Chapter 7  Mechanistic evaluation of selected sub-fractions/compounds

7.1 Introduction

Cancer treatment involves surgery, radiation and drug therapy, either in single or in combination and provides temporary relief from symptoms, prolongation of life and occasionally, cures. A successful anticancer drug should be able to selectively kill or debilitate cancer cells without causing excessive harm to the normal cells.

7.1.1. Apoptosis

Apoptosis is the preferred mode of action for anticancer compounds. The life span of the cells, normal and cancer, is highly affected by the rate of apoptosis. Thus, modulation of apoptosis might be extremely useful in the management and therapy or cancer chemoprevention. There is an unmet need for new compounds or formulations for potential chemotherapeutic agents. It has become evident based on recent studies that apoptosis is an important mode of action for many anti-cancer agents, including ionizing radiation, alkylating agents such as cisplatin, topoisomerase inhibitor etoposide, cytokine tumour necrosis factor (TNF), taxol, and N-substituted benzamides such as metoclopramide. An in-depth understanding of the modes of action of these compounds should provide essential information for their use in cancer prevention and in cancer therapy (Taraphdar et al., 2001).

Apoptosis or programmed cell death is characterized by cell shrinkage, plasma membrane blebbing, maintenance of organelle integrity, condensation and fragmentation of DNA, followed by removal by phagocytes causing minimal damage to the surrounding tissues. Apoptosis is classified into two types of death pathways: extrinsic pathway and the intrinsic mitochondria-mediated pathway. These processes are not exclusive and they can be linked; molecules in one pathway can influence the other.

7.1.2 Cell cycle checkpoint inhibitors

Cell cycle checkpoints are one of the most sought after mechanisms for the development of anticancer agents. Cell cycle checkpoints are commonly defective in cancers. One of the hallmarks of cancer is uncontrolled proliferation, a consequence of loss of normal cell cycle control that underlies tumor progression. High proliferative index of cancers compared to the surrounding normal tissue has been recognized as a common feature in
all cancers. Therefore, targeting the higher proliferation rate of cancer cells can be used to inhibit tumor growth by preferential killing of rapidly proliferating cells.

7.2 Materials and Methods

7.2.1 Analysis of cell cycle distribution profile

The effect of extract/fractions on cell cycle distribution was assessed by flow cytometry after propidium iodide staining. Cells (MDA-MB-231 cells/HCT-116/MCF-7: 1 × 10^5 cells/well) were treated with fractions/sub-fractions/isolated compounds at the IC_{50} values for 48 h. Cells in the control group received only media containing 0.1% DMSO and doxorubicin was used as positive control. Cells were harvested, washed with PBS, fixed with ice-cold 70% ethanol and kept at 4 °C for 12 h. The cells were again washed with cold PBS and stained with propidium iodide (5 µg/mL) solution containing 0.1 mg/mL RNase and incubated in dark for 30 min. The cellular DNA content was analyzed by flow cytometry (Becton Dickinson Accuri, San Diego, CA, USA) and percentage of cells determined in G₀/G₁, S, and G₂/M phases of cell cycle using the BD software after exclusion of cellular debris and aggregates (Lima et al., 2014).

7.2.2 Clonogenic assay

Clonogenic assay was carried out for NJM extract and column sub-fractions in MDA-MB-231 cells. The MDA-MB-231 cells (400 cells/well) were added in a 6-well plate and treated with NJM and NJDE at 20 µg/mL and NJPE and NJEA at 35 µg/mL for 48 h. Cells in the control group received only media containing 0.1% DMSO and doxorubicin was used as positive control. The media was removed after treatment and cells were incubated with fresh media for 12 days, fixed with 70% ethanol and stained with crystal violet (0.5% in ethanol). Cell colonies with more than 50 cells were counted. The treatments were carried out in triplicate.

7.2.3 Apoptosis by Hoechst 33258 and AO/EB (dual) nuclear staining

Apoptotic activity of extract/fractions was determined by Hoechst 33258 staining as described by Harada et al (2005) and AO/EB staining as described by Reddy et al 2015. Cells (MDA-MB-231 cells/HCT-116/MCF-7: 1 × 10^5 cells/well) were treated with fractions/sub-fractions/isolated compounds at the IC_{50} values for 48 h. Cells in the
control group received only media containing 0.1% DMSO and doxorubicin was used as positive control. Cells were washed with PBS and fixed with 70% ethanol for 5 min. After fixation, cells were incubated with Hoechst 33258 stain in PBS (5 µg/mL) or AO/EB (20/30 µg/mL) for 30 min at 37 °C in the dark. Cells were thoroughly washed with PBS and examined under a fluorescent microscope. Apoptotic cells were identified by nuclear condensation, formation of membrane blebs and apoptotic bodies. The mean number of apoptotic cells was determined by counting apoptotic cells in six different fields.

7.2.4 Cell viability assay in MCF-10A normal breast cells

Cell viability was tested for the active fractions in normal MCF-10A cells by SRB assay as described before.

7.2.5 Caspase-Glo® 3/7 Assay

The Caspase-Glo® 3/7 Assay is a luminescent assay that measures caspase-3 and -7 activities in cell cultures. The assay provides a proluminescent caspase-3/7 substrate, which contains the tetrapeptide sequence DEVD. This substrate is cleaved to release aminoluciferin, a substrate of luciferase used in the production of light. The Caspase-Glo® 3/7 Reagent is optimized for caspase activity, luciferase activity and cell lysis. Addition of the single Caspase-Glo® 3/7 Reagent in an "add-mix-measure" format results in cell lysis, followed by caspase cleavage of the substrate and generation of a “glow-type” luminescent signal. The Caspase-Glo® 3/7 Assay is designed for use with multiwell plate formats, making it ideal for automated high-throughput screening of caspase activity or apoptosis (Benchimol 2001).

7.2.6 CellTiter-Glo® Luminescent Cell Viability Assay

The CellTiter-Glo® Luminescent Cell Viability Assay is a homogeneous method of determining the number of viable cells in culture based on quantitation of the ATP present, an indicator of metabolically active cells. The CellTiter-Glo® Assay is designed for use with multiwell formats, making it ideal for automated high-throughput screening (HTS), cell proliferation and cytotoxicity assays. The homogeneous assay procedure involves adding the single reagent (CellTiter-Glo® Reagent) directly to cells cultured in serum-supplemented medium. Cell washing, removal of medium and multiple pipetting
steps are not required. The system detects as few as 15 cells/well in a 384-well format in 10 minutes after adding reagent and mixing. The homogeneous "add-mix-measure" format results in cell lysis and generation of a luminescent signal proportional to the amount of ATP present. The amount of ATP is directly proportional to the number of cells present in culture. The CellTiter-Glo® Assay generates a "glow-type" luminescent signal, which has a half-life generally greater than five hours, depending on cell type and medium used. The extended half-life eliminates the need to use reagent injectors and provides flexibility for continuous or batch mode processing of multiple plates. The unique homogeneous format avoids errors that may be introduced by other ATP measurement methods that require multiple steps (Benchimol 2001).

7.3 Results and Discussion

7.3.1 Cell cycle

7.3.1.1 NJM/fractions induce cell cycle arrest in MDA-MB-231 cells by flow cytometry

The antiproliferative effects of NJM and active fractions were investigated on the cell cycle distribution in various cancer cells by propidium iodide staining and flow cytometry.

MDA-MB-231 cells treated with NJM showed an increase in the percentage of cells in G2/M phase as compared to control cells treated with 0.1% DMSO (18.5% vs 14.7%). Interestingly, treatment with NJDE increased the proportion of cells in G0/G1 phase when compared to negative control cells (79.3% vs 76.3%) while NJPE and NJEA showed G2/M arrest as compared to negative control cells (20.7% and 19% respectively vs 14.7%). Doxorubicin showed a prominent increase in cells in G2/M phase (22.7% vs 14.7%) which was in accordance with the published data that doxorubicin causes cell cycle arrest at G2/M phase in MDA-MB-231 cells (Barr On et al., 2007).
Figure 7.1 NJM/fractions induce cell cycle arrest in MDA-MB-231 cells analyzed by flow cytometry.
7.3.1.2 NJDE fraction/sub-fraction/nardin induce cell cycle arrest in HCT-116 cells

HCT-116 cells treated with NJDE showed an increase in the percentage of cells in the G0/G1 phase as compared to control cells treated with 0.1% DMSO (78.5% vs 69.1%). Treatment with nardin increased the proportion of HCT-116 cells in the G0/G1 phase when compared to negative control cells (80.6% vs 69.1%) while treatment with PA-rich oil showed G0/G1 arrest as compared to negative control cells (75.6% vs 69.1%).

Figure 7.2 NJDE fraction/sub-fraction/nardin induce cell cycle arrest in HCT-116 cells analyzed by flow cytometry

A: Normal Control B: Nardin C: PA-rich oil D: NJDE
7.3.1.3 MUEA fraction/sub-fractions/isolated compounds induce cell cycle arrest in HCT-116 cells

HCT-116 cells treated with MUEA showed an increase in the percentage of cells in the G₀/G₁ phase as compared to control cells treated with 0.1% DMSO (79.0% vs 69.1%). Treatment with the fraction MUEA/C2/F15/70/30 increased the proportion of HCT-116 cells in the G₀/G₁ phase when compared to negative control cells (73.3% vs 69.1%) while treatment with quercetin showed G₂/M arrest as compared to negative control cells (17.0% vs 19.4%).

Figure 7.3 MUEA fraction/sub-fractions/isolated compounds induce cell cycle arrest in HCT-116 cells analyzed by flow cytometry

A: Normal Control  B: Quercetin  C: MUEA/C2/F15/70/30  D: MUEA
7.3.1.4 MUEA active sub-fraction/isolated compounds induce cell cycle arrest in MCF-7 cells

Treatment with the fraction MUEA/C2/F15/70/30 increased the proportion of MCF-7 cells in the S phase when compared to negative control cells (13.2% vs 10.5%). MCF-7 cells treated with pyrogallol showed an increase in the percentage of cells in the G2/M phase as compared to control cells treated with 0.1% DMSO (28.4% vs 24.0%) while treatment with quercetin increased the proportion of MCF-7 cells in the S phase when compared to negative control cells (15.9% vs 10.5%) and G2/M arrest as compared to negative control cells (26% vs 24%). It has been previously reported that quercetin causes G2/M arrest in MCF-7 cells (Choi et al., 2001).

Figure 7.4 MUEA sub-fraction/isolated compounds induce cell cycle arrest in MCF-7 cells analyzed by flow cytometry

A: Normal Control B: MUEA/C2/F15/70/30 C: Pyrogallol D: Quercetin
7.3.1.5 NJDE active sub-fraction/isolated compounds induce cell cycle arrest in MCF-7 cells

MCF-7 cells treated with NJDE/C1/F4/85/15 showed an increase in the percentage of cells in the $G_0/G_1$ phase as compared to control cells treated with 0.1% DMSO (68.5% vs 63.6%). Treatment with nardin increased the proportion of HCT-116 cells in the $G_2/M$ phase when compared to negative control cells (29.6% vs 24%) while treatment with PA-rich oil showed $G_2/M$ arrest as compared to negative control cells (31.5% vs 24%).

Figure 7.5 NJDE fraction/sub-fraction induce cell cycle arrest in MCF-7 cells analyzed by flow cytometry
7.3.1.6 NJM/fractions induce apoptosis in MDA-MB-231 cells by Hoechst 33258 staining

To confirm the apoptotic effects of fractions, Hoechst 33258 stain was used to study the morphological changes in MDA-MB-231 cells under microscope. After treatment with NJM/fractions for 48 h, significant \((P < 0.001)\) increase in apoptotic cells were observed as compared to negative control cells which showed regular contours with uniform fluorescence intensity. The treated cells exhibited characteristic apoptotic changes for example cell shrinkage, nuclear condensation, and formation of round apoptotic bodies which appeared as round spherical beads. Our findings suggest that NJM, NJPE, NJDE and NJEA cause cell death by inducing apoptosis in MDA-MB-231 cells which is a highly desirable feature of anticancer agents (Figure 7.6).

7.3.1.7 NJM/fractions induce apoptosis in MCF-7 cells by AO/EB staining

In AO/EB staining method, the cells appeared as green colored fluorescent spots for viable cells, yellow color for early apoptotic cells and reddish to orange for the late apoptotic cells. The fractions NJDE, NJDE/C1/F4/85/15, PA-rich oil and the isolated compound nardin exhibited a significant \((< 0.05)\) increase in the apoptotic index after 48 h of treatment in MCF-7 cells. Thereby, suggesting apoptosis as the mode of cell death by NJDE fractions and isolated compounds (Figure 7.7).

7.3.1.8 MUEA/fractions induce apoptosis in MCF-7 cells by AO/EB staining

The fractions MUEA, MUEA/C2/F15/70/30, and the isolate compounds, pyrogallol, gallic acid, and quercetin exhibited a significant \((< 0.05)\) increase in the apoptotic index after 48 h of treatment in MCF-7 cells. Thereby, suggesting apoptosis as the mode of cell death by MUEA fractions and isolated compounds (Figure 7.8).
Figure 7.6 NJM/fractions induce apoptosis in MDA-MB-231 cells by Hoechst 33258 staining

(A) Representative images of cells after treatment for 48 h. Apoptotic morphology was confirmed by nuclear condensation, formation of membrane blebs, round apoptotic bodies (orange arrows).

(B) Number of apoptotic cells were estimated by counting apoptotic cells in six different fields. Results are expressed as mean ± SEM. *P < 0.001 compared with negative control. The treatments were carried out in triplicate.
Figure 7.7 NJM/fractions induce apoptosis in MCF-7 cells by AO/EB staining

(A) Representative images of cells after treatment for 48 h. Apoptotic morphology was confirmed by morphological changes of apoptosis: Living cells have a normal green nucleus; early apoptotic cells have bright green nucleus with condensed or fragmented chromatin; late apoptotic cells display condensed and fragmented orange chromatin; cells that have died from direct necrosis have a structurally normal orange nucleus nuclear condensation, formation of membrane blebs, round apoptotic bodies.

(B) Apoptotic index (AI) was calculated using was calculated as % of apoptotic cells from randomly counted 100 cells in each treatment group. Results are expressed as mean ± SEM. *P < 0.001 compared with negative control. The treatments were carried out in triplicate.
Figure 7.8 MUEA/fractions induce apoptosis in MCF-7 cells by AO/EB staining

(A) Representative images of cells after treatment for 48 h. Apoptotic morphology was confirmed by nuclear condensation, formation of membrane blebs, round apoptotic bodies (orange arrows).

(B) Number of apoptotic cells were estimated by counting apoptotic cells in six different fields. Results are expressed as mean ± SEM. *P < 0.001 compared with negative control. The treatments were carried out in triplicate.
7.3.1.9 NJM/fractions reduce clonogenic capacity of MDA-MB-231 cells

Clonogenic assay is considered as the gold standard to determine the anticancer activity of drugs. NJM and fractions, NJPE, NJDE, and NJEA significantly ($P < 0.001$) reduced the colony formation of MDA-MB-231 cells over a period of 12 days thereby suggesting the long-term antiproliferative effect of NJM/fractions. Owing to their ability to inhibit colony formation of MDA-MB-231 cells, we speculate that NJM/fractions could significantly contribute to the reduction of metastases.

**Figure 7.9 NJM/fractions reduce clonogenic capacity of MDA-MB-231 cells**

7.3.1.10 Cell viability and Caspase 3/7 fold change expression in MDA-MB-231 cells

The effect of the MUEA sub-fractions and isolated compound from NJDE demonstrated significant dose-dependent reduction in cell viability of MDA-MB-231Br cells as observed in the CellTiter-Glo® Luminescent Cell assay. The results were in accordance with the cell viability assays reported earlier by MTT or SRB method.

Caspases are the central regulators of programmed cell death. The effector caspase 3/7 activation plays a very important role in the initiation of apoptosis. Our study on caspase activation revealed that the test compound, MUEA at both doses 75 and 150 µg/mL increased the fold expression of caspase 3/7 by 1.67 and 5.52 fold respectively. The MUEA/C2/F15/70/30 active fraction at 100 µg/mL exhibited 1.53 fold change expression of caspase 3/7.
Nardin at 100 µg/mL exhibited a 2.65 fold change of caspase 3/7 while PA-rich oil 1.13 fold and 3.86 fold change in caspase 3/7 expression at 25 at 100 µg/mL and 50 at 100 µg/mL. NJDE/C1/F2/95/5 exhibited a 1.46 fold change at 50 µg/mL. NJDE/C1/F4/85/15 showed 5.89 and 9.65 fold change of caspase 3/7 expression at 25 µg/mL and 50 µg/mL. NJDE/C1/F10/91/9 exhibited a 2.67 and 12.21 fold change at 25 and 50 µg/mL.

Based on the caspase 3/7 activation studies, it can be postulated that the active sub-fraction of MUEA and NJDE sub-fractions and isolated compounds exhibited significant activation of the apoptotic pathway in MDA-MB-231 Br cells.

**Figure 7.10 Effect of treatments on cell viability of MDA-MB-231Br cells by CellTiter-Glo® Luminescent Cell assay**
Figure 7.11 Effect of treatments on fold expression of Caspase 3/7 in MDA-MB-231Br cells by CellTiter-Glo® Luminescent Cell assay

7.3.1.10 Effect of treatments on normal human breast cells

The effect of the active fractions and column sub-fractions were evaluated on normal MCF-10A cells. The fraction MUEA and active column sub-fractions I, MUEA/C1/F7/40/60 and active column sub-fraction II exhibited an IC<sub>50</sub> values of 348.50 μg/mL, 87.40 μg/mL and 36.40 μg/mL respectively. The fractions exhibited selectivity towards breast cancer cells as compared to normal MCF-10A cells that is a highly desirable feature of anticancer drugs.

The fraction NJDE and active column sub-fractions I, NJDE/C1/F2/95/5 and NJDE/C1/F4/85/15 exhibited IC<sub>50</sub> values of 28.42 μg/mL, 30.66 μg/mL, and 16.74 μg/mL respectively. All these fractions exhibited higher cytotoxicity towards breast cancer cells as compared to normal MCF-10A cells that is a highly desirable feature of anticancer drugs.
Table 7.1 *In vitro* cytotoxicity of selected fractions of *M. umbellatum* in MCF-10A cells by MTT assay

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Percentage cell viability at concentrations (µg/mL)</th>
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<tbody>
<tr>
<td></td>
<td>25</td>
</tr>
<tr>
<td>MUM</td>
<td>113.18 ± 10.09</td>
</tr>
<tr>
<td>MUEA</td>
<td>102.33 ± 3.91</td>
</tr>
<tr>
<td><strong>12.5</strong></td>
<td></td>
</tr>
<tr>
<td>MUEA/C1/F7/60/40 (C1)</td>
<td>112.41 ± 6.10</td>
</tr>
<tr>
<td>MUEA/C2/F15/70/30 (C2)</td>
<td>97.69 ± 3.84</td>
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</tbody>
</table>

Values are expressed as mean ± SEM. Experiment was done in triplicate.

Table 7.2 *In vitro* cytotoxicity of selected fractions of *N. jatamansi* in MCF-10A cells by MTT assay

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Percentage cell viability at concentrations (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2.5</td>
</tr>
<tr>
<td>NJM</td>
<td>82.96 ± 6.43</td>
</tr>
<tr>
<td>NJDE</td>
<td>111.02 ± 4.7</td>
</tr>
<tr>
<td>NJEA</td>
<td>109.40 ± 0.96</td>
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<tr>
<td>NJDE/C1/F2/95/5</td>
<td>96.97 ± 12.24</td>
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<tr>
<td>NJDE/C1/F4/85/15</td>
<td>83.67 ± 5.65</td>
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Values are expressed as mean ± SEM. Experiment was done in triplicate.
Table 7.3 IC$_{50}$ values of selected sub-fractions of $M$. umbellatum and $N$. jatamansi in MCF-10A cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>IC$_{50}$ (μg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MCF-10A</td>
</tr>
<tr>
<td>MUM</td>
<td>&gt;500</td>
</tr>
<tr>
<td>MUEA</td>
<td>348.50</td>
</tr>
<tr>
<td>MUEA/C1/F7/60/40 (C1)</td>
<td>87.40</td>
</tr>
<tr>
<td>MUEA/C2/F15/70/30 (C2)</td>
<td>36.40</td>
</tr>
<tr>
<td>NJM</td>
<td>110.4</td>
</tr>
<tr>
<td>NJDE</td>
<td>28.42</td>
</tr>
<tr>
<td>NJEA</td>
<td>24.05</td>
</tr>
<tr>
<td>NJDE/C1/F2/95/5</td>
<td>30.66</td>
</tr>
<tr>
<td>NJDE/C1/F4/85/15</td>
<td>16.74</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>0.01</td>
</tr>
</tbody>
</table>

7.4 Summary of mechanistic evaluation

Our results demonstrated that the NJDE fraction of $Nardostachys$ jatamansi acts through the activation of apoptotic pathway evident through increased fold expression of Caspase 3/7 in MDA-MB-231Br cells. The effect of NJDE fraction could be attributed to the synergistic activities of PA-rich oil and nardin. In cell cycle analysis, NJDE fraction caused cell cycle arrest in MDA-MB-231 cells in the G$_0$/G$_1$ phase. In colon cancer cells, NJDE fraction caused G$_0$/G$_1$ arrest that could be due to the cumulative effect of PA-rich oil and nardin. Both PA-rich oil and nardin exhibited G$_0$/G$_1$ arrest in HCT-116 cells and G2/M arrest in MCF-7 cells.

In addition, lupeol and β-sitosterol that were identified and quantified by HPTLC studies could be responsible partly for the anticancer activity of NJPE and β-sitosterol for NJEA fraction. Lupeol and β-sitosterol are reported to have multi-target action with immense anticancer potential modulating key signalling pathways that are implicated in various types of cancer, modulation of antioxidant enzyme levels in disease states, and reducing free radical generation (Prasad et al, 2008; Saleem, 2009; Baskar et al, 2010).
The MUEA fraction of Memecylon umbellatum acts through the activation of apoptotic pathway through increased fold expression of Caspase 3/7 in MDA-MB-231Br cells. The effect of MUEA fraction could be attributed to the phenolic compounds pyrogallol, quercetin, and gallic acid. All compounds exhibited significant induction of apoptosis as evident through the nuclear staining methods. In cell cycle analysis of MCF-7 cells, the active fraction MUEA/C2/F15/70/30 increased the proportion of cells in the S phase. MCF-7 cells treated with pyrogallol showed an increase in the percentage of cells in the G2/M phase as while treatment with quercetin increased the proportion of MCF-7 cells in the S phase and G2/M arrest as compared to negative control cells.

MUEA fraction demonstrated G0/G1 arrest in HCT-116 cells. Quercetin exhibited G2/M arrest in HCT-116 cells that is in accordance with the published literature.

Based on the caspase 3/7 activation studies, it can be postulated that the active sub-fraction of MUEA and NJDE sub-fractions and nardin exhibited significant activation of the apoptotic pathway in MDA-MB-231 Br cells.

Overall, MUEA fraction and its active components: Pyrogallol, quercetin, and gallic acid act by the preferred mode of cell death i.e. induction of apoptosis and cell cycle arrest in colon and breast cancer cells (both ER+ and triple negative). NJDE fraction and its active components: PA-rich oil and sesquiterpene acid nardin act by the preferred mode of cell death i.e. induction of apoptosis and cell cycle arrest in colon and breast cancer cells.

Moreover, the active column sub-fractions of both plants demonstrated significant selectivity towards breast cancer cells as compared to normal MCF-10A cells, a highly desirable feature of anticancer therapeutics.

7.5 References


