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Original Article

Antioxidant role of plumbagin in modification of radiation-induced oxidative damage

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ABSTRACT

Objective: Plumbagin is a well-known ingredient of Plumbago zeylanica, which is used in Ayurveda for its multiple therapeutic actions. We have attempted to understand protective effects of plumbagin against gamma radiation-induced damage to lipids, proteins, and enzymes of rat liver mitochondria in vitro. **Methods:** Rat liver mitochondrial preparation of female Wistar rats has been exposed to gamma radiation (450 Gy) to induce oxidative damage of its components. The protection offered by increasing concentration of plumbagin during irradiation for lipids, proteins and enzymes of rat liver mitochondrial preparation has been studied. The protection exerted by plumbagin (50-150 μM) has been studied using different standard assays for lipids (thiobarbituric acid reactive substance and ferrous oxidation in xylenol orange), proteins (dinitrophenylhydrazine and dithiobisnitrobenzoic acid), glutathione (fluorescence), superoxide dismutase (epinephrine oxidation) and succinate dehydrogenase (dichlorophenolindophenol reduction). **Results:** Plumbagin has been found to inhibit lipid peroxidation and protein carbonyl formation; protect thiols (glutathione and protein) and enzyme activity (superoxide dismutase and succinate dehydrogenase) in a concentration dependent manner. **Conclusion:** The protection provided by plumbagin to lipids, proteins and enzymes of rat liver mitochondrial preparation against gamma radiation-induced damage suggest that it can provide radioprotection in the living systems.

KEY WORDS: Gamma radiation, lipid peroxidation, plumbagin, protein damage

INTRODUCTION

Traditional Indian medicinal system (Ayurveda) makes use of many natural products from plants to cure several diseases and pathological conditions [1]. Ayurvedic herbal preparations are assumed to have advantage over modern medicines because of their natural origin and traditional use. Generally, different parts of the plants including root, stem, bark, leaf, seed and secretion are used alone or in combination in Ayurveda for their various therapeutic actions. The medicinal preparations of *Plumbago zeylanica* (*Chitrak* in Hindi) are commonly used in Ayurveda against diarrhea, fever, microbial infection, and dysentery [2]. Further, *Plumbago zeylanica* plant is known to have several pharmacological actions including antidiabetic, hepatoprotective, anticancer, antiproliferative, cardiotonic, neuro-protective, anti-atherogenic, anti-inflammatory, anti-bacterial, and anti-fungal activity [2-8]. Naphthoquinone, coumarin, and anthraquinone derivatives are major constituents of the roots of *Plumbago zeylanica* plant. Among these constituents, plumbagin (5-hydroxy-2-methyl-1,4-naphthoquinone, Figure 1) is one of the major and pharmacologically active constituent of roots of *Plumbago zeylanica*. Plumbagin (Plm) can scavenge both oxidizing and reducing free radicals because its chemical structure contains both quinone and phenolic groups.

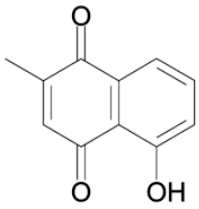

Figure 1. Plumbagin (5-hydroxy-2-methyl-1,4-naphthoquinone)

A survey of literature shows that Plm scavenges oxidizing free radicals, takes part in redox cycling, undergoes Michael addition with thiols, and chelate metal ions in addition to other pharmacological actions [2, 7-10]. Such quinonic compounds, including coenzyme Q10, are also known to scavenge superoxide radical anion ($O_2^{\bullet -} + Q \Leftrightarrow Q^{\bullet -} + O_2$) and undergo redox reactions depending on the pH, quinone reduction potential, presence of reducing agent, oxygen ($E^0_{O_2,aq/O_2,aq^{\bullet -}} = -0.18$ V), and other environmental conditions [11-15]. Recently, naphthoquinone (parent compound of plumbagin) was also shown to impart radioprotection in lymphocytes by inhibiting caspase activation [16].

The deleterious free radicals that are generated in oxidative stress, accidental radiation exposure and during radiation therapy can be generated *in situ* in biological systems using

^{60}Co gamma (Eγ(av.) = 1.25 MeV) irradiation. Further, it is known that high-energy radiation (E = MeV order) deposits energy on passage through the medium to cause non-selective excitation and ionization of the atoms and molecules (E = eV order) present in it [17]. Direct action/damage of molecules is predominant with high linear energy transfer (LET) radiation like alpha particles, heavy charged particles and neutrons. Indirect action/damage of molecules is predominant with low LET radiation like x-rays and gamma rays. Indirect action is through lysis of the molecules of major component (solvent) producing its free radicals. Irradiation of biological systems (~60% water) to low LET gamma radiation causes lysis of water molecules to produce hydrated electron (e^-_{aq}), H-atom, hydroxyl radical (•OH), *etc*. At neutral pH, hydroxyl radical is a strongly oxidizing species (E° +1.8 V). However, reducing species hydrated electron (e^-_{aq}) and H-atom convert to superoxide radical (O_2•-) and its protonated form hydroperoxyl radical (HO_2•) depending on the pH of the medium (pK_a of HO_2• = 4.8). These free radicals, generated on exposure to low LET radiation, react with biological molecules (RH) causing their oxidation and set up a chain of oxidation reactions depending on the conditions. Lipids (LH) react with hydroxyl radical to produce lipid radical (L•), which in turn reacts with oxygen to start a chain of free radical damage involving lipid peroxyl radical (LOO•) (Equations 1 to 3). Similarly, proteins (PH) are oxidized to produce carbon-centered radicals predominantly in the first step, which results in formation of protein carbonyls mostly (Equations 4 and 5). It is to be noted that sulfur- and oxygen-centered radicals of proteins are also produced in their oxidation reactions to some extent, which can result in folding/unfolding and formation of protein cross-links resulting in changes in functionality of proteins/enzymes. The lipid and protein radicals produced on radiation-induced oxidation can be scavenged by an antioxidant (AH) to inhibit further oxidation reactions of biomolecules (Equation 6).

$$LH + \bullet OH \rightarrow L\bullet + H_2O \quad (1)$$

$$L\bullet + O_2 \rightarrow LOO\bullet \quad (2)$$

$$LOO\bullet + LH \rightarrow LOOH + L\bullet \quad (3)$$

$$PH + OH \rightarrow P(\text{-}CH\bullet\text{-}) + H_2O \quad (4)$$

$$P(\text{-}CH\bullet\text{-}) + O_2 \rightarrow P(>C=O) \quad (5)$$

$$L\bullet / P(\text{-}CH\bullet\text{-}) + AH \rightarrow LH / PH + A\bullet \quad (6)$$

As mentioned earlier, ionizing radiations (heavy charged particles, neutrons, x-ray, γ-rays, *etc*) produce highly reactive free radicals that cause damage to biological molecules resulting in radiation injury. Since antioxidants scavenge these free radicals to protect against them, they may be also called radioprotector [18]. Plm has both quinone and phenolic groups (Figure 1), which scavenge reducing as well as oxidizing radicals, respectively to produce less reactive semiquinone radical and phenoxyl radical [19]. We have suggested that Plm may act as electron buffer (Equations 7 and 8) because of scavenging of both oxidizing and reducing radical [19].

$$\text{Reduction path:} \quad Plm\ (Q) \leftrightarrows Plm(QH)\bullet \leftrightarrows Plm\ (QH2) \quad (7)$$

$$\text{Oxidation path:} \quad Plm\ (QH_2) \leftrightarrows Plm(QH)\bullet \leftrightarrows Plm\ (Q) \quad (8a)$$

$$Plm\ (OH) + ox\bullet \rightarrow Plm(O\bullet) + ox- \quad (8b)$$

This suggests that Plm may protect biological system from damage induced by both reducing and oxidizing free radicals produced on exposure to gamma radiation. Therefore, we have attempted to understand the effect of presence of Plm during gamma radiation-induced damage to components of rat liver mitochondrial preparation.

MATERIALS AND METHODS

Chemicals

Plm purchased from Sigma-Aldrich was used without further purification. All other chemicals were of analytical reagent grade or highest purity available. Aqueous solutions were freshly prepared in deionized 'nanopure' water (conductivity < 0.06 μS/cm) from a Barnstead™ nano-pure system.

Animal maintenance and rat liver mitochondrial preparation

Female Wistar rats (250-300 g) of approximately ~14 weeks old, housed in humidity and temperature controlled room (24 ± 2C) with 12/12 h light/dark cycle, fed standard laboratory diet and water *ad libitum* were used. The guidelines issued by the Institutional Animal Ethics Committee of the parent institute (BARC) for the maintenance and dissection of small animals were strictly followed. Rat liver mitochondrial preparation (RLM) in aqueous solution was prepared as described earlier at 4°C [20].

In brief, rat livers were homogenized in 0.25 M sucrose containing 1 mM EDTA. The homogenate was centrifuged at 3000g for 10 min to remove cell debris and the nuclear fraction. The resultant supernatant was centrifuged at 10,000g for 10 min. This mitochondrial pellet was washed thrice with 0.15 M Tris-HCl buffer (pH 7.4) to remove sucrose. Protein was estimated by Lowry method and pellets were suspended in the above buffer at a concentration of 10 mg protein/ml [21].

Sample irradiation and measurements

The biological samples (RLM with or without Plm) were exposed to gamma radiation in a ^{60}Co gamma chamber. RLM samples prepared at at 4°C were taken in 2.5 ml microfuge

tubes, closed in air and irradiated in a cylindrical cavity in ^{60}Co gamma chamber. A relatively higher radiation dose (450 Gy) and concentration of Plm (up to 150 μM) was chosen to get measurable effect of radiation on RLM and significant protective effect of Plm on it. Generally, gamma irradiation-induced damage of lipids and proteins are measured in terms of formation of their oxidized products as lipid hydroperoxides, aldehydes, protein carbonyls and thiol oxidation.

Estimation of lipid damage

Lipid hydroperoxides (oxidized lipid products) were quantified using modified ferrous oxidation in xylenol orange (FOX) assay for the measurement of hydrogen peroxide [22]. Malondialdehyde (MDA) and other aldehyde products of lipid peroxidation were estimated by standard thiobarbituric acid reactive substance (TBARS) method [20].

Estimation of protein damage

Oxidation of protein results in formation of carbonyl groups on it. Reactions of these carbonyl groups of protein with 2,4-dinitrophenylhydrazine (DNPH) produce a colored complex (2,4-dinitrophenylhydrazone), which was measured to study protein oxidation [23]. Further, formation of 2-nitro-5-thiobenzoic acid (NTB) on reaction of protein thiol with 5,5'-dithiobis-(2-nitrobenzoic acid) or DTNB was used to measure protein thiol [24].

Assay of enzymes

Loss of enzyme activity of superoxide dismutase (SOD) on gamma radiation exposure in the presence and absence of Plm was measured using epinephrine oxidation method [25]. Similarly, loss of enzyme activity of succinate dehydrogenase (SDH) was estimated using phenazine methosulfate (PMS) and 2,6-dichlorophenolindophenol (DCIP) as described earlier [26]. SDH enzyme reduces DCIP dye whose absorbance is in turn a measure of the activity of SDH.

Estimation of glutathione

Fluorescence of o-phthalaldehyde was used for the estimation of glutathione as described earlier [27].

Statistical analysis

The experimental values obtained in different assays are reported as mean ± SEM from six experiments. ANOVA was performed using 'Origin' scientific graphing and data analysis software (version 6.1). The level of statistical significance was chosen to be $P < 0.05$.

RESULTS

It is known that exposure of biological samples to gamma radiation leads to radiolysis of the major component (H_2O) generating oxidizing (hydroxyl radical) and reducing (hydrated electron: e^-_{aq}; hydrogen atom: H) free radicals, which initiate oxidative stress. The reaction of hydroxyl radical with lipids is known to produce lipid radicals by H-atom abstraction, in addition to other reactions [17]. These lipid radicals react with oxygen to produce lipid peroxidation products in RLM, which are generally measured by TBARS and lipid hydroperoxide (LOOH) assay. In the present work, extent of radiation induced-lipid peroxidation in RLM has been found to decrease systematically on increasing the concentration of the Plm from 50 to 150 μM. The reduction in formation of radiation-induced TBARS and LOOH are shown in Figure 2 on right and left vertical axis, respectively. It is evident that Plm significantly inhibited the formation of these lipid peroxidation products. At 150 μM, Plm exerted ~74% and ~83% protection in terms of inhibition of lipid peroxidation as measured using TBARS and LOOH estimation, respectively. The concentration dependent inhibition of lipid peroxidation suggests scavenging of lipid peroxyl radicals by Plm. Earlier, we have reported that direct scavenging of lipid peroxyl radicals by Plm (LOO• + Plm → LOOH + Plm(-H)•) takes place efficiently ($k = 1.6 \times 10^8$ $M^{(-1)}$ $s^{(-1)}$) in chemical system [19]. Further, the high rate constant clearly suggests direct scavenging of lipid radicals by Plm to be the major path of lipid protection.

The effect of radiation in terms of loss of activity of enzymes and their protection by Plm has been studied. Superoxide dismutase (SOD) is an important antioxidant enzyme and has a key role in the cellular redox cycle. Loss of SOD or its activity by gamma irradiation may result in alteration in redox processes in the cell leading to oxidative stress. Radiation exposure has been found to decrease the level of SOD activity in RLM. However, Plm has been found to protect SOD activity in a concentration dependent manner with ~82% restoration at 150 μM (Figure 3A). Another enzyme having a key role in the functions of mitochondria is the succinate dehydrogenase (SDH), which is also a mitochondrial marker enzyme. SDH catalyzes the oxidation

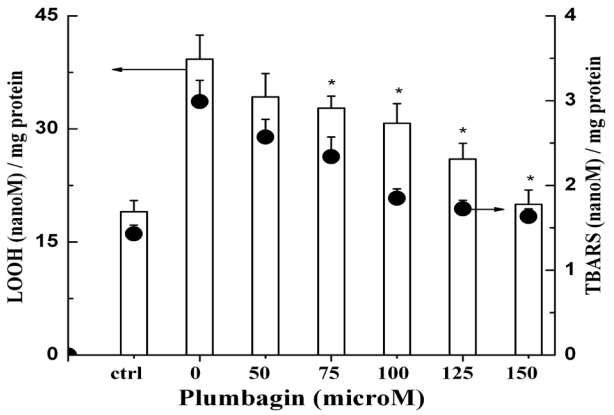

Figure 2. Gamma radiation-induced oxidation of lipid and its inhibition by plumbagin as measured by thiobarbituric acid reactive substance (TBARS) method (•, right axis), and lipid hydroperoxide (LOOH) formation (bars, left axis). *P < 0.05 compared to 0 μM dose.

of succinate to fumarate with concurrent reduction of ubiquinone to ubiquinol in the citric acid cycle. Exposure of RLM to gamma radiation resulted in loss of SDH activity, which was inhibited by the presence of Plm in a concentration dependent manner. Plm has been found to confer ~41.5% protection to SDH activity at 120 μM (Figure 3B).

Among cellular antioxidants, glutathione (GSH) is a well-known endogenous tripeptide antioxidant and its role is well established in physiology [17]. Plm has been found to act as a moderate protector for restoration of gamma radiation-induced depletion of glutathione, though at higher concentration (> 100 μM). Plm is assumed to protect GSH by direct scavenging of oxidizing free radicals as a competitive scavenger alone and/or as Plm-GS Michael adduct. The Plm-GS adduct is assumed because Plm may react with GSH by reductive addition (Michael adduct formation), which is known for other quinones [9, 15]. Therefore, the experimental measurement of GSH may not directly correspond to the extent of protection conferred by Plm (Figure 4A). Further, redox properties of Plm–GS adduct and consequently its free radical scavenging reactions are expected to be different as compared to its components Plm and GSH [28, 29]. Similarly, exposure of RLM to gamma radiation has been found to deplete protein thiols. In this case, the presence of Plm during irradiation inhibited depletion of protein thiols, as measured by DTNB assay, but at concentration beyond 100 μM only. At 120 μM, Plm restored good amount of protein thiol level back towards normal (Figure 4B). The experimental data suggest that at concentrations up to 100 μM almost all plumbagin is probably bound to thiol group of glutathione or protein. This Plm-SR Michael adduct probably behaves differently in scavenging oxidizing radicals as compared to thiol (RSH) and Plm. However, Plm provided significant protection to GSH and protein thiol at high concentration (> 100 μM). It is to be noted that it is difficult to separate and assign biological effects of quinone and quinone-thiol adduct as they can form different products in presence of oxygen and because of cross-reactions [28, 29]. Further, at higher Plm concentration as compared to thiols, the hydroquinone form can be also present that can serve as electron donor to oxidizing radicals (Equation 9).

$$2Q + RSH \rightarrow Q\text{-}SR + QH_2 \quad (9)$$

Generally, radiation-induced damage to proteins results in formation of protein carbonyls, oxidation of cysteine thiol and oxidation of aromatic amino acids (tyrosine, tryptophan, phenyl alanine). Gamma radiation exposure resulting in formation of protein carbonyls in RLM has been studied. The protein carbonyls have been estimated by

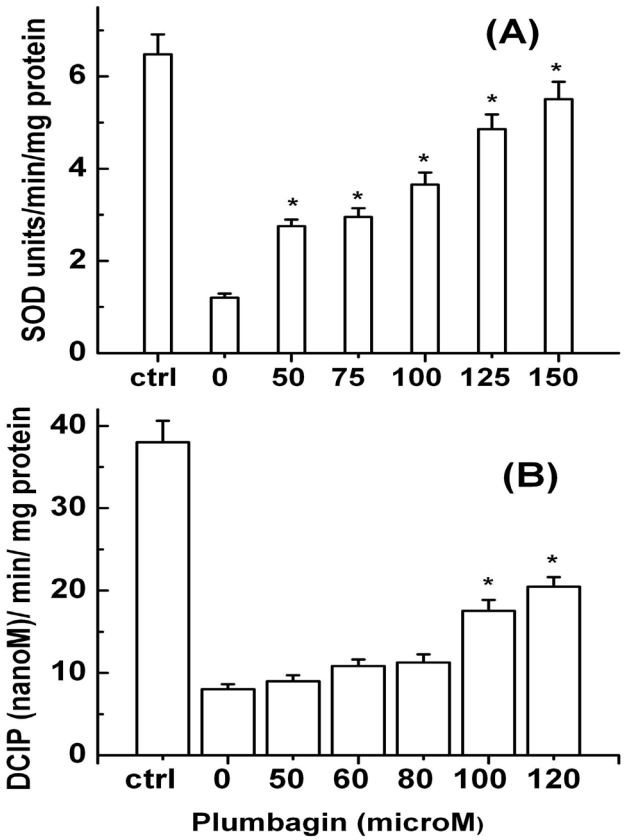

Figure 3. (A) Prevention of loss of superoxide dismutase (SOD) activity; and **(B)** protection of succinate dehydrogenase (SDH) activity (estimated using 2,6-dichlorophenolindophenol - DCIP) by plumbagin on gamma irradiation. *P < 0.05 compared to 0 μM dose.

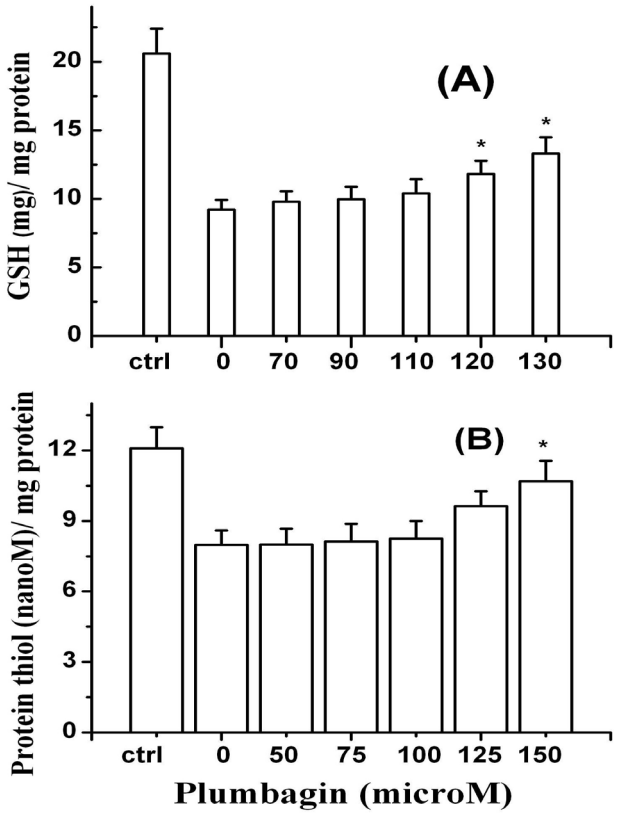

Figure 4. Radiation-induced depletion and its restoration by plumbagin for **(A)** glutathione (GSH), and **(B)** protein thiol. *P < 0.05 compared to 0 μM dose.

using dinitrophenylhydrazine in the presence and absence of Plm during irradiation. The presence of Plm reduced the radiation-induced protein carbonyl formation in a concentration dependent manner and ~56% protection has been observed at about 150 μM Plm (Figure 5). Earlier, Plm is reported to scavenge tyrosine radical (a model protein radical) with sufficiently high rate constant (7.1×10^6 $M^{(-1)}$ $s^{(-1)}$ [19]. Therefore, protection provided by Plm to proteins can be attributed to scavenging of protein radicals.

DISCUSSION

The present study clearly shows protection offered by Plm against gamma radiation-induced damage to lipids, proteins and enzymes in RLM. The protection by Plm observed in the present study agrees well with similar protection against chemically induced oxidative damage reported by Tilak *et al* [8]. It is to be noted that Tilak *et al* [8] showed protection provided by plant extract and Plm against oxidative damage induced by chemical method instead of radiation. Radiation method has advantage over other methods as it is done at room temperature, without addition of chemicals and involves in situ homogenous production of free radicals. Further, studies with pure chemical instead of extract give exclusive information on the effect of the particular chemical on the studied process.

The collective radiation chemical yield of free radicals in aqueous solution is ~0.6 μM per Gy of absorbed radiation dose. Even though biological systems are not like chemical system but a competition kinetic approach can be used to calculate Plm concentration required for competitive scavenging of free radicals. The calculation suggests that for any significant effect of the added chemical (Plm) in RLM, its concentration should be ~50 μM or more, which is used in the present study.

Plm has high partition coefficient (LogP = 2.3) at pH 7 which results in its partitioning mostly in lipid phase. Therefore, it has been found to provide more protection to lipids as compared to water-soluble thiols, protein and enzymes. Moreover, due to the same reason protection of lipids is significant and linear from 50 to 150 μM. Further, due to partitioning of Plm in the lipid phase its concentration is less in aqueous phase, which results in protection of proteins at 100 μM and beyond only. It is to be noted that Plm quinone (Q) can also make Michael adduct with thiol functional groups of GSH and proteins (CySH) namely, QSG and QSCy, respectively. This may result in loss of thiols functional group and decrease in availability of free Plm at low concentrations of Plm quinone. It has been taken care of in the present study by using a little higher Plm concentrations (≥ 50 μM). However, free radicals could be scavenged by hydroquinone form of Plm at [Plm]/[Q] ratio > 2 (Equation 9). Further, the Plm-SR adduct produced may have different scavenging capacity for free radicals. These effects are manifested in terms of significant protection of GSH and protein thiols only with Plm concentration beyond 100 μM.

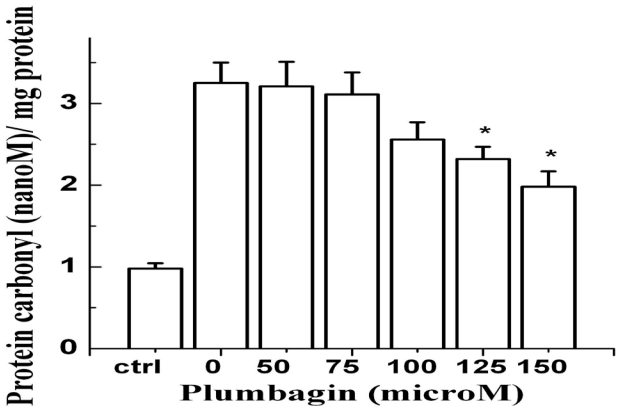

Figure 5. Radiation-induced protein carbonyl formation and its inhibition by plumbagin. *P < 0.05 compared to 0 μM dose.

The protection of lipids, thiols, protein and enzymes in RLM by Plm on gamma irradiation suggests that it is not causing damage to biomolecules by producing superoxide radical anion as suggested for some quinone compounds. On the contrary, Plm can be assumed to act as electron buffer by the involvement of its quinone/hydroquinone couple in addition to oxidation of its phenolic group and Micahel adduct formation with thiols. Recently, we have provided a clear evidence of scavenging of reducing radicals ($O_2^{\bullet -}$ and $\bullet C_2H_4OH$) by Plm producing its semiquinone radical anion followed by formation of hydroquinone [19]. The phenol and quinone groups are known to scavenge oxidizing and reducing free radicals, respectively, in addition to other biochemical reactions. Therefore, it can be safely assumed that some quinones (Q) may also act as electron buffers, like coenzyme Q10, by accepting electrons or reacting with reducing agents to produce hydroquinone (QH_2), which in turn can donate electrons to oxidants to regenerate quinone (Q). Further, hydroquinone (QH_2) can scavenge oxidizing radicals to produce less reactive semiquinone ($QH\bullet$) radical. Moreover, it can be said that the hydroxyquinones are interesting molecules that can scavenge both oxidizing and reducing free radicals in the conditions of oxidative stress. Earlier, we have shown that another hydroxyquinone molecule, embelin, scavenges both oxidizing and reducing free radicals, and acts as antioxidant in addition to known pharmacological activities [30, 31]. The protection of biomolecules of RLM in the present study and previously reported anti-inflammatory and antioxidant activity of Plm may be partly attributed to free radical scavenging activity of it [8, 32, 33].

As mentioned earlier, Plm is widely used in Ayurvedic medicine though some studies have also shown its toxic effects when used in combination with potent cytotoxic drug [34, 35]. SivaKumar et al [34] have found that Plm induced micronuclei at the doses 4, 8 and 16 mg/kg body weight for Swiss albino mice. It has been shown to be toxic to bone marrow cells of

Swiss albino mice. In another study, no significant change in glutathione S-transferase (GST) activity was observed with Plm, whereas GST activity was significantly inhibited by higher doses of plumbagin in combination with cytotoxic drug. Further, Demma et al [35] have found that plumbagin significantly reduced catechol-induced DNA damage.

In summary, plumbagin, 5-hydroxy-2-methyl-1,4-naphthoquinone, has been found to protect lipids, proteins and enzymes in rat liver mitochondrial preparation from gamma radiation-induced oxidative damage as measured using several assays. The presence of both quinone and phenol groups in Plm makes it an efficient scavenger of reducing as well as oxidizing free radicals, respectively. The antioxidant activity exerted by Plm against gamma radiation also suggests its radioprotection activity. Therefore, prevalent use of Plm in traditional medicine along with antioxidant activity makes it a suitable candidate for trials in combination therapies during radiation therapy.

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Conflict of Interest: None declared