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Original Article

Protective effects of silybin and analogues against X-ray radiation-induced damage

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Silybin (SLB) and similar analogues, namely, hesperetin (HESP), naringenin (NAN) and naringin (NAR), are believed to be active constituents of natural flavonoids that have been reported as chemopreventive agents for certain cancers. Moreover, SLB and analogues have been determined to fast repair DNA bases from oxidative damage by pulse radiolysis techniques. The present study was designed to evaluate the protective effects of SLB and analogues on soft X-ray-induced damage to plasmid DNA *in vitro*. The DNA damage was determined by agarose gel electrophoresis. SLB and analogues were found to protect DNA from radiation damage at micromolar concentrations. Among the compounds tested, HESP and SLB were the most effective in preventing X-ray-induced formation of DNA single-strand breaks (SSB). A comparison of these results with other experiments showed that the ability of SLB and analogues to inhibit DNA damage *in vitro* correlated with the ability of the compounds to scavenge free radicals. Our work revealed that natural flavonoids, SLB and analogues may be used as potent radioprotectors against radiation damage.

Keywords silybin; DNA; free radicals; antioxidant; protection

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Introduction

In the past decade, epidemiological, biological, and clinical studies have provided evidence that free radical-induced oxidative damage of cell membranes, DNA, and proteins might play a causative role in aging and degenerative diseases, such as cancer, atherosclerosis, and cataracts. Additionally, these studies have found that antioxidants, such as α-tocopherol, L-ascorbic acid, and β-carotene, might have beneficial effects by protecting against these diseases [1–3]. Therefore, the inhibition of free radical-induced oxidative damage by supplementation with antioxidants has become an attractive therapeutic strategy for reducing the risk of these diseases [4,5]. One class of antioxidants that has emerged from epidemiological studies is the flavonoids. Flavonoids are representatives of a large and complex group of phenolic compounds that occur throughout the plant kingdom and are synthesized in most plant tissues. Flavonoids provide colour, flavour, antifungal, and antibacterial activities [6,7]. These polyphenols also function as free radical scavengers, leading to beneficial action in cardiovascular disorders [8], prevention of DNA SSB [9], anticancer activity [10], etc. The antioxidant activities of flavonoids depend on the structure of the molecules, the initiation conditions, and the microenvironment of the reaction medium. The desirable therapeutic properties of polyphenols have also been considered to depend on their ability to scavenge reactive oxygen species (ROS) [11]. Although oxygen-derived free radicals can be beneficial, much of the interest in these species is related to their potential to cause damage to DNA, thereby altering gene expression, cell growth, and differentiation [12–14]. The most reactive, and therefore potentially hazardous, oxygen-derived radical is the hydroxyl radical (•OH). ROS such as superoxide (O_2^-) and hydrogen peroxide are relatively stable and their connection to cellular damage is derived from their decomposition to OH radical.

The oxidation of substrates such as DNA is of interest, because DNA is an important target for free radical attack. Plasmids are a convenient model system for studying DNA damage, because they have a well-defined size, are

relatively easy to prepare in milligram quantities of high purity for radiation chemistry and SSB are readily revealed by gel electrophoresis. In an attempt to better understand the interaction of DNA and antioxidants associated with ionizing radiation, we examined SSB formation in DNA irradiated in diluted aqueous solution containing plasmid DNA and target flavonoids such as silybin (SLB), hesperetin (HESP), naringenin (NAN), and naringin (NAR) (**Fig. 1**). SLB, extracted from the seeds of *Silybum marianum*, is the major active constituent of silymarin, which possesses a wide range of medicinal properties. SLB is a mixture of two diastereomers A and B at approximately 1:1 ratio. Recently, SLB has received great attention due to its beneficial activities such as anticancer, neuroactive, and neuroprotective activities [15]. Like most plants and herbs, SLB has medicinal properties and is used in traditional Chinese medicine to treat various ailments in humans. Silybin has been proved to show the protective effects against chemically and UVB-induced skin damages both in cell cultures and animal experiments. Very recently, we reported that SLB could protect DNA from radiation damage at very low concentrations [16] and the other natural flavonoids with similar structure of SLB all showed fast reparation of DNA base using pulse radiolysis techniques [17]. When pulse radiolysis technique was used to elicit damage by oxidizing radicals, these flavonoids all showed fast repair of damaged DNA bases [18]. To further explore the potential protective effect from DNA radiation damage, the degree of DNA strand breakage with or without SLB and analogues was evaluated by gel electrophoresis. Additionally, the possible relationship between the scavenging action of the flavonoids and the protection of DNA against radiation damage was also investigated *in vitro*.

Materials and Methods

Materials

Silybin (SLB), HESP, NAN, NAR, and ethidium bromide were purchased from Sigma-Aldrich (Tokyo, Japan). Plasmid DNA (pUC18, 2686 base pairs) was prepared and purified as previously described [19]. The plasmid, which is over 95% in the closed circular form, was subsequently stored at −20°C in potassium phosphate buffer (PBS) (pH 8.0) at a concentration of 20 μg/ml. The plasmid DNA was used within two weeks. All other reagents were obtained from Wako Pure Chemical Industries, Ltd. (Tokyo, Japan). All solutions were freshly prepared with triply distilled water before each experiment.

DNA irradiation

The plasmid substrate was irradiated by soft X-ray in air-saturated aqueous solution. These solutions contained pUC18 DNA (6 μg/ml), sodium phosphate buffer solution (PBS buffer) (20 mM, pH 7.4), and an antioxidant (0.1–2 mM). The antioxidant was one of the following: SLB, HESP, NAN, or NAR. Each aliquot was 25 μl in volume. Depending on the antioxidant concentration, the maximum dose was 20–300 Gy. The dose rate of 0.23 Gy/s was quantified by means of the Fricke method. During irradiation, the samples were kept at 4°C.

Under these conditions, the radiation decomposes water into free radicals (reaction (1)), of which the OH radical is the main oxidizing species. The reducing H-atoms and e^-_{aq} are converted to O_2^- (reaction (2)), which does not damage the DNA to any significant extent [20].

$$H_2O \rightsquigarrow e^-_{aq},\ {}^{\bullet}OH,\ H^{\bullet},\ H_2O_2,\ HO_2,\ H_2, \qquad (1)$$

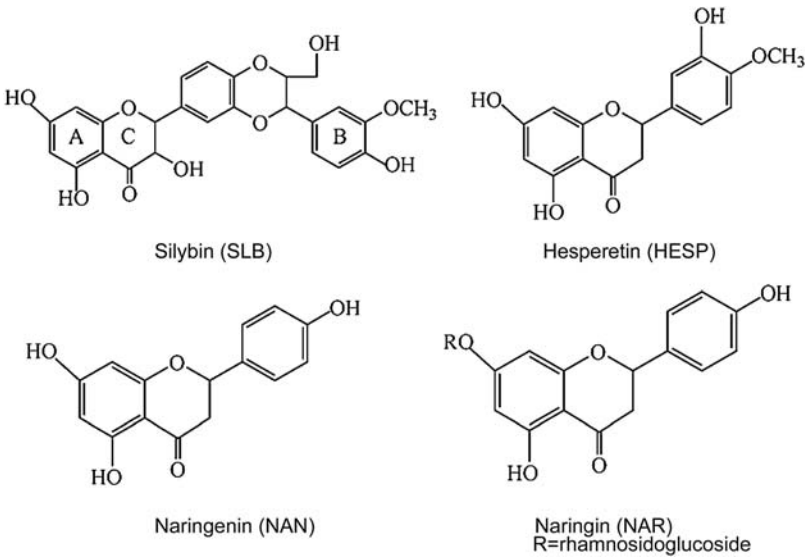

Figure 1 Chemical structure of the test flavonoids.

$$e^-_{aq}/H^\bullet + O_2 \rightarrow O_2^{\bullet -}/HO_2^\bullet. \quad (2)$$

The effects of flavonoids on DNA radicals, which include altering the yield of residual DNA damage, were studied by maintaining a constant OH radical scavenging capacity. The protection of the plasmid from SSB formation and other damage through direct radical scavenging was maintained by increasing the concentration of flavonoids. In this medium, the radical scavenging includes intercepting OH radicals by PBS buffer (pH 7.4, <20 mM) (reaction (3)) and flavonoids (abbreviated F, reaction (4)) before they react with DNA. The rate constant for phosphate is about $10^5\ M^{-1}\ s^{-1}$ [21], while for flavonoids the values were more than $10^{10}\ M^{-1}\ s^{-1}$ (**Table 1**), which indicates that PBS buffer has a very weak scavenging capacity at the same concentration as flavones. By maintaining a low scavenging capacity, DNA strand breakage and base damage arise almost exclusively by a small proportion of the OH radical, which is produced directly or indirectly, by the irradiation of water, formed in close proximity to the DNA [22] (reaction (5)).

$$^\bullet OH + HPO_4^- \rightarrow OH^- + HPO_4^\bullet \quad [22], \quad (3)$$

$$^\bullet OH + F \rightarrow H_2O + F(-H)^\bullet, \quad (4)$$

$$^\bullet OH + DNA + H^+ \rightarrow\rightarrow H_2O + DNA^{\bullet +} \rightarrow\rightarrow \text{base damage/strand break}. \quad (5)$$

Agarose gel electrophoresis investigation of DNA strand breaks

Radiation-induced damage in DNA was determined by the conversion of supercoiled pUC18 plasmid DNA to open circular (oc) and linear forms, according to the procedure described previously [23]. Immediately after irradiation, the different forms of DNA were separated by agarose gel electrophoresis. The gels, in a horizontal slab gel apparatus, were stained with ethidium bromide at 140 V for 5 h at 5.7°C, and DNA bands were photographed under ultraviolet illumination.

Table 1 The rate constants and the yield of SSB ($G_{(SSB)}$) of SLB and analogues.

Flavonoid	k_{OH} ($10^{10}\ M^{-1}\ s^{-1}$)	$G_{(SSB)}$ (10^{-4} μmole/J)	
		0.02 mM[b]	0.2 mM[b]
DNA[a]	0.11	6.21 ± 0.41	
SLB	2.12 ± 0.05	3.01 ± 0.25	0.45 ± 0.11
HESP	2.02 ± 0.04	3.12 ± 0.16	0.52 ± 0.13
NAN	1.96 ± 0.04	3.08 ± 0.12	0.58 ± 0.09
NAR	1.78 ± 0.04	3.41 ± 0.16	0.62 ± 0.12

$G_{(SSB)}$ means radiation chemical yield of single-strand breaks per absorbed dose (Gy). $G_{(SSB)}$ is equal to (DNA concentration/μmole·dm^{-3})/[(D_0/Gy) × (ρ/kg dm^{-3})]. *Note*: Data from 'a' ref. [32], 'b' means the concentrations of tested flavonoids.

Calculation of the radiation chemical value for SSB formation ($G_{(SSB)}$)

A dose–response was determined from the logarithmic loss of closed circular plasmid DNA with the radiation dose. Plotting the logarithm of the fraction of intact supercoiled DNA against the radiation dose provided a straight line. The D_0 was calculated from the reciprocal of the slope of the straight line. At least seven points were used in the estimation of each D_0, where $D_0 = (\log_{10}37 - 2)/slope$. The highest dose was usually two or three times the D_0 [24].

At D_0, the concentration of SSBs is equal to the concentration of DNA, expressed as plasmid molecules (relative molecular mass = 650 g mole^{-1} bp^{-1} × number of base pairs). For pUC18 DNA, the number of base pairs is 2686. From the definition of the radiation chemical value (G value) (in units of μmole/J), the G value for SSB formation, $G_{(SSB)}$ is equal to (DNA concentration/μmole dm^{-3})/[(D_0/Gy) × (ρ/kg dm^{-3})]. The density of the solution (ρ) was assumed to be unity as previously described [25].

From the slope of this response, a D_{37} value was obtained, assuming Poisson statistics for SSB induction, represents the radiation dose required to give an average one SSB per plasmid molecule. Using the D_{37} value, an average number of SSB/Gy/Da ($n_{(SSB)}$) was obtained: $n_{(SSB)} = 1/(2686 \times 650 \times D_{37})$. The $n_{(SSB)}$ is used to quantitativly evaluate the single-strand breakage after irradiation.

Determination of the rate constants with OH radicals

To further clarify the relationship between the different protective efficiencies and radical scavenging capacities, the rate constants of flavonoids reacting with OH radials were measured by pulse radiolysis techniques. Pulse radiolysis studies and the associated dosimetry were carried out using a 35 MeV linear accelerator at the University of Tokyo. Detailed descriptions of the set-up and experimental conditions have been described elsewhere [26]. The rate constants for OH radical with the flavonoids were determined as previously described, where the competition plots were constructed for the yield of each of the flavonoid radicals in the mixtures containing KSCN in N_2O-saturated solution [27]. The rate constants for reaction (4), calculated relative to the rate constant of the scavenging OH radicals by KSCN of $1.1 \times 10^{10}\ M^{-1}\ s^{-1}$[28] are presented in **Table 1**.

Results

DNA strand breaks induced by X-ray radiation

Conversion of the supercoiled form (sc) of plasmid DNA to the open circular (oc) and linear forms has been used as an index of DNA damage. The formation of the circular form of DNA is indicative of single-strand breaks, and the formation of a linear form of DNA is indicative of double-strand breaks [29]. **Figure 2(A)** shows X-ray-induced pUC18 DNA strand breaks. In each lane, the lower bands are attributed to the supercoiled DNA, the upper bands indicate the open circular DNA and the middle bands that sometimes appear, are the linear form of DNA. It is generally assumed that it takes only the single-strand scission event to convert supercoiled DNA to open circular DNA [30], and the latter always seems to be present in supercoiled DNA. A second strand scission event converts open circular DNA into linear DNA, provided this event occurs on the other (i.e. the uncut) strand and within about 5 bp of the break in the first strand.

As shown in **Fig. 2(A)**, the supercoiled DNA was gradually converted to open circular DNA with an increase in dose (from 2 to 10 Gy), and the open circular DNA was gradually converted to linear DNA with a further increase in dose (from 10 to 50 Gy). It can be concluded that the damage of pUC18 DNA is dose dependent.

Protection of DNA strand breaks

In **Fig. 2(A)**, lanes 8–13 showed the effect of 0.2 mM SLB on the formation of strand breaks in plasmid pUC18 DNA by different doses of X-ray radiation (0–120 Gy). Compared with DNA without SLB, at a dose of 50 Gy, 100% of the plasmid DNA was converted into open circle and linear forms [**Fig. 2(A), lanes 6, 7**]. The presence of 0.2 mM SLB inhibited this conversion from the supercoiled form (sc) to the open circle form, almost 40–70% at different doses of X-ray radiation. The percent of the sc form of plasmid DNA remaining (% sc) after exposure to various doses of X-ray radiation, with and without 0.2 mM SLB, was plotted against the radiation dose in **Fig. 2(B)**.

As shown in **Fig. 3(A)** lane 2, pure DNA was irradiated at 50 Gy, resulting in the formation of open circular and linear forms. This confirmed both single-strand and double-strand DNA breakage upon irradiation. Addition of SLB at 0.01–1.5 mM to solutions of DNA caused a partial or complete inhibition of the conversion of supercoiled DNA to open circular and linear forms, indicating that SLB is able to protect plasmid DNA against radiation-induced damage. With increasing concentrations

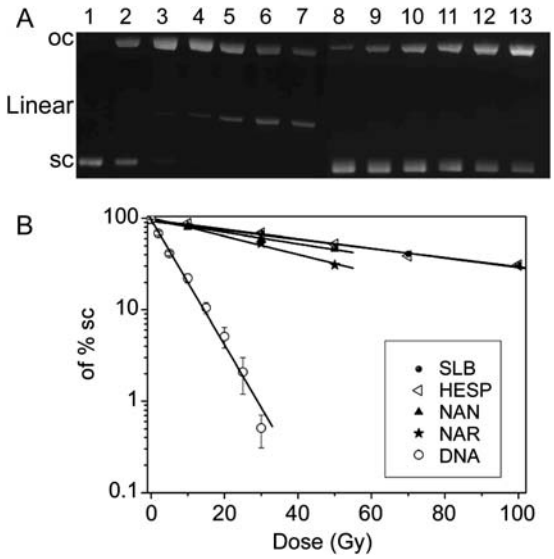

Figure 2 SLB and analogues prevent DNA damage induced by difference doses of irradiation (A) Agarose gel electrophoretic pattern of 25 μg/ml DNA in the presence or absence of 0.2 mM SLB after irradiation with different doses in PBS at pH 7.4. Lanes 1–7, DNA alone; lanes 8–13, DNA + 0.2 mM SLB. Lane 1, 0 Gy; lane 2, 2 Gy; lane 3, 6 Gy; lanes 4 and 8, 10 Gy; lane 5, 20 Gy; lanes 6 and 9, 30 Gy; lanes 7 and 10, 50 Gy; lane 11, 70 Gy; lane 12, 100 Gy; lane 13, 120 Gy. (B) Presentation of log of % sc form of DNA against various doses of soft X-ray radiation. When plasmid DNA was irradiated, there exist three different formation: open circle (oc), linear and supercoiled (sc) form. % sc means the percent of the supercoiled form of plasmid DNA remaining after X-ray radiation.

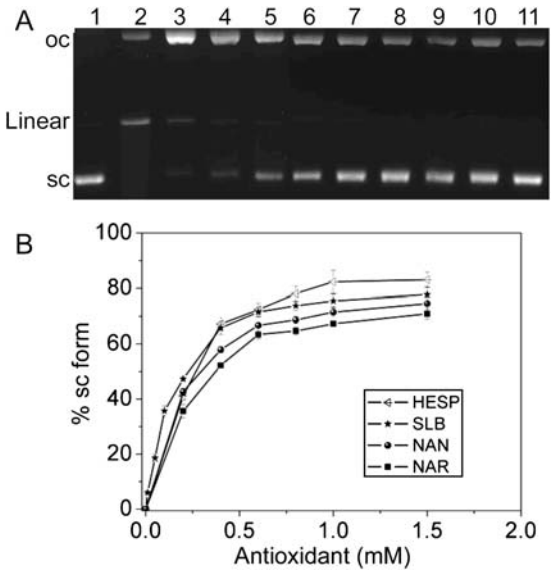

Figure 3 SLB and analogues dose dependently prevent DNA damage (A) Effects of SLB on 25 μg/ml DNA strand breaks after 50 Gy radiation. Lane 1, DNA 0 Gy; lanes 2–11, 50 Gy. Lane 2, DNA + 50 Gy; lane 3, DNA + 0.01 mM SLB; lane 4, DNA + 0.05 mM SLB; lane 5, DNA + 0.1 mM SLB; lane 6, DNA + 0.2 mM SLB; lane 7, DNA + 0.4 mM SLB; lane 8, DNA + 0.6 mM SLB; lane 9, DNA + 0.8 mM SLB; lane 10, DNA + 1.0 mM SLB; lane 11, DNA + 1.5 mM SLB. (B) Graph plotted for % sc form of pUC18 DNA vs. concentration of SLB and analogues. Experiments were carried out in three separate experiments with error bars representing the standard deviation.

of SLB, the formation of the open circular and linear forms of DNA decreased (lanes 3–11). The percentage of sc form remaining after different treatments is plotted against the concentration of SLB in **Fig. 3(B)**. SLB protects the supercoiled form of DNA up to almost 80%, in a range of 0.01–2 mM. The radioprotection offered by SLB at 50 Gy dose of X-ray radiation began to plateau after the concentration of 0.6 mM. This tendency was observed by the SLB analogues as well (data not shown). Thus, it is evident that SLB can offer protection to DNA against gamma radiation-induced damage *in vitro*, by significantly reducing the formation of strand breaks.

The yield of single-strand breakage ($G_{(SSB)}$ value and $n_{(SSB)}$)

DNA strand breakage after irradiation in the presence and absence of each of the four flavonoids was followed by gel electrophoresis. Examples of the radiation dose–response curves for the loss of the supercoiled form in the presence and absence of SLB are presented in **Fig. 2(B)**. D_0 values from such plots were used to calculate the yield of strand breaks ($n_{(SSB)}$) for each SLB and are presented in **Fig. 4**. The scavenging capacity was used to estimate the effects of different flavonoids concentrations, which is defined as the product of the solute concentration and the rate coefficient for the scavenging reaction. Scavenging capacity is essentially the pseudo-first-order rate coefficient. It was calculated via the rate constant multiplying the concentration. Six concentrations of SLB were tested, respectively, i.e. 2, 10, 20, 100, 200, and 400 μM level, for DNA strand breaks. While the low concentrations were at physiological levels, the highest concentration resulted in the maximum, or near maximum, reduction of single-strand breaks when present during irradiation. As the SLB concentration increased from 0.01 to 0.4 mM, the G value for SSB information decreased from 2.2 to 40×10^{-4} μM/Gy. At SLB concentrations >0.1 mM, the variation of SSB yield with SLB concentration was less pronounced than that at lower SLB concentrations.

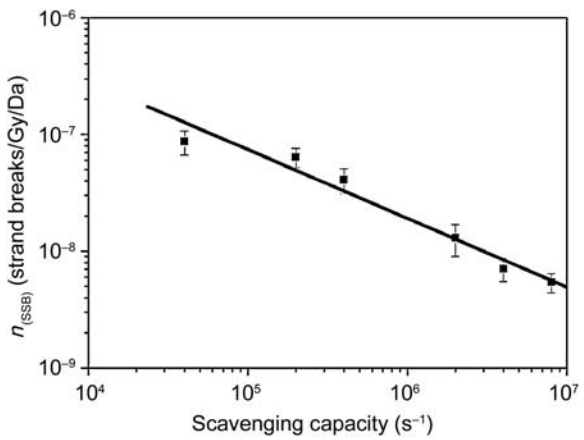

Figure 4 A double-logarithmic plot of the SSB yield $n_{(SSB)}$ vs. scavenging capacity of SLB Scavenging capacity is defined as the product of the solute concentration and the rate coefficient for the scavenging reaction. It was calculated via the rate constant multiplying the concentration.

Protective effect of SLB and analogues against DNA damage

We recently demonstrated that SLB and analogues can scavenge oxidizing radicals and repair dGMP-OH adducts [17]. The kinetics and transient procedure has been confirmed by pulse radiolysis. Therefore, it is desirable to investigate whether SLB and analogues show similar effects on DNA damage.

Six concentrations (0.2, 0.4, 0.6, 0.8, 1.0, and 1.5 mM) were used to compare the efficiency of SLB and the analogues [**Fig. 3(B)**]. The lowest concentration has been observed to correspond to the distinct effect of SLB. SLB, at concentration of 0.2 mM, had a somewhat higher protective ability than HESP, while NAR had the lowest protective ability. Thus, at lower concentrations (less than 0.6 mM), the studied compounds have the following order of protective action: SLB > HESP > NAN > NAR. The effects of all four flavonoids became equal at concentrations up to 1.0 mM. NAN had a lower efficiency and NAR was found to be the least effective. As for the $G_{(SSB)}$ at the two concentrations of tested flavonoids, namely 0.02 and 0.20 mM, the four flavonoids give the similar tendency (**Fig. 5**). For HESP, at 0.02 mM the $G_{(SSB)}$ was 3.12×10^{-4} μmole/J while at 0.20 mM the value was 0.52×10^{-4} μmole/J. The higher concentration means the higher protective effect, which shows the lower yield of single-strand breakages. The different rate constants of SLB and analogues indicate a different scavenging capacity for OH radicals under the same conditions. As seen in **Table 1**, by way of comparison, the rate constant of NAR is the lowest, $1.78 \times 10^{10}\ M^{-1}\ s^{-1}$, which indicates the lowest scavenging capacity. However, at higher concentrations (≥1.0 mM),

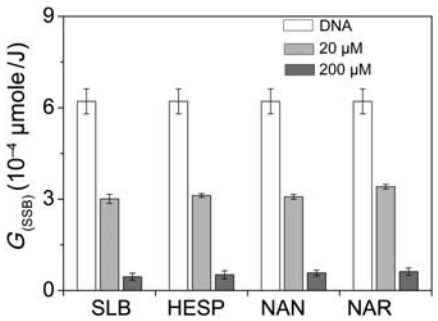

Figure 5 Yields of DNA strand breaks in the presence or absence of SLB and analogues $G_{(SSB)}$ means the radiation chemistry yield of DNA strand breaks. Error bars present the standard deviation derived from the average of the D_0 values.

the remaining of % sc gradually tends to unanimity. This is due to both aspects: DNA can be oxidized by certain oxidizing radicals and the direct radiolysis of scavengers may lead to the formation of some highly reactive species.

Discussion

Some natural and synthetic chemicals have been investigated in the recent past decade for their efficiency to repair DNA bases and protect against radiation-induced damage in biological systems [31]. We have demonstrated that they can directly interact with DNA bases to repair DNA by electron transfer [17]. On the other hand, SLB and analogues also repair DNA radicals from the damage caused by ionizing radiation, which is predominantly mediated by $H^{\bullet}$, $^{\bullet}OH$, and hydrated electrons. In the present system, the rate constants of SLB and other flavonoids are about $2 \times 10^{10}\ M^{-1}\ s^{-1}$ while for DNA that is $1.1 \times 10^{9}\ M^{-1}\ s^{-1}$ [32]. And the concentrations of SLB and analogues were 10 times higher than that of DNA. So the rate of $^{\bullet}OH$ reacting with test flavonoids is 20 times higher than that with DNA, which means SLB and analogues scavenge oxidizing free radicals, especially OH radical, generated by ionizing radiation. A similar mechanism has been proposed to explain the antioxidant activities of vanillin [33].

This *in vitro* study showed that SLB and analogues are highly active in reducing the amount of oxidative damage sustained by DNA through OH radical attack. SLB, when compared with other classes of flavonoids, e.g. green tea catechin [25], quercetin [34], was found to be as active as these in reducing the amount of strand breakage and residual base damage. The pulse radiolysis data support the mechanism of electron transfer from SLB to radical sites on DNA bases. Both a high percentage and increased rate of electron transfer qualitatively correlated with increased efficiency in reducing DNA damage. In our present work, SLB and analogues exert antioxidant effect in the protection of DNA. The reaction of OH radicals with DNA gives rise to a wide range of radical intermediates on all of the DNA bases as well as H-atom abstraction from different sites on the ribose moiety.

Our findings await definitive studies on the uptake of the different classes of flavonoids and their distribution in cells. Flavonoids are relatively small uncharged molecules that should pass through cellular membranes, but it is presently unknown if they can be concentrated inside the cell. Modest binding to DNA has been reported for some polyphenol, e.g. catechin [26], quercetin [34]. However, even if there is little increase in the uptake by cells over the concentrations measured in plasma, our data suggested that SLB and analogues can be active against DNA damage at the micromolar level. A concentration of antioxidants in the micromolar range cannot compete with cellular constituents, which are present at much higher concentrations for the scavenging of highly reactive ROS species such as OH radicals. Ionizing radiation-induced damages to cellular DNA are of prime biological significance. Protecting DNA from radiation damage might result in prevention of the cancer/mutations induced by radiation. The present study showed plasmid DNA was protected from deleterious effects of X-ray radiation by silybin and its analogues *in vitro* condition of radiation exposure. Silybin and the analogues (0.2 mM) offered different ability to protect plasmid DNA against radiation-induced strand breaks. The higher protection and rapid reparation activity of silybin agree well with the rate constant reacting with OH radical. Although plasmid DNA was chosen as model systems, the results may give us useful information for potential radioprotectors.

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