CHAPTER 4

In vitro anti-cancer studies

4.0.1. KEYWORDS: - Anti-cancer, hepatocellular, apoptosis, MAPK signaling


4.1. INTRODUCTION

Hepatocellular carcinoma (HCC), the most predominant primary liver cancer, accounts for nearly 80% cases of liver cancer (Boyle and Levin, 2008). Despite the decline in the incidence rate of most cancers, the incidence rate of liver cancer alone has risen by 2.7% in the recent years (Ryerson et al., 2016). Chief risk factors for HCC include cirrhosis, chronic hepatitis B virus (HBV) and hepatitis C virus (HCV) infection, alcoholism, continued aflatoxin exposure etc. (Stagos et al., 2012). Being the most commonly reported chemo-resistant solid cancer (EASL-EORTC, 2012), associated with substantial mortality (Zhu et al, 2016), efforts to obstruct HCC development at the initial phase are on the rise. Increasing evidence suggests the numerous benefits of using chemo preventive phytochemicals for the suppression of hepatocarcinogenesis (Stagos et al., 2012; Nishino, 2009).

Accumulating evidence advocates apoptotic mode of cell death to be a promising target for cancer therapy devoid of severe side effects (Ghobrial et al., 2005). Several findings have indicated the potential role of apoptotic markers such as tumor suppressor p53, caspases, PARP
(Poly ADP ribose polymerase) etc. in instigating various cellular responses which eventually initiates apoptosis (Li et al., 1997). For instance, the genetic profiling of human HCC’s identified alteration in the expression of tumor suppressor gene p53 in liver carcinogenesis (Farazi et al., 2006). Consequently, apoptosis dysregulation due to the underlying mutation in key signaling proteins is a chief cause for the malignant phenotype and multi-drug resistance of HCC toward chemotherapy (Carbajo-Pescador et al., 2013). Deregulated functioning of mitogen-activated protein kinase (MAPK) signaling pathway is also found to contribute to liver tumor development (Huang et al., 2010). Hence, drug discovery and development of compounds which modulate these signaling pathways could be valuable in HCC therapy.

4.2. OBJECTIVE

The chapter is focused on the antitumor effects of *Elytranthe parasitica* (L.) Danser (EP) bioactive fractions on the expression of apoptosis and MAPK (mitogen-activated protein kinase) markers deregulated in HCC.

4.3. MATERIAL AND METHODS

4.3.0.1 Chemicals and reagents

Ethanol was procured from Hayman Ltd, Essex, UK. Diethyl ether was purchased from Finar Limited, Ahmedabad, India. Crystal violet (# C3886), Dulbecco’s Modified Eagle’s medium (DMEM), Gentamycin and Sulforhodamine B (SRB) were purchased from Sigma Aldrich, St Louis, USA. PathScan® Apoptosis Multi-Target Sandwich ELISA kit (#7105) and PathScan® MAPK Multi-Target Sandwich ELISA kit (#7274) were obtained from Cell Signaling Technology Inc., Massachusetts, USA. Cellular Reactive Oxygen Species Detection Assay Kit (#ab113851) was procured from Abcam, Cambridge, UK. Doxorubicin was procured from Fresenius Kabi Oncology Ltd., Solon, Himachal Pradesh, India. Methanol was purchased from Merck Specialties Pvt Ltd, Mumbai, India. All reagents and chemicals used were of analytical grade.

4.3.0.2 Cell culture and conditions

Human hepatocellular epithelial carcinoma cell line (HepG2) was procured from National Centre for cell Sciences (NCCS), Pune, India and cultured in DMEM supplemented with 10% Fetal Bovine Serum (FBS) and 1% penicillin-streptomycin. The cells were maintained at 37°C under
humidified conditions (in 5% CO₂). Cell viability was ascertained using the trypan blue dye exclusion technique.

4.3.1. **In vitro anti-cancer evaluation of the most active fractions**

4.3.1.1 **Cytotoxicity assay**

Cytotoxicity of the EP column fractions obtained via bioactivity guided fractionation were determined by Sulforhodamine B (SRB) assay according to a standardized protocol (Vichai & Kirtikara, 2006). Briefly, exponentially growing cells were seeded into 96-well plates (5,000 cells/well in 100 µL of media) and incubated for 24 h. Prior to the experiment, the test compounds were prepared in dimethyl sulfoxide, DMSO and diluted with media serially to obtain appropriate concentrations. Cells were treated with the extracts and fractions at concentrations of 3.125, 6.25, 12.5, 25, 50, 100 and 200 µg/mL and incubated for 48 h. Cells in control group were treated with media containing 0.25% DMSO. All treatments were carried out in triplicate. After 48 h of incubation from treatment period, to each well 100 µL of 10% ice – cold TCA was added gently. The plate was subsequently incubated for 1 h at 4°C. Following this, the plate was sharply flicked, washed four times with 200 µL of deionized water and air dried. The plate was stained by adding 100 µL Sulforhodamine B (0.057% w/v in 1% v/v acetic acid) and kept for 30 min in the dark at room temperature. Subsequently, the unbound dye was rapidly removed by rinsing four times with 1% v/v acetic acid. The plate was air dried and excess water was removed by tapping on a tissue paper. 100 µL of 10 mM unbuffered Tris base was added to each well to solubilize the dye. The plate was shaken in a gyratory mixer with agitation for 10 minutes. Finally, absorbance was measured using microplate ELISA reader at 570 nm, subsequently percentage cell viability was estimated using the following formula:

\[
\text{Percentage cell viability} = 100 - \frac{[(A-B)/A] \times 100}{},
\]

where A = Absorbance of cells treated with 0.25 % DMSO medium, B = Absorbance of cells treated with EP extract/fractions. Doxorubicin was used as the standard drug. % cell viability was plotted against concentration and IC₅₀ values were determined using Prism 5.03 Demo Version (GraphPad Software Inc., California, Virginia, USA). Doxorubicin (DOX) was used as the standard drug. Percentage cell viability was plotted against concentration and IC₅₀ values were calculated using GraphPad Prism 5.03 (GraphPad Software Inc., California, USA).
4.3.1.2 Colony formation assay

Colony formation assay was used to observe the ability of the hepatocellular cells to undergo “unlimited division” after treatment with EP fractions (Franken et al., 2006). In brief, HepG2 cells were seeded in 12-well plates (200 cells/well in 3 mL media). Once the cells were attached, treatments were added. After treatment, the cells were placed for incubation for a time-period equivalent to six potential divisions. After approximately 48 hours, media was removed and fresh media was added. This was continued for 10-12 days, after which the cells were fixed and stained using crystal violet (0.5% w/v in ethanol). Cells in the control group and the standard group were treated with media and doxorubicin respectively. All treatments were carried out in triplicate. Cell colonies containing more than 50 cells were counted. Plating efficiency (PF) and surviving fraction (SF) were determined as follows:

\[
\text{Plating Efficiency} = \frac{\text{Number of colonies formed}}{\text{Number of cells seeded}} \times 100
\]

\[
\text{Surviving fraction} = \frac{\text{Number of colonies formed}}{\text{Number of cells seeded}} \times \text{PE}
\]

4.3.1.3 Migration assay

Cell migration was measured in vitro using scratch wound assay in accordance with the standard protocol with slight modification (Liang et al., 2007). HepG2 cells (5 x 10^5 cell/well) were seeded in six well plates and cultured in DMEM growth media supplemented with 10% Fetal Bovine Serum. Once monolayer formation occurred, the cells were serum starved for 16-18 hours before treatment to prevent cell proliferation. A “scratch wound” was created on the cell monolayer using a 200 μL pipette tip. Two such parallel scratches were made per well. The wells were washed with growth media (1 mL) to remove debris and to even the edges of the wound. The wounded edges were photographed right away. This was considered as the zero-hour image and the wounds were considered as 100% open. Subsequently, the cells were incubated with media comprising treatment. At selected time points of 24, 48 and 72 hours, the wounded edges were photographed at 4x magnification. Wound size was measured using ImageJ 1.43u software (National Institutes of Health, Maryland, USA). Results were expressed as mean % migration ± SD. The percentage migration (i.e. the distance the cells migrated into the scratch wound area over time) was assessed as follows:
Percentage migration = \[
[(\text{Difference in Wound area at 0 h and n}\text{-th h})/\text{Wound area at 0 h}] \times 100
\]

Where nth h refers to the selected time points (i.e. 24, 48 and 72 h)

### 4.3.1.4 Detection of Reactive Oxygen Species

Cellular Reactive Oxygen Species Detection Assay Kit (#ab113851) was used to estimate ROS generation according to the kit manufacturer’s ELISA protocol. Briefly, adherent HepG2 cells were grown in DMEM media. Harvested cells were seeded in a dark, clear bottom 96 well microplate at a density of \(2.5 \times 10^4\) well and allowed to adhere overnight. After 24 h, the media was removed and treated with test compounds (100 µL/well). The plates were incubated for 3 h (since maximum ROS formation was observed at this specific time point). The cells were stained by adding 100 µL/well of the 25 µM DCFDA solution. The plate was incubated at 37˚C in the dark for 45 minutes. The DCFDA solution was discarded and 100 µL/well of 1 X buffer was added. Optical Density was recorded at an excitation WL at 485 nm and emission WL at 535 nm. Untreated wells containing media alone was used as blank or control wells. 50 µM Tert-butyl hydrogen peroxide (TBHP) treated wells were employed as the positive control. Doxorubicin (0.5 µM) was used as the standard drug.

### 4.3.1.5 Detection of apoptosis (Annexin V/PI staining)

Exponentially growing HepG2 cells (1 \(\times 10^6\)) were treated with EP fractions for 48 h. Following this, the cells were trypsinized, washed with PBS (Phosphate Buffer Saline) and centrifuged. The cells were subsequently stained with Annexin V/PI (Propidium iodide) solution and incubated for 20 min in the dark. The cells were then analyzed by flow-cytometry (Muse Cell Analyzer) for detection of live, dead, early and late apoptotic cells (Liang et al., 2016).

### 4.3.1.6 Regulation of apoptosis related proteins

PathScan® Apoptosis Multi-Target Sandwich ELISA kit (#7105) was used to investigate change in the expression of apoptosis related proteins phosphorylated p53, total p53, caspase-3, PARP, phosphorylated Bad, total Bad following treatment with EP active fractions in accordance with the manufacturer’s standardized procedure. In brief, HepG2 cells (1 \(\times 10^6\)) were seeded in sterile tissue culture flasks, incubated and treated with EP fractions. After 48 h, cells were scraped and
added cell lysis buffer with protease inhibitor. Cell lysates were sonicated and centrifuged for 10 min at 4°C. Supernatant obtained was screened for alteration in expression of apoptotic markers.

4.3.1.7 Expression of mitogen activated protein kinases (MAPK) proteins
As per the standard protocol, PathScan ® MAPK Multi-Target Sandwich ELISA kit (#7274, Cell Signaling Technology Inc., Danvers, Massachusetts) was used to study changes in the expression of MAPK related proteins phospho-p44/42 MAPK, phosphorylated p38α MAPK, MEK and phosphorylated MEK, SAPK/JNK and phosphorylated SAPK/JNK following treatment with EP active fractions. Briefly, 1 x 10⁶ HepG2 cells were seeded in sterile tissue culture flasks, incubated and treated with EP fractions. After 48 h, cells were scraped and added cell lysis buffer with protease inhibitor. Cell lysates were sonicated and centrifuged for 10 min at 4 ºC. Supernatant obtained was screened for alteration in MAPK protein expression.

4.3.2. Statistical Analysis
Results are represented as Mean ± Standard Deviation (SD). IC50 was estimated using GraphPad Prism 5.0 software package (GraphPad Software, Inc., California, USA). Level of statistical significance was determined by one-way ANOVA, followed by Tukey’s post hoc test between different groups and set at p < 0.05 and p < 0.001.

4.4. RESULT AND DISCUSSION
4.5.1. EP suppresses the growth and proliferation of HepG2 cells
Bioactivity guided separation of the plant extract afforded bioactive fractions EP.DEE and FR 3A. Anti-proliferative effects of the bioactive fractions on the growth and proliferation ability of HepG2 hepatocellular carcinoma cells was assessed by SRB assay, which primarily estimated the total cellular protein content of the cell. Doxorubicin, a commonly used chemotherapeutic drug against liver cancer was employed as the standard. Of the EP fractions obtained via bioactivity guided fractionation, considerable decrease in HepG2 cell viability was observed following treatment with bioactive fractions EP.DEE (IC50:56.7 µg/mL ± 7.8) and EP fraction FR 3A (IC50:12.5 µg/mL ± 0.60). Additionally, dose dependent decrease in cell viability was observed following treatment with EP.DEE and FR 3A fractions. Standard drug doxorubicin exhibited strong inhibitory effects on the growth of HepG2 cells (IC50: 0.5 µM ± 0.20), which
was in accordance with earlier studies (Choi et al., 2008; Capone et al., 2014; Qiu et al., 2015). Therefore, the most bioactive fractions FR 3A and EP.DEE, obtained via bioactivity guided fractionation were selected for further studies.

4.5.2. **EP bioactive fractions obstructs the formation of HepG2 hepatocellular colonies**

Reproductively active cell forms large colonies and is known to be clonogenic. Treatment with anti-cancer agents considerably reduces the reproducible integrity and hence the clonogenic capacity of cancer cells (Blumenthal, 2005). *Per se*, clonogenic survival assay is considered the "gold standard" as it detects all cells that have undergone cell death following treatment with anticancer agent due to DNA or chromosomal damage caused by apoptosis or necrosis (Brown & Attardi, 2005). Colony formation assay was performed to assess the effect of EP bioactive fractions on the clonogenic capacity of HepG2 cells. After 12 days of incubation, the number of colonies that arose in the control group was observed to be considerably high (69.3 ± 0.22). Following treatment with EP bioactive fractions for 12 days, the ability of HepG2 hepatocellular cells to undergo "unlimited" cellular division by forming colonies was significantly reduced (p > 0.001), when compared to the control. Percentage plating efficiency and surviving fraction were assessed for ascertaining the clonogenic capacity of the cell. Plating efficiency refers to the percentage of cells that form colonies whereas surviving fraction is the percentage of cells that have retained their capacity to divide and form colonies after treatment (Munshi et al., 2005). Relatively high percentage plating efficiency was detected in the control group (83.8 ± 1.75). In contrast, EP.DEE and FR 3A treated groups exhibited % plating efficiency of 26.2 ± 0.75 and 12.0 ± 0.50 respectively (Figure 4.1). Our findings indicate the EP bioactive fractions exert long term anti-proliferative effects on HepG2 cells and comprises of phytochemicals which have strongly obstructed the ability of the hepatocellular cells to form colonies and metastasize.
4.5.3. EP bioactive fraction hinders cell migration \textit{in vitro}

The major cause of cancer lethality is metastasis; over 90% of cancer fatality is attributed to metastatic dissemination. Cellular metastasis and invasion (a major hallmark of cancer cells) occurs if the cells have gained the ability to migrate (Kramer et al., 2009); novel anticancer agents which could obstruct cellular metastasis and invasion are highly desirable. Cellular migration was examined using \textit{in vitro} anti-metastatic scratch wound assay, which considerably recapitulates cell migration that occurs \textit{in vivo} (Franken et al., 2006). In dense monolayers of HepG2 cells, scratch wounds were made to produce a cell free area and cell migration was documented with the treatments groups, EP.DEE and FR 3A at chosen time points of 0, 24, 48 and 72 h alongside normal control and doxorubicin. With increasing time, more cells were observed to migrate into the scratched areas. At 24, 48 and 72 h, percentage migration of HepG2 cells in the control group was considerably high when compared to the treated groups. At 72 hours, the wound gap in the control group closed in whereas the wound gap in the treated groups was still intact (Figure 4.2). In metastatic scratch wound assay, the migrating cells on the wounded edge directionally polarize and migrate towards the center of the scratch (Cory, 2011). Hence, percentage migration was documented at different time points and it was observed that EP bioactive fractions considerably decreased the invasion and migration of HepG2 cells.

\textbf{Figure 4.1.} EP bioactive fractions reduces HepG2 colonization in colony formation assay. HepG2 cell (200 cells/well) were treated with test compounds for 12 days, stained with crystal violet (0.5% w/v in ethanol) and observed colony formation. Results are represented as mean ± SD (n=3). **p < 0.001 vs. control. All treatments were performed in triplicate.
4.5.4. EP bioactive fractions increases ROS levels

The effect of bioactive fraction on the generation of ROS radicals in HepG2 cells was evaluated using DCFDA assay. A slight increase in ROS levels were observed at 0.5 h following treatment, in comparison to the control group. However, mean fluorescence intensity was observed to be

**Figure 4.2.** EP bioactive fractions impeded the migration of HepG2 migration. Monolayer of HepG2 cells were scratched and subsequently micrographed (0 h reading). Treatments were added, and wound images were photographed at chosen timepoints. Wound size was calculated, and percent migration was determined. (a) Representative scratch wound images exemplifying the effect of EP enriched fractions on HepG2 cell migration over a time period of 24, 48 and 72 h. (b) Percentage migration of EP enriched fractions (EP.DEE, FR 3A), doxorubicin, control group at set time points. Results are expressed as mean ± SD.
highest at 3 h, there was a gradual decline in the fluorescence intensity at 6 h. Dose dependent increase in ROS generation was observed in HepG2 cells treated with EP bioactive fractions EP.DEE and FR3A when comparative to the negative control group (Figure 4.3). Significant increase in fluorescence was detected in Doxorubicin treated group.

![Graph](image)

**Figure 4.3.** Effect of EP bioactive fraction on intracellular ROS generation (DCFDA assay). Intracellular ROS production was estimated following treatment with test compounds at 3 h. Results are expressed as means ± SD (n=3). *p < 0.05, compared to control group.

Maintenance of optimum levels of ROS is vital for normal growth survival. Accumulating evidence from numerous studies have detected highly elevated levels of ROS in cancer cells (Szatrowski and Nathan, 1991). Increasing evidence suggests ROS plays a chief role in determining cell fate by regulating various mechanisms including activations of apoptosis and MAPKs (mitogen-activated protein kinases). Crucially, ROS are instrumental in triggering the release of pro-apoptotic proteins in the process of mitochondrial mediated apoptosis (Wang, 2001). DCFH-DA assay employs the fluorogenic cell permeating dye 2’,7’–dichlorofluorescein diacetate (DCFDA), which gives an accurate estimate of the free radical activity (e.g. hydroxyl, peroxyl) within the cell. After diffusion into the cell, DCFDA is initially deacetylated by esterases and subsequently oxidized by ROS into 2’, 7’–dichlorofluorescin (DCF). Being a highly fluorescent compound, DCF levels can be detected by fluorescent spectroscopy. Therefore, high fluorescence intensity would indicate elevated ROS levels and vice versa. In the current study, intracellular ROS generation increased considerably in HepG2 cells treated with EP bioactive fractions.
4.5.5. **EP bioactive fractions induce apoptosis**

Apoptotic cells demonstrate an “eat-me” signal by exposing phosphatidylserine (PS) on the outer leaflet of the cell plasma membrane, which causes these cells to be engulfed by phagocytes (Segawa K, 2015). Following treatment with the bioactive fractions, the number of cells in the apoptotic phase significantly in comparison to the control group (p < 0.05). Apoptotic induction in normal cells was 23.25%. Treatment with EP.DEE and FR 3A fractions increased the apoptotic induction in the late stage of apoptosis to 63.95% and 65.25% respectively. This was comparable with standard doxorubicin, which had 67.30% cells in the late apoptotic stage. Figure 4.4 illustrates the Annexin V/PI detection of apoptosis in HepG2 cells following treatment with EP fractions and doxorubicin. A combination of Annexin V (a cellular protein with high affinity for PS) and Propidium iodide (PI), (a vital dye that exclusively permeates damaged cells) helps differentiate between viable, early apoptotic, late apoptotic and dead cells. In early apoptosis, Annexin V binds with PS and detects cells which are in the early apoptotic phase. In the late phase of apoptosis, when cellular membrane integrity is compromised, both Annexin V and PI permeates into the cells, and therefore detects late apoptotic and dead cells. Following treatment with bioactive fractions for 48 hours, apoptosis was triggered in HepG2 cells, evidenced by the increased percentage of cell in the apoptotic phases.
4.5.6. EP bioactive fraction induces apoptosis by modulating the expression of apoptosis related proteins

Accruing evidence from comprehensive studies on cancer samples (in mouse models, in vitro cultured cells, human tissues) indicate that evasion of programmed cell death (apoptosis) is a hallmark found in virtually all cancer cells (Hanahan and Weinberg, 2000). Therefore, using the sandwich ELISA method, the effect of bioactive EP fractions on the expression of chief

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**Figure 4.4.** EP bioactive fractions triggers apoptosis in HepG2 cells. Exponentially growing HepG2 cells were treated with test compounds (A) Control (B) EP.DEE fraction (C) FR 3A fraction (D) DOX for 48 h, trypsinized, centrifuged and stained with Annexin V/PI. Apoptosis profile was estimated as % live, early apoptotic, late apoptotic and dead cells by flow cytometry.
apoptotic protein markers p53, phosphorylated p53, cleaved PARP, cleaved caspase-3, bad and phosphorylated bad was assessed.

p53, also known as “the guardian of the genome” plays a vital role in regulating cell cycle events and is the most significant tumor suppressor (Lane & Levine, 2010). Cellular stress stimuli such as DNA damage, hypoxia, oxidative stress and activated oncogenes triggers p53 activation via a cascade of phosphorylation events. Subsequently, p53 modulates the expression of numerous p53 responsive genes, involved in DNA repair, cell cycle arrest and apoptosis, if the DNA damage is beyond repair (Vousden & Prives, 2009). Additionally, p53 impedes the formation of new blood vessels or angiogenesis, which drastically suppresses tumor growth (Teodoro, 2007). In 50% of cancers, including hepatocellular carcinoma, mutant allele of TP53 gene is found (Leroy et al., 2014). Mutant p53 proteins known as “Gain of function” drive cancer associated functions such as hyperproliferation, invasion, migration, drug resistance, neo-angiogenesis (Muller et al., 2014). EP bioactive fractions considerably upregulated (p<0.001) the expression of p53, revealing its significant effect in retarding the growth of HepG2 cells and subsequently obstructing HCC development. Phosphorylation of p53 at Ser15 position triggers the activation of p53 and sets off a cascade of further phosphorylation events (Saito et al., 2002), ultimately ensuing in the uncoupling of p53 from with its negative regulator, MDM2, thereby stabilizing its activity. In the present study, significant upregulation (p<0.001) of phosphorylated p53 expression was observed in EP bioactive fraction treated HepG2 cells when compared to negative control group cells.

Poly ADP-Ribose (PAR) Polymerase or PARP, an enzyme located in the cell nucleus plays a significant role in repairing damaged DNA (Herceg and Wang, 1999). Structurally, it consists of three domains: an amino terminal domain through which it binds to DNA, a central regulating domain and a carboxy terminal domain via which it binds to NAD (Nucleotide Adenine Dinucleotide). Poly ADP-Ribosylation or PARylation occurs when PARP employs NAD to covalently attach ADP ribose units to glutamine residues of an extensive array of proteins. Coincidentally, PARylation occurs 500-fold more in the vicinity of DNA strand breaks (Jankevicius et a., 2013). Being a DNA nick sensor, PARP repairs single stranded breaks in DNA by PARylation. PARP binds to the DNA, synthesizes PARP chains across a sequence of substrate proteins and recruit DNA repair effectors such as XRCC1 (X-ray complementing cross gene) to the site of DNA damage and orchestrates their activity (Satoh and Lindahl, 1992).
Hence, in the non-cleaved form, PARP helps in the repair of damaged DNA and thus hinders the cells from undergoing apoptosis (Patel et al., 1996; Wang et al., 1997). However, cellular stress signals cause the cleavage of the 113 kDa PARP into two polypeptide fractions of 89 and 24kDa by various caspases (notably caspase 3) at Asp214 and Gly215 (Nicholson et al., 1995; Tiwari et al., 1995), thus initiating apoptosis. In the present study, EP bioactive fraction and doxorubicin treated cells upregulated cleaved PARP expression, strongly suggesting the initiation of apoptosis in these groups. Cleaved PARP (Asp214) expression was increased in EP bioactive fraction EP.DEE and doxorubicin group. Relatively less increase in cleaved PARP expression was noted in FR 3A fraction.

Cysteiny1 aspartic acid specific proteases or caspases, are enzymes which cleave proteins at their aspartic acid residues. The two major apoptotic pathways, extrinsic and intrinsic pathway culminate at the execution pathway, whose initiation occurs through the activation of executioner caspases. There are primarily 3 executioner caspases, caspase-3, 6, and 7 (Sakahira et al., 1998). Of these, caspase 3, the most significant executioner caspase cleaves numerous substrates such as PARP, gelsolin, ICAD (Slee et al., 2001), eventually causing morphological and biochemical changes leading to apoptosis. In the present study, EP bioactive fractions increased cleaved caspase 3 (Asp175) expression, although not significantly.

Bad or Bcl-2 associated death promoter is a pro-apoptotic protein belonging to the Bcl-2 (B cell lymphoma) family of proteins, which hetero-dimerizes the anti-apoptotic Bcl-2 family members, Bcl-XI and Bcl-2 and thus prevents their anti-apoptotic effects (Yang et al., 1995). In their free state, Bcl-XI and Bcl-2 protects the cell from undergoing apoptosis by inhibiting the release of cytochrome c from mitochondria and regulating caspase activation (Newmeyer et al., 2000). In its phosphorylated form, bad is unable to release cytochrome c from mitochondria due to entrapment by 14-3-3 phosphoserine binding protein in the cytosol (Zha et al., 1996). In the present study, EP bioactive fraction treated HepG2 cells upregulated the expression of bad. However, phosphorylated bad expression remained unchanged in both doxorubicin and the bioactive EP fractions. Figure 4.5 illustrates the effect of EP bioactive fractions on apoptotic protein markers.
Figure 4.5. EP Bioactive fractions modulate the expression of apoptosis marker proteins. HepG2 cells were incubated and treated with test compounds for 48 h, subsequently cells were scraped, and cell lysates were prepared. Following centrifugation, supernatant was tested for the expression of apoptotic markers using PathScan ® Apoptosis Multi-Target Sandwich ELISA kit. Treatments include EP bioactive fractions EP.DEE, FR 3A, DOX and control group. Values are represented as mean ± SD (n=3), * p< 0.05 vs. control, **p<0.001 vs. control
4.5.7. EP bioactive fraction modulates the expression of MAPK proteins

Recent studies have implicated MAPK (Mitogen Activated Protein Kinase) signaling pathway in liver tumor development (Liu et al., 2011). *Per se*, our study focused on the alteration in the expression levels of MAP Kinase proteins: phosphorylated p44/42 MAPK (or ERK1/2), MEK, phosphorylated MEK, SAPK/JNK, phosphorylated SAPK/JNK and p38α MAPK following treatment with EP bioactive fractions was assessed using sandwich ELISA method.

Being a survival pathway, signals from cell surface receptors are transmitted via cascade events, culminating in effects such as cell growth, proliferation, migration, apoptosis and differentiation. Extracellular-signal Regulated Kinase (ERK) is one of the protein serine threonine kinase belonging to the MAPK family. Very often, ERK is overexpressed in numerous cancers and is therefore a promising target for cancer treatment (Khavari et al., 2007). Accumulating evidence implicates the higher expression of ERK 1/2 in human HCC cell lines than their counterpart normal cell lines (Hoshino et al., 1999). Recent studies in animal models have shown the involvement of ERK 1/2 signaling in rat hepatoma cell proliferation (Schmidt et al., 1999). Schmitz *et al* have reported significant negative correlation between phosphorylated ERK1/2 expression and overall survival (Schmitz et al., 2008). In the present study, EP bioactive fractions decreased the expression of phosphorylated ERK 1/2 significantly in comparison to the negative control. In contrast, doxorubicin treated HepG2 cells significantly upregulated (p < 0.0001) the expression of phosphorylated ERK in comparison to the control group. Choi *et al* reported doxorubicin-induced apoptosis in HepG2 cell lines via upregulation of phosphorylated ERK at 24 hours (Choi et al., 2008). Similar observations have been reported in other studies as well (Chen et al., 2003; Choi et al., 2004). Activation of ERK 1/2 occurs through the phosphorylation of threonine and tyrosine residues by MEK 1/2, proteins upstream to ERK in the Ras-Raf-MEK-ERK pathway (Mehdizadeh et al., 2016). In the present study, expression of total MEK and phosphorylated MEK was decreased in EP bioactive fractions when compared to untreated cells. Likewise, doxorubicin treated cells downregulated MEK and phosphorylated MEK in comparison to control.

p38α MAPK, a potent inhibitor of cell proliferation suppresses liver tumor cell proliferation by antagonizing JNK pathway (Hui et al., 2007). However, EP bioactive fraction did not alter the expression of p38α MAPK in comparison to control cells. Doxorubicin treated cells mildly upregulated p38α MAPK expression in comparison to control group.
JNK (c-jun N-terminal kinase) or SAPK (Stress Activated Protein Kinase) play a crucial role in determining the fate of the cell i.e. deciding whether the cell dies or survives (Singh et al., 2007). Several studies suggest JNK to exert pro-tumorigenic role in liver carcinogenesis. For instance, JNK1 promotes cellular proliferation in diethylnitrosamine (DEN)-induced liver carcinogenesis (Hui et al., 2008). In many human HCC samples, augmented activation of JNK and c-jun was reported (Chang et al., 2009). In the current study, there was slight downregulation in the expression of total JNK and phosphorylated JNK in EP bioactive fraction EP.DEE treated HepG2 cells when compared to untreated HepG2 cells. Significant downregulation was observed in FR 3A and Doxorubicin treated cells. Figure 4.6 depicts the effect of EP bioactive fractions on MAPK signaling protein markers.

**Figure 4.6.** EP bioactive fractions modulate the expression of MAPK proteins. HepG2 cells were incubated and treated with test compounds for 48 h, subsequently cells were scraped and cell lysates were prepared. Following centrifugation, supernatant was tested using PathScan® MAPK Multi-Target Sandwich ELISA kit ELISA kit. Treatments include EP bioactive fractions EP.DEE, FR 3A, Doxorubicin and control group. Results are expressed as mean ± SD (n=3), * p< 0.05 vs. control, **p<0.001 vs. control.
4.5. SUMMARY AND CONCLUSION

This chapter highlights the effect of EP bioactive fractions on the expression of apoptosis and mitogen-activated protein kinase (MAPK) markers deregulated in HCC. EP bioactive fractions showed significant anti-tumor potential, reduced clonogenicity, considerably inhibited cell migration and induced ROS generation in HepG2 cells in vitro. The fractions augmented annexin V binding and induced apoptosis via cell cycle arrest at G2/M and S phase checkpoints. The fractions increased expression levels of p53, bad and increased PARP (Poly ADP ribose polymerase) and Caspase-3 cleavage. Additionally, these effects were associated with significant downregulation of phosphorylated ERK. Our findings present insight into induction of HepG2 cell cycle arrest and apoptosis by EP fractions. Anti-tumor efficacy of the fractions can be evaluated in vivo and further mechanistic studies could be undertaken to comprehend the precise mechanism by which EP active fractions obstructs the development of HCC.

4.6. REFERENCE


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**CHAPTER 4- IN VITRO ANTI-CANCER STUDIES**


