Summary

The chapter deals with the *in vitro* radioprotective mechanism of protection of quercetin-3-*O*-rutinoside (PMC-1), the major constituent isolated from PM. The reaction of radiolytically generated hydroxyl radical with protein, lipid or DNA molecules was prevented by PMC-1. Further, to understand the reactivity of these radicals, pulse radiolysis studies were performed. The determined rate constants for the reactions with different free radicals [hydroxyl (•OH) and trichloromethyl peroxyl (CCl₃O₂•)] indicated antioxidant activity of PMC-1. The apoptotic effect of PMC-1 was further established by monitoring levels of phospho-p53, p53, Bad, cleaved caspase-3 and cleaved PARP in irradiated V79 cells. PMC-1 pretreatment significantly (*p*<0.05) decreased the radiation-induced increase in of phospho-p53, p53, Bad, cleaved caspase-3 and cleaved PARP [Poly (ADP-ribose) polymerase] in V79 cells. PMC-1, by itself did not produce any change in apoptotic markers compared to normal control.

3.1. Introduction

The role of free radicals in disease manifestation has drawn considerable attention over the past two decades. Free radicals can be defined as molecules (or molecular fragments) with an unpaired electron in the outer orbital [1]. Due to the unpaired electron, free radicals have high chemical reactivity and short half-life [2]. They can react with macromolecules such as proteins, DNA, and lipids to produce various damaging effects depending upon their reactivity [3].

To understand the reactivity of radicals which occur on timescales faster than approximately $10^{-4}$-$10^3$ s, pulse radiolysis technique were used. Pulse radiolysis is a method for studying events occurring between $10^{-11}$ and $10^{-2}$ s after energy absorption [4]. This provides the extent and timescale of radiation damage in dilute aqueous solutions and a simplistic use of experimental conditions to isolate a single reactive species [5].
As described in chapter 2, we isolated and characterized PMC-1, as the most potent compound based on its antioxidant potential, protection against radiation-induced ROS and DNA damage in V79 cells. Based on the results obtained from chapter 2, we focused on evaluating the in vitro radioprotective mechanism of PMC-1.

3.2. Materials and Methods

3.2.1. Chemicals

2-propanol, Bovine serum albumin (BSA), carbon tetrachloride (CCl₄), dinitrophenylhydrazine (DNPH) and guanidine hydrochloride were purchased from Sigma Chemicals (St.Louis, MO, USA). BCA protein kit was purchased from Thermo Fisher Scientific Inc., (MA, USA). Thiobarbituric acid (TBA) and trichloroacetic acid (TCA) were purchased from HiMedia, Mumbai. Butylated hydroxytoluene (BHT) was purchased from Merck, Mumbai. Plasmid DNA (pBR322) and loading buffer were obtained from Bangalore Genei, India. All the other chemicals and solvents used were of analytical grade. The reagent solutions were prepared in nanopure water from a Millipore Milli-Q system just before the use.

3.2.2. Protein carbonylation assay

One milligram of protein was dissolved in 1 ml of 10mM phosphate buffer (pH 7.4). Different concentrations of PMC-1 (0-100μM) were added to the protein solution. Control sample was prepared by substituting test compound with buffer. Samples were exposed to γ-radiation at an absorbed dose of 50 Gy (dose rate 40 Gy/min). Following irradiation, the protein was precipitated with ice chilled 40% TCA. The pellet was suspended in DNPH and incubated at room temperature for 4 h. Proteins were reprecipitated with 10% TCA. Excess DNPH was removed by washing with
ethyl acetate in ethanol (1:1) till the pellet decolorized. The protein pellet was then
dissolved in 6N guanidine hydrochloride and the absorbance was measured at 370
nm. The results are expressed in terms of percentage inhibition in radiation-induced
protein carbonylation [6].

3.3.3. Lipid peroxidation assay

Lipid peroxidation (LPO) studies were carried out in rat brain homogenate in
phosphate buffer (pH 7.4). Samples were exposed to γ-radiation at an absorbed dose
of 200 Gy (dose rate 40 Gy/min) both in the absence and presence of different
concentrations of PMC-1 (0-100µM). The extent of LPO was estimated in terms of
thiobarbituric acid reactive substances (TBARS) using TBA reagent (15% w/v TCA,
0.375% w/v TBA, 0.25 N hydrochloric acid, 0.05% w/v BHT). The absorbance was
measured at 532 nm. The results are expressed in terms of percentage inhibition in
radiation-induced LPO [6].

3.2.4. pBR322 DNA damage assay

Agarose gel electrophoresis was employed to assess the radiation-induced damage to
pBR322 DNA. Agarose gel (1%) was prepared in 130 mM tris-borate/2.5 mM EDTA
(TBE) buffer, ethidium bromide was added to the gel (for visualization of the DNA
bands in a UV transilluminator). About 400 ng of pBR322 DNA was suspended in 20
µl of 10 mM sodium phosphate buffer (pH 7.4) and exposed to an absorbed dose of
50 Gy (dose rate 40 Gy/min) both in the absence and presence of different
concentrations of PMC-1 (0-50 µM). Control and test samples were mixed with
loading dye (0.25% bromophenol blue and 30% glycerol) and loaded into the wells.
The gel was submerged in an electrophoresis tank filled with tris borate EDTA (TBE)
buffer. Electrophoresis was carried out at 60 V for one and half hours to separate the open circular (OC) and the super coiled (SC) form of DNA. The movement of the DNA bands was visualized on a UV transilluminator. The band intensity was quantified by uvitec software (Gel documentation system) [6, 7].

3.2.5. Pulse radiolysis studies

3.2.5.1. Reaction of hydroxyl (\(^{\cdot}\)OH) and trichloromethyl peroxyl (CCl\(_3\)O\(_2\)^{\cdot}\)) radical

Pulse radiolysis experiments were carried out with high-energy electron pulses (7 MeV, 500 ns) obtained from linear electron accelerator (LINAC) assembly at BARC, Mumbai. [8]. The transients were detected by absorption spectrometry. The absorbed dose was measured by using aerated thiocyanate dosimeter by monitoring the (SCN\(_2\)^{\cdot}\) species at 475 nm with \(G_e\) value of 2.59 x 10\(^{-4}\) m\(^2\)J\(^{-1}\) [9]. Here, \(G\) denotes the radiation chemical yield in mol/J and \(e\), the molar absorption coefficient in m\(^2\)/mol.

Typical dose/pulse used for these studies varied from 8 Gy to 100 Gy. Radiolysis of water produces \(e_{aq}^{\cdot}\), \(H^{\cdot}\) and \(^{\cdot}\)OH radicals in addition to the molecular products \(H_2\), \(H_2O_2\), and \(H_3O^{\cdot}\) [10, 11].

\[
H_2O \rightarrow ^{\cdot}H, ^{\cdot}OH, e_{aq}^{\cdot}, H_2, H_2O_2, H_3O^{\cdot} \tag{1}
\]

The reaction with \(^{\cdot}\)OH radicals was carried out in the presence and absence of PMC-1 (30\(\mu\)M) in \(N_2O\)-saturated solutions, where \(e_{aq}^{\cdot}\) is quantitatively converted to \(^{\cdot}\)OH radicals

\[
N_2O + e_{aq}^{\cdot} \rightarrow ^{\cdot}OH + OH^- + N_2 \tag{2}
\]

CCl\(_3\)O\(_2\)^{\cdot}\) radicals were generated by irradiating the aerated aqueous solution in the presence and absence of PMC-1 (130\(\mu\)M) containing 48% 2-propanol, 4% of CCl\(_4\)
and 48% 20 mM phosphate buffer (pH = 8.5) [12, 13]. The formation of \( \text{CCl}_3\text{O}_2^- \) radical was as follows.

\[
\begin{align*}
(\text{CH}_3)_2\text{CHOH} + \text{H}^+ + \cdot \text{OH} & \rightarrow (\text{CH}_3)_2\text{C}^\cdot \text{OH} + \text{H}_2\text{O} & (3) \\
(\text{CH}_3)_2\text{C}^\cdot \text{OH} + \text{CCl}_4 & \rightarrow \text{CCl}_3^- + (\text{CH}_3)_2\text{CO} + \text{H}^+ + \text{Cl}^- & (4) \\
\text{CCl}_4 + e^-_{\text{aq}} & \rightarrow \text{CCl}_3^- + \text{Cl}^- & (5) \\
\text{CCl}_3^- + \text{O}_2 & \rightarrow \text{CCl}_3\text{O}_2^- & (6)
\end{align*}
\]

Based on the above studies and results obtained from chapter 2, PMC-1 was further taken up to understand the mechanism of action.

### 3.2.6. Mechanism of protection

#### 3.2.6.1. Apoptosis assay in V79 cells by kit method

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

#### 3.2.6.1.1. Experimental design

- **Group 1** – Normal control
- **Group 2** – Drug control (PMC-1 alone)
- **Group 3** – Radiation control (10 Gy)
- **Group 4** – PMC-1 + Radiation: Cells treated with PMC-1 (50µM) for 30 min prior to \( \gamma \)-radiation (10 Gy).
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**In vitro radioprotective mechanism of PMC-1**

3.2.6.1.2. Preparation of cell lysate

1. V79 cells were incubated with PMC-1 (50 μM) for 30 min and then exposed to γ-radiation (10 Gy).
2. After irradiation, cells were harvested under nondenaturing conditions, medium was removed and cells were rinsed once with ice-cold phosphate buffer saline (PBS).
3. PBS was removed and 0.5 ml ice-cold 1X cell lysis buffer plus 1mM phenylmethylsulfonyl fluoride (PMSF) was added to each plate and incubated on ice for 5 minutes.
4. Cells were scraped off the plate and transferred to appropriate tubes.
5. Lysates were sonicated on ice, microcentrifuged for 10 minutes at 4°C and transferred the supernatant to a new tube.
6. The supernatant is the cell lysate. Single-use aliquots were stored at –80°C.

3.2.6.1.3. Total protein content

Total protein in cell lysate was estimated by the BCA Protein Assay Kit (Thermo Fisher Scientific Inc., MA, USA). The protocol was followed by the standard procedure supplied by the manufacturer of the kit as described in chapter 2.2.8.8.4.

3.2.6.1.4. Test Procedure

The method employed follows PathScan® Apoptosis Multi-Target Sandwich ELISA Kit #7105 (Cell Signaling Technology Inc., Danvers, MA, USA). The protocol was followed by the standard procedure supplied by the manufacturer of the kit as shown below.
1. 100 μl of sample diluent (0.1% Tween 20 in 20 X PBS) is mixed with 100 μl of cell lysate in a microcentrifuge tube and then vortexed for a few seconds.

2. 100 μl of each diluted cell lysate was added to the appropriate well, sealed with tape and pressed firmly onto top of microwells. The plates were incubated overnight at 4°C, which gives the best detection of target protein.

3. Gently removed the tape and washed the wells following the steps below.
   a. Discarded plate contents into a receptacle.
   b. Washed 4 times with 1X wash buffer, 200 μl each time for each well.
   c. After each wash, plates were tapped on fresh tissue papers to remove the residual solution in each well, without allowing the wells to completely dry.
   d. Cleaned the underside of all wells with a lint-free tissue.

4. 100 μl of detection antibody (green-colored bottle matching with the corresponding marker) was added to each well, sealed with tape and incubated the plate for 1 hour at 37°C.

5. Repeated wash procedure as in Step 3.

6. 100 μl of horseradish peroxidase (HRP)-linked secondary antibody (red-colored solution) was added to each well, sealed with tape and incubated the plate for 30 minutes at 37°C.

7. Repeated wash procedure as in Step 3.

8. 100 μl of 3,3′,5,5′-tetramethylbenzidine (TMB) substrate was added to each well, sealed with tape and incubated the plate for 30 minutes at 25°C.

9. 100 μl of STOP solution was added to each well and shook gently for a few seconds.
10. Absorbance of each well was read at 450 nm using an ELISA microplate reader and following markers were assessed; phospho-p53, p53, Bad, phospho-Bad, cleaved caspase-3 and cleaved PARP.

3.2.7. Statistical analysis

Statistical significance between the groups was determined by one-way analysis of variance (ANOVA) followed by post hoc Tukey’s test using GraphPad Prism version 5.02. \( p < 0.05 \) was considered to be significant. All values were expressed as mean ± standard error of mean (SEM).

3.3. Results

3.3.1. Inhibition of radiation-induced protein carbonylation and lipid peroxidation by PMC-1

Fig. 3.1 (A & B) represents percentage inhibition of protein carbonylation and lipid peroxidation with increasing concentration of PMC-1. IC\(_{50}\) values for protein carbonylation and lipid peroxidation were found to be 51.1 ± 1.9\( \mu \)M and 42.5 ± 1.2 \( \mu \)M respectively.
Fig. 3.1. % inhibition of protein carbonylation (A) and LPO (B) with varying concentrations of PMC-1. All values are expressed as mean ± SEM and experiments were carried out in triplicate.

3.3.2. Inhibition of radiation-induced pBR322 DNA damage by PMC-1

Fig. 3.2 shows agarose gel electrophoresis pattern. Lanes 1-6 show the pattern of separation for the irradiated plasmid DNA in the presence/absence of varying concentrations of PMC-1 (0-50 µM). On exposure to γ-radiation, the SC form of DNA was converted to the OC form. In presence of PMC-1, the damage to the SC form was reduced. The intensities of OC and SC form of plasmid DNA were measured and the ratio of OC/SC was plotted as a function of the concentrations of PMC-1 (Fig. 3.3). IC₅₀ values were estimated to be 25.8 ± 2.5 µM.
Fig 3.2. Agarose gel electrophoretic pattern, depicting super coiled and nicked open circular forms of plasmid DNA protection, by varying concentrations of PMC-1 against 50 Gy $\gamma$-radiation induced strand breaks. Electrophoresis was carried out at 60 V for 90 min to separate the open circular (OC) and the super coiled (SC) form of DNA. The arrow indicates the direction of electrophoretic mobility.

**Lane 1**: pBR322 control

**Lane 2**: pBR322 treated with PMC-1 alone

**Lane 3**: pBR322 exposed to $\gamma$-radiation of 50 Gy

**Lanes 4-6**: pBR322 exposed to $\gamma$-radiation dose of 50 Gy in presence of 10, 25 and 50 $\mu$M of PMC-1 respectively

Fig. 3.3. Line graph showing ratio of OC/SC form as a function of the concentrations of PMC-1.

$IC_{50} = 25.8 \pm 2.5 \mu$M
3.3.3. Pulse radiolysis

3.3.3.1. Reaction of hydroxyl radical (\(^{\cdot}\)OH) with PMC-1

Pulse radiolysis experiments revealed that the reaction of \(^{\cdot}\)OH radical with PMC-1 (30\(\mu\)M) produced a transient which has absorption maxima at 470 nm, and a bleaching signal at 390 nm, due to the parent absorption. The transient spectra for the reaction of \(^{\cdot}\)OH radical with PMC-1 are shown in Fig. 3.4.

![Transient spectrum obtained during reactions of \(^{\cdot}\)OH radical with PMC-1.](image)

**Figure 3.4.** Transient spectrum obtained during reactions of \(^{\cdot}\)OH radical with PMC-1.

The reaction of \(^{\cdot}\)OH radical with aromatic substrate proceeds through different ways, such as addition to ring, one-electron oxidation and hydrogen atom abstraction. To understand the mode of reaction for \(^{\cdot}\)OH radical, the transient spectra with specific one electron oxidant, azide radical (\(N_3^{\cdot}\)) was carried out.

The transient spectra for the reaction of \(N_3^{\cdot}\)radical with PMC-1 are shown in Fig. 3.5 which also has absorption band at 470 nm and bleaching at 390 nm. By comparing
both the spectra obtained from \(*\text{OH}\) radical and \(N_3^*\) it is evident that the species absorbing at 470 nm was due to one electron oxidized product of PMC-1 that is the corresponding phenoxy radical derived from phenolic OH groups present in the molecules.

The phenoxy radicals thus produced are easily stabilized throughout the flavan nucleus. The bimolecular rate constant for the reaction of \(N_3^*\) radical with PMC-1 was determined from the buildup kinetics of the transient at 470 nm in presence of different concentration of PMC-1. The bimolecular rate constant (\(k\)) was determined from the slope of linear regression plot of solute concentration against \(k_{\text{obs}}\) using equation 1, as shown in Fig. 3.5.

\[
k_{\text{obs}} = k[solute]
\]

The bimolecular rate constant of PMC-1 for the reaction with \(N_3^*\)radical was estimated as \(4.15 \times 10^9 \text{ M}^{-1}\text{s}^{-1}\).

![Figure 3.5](image-url)

**Figure 3.5.** Transient spectrum obtained during reaction of \(N_3^*\) radical with PMC-1. Inset shows the slope of linear regression plot of PMC-1 concentration against \(k_{\text{obs}}\).
3.3.3.2. Reaction of trichloro methyl peroxyl radical (CCl₃O₂•) with PMC-1

Peroxyl radicals are one of the common free radicals produced during oxidative stress. Halogenated peroxyl radicals are model peroxyl radicals, commonly used to study the reaction with antioxidants or free radical scavengers. Fig. 3.6 shows the transient absorption spectra of PMC-1 after reaction of CCl₃O₂• radical at pH 7.

The transient formed by CCl₃O₂• radical reaction with PMC-1 has weak broad absorption band from 470 nm along with a bleaching signal at 380 nm. The reaction of CCl₃O₂• radical with PMC-1 was relatively slower than that of *OH radical because CCl₃O₂• radical is less powerful oxidant than *OH radical. As discussed earlier for *OH and N₃• radicals, the band at 470 nm in Fig. 3.6 is assigned for oxidation of PMC-1 by CCl₃O₂• radical.

The bimolecular rate constant of PMC-1 for the reaction with CCl₃O₂• radical was estimated as 3.47 x 10⁷ M⁻¹s⁻¹ using equation 1.

![Fig. 3.6](image-url)

**Fig. 3.6.** Transient spectrum obtained during reactions of CCl₃O₂• radical with PMC-1. Inset shows the slope of linear regression plot of PMC-1 concentration against k_{obs}.
3.3.4. Mechanism of protection

3.3.4.1. Evaluation of apoptotic markers in irradiated V79 cells

Fig. 3.7 shows γ-radiation-induced changes in apoptotic markers in V79 cells. PMC-1 pretreatment significantly ($p<0.05$) decreased the radiation-induced increase in phospho-p53 (2.2 fold), p53 (2.0 fold), Bad (1.6 fold), cleaved caspase-3 (1.4 fold) and cleaved PARP (1.6 fold) in V79 cells, compared to radiation control.

However, there was no significant change observed in radiation-induced phospho-Bad level in V79 cells. PMC-1, itself did not produce any change in apoptotic markers compared to normal control.
Fig. 3.7. Effect of PMC-1 on apoptotic markers in irradiated (10 Gy) V79 cells. (A) p53, (B) phospho-p53, (C) Bad, (D) phospho-BAD, (E) cleaved caspase-3 and (F) cleaved PARP. Results are presented as mean ± SEM (n=3). *p < 0.05 as compared to normal control, #p < 0.05 as compared to radiation control.
Discussion

Free radicals, generated by radiation, attack bio-molecules such as the fatty acid component of membrane lipids and DNA, leading to lipid peroxidation, strand breaks and ultimately cell death [14]. The reaction of radiolytically generated hydroxyl radical with protein, lipid or DNA molecules was prevented by PMC-1. On exposure to γ-radiation, the SC form of DNA was converted to the OC form. In presence of PMC-1, the damage to the SC form was reduced. This indicates that radiation-induced DNA damage which is primarily mediated by hydroxyl radicals is attenuated effectively by PMC-1. Further, pulse radiolysis studies of PMC-1 were performed to determine rate constants towards reactions with different free radicals, indicating radical scavenging property of PMC-1. The ability of PMC-1 to inactivate these radicals may be attributed to the presence of phenolic OH group. The above activities may be probably due to 3’,4’-catechol ortho-dihydroxy moiety present in the B-ring of PMC-1, which has a strong affinity for phospholipid membranes as well as due to the presence of multiple hydroxyl groups that make these molecules excellent free radical scavengers [15, 16]. Additionally, oxidation of B ring present in the catechol structure of PMC-1 results the formation of an ortho-semiquinone radical, which has been reported to be involved in the anti-lipid peroxidation activity [17]. Therefore, the strong antioxidant potential probably depends on the structure of PMC-1, especially the number and arrangement of hydroxyl groups and the extended conjugation in the molecular structure.

Another important aspect of radioprotective potential of PMC-1 at cellular level is its ability to modulate the cytoprotective and cytotoxic pathways. V79 cells have been widely used in the studies of DNA damage and DNA repair [18]. Radiation treated V79 cells showed increased levels of phospho-p53, p53, BAD, cleaved caspase-3 and
cleaved PARP, indicating apoptosis. Apoptosis occurs either through the intrinsic mitochondrial or extrinsic death receptor pathway [19, 20]. In the mitochondrial pathway, death stimuli target mitochondria either directly or through transduction by proapoptotic members of the Bcl-2 family [21]. Cell sensitivity to apoptotic stimuli depends on the interaction between pro-apoptotic and anti-apoptotic Bcl-2 proteins [22, 23]. The pro-apoptotic Bcl-2 proteins (Bad, Bax, Bak, etc.) in the cytosol act as sensors of cellular damage and relocate to the surface of the mitochondria due to cellular stress [24]. In the mitochondrion, Bax and Bak oligomerization is induced by p53 which then physically interacts with protective Bcl-xL and Bcl-2 on mitochondrial surface [25]. The interaction between pro-apoptotic and anti-apoptotic proteins disrupts the normal function of the anti-apoptotic Bcl-2 proteins, thus resulting in the formation of pores in the mitochondria through which cytochrome C and other pro-apoptotic molecules are released [26]. This event eventually leads to the activation of effector caspases, which are responsible for the cleavage of the key cellular proteins, that leads to characteristic morphological changes observed in cells undergoing apoptosis [27].

The cleavage of chromosomal DNA into nucleosomal units is one of the signals of apoptosis [28]. The caspases play critical role in the process of apoptosis by activating DNAses, inhibiting DNA repair enzymes and breaking down structural proteins in the nucleus [29]. As observed from DNA ladder assay, radiation induced fragmentation of DNA into nucleosomal units in V79 cells caused by an enzyme known as caspase activated DNase (CAD). This CAD usually exists as an inactive complex with ICAD (inhibitor of CAD), which is cleaved by caspases (caspase 3) during apoptosis releasing CAD, that triggers rapid fragmentation of the nuclear DNA [28, 30]. In the present study, the elevated level of cleaved caspase-3 (active) with radiation treatment
correlated the events of DNA damage. PARP, a protein involved in repair of DNA damage, was found reduced in radiation treated cells, indicated by increased levels of cleaved PARP by active caspase 3 [31]. The ability of PARP to repair DNA damage was thus arrested. All these parameters were reversed with PMC-1 pretreatment in V79 cells exposed to radiation.

3.5. Conclusions

- PMC-1 attenuated radiation-induced protein carbonylation, lipid peroxidation and DNA damage in pBR322. The determined rate constants for the reactions of PMC-1 with different free radicals [(•OH) and (CCl₃O₂•)] indicated antioxidant properties of PMC-1 carried out by pulse radiolysis.
- PMC-1 pretreatment decreased the radiation-induced increase in phospho-p53, p53, Bad, cleaved caspase-3 and cleaved PARP in irradiated V79 cells compared to radiation control.
3.6. References


