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AMELIORATION OF γ-RADIATION-INDUCED GENOTOXICITY BY NANOSILYMARIN: A COMPARATIVE STUDY INDICATES POSSIBLE IMPLICATIONS FOR CHEMICAL BIOLOGICAL RADIOLOGICAL AND NUCLEAR (CBRN) DEFENCE

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ABSTRACT

Purpose: To check the comparative efficacy of DNA protection by silymarin and its nanoformulation as an effective radiation countermeasure agent in ameliorating γ-radiation-induced Genotoxicity.

Methods: The study performed suggests the efficacy of silymarin and its nanoformulation specifically in ameliorating γ-radiation-induced genotoxic effects at cellular, plasmid DNA levels etc.

Results: The retention of super-coiled DNA following treatment of DNA with various concentrations of silymarin (parent compound) was found to be maximum at 25µg/ml, whereas better retention was seen at 10µg/ml in case of silymarin nanoformulation. Micronuclei count also reduced maximally at 10µg/ml when treated with silymarin nanoformulation as compared to 25µg/ml using parent compound.

Summary : Silymarin and its nanoformulation showed no toxic effects on DNA. The nanoformulation demonstrated better results in terms of protection of genetic material against γ-radiation due to increase in surface area and hence improved bioavailability. The nanoformulation can be of use in mitigating the deleterious effects of radiation and plausible biothreat agents.

Key words: Silymarin, γ-radiation, nanoformulation, HEK cells, micronuclei, pUC19

INTRODUCTION

Ionizing radiation inflicts substantial damage to living tissues through a cascade of molecular events (1). Radiation exposure of biological systems results in oxidative stress due to hydrolysis of water and generation of ROS (*e.g.*, superoxide radicals [$O_2^{\bullet}$], hydrogen peroxide [H_2O_2] and hydroxyl radicals [$^{\bullet}OH$]) which initiates a plethora of chemical peroxidative processes (2). Interaction of ionizing radiation directly with macromolecules results in breakage of covalent bonds (3). The damage to protein molecules has also been examined in great detail and has been found throughout polypeptides regardless of the site of the primary ionization (4). One of the most susceptible targets in a living cell is DNA. The radiation-induced DNA damage can be of various types e.g., single and double-strand breaks, base damage, damage to sugar moiety and cross-linkages of the intra and inter-strand types etc(5). These damages are a result of oxidative stress which can be induced by free radicals (6-8).

Several compounds with antioxidant properties have the ability to alleviate deleterious effects of

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ionizing radiation in living systems and bio-molecules (9, 10). In view of this, efforts have been made to improve the therapeutic effect of radiotherapy by minimizing normal tissue injury and by-stander damage to acceptable levels using radioprotective compounds mainly containing sulfhydryl groups like cysteine, cysteamine and WR-2721 (11). The radioprotective ability of these compounds has been substantially attributed to their reactive oxygen species (ROS) scavenging ability (12). The search for effective and non-toxic radiation countermeasures is a necessity and consequently attention has been diverted towards the naturally occurring antioxidants. Additionally, a plethora of medicinal plants, endophytes and other microbial products have also been investigated for their radioprotective efficacy (13-21). In view of the proven medicinal value of a number of natural products in treating various ailments, interest in medicinal and aromatic plants is increasing worldwide (15). Radioprotection being a multifaceted phenomenon, it is essential to investigate and elucidate the mechanisms involved in radioprotective action of the herbal drugs.

A number of natural plant products (polyphenols, flavonoids, vitamins and carotenes) are known to exhibit antioxidant properties (22). Silymarin is a non-toxic bioactive flavonoid, an approved herbal hepatoprotectant and is consumed worldwide as a dietary antioxidant for prevention and treatment of a number of diseases (23, 24). The antioxidant, anti-inflammatory and anti-carcinogenic properties of silymarin has been demonstrated in various *in vitro* and *in vivo* models against oxidative stress, inflammatory responses and chemical carcinogen-induced tumor promotion (25, 26). Silymarin has also been reported to increase aspartate aminotransferase (ALT) and -glutamyltranspeptidase (GGT) in plasma (27). It has also beenwidely used as a topical ointment for the treatment of breast cancer (28). Silymarin is known to modulate proinflammatory pathways via downregulation of cyclooxygenase-2 (COX-2) (29) and 5-lipooxygenase (LOX), thereby inhibiting hepatic cytochrome P450 detoxification system involving hepatic cytochrome P450 enzyme activities (30). Silymarin is also known to be able to protect hepatic tissues from radiation directly by stabilizing membrane permeability and preventing liver glutathione depletion (31). In addition to its hepatoprotective properties, silymarin also exhibits protective effects against various drugs of nephrotoxic nature (32). The compound silibinin has been reported to protect hepatocytes by blocking the hepatotoxin receptors present on the hepatocyte membrane, thus preventing apoptosis and cell death (33). It has been documented that silymarin protects from hepatotoxins by reducing the levels of oxidized Glutathione (GSH) in the liver and intestine; stimulate the ribosomal RNA polymerase and protein synthesis resulting in enhanced regeneration of hepatocytes. Silymarin has been shown to protect liver against fumonisin B1 –induced damage (34) and sepsis-induced acute lung and brain injury in mice. The powerful hepatoprotective effects of silibinin have been reported in cultured primary rat hepatocytes against apoptosis and cytotoxicity caused by Ochratoxin A (OTA) (35), not only in OTA but hepatoprotective effects in Aflatoxin B1 have also been studied in case of albino male Wistar rats (36). It has been reported that silymarin derived -phospholipid complex is involved in reducing the toxic effects of aflatoxin B1 (AFB1), as studied in broiler chickens (37). Potential ameliorative effects of Silymarin in combination with vitamin E against (OTA)-induced immunotoxic effects in White Leghorn cockerels have also been reported (38). In addition, protective effects of silymarin on L-arginine-induced genotoxicity in *in vitro* lymphocyte culture have also been elucidated (39).

The phytochemicals e.g., lignans and flavonoids present in silymarin have been individually reported to be of prominent efficacy against various pathological symptoms and also possess radioprotective properties (40)

Nanoparticles have been recently used as efficient drug delivery systems in recent years to protect against radiation injuries. Nanomedicines are emerging as one of the new treatment options, (41, 42) since they are novel in their mode of action (43, 44). Silymarin is a known natural lipophilic agent and despite its prominent properties, it has the limitation of low bioavailability in living organisms. A nanotechnology based approach can lead to the development of novel drug delivery systems to increase the solubility and oral absorption of various drugs for achieving better bioavailability and therapeutic activity (45-48). Keeping this in mind, a nanoformulation of silymarin was

prepared and utilized for experimentation in the present study comparing it with the parental compound silymarin.

Irradiation of purified DNA molecules has been extensively used during the last decade for studying the interaction of ionizing radiation with DNA (49-53). Plasmid DNA is considered a useful model for investigating interactions between topologically constrained DNA and radiation in addition to their role as vectors (54-56). Micronuclei estimation can effectively serve as a biological dosimeter to estimate *in vitro* ionizing radiation exposure (57). Keeping these key observations in view, this paper reports comparative protection of DNA against gamma radiation-induced damage using silymarin and its nanoformulation. Protection to DNA under *in vitro* conditions of irradiation was estimated in plasmid DNA by plasmid relaxation assay and micronuclei estimation in order to evaluate the potential of silymarin and its nanoformulation. It has been hypothesized that silymarin and the nanoformulation interacts directly with plasmid DNA to exhibit its shielding effect against radiation exposure. Elucidation of the mechanism of action of silymarin in radiation protection can aid in forming strategies for the development of an ideal radioprotector for human use.

MATERIALS AND METHODS

Chemicals

Low melting agarose, Tris-base, Ethidium bromide, Tris-HCL, bromophenol blue, xylene cyanol, glycerol, sucrose, high glucose Dulbecco Modified Eagle Medium (HG-DMEM), Trypsin-EDTA, Bovine serum albumin (BSA) and Hoechst-33258 etc. were obtained from Sigma Aldrich St. Louis MO, USA). pUC 19 plasmid was obtained from Thermoscientific, PA, USA. Methanol, Acetic acid, Tween-20 and citric acid were purchased from Merck India Pvt. Ltd, Mumbai, India.

Herbal extract

Silymarin was procured from Wuxi Gorunjie Technology Co. Ltd, Jiangsu, China. The sample was stored in a cool and dry place away from strong light and heat as per the prescribed information of the manufacturer.

Irradiation

A ^{60}Co gamma irradiator (Gamma cell 5000, Board of Radiation and Isotope Technology, Mumbai, India) with a dose rate of 1.12kGy/h was used as irradiation source. HEK cells were grown in 10% FBS supplemented with MEM at 37ºC in humidified 5% CO_2: 95% Air envrionment. Logarithmically growing cells were exposed to γ-radiation using ^{60}Co gamma chamber (Bhabhatron-II Telecobalt unit – BARC, Mumbai, India; Dose rate of 1.4 Gy/min) at room temperature.

DNA damage assay

The effect of silymarin and its nanoformulation on radiation-induced relaxation of plasmid (pUC19) DNA was evaluated (53). 200ng of plasmid DNA, in potassium phosphate buffer (0.1mM, pH 7.4), was exposed to γ-radiation (250Gy) in the presence of different concentrations of silymarin and its nanoform (10-500µg/ml). After irradiation, the plasmid and silymarin reaction mixture was suspended in TE buffer (Tris-EDTA, pH 8.0) and resolved on 1% agarose (w/v) submarine gels in electrophoresis buffer (TBE; pH 8.0) at 45V (3V/cm). The gel was subsequently stained with 0.5µg/ml ethidium bromide for 15-30min and photographed under UV illumination. The comparative amount of relaxed and supercoiled DNA was determined by scanning the gel with the Bio Rad GEL-DOC system (Bio Rad, Hercules, California, USA).

Miconucleus assay

The micronucleus technique was used as a method for measurement of radiation-induced chromosomal damage. HEK cells were treated with silymarin and nanoformulation 0.5 h prior to γ-radiation (2Gy) and incubated for different time intervals for evaluation of protection against micronucleus formation. Upon completion of incubation period, cells were washed twice in phosphate buffered saline (PBS, pH 7.2) and fixed in Carnoy‘s fixative (Methanol: Acetic acid; 3:1) at 4°C for 24h. Fixed cells were spread on clean pre-chilled microscopic slides. Following overnight air drying, slides were stained with 10µg/ml Hoechst-33258 in phosphate buffer ($Na_2HPO4.2H_2O$, 0.5% tween-20, and 0.1M citric acid) in the ratio 9:1, final pH 7.4 for 30min, in dark at room temperature (54). After washing off excess stain with distilled water followed by PBS, the slides were mounted in PBS-glycerol (1:1) and observed under fluorescence microscope (Olympus BX60, Tokyo, Japan) using UV excitation filter. A total of 500 cells in triplicates were scored per group.

The frequency of cells with micronuclei, called the M-fraction (MF) was calculated as:

$$MF(\%) = N_m/N_t \text{ X } 100$$

where Nm is the number of cells with micronuclei and Nt is the total number of cells analyzed.

RESULTS

DNA Protective Effect of Silymarin and its nanoformulation Radiation exposure causes DNA strand breaks resulting in conformational changes in terms of relaxation of plasmid DNA from supercoiled form to open circular form. The DNA protecting ability of silymarin was investigated using the plasmid relaxation assay which is a method of semi-quantitative assessment of ionizing radiation-induced oxidative damage to DNA (52). It was observed **(Figure 1)** that silymarin reduced γ-radiation-induced appearance of open circular form of the plasmid DNA significantly ($p < 0.05$). Different doses of silymarin in the range of 5-50µg/ml were evaluated for assessing their protective efficacy against γ-radiation in terms of percentage of supercoiled form retained. It is evident from **Figure 1** and **Figure 2** that untreated control (positive control, lane 1) comprised of more than 70% supercoiled form, while upon exposure to γ-radiation (250Gy) (negative control, lane 2) nearly 65% of plasmid DNA converted to its relaxed form (open circular DNA). Densitometric analysis of pUC 19 DNA pre-treated for 1h with silymarin and then irradiated revealed that silymarin could aid in retaining the supercoiled form of DNA by more than 70% in the range of 25–50µg/ml, which was significantly higher ($p < 0.05$) than that produced in the pre-treated silymarin lane (5µg/ml, lane 7), which retained only 58% supercoiled form. Results indicate that silymarin significantly shielded plasmid DNA from strand breaks induced by γ-radiation and it may be attributed to the higher level of flavonolignan content of silymarin.

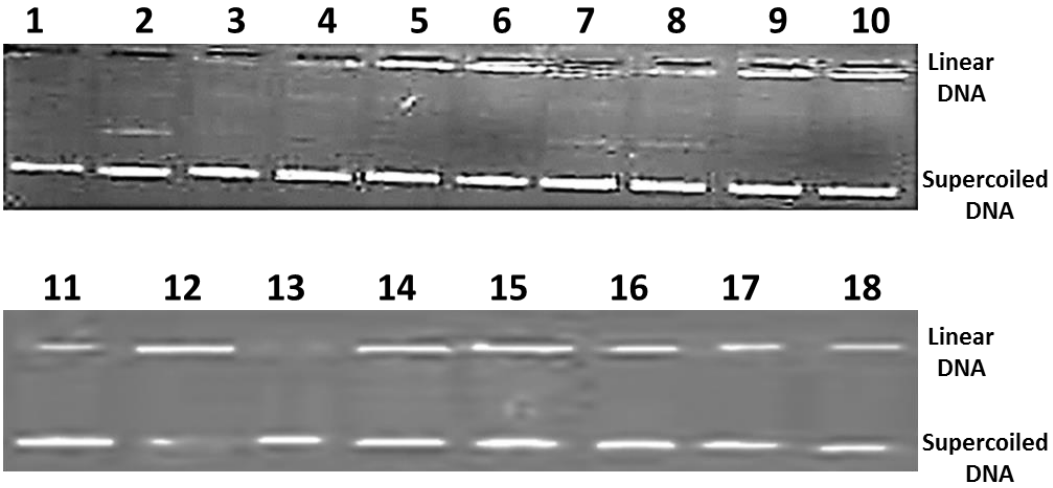

Figure 1. Evaluation of DNA protective ability of silymarin and its nanoformulation. First Gel image represent silymarin alone with different concentrations along with 250Gy-radiation. Lane 1- Untreated, 2 - 250Gy, 3 - 5µg/ml, 4 - 10µg/ml, 5 - 25 µg/ml, 6 - 50 µg/ml, 7 - 5µg/ml+250Gy, 8 - 10µg/ml+250Gy, 9 - 25µg/ml+250Gy, 10 - 50µg/ml+250Gy. Second Gel Image represents Silymarin nanoformulation group in different concentrations along with 250Gy -radiation. Lane 11 - Untreated, 12 - 250Gy, 13 - 10 µg/ml, 14 - 25 µg/ml, 15 - 50 µg/ml, 16 - 10 µg/ml+250Gy, 17 - 25 µg/ml+250Gy, 18 - 50 µg/ml+250Gy.

The DNA protecting ability of silymarin nanoformulation was also investigated using the plasmid relaxation assay. The untreated control (positive control, lane 1) comprised more than 70% supercoiled form, while upon exposure to 250Gy γ-radiation (negative control, lane 2), nearly 70% of plasmid DNA was observed in relaxed form (open circular DNA).

Densitometric study of pUC19 DNA pre-treatment for 1h with silymarin nanoformulation and then radiation treatment revealed that silymarin nanoformulation at a concentration of 10µg/ml (lane 6), 25µg/ml (lane 7), 50µg/ml (lane 8) led to a decrease of 32.58%, 38.43% and 40.51% in the open circular form respectively. Silymarin nanoformulation could shield the

supercoiled form of DNA by more than 70% in the range of 10µg/ml, which was significantly higher ($p < 0.05$) than that obtained in the pre-treated silymarin group (25µg/ml, lane 7), which retained only 62% supercoiled form **(Figure 2).** Silymarin nanoformulation showed better performance at lower concentration range and the supercoiled DNA decreased in an increasing drug concentration-dependent manner.

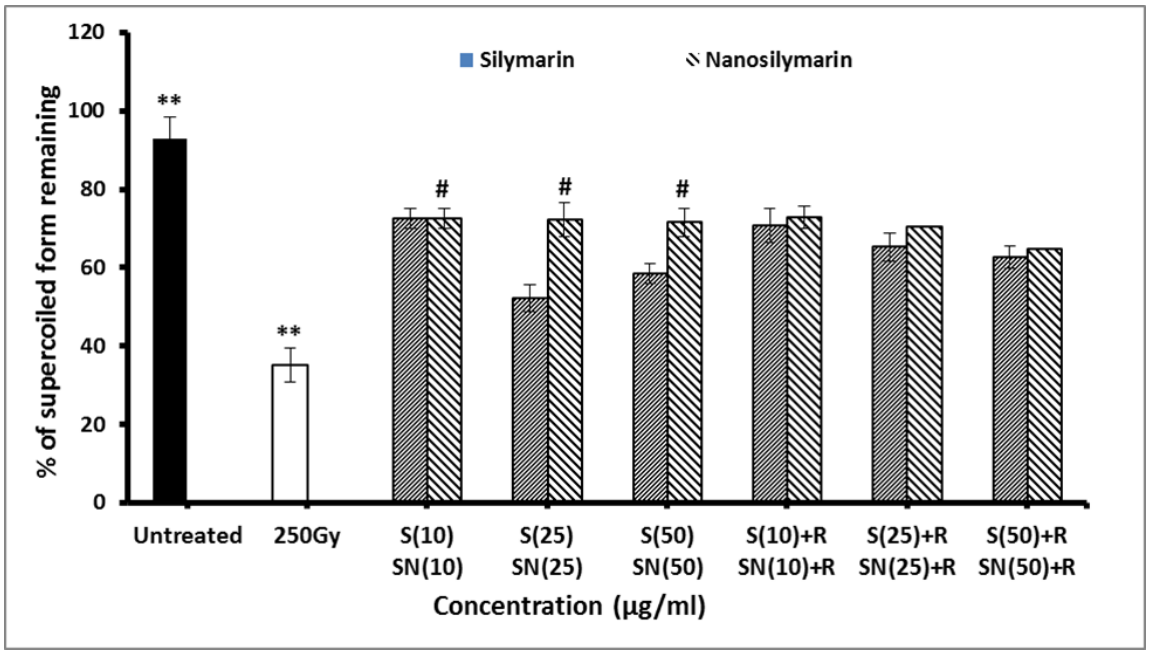

Figure 2. Effect of silymarin and its nanoformulation (10-50 µg/ml) on γrradiation (250Gy)-induced pUC19 plasmid DNA damage. Treatment of pUC 19 by 10µg/ml against 250Gy -irradiation shows significant decrease amount of supercoiled DNA in both form of silymarin significantly, as compared to the untreated. Silymarin alone showed dose-dependent reduction in supercoiled form. (Significant levels: $^{**}p < 0.05$ Untreated group vs Radiation (250Gy); $^{\#}p < 0.001$Silymarin (N) 10µg/ml vs silymarin (N) 50µg/ml.

Table 1. *Micronucleus frequency in HEK cells after pre-incubation with silymarin (25µg/ml) and silymarin nanoformulation (10µg/ml) at different time-intervals. Micronuclei frequency (MN) was calculated in HEK cells per 500 cells. Both silymarin groups show a significant decline in MN frequency as compared to the irradiated group in all time-points. Results are expressed as a mean of replicates (in thrice) from three independent experiments. Results were compared using two-tailed student's t-test with Bonferroni correction following analysis of variance. All data expressed as mean ± standard error of mean.*

	24h	**48h**	**72h**
Control	6 2.3	8 3.78	5 3.58
2Gy	46 1.5	51.5 1.78	63 .85**
Silymarin (25µg/ml)	8 0.58	11 1.96	10 1.75
Silymarin (25µg/ml)+2Gy	9 1.58	10 0.48	6 1.96
Silymarin (N) (10µg/ml)	6 2.48	5 0.79	6 2.74
Silymarin (N) (10µg/ml) +2Gy	6 2.75	3 0.96	3 3.85**

Effect of silymarin and its nanoformulation pretreatment on γ-radiation-induced DNA damage and micronuclei formation

γrradiation possesses the potential to induce micronuclei formation in HEK cells even at a low dose. Radiation exposure (2Gy) significantly induced MN formation by 8.0, 7.0 and 12.5-fold at 24 h, 48 h and 72 h time intervals respectively ($p < 0.05$) as compared to control **(Table 1).**

Pretreatment with silymarin followed by irradiation of cells with γ-rays resulted in a significant ($p < 0.05$) decrease in the percentage of micro-nucleated cells and total MN in comparison to the radiationalone group. Silymarin alone (25μg/ml) decreased MN frequency by nearly three-times as compared to irradiated control, hence exhibiting its non-toxic nature. Silymarin elicited maximum reduction (63.85 %) in the radiation-induced MN formation at 72 h time-point.

Silymarin nanoformulation when given alone (10μg/ml), also caused a significant decrease ($p < 0.05$) in MN numbers as compared to radiation exposed group at all-time intervals and also when compared to control, exhibiting its non-toxic nature. At later time intervals, pre-treated irradiated silymarin groups exhibited decreased MN level much efficiently with maximum reduction (61%).

DISCUSSION

Both ionizing-radiation and mycotoxins are known to have mutagenic and carcinogenic effects via generation of reactive oxygen species (55-57). Various compounds possessing antioxidant properties have been evaluated in recent years to shield DNA against the harmful effects of environmental genotoxins. Silymarin has been shown to protect against ribavirin-induced genotoxicity (58). The present study provides direct evidence of protection against genotoxic effects by silymarin and its nanoformulation. Protecting cellular DNA from radiation damage might aid in the prevention of cancers/mutations induced by radiation. This approach also has implications in reducing the undesirable side effects caused by ionizing radiation injury (64) In our previous report on radioprotective studies, we have provided direct evidence of free-radical scavenging ability of silymarin (40). The present study shows that under *in vitro* conditions, silymarin exhibits the ability to shield DNA against exposure to γ-radiation induced damage.

Most of the radiation damage to biomolecules is mediated through ROS generation by radiolysis of water. Therefore, the effect of silymarin on DNA irradiated under aqueous condition, where indirect effect becomes dominant, was evaluated. It was observed that under acellular conditions of irradiation, silymarin protected plasmid pUC19 DNA against γ- radiation-induced damage significantly **(Figure 1 and 2)**. It prevented the occurrence of radiation-induced strand breakage events in the plasmid DNA as is evident from conservation of supercoiled form of DNA. This fact can also be attributed to the physical interaction of silymarin with plasmid DNA in order to prevent free radicals from damaging the DNA supercoiled conformations. Exposure of pUC19 to 250Gy γ-radiation caused conversion of supercoiled pUC19 DNA (fast mitigating), into open circular form (slow mitigating) but maximal retention of supercoiled form was achieved at 25μg/ml range (lane 9). Silymarin (25μg/ml) + 250Gy showed maximum protection (< 72% supercoiled retention form) as compared to radiation alone group (35% supercoiled form) **(Figure 1)**. Level of supercoiled DNA retention in percent in silymarin nanoformulation was also compared and it showed enhanced supercoiled retention at lower concentration range (10μg/ml) and it decreased in a concentration-dependent manner. This may be due to its DNA protective ability at 10μg/ml, which is lesser than parent silymarin concentration, which protects pUC19 DNA at 25μg/ml **(Figure 2).**

The present study also provides evidence that silymarin and its nanoformulation possess the ability to inhibit radiationinduced micronuclei formation and DNA damage in human embryonic kidney cells (Figure 3). Studies on radiation-induced DNA damage by Micronuclei (MN) assay carried out in HEK cells as per the method reported by Schmid, 1975, revealed that irradiation (2Gy) significantly enhanced the frequency of MN in HEK cells as compared to control. Pre-irradiation (0.5h) treatment with silymarin substantially countered this upsurge in MN frequency clearly indicating its role in reduction of radiation-induced DNA damage. However, treatment with silymarin nanoformulation 0.5h before irradiation showed high reduction in micronuclei count in a significant way in later time-intervals as compared to radiation alone showing its radioprotective nature (Figure 3b). Also, it was found that the most effective concentration of

silymarin nanoformulation for minimizing micronuclei count was lower than that of the parent silymarin compound.

Our results have clearly shown that the radioprotective efficacy of silymarin nanoformulation is better than silymarin parent compound. It may be due to the fact that silymarin is orally absorbed but has very poor bioavailability due to its poor water solubility (65). The formulation work has been performed to enhance its solubility so as to increase its bioavailability and thus, its radioprotective property as compared to its parent compound. Under *in vitro* conditions of radiation exposure, it was found that silymarin nanoformulation significantly reduced DNA damage induced by radiation as is evident from plasmid relaxation assay and micronuclei count in HEK cells, which is a sensitive technique to measure radiation damage and can be a reliable method for biodosimetry. Hence, in conclusion, it can be stated that both silymarin and its nanoformulation aid in preserving the structural and functional integrity of DNA upon exposure to ionizing radiations. However, the nanoformulation is more efficient than its parent compound in shielding the genetic material against radiation-induced damage. Based on the leads, and preliminary *in vitro* studies vis-à-vis mycotoxins, it would be interesting to further study the effects of silymarin, including nanosilymarin formulation, in mitigating the deleterious effects of mycotoxins particularly in liver and kidneys of higher animal models. With the preliminary results indicating promise, the possible implications of silymarin and nanosilymarin for CBRN defence is an area that needs further exploration.

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