Chapter 5

**Comparative studies of PM1 with its active constituents against γ-radiation-induced cytotoxicity and genotoxicity in V79 cells**

**Summary**

This chapter deals with the studies carried out to compare cytoprotective and antigenotoxic activity of the active fraction of *Pilea microphylla* (PM1) with that of its active polyphenolic constituents against γ-radiation in V79 cells. PM1 was evaluated for its free radical scavenging potential using Fenton reaction-induced DNA damage and lipid peroxidation. Further, PM1 was subjected against γ-radiation-induced cytotoxicity and genotoxicity in V79 cells.

PM1 significantly reduced free radical-mediated calf thymus DNA damage and lipid peroxidation. Among the concentrations tested (12.5, 25 and 50 µg/ml) for radioprotection, PM1 at 25 µg/ml exhibited maximum protection. Further, when compared with constituent polyphenols viz., quercetin-3-O-rutinoside, quercetin and 3-O-caffeoylquinic acid (equivalent to quantities present in PM1-25 µg/ml), both individually and collectively, the combination of polyphenols was found more effective than individual constituents in preventing γ-radiation-induced cytotoxicity and genotoxicity. To conclude, radioprotection is possibly a synergistic effect of the phytochemicals present in the herbal extract, rather than any single component.
Chapter 5  Comparative studies of PM1 with its active constituents against γ -radiation-induced cytotoxicity and genotoxicity in V79 cells

5.1. Introduction

Ethnobotanical research has been focused from decades on the discovery of evaluating new constituents from plant products [1]. Active constituents isolated from plant products have been selected for their high activity against human diseases. However, they do not possess the same amount of activity as crude extracts at comparable concentrations [2]. This phenomenon is attributed to the absence of interacting constituents present in the crude extract. These isolated compounds are more expensive to produce. In contrast, traditional medicines can be grown and produced locally at lower cost [2]. There are reports that crude extracts possess better in vitro or in vivo activity than isolated compounds at an comparable dose [3].

*Pilea microphylla* (PM) is one such plant which has been studied for its free radical scavenging and radioprotective properties in Swiss albino mice[4]. Its significant antioxidant and in vivo radioprotective potential prompted us to isolate and characterize the plant for its active constituents. In chapter 2, we observed the presence of polyphenols namely quercetin-3-O-rutinoside, 3-O-caffeoylquinic acid, luteolin-7-O-glucoside, apigenin-7-O-rutinoside, apigenin-7-O-β-D-glucopyranoside and quercetin. Quercetin-3-O-rutinoside, 3-O-caffeoylquinic acid and quercetin have been studied for its radioprotective potential [5, 6]. Therefore, their availability in PM1 prompted us to design our present study, wherein we have evaluated the cytoprotective and antigenotoxic potential of PM1 against radiation-induced damage in V79 cells and compared that with its constituent polyphenols (quercetin-3-O-rutinoside, quercetin and 3-O-caffeoylquinic acid). Isolated pure polyphenols were used at equivalent concentrations as present in PM1, in order to compare the radioprotective activity of the combined fraction vis-à-vis its active constituents.
5.2. Materials and methods

5.2.1. Chemicals

Dulbecco’s minimum essential medium (DMEM), 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), cytochalasin-B, normal melting agarose (NMA), low melting agarose (LMA), Triton X-100, trizma base, ethidium bromide and RNAse were purchased from Sigma-Aldrich (St. Louis, MO, USA). Fetal bovine serum (FBS), trypsin, sodium bicarbonate, thiobarbituric acid and sodium dodecyl sulphate (SDS) were purchased from HiMedia Laboratories Pvt. Ltd., Mumbai, India. Acridine orange and crystal violet were procured from SD Fine Chem Ltd., Mumbai, India. All the other chemicals and solvents used were of analytical grade.

5.2.2. Calf thymus DNA protection assay

An initial experiment was designed to assess the free radical scavenging potential of PM1 against Fenton reaction-induced damage to calf thymus DNA. The following groups were employed.

- **Group 1** – Normal control: Calf thymus DNA alone.
- **Group 2** – Drug control (PM1 alone): Calf thymus DNA treated with PM1 (50 µg/ml) for 30 min.
- **Group 3** – Fenton reaction (Fenton reaction alone): Calf thymus DNA exposed to H₂O₂-induced Fenton reaction.
- **Group 4** – PM1 + Fenton reaction: Calf thymus DNA treated with different concentrations (12.5, 25 and 50 µg/ml) of PM1 for 30 min and then subjected to H₂O₂-induced Fenton reaction.

The method assessment of Calf thymus DNA protection is described in chapter 2, section 2.2.7.
5.2.3. Cell culture conditions and Irradiation protocol

Cell culture conditions and Irradiation protocol are described in chapter 2; section 2.2.8.1 and 2.2.8.2.

The radiation doses used for cell viability, clonogenic and micronuclei assay were 2, 4, 6, 8 and 10 Gy. A single radiation dose of 10 Gy was used for lipid peroxidation, DNA ladder assay, comet assay and nuclear staining assay.

5.2.4. Cytotoxicity assay of PM1 on V79 cells

The effect of PM1 on the viability of V79 cells was determined using MTT assay, which is based on the mitochondrial dehydrogenase-induced reduction of tetrazolium salt to formazan by viable cells [7]. Briefly, V79 cells were incubated with PM1 (0.1–1 mg/ml) for 24h and 48h. After incubation, medium was removed and 100 µl of the MTT stock solution (1 mg/ml) was added to each well. After incubating for 4 h, the formazan crystals in each well were dissolved in 100 µl of DMSO, absorbance read at 540 nm on a scanning multi-well plate reader (ELx800, BioTek Instruments Inc., Winooski, VT, USA) and % cytotoxicity calculated from the formula:

\[
\frac{\left(A_C - A_B\right) - \left(A_T - A_B\right)}{A_C - A_B}
\]

Where \(A_C\), \(A_T\) and \(A_B\) are absorbance of control, test and blank respectively. IC\(_{50}\) values were calculated [7].

5.2.5. Optimisation of cytoprotective and antigenotoxic dose of PM1 in \(\gamma\)-irradiated V79 cells

5.2.5.1. Experimental design I

This experiment was designed to select the optimum radioprotective concentration of PM1 with the help of with the help of different methods listed below.
a) Cytoprotective studies: Cell viability assay and Clonogenic assay
b) Antigenotoxic studies: Micronuclei assay and DNA ladder assay
c) Anti-lipid peroxidation study

V79 cells were divided into four different groups, which were treated as shown below.

- **Group 1** – Normal control
- **Group 2** – Drug control (PM1 alone): Cells treated with PM1 (50 µg/ml) for 30 min.
- **Group 3** – Radiation control (Irradiation alone): Cells exposed to γ-radiation.
- **Group 4** – PM1 + Radiation (R): Cells treated with different concentrations (12.5, 25 and 50 µg/ml) of PM1 for 30 min before exposure to γ-radiation.

### 5.2.5.2. Cell viability assay

The experimental design for the assessment of cell viability is described under section 5.2.5.1.

% cytotoxicity was calculated as mentioned in section 5.2.4. The % cell viability, considered as radio-protective effect, was calculated from the formula: \((100 - \% \text{ cytotoxicity})\)

### 5.2.5.3. Clonogenic assay

The experimental design for the assessment of survival and cloning efficiency of V79 cells is described under section 5.2.5.1. [8]. In brief, following treatments, cells were trypsinised. A suspension of approximately 100 cells was added to 60 mm petriplate and allowed to form colonies for one week. Cell growth was terminated and colonies
stained with 1% w/v crystal violet in methanol. Survival fraction (SF) was calculated relative to the respective controls.

\[ SF = \frac{\text{colonies}}{\text{Number of cells seeded}} \times \frac{100}{\text{PE}} \]

Where plating efficiency (PE) is calculated as

\[ \text{PE} = \frac{\text{colonies in normal control}}{\text{Number of cells seeded in normal control}} \times 100 \]

5.2.5.4. Micronuclei assay

The experimental design for the assessment of micronuclei assay in V79 cells is described under section 5.2.5.1. [9]. At the sixth hour after irradiation, 3 µg/ml of cytochalasin-B was added to block cytokinesis. Cells were trypsinised after 16 h, centrifuged (3,500 g for 10 min), and the supernatant removed. The cell pellet was subjected to a mild hypotonic (0.56 % potassium chloride) treatment followed by fixation at 4°C overnight. The cells were finally re-suspended in a small volume of Carnoy’s fixative (acetic acid: methanol, 1:3) and spread on pre-cleaned, chilled, coded slides to avoid observer bias. The slides were stained with 0.125 % w/v acridine orange in Sorensen’s buffer (pH 6.8) and observed under a fluorescence microscope using 450-490 nm filters. From each culture group, 1000 binucleated cells (BNC) with preserved cytoplasm were counted and the percentage frequency of micronucleated binucleate (%MNBNC) was determined. Cell proliferation in vitro was evaluated by counting the frequencies of mono, bi, and polynucleate cells and the results were presented as cytokinesis-block proliferation index (CBPI).

\[ \text{CBPI} = M_1 + 2M_{II} + 3(M_{III} + M_{IV})/N \]

Where \( M_1, M_{II}, M_{III} \) and \( M_{IV} \) represent the number of cells with subscripts representing number of nuclei respectively and \( N \) is the number of cells scored.
5.2.5.5. DNA ladder assay

The experimental design for the assessment of DNA fragmentation assay in cells is described under section 5.2.5.1. In brief, cells were lysed and treated with RNase for 1 h. The lysates were then loaded into the wells of 1.5 % w/v agarose gel and subjected to electrophoresis at 60 V for 2 h. The pattern of DNA fragmentation was visualized and compared with a reference DNA marker (3kb). Photographs were taken under UV light by a gel documentation unit (UVItec Limited, Cambridge, UK).

5.2.5.6. Lipid peroxidation assay in V79 cells

The experimental design for the assessment of lipid peroxidation in cells is described under section 5.2.5.1., and performed according to the method described in chapter 2, section 2.2.8.6.

5.2.6. Comparison of radioprotective potential of PM1 with constituent polyphenols

5.2.6.1. Experimental design II

The optimum radioprotective concentration of PM1 was compared with its constituent polyphenols [quercetin-3-O-rutinoside (Ru), 3-O-caffeoylquinic acid (C) and quercetin (Q) both individually and in combination] with the help of different methods listed below.

a) Cytoprotective studies: Cell viability assay and Clonogenic assay

b) Antigenotoxic studies: Comet assay and Apoptosis by nuclear staining

Concentrations of Ru, C and Q equivalent to the respective concentrations in PM1 (25 µg/ml), i.e. 27.25, 14.25 and 4.25 ng/ml, respectively, were selected individually and in combination (Ru, C and Q = RuQC).
V79 cells were divided into four different groups, which were treated as shown below.

- **Group 1** – Normal control
- **Group 2** – Drug control [PM1 (25 µg/ml)/Ru/Q/C/RuQC alone]: Cells treated with PM1 (25 µg/ml)/Ru/Q/C/RuQC for 30 min.
- **Group 3** – Radiation control (Irradiation alone): Cells exposed to γ-radiation.
- **Group 4** – PM1/Ru/Q/C/RuQC + Radiation (R): Cells treated with PM1 (25 µg/ml)/Ru/Q/C/RuQC for 30 min before exposure to γ-radiation.

### 5.2.6.2. Cell viability assay

The experimental design for the assessment of cell viability in cells is described under section 5.2.6.1., was determined using the MTT assay [7] and the absorbance read at 540 nm. % cytotoxicity was calculated as mentioned in section 5.2.4. The % cell viability, considered as radio-protective effect, was calculated from the formula: \(100 - \% \text{ cytotoxicity}\)

### 5.2.6.3. Clonogenic assay

The experimental design for the assessment of survival and cloning efficiency of V79 cells is described under section 5.2.6.1., and performed according to the method described under section 5.2.5.3.

### 5.2.6.4. Comet assay

The experimental design for the assessment of alkaline comet assay in cells is described under section 5.2.6.1., and performed according to the method described in chapter 2, section 2.2.8.7.
5.2.6.5. Apoptosis by nuclear staining assay

Microscopic staining to detect apoptosis was carried out with modifications of method described [10]. The experimental design for this assay is described under section 5.2.6.2., Cells were gently washed with PBS, fixed with 90 % methanol for 20 min at –20 °C and immediately stained with acridine orange (100 µg/ml). Each well was then examined under fluorescence microscope (excitation wavelength 455 nm) for any nuclear changes. The percentage of apoptotic cells (% APC) was calculated with respect to total number of cells examined. Three slides were prepared for each treatment group and 1000 cells were scored per slide.

5.2.7. Statistical analysis

LD_{50} is defined as the radiation dose at which 50 % cell death was observed. A graph was plotted between different radiation doses vs. effect (for both cell viability assay and clonogenic assay) and LD_{50} was calculated by non-linear regression \( y = a + b \ln(x) \).

Statistical comparisons were performed using GraphPad Prism 5.02 by one-way analysis of variance (ANOVA), followed by post hoc Tukey’s test. Results were expressed as mean ±S.E.M. and \( p < 0.05 \) was considered significant.
5.3. Results

5.3.1. Calf thymus DNA protection

All tested doses of PM1 protected against hydroxyl radical-induced calf thymus DNA damage (Fig. 5.1 – lanes 4, 5 and 6), with greater protection at 25 µg/ml. PM1 drug control (50 µg/ml) did not cause any damage to the DNA (Fig. 5.1 – lanes 2).

Fig. 5.1. Calf thymus DNA protection studies by PM1.

Lane 1: DNA control; Lane 2: PM1 drug control (DNA + PM1-50 µg/ml); Lane 3: Fenton reaction (FR)-induced DNA damage; Lane 4: DNA + PM1-12.5 µg/ml + FR; Lane 5: DNA + PM1-25 µg/ml + FR; Lane 6: DNA + PM1-50 µg/ml + FR.
5.3.2. Cytotoxic effects of PM1 on V79 cells

PM1 in the concentration range of 0.1–1 mg/ml, after an incubation period of 24 and 48 h, was found to be safe in V79 cells. IC$_{50}$ for % cytotoxicity computed from the dose-response curves at 24 and 48 h were found to be > 1 mg/ml and 0.84 mg/ml, respectively. Thus, a range of concentrations (12.5, 25 and 50 µg/ml) was selected for radioprotective studies of PM1.

5.3.3. Optimisation of cytoprotective and antigenotoxic potential of PM1 in $\gamma$-irradiated V79 cells

5.3.3.1. Cell viability assay

In V79 cells, $\gamma$-radiation resulted in a dose dependent reduction in cell viability (Fig. 3) with an LD$_{50}$ of 6.73 ± 0.01 Gy (Table 5.1). PM1 at both 12.5 and 25 µg/ml showed radioprotection against all the radiation doses (Fig. 5.2), whereas, 50 µg/ml of PM1 was protective only at higher radiation doses (8 and 10 Gy). However, PM1 at 25 µg/ml increased the LD$_{50}$ of radiation dose significantly ($p < 0.05$), as indicated by the DMF (Table 5.1), thus conferring maximum radioprotection among the concentrations tested.
**Fig. 5.2.** Effect of various doses of PM1 on percentage viability of V79 cells treated with different radiation doses in cell viability assay. All values are expressed as mean ± SEM and experiments were carried out in triplicate. *p < 0.05 compared with radiation control.

**Table 5.1.** Effect of PM1 (12.5, 25 and 50 µg/ml) on LD_{50} (Gy) of γ-radiation and corresponding dose modifying factors (DMF) assessed by cell viability assay.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>LD_{50} (Gy)</th>
<th>DMF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Radiation control</td>
<td>6.73 ± 0.01</td>
<td>1</td>
</tr>
<tr>
<td>PM1 (12.5 µg/ml)</td>
<td>10.58 ± 0.53 *</td>
<td>1.57 ± 0.08</td>
</tr>
<tr>
<td>PM1 (25 µg/ml)</td>
<td>13.86 ± 1.42 *</td>
<td>2.06 ± 0.21</td>
</tr>
<tr>
<td>PM1 (50 µg/ml)</td>
<td>10.21 ± 0.51 *</td>
<td>1.51 ± 0.08</td>
</tr>
</tbody>
</table>
All values are expressed as mean ± SEM and experiments were carried out in triplicate. * $p < 0.05$ compared with radiation control.

### 5.3.3.2. Clonogenic assay

While all the tested doses of PM1 (12.5, 25 and 50 $\mu$g/ml) significantly ($p < 0.05$) increased the radiation LD$_{50}$, it was maximally elevated at 25 $\mu$g/ml as indicated from the DMF (Table 5.2). As seen in Fig. 5.3, with increasing concentration of PM1 from 12.5 to 25 $\mu$g/ml, there was a reduction in the cytotoxic effect, produced across the radiation dose range. However, on further increasing PM1 dose to 50 $\mu$g/ml, survival declined and no protection was observed against any radiation dose.

**Fig. 5.3.** Effect of various doses of PM1 on the survival fraction (SF) of V79 cells treated with different radiation doses in clonogenic assay.

The number of colonies per group was counted and their survival fraction was calculated relative to normal control group. Cell survival curves were normalized for
the plating efficiency of normal control cells with/without PM1. SF for normal control was 0.97 ± 0.05 indicating plating efficiency.

All values are expressed as mean ± SEM and experiments were carried out in triplicate. *$p < 0.05$ compared with radiation control.

**Table 5.2.** Effect of PM1 (12.5, 25 and 50 µg/ml) on LD$_{50}$ (Gy) of γ-radiation and corresponding dose modifying factors (DMF) assessed by clonogenic assay.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>LD$_{50}$ (Gy)</th>
<th>DMF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Radiation control</td>
<td>2.37 ± 0.08</td>
<td>1</td>
</tr>
<tr>
<td>PM1 (12.5 µg/ml)</td>
<td>3.85 ± 0.29 * #</td>
<td>1.62 ± 0.12</td>
</tr>
<tr>
<td>PM1 (25 µg/ml)</td>
<td>7.66 ± 0.01 *</td>
<td>3.23 ± 0.01</td>
</tr>
<tr>
<td>PM1 (50 µg/ml)</td>
<td>3.65 ± 0.12 * #</td>
<td>1.54 ± 0.05</td>
</tr>
</tbody>
</table>

The changes in radioprotection were quantified from the DMF.

All values are expressed as mean ± SEM and experiments were carried out in triplicate. *$p < 0.05$ compared with radiation control and #$p < 0.05$ compared with PM1 (25 µg/ml).

**5.3.3.3. Micronuclei assay**

γ-radiation (2–10 Gy), dose dependently, increased %MNBNC up to 6 Gy, followed by a decline at higher radiation doses (Fig. 5.4). However, there was a reduction in CBPI (Table 5.3) with increasing dose of radiation. Pre-treatment of V79 cells with PM1 – 12.5 and 25 µg/ml resulted in a significant ($p < 0.05$) decrease in %MNBNC at all radiation doses, whereas PM1 – 50 µg/ml was least effective. While all the tested
doses of PM1 (12.5, 25 and 50 μg/ml) significantly improved the CBPI. PM1 (25 μg/ml) was consistently effective in improving the indices against all radiation doses (Table 5.3).

Further, microscopic examination revealed decline in the frequency of micronuclei with PM1 (25 μg/ml) pre-treatment compared to irradiated cells (Fig. 5.5 C). PM1 drug control did not show any clastogenic effect in V79 cells (Table 5.3).

**Fig. 5.4.** Effect of PM1 pre-treatment on percentage frequency of micronucleated binucleate cells (%MNBNC) in irradiated V79 fibroblasts.

All values are expressed as mean ± SEM and experiments were carried out in triplicate. *p < 0.05 compared with radiation control and #p < 0.05 compared with PM1 (25 μg/ml).
Fig. 5.5. Changes in the frequency of micronuclei (MN) indicated by yellow arrows, in (A) Normal control cells with no micronuclei; (B) γ-irradiated V79 cell with three micronuclei; (C) PM1 pre-treated V79 cell with reduced frequency of micronuclei.

Table 5.3. Effect of PM1 pre-treatment on cytokinesis-block proliferation index (CBPI) in irradiated V79 fibroblasts assessed by micronuclei assay.

<table>
<thead>
<tr>
<th>Radiation Dose (Gy)</th>
<th>CBPI (Cytokinesis Block Proliferation Index)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Radiation control</td>
</tr>
<tr>
<td>0</td>
<td>1.958 ± 0.004</td>
</tr>
<tr>
<td>2</td>
<td>1.722 ± 0.002</td>
</tr>
<tr>
<td>4</td>
<td>1.669 ± 0.001</td>
</tr>
<tr>
<td>6</td>
<td>1.550 ± 0.001</td>
</tr>
<tr>
<td>8</td>
<td>1.460 ± 0.002</td>
</tr>
<tr>
<td>10</td>
<td>1.429 ± 0.004</td>
</tr>
</tbody>
</table>
All values are expressed as mean ± SEM and experiments were carried out in triplicate. *p < 0.05 compared with radiation control and #p < 0.05 compared with PM1 (25 µg/ml).

5.3.3.4. DNA ladder assay

The damaged DNA in the radiation control appeared as ladder of low molecular weight fragments (Fig. 5.6, lane 4). Pre-treatment with PM1 (25 µg/ml) decreased the number and intensity of the bands (Fig. 5.6, lane 4), as compared to the radiation control, indicating inhibition of apoptosis. PM1 – 12.5 and 50 µg/ml were ineffective against γ-radiation-induced apoptosis.

Fig. 5.6. DNA fragmentation in PM1 pre-treated V79 cells exposed to radiation (10 Gy) by agarose gel electrophoresis.
Lane 1: DNA marker; Lane 2: Normal control; Lane 3: PM1 drug control (50 µg/ml); Lane 4: 10 Gy (Radiation control); Lane 5: PM1 (12.5 µg/ml) + 10 Gy; Lane 6: PM1 (25 µg/ml) + 10 Gy; Lane 7: PM1 (50 µg/ml) + 10 Gy.

5.3.3.5. Lipid peroxidation assay in V79 cells

γ-radiation significantly \((p < 0.05)\) increased the level of TBARS in V79 cells. Pretreatment with PM1 (12.5, 25 and 50 µg/ml) significantly \((p < 0.05)\) reduced the elevated TBARS level in γ-irradiated cells. PM1 (25 µg/ml) showed maximum effect compared to PM1 – 12.5 and 50 µg/ml (Fig. 5.7). PM1 drug control (50 µg/ml) showed no significant change in the levels of TBARS compared to normal control.

![Figure 5.7](image)

**Fig. 5.7.** Protective effect of various concentration of PM1 upon γ-radiation (10 Gy)-induced membrane damage in V79 cells.

Lipid peroxidation was assayed by measuring amount of TBARS. The measurement was made in triplicate and values are expressed as mean ± SEM. \#\(p < 0.05\) compared with normal control; \*\(p < 0.05\) compared with radiation control.
5.3.4. Comparison of radioprotective potential of PM1 (25 µg/ml) with constituent polyphenols

For comparative cytoprotective and antigenotoxic studies, 25 µg/ml of PM1 was selected, as this concentration exhibited maximum protection against radiation effects in the aforementioned experiments. An equivalent concentration of quercetin-3-O-rutinoside (Ru), 3-O-caffeoylquinic acid (C) and quercetin (Q) present in PM1 (25 µg/ml) along with a mixture of these standards (RuQC) was employed for comparison.

5.3.4.1. Effect of PM1 (25 µg/ml) versus Ru, C, Q and RuQC on cell viability

RuQC significantly ($p < 0.05$) arrested radiation-induced decline in cell viability comparable to PM1 (25 µg/ml) (Fig. 5.8). However, individually, Ru, C and Q failed to show significant effect against $\gamma$-radiation (6 Gy)-induced cytotoxicity.

![Figure 5.8](image)

**Fig. 5.8.** Percentage cell viability with pure polyphenols (Ru, C and Q) / mixture of polyphenols (RuQC) / PM1 pre-treatment on $\gamma$-radiation (6 Gy)-induced V79 cells.
All values are expressed as mean ± SEM and experiments were carried out in triplicate. \( #p < 0.05 \) compared with normal control; \( *p < 0.05 \) compared with radiation control.

### 5.3.4.2. Effect of PM1 (25 µg/ml) versus Ru, C, Q and RuQC on cell clonogenicity

Since clonogenic assay is considered as the gold standard for cytotoxic activity [11], the results of MTT assay were further confirmed by this method. Colony forming ability of V79 cells, assessed as SF, was significantly \( (p < 0.05) \) improved in RuQC (combination of polyphenols) pre-treated group and found comparable to that of PM1 (25 µg/ml) (Fig. 5.9). However, Ru, C and Q, individually failed to show significant effect against \( \gamma \)-radiation (2 Gy)-induced cytotoxicity.

![Fig. 5.9](image.png)

**Fig. 5.9.** Survival fraction (SF) with pure polyphenols (Ru, C and Q) / mixture of polyphenols (RuQC) / PM1 pre-treatment on \( \gamma \)-radiation (2 Gy)-induced V79 cells. All values are expressed as mean ± SEM and experiments were carried out in triplicate. \( #p < 0.05 \) compared with normal control; \( *p < 0.05 \) compared with radiation control.
5.3.4.3. Effect of PM1 (25 µg/ml) versus Ru, C, Q and RuQC on DNA damage by comet assay

Nucleoids of the cells in the normal control appeared circular, whereas those in the radiation control looked like comets, with fluorescence intensity diminishing from the head to the tail, indicating DNA damage (Fig. 5.11). Fig. 5.10 shows the frequency distribution histograms of tail length (TL) and olive tail moment (OTM) of the treatment groups. All treatment groups, except Q, showed decline in TL and OTM compared to radiation control. However, maximum reduction in TL and OTM was observed with both RuQC and PM1 (25 µg/ml) pre-treatment.

![Fig. 5.10. Effect of pure polyphenols (Ru, C and Q) / mixture of polyphenols (RuQC) / PM1 pre-treatment on γ-radiation (10 Gy)-induced DNA damage.](image)

Comet parameters such as (A) Tail length (TL) and (B) Olive tail moment (OTM) are represented as bar graph (means ± SEM), obtained by analyzing 100 cells. \( \# p < 0.05 \) compared to the normal control, \(* p < 0.05 \) compared to the radiation control.

Ru+R = quercetin-3-O-rutinoside + radiation; Q+R = quercetin + radiation; C+R = 3-O-caffeoylquinic acid + radiation; RuQC+R = quercetin-3-O-rutinoside + quercetin + 3-O-caffeoylquinic acid + radiation.
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Fig. 5.11. Typical photomicrographs of (A) Intact DNA in normal V79 cells; (B) $\gamma$-
radiation-induced DNA damage (yellow arrow indicating tail of DNA); (C) represents 
DNA of PM1 (25 µg/ml) drug control cells; (D) represents DNA of irradiated cells 
pre-treated with 25 µg/ml of PM1; (E) DNA in quercetin-3-O-rutinoside pre-treated 
cells exposed to radiation; (F) DNA in quercetin pre-treated cells exposed to 
radiation; (G) DNA in 3-O-caffeoylquinic acid pre-treated cells exposed to radiation; 
(H) DNA in irradiated cells pre-treated with combination of quercetin-3-O-rutinoside, 
quercetin and 3-O-caffeoylquinic acid.

5.3.4.4. Effect of PM1 (25 µg/ml) versus Ru, C, Q and RuQC on apoptosis by nuclear staining

$\gamma$-radiation resulted in apoptosis where percentage apoptotic cells (% APC) was 
found to be 59.40 ± 2.47 in radiation control group. Pretreatment with PM1 and 
RuQC reduced % APC to 23.05 ± 2.91 and 22.47 ± 1.77, respectively (Table 5.4). 
Among the reference standards (Ru, Q and C), Ru and C reduced the % APC, 
whereas, quercetin did not show any effect.
Table 5.4. Effect of pre-treatment with pure polyphenols/mixture of polyphenols/PM1 on percentage apoptotic cells (% APC) assessed by nuclear staining.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Percentage apoptotic cells (％APC)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal control</td>
<td>8.92 ± 1.81</td>
</tr>
<tr>
<td>RuQC control</td>
<td>11.33 ± 1.12</td>
</tr>
<tr>
<td>PM1 drug control</td>
<td>10.06 ± 2.78</td>
</tr>
<tr>
<td>Radiation control</td>
<td>59.40 ± 2.47&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Ru + R</td>
<td>32.69 ± 4.90&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>Q + R</td>
<td>47.24 ± 5.93&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>C + R</td>
<td>39.66 ± 1.94&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>RuQC + R</td>
<td>22.47 ± 1.77&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>PM1 (25 μg/ml) − R</td>
<td>23.05 ± 2.91&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

All values are expressed as mean ± SEM and experiments were carried out in triplicate.

<sup>a</sup>p < 0.05 compared with normal control; <sup>b</sup>p < 0.05 compared with radiation control.
Chapter 5  Comparative studies of PM1 with its active constituents against $\gamma$-radiation-induced cytotoxicity and genotoxicity in V79 cells

5.4. Discussion

The focus of the present study was to elucidate the radioprotective potential of the active fraction of *Pilea microphylla* (PM1) towards normal cells (V79 cells) and compare that with its constituent polyphenols. It has been often observed that the polyphenol-rich crude plant extracts elicit greater biological activity than individual constituents, at an equivalent dose [3, 12]. Thus, we designed the study to explore this property of whole plant extracts, from the perspective of radioprotection. Here, we have correlated the cytoprotective and antimutagenic effect of PM1 with its corresponding phytochemical constituents at equivalent doses against radiation-induced cellular injury in V79 cells.

PM1, as evaluated by cytotoxicity assay, was found to be non-toxic to V79 cells. Fenton reaction is known to generate hydroxyl radicals, which can produce DNA strand breaks [13] and membrane damage by inducing lipid peroxidation [14], an effect similar to radiation exposure. PM1 pre-treatment reduced the intensity of calf thymus DNA damage (induced by *in vitro* Fenton reaction) and decreased lipid peroxidation by 71.2 % in $\gamma$-irradiated V79 cells, thus conferring protection against oxidative damage. This could be attributed to the free radical scavenging action of PM1.

PM1 was then subjected to a set of cytoprotective and antigenotoxic studies in V79 cells. At 25 µg/ml of PM1, there was a 2-3 fold increase in cytoprotection as seen from the dose modifying factors (DMFs) in cell viability assay and clonogenic assay. This could be due to the reduction in radiation-induced oxidative stress by both free radical scavenging and improved endogenous antioxidant/enzyme status conferred by PM1 [4]. When subjected to antigenotoxic studies, treatment with PM1 at 25 µg/ml
significantly inhibited radiation-induced DNA breaks in micronuclei assay as evident from the reduced % MNBNC (Fig. 5.5 and Fig. 5.6), by as much as 57%. PM1 (25 µg/ml) also reduced both the number and intensity of DNA bands as observed from DNA ladder assay. Thus, the effective dose of PM1, taken forward for comparison studies, was optimised at 25 µg/ml.

The concluding segment of our study was to compare the activity of PM1 at the best radioprotective dose (25 µg/ml) with its constituent polyphenols (quercetin-3-O-rutinoside, 3-O-caffeoylquinic acid and quercetin) at equivalent concentrations present in 25 µg/ml of PM1. Since, quercetin-3-O-rutinoside, quercetin and 3-O-caffeoylquinic acid are the polyphenols reported with radioprotective activity [5, 6], cytoprotective and antigenotoxic studies were carried out for comparing the effect of PM1 (at the optimum dose of 25 µg/ml) with its constituent polyphenols. The cell survival data obtained from cytoprotective studies showed that the individual polyphenols failed to elicit any protection, whereas, the mixture of polyphenols (RuQC) rendered protection comparable to PM1. Additionally, it was observed from the antigenotoxic studies that quercetin-3-O-rutinoside and 3-O-caffeoylquinic acid treatment per se conferred protection, though not as significantly as RuQC. Quercetin, however, failed to show any antigenotoxic response.

Earlier reports suggest that quercetin is effective as a radioprotector only at microgram concentrations [6]. We found that the polyphenol mixture containing nanogram quantities of quercetin in combination with quercetin-3-O-rutinoside and 3-O-caffeoylquinic acid (two major constituents) conferred significant ($p < 0.05$) overall protection to V79 cells, which was comparable to PM1. There are several studies endorsing this concept, where synergism by plant polyphenols have elicited improved
radioprotection [15-17]. Besides synergism playing a pharmacodynamic role in this scenario, there are reviews which highlight that antioxidant polyphenols when administered in pure form sometimes become more harmful than beneficial in pathological situations. Antioxidant-rich foods such as fruits, vegetables, and spices provide both protective and prudent preventive strategy in a pathological state than supplementation solely with a pure antioxidant [18-21]. This statement strengthens our findings in radiation-mediated pathological condition, where the plant extract PM1, rich in polyphenols, conferred better protection than the individual polyphenols.

Herbal extracts efficiently restore the disturbed equilibrium during radiation injury, in a collective and holistic manner [4, 22-25] owing to their varied phytochemical spectra. Not only radioprotection, but other biological activities have also improved using combination therapies with plant polyphenols, functioning via multifarious mechanisms in a synergistic manner. For example, it has been observed that the effectiveness of artemisinin, a major bioactive component present in the traditional Chinese herbal preparations (tea), is augmented by the co-existing polyphenols against malaria and cancer [26]. Our study, in line with previous reports, establishes that polyphenols are antioxidants with radioprotective potential. However, the overall activity of herbal extracts is a result of several polyphenols acting in concert, encountered at sub therapeutic concentrations in the extracts.

To conclude, PM1 appears to protect V79 cells from radiation-induced cytotoxicity and genotoxicity by three interdependent mechanisms, namely free radical scavenging, augmentation of antioxidant status, and inhibition of lipid peroxidation. These properties are conferred synergistically by the constituent polyphenols, rendering PM1 more effective than the individual constituents at equivalent
quantities. Further studies are warranted to unravel the mechanism that leads to such synergistic effects in herbal extracts.

5.5. Conclusion

- Active fraction of *Pilea microphylla* (PM1) was evaluated for its free radical scavenging potential using Fenton reaction-induced DNA damage and lipid peroxidation. Further, study was designed to compare cytoprotective and antigenotoxic activity of PM1 with that of its active polyphenolic constituents against \( \gamma \)-radiation in V79 cells.

- PM1 significantly reduced free radical-mediated calf thymus DNA damage and lipid peroxidation. Among the concentrations tested (12.5, 25 and 50 \( \mu g/ml \)) for radioprotection, PM1 at 25 \( \mu g/ml \) exhibited maximum protection. Further, when compared with constituent polyphenols viz., quercetin-3-\( O \)-rutinoside, quercetin and 3-\( O \)-caffeoylquinic acid (equivalent to PM1-25 \( \mu g/ml \)), a combination of polyphenols was found most effective in preventing \( \gamma \)-radiation-induced cytotoxicity and genotoxicity.

- Synergism by polyphenols/flavonoids has elicited improved radioprotection. Therefore, radioprotection is possibly a synergistic effect of the phytochemicals present in the herbal extract, rather than any single component.
5.6. References


Chapter 5  Comparative studies of PM1 with its active constituents against γ-radiation-induced cytotoxicity and genotoxicity in V79 cells


