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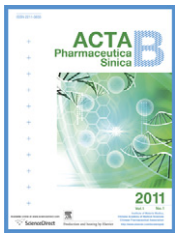

ORIGINAL ARTICLE

Phenolic compounds isolated from *Pilea microphylla* prevent radiation-induced cellular DNA damage

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Abstract Six phenolic compounds namely, quercetin-3-*O*-rutinoside **(1)**, 3-*O*-caffeoylquinic acid **(2)**, luteolin-7-*O*-glucoside **(3)**, apigenin-7-*O*-rutinoside **(4)**, apigenin-7-*O*-*β*-D-glucopyranoside **(5)** and quercetin **(6)** were isolated from the whole plant of *Pilea microphylla* using conventional open-silica gel column chromatography and preparative HPLC. Further, these compounds were characterized by 1D, 2D NMR techniques and high-resolution LC–MS. Compounds **1–3** and **6** exhibited significant antioxidant potential in scavenging free radicals such as DPPH, ABTS and SOD with IC_{50} of 3.3–20.4 μmol/L. The same compounds also prevented lipid peroxidation with IC_{50} of 10.4–32.2 μmol/L. The compounds also significantly prevented the Fenton reagent-induced calf thymus DNA damage. Pre-treatment with compounds **1–3** and **6** in V79 cells attenuated radiation-induced formation of reactive oxygen species, lipid peroxidation, cytotoxicity and DNA damage, correlating the antioxidant activity of polyphenols with their radioprotective effects. Compounds **1**, **3** and **6** significantly inhibited lipid peroxidation, presumably due to 3′,4′-catechol *ortho*-dihydroxy moiety in the B-ring, which has a strong affinity for phospholipid membranes. Oxidation of flavonoids, with catechol structure on B-ring, yields a fairly stable *ortho*-semiquinone

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radical by facilitating electron delocalization, which is involved in antioxidant mechanism. Hence, the flavonoid structure, number and location of hydroxyl groups together determine the antioxidant and radioprotection mechanism.

1. Introduction

Damage to normal tissues and the consequent side effects are critical problems associated with cancer radiotherapy. Radiation-induced normal tissue injuries are manifestations of increased production of reactive oxygen species (ROS) such as, superoxide radical ($O_2^{\cdot-}$), hydroxyl radical ($OH^{\bullet}$), hydrogen peroxide (H_2O_2), etc[1]. These radicals emerge as by-products of Fenton reaction in the intracellular compartments[2]. ROS are highly mutagenic and cause oxidative damage to cellular macromolecules resulting in DNA strand breaks, base modifications and genetic alterations[3]. These changes activate poly (ADP-ribose) polymerase, involved in a reaction that leads to apoptosis[4]. Therefore, protection against Fenton reagent-induced calf thymus DNA damage serves as a useful tool in testing bio-molecules that mitigate radiation injury. Plant metabolites exhibit a diverse array of biological activities that may be relevant in alleviation of ionizing radiation-induced damage in mammalian systems. The role of plant phenolics as modulators of cell signaling and gene expression, in addition to their antioxidant properties, has been reported[5]. Various mechanisms have been proposed to account for the antioxidant ability of phenolic compounds, which include their radical scavenging ability, metal chelating property and hydrogen donating ability. A correlation between the antioxidant property and radioprotection by phenolic compounds was first proposed by Shimoi and Emerit[6–9]. In principle, plant metabolites delay the oxidation of bio-molecules by inhibiting the initiation and propagation of oxidizing chain reactions, thereby interfering with the initiation of apoptosis[10].

Pilea microphylla (PM) is a traditionally used medicinal plant, with potential uses as antimicrobial and antioxidant in food industry[11]. PM is also reported to possess significant antioxidant and radioprotective properties[12]. Previously, we demonstrated that the flavonoid-rich fraction of *P. microphylla* (PM1) elicits free radical scavenging and exerts radioprotection to highly proliferative organs such as gastrointestinal tract (GIT) and spleen during acute radiation toxicities[12]. We have characterized PM1 by RP-HPLC and quantified the major constituent polyphenols[13]. In the present study, we have isolated six phenolic compounds **(1–6)** (Fig. 1) 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, in order to identify the biologically potent constituents responsible for conferring radioprotection to PM. All compounds were isolated using conventional open-silica gel column chromatography, prep HPLC and were fully characterized by extensive use of 1D, 2D NMR techniques, high-resolution LC-MS and evaluated by comparing with data in the literatures. Further, we investigated antioxidant potential of the compounds using *in vitro* free radical scavenging assays. Based on the observations, the compounds were screened for their radioprotective efficacy against γ-radiation-induced damage in V79 cells.

2. Materials and methods

2.1. Chemicals and instruments

The PM1 fraction was filtered through a Millex HA 0.45 μm filter (Millipore Co., Billerica, MA, USA), before injection. In the HPLC analysis, each peak was scanned in the range of 190–370 nm with the diode-array UV detector. Chromatographic data were collected and analyzed using the Shimadzu Class-VP software. Semi-preparative RP-HPLC was established on HPLC unit (Shimadzu Co., Kyoto, Japan) using a Phenomenex Onyx C_{18} Semi-Prep (100 mm × 10 mm) column with acetonitrile in 0.1% aqueous formic acid as mobile phase and a flow rate of 5 mL/min based on the analytical method reported earlier[13]. Conventional column chromatography was conducted with silica gel 100–200 mesh (Merck). Preparative TLC was performed on silica gel 60 GF_{254} plates (Merck) and plates were observed under a UV-CAMAG spectrometer (254 nm). NMR spectra were taken on an Inova-500 NMR spectrometer (Varian Medical Systems Inc., Palo Alto, CA, USA) at 499.8 and 125.7 MHz, respectively, in CD_3OD or DMSO-d_6. High resolution mass spectra (HRMS) were recorded by ESI-MS on an Apex IV 7 T Fourier-Transform Ion-Cyclotron-Resonance (FT-ICR) Mass Spectrometer (Bruker Daltonics, Billerica, MA, USA) with HP-Mix as standard. Chemical shifts are expressed in a δ (ppm) scale with tetramethylsilane as an internal standard and coupling constants values J in Hz. All chemicals and reagents used were purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA). For cell culture studies, Dulbecco's minimum essential medium (DMEM), 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), normal melting agarose (NMA), low melting agarose (LMA), Triton X-100, Trizma base, ethidium bromide and dichlorofluorescin di-acetate (DCFH-DA) purchased from Sigma-Aldrich were used. Fetal bovine serum (FBS), trypsin, sodium bicarbonate, thiobarbituric acid and sodium dodecyl sulfate (SDS) were purchased from HiMedia Laboratories Pvt. Ltd. (Mumbai, India). Tissue culture accessories were from Tarsons Products Pvt. Ltd. (Bangalore, India). All the other chemicals and solvents used were of analytical grade.

2.2. Plant material

The whole plant of PM was collected in the month of July to September from the mossy locations in and around Manipal, Udupi district, Karnataka, India. The specimen was authenticated by Dr. G.K. Bhat, (Department of Botany, Poornaprajna College, Udupi, India). A voucher specimen (MCOPS/PHCOL/2008/06) has been deposited in the herbarium of the Department

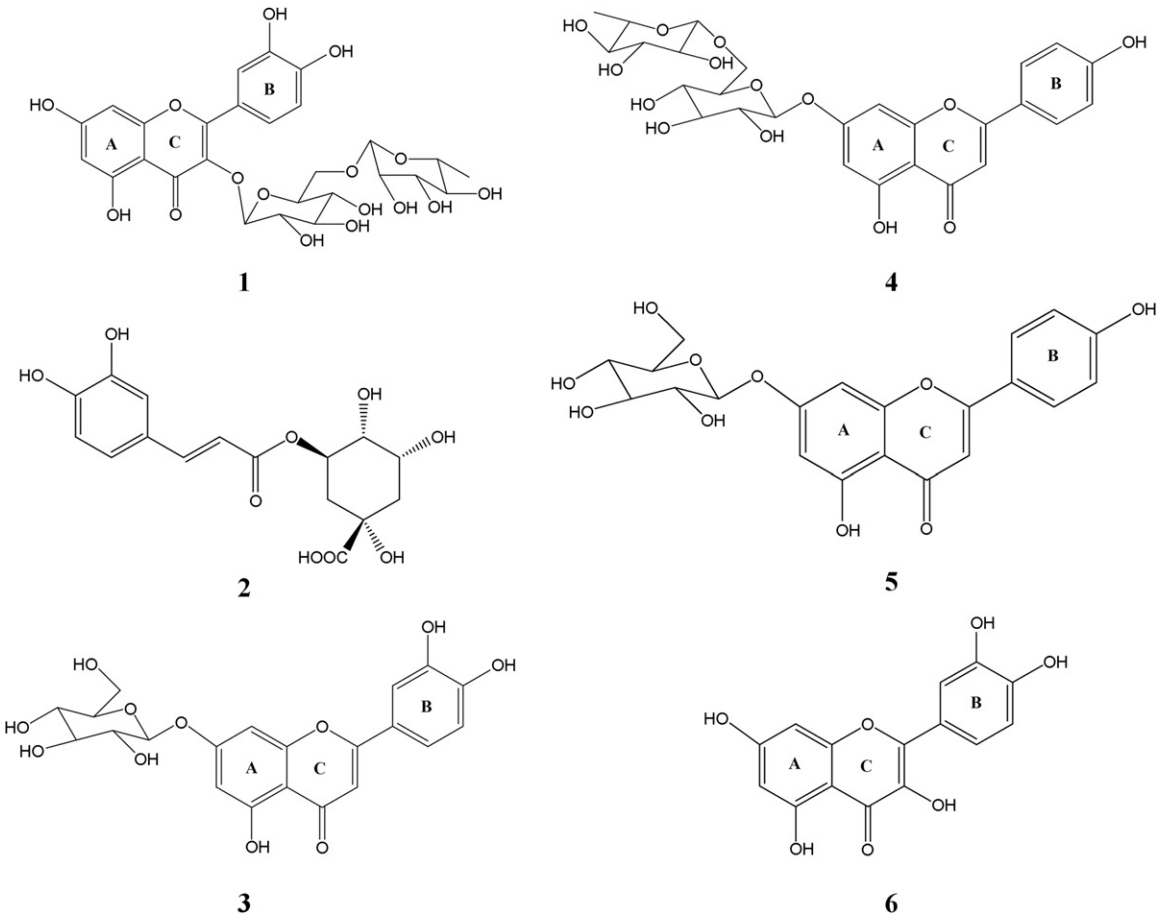

Figure 1 Structures of isolated phenolic compounds from *Pilea microphylla*. **1**: Quercetin-3-*O*-rutinoside; **2**: 3-*O*-Caffeoylquinic acid; **3**: Luteolin-7-*O*-glucoside; **4**: Apigenin-7-*O*-rutinoside; **5**: Apigenin-7-*O*-*β*-D-glucopyranoside; **6**: Quercetin.

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2.3. Extraction and isolation

Dried plant material (200 g) of *P. microphylla* was extracted with EtOH (1 L × 3) for 3 h to afford the EtOH extract (29 g). A portion of the EtOH extract (28 g) was dissolved in water (150 mL) and extracted with petroleum ether (750 mL × 3) to yield a petroleum ether-soluble fraction. The remaining residue was further extracted with acetone (500 mL × 3) to afford the acetone soluble fraction, which on concentration under reduced pressure yielded a residue (10 g) (PM1).

PM1 was found to be most active fraction and therefore was taken up for systematic isolation.

The residue (10 g) was subjected to silica gel column (60 mm × 6 mm) with a $CHCl_3$–MeOH–H_2O (7:2.5:0.5) isocratic gradient system to give fourteen (1–14) fractions. Fraction 8 (60 mg) was subjected to HPLC separation (Phenomenex Onyx C_{18} Semi-Prep (100 mm × 10 mm)), CH_3CN-0.1% HCOOH (1:9, flow rate 5 mL/min) to afford compound **2** (5.3 mg). Fractions comprising of 5–7 were combined (410 mg) and subjected to semi-prep HPLC, CH_3CN-0.1% HCOOH (3:7 and finally, 4:6; flow rate 5 mL/min) to yield compounds **1** (10.2 mg), **3** (2.8 mg), **4** (2.6 mg) and **5** (2.4 mg). Finally, fractions 2&3 (320 mg) afforded compound **6** using CH_3CN-0.1% HCOOH (1:1, 16.0 mg).

Quercetin-3-*O*-rutinoside (1): Pale yellow amorphous powder from methanol, mp 208–210 °C (dec); (−)-ESI MS: *m*/*z* 609 $[M–H]^-$, (+)-ESI MS: *m*/*z* 633.02 $[M+Na]^+$, 633.02 calculated for $C_{27}H_{30}O_{16}$. ^1H and ^{13}C NMR (DMSO-d_6) spectral data were in full agreement with literature[14].

3-*O*-Caffeoylquinic acid (2): White powder from methanol, mp 207–209 °C; (+)-ESI MS: *m*/*z* 353.07 $[M–H]^-$ (calculated 377.07 for $C_{16}H_{18}O_9Na$). ^1H and ^{13}C NMR (CD_3OD) spectral data were in agreement with literature[15].

Luteolin-7-*O*-glucoside (3): Yellow amorphous powder, mp 201–206 °C; (−)-ESI MS: *m*/*z* 447.05 $[M-H]^-$, (+)-ESI MS: *m*/*z* 449.09 $[M+H]^+$ (calculated 448.09 for $C_{21}H_{20}O_{11}$); ^1H and ^{13}C NMR (DMSO-d_6) spectral data were in agreement with literature[16].

Apigenin-7-*O*-rutinoside (Isorhoifolin) (4): Amorphous powder from methanol, mp 270–275 °C; (−)-ESI MS: *m*/*z* 577.04 $[M-H]^-$, (+)-ESI MS: *m*/*z* 579.08 $[M+H]^+$ (calculated 579.08 for $C_{27}H_{30}O_{14}$); ^1H and ^{13}C NMR (DMSO-d_6) spectral data were in agreement with literature[16].

Apigenin-7-*O*-*β*-D-glucopyranoside (5): Amorphous powder from methanol, mp 219–225 °C; (–)-ESI MS: *m*/*z* 431.04 $[M-H]^-$, (+)-ESI MS: *m*/*z*=433.08 $[M+H]^+$ (calculated 431.04 for $C_{21}H_{10}O_{10}$); ^1H and ^{13}C NMR (DMSO-d_6) spectral data were in agreement with literature[16].

Quercetin (6): Yellow powder from methanol, mp 315–316 °C; (−)-ESI MS: *m/z* 301.04 $[M–H]^-$, (+)-ESI MS: *m/z* 303.08 $[M+H]^+$ (calculated 301.04 for $C_{15}H_{10}O_7$); ^1H and ^{13}C NMR (DMSO-d_6) spectral data were in agreement with literature[17].

2.4. *In vitro free radical scavenging assays*

2.4.1. *Reaction with DPPH and ABTS*

Reactions of compounds were carried out with DPPH radical and ABTS radical anion. The reaction of DPPH with compounds (**1–6**) was followed by steady state[18]. For steady state measurements, 100 μmol/L DPPH in methanol was mixed with different concentrations of compounds (1–50 μmol/L) in methanol, and kept in dark for 20 min. The absorbance at 517 nm was monitored both in the presence and absence of compounds. The reaction of $ABTS^{\bullet-}$ with compounds was also followed by steady state methods. For steady state measurements, 100 μmol/L ABTS [produced by the reaction of 2 mmol/L $ABTS^{2-}$ with potassium persulfate (0.17 mmol/L) in phosphate buffer (pH 7.4, 20 mmol/L)] was mixed with compounds (**1–6**) (1–50 μmol/L), mixed well and the absorbance was monitored at 734 nm in the presence/absence of compounds. Experiment was performed in triplicate and ascorbic acid was used as standard.

2.4.2. *Estimation of superoxide dismutase (SOD)*

Superoxide was generated by NADH/phenazine methosulfate and was measured by NBT reduction method[19]. Reaction mixture contained phosphate buffer (100 mmol/L, pH 7), 20 μmol/L phenazine methosulfate, 156 μmol/L NADH, 600 μmol/L NBT and compounds (**1–6**) in different concentration (1–50 μmol/L). After incubating at room temperature for 10 min, absorbance was recorded at 560 nm; experiment was performed in triplicate and ascorbic acid was used as standard.

2.4.3. *Lipid peroxidation studies*

Egg phosphatidylcholine (20 mg) in chloroform (2 mL) was dried under vacuum in a rotary evaporator to give a thin homogenous film, and further dispersed in normal saline (5 mL) with a vortex mixer. The mixture was sonicated to get a homogeneous suspension of liposomes. Lipid peroxidation (LPO) was initiated by adding 50 μmol/L ascorbic acid to a mixture containing liposome (0.1 mL), 150 mmol/L potassium chloride, 200 μmol/L ferric chloride and different concentrations of compounds (1–50 μmol/L) in a total volume of 0.4 mL. The reaction mixture was incubated for 40 min at 37 °C. After incubation, 1 mL of ice-cold 0.25 mol/L hydrochloric acid containing 20% (*w/v*) trichloroacetic acid, 0.4% (*w/v*) thiobarbituric acid and 0.05% (*w/v*) butylated hydroxytoluene was added to terminate the reaction. The samples were then heated at 80 °C for 20 min, cooled, extracted with a constant amount of butanol, and the absorbance of the upper organic layer was measured at 532 nm[20].

2.5. *Calf thymus DNA protection studies*

Fenton reaction-induced calf thymus DNA protection studies were carried out according to previously described method[21]. In brief, reaction mixture with different concentration ranges (16–165 μmol/L) of compounds **1–3** and **6** were incubated for 20 min, loaded into wells followed by electrophoresis. Photographs were taken under UV (312 nm) transillumination to visualize DNA mobility.

2.6. *Radioprotection bioassays*

2.6.1. *Cell line and culture condition*

V79 cells were selected because of their stable karyotype, short generation time, and easy maintenance[22]. This cell line is well characterized and commonly used in mutagenicity and cytotoxicity studies[23]. V79 cells were procured from National Centre for Cell Science (NCCS), and maintained at 37 °C in an incubator with a humidified atmosphere of 5% CO_2. The cells were routinely cultured in 25 cm^2 flasks (Greiner Bio-One GmbH, Frickenhausen, Germany) containing Eagle's minimum essential medium (MEM), 10% heat inactivated fetal bovine serum (FBS) and Gentamycin (12 μg/mL). Nanopure water from Milli-Q system (Millipore Corp., Billerica, MA, USA) was used to prepare the media.

2.6.2. *Irradiation assay*

A monolayer of exponentially growing cells was harvested using trypsin–EDTA solution and cell suspensions were prepared for experiments. The cell cultures were irradiated with γ-radiation from a ^{60}Co source using a Theratron Tele Cobalt therapy source (Department of Radiotherapy, Shirdi Sai Baba Cancer Hospital, Manipal) at a dose rate of 1 Gy/min, calculated by ionization chamber dosimetry. The source to surface distance was adjusted as per dosimetric requirements. After exposure, the cell cultures were transferred to a 5% CO_2 incubator and allowed to grow up for the stipulated time period pertaining to the assay at 37 °C.

2.6.3. *Measurement of reactive oxygen species (ROS)*

Intracellular levels of ROS were estimated using a cell permeable oxidation sensitive probe DCF-DA whose fluorescence intensity increases after oxidation to dichlorofluorescein (DCF) by ROS[24]. Approximately, 25,000 cells were seeded in each well of a sterile 96-well black plate and incubated overnight. The following day, cells were incubated with oxidation sensitive DCF-DA (a final concentration of 50 μmol/L in media) for 1 h at 37 °C. After 1 h, the contents of the wells were aspirated out and replaced with same volume of media containing different concentrations (10–300 μmol/L) of compounds **1–3** and **6**. Plates were further incubated for 3–4 h and then irradiated with 10 Gy γ-radiations[25]. After 20 min, the plate was read at excitation wavelength 488 nm and emission wavelength 525 nm using fluorescence microplate reader (FL × 800, BioTek Instruments Inc., Winooski, VT, USA). The percentage inhibition of reactive oxygen species was calculated using the following formula: Percentage inhibition of ROS = (Fluorescence of irradiated cells − Fluorescence of compound treated cells)/(Fluorescence of irradiated cells) × 100%.

2.6.4. *Assessment of lipid peroxidation in V79 cells*

Lipid peroxidation (LPO) in V79 cells was assessed following previously reported method[26]. In brief, V79 cells were treated with compounds **1–3** and **6** (10–100 μmol/L) and after 1 h, irradiated with 10 Gy γ-radiation[26]. The cells were then

incubated for 24 h at 37 °C, followed by washing with PBS, scraping and homogenizing in ice-cold 1.15% KCl 100 μL of the cell lysates was mixed with 8.1% SDS, 20% acetic acid (adjusted to pH 3.5) and 0.8% thiobarbituric acid (TBA), followed by addition of distilled water to a final volume of 4 mL. The mixture was heated at 95 °C for 2 h. After cooling the mixture to room temperature, 5 mL of *n*-butanol and pyridine mixture (15:1, *v*/*v*) was added to each sample and shaken gently. The mixture was centrifuged at $1000 \times g$ for 10 min and the supernatant was collected. Thiobarbituric acid reactive substances (TBARS) in the supernatant were assayed from absorbance measured spectrophotometrically at 532 nm, using extinction coefficient of 1.56×10^5 L/mol/cm at 532 nm.

2.6.5. *Assessment of cell viability by MTT assay*

The effect of compounds **1–3** and **6** on the viability of V79 cells was determined using MTT assay, which is based on the reduction of a tetrazolium salt by mitochondrial dehydrogenase in viable cells[27]. V79 cells were incubated with compounds **1–3** and **6** (10–100 μmol/L) and then irradiated with 10 Gy γ-radiation[25,28]. Twenty four hours later, media was removed and 100 μL of the MTT stock solution (2 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 (EL × 800, BioTek Instruments Inc., Winooski, VT, USA) and % cytotoxicity calculated from the formula:

$$[(A_C - A_B) - (A_T - A_B)]/(A_C - A_B)$$

where A_C, A_T and A_B are absorbance of control, test and blank, respectively.

The % cell viability, considered as radio-protective effect, was calculated from the formula: (100% cytotoxicity).

2.6.6. *Evaluation of DNA damage by comet assay*

Oxidative DNA damage was induced by γ-radiation (10 Gy) in V79 cells[29], in the presence/absence of compounds **1–3** and **6** (50 μmol/L). The cells were processed for alkaline comet assay as described previously[30,31]. The slides were immersed in lysis buffer for 1 h at 4 °C and equilibrated in alkaline solution for 20 min, followed by electrophoresis at 18 V, 300 mA (Sub-Cell GT system with PowerPac basic power supply, Bio-Rad Laboratories Inc., Hercules, CA, USA). After electrophoresis, the slides were neutralized and stained by ethidium bromide. The images were captured using a fluorescence microscope (Eclipse TS100, Nikon Instruments Inc., Melville, NY, USA). Fifty images per slide were analyzed for tail length (TL), and olive tail moment (OTM) using image analyzer CASP software version 1.2.2.

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), $n = 3$.

3. Results

3.1. *In vitro free radical scavenging activity*

Compounds **1–3** and **6** scavenged 2, 2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) ($ABTS^{\bullet -}$) radicals in a concentration dependent manner. Their IC_{50} values for DPPH radicals were found to be 20.4 ± 0.3, 18.6 ± 0.1, 9.7 ± 1.0, 8.0 ± 0.2 and for $ABTS^{\bullet -}$ radicals, 5.9 ± 0.1, 8.0 ± 0.1, 6.7 ± 0.2 and 3.3 ± 0.1 μmol/L, respectively (Table 1). All the above compounds also scavenged superoxide radical at low concentrations (Table 1). However, compounds **4** and **5** did not exhibit any activity at the tested concentrations.

3.2. *In vitro lipid peroxidation assay*

The ability of the phenolic compounds **1–3** and **6** to inhibit iron-induced lipid peroxidation was studied using phosphatidylcholine liposomes with *a*-tocopherol as a reference standard. Quercetin-3-*O*-rutinoside (**1**), 3-*O*-Caffeoylquinic acid (**2**), luteolin-7-*O*-glucoside (**3**) and quercetin (**6**) were found to possess an IC_{50} of 32.2 ± 0.4, 10.4 ± 0.3, 23.7 ± 0.6, 17.2 ± 0.4 μmol/L, respectively, while that of *a*-tocopherol was found to be 364.1 ± 24.1 μmol/L (Table 1). However, compounds **4** and **5** did not exhibit any activity at the tested concentrations.

3.3. *Calf thymus DNA protection studies*

Hydroxyl radical-induced calf thymus DNA damage was studied (lanes 6 and 13). All tested doses of the phenolic compounds **1**, **2** (lanes 7–12), **3** and **6** (lanes 14–19) significantly showed protection against DNA damage. The drug controls by itself did not cause any damage to the DNA (lane 2–5) (Fig. 2). However, compounds **4** and **5** did not exhibit any activity at the tested concentrations.

Table 1 Steady state free radical scavenging of phenolic compounds expressed in IC_{50} (μmol/L).

Compound	DPPH	ABTS	SOD	LPx
1	20.4 ± 0.3	5.9 ± 0.1	10.6 ± 0.1	32.2 ± 0.4
2	18.6 ± 0.1	8.0 ± 0.1	5.7 ± 0.3	10.4 ± 0.3
3	9.7 ± 1.0	6.7 ± 0.2	7.6 ± 0.1	23.7 ± 0.6
6	8.0 ± 0.2	3.3 ± 0.1	8.9 ± 0.1	17.2 ± 0.4
Ascorbic acid	34.2 ± 0.4	23.8 ± 0.3	102.4 ± 0.6	–
a-tocopherol	–	–	–	364.1 ± 24.1

Results are expressed as mean ± SEM.

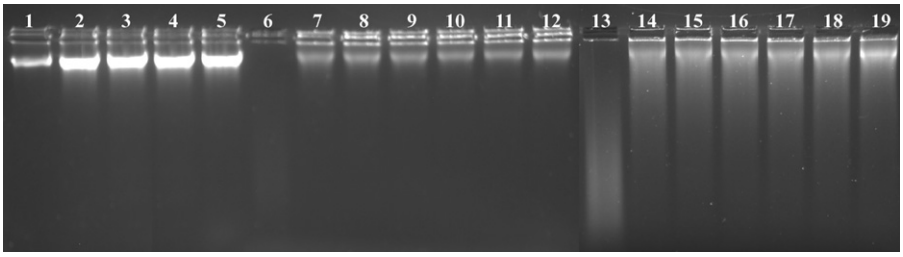

Figure 2 Calf thymus DNA protection studies by compounds **1–3** and **6**. Lane 1: DNA control; Lane 2: **1** (80 μmol/L)+DNA (**1** control); Lane 3: **2** (140 μmol/L)+DNA (**2** control); Lane 4: **3** (112 μmol/L)+DNA (**3** control); Lane 5: **6** (165 μmol/L)+DNA (**6** control); Lane 6: Fenton reaction (FR)-induced DNA damage; Lane 7: (16 μmol/L) **1**+DNA+FR; Lane 8: (41 μmol/L) **1**+DNA+FR; Lane 9: (80 μmol/L) **1**+DNA+FR, Lane 10: (28 μmol/L) **2**+DNA+FR; Lane 11: (70 μmol/L) **2**+DNA+FR; Lane 12: (140 μmol/L) **2**+DNA+FR, Lane 13: Fenton reaction (FR)-induced DNA damage; Lane 14: (22 μmol/L) **3**+DNA+FR; Lane 15: (56 μmol/L) **3**+DNA+FR; Lane 16: (112 μmol/L) **3**+DNA+FR, Lane 17: (33 μmol/L) **6**+DNA+FR; Lane 18: (83 μmol/L) **6**+DNA+FR; Lane 19: (165 μmol/L) **6**+DNA+FR.

Table 2 Inhibition (IC_{50} in μmol/L) of γ-radiation induced reactive oxygen species by phenolic compounds.

Compound	IC_{50} (μmol/L)
1	22.9±4.0
2	66.0±5.8
3	48.3±5.3
6	79.0±5.7

Results are expressed as mean±SEM.

3.4. *Effect of compounds **1–3** and **6** on intracellular ROS against 10 Gy γ-radiation*

Non fluorescent DCF-DA is hydrolyzed by intracellular esterases and readily oxidized to highly fluorescent dichlorofluorescein (DCF) in the presence of ROS. ROS induction in irradiated cells was found to be 86.52±6.81%. A dose dependent inhibition was observed at all tested concentrations (10–300 μmol/L) of the phenolic compounds (**1–3** and **6**). The IC_{50} for compounds **1**, **2**, **3** and **6** were determined as 22.9±4.0, 66.0±5.8, 48.3±5.3 and 79.0±5.7 μmol/L, respectively (Table 2).

3.5. *Effect of compounds **1–3** and **6** on cellular lipid peroxidation against 10 Gy γ-radiation*

Irradiation significantly ($P<0.05$) increased the level of TBARS in V79 cells. Pretreatment with compounds **1–3** and **6** (10–100 μmol/L) reduced the elevated TBARS level, in γ-irradiated cells, in a dose dependent manner (Fig. 3). Compound **1** effectively reduced TBARS at all concentrations tested. Compounds **2** and **3** attenuated lipid peroxidation at 50 and 100 μmol/L, whereas compound **6** showed significant effect at concentrations 25 μmol/L onwards. None of the compounds, by itself induced lipid peroxidation.

3.6. *Effect of compounds **1–3** and **6** on cell viability assessed by MTT assay against 10 Gy γ-radiation*

The radioprotective effects of compounds **1–3** and **6** on the survival rate of V79 cells, exposed to γ-radiation, were determined. As demonstrated in Fig. 4, compounds **1–3** and **6** increased percentage cell viability in the irradiated cells. Treatment of V79 cells with various concentrations (10–100 μmol/L) of compounds **1–3** and **6** before exposure to 10 Gy γ-irradiation resulted in a gradual increase in the cell viability (MTT formazan formation). Compounds **1**, **2**, **3** and **6** exhibited a maximum viability of 85.9%, 71.1%, 67.4% and 82.1%, respectively, against 10 Gy at 24 h post-irradiation (Fig. 4).

3.7. *Effect of compounds **1–3** and **6** on DNA damage assessed 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 head to tail, indicating DNA damage. Fig. 5 showed the frequency distribution histograms of tail length (TL) and Olive tail moment (OTM) of the treatment groups. All treatment groups showed significant ($P<0.05$) decline in TL and OTM compared to radiation control. However, maximum reduction in comet parameters TL and OTM was observed with compounds **1** and **6** pre-treatment.

4. Discussion

Most of the cellular alterations induced by ionizing radiations are mainly caused by free radicals, derived from oxygen[32–34]. These free radicals not only disturb the endogenous antioxidant milieu by altering cellular biochemistry, but also interfere with the genetic structure leading to apoptosis and cell death. In this context, free radical scavengers play a major role in radioprotection.

In our study, phenolic compounds (**1–6**) were isolated from PM using prep RP-HPLC and screened for *in vitro* free radical scavenging and radioprotective potential. These compounds have been previously investigated for their free radical scavenging potential in different testing environments[35,36]. Since, these polyphenols were isolated for the first time from *P. microphylla*, we carried out their detailed antioxidant activities both in cell-free as well as cell-based systems and correlated their structural aspects with their behavior towards oxidative stress. Compounds **1–3** and **6** scavenged DPPH and $ABTS^{\bullet-}$ radicals in a concentration dependent manner.

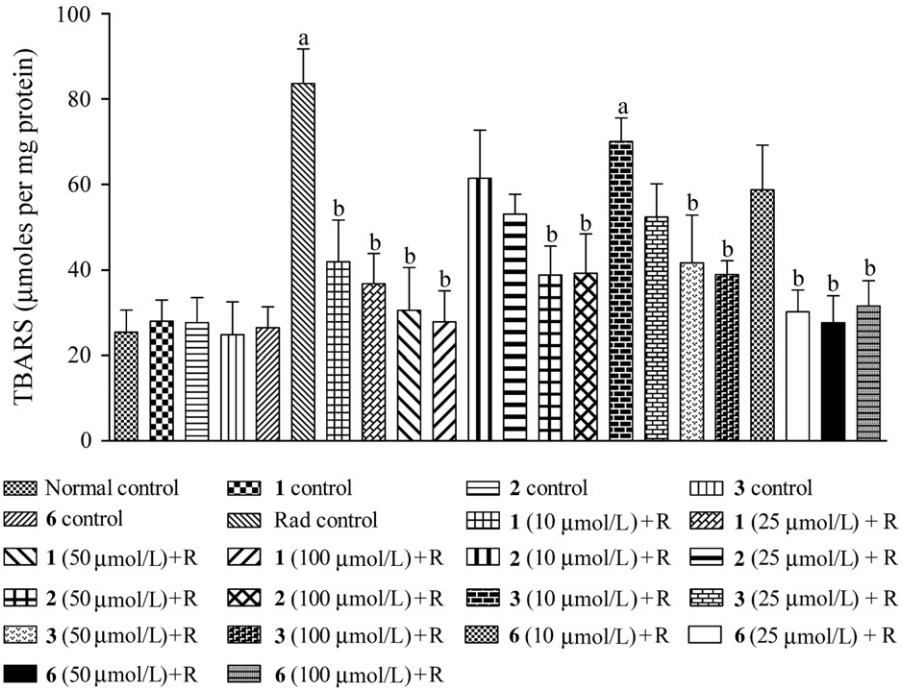

Figure 3 Effect of compounds (**1–3** and **6**) on cellular lipid peroxidation against 10 Gy γ-radiation. Lipid peroxidation was assayed by measuring amount of TBARS. All values are expressed as mean ± SEM and experiments were carried out in triplicate. [a]$P<0.05$ compared with normal control; [b]$P<0.05$ compared with radiation control.

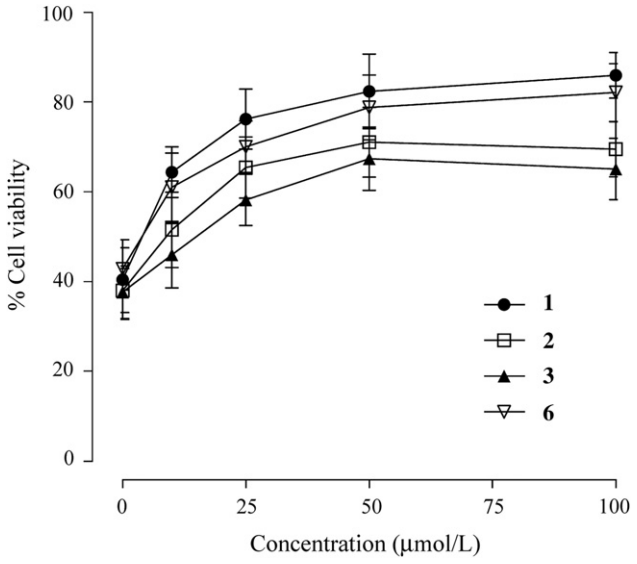

Figure 4 Effect of compounds (**1–3** and **6**) on cell viability assessed by MTT assay against 10 Gy γ-radiation. All values are expressed as mean ± SEM and experiments were carried out in triplicate.

Scavenging of DPPH and $ABTS^{\bullet-}$ free radicals point to reliable proton accepting and electron donating ability[37]. There are reports on the antioxidant activities of compounds, quercetin-3-*O*-rutinoside (rutin), 3-*O*-caffeoylquinic acid (chlorogenic acid) and quercetin in cell-free systems[38]. In these studies, the trolox equivalent antioxidant capacity of pure compounds revealed that quercetin-3-*O*-rutinoside (rutin), 3-*O*-caffeoylquinic acid (chlorogenic acid) and quercetin scavenged DPPH and $ABTS^{\bullet-}$ [39]. Our investigation on the compounds **1–3** and **6** for radical scavenging assays was consistent with previous observations, as the order of activity of the compounds differed in different testing environments (DPPH, ABTS, superoxide scavenging and inhibition of lipid peroxidation). The results showed that the tested flavonoids exhibited a much wider variation in the radical scavenging activity. Systematic analysis has revealed that the radical scavenging activities of polyphenols are greatly influenced by the number and configuration of phenolic hydroxyl groups in the molecules and also by glycosylation and configuration of other substituents[35]. The ability of compounds **1–3** and **6** to scavenge superoxide radicals at high potency probably stems from the B-ring *ortho*-dihydroxy configuration, which has been reported to enhance superoxide scavenging[40]. On the other hand, substitution on rings A and C has little impact on superoxide scavenging[41,42]. Superoxide is known to produce oxidative injury to enzymes, lipid membranes and DNA, which is attenuated by superoxide dismutase (SOD), a key antioxidant enzyme[43].

As observed from lipid peroxidation studies, compounds **1–3** and **6** markedly inhibited malondialdehyde (MDA) formation, indicating free radical scavenging potential. Lipid peroxidation is one of the important contributing factors for inflammatory responses[44]. Lipid peroxidation is also reported to increase phospholipase A activity, which further contributes to cell injury[45]. *In vitro* Fenton reaction is known to generate oxidizing species similar to γ-radiation and damage isolated DNA. Degradation of DNA by oxygen radicals is an important genotoxic mechanism associated with ageing[46]. Compounds **1–3** and **6** prevented Fenton reagent-induced calf thymus DNA damage. The probable mechanisms of protection to DNA could be mediated by the binding of the phenolic

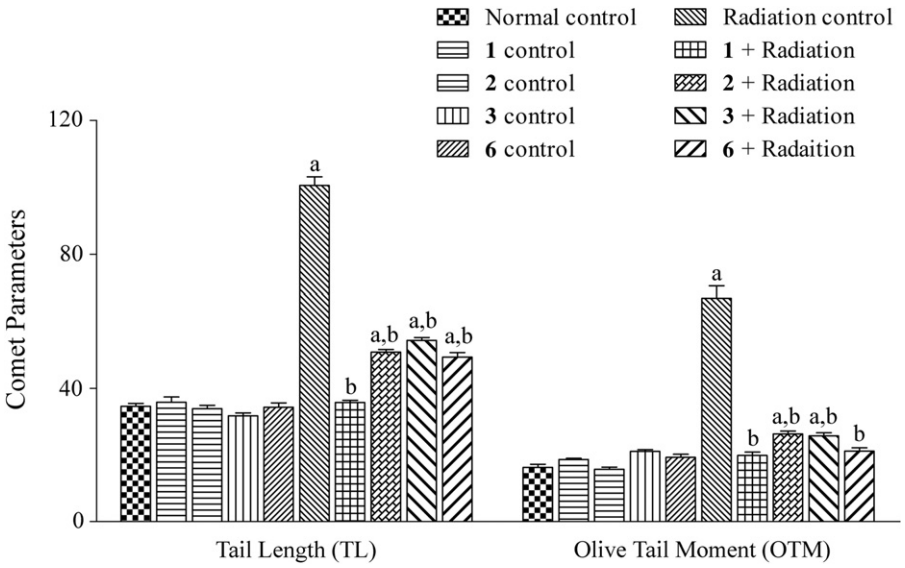

Figure 5 Effect of compounds (**1–3** and **6**) on γ-radiation-induced DNA damage at 10 Gy γ-radiation. Comet parameters such as Tail length (TL) and Olive tail moment (OTM) are represented as bar graph (means ± SEM), obtained by analyzing 100 cells. [a] $P<0.05$ compared to the normal control, [b] $P<0.05$ compared to the radiation control.

compounds to DNA either via the iron bound to the DNA or directly with the phosphate backbone[47,48]. This supports the protective role of phenolic compounds in plants and UV screen filters. These compounds present in plant epidermal layers strongly absorb in the UV region and protect DNA from oxidative damage[48].

Compounds **4** and **5** did not exhibit any antioxidant potential at tested concentrations. This could be attributed to the absence of catechol *ortho*-dihydroxy substitution in the B-ring of these compounds. The *ortho*-dihydroxy moiety seems to be the main requisite for the scavenging activity of catechol-containing derivatives[49–51].

Compounds **1–3** and **6**, which were found active in preliminary antioxidant screening were further subjected to ROS scavenging and radioprotection studies. Intracellular ROS in irradiated V79 cells were measured using a DCF-DA fluorescence assay. This was to evaluate the role of oxidative damage in cells and find possible correlations between oxidative stress modification and extent of DNA damage reduction. Pretreatment with the compounds **1–3** and **6** prevented γ-radiation-induced ROS formation in V79 cells. We also observed a significant increase in TBARS in irradiated V79 cells. A significant consequence of ROS-induced damage to cells or mitochondria is the generation of lipid hydroperoxides, conjugated dienes and malondialdehyde[52]. Membrane phospholipids being major targets of oxidative damage, lipid peroxidation is often the first parameter analyzed for proving the involvement of free radical damage[53,54]. Compounds **1–3** and **6** showed a dose-dependent reduction in radiation-induced lipid peroxidation.

Compounds **1–3** and **6** significantly protected V79 cells from γ-radiation-induced cytotoxicity, as observed from MTT assay. The reduction in intracellular ROS is possibly responsible for attenuating radiation-induced cytotoxicity, thereby improving survival rate. Compounds **1–3** and **6** also reduced DNA strand breaks conferring significant protection as assessed by comet assay. There is a considerable literature suggesting that free radical scavengers can be used to prevent apoptosis caused by ionizing radiation[55]. This property can be explored in the therapeutic application of phenolics in ameliorating radiation-induced genotoxicity.

Structure activity relationship (SAR) of test compounds provides clues to the antioxidant mechanisms of phenolic compounds in general. Phenolic compounds can easily donate electrons to reactive radicals. The resonance stability of phenoxy radical formed, retards radical chain reactions[36]. By a similar approach, compounds **1–3** and **6** containing dihydroxy moieties exhibit strong anti-lipid peroxidation and cytoprotective effects against radiation-induced injuries. There is a correlation between the inhibition of lipid peroxidation and cytoprotective capacity of these compounds. This correlation may be attributed to the presence of both hydrophilic and hydrophobic compartments in both living cells and lipid membranes. Quercetin-3-*O*-rutinoside (**1**), luteolin-7-*O*-glucoside (**3**) and quercetin (**6**) significantly inhibited lipid peroxidation, presumably due to 3,4-catechol *ortho*-dihydroxy moiety in the B-ring, which has a strong affinity for phospholipid membranes. Oxidation of the B-ring in the catechol structure of a flavonoid yields a fairly stable *ortho*-semiquinone radical, by facilitating electron delocalization, involved in antioxidant mechanism[56]. Thus, the structural criteria contributing to high antioxidant activity of the polyphenols include the *ortho*-dihydroxy groups (catechol structure) in the B-ring or in the A-ring, the 3-hydroxyl group or the 3-galloyl group (catechol structure) in the C-ring, and the 2,3-double bond in conjugation with 4-oxo function (carbonyl group) in the C-ring[35]. In previous studies it has been shown that flavonoids (quercetin-3-*O*-rutinoside, luteolin-7-*O*-glucoside and quercetin) containing the 3,4-catechol *ortho*-dihydroxy structure are more active than those containing the C-4′ position alone (kaempferol) or containing the dihydroxyl groups with meta-arrangement (C-2′ and C-5′) positions (morin). Thus, while lipophilicity facilitates compound permeation through the bilayer, specific structural configurations (catechol *ortho*-dihydroxy) are also

required for optimal antioxidant potency *in vitro* and in cell-based systems. Therefore, the interplay of hydrophilic and hydrophobic characteristics, together with the flavonoid structure, especially the number and location of hydroxyl groups, study the mechanism of antioxidant activities in the liposomal system and the cytoprotective effects in an oxidative stress-induced cell model[49,57]. In addition to the antioxidant potential, another reason contributing to radioprotection in the cell system is probably the increased bioavailability of the compounds. There are reports that suggest that glycoside forms of flavonoids have better absorption profiles compared to their aglycone forms. For example, quercetin glycosides contained in onions have higher absorption (52%) than quercetin aglycones (24%)[58]. The sugar portion bound to the aglycone portion increases the solubility in polar solvents and consequently improves absorption, responsible for increased cellular bioavailability[59]. Comparative radioprotective studies on plant polyphenols have also shown that the flavonoids quercetin-3-*O*-rutinoside and quercetin 3-*O*-glucoside maximally scavenged hydroxyl and superoxide radicals, thereby protecting rat liver mitochondrial lipids, proteins and plasmid pBR322 DNA from radiation induced damage[34]. Further, luteolin derivatives containing sugar group have been reported with greater lipid peroxidation inhibition compared to luteolin. All these evidences of increased antioxidant ability of the polyphenols in liposome system could be attributed to the sugar group[36]. A reasonable mechanism reported suggests that sugar group addition increases the polarity of the molecule. This facilitates the polar antioxidants to retard lipid peroxidation by scavenging water soluble oxygen species more effectively[36].

In conclusions, the radioprotective effect of phenolic compounds strongly correlated to their antioxidant activity in V79 cells. The radioprotective effects and antioxidant activity of the phenolic compounds were dependent on the arrangement of functional groups on core structure of the compounds. All these findings support the utilization of phenolic compounds in the mitigation of radiation injury and thus necessitate the *in vivo* optimization of isolated phenolic compounds for their viable use in radioprotection.

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