Protective effect of silymarin on erythrocyte haemolysate against benzo(a)pyrene and exogenous reactive oxygen species (H$_2$O$_2$) induced oxidative stress

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Abstract

The present study was carried out to evaluate the in vitro antioxidant properties and protective effects of silymarin (milk thistle) in human erythrocyte haemolysates against benzo(a)pyrene [B(a)P], a potent carcinogenic chemical. Protective effect of silymarin was assessed in vitro by monitoring the antioxidant enzymes and malondialdehyde in three groups of haemolysates—(I) vehicle control (II) B(a)P incubated group and (III) B(a)P co-incubated with silymarin. The effects of silymarin on lipid peroxidation (LPO) and antioxidant enzymes [superoxide dismutase; SOD, catalase; CAT, glutathione peroxidase; GPx, glutathione reductase; GR and glutathione-S-transferases; GST] were assessed on haemolysates. It was observed that specific activity of antioxidant enzymes were significantly decreased and the malondialdehyde levels were elevated when haemolysates were incubated with B(a)P. The protective effect of silymarin is elucidated by the significant reversal of the antioxidant enzymes and reduction in the levels of malondialdehyde. In addition, haemolysates were incubated with B(a)P for 45 min and the B(a)P metabolite, 3-hydroxy benzo(a)pyrene (3-OH–B(a)P) was detected using HPLC. An increased level of the metabolite was detected in group II. Whereas, when haemolysates were co-incubated with silymarin, the reactive metabolite 3-OH–B(a)P was not detectable which further confirms the protective role of silymarin. Generation of 3-OH–B(a)P in group II implicates the possibility of reactive oxygen species (O$_2^·$/C0$_2$ and H$_2$O$_2$) production in haemolysates during cytochrome P450 1A1 (CYP1A1) mediated Phase-I-metabolism. Hence, we incubated the haemolysates with exogenous reactive oxygen species H$_2$O$_2$ and assessed the protective role of silymarin against H$_2$O$_2$. From the results of our study, it was suggested that silymarin possess substantial protective effect and free radical scavenging mechanism against environmental contaminants induced oxidative stress damages.

Keywords: Oxidative stress; Silymarin; Benzo(a)pyrene; Malondialdehyde; Antioxidant enzymes

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are a ubiquitous class of environmental contaminants that have large number of hazardous consequences on human health (Hanachi et al., 2003). An important and very extensively studied prototype of this environmental contaminant is benzo(a)pyrene (B(a)P) (Uno et al., 2004). B(a)P undergoes a metabolic activation to form reactive intermediates before it is capable of inducing mutagenic, cytotoxic, and teratogenic effects in various species and tissues (Buening et al., 1978; Gelboin, 1980; Borhoumi et al., 2002). It has been revealed that the carcinogenic potency of B(a)P is ensued by cytochrome P450 1A1 (CYP1A1) mediated reaction, forming carcinogenic B(a)P-metabolites that play a main role in the mutagenesis and carcinogenesis of B(a)P (Weinstein et al., 1976). The B(a)P metabolites like quinines and phenols are important redox-cycle compounds
that are produced during the metabolic processing of \( \text{B(a)P} \) by a one-electron oxidation. The toxicity of these compounds is related to reactive oxygen species (ROS) such as, superoxide anion radicals (\( \text{O}_2^- \)) and hydrogen peroxide (\( \text{H}_2\text{O}_2 \)) (Penning et al., 1996), which causes oxidative stress in humans.

ROS are a double-edged sword – they serve as key signal molecules in physiological processes but also have a role in pathological processes involving chronic fatigue, premature aging, skin disorders, hormonal imbalances, cancer, atherosclerosis, heart diseases and all sorts of autoimmune disorders. (Agarwal et al., 2005). It has been reported that \( \text{B(a)P} \) induces oxidative stress in blood monocytes, erythrocytes and macrophages and other tissues (Vayssier-Taussat et al., 2001; Grevenynge et al., 2003). Red blood cells can be regarded as circulating antioxidant carriers, reflecting exposure to ROS. Indeed, they have been used as a model for the investigation of free-radical induced oxidant stress because of several reasons; they are continually exposed to high oxygen tensions, they are unable to replace damaged components, the membrane lipids are composed partly of polyunsaturated fatty acid side chains which are vulnerable to peroxidation, and they have antioxidant enzyme systems (Konyalioglu and Karamenderes, 2005).

Under normal conditions, the continuous production of free radicals is compensated by the powerful action of protective enzymes like superoxide dismutase, catalase and glutathione peroxidase that are believed as major antioxidant enzymes present in the human body that protect against the oxygen toxicity. But large number of drugs and other xenobiotics can stimulate the generation of reactive oxygen species by redox cycling and it could be more vulnerable compared to normal condition (Abraham et al., 2005).

Though several synthetic antioxidants are available, a growing trend has been targeted toward the use of natural products as antioxidants (polyphenols, flavonoids, vitamins, carotenols and lycopene) in view of toxicity condemn (Zeisel, 2004). Antioxidants provide necessary defence against the oxidative stress induced damages. Bioflavonoids are ubiquitous group of polyphenolic substances that are present in most plants (Nijveldt et al., 2001).

Silymarin [synonyms – Silybin, Silibinin, Silliver], a plant derived flavonoids, which is classified as benzopyranones, (Frascini et al., 2002) is isolated from the fruits and seeds of the milk thistle (\( \text{Silymarin marianum} \)) and in reality is a mixture of three structural components: silibinin, silydianine and silychristine (Fig. 1). Milk thistle is a member of the \( \text{Asteraceae} \) family (Koen and Walterovab, 2005). It has been reported as having multiple pharmacological activities including antioxidant, hepatoprotectant and anti-inflammatory agent, antibacterial, antiallergic, antimitagenic, antiviral, antineoplastic, anti thrombotic agents and vasodilatory actions (Abascal and Yarnell, 2003).

There are reports that flavones inhibit CYP1A1 induction by competitively binding with aryl hydrocarbon receptor (\( \text{AhR} \)) (receptor for PAH’s) and thus prevent the metabolism of \( \text{PAH} \) (Plı’sˇkova´ et al., 2005). Since many flavonoids are reported to be \( \text{AhR} \) ligands (Reiners et al., 1999), it was assessed whether silymarin could act as a competitor of \( \text{B(a)P} \) and suppress the toxicity induced by the environmental toxin \( \text{B(a)P} \). Silymarin is already reported to prevent damages induced by other environmental toxins like benzoyl peroxide (Zhao et al., 2000; Jeong et al., 2005).

The aim of our study is to investigate oxidative damage in erythrocytes during \( \text{B(a)P in vitro} \) incubation and the

Fig. 1. The structural components of silymarin: silibinin, silydianine and silychristine.
2. Materials and methods

2.1. Flavonoid material

Silymarin, the commercially available plant flavonoid was purchased from Ranbaxy Laboratory Limited, Manipur, India.

2.2. Chemicals

3-Hydroxy benzo(a)pyrene and benzo(a)pyrene were purchased from National Cancer Institute’s Chemical Repository, USA and Alfa Aesar, MA, respectively. The other chemicals and reagents used were of analytical grade.

2.3. Preparation of haemolysates from human erythrocytes

Blood from healthy volunteers was collected in vacutainer polystyrene tubes containing EDTA as anticoagulant. The samples were centrifuged at 3000 × g for 15 min. Plasma and buffy coat were removed by aspiration and the erythrocytes were washed three times with cold (4 °C) saline and suspended in equal volumes of deionised water. The haemolysate were frozen in 500 μl aliquots and were stored at −80 °C in deep-freezer, until used for analysis of antioxidant enzyme activities and lipid peroxidation. Erythrocyte suspensions were haemolysed by adding equal volume of distilled water and vigorous vortexing to get 50% haemolysate.

2.4. Determination of haemoglobin concentration

Haemoglobin concentration was quantified in Haemoglobinometer using Sahil’s pipette. Prior to enzyme analysis 50% haemolysates were diluted to 5:1 volume with water and further 2:1 by volume with phosphate buffer containing 0.05 mM dithiothreitol to a final dilution of 1:20 (5%) (Bukowska, 2004).

2.5. Preparation of incubations with silymarin

Erythrocyte haemolysates (5%) obtained from healthy donors was divided into three groups as follows:

* Group I Vehicle control
  Erythrocyte haemolysate, DMSO (less than 0.2%) and PBS

* Group II B(a)P
  Erythrocyte haemolysate, B(a)P (300 μM dissolved in less than 0.2% DMSO) and PBS

* Group III B(a)P + Silymarin
  Erythrocyte haemolysate, B(a)P (300 μM), silymarin (500 μM dissolved in less than 0.2% DMSO) and PBS

Enzyme assays and the other parameters were assessed in the erythrocyte hemolysates, after incubating B(a)P and silymarin for 45 min.

2.6. Assays of antioxidant enzymes

2.6.1. Assay of CAT activity

Catalase activity was measured according to Aebi (1984) by using UV/visible spectrophotometer. At 37 °C, 25 μl of haemolysates and 1 ml of H2O2 (30 mM) were added to 1.975 ml PBS. The rapid decomposition of H2O2 was determined for every 30 s from the decrease in absorbance at 240 nm for 3 min. Enzyme activity was expressed as units/g Hb [1 unit = 1 mM of H2O2 decomposed min⁻¹ calculated using a molar extinction coefficient for H2O2 of 0.0394 mM⁻¹ cm⁻¹].

2.6.2. Assay of GR activity

Activity of the glutathione reductase was determined as described by Carlberg and Mannervik (1975). The reaction mixture consisted of 0.1 M phosphate buffer (pH 7.6), containing 0.5 mM EDTA, 1.0 mM GSSG, 0.1 mM NADPH and 100 μl aliquots of the supernatant in a final volume of 1.0 ml. The consumption of NADPH was monitored spectrophotometrically at 340 nm. The enzyme activity was expressed as units/g Hb (1 unit = 1 mM of NADPH oxidized min⁻¹ calculated using an extinction coefficient of 6.22 mM⁻¹ cm⁻¹).

2.6.3. Assay of GST activity

Glutathione-S-transferase was assayed by measuring at 340 nm the rate of formation of a GSH conjugate with 1-chloro-2,4-dinitrobenzene (CDNB) (Habig and Jakoby, 1981). The reaction mixture contained 50 μl aliquots of the supernatant, 0.3 mM GSH, 0.17 mM CDNB and 0.1 M phosphate buffer, pH 6.5, in a final volume of 1.0 ml. Phosphate buffer–CDNB mixture was preincubated for 10 min at 37 °C and the reaction was started by adding GSH followed immediately by an aliquot of the haemolysate. The activity was expressed as units/g Hb [1 unit = 1 μM of GSH decomposed min⁻¹ using an extinction coefficient for GSH of 9.6 M⁻¹ cm⁻¹].
2.6.4. Removal of contaminating hemoglobin from the hemolysate and Assay of SOD Activity

To extract Cu–Zn SOD, the hemoglobin present in the hemolysate was removed according to the method of McCord and Fridovich (1969). Briefly, 2.5 ml of the hemolysates were warmed at 37 °C and treated with 1 ml of ethanol–chloroform (2:1, v/v) and mixed thoroughly to obtain a thick precipitate. 2 ml of deionized water was added, mixed again and incubated at 37 °C for 15 min with occasional stirring. The mixture was then centrifuged to spin down the precipitate. The colorless supernatant, thus obtained, was diluted with deionized water in a ratio of 1:1. This hemoglobin-free preparation was then used for superoxide dismutase (SOD) assay.

Superoxide dismutase was assayed according to the method of Paoletti and Mocali (1990), based on NADPH oxidation. The method consists of a purely chemical reaction sequence, which generates superoxide anion from molecular oxygen in the presence of EDTA, manganese(II) chloride and mercaptoethanol. The reaction mixture in a final volume of 1 ml contained 1.9 ml of 100 mM triethanolamine diethanolamine–HCl buffer (TDB), 10 mM EDTA–MnCl2, and 0.2 ml of hemolysate (sample solvent for control). The contents were mixed and allowed to stand for 5 min for a stable baseline. The reaction was initiated by the addition of 0.2 ml of 10 mM mercaptoethanol. The contents of the cuvette were mixed and the decrease in absorbance at 340 nm was followed for 10 min to allow NADPH oxidation. The enzyme activity was expressed as units/g Hb [1 unit = mM of NAPDH oxidized min⁻¹ using an extinction coefficient of 6.22 mM⁻¹ cm⁻¹].

2.7. Estimation of lipid peroxidation

This method evaluates oxidative stress by measuring malondialdehyde (MDA), the last product of lipid breakdown caused by oxidative stress (Yagi and Rastogi, 1979). Hemolysates were mixed with 20% TCA (1:1) and centrifuged at 3000 rpm for 15 min. TBA (15%) was added to the supernatant and the samples were heated to 100 °C for 15 min. The absorbance of the supernatant was measured at 532 nm. The results were expressed as μM of TBARS/g Hb determined using the calibration curve prepared with different concentrations of MDA standards.

2.8. Determination of total protein content

Total protein content, in erythrocyte haemolysates was evaluated using BSA as standard (Lowry et al., 1951).

2.9. GPx and CAT activity gel assays

GPx and CAT activities were measured using activity gels as described by Sun et al. (1988). For GPx, the cell homogenates were run on 7.5% polyacrylamide gel, and the gel was soaked in 1 mM reduced glutathione. The substrate, cumene hydroperoxide (0.008%), was added to the solution, and the gel was soaked for 10 min. The gel was rinsed twice with water and stained in 1% ferric chloride/1% potassium ferricyanide solution. After staining, the gel became dark green/blue with yellow bands, indicating the presence of GPx activity. CAT activity was measured by separating samples on a native 5% or 8% polyacrylamide gel. For detection of catalase activity, the gel was soaked in a solution containing 0.03% hydrogen peroxide for 10 min and then stained with the same staining solution. The gels were photographed in a Vilber Lourmet (France) gel documentation system.

2.10. Identification of B(a)P metabolite [3-OH–B(a)P]

Erythrocyte haemolysates were incubated in 1 ml reaction mixtures containing 100 mM potassium phosphate buffer pH 7.4; 10 mM MgCl2, 0.1 mM EDTA and 300 μM B(a)P at 37 °C for time period of 45 min in the presence of 2 mM NAD(P)H. The reactions were terminated with 1 ml of ice-cold acetone. B(a)P metabolites were extracted with ethyl acetate, dried with Na2SO4 and evaporated. Metabolite residue was re-dissolved in 60 μl methanol. In vitro metabolism of B(a)P in erythrocytes was investigated with the help of HPLC. B(a)P and B(a)P metabolite (3-hydroxy benzo(a)pyrene; 3-OH B(a)P) were separated by HPLC using ODS-C-18 column with a linear solvent methanol for 15 min at 1 ml/min flow rate and monitored at 254 nm. Retention time of these metabolites was compared to authentic standards obtained from the National Cancer Institute’s Chemical Repository, USA.

2.11. Statistical analysis

All the experiments were performed in triplicates and the results were analyzed using Student’s t-test. Comparisons were made between Group I Vs group II and Group III vs group II.

3. Results and discussion

In the present study, the levels of lipid peroxidation products were evaluated in B(a)P and silymarin incubated erythrocyte haemolysates to elucidate the protective role of silymarin. TBARS represent a measurement of oxidative damage and have been utilized in analysis of xenobiotic induced oxidative damage. MDA a highly reactive, bifunctional molecule has been shown to cross-link erythrocyte and leucocyte phospholipids and proteins to impair a variety of the membrane-related functions, and ultimately leading to diminished erythrocytes and leucocytes survival (Chiu et al., 1989; Sugihara et al., 1991; Ault and Lawrence, 2003). TBARS were assayed in haemolysates as a conventional index for lipid peroxidation products. Haemolysates were incubated with 300 μM of B(a)P and co-incubated with 500 μM of silymarin. The amount of TBARS formed from 300 μM of B(a)P alone incubated cells were
1.184 ± 0.441 µM/g Hb whereas the haemolysates which were co-incubated with 500 µM of silymarin along with 300 µM B(a)P showed 0.494 ± 0.23 µM/g Hb (TBARS levels in vehicle control was 0.592 ± 0.257 µM/g Hb). From vehicle control, the percentage of inhibition for B(a)P treatment was 50.04% and percentage of protection for silymarin was 83.45% (Fig. 2).

It has been already reported that increase in MDA levels in haemolysates during exogenous reactive oxygen species (H₂O₂) treatment enhances lipid peroxidation products that leads to erythrocyte damage (Tavazzi et al., 2000). In our study, co-incubation with silymarin significantly reversed these changes and hence it may be possible that the silymarin gives protection against B(a)P induced lipid peroxidation. Gerhäuser et al. (2003) also reported that silymarin is involved in the free radical scavenging activity. Since, reactive oxygen species and reactive metabolites of xenobiotic compounds play a main role in lipid peroxidation (Tedesco et al., 2000), we made an attempt to find out the status of antioxidant enzymes such as SOD, CAT, GSH-Px, GST and GR in haemolysates when incubated with B(a)P, which produces reactive metabolites and ROS during CYP1A1 mediated metabolism. In addition, protective effect of silymarin against these ROS and B(a)P metabolites were also studied by measuring these antioxidant enzymes.

SOD activity in 300 µM treated B(a)P (2.45 ± 0.67 U/g Hb) group was observed to be significantly lower (P < 0.10) than compared to vehicle control group (3.61 ± 0.05 U/g Hb) (Fig. 3). Decreased erythrocyte SOD activity observed during B(a)P incubation denotes an increased level of ROS generation. This is confirmed by the increased level of TBARS observed during B(a)P exposure (see Fig. 2). Mates et al. (1999) also explained that increased levels of TBARS pave decreased SOD activity in haemolysates during toxin treatment. In our study, SOD activities were not affected when haemolysates were co-incubated with 500 µM of silymarin (2.95 ± 0.52 U/g Hb). This indicates that silymarin is involved in the removal of superoxide anion radicals and hence, available SOD in the haemolysates were not changed by radicals which were produced during B(a)P incubation. Gerhäuser et al. (2003) and Gazak et al. (2004) proved the ability of silymarin and oxidized silybin in the removal/scavenging of O₂⁻ radicals. From vehicle control, the percentage of SOD inhibition for B(a)P and percentage of SOD protection by silymarin were determined as 32.13% and 81.7%, respectively (Fig. 3).

The effect of B(a)P on CAT activities in erythrocyte haemolysate is shown in Fig. 2. CAT activity of 300 µM B(a)P incubated group was measured to be significantly lower (P < 0.1) than in vehicle control group. Erythrocyte haemolysate’s CAT activity in Group III (300 µM B(a)P + 500 µM silymarin) indicates that silymarin completely restored the enzyme activity to the normal level. In B(a)P incubated haemolysates, SOD plays a main role in the removal of O₂⁻ radicals during which it will generate H₂O₂ (Saito, 1987). In order to remove H₂O₂, CAT were consumed in the incubation mixture and hence we obtained reduced level of CAT in B(a)P incubated haemolysates. The percentage of CAT inhibition for B(a)P and percentage of CAT protection by silymarin were determined as 33.2% and 92.13%, respectively.

Glutathione reductase activity was also found to be decreased in B(a)P treated groups (8.44 ± 2.459 U/g Hb) compared to vehicle control (15.66 ± 4.66 U/g Hb) group, whereas during co-incubation with silymarin the enzyme activity remained unchanged (13.88 ± 4.68 U/g Hb). From vehicle control, the percentage of inhibition for B(a)P was 46.1% and percentage of protection for 500 µM of silymarin was 88.63% as shown in Fig. 4. Similarly decreased activities of GSH-Px was observed in B(a)P incubated group-II. It has been reported that decreased activity of GR, GPx and CAT in haemolysates indicates oxidative stress (Mates et al., 1999; Das et al., 2004). On the other hand, all these enzymes were unchanged when co-incubated with silymarin. It has been already suggested that CAT, GSH-Px and GR have amelioratory role against peroxidative insult to erythrocyte membrane (Gaetani et al.,
and in our study, the unchanged activities of these enzymes during silymarin co-incubation may result in removing H$_2$O$_2$ and peroxides efficiently to protect the erythrocyte membrane lipids from peroxidation. This shows that silymarin is capable of protecting the cells from the damage caused by B($a$)P. To substantiate the current results and as a supportive evidence for the quantitative assay for GPx and CAT as reported earlier, we detected the antioxidant enzymes in native gel electrophoresis. It demonstrated that GPx and CAT activity was significantly decreased during toxin treatment compared to vehicle control group (Fig. 5). Alteration in the intensity of band pattern during silymarin treatment as reflected by native PAGE analysis shows normalisation of antioxidant defense system. All these changes demonstrate protection against xenobiotic toxicity on treatment with plant antioxidant silymarin.

During B($a$)P metabolism, B($a$)P metabolites were removed by phase II enzymes like GST (Spiff and Uwakwe, 2003). In our study, GST activity was significantly decreased during toxin treatment compared to vehicle control group (Fig. 5). Alteration in the intensity of band pattern during silymarin treatment as reflected by native PAGE analysis shows normalisation of antioxidant defense system. All these changes demonstrate protection against xenobiotic toxicity on treatment with plant antioxidant silymarin.

During B($a$)P metabolism, B($a$)P metabolites were removed by phase II enzymes like GST (Spiff and Uwakwe, 2003). In our study, GST activity was significantly decreased ($P < 0.05$) in B($a$)P incubated haemolysates (1.98 ± 0.58 U/g Hb) when compared to vehicle control group (5.7 ± 0.49 U/g Hb) (Fig. 3). Our in vitro results showed that GST decrease in haemolysates could be a valuable indicator of oxidative stress during B($a$)P treatment. The functional role of GST is to participate in the detoxification of lipid peroxidation products as well as lipophilic drugs and other xenobiotics. Neefjes et al. (2006) also found the inhibition of erythrocyte GST in human adults and

found that in vitro GST can be used as indicator to oxidative stress. Simultaneous co-incubation of 500 µM silymarin with 300 µM B($a$)P prevented the decreased activity of GST (4.5 ± 0.67 U/g Hb). From vehicle control, the percentage of inhibition for B($a$)P was 65.3% and percentage of protection for 500 µM of silymarin was 78.75%.

In our study, all the measured antioxidant enzymes were decreased in B($a$)P incubated haemolysates. On the contrary, when haemolysates were co-incubated with B($a$)P and silymarin, antioxidant enzymes were not altered. There are two possible reasons for the unchanged status of antioxidant enzymes in silymarin + B($a$)P incubated reaction mixture. One is, silymarin itself can act as a radical scavenger and hence available antioxidant enzymes in the reaction mixture were not changed. Other is, silymarin would inhibit CYP1A1 metabolism of B($a$)P and further generation of ROS so that B($a$)P metabolites are blocked. In order to find out the exact role of silymarin, i.e., whether it act as B($a$)P metabolite inhibitor or antioxidant, we measured B($a$)P metabolites in B($a$)P + silymarin reaction mixture and measured CAT activity in exogenous ROS (H$_2$O$_2$) exposed reaction mixture.

Haemolysates were incubated with 300 µM of B($a$)P alone and 300 µM B($a$)P along with 500 µM silymarin for 45 min. B($a$)P (0.19 µg) metabolite, 3-hydroxy benzo($a$)pyrene was observed in B($a$)P incubated haemolysate. Retention time for 3-OH B($a$)P was 4.6 min (Fig. 6). On the contrary, 3-OH B($a$)P was not observed in the haemolysates when co-incubated with silymarin. This result clearly indicates that silymarin plays an inhibitory role on CYP enzymes and hence, metabolites were not produced or blocked. Gerhäuser et al. (2003) also reported that silymarin is involved in the inhibition of CYP1A1 activity. In order to know the ability of silymarin in ROS elimination, exogenous reactive oxygen species H$_2$O$_2$ was incubated with haemolysates and co-incubated with silymarin. Incubation of 10 mM H$_2$O$_2$ to haemolysate and co-incubation with 400 µM silymarin showed that H$_2$O$_2$ incubated haemolysate had significantly decreased ($P < 0.05$) level of catalase activity (0.210 ± 0.012 U/g Hb) compared to the vehicle control (0.314 ± 0.012 U/g Hb). On the other hand, the haemolysate co-incubated with 400 µM silymarin showed near normal CAT activity (0.288 ± 0.04 U/g Hb)
as shown in the Fig. 7. The percentage of inhibition from vehicle control for H$_2$O$_2$ was 33.12% and percentage of protection from vehicle control for 400 µM of silymarin was 91.72%. From the above results it is discernible that silymarin contains both antioxidant properties and carcinogenic metabolite [3-OH–B(a)P] inhibiting properties.

Our results allow us to draw a conclusion that silymarin would protect the haemolysates against environmental toxins induced oxidative stress. These finding provide a basis for the development of novel therapeutic strategies, such as antioxidant supplementation like a flavonoid silymarin for protection against the damages caused by environmental contaminants.

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References


