respectively. It was observed that the compound induced the cell death by arresting the cells in G1 and G2/M phase of the cell cycle respectively and the loss of mitochondrial membrane potential ($\Delta\Psi_m$) is largely due to activation of mitochondrial permeability transition pore which leads to the subsequent release of Cytochrome C from mitochondria and consequently triggers other apoptotic factors. Mitochondrial membrane potential disruption and G2/M arrest has previously been described in leukemia cellsupon treatment with emodin or aloe-emodin and rhein fromrhubarb [20, 23-25]. Moreover a report has shown that emodin could antagonize the signaling pathways ERK and AKT, which are essential for cell survival in A-549 lung carcinoma cells [26]. Some reports showed that emodin suppressed lung cancer cells proliferation via inactivating ERK1/2 signaling pathway [27,28]. Ko *et al.* [29] demonstrated that emodin induced cytotoxicity occurs via ERK1/2 inactivation and via ERCC1 and Rad51 could cause down regulation in human lung cancer cells.

CONCLUSION

In summary, we demonstrated that emodin caused an inhibition of cell growth with cycle arrest and apoptosis induction in different human cancer cell lines. Moreover, a large part of our study essentially focused on the mitochondrial pathway and we investigated loss of mitochondrial membrane potential ($\Delta\Psi$m) is largely due to activation of mitochondrial permeability transition pore which leads to the

subsequent release of Cytochrome C from mitochondria and consequently triggers other apoptotic factors. These new findings suggest that emodin-induced effects may have novel therapeutic applications for the treatment of lung cancer.

Conflict of interests

None declared.

Acknowledgement

We are thankful for the CSIR New Delhi for providing the financial assistance during the tenure of the work. The authors are also highly thankful Dr. Ram A. Vishvakarma Director IIIM, for providing the lab facilities during this study.

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HOW TO CITE THIS ARTICLE

Dar AR, Majeed R, Sheikh AA, Shakeel-u-Rehman, Hamid A, Hassan QP. Emodin, isolated and characterized from an endophytic fungus *Polyporales sp*., induces apoptotic cell death in human lung cancer cells through the loss of mitochondrial membrane potential. J Phytopharmacol 2017;6(5):288-292.