Chapter - III
CHAPTER 3

Emodin (1, 3, 8-trihydroxy-6-methylanthraquinone), a natural anthraquinone derivative inhibits growth and induces apoptosis in human liver cancer cells through reactive oxygen species generation

3.1. ABSTRACT

Hepatocellular carcinoma (HCC) is leading cause of death worldwide with median survival time where the surgical ablation is impracticable. Emodin, an active natural anthraquinone derivative found in the roots and rhizomes of numerous Chinese medicinal herbs, proved to play a major role as chemotherapeutic agent with various kinds of cancer cells without affecting normal counterparts. Oxidative stress is characterised by an increased level of reactive oxygen species (ROS) that disrupts the intracellular reduction-oxidation (redox) balance and has been implicated in various diseases including cancer. In the present investigation we are focusing on induction of apoptosis and growth arrest in liver cancer cells (HepG2) through oxidative stress mediated mechanism by emodin, an ROS producer. Higher production of intracellular ROS (DCFH-DA dye method) and membrane LPO in emodin treated cancer cells, along with depletion of antioxidant components, suggests that ROS generation and oxidative stress might be the primary mechanism for toxicity of emodin to human liver cancer cells (HepG2) in dose dependent manner.

Emodin caused cell death were carried out by MTT assay and live/dead assay method. SubG1 arrest marker for apoptosis also was found in treated cells through the FACS analysis revealed that dose dependent increase in apoptotic population in cells treated with emodin in various concentrations. DNA damage in HepG2 cells was observed the emodin treated groups through COMET assay, Micronucleus assay and γH2AX foci detection confirmed the apoptotic efficacy of emodin. Western blot analysis indicated that the protein levels of cytochrome c and the ratio of Bax/Bcl-2 were increased in concentration dependent manner HepG2 cells treated with emodin. Oxidative stress and apoptotic efficacy of emodin were significantly blocked on pre-treatment with the ROS inhibitor N-acetylcysteine (10 mM NAC), showing the association of increased ROS in the pro-apoptotic activity of emodin. It is confirmed through phase contrast microscopic analysis, MTT assay and DCFH-DA dye
method (fluorimetry). These studies suggest emodin inhibits the growth of liver cancer cells in part, by inducing ROS production, oxidative DNA damage and apoptosis and has the potential to treatment option for liver cancer. Overall, our data demonstrated that emodin induce apoptosis in cancer cells, which is likely to be mediated by ROS mediated apoptosis and mitochondrial dysfunction which cause cytochrome c release finally lead to apoptosis by which most of the anticancer drugs trigger apoptosis. Emodin is therefore likely a promising chemotherapeutic agent against hepatoma in the future.
3.2. INTRODUCTION

Hepatocellular carcinoma (HCC) is a malignant tumor that arises from hepatocytes, a major cell type in the liver and it accounts for >90% of all primary liver cancers and a major cause of morbidity and mortality (El-Serag and Rudolph, 2007). HCC is the fifth most common human cancer, with approximately 750,000 new cases occurring worldwide each year (Ferlay et al., 2010) and it ranks third in annual global cancer mortality rates and has the shortest survival time of any cancer in both males and females. The HCC is distinguished as a highly chemoresistant cancer with several aetiological factors being classified as high-risk factors, including exposure to aflatoxin B1, alcohol abuse, infection with hepatitis B virus and hepatitis C virus (Kern et al., 2002; Llovet, 2003; Farazi and DePinho, 2006).

Surgical treatment is considered to be the best choice for HCC (Takayama et al., 2010), however tumor size, hepatic functional reserve and/or portal hypertension may all restrict surgical ablation (Kerr and Kerr, 2009). Chemotherapy is the most common treatment for advanced cancers (Cascinu et al., 1999). Compared with local treatments, such as radiation and surgery, chemotherapy is a systemic treatment that may reach cancer cells wherever they have spread (Ramalingam and Belani, 2008). Chemotherapy using conventional cytotoxic drugs, such as doxorubicin, cisplatin and fluorouracil is one of the commonly treatment options, especially for patients with unresectable tumors. Though, because of poor response rates, severe toxicities and high recurrence rates, the mean survival time is approximately 6 months (Llovet and Bruix, 2003). The exploration for new chemo-preventive and anti-tumor agents that are more effective and less toxic has magnetized enormous interest in phytochemicals. Emodin (1, 3, 8-trihydroxy-6-methylanthraquinone), a natural anthraquinone derivative isolated from root and rhizome of Rheum palmatum L (Liang et al., 1995) is one such compound.

Emodin has been demonstrated to display a number of biological activities such as antiviral, antimicrobial, immunosuppressive hepatoprotective, anti-inflammatory and antiulcerogenic (Lin et al., 1996). Emodin has been exhibited to regulate many gene expression associated with cell proliferation, cell apoptosis, oncogenesis, DNA repair and cancer cell invasion and metastasis (Cha et al., 2005; Huang et al., 2006; Kwak et al., 2006; Lu et al., 2009; Muto et al., 2007; Shieh et al., 2004). Recent studies confirm that emodin can induce apoptosis in several human cancer cells such as the human lung cancer
(Ko et al., 2010), leukemia (Chun-Guang et al., 2010), ovarian cancer (Li et al., 2009), colon cancer (Ma et al., 2012) cervical cancer, hepatoma and prostate cancer (Chen et al., 2002; Shieh et al., 2004). Emodin, as a tyrosine kinase inhibitor, can inhibit the kinase activity of HER-2/neu and suppress the proliferation of HER-2/neu–overexpressed non-small cell lung cancer (NSCLC) and breast cancer cells (Zhang et al., 1999; Zhang et al., 1995).

Results in HeLa cells demonstrated that the emodin-mediated generation of ROS inhibits the prosurvival transcription factors NF-kB, thus accounting for the cytotoxicity (Yi et al., 2006). The molecular mechanism by which emodin induces cell apoptosis is through inactivation of ERK and AKT and the decrease of anti-apoptotic protein Bcl-2 levels in human A549 cells (Su et al., 2005). Emodin has been proposed as a potential agent in the management of tumors. Also emodin is considered as a strong ROS-producing agent (Jing et al., 2006) and induction of DNA damage (Wang et al., 2006). Emodin-mediated oxidative injury triggers mitochondrial dysfunction, cytochrome c release and caspase activation, which leads to apoptosis in Lung cancer A549 cells (Su et al., 2005). The generation of ROS may contribute to mitochondrial damage, reduction of mitochondrial trans-membrane potential, release of cytochrome c and Smac, and subsequent caspase activation including apoptosis (Huang et al., 2008). Even under condition of a long-term diet of emodin, no evidence of carcinogenic activity in rats and mice was observed (NTP Toxicology and Carcinogenesis Studies of EMODIN (CAS NO. 518–82–1).

In the case of rats or mice given emodin during pregnancy period, neither prenatal mortality nor morphologic development was affected and genotoxicity of emodin would be distinct (Jahnke et al., 2004; Heidemann et al., 1996). It has been discovered that cytotoxicity of emodin on human fetal hepatic cell line L-2α and found that emodin lacks significant effect on non-cancer liver cells. These data suggest that normal cells are more resistant to emodin-induced cytotoxicity than cancer cells (Huang et al., 2008). Above observations made clear that emodin can serve as a best chemotherapeutic drug.

It is well recognized that both growth inhibition and apoptosis are interconnected in establishing the response of cancer cells to chemotherapeutic agent. A moderate increase in ROS can promote cell proliferation and differentiation (Boonstra and Post, 2004; Schafer and Buettner, 2001), whereas excessive amounts of ROS can cause oxidative damages to lipids,
proteins and DNA and lead to cell death (Szatrowski and Nathan, 1991; Kawanishi, 2006). Several chemotherapeutic drugs exert their cytotoxic effects through the generation of ROS (Alexandre et al., 2006). It is now clear that the generation of ROS can be exploited therapeutically in the treatment of cancer. Apoptosis, a form of programmed cell death, is a critical defense mechanism against the formation and progression of cancer and displays distinct morphological and biochemical traits (Lin et al., 2010).

Apoptosis can be divided into two pathways: the extrinsic (death receptor) and intrinsic (mitochondria) pathway (MacKenzie and Clark, 2008; Repicky et al., 2008). The extrinsic pathway involves Fas and tumor necrosis factor receptor 1 and an initiator caspase such as caspase-8 which drives its activation through self-cleavage and then activates downstream caspases such as caspase-9 and caspase-3 (Falschlehner et al., 2007). The intrinsic pathway involves the death signals to mitochondria, which lead to the release of several mitochondrial inter membrane space proteins such as cytochrome c, which then associate with Apaf-1 and procaspase-9 to form the apoptosome (Labeledzka et al., 2006). The mitochondrial pathway is controlled and regulated by Bcl-2 family proteins such as the antiapoptotic subfamily comprising Bcl-2 and Bcl-xl and the proapoptotic subfamily comprising Bax and Bak (Tsujimoto, 2003; Chipuk and Green, 2008).

Emodin mediated chemotherapeutic response to HCC cells remains obscure. Here we have trying to explore the possible mechanism that emodin could induce oxidative stress and apoptosis is demonstrated.
3.3. OBJECTIVE

1) To investigate growth inhibitory and apoptotic efficacy of emodin via ROS generation in human liver cancer cells.

2) To confirm the efficacy of emodin on ROS generation through supplementation of antioxidants in HCC cells.
3.4. MATERIALS AND METHODS

3.4.1. Materials

3.4.1.1. Chemicals

Emodin, 5,6-carboxy2,7-dichlorofluorescein diacetate (DCFH-DA), N-acetylcysteine (NAC), penicillin, streptomycin, DMEM media and fetal bovine serum purchased from Sigma Aldrich, Bangalore. Live/Dead assay kit purchased from Molecular Probes, Eugene, OR, USA and SYBR Green is purchased from Trevigen. The antibodies for cytochrome c, Bax, Bcl-2, β-actin and COX IV (Cytochrome c oxidase) were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Secondary antibodies conjugated with horseradish peroxidase (HRP) were bought from GE Healthcare (Piscataway, NJ, USA)

3.4.1.2. Cell culture

Human hepatocellular carcinoma cell line HepG-2 were obtained from NCCS Pune India, were grown to confluence in 25 cm² and 75 cm² flasks supplemented with Dulbecco’s modified eagle’s medium (DMEM) and 10% fetal bovine serum (FBS) (v/v), containing, 100 units/ml penicillin, 30 µg/ml streptomycin and 20 µg/ml gentamycin in a CO₂ incubator with 5% CO₂. Cells at 80% confluence were used for all the experiments. All experiments were carried out within 20 passages, between passage no. 25 to passage no. 45 to ensure uniformity of cell population and reproducibility.

3.4.1.3. Drug treatment

A 60 mM solution of emodin (from Aldrich), with purity of 99%, was prepared in DMSO, stored as small aliquots at -20°C and then diluted further in cell culture medium as needed. Final concentrations were between 5, 10, 25 50 and 60 µM respectively. The final concentration of DMSO was 0.1%. Cells were seeded and cultured for 24h and then replenished with medium containing drugs for stipulated time intervals and harvested for further analysis.
3.4.2. Methods

3.4.2.1. Cytotoxicity by MTT assay

Cell viability was assayed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, because the reduction of tetrazolium salts is widely accepted as a reliable method to examine cell viability/proliferation. Briefly, the cells (5×10^3/ml) were incubated in triplicate in a 96-well plate in the presence or absence of different concentrations of emodin (0, 5, 10, 25, 50 and 60 µM) in a final volume of 0.2 ml for 24, 48 and 72h intervals at 37°C. After the exposure times, the wells were treated with a final concentration of 0.5 mg/ml MTT and incubated at 37°C for 4h. The purple formazan crystals formed were dissolved in 0.2 ml DMSO and read at 570 nm in a microquant plate reader (Bio-Tek Instruments).

3.4.2.2. Biochemical evaluation for oxidative stress and antioxidant enzymes

Cells were cultured in a 75 cm² culture flask and exposed to emodin at the concentration of (0, 5, 10, 25 and 50 µM) for 24h. After the treatment, cell extracts were prepared by sonication in 50 mM Tris, 5 mM ethylene diamine tetraacetic acid (EDTA), 10 µg/ml phenyl methyl sulfonyl fluoride (PMSF), pH 7.6. The cell debris was removed by centrifugation at 4000 rpm for 5 min at 4°C and the protein content of the supernatant was determined by the Lowry method and used for antioxidant enzyme assays. Both reagent blanks and enzyme blanks were measured for all assays. All assays were carried out in the linear range and expressed as specific activities. Lipid peroxidation (LPO) was determined by Thiobarbituric acid (TBA) reaction with malondialdehyde (MDA) as per the method of Devasagayam et al. (1987). The activities of antioxidant enzymes SOD (Marklund et al., 1974), CAT (Aebi et al., 1984), GPx (Rotruck et al., 1973) and levels of total protein (Lowry et al., 1951) was measured with previously described standard methods.

3.4.2.3. Live/Dead Assay

Apoptosis of cells was also determined by Live/Dead assay (Molecular Probes, Eugene, OR, USA) that measures intracellular esterase activity and plasma membrane integrity. It is a two-color fluorescence assay that simultaneously determines: Live cell number—Live cells have intracellular esterases that convert nonfluorescent, cell-permeable...
calcein acetoxyethyl (calcein AM) to the intensely fluorescent calcein. Cleaved calcein is retained within cells. Dead cell number—Dead cells have damaged membranes; the ethidium homodimer-1 (EthD-1) enters damaged cells and is fluorescent when bound to nucleic acids. EthD-1 produces a bright red fluorescence in damaged or dead cells. Briefly, 1 X10^6 HepG2 cells were incubated with emodin in various concentrations (0, 5, 10, 25 and 50 µM for 24h) at 37°C. Cells were stained with the Live/Dead reagent (5 µM ethidium homodimer, 5 µM calcein-AM) and then incubated at 37°C for 30 min. Cells were analyzed under a fluorescence microscope (DP 70, Olympus, Tokyo, Japan) by counting live (green) and dead (red) cells in 20 random fields.

3.4.2.4. Propidium Iodide (PI) Staining for DNA Fragmentation

Following drug treatment, cells were harvested, washed in 1 x Phosphate Buffered Saline and centrifuged at 260g for 5 min, fixed in 70% cold ethanol : 1 x PBS and stored at 4°C. Prior to flow cytometric analysis, cells were washed twice in 1x PBS at 720g for 5 min. Cells were then stained using 500 l of Propidium Iodide (PI, Sigma): RNase A solution (2 mg PI, 2 mg RNaseA/100ml 0.1% BSA in 1x PBS) and incubated at 37°C for 30 min. Flow cytometric analysis (BD X Calibur Flow cytometer) was carried out at an excitation wavelength of 488 nm and emission wavelength of 610 nm and the area of sub-G1phase (apoptosis peak) is located as the gate to determine the damage of DNA. Usually the area before the G0/G1 phase is set for a gate that can be used to determine DNA damage while the apoptosis peak can represent the amount of apoptosis cell in all treated cells. Approximately 10,000 events were collected. Obtained data was analyzed with WINMDI software.

3.4.2.5. Cytokinesis Blocked Micronucleus (CBMN) Analysis

After treatment with emodin with indicated doses (0, 5, 10, 25 and 50 µM), cells were incubated in fresh medium with 4.0 µg/ml cytochalasin B (Sigma) for 22h. The protocol used is adapted from Hande et al. (1996). One thousand binucleated cells with/without the presence of micronuclei were scored under a fluorescence microscope (Olympus) with an appropriate triple band filter. The number of micronuclei in double nuclear cells was counted. At least 1000 double nuclear cells were calculated. Each dosage had three parallel exponents and the experiment was repeated thrice.
3.4.2.6. Alkaline single cell gel electrophoresis (Comet) assay

Harvested cells were resuspended in Hank’s balanced salt solution (Sigma) were mixed with 0.7% low melting point agarose, applied on Comet slides and subjected to lysis (2.5 M NaCl, 0.1 M pH 8 EDTA, 10 mM Tris base, 1% Triton X) at 42°C for 1h. The slides were loaded into a gel electrophoresis tank in 0.3 M NaOH, pH 13 with EDTA, allowed to denature for 40 min and run at constant 25 V/300 mA for 20 min. Samples were neutralized with 0.5 M Tris–HCl pH7.5 for 15 min, dehydrated in 70% ethanol and dried at 37°C. DNA was stained with SYBR Green. One hundred randomly selected cells were examined per sample using Komet Imager Software. Extent of DNA damage was expressed as a measure of comet tail moments, which corresponds to the fraction of DNA in the comet tail.

3.4.2.7. Measurement of intracellular ROS

Intracellular production of ROS was measured using 2,7-dichlorofluorescein diacetate (DCFH-DA). The DCFHDA passively enters the cell, where it reacts with ROS to form the highly fluorescent compound dichlorofluorescein (DCF). In brief, 10 mM DCFH-DA stock solution (in methanol) was diluted in culture medium without serum or another additive to yield a 100 µM working solution. HepG2 cells were treated with emodin with increasing concentration (0, 5, 10, 25 and 50 µM of emodin and 50 µM of H2O2) for 24h. At the end of exposure, cells were washed twice with HBSS and then incubated in 1 ml of working solution of DCFH-DA at 37°C for 30 min. Cells were lysed in alkaline solution and centrifuged at 2300 × g for 10 min. A 200 µl supernatant was transferred to a 96-well plate and fluorescence was measured at 485 nm excitation and 520 nm emission using a microplate reader (FLUO star Omega). The values were expressed as a percent of fluorescence intensity relative to control wells.

3.4.2.8. γH2AX foci detection

γH2AX foci detection is a sensitive assay indicative of double strand breaks sustained by the cells. For this experiment 10, 000 cells were seeded on 22 mm x 22 mm cover slips. The cells were fixed with 4% formaldehyde following treatment. The cells were then washed with PBS and subsequently permeabilised with 0.2% of Triton-X 100. Cells were blocked in 5% BSA. Cells were then incubated with mouse monoclonal...
γH2AX primary antibody at 37°C for 1h. Cells were washed in 1X PBS and incubated with goat anti mouse IgG conjugated with fluorescein isothiocyanate. The cover slips were then mounted on slides with DAPI in vecta shield. γH2AX foci were quantitated in 50 nuclei per sample under the fluorescence. Triplicates were performed.

3.4.2.9. Determination of Oxidative Damage

Oxidative damage was measured using DCFH-DA oxidation through flow cytometry and fluorescence microplate reader.

DCFH-DA was used as ROS capture in the cells. The average fluorescent intensity DCF stands for intracellular ROS levels. Cultured cells were exposed to the 25 µM emodin for 12h and with pre-incubation of 10 mM NAC for 4h followed by 10 µM of DCFH-DA at 37°C for 15 min, stained samples were fluorescence intensity was measured at 485 nm excitation and 520 nm emission using a microplate reader (FLUOstar Omega). 50 µM of H2O2 is used as positive control. The values were expressed as a percent of fluorescence intensity relative to control wells. Cellular ROS contents were also measured by preincubating cells at 4h with 10 mM NAC for emodin treated cells, followed by flow cytometry (BD X Calibur Flow cytometer) was carried out at an excitation wavelength of 488 nm and emission wavelength of 535 nm and the area of sub-G1phase (apoptosis peak) is located as the gate to determine the damage of DNA.

3.4.2.10. Evaluation of morphology by phase contrast microscopy

To further confirm the oxidative stress mediated cell damage in HCC cells, HepG2 cells with a density 1 × 10^5 cells/ml were plated in 6-well plates and the next day it was treated with emodin (25 µM) or without emodin and with pre-incubation of 10 mM NAC for 4h along with or without emodin (25 µM) for 12h. The cells were observed for the morphological characteristics under phase contrast inverted microscope. Morphological changes like rounding up of cells, apoptotic body formation and cell detachment were observed.

3.4.2.11. Western Blot Analysis:

1×10^5 cells exposed with various concentration emodin were lysed and protein concentrations were determined by BCA method. For protein determination of each sample, the cell lysates (40 µg of each) were separated by SDS–PAGE on a polyacrylamide
gel followed by electrotransfer onto a PVDF membrane (Immobilon-P; Millipore, Bedford, MA, USA). The blots were then incubated with primary antibodies (1:1000 dilutions in blocking buffer) overnight at 4°C. After being washed, secondary antibodies-conjugated with horseradish peroxidase (HRP) were applied at a dilution of 1:20,000 in blocking buffer for 1 h at room temperature. HRP-conjugated goat anti-rabbit or anti-mouse IgG was used as a secondary antibody for enhanced chemiluminescence (ECL Kit, Millipore, Billerica, MA, USA). The protein levels of cytosolic and mitochondrial cytochrome c were carried out according to the manufacturer’s protocol (Mitochondria/Cytosol Fractionation Kit, BioVision, Inc., Mountain View, CA, USA). Western blotting for examining the effects of emodin on the levels of Bax, Bcl-2, β-actin, cytochrome c and COX IV were performed for emodin-treated HepG2 cells.

3.4.2.12. Statistical analysis

All data are expressed as mean ± S.E.M of 3 independent experiments. Statistical significance was determined using unpaired Student’s t-test and the probability p value less than 0.001 were considered statistically significant.
3.5. RESULTS

3.5.1. Exposure of emodin resulted in cell morphological changes and loss of viability in HepG2 cells

Growth inhibition of HepG2 cells was assessed following 24, 48 and 72 h treatment with emodin by the MTT assay. As shown in Fig. 3.1 emodin inhibited the proliferation of HepG2 cells in a dose-dependent manner with various time courses. Treatment of emodin at 60 µM concentration inhibited the cell proliferation to 50% at 24 h time point. 50 µM of emodin significantly decreased almost 50% the viable cells at 48h and the 50% of the cell death were detected in the treatment of 25 µM emodin with 72h incubation (Fig. 3.1).

![Fig.3.1. Emodin induced loss of total cell viability in HepG2 human liver cancer cells.](image)

HepG2 cells (5×10³/ml) were plated in triplicate, treated with indicated concentrations of emodin and then subjected to MTT assay after 24, 48 and 72 h to analyzed proliferation of cells. Each bar represents mean ± SEM of 3 experiments. *P < 0.001 compared to control (One way ANOVA followed by Tukey’s multiple comparison tests).
3.5.2. Emodin altered the oxidant/antioxidant balance of human liver cancer cells in concentration dependent manner

It has been suggested that oxidant generation and antioxidant depletion are the common pathways through which anticancer drugs trigger apoptosis in cancer cells. In order to investigate whether the cytotoxic effect of emodin are related to the induction ROS as well as significant fall in antioxidant enzymes, HepG2 cells were treated with emodin with increasing concentration (0, 5, 10, 25 and 50 µM) and the activities of LPO and antioxidant enzymes including SOD, GPx and CAT were measured. MDA levels, the end product of LPO were used to reflect the level of oxidative damage in cells, were significantly (P<0.001) increased in dose dependent manner. As the evidence of dose dependent increase in lipid peroxidation, MDA content showed a significant (P<0.001) elevation of 61.55% in 10 µM, 94.96% in 25 µM and 155% in 50 µM of emodin treated groups when compared to untreated group in a dose dependent manner (Fig. 3.2a). However, 13.34% in 5 µM emodin treated group was not shown significant difference in MDA content when compared to that of control group.

Increased SOD, GPx and CAT enzymes are regarded as the first line of the antioxidant defense system against ROS generated during oxidative stress. Significant (P<0.001) dose dependent decrease in SOD, catalase and GPx activities were observed in the cells challenged with emodin at various concentration. A dose-dependent decrease was observed in the activity of GPx. 7% in 5 µM, 42.5% in 10 µM, 70.7% in 25 µM and 84.4% in 50 µM of emodin treated groups were observed (Fig. 3.2b). SOD activity was found to be decreased in dose dependant manner in emodin treated groups with various concentration of emodin when compare to the control cells (10.45% in 5 µM, 55.74% in 10 µM, 68.16% in 25 µM and 81.11% in 50 µM) (Fig. 3.2c). Emodin induced depletion in catalase activity 9.56%, 32.23%, 57.77%, 71.43% in 5 µM, 10 µM, 25 µM and 50 µM respectively (Fig. 3.2d). The results were found to be statistically significant (p<0.001) when compared to the control group.
Fig. 3.2. Emodin induced oxidant generation and diminishes anti-oxidant defence system in human liver cancer (Hepg2) cells in a dose dependent manner. Lipid peroxidation (a) Glutathione peroxidase (b) SOD (c) and Catalase (d) in the cells treated with various concentration of emodin for 24h (0, 5, 10, 25 and 50 µM of emodin). Each bar represents mean ± SEM of 3 experiments * P < 0.001 compared to control (One way ANOVA followed by Tukey’s multiple comparison test).
3.5.3. Emodin Induces ROS generation determined by DCF-DA fluorescent dye fluorimetry

ROS play critical roles in the regulation of diverse functional pathways involved in apoptosis, proliferation and survival in cancer cells. Therefore, we investigated whether generation of intracellular ROS is part of the mechanism by which emodin induces apoptosis through oxidative stress in HepG2 cells. The generation of ROS by emodin was assessed by using the fluorescent probe H$_2$O$_2$ DCFDA to detect H2. H$_2$DCFDA is cleaved intracellularly by non-specific esterase to non-fluorescent 2,7-dichlorofluorescin (DCFH), which leads to the fluorescence compound 2,7-dichlorofluorescein (DCF) upon oxidation by ROS. Treatment with 0, 5, 10, 25 and 50 µM of emodin significantly increased ROS (p<0.001) in a dose dependent manner (Fig. 3.3). The value of ROS was expressed as relative fluorescence compared to control value which was taken as 100%. The cells were also treated with 50 µM of H$_2$O$_2$ as a positive control, which showed a significant increase in relative fluorescence (p<0.001).

![Fig.3.3. Induction of ROS generation in HepG2 cell lines.](image)

**Fig.3.3. Induction of ROS generation in HepG2 cell lines.** Cells were treated with emodin in various concentration for 24h and H$_2$O$_2$ (50 µM) was used as positive control. These cell lines were then labelled with H2DCFH-DA and ROS production was quantified by fluorimetry. Each bar represents mean ± SEM of 3 experiments. * P < 0.001 compared to control (One way ANOVA followed by Tukey’s multiple comparison test).
3.5.4 Emodin enhances % of apoptotic population with increasing concentration

We also determined whether emodin can enhance apoptosis using esterase staining Live and Dead assay. We found that emodin induced apoptosis in HepG2 cells in concentration dependent manner. Control cells have very few numbers of dead cells about 2%. The percentage of dead cell population increased with increasing dose of emodin. 0-2%, 5-4%, 10-11%, 25-25%, 50-34% and 60-48% respectively.

![Fig.3.4. Emodin induced apoptotic cell death in HepG2 cells treated with increasing concentration](image)

*Fig.3.4. Emodin induced apoptotic cell death in HepG2 cells treated with increasing concentration: HepG2 cells were treated emodin (0, 5, 10, 25, 50 and 60µM for 24h) and the cytotoxicity was determined by the live/dead assay and 20 random fields were counted.*

3.5.5 Emodin induces subG1 arrest in dose dependent manner

The propidium iodide (PI) flow cytometric assay has been broadly used for the evaluation of apoptosis in different experimental models. It is based on the principle that apoptotic cells, among other typical features, are characterized by DNA fragmentation and subsequently, loss of nuclear DNA content. Use of a fluorochrome, such as PI, that is capable of binding and labeling DNA makes it possible to obtain a rapid and precise evaluation of cellular DNA content by flow cytometric analysis (Riccardi and Nicoletti, 2006). Apoptosis is also an important mechanism which is supported by presence of Sub G1 peak in flow cytometry results (Vousden, 2000). To look into the mechanism leading to the loss of cell proliferation by emodin, flow cytometric analysis of cell cycle was conducted to know if there is any cell cycle arrest taking place. We found that emodin can
cause significant increase (Fig. 3.5B) in accumulation of cell population in subG1 phase of cell cycle with increasing concentration of dose (0-2.01%, 5 µM-4.85%, 10 µM-9.72%, 25 µM-18.3% and 50 µM-28.46%), which would indicate the occurrence of apoptosis (Fig. 3.5A). To discover if cell cycle arrest were associated with increase in DNA damage, we looked at DNA damage markers: CBMN and Comet tails.

A.

B.

Fig. 3.5. Emodin changed the DNA content and induced apoptosis in HepG2 cells. (A). Cells were incubated with or without 0, 10, 20, 30, 40 or 50 µM of emodin for 24h and then were harvested for determination the sub-G1 phase (apoptosis); (B). Quantitative comparison of M1 population (%) induced by various concentration of emodin for 24h. Each bar represents mean ± SEM of 3 experiments. * P < 0.001 compared to control (One way ANOVA followed by Tukey’s multiple comparison test).
3.5.6. Emodin induces oxidative stress induced DNA damage in HepG2 cells treated with increasing concentration

We were next interested to investigate whether the emodin caused DNA damage HepG2 cells treated with various concentrations. The extent of DNA damage accumulated in HepG2 cells in response to Emodin induced oxidative stress was studied using DNA markers - CBMN and comet. Binucleated (BN) cells were scored for the presence and distribution of micronuclei (MN) (Fig. 3.6A). The frequency of MN and percent BN with MN increased significantly in a dose-dependent manner (10, 25 and 50 µM) in HepG2 cells (p < 0.001). But at the lower dose (5 µM) there is no significant increase was observed (Fig. 3.6B). Comet assay (also known as SCGE assay) is an uncomplicated and sensitive technique for the detection of oxidative stress induced DNA damage at the level of the individual cell. HepG2 exhibited a dose-dependent increase in tail moments (Fig. 3.6C) following the 24h treatment (p < 0.001) demonstrated that DNA damage had occurred in response to treatment with emodin. At lower dose (5 µM) there is no significant damage was occurred (Fig. 3.6D).

A.
B.

![Graph showing percentage of MN with Emodin concentration]

C.

![Images showing green fluorescent proteins under different conditions]
D.

**Fig. 3.6. Emodin induced DNA damage is oxidative stress mediated.** (A). Acridine orange stained cytokinesis blocked binucleated cells a. Binucleated cell with no micronuclei from a control sample, b. Binucleated cell with one micronuclei as indicated by the arrow and c. Binucleated cell with two micronuclei as indicated by the arrows; (B), a. Percentage of binucleated cells (BN) with micronuclei (MN) and (b). Percentage of MN in cytokinesis-blocked binucleate HepG2 cells following treatment with various concentration of emodin. Each bar represents mean ± SEM of 3 experiments. * $P < 0.001$ compared to control (One way ANOVA followed by Tukey’s multiple comparison test). (C). Sybr Green-stained comets of a typical comet from nuclei of control and cells treated with increasing concentration of emodin (a: 0 or control; b: 5 µM of emodin; c: 10 µM of emodin; d: 25 µM of emodin; e: 50 µM of emodin). (D). Comet tail moments following emodin treatment. Each bar represents mean ± SEM of 3 experiments. * $P < 0.001$ compared to control (One way ANOVA followed by Tukey’s multiple comparison test).

**3.5.7. Emodin induced DNA double strand break is mediated through oxidative stress with increasing concentration**

The oxidatively induced DNA damage associated with ROS typically are apurinic/apyrimidinic (abasic) DNA sites, oxidized purines and pyrimidines, single strand (SSBs) and double strand (DSB) DNA breaks (Altieri et al., 2008). $\gamma$H2AX foci are indicative of double strand breaks sustained by the cell. A significant increase in foci formation is observed between the untreated cells and 10, 25 and 50 µM of emodin ($p<0.001$) in the HepG2 cells.
Fig. 3.7. Emodin induces DNA double strand breaks. (A). DNA DSBs evaluation using phosphorylation of histone H2AX in HepG2 cells after treatment with emodin in various concentrations (a: 0 or control; b: 5 µM of emodin; c: 10 µM of emodin; d: 25 µM of emodin; e: 50 µM of emodin). Immunocytochemistry detecting phosphorylated histone H2AX foci (green spots). Nuclei were stained with DAPI (blue). (B). γH2AX foci detection experiment depicting average foci versus treatment with emodin in different dose. Each bar represents mean ± SEM of 3 experiments. * P < 0.001 compared to control (One way ANOVA followed by Tukey’s multiple comparison test).
3.5.8. Emodin induced apoptosis is mediated through oxidative stress mechanism in liver cancer cells

To confirm that the effect of emodin induced DNA damage in HepG2 cells as noted in MNA, COMET assay and DNA double strand break was due to oxidative stress, HepG2 cells were treated with 0, 5, 10, 25 and 50 µM for 12h. Cells were harvested from each treatment and then were lysed and total proteins from each sample were prepared for Western blotting analysis for apoptosis associated proteins expression. The results from Western blots are shown in Fig. 3.8A and B and indicated that the levels of Bax and cytosolic cytochrome c were increased and the level of Bcl-2 and mitochondrial cytochrome c was decreased, which led to cell apoptosis in emodin-treated HepG2 cells.

![Fig. 3.8A and B](image)

**Fig.3.8. Emodin affected the apoptosis-associated protein levels on HepG2 cells.** Cells were incubated with various concentrations of emodin (0, 5, 10, 25 and 50 µM) for 12 h and the cells were collected for Western blotting as described in Materials and methods. (A). The protein levels of Bcl-2 and Bax were shown and (B). The fractionate cytosolic (top) and mitochondrial (bottom) cellular cytochrome c protein were performed. β-actin and COX IV are used as an internal control, respectively.
3.5.9. Oxidative stress is involved in emodin-induced apoptosis

Emodin-mediated oxidative stress was studied by pre-treating the cells with water soluble thiol antioxidant 10 mM NAC to quench ROS. Morphological changes by treatment with emodin (25 µM) were also examined using phase-contrast microscopy, emodin treatment led to increase the cell morphological changes, including early effects like rounding up of cells, cell shrinkage, cells floated on the well and cell number were reduced compared to the control after 24 h exposure (Fig. 3.9A) while the addition of NAC almost completely eliminated the floating cells. These results suggest that emodin significantly inhibits the growth of HepG2 cells through ROS generation. Emodin induced cell death via oxidative stress was confirmed with cell proliferation assay (MTT). N-acetylcysteine (NAC, a ROS Quencher) decreased emodin-induced cell death when compared with emodin-treated cells alone (Fig. 3.9B) and this result is in agreement with Ma et al. (2011) in LS1034 human colon cancer cells treated with emodin and NAC.

HepG2 cells treated with 25 µM of emodin resulted in 28.8% apoptotic cell population in 12h treatment. Interestingly, pre-treatment with NAC (10 mM) inhibited the pro-oxidant effects of emodin to 3.83% from 28.8% by the FACS analysis (Fig. 3.9C). From the results it is understand that emodin induced ROS generation and that ROS could induce oxidative stress in HepG2 cells. The thiol NAC is a source of cysteine for the synthesis of the endogenous antioxidant glutathione (GSH), thus, NAC is postulated to protect cells by increasing intracellular GSH levels and scavenging ROS. NAC was able to completely abrogate emodin-induced apoptosis. In contrast, emodin induced cell death significantly increased in HepG2 cells with the addition of H2O2 (Fig.3.9D). These data suggest that emodin induced cell death might be triggered by oxidative stress. Thus, the protection afforded by antioxidants against the induction of cell death by emodin suggests that free radicals may be involved in this phenomenon. To examine this possibility, we measured the levels of intracellular free radicals before and after exposure to emodin using the cell permeate dye DCFH-DA. Measurements of cellular fluorescence revealed that emodin generated 5 fold increases of intracellular ROS in HepG2 cells. A non-cytotoxic dose of NAC completely suppressed emodin-induced
ROS generation (Fig.3.9D). Emodin substantially enhanced oxidative stress mediated apoptosis in HepG2 cells and pre-treatment of cells with NAC markedly reduced this emodin-induced enhancement. These results suggest that ROS is needed for the oxidative stress mediated apoptosis by emodin in HepG2 cells.

A.
B.

![Bar graph comparing % viability across different conditions.]

C.

![Histograms showing SubG1 distribution across different conditions.]

SubG1 - 2.60

SubG1 - 28.84

SubG1 - 2.05

SubG1 - 4.66
Fig. 3.9. Oxidative stress-mediated apoptotic cell execution after treatment with emodin. Emodin induces cell death in HepG2 cells. (A). Cells were treated with emodin for 12h and pre-treated with NAC (10 mM) for 4h and visualized by phase contrast microscopy (×100 magnification). Data is representative of at least 3 independent experiments. (B). Cells were treated with 10 mM NAC, emodin (25 µM) or emodin combined with NAC for 4h prior to analysis cell viability. (C). emodin-induced apoptosis is abrogated or inhibited by antioxidants. Shown is flow cytometric DNA analysis of HepG2 cells: histograms were generated from analysis of propidium iodide-stained cells pre-treated for 4h with antioxidant NAC and then for a further 12 h with 25 µM emodin. (D) The intracellular generation of ROS was measured using the oxidation-sensitive fluorescein DCFH-DA. Exponentially growing cells (2x10⁵/ml) were treated with or without 25 µM emodin, pre-treated with NAC (10 mM), treated with NAC along with 25 µM emodin and with 50 µM H₂O₂ for 1h. The cells were collected and loaded with DCFH-DA for 30 min at 37°C and then incubated for 4h with antioxidant before loading with DCFH-DA. The fluorescence intensity of the cell suspension was measured using a fluorescence spectrophotometer with excitation at 488 nm and emission at 525 nm. Each value represents the mean S.E. of duplicate assays in three independent experiments (*, p< 0.001 when emodin compared with control and Emodin + NAC compared with emodin treated cells), (a: Control; b: Emodin 25 µM; c: NAC; d: NAC+Emodin).
3.6. DISCUSSION

Current developments in cancer therapeutic research suggest that a number of apoptotic stimuli share common mechanistic pathways characterized by the generation of ROS through oxidative stress (Yip et al., 2011; Ryter et al., 2007). ROS played a very important role in apoptosis induction under both physiological and pathological conditions. Interestingly, mitochondria were both the source and target of ROS. ROS typically include the superoxide radical (O$_2^-$), hydrogen peroxide (H$_2$O$_2$) and hydroxyl radical (OH), which cause damage to cellular components including DNA, proteins and lipids (Ott et al., 2007). Exploiting the cancer cell killing potential of ROS could be performed by two means, namely, (1) inducing the generation of ROS directly in tumor cells and (2) inhibiting the antioxidative enzyme (defense) system of tumor cells. Many studies have shown that diverse chemotherapeutic agents can induce apoptosis in cancer cell lines by elevating oxidative stress (Trachootham et al., 2009). Several natural compounds such as gallic acid (Chen et al., 2009), β-phenylethyl isothiocyanate (Trachootham et al., 2009) and sulforaphane were also reported to induce apoptosis in cancer cell lines through ROS generation.

Previous treatments of diseases with herbs were experimental more than theoretical. Therefore, clarification of the mechanisms of action of components in herbs may be important in developing their applications. Emodin (1,3,8-trihydroxy-6-methyl-anthraquinone), a natural anthraquinone derivative isolated from Rheum palmatum L, has been reported to exhibit anti-cancer effect on several human cancers such as liver cancers and lung cancers. However, the molecular mechanisms of emodin-mediated tumor regression have not been fully defined. Emodin, a natural anthraquinone derivative, was selected because of its derived semiquinone structure which is likely to increase the generation of intracellular ROS. It is a type of natural anthraquinone with a molecular structure similar to that of DMNQ (Kamei et al., 1998). DMNQ is considered as a ROS generator because its property of quinone and derived semiquinone, like mitochondrial ubiquinone, allows it to transfer electrons (Kovacic et al., 2000). Emodin has a structure similar to those of DMNQ and ubiquinone. Quinones are well known redox active molecules capable of forming a redox cycle with their semiquinone radicals leading to the formation of ROS. Thus, it has been suggested that the quinoid structure of emodin could be activated to the semiquinone radical intermediate which in turn could react with oxygen to produce ROS and ROS-induced apoptosis (Chan et al., 1993).
In the present report, we found that emodin decreased the percentage of viable HepG2 cells and these effects are a concentration-dependent manner, which is in agreement with other reports showed that emodin induced cytotoxic effects in human prostate cancer LNCaP cells and LS1034 colon cancer cells (Yu et al., 2008; Ma et al., 2012).

A previous study demonstrated that treatment with emodin rapidly increases reactive oxygen species generation in vascular smooth muscle cells (Wang et al., 2007). Cai et al. (2008) provided evidence that the inhibition of RhoA activation and induction of apoptosis is associated with an increase in oxidative stress in emodin-treated gastric carcinoma cells. Emodin has been characterized as a strong ROS-producing agent that can generate superoxide radical anions, hydrogen peroxide and the hydroxyl radical (Rahimipour et al., 2001), which eventually cause DNA strand break and apoptosis.

In the present study, emodin significantly altered the oxidant/antioxidant status of human liver cancer cells. ROS generation and membrane LPO were significantly higher, whereas antioxidant enzymes SOD, CAT and GPx were significantly lower in emodin-treated cells. The activity of GPx can provide important clues about the consumption rate of GSH during detoxification of organic hydroperoxides by it. SOD is specialized to convert highly reactive $O^{2-}$ to less toxic H$_2$O$_2$. CAT enzyme reduces H$_2$O$_2$ to H$_2$O (Ahamed et al., 2010; Ahamed, 2011). Higher production of intracellular ROS and membrane LPO in emodin-treated cancer cells, along with depletion of antioxidant components, suggests that ROS generation and oxidative stress might be the primary mechanisms for toxicity of emodin to human liver cancer HepG2 cells. Results from flow cytometric assay also showed that emodin induced sub-G1 phase in cell cycle distribution of HepG2 cells, which is in agreement with report addressing anticancer effects in human prostate cancer cells and colon cancer cells in vitro and in vivo (Yu et al., 2008; Ma et al., 2012).

In agreement with sub-G1 population, the results from live and dead assay revealing that increasing dead cell population along with increasing concentration of emodin confirmed the apoptotic efficacy of emodin in dose dependent manner.

Oxidative stress in cell cultures may lead to chemical modification of proteins, lipid peroxidation and DNA lesions such as single-stranded breaks and double stranded breaks (Brawn and Fridovich, 1981; Sies and de Groot, 1992; Stadtman, 2006), while CBMN demonstrate the clastogenic effects of oxidative stress, SCGE reveals the overall
DNA damage that result in a comet tail. Presence of MN and COMET are indicative marker of DNA damage induced by emodin in a dose dependent manner. DNA double-strand break (DSB) is a type of DNA damage in which two complementary strands of the double helix of DNA are damaged simultaneously, in locations close to each other. DSB is the most dangerous type of DNA damage, because it is believed that a single unrepaired DSB is sufficient for the induction of cell death process (Jackson, 2002; Sonoda et al., 2006). γ-H2AX – A Novel Biomarker for DNA Double-strand Breaks. Obvious γ-H2AX foci formation was observed in cells challenged with emodin. The phosphorylation of H2AX and organization of γ-H2AX into nuclear foci indicated the existence of emodin-induced DSBs in dose dependent manner.

It is reported that apoptosis can be divided into mitochondrial dependent and -independent pathways. Furthermore, it was reported that if the loss of the outer mitochondrial membrane integrity and the release of cytochrome c from the mitochondria to the cytosol of cells after exposed stimulator then the cells are committed to apoptosis (Di Giovanni et al., 2001). In the present study, our results already showed that emodin altered the levels of cytochrome c release. In order to investigate the possible molecular signal pathway of apoptosis in HepG2 cells after exposure to emodin, western blotting analysis was used for examining the apoptotic protein levels, which indicated that anti-apoptotic Bcl-2 significantly decreased in dose dependent manner in HepG2 cells and pro-apoptotic Bax was increased in comparison to control, which led to the decrease the ratio of Bax/Bcl2 then caused mitochondrial dysfunction then induced apoptosis. It was reported that Bax/Bcl-2 ratio indicates whether and how a cell will respond to an apoptotic signal (Yang et al., 2010). Our results showed that the Bcl-2/Bax ratio therefore decreased with increasing emodin concentration. This decrease may contribute to the oxidative stress mediated apoptosis via the mitochondrial apoptosis pathway. This result is agreement with previous results in various cancer cells treated with emodin (Ma et al., 2012; Wang et al., 2011; Yu et al., 2008; Su et al., 2005, Zhang et al., 1999; Jing et al., 2006).

There is evidence which suggests that ROS may act as signalling molecules for the initiation and execution of the apoptotic cell death program (Thannickal et al., 2000). In this investigation, we studied the role of ROS formation in emodin-induced apoptosis of HepG2 cells. NAC (10 mM) was shown to inhibit emodin-induced cytotoxicity and
morphological changes in HepG2 cells treated with 25 µM of emodin. Oxidative stress is implicated in a number of cellular processes including apoptosis and many chemotherapeutic agents are known to induce their cytotoxic effects to tumor cells by an ROS mediated mechanism. Quinones are known to modulate oxidative stress as part of their mechanism of anticancer action and thus we examined ROS production by emodin, an anthraquinone. We report in that emodin (25 µM, 12h) induces apoptosis and pre-treatment of HepG2 cells with 20 mM NAC inhibited this effect. Measurements of cellular fluorescence revealed that emodin generated a 5-fold increase of intracellular ROS in HepG2 cells. A non-cytotoxic dose of NAC completely suppressed emodin-induced ROS generation.

3.7. CONCLUSION

In conclusion, we found that Emodin induced oxidative stress and apoptosis through generating ROS and apoptosis in HepG2 cells. Thus, we proposed that Emodin treatment results predominantly in ROS generation through mitochondrial cytochrome c release and apoptosis induction via altering Bel-2/Bax ratio, a mechanism drawing much current interest with respect to its therapeutic potential in HepG2 cells in the future. These findings may aid in the understanding of the mode of actions of the emodin and provide a theoretical basis for the therapeutic use of this compound in further investigations.
REFERENCES


