Evaluation of Silymarin as a Promising Radioprotector

Manish Adhikari[a], Rajesh Arora[a,b], Raman Chawla[a], Jyoti Sharma[a], Atlar Singh Dhaker[a], Damodar Gupta[a], Neha Dubey[a], Raj Kumar[a], Veselin Ivanov[b], Veselina Gadjeva[b], Renata Gevrenova[c], and Rakesh Kumar Sharma[a,*]

[a] Institute of Nuclear Medicine and Allied Sciences, Brig SK Mazumdar Marg, Delhi-110054, India. E-mail: rks@inmas.drdo.in
[b] Department of Chemistry and Biochemistry, Medical Faculty, Trakia University, Armeiska 11, Stara Zagora 6000, Bulgaria
[c] Faculty of Pharmacy, Medical University Sofia, Dunav Str. 2, Sofia 1000, Bulgaria

* Author for correspondence and reprint requests

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Silymarin, a purified extract of seeds of *Silybum marianum* L. and well known for its hepatoprotective abilities, has been evaluated for inherent utility as a radioprotective agent. A fraction (INM-7035) was authenticated by characterizing the percentage composition of silybin A and B (39.9% and 57.4%). Free radical scavenging activities of INM-7035 against superoxide radicals (>68%), hydroxyl radicals (>33.75%), DPPH (67.2%), and ABTS (32.4%) were also evaluated. The fraction chelated (>30%) ferrous ions, thereby able to restrict amplification. INM-7035 exhibited >50% peroxyl radical scavenging activity in the lipid phase along with dose-dependent (R^2 = 0.990) reducing power in the aqueous phase. Radiation-induced free radical flux can lead to disruption of biomolecules like membrane lipids. INM-7035 completely inhibited lipid peroxidative stress in case of membranes against supralethal radiation stress in the liposomal system. The ability of INM-7035 to modulate the levels of NF-κB, indicated its inherent potential as a radioprotective bioactive constituent.

Key words: Silymarin, Radioprotection, Antioxidant

Introduction

The deleterious impact of oxidative damage mediated by radiation-induced free radicals on living organisms is well established (Sies, 1985). Damage induced by reactive oxygen species (ROS) and reactive nitrogen species (RNS) in mammalian systems can be reduced by exogenous supplementation usually with antioxidants as a preventive strategy to boost the endogenous antioxidant defence (Arora *et al*., 2005a; Sagar *et al*., 2006) since the biological system is overwhelmed by the radiation damage. The ROS/RNS generated as a consequence of irradiation interact with various macromolecules like DNA and the sulfhydryl group of cellular proteins and lipids leading to protein fragmentation and denaturation (Huie and Padmaja, 1993). A number of natural plant products used in traditional Indian medicine possess potential antioxidants that are yet to be explored for various medicinal purposes (Priyadarshini *et al*., 2005; Arora *et al*., 2005a,b,c, 2007).

Silymarin, a purified extract of seeds of milk thistle (*Silybum marianum* L., Asteraceae), contains flavolignans like silybinin (60–70%) alongwith isosilybin (5%), silydianin (10%), and silychristin (5%) (Saller *et al*., 2001; Khan *et al*., 2006). Silybin has structural similarity with steroidal hormones and thereby acts in protein synthesis (Kosina *et al*., 2002). It has been reported that it exhibits curative potential in alcoholic liver disease, acute and chronic viral hepatitis, and toxins-induced hepatic damage, and plausible beneficial effects in radiation-induced damage to the membrane of liver cells (Kren and Walterová, 2005; Ball and Kowdley, 2005; Ramadan *et al*., 2002). Such potential was attributed to its ability to maintain the cell fluidity (Muriel and Mourelle, 1990), to the hepatocyte Ca^{2+} content (Farghali *et al*., 2000), to enhanced protein and DNA synthesis, and to its anti-inflammatory ability (Dehmlow *et al*., 1996) and ability to modulate the hepatic detoxification machinery (Baer-Dubowska *et al*., 1998). In addition, its dehydrosuccinate sodium salt acts as an

Table I. Standardization and quality control of herbal material of silymarin[a].

Analysis	Specification	Result
Assay (UV)	Min. 80%	81.0%
Assay (HPLC)	Min. 70%	70.2%
Chemical physical control		
Characters/appearance	Fine powder	Conforms
Colour	Litter yellow	Conforms
Odour	Characteristic	Conforms
Mesh size/sieve analysis	NLT[b] 100% through 80 mesh	Conforms
Loss on drying	NMT[b] 5.0%	2.8%
Sulfated ash	NMT 1.0%	0.5%
Heavy metals	NMT 10 ppm	Conforms
Arsenic (As)	NMT 1 ppm	Conforms
Lead (Pb)	NMT 1 ppm	Conforms
Sterilization method	High temperature and pressure	Conforms
Total plate count	NMT 1,000 cfu/g	210 cfu/g
Total yeast and mold	NMT 100 cfu/g	30 cfu/g
E. coli	Negative	Conforms
Salmonella	Negative	Conforms
Staphylococcus	Negative	Conforms

[a] 2 years shelf life if properly stored; sample name, silymarin-milk thistle extract powder; batch number, GRJ-Sil-20070620.
[b] NLT, not lower than; NMT, not more than.

inhibitor of linoleic acid (lipid phase) catalyzed by Fe^{2+} salts (Ferenci *et al*., 1989), inhibits hydroxyl radicals generation in the liposomal system (under chemically induced peroxidative stress) (Bosisio *et al*., 1992), and protects hepatocytes and erythrocytes against chemically induced and osmotic shock-induced damage, respectively (Davila *et al*., 1989; Valenzuela and Garrido, 1994). In view of such an inherent potential silymarin was studied against radiation-induced oxidative stress *in vitro*, and its utility as radioprotective constituent for the development of herbal radioprotective formulations in future was established.

Material and Methods

Herbal material

The milk thistle powder was procured in the form of fine, light yellow powder from Wuxi Gorunjie Technology Co. Ltd, Jiangsu, China. A detailed analysis of various physicochemical characteristics and microbiological control tests is given in Table I. The sample was stored in a cool and dry place away from strong light and heat as per the prescribed information of the manufacturer. The sample is referred to as INM-7035 in the present study.

Irradiation

A ^{60}Co gamma source (Gamma cell 5000, Board of Radiation and Isotope Technology, Mumbai, India) at a dose rate of 1.8 kGy/h was used as source of irradiation in the liposomal based membrane protection assay. Dosimetry was carried out using Baldwin Farmer's secondary dosimeter and Fricke's chemical dosimetry method.

Authentication of silymarin using HPLC analysis

HPLC analysis was carried out as per European Pharmacopoeia 5.0. The level of standard phytoconstituents, *i.e.* silibin A/B, was analysed using a Varian Microsorb MV 100-5 column (150 × 4.6 mm i.d., 5 μm; Varian, Walnut Greek, CA, USA). The mobile phase was composed of solvent A [phosphoric acid/methanol/water (0.5:35:65, v/v/v)] and solvent B [phosphoric acid/methanol/water (0.5:50:50, v/v/v)]. The gradient program began at 100% A, followed by a linear gradient to 100% B within 28 min, an isocratic step for 7 min, and then return to the initial conditions within 1 min. The calibration time was 15 min. The flow rate was 0.8 ml/min. The peak analysis was compared with the standard run, and cospiking analysis was performed to authenticate the sample. The chromatographic analysis was performed on a Varian

chromatographic system, which consisted of a tertiary pump model 9012, a Rheodyne injector with a 20-μl sample loop, and a UV-VIS detector model 9050. The chromatograms were recorded at 288 nm.

Estimation of total polyphenolic content

The polyphenolic content was estimated using the standard method (Singleton and Rossi, 1965). 10 ml of water and 1.5 ml of Folin Ciocalteu reagent (FCR) were added to an aliquot (10 μl) taken from the stock solution (1 mg/ml) of INM-7035. The mixture was kept for 5 min at room temperature, and then 4 ml of 20% sodium carbonate solution were added, and the volume was made up to 25 ml with double distilled water. After 30 min of absorbance the developed colour was recorded at 765 nm using a μQuant™ microplate spectrophotometer (BioTek Instruments Inc., Highland Park, Whinooski, VT, USA), and the polyphenolic content was estimated against quercetin as standard.

Estimation of total flavonoid content

The total flavonoid content was determined with aluminium chloride ($AlCl_3$) according to the standardized method (Zhishen *et al*., 1999) using quercetin as a standard. INM-7035 was diluted 1:3 with distilled water followed by mixing with sodium nitrite (300 μl, 5%). After 5 min at 25 °C, aluminium chloride (300 μl, 10%) was added. After an incubation time of 5 min, the reaction mixture was treated with 200 μl of 1 mM sodium hydroxide. Finally, the reaction mixture was diluted to 1 ml with water and the absorbance measured at 510 nm.

DPPH radical scavenging assay

The free radical scavenging activity utilizing DPPH was evaluated (Cuendet *et al*., 1977). Assays were performed in 3 ml reaction mixture containing of 0.05 mM methanolic DPPH (2 ml) solution with varying concentrations of INM-7035 and methanol. The inhibitory effect of different concentrations of INM-7035 (1–2000 μg/ml) on DPPH was measured by recording the absorbance of the reaction mixture at 517 nm, continuously for 5 min. The IC_{50} value represents the level where 50% of the radicals were scavenged by the test samples.

ABTS radical decolourization assay

The ABTS diammonium salt radical cation decolourization test was performed according to Pellegrini *et al.* (1999). The reaction mixture contained 0.45 mM ABTS with varying concentrations of INM-7035 (0.1–500 μg/ml). An ABTS stock reagent mixture was prepared with 1.8 mM ABTS. The ABTS working solution with an equal volume of PBS served as control. The reaction mixtures were incubated at room temperature (28 °C) for 30 min, and the absorbance was measured at 734 nm against PBS as control.

Primary free radical scavenging activity

The superoxide radical scavenging activity of INM-7035 was measured using the nitroblue tetrazolium (NBT) reduction assay described by Kakkar *et al.* (1984). Varied concentrations of silymarin (0–25 μg/ml) were taken and mixed with sodium pyrophosphate buffer (0.052 M, pH 8.3) and phenazine methosulfate (186 μM). NBT (300 μM) was added to the above solution and the final volume adjusted to 3 ml. The reaction was initiated by adding NADH (780 μM), and the solution was incubated at 37 °C for 3 min. The reaction was terminated by adding glacial acetic acid to the resultant mixture, following by 4 ml of *n*-butanol and vigorous mixing. The reaction mixture was allowed to stand for 10 min at room temperature, the *n*-butanol layer was separated by centrifugation (1000 × *g*), and the intensity of chromogen (in the *n*-butanol layer) was measured at 560 nm. Quercetin was used as positive control.

Secondary free radical scavenging activity

The hydroxyl radical scavenging activity was evaluated using the 2-deoxy-D-ribose degradation assay (Green *et al*., 1982). The reaction mixture contained, in a final volume of 1 ml, 2-deoxy-2-ribose (2.8 mM), KH_2PO_4/KOH buffer (20 mM, pH 7.4), $FeCl_3$ (100 μM), EDTA (100 μM), hydrogen peroxide (1.0 mM), ascorbic acid (100 μM) and various concentrations (0–100 μg/ml) of the test sample or reference compound. 1 ml each of trichloroacetic acid (10% w/v) and thiobarbituric acid (0.5% w/v in 0.025 M NaOH) were added to each sample, and the mixture was re-incubated in a hot water bath (Yorco Instruments, India) at 55 °C for 15 min. The tubes were cooled to room

temperature and the absorbance was recorded at 532 nm against the blank with ascorbic acid as a positive control. The percentage inhibition of degradation of deoxyribose or hydroxyl radical scavenging potential was evaluated as follows:

$$\% \text{ inhibition} = (OD_{control} - OD_{sample}/OD_{control}) \cdot 100,$$

where OD_{sample} is the absorbance of sample at 532 nm and $OD_{control}$ is the absorbance of control at 532 nm.

Antioxidant activity in the aqueous phase

The reducing power of INM-7035 was evaluated using the potassium ferricyanide assay (Oyaziu, 1986). Different concentrations of INM-7035 (50 μl) were mixed with 200 μl each of 0.2 M phosphate buffer (pH 6.5) and 0.1% potassium ferricyanide and incubated at 50 °C for 20 min. About 250 μl of 10% trichloroacetic acid were added to the above mixture and centrifuged. The resulting supernatant was then taken and mixed with 500 μl of double distilled water and 100 μl of 0.1% ferric chloride, and further incubated at 37 °C for 10 min. The absorbance was recorded at 700 nm.

Antioxidant activity in the lipid phase

Varied concentrations of INM-7035 (500 μl) mixed with 5 ml of pre-emulsion [three volumes of linoleic acid and an equal volume of Tween-20 in 200 volumes of 30% (v/v) ethanol] with/without 0.25 kGy γ-irradiation exposure, referred as treatment mixture, were incubated at 37 °C. Aliquots from treatment mixtures were taken for assaying the products of peroxidation using the ammonium thiocyanate assay (Asamari *et al.*, 1996). The assay mixture contained 2.5 ml of 75% ethanol, 50 μl of ferrous chloride (0.1%, w/v), 50 μl of ammonium thiocyanate (30%) along with 50 μl of an aliquot in the respective sample. Development of colour was measured at 500 nm against ethanol in a reference cell.

Membrane protection index

Soya lecithin (phospholipid) and cholesterol (1:1 molar ratio) were suspended in an appropriate amount of chloroform. A thin film was developed by complete evaporation of chloroform in a rotary evaporator (Buchi, New Castle, USA) at an ambient temperature of 40 °C. The film was subjected to hydration in phosphate buffer saline (0.1 M, pH 7.4) and incubated in a shaking water bath (40 °C) for 4 h. The stock solution was then diluted using phosphate buffer saline (0.1 M, pH 7.4) to 5 mg/ml final concentration of the working solution (in terms of phospholipid content) (New *et al.*, 1990). Four different groups, *i.e.*, liposome only, liposome + INM-7035, liposome + INM-7035 + 200 Gy, and liposome + 200 Gy, were evaluated for the levels of malonedialdehyde (MDA), an end product of membrane degeneration. A radiation dose of 0.4 kGy at a dose rate of 1.93 kGy/h was used as a source of stress, and the groups chosen above were exposed followed by an incubation of 1 h at 37 °C. The equivalent volume of 10% trichloroacetic acid (TCA) and 0.5% thiobarbituric acid (prepared in 0.025 M NaOH) were added to the reaction mixture. The resultant mixture was then subjected to 80 °C for 1 h in a water bath. A pink coloured chromogen complex was formed, readable at 535 nm.

Reporter gene assay

293T cells (HEK cells) obtained from ATCC, USA were used for the NF-κB (nuclear factor κ-B) reporter assay (Smilovic *et al.*, 2008). Cells were infected with Lenti NF-κB reporter conjugated with LacZ (SA Biosciences, Frederick, MD, USA), and stable clones were obtained using puromycin selection (1 μg/ml). Cells were treated with increasing concentrations of INM-7035 for 6 h at 37 °C to check the activation of NF-κB after incubation media were replaced and attached cells were washed gently with phosphate buffered saline followed by lysis of the cells in lysis buffer [0.25 M tris (2-amino-2-hydroxymethyl-propane-1,3-diol), pH 8.0]. After lysis cleavage buffer (0.6 M $Na_2HPO_4 \cdot 7H_2O$, 0.4 M $NaH_2PO_4 \cdot H_2O$, 0.1 M KCl, 0.01 M $MgSO_4 \cdot 7H_2O$, pH 7) with β-mercaptoethanol and *ortho*-nitro-phenyl-β-galactoside (ONPG) was added for the measurement of lac-z reporter at 420 nm.

Results and Discussion

The continued quest to develop ideal radioprotective agents has, with reference to designing appropriate modifiers of radiation response, led to an enhanced understanding of free radical biology. Agents to remove internal radionuclides with therapies using granulocyte colony stimulating factors have also been recommended (Weiss and Landauer, 2009). However, the γ-radiation-

induced damages still require development of novel radioprotective agents. This led to the screening of various natural plant products as bioactive constituents for the development of future radioprotective formulations. The focus of the present study was to evaluate the antioxidant and radiomodulatory potential of silymarin referred as INM-7035. The HPLC analysis followed by cospiking analysis confirmed the presence of silibin A (39.9%) and silibin B (54.7%) in the sample (Fig. 1). The polyphenolic content of INM-7035 was found to be 7.022 mg/ml while the flavonoid content was found to be 0.76751 mg/ml.

An imbalance of generation of ROS with respect to inactivation caused by internal/external effects/stimuli or by radiation stress may lead to oxidative stress. INM-7035 has been evaluated for both primary (superoxide) and secondary (hydroxyl radical) free radical scavenging activity. INM-7035 exhibited a significantly ($p < 0.01$) higher activity as compared to quercetin in a concentration-dependent manner ($R^2 = 0.993$) in the nano-concentration range (Fig. 2C). Secondary free radicals, *i.e.* hydroxyl radicals that primarily target the biomolecules, were also tested. INM-7035 exhibited comparable site-specific and non-site-specific activities with maximal activity >33.75% at 100 μg/ml in a concentration-dependent manner (Fig. 2D, $R^2 = 0.983$, $R^2 = 0.988$). These observations revealed that INM-7035 restricts the initiation process of the free radical cascade by significantly ($p < 0.05$) removing superoxide radicals (Fig. 2C) and also, at the level of amplification, by chelating-redox active Fe^{2+} (site-specific activity: >30%) indicating its inherent radioprotective potential. Amifostine, a well known radioprotective molecule with inherent toxicity, has been reported to suppress the reactivity of intralysosomal iron (Yu *et al*., 2003).

The underlying basis of free radical mechanism was evaluated using ABTS and DPPH free radical scavenging activity models. In the former case, INM-7035 exhibited 32.4% activity at 500 μg/ml

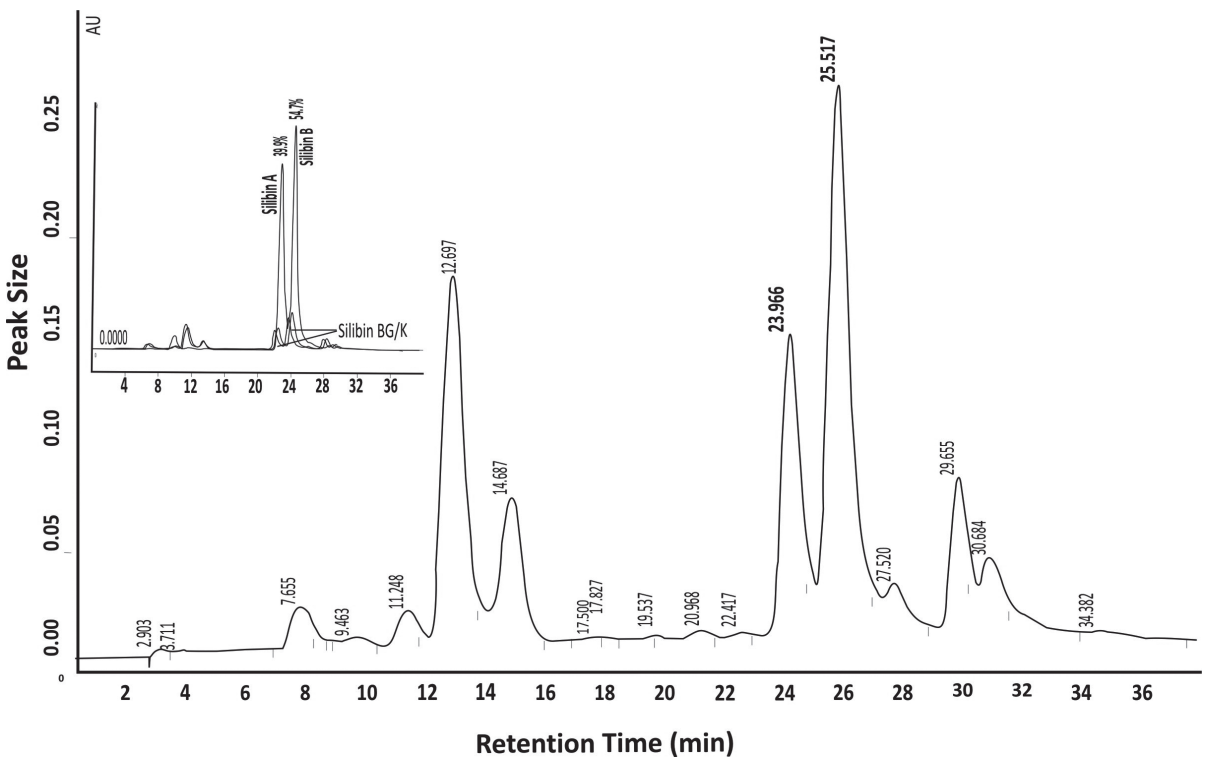

Fig. 1. HPLC profile of INM-7035, a purified sample of silymarin, milk thistle extract powder. Inset: Cospiking analysis revealing the presence of silibin A (39.9%) and silibin B (54.7%).

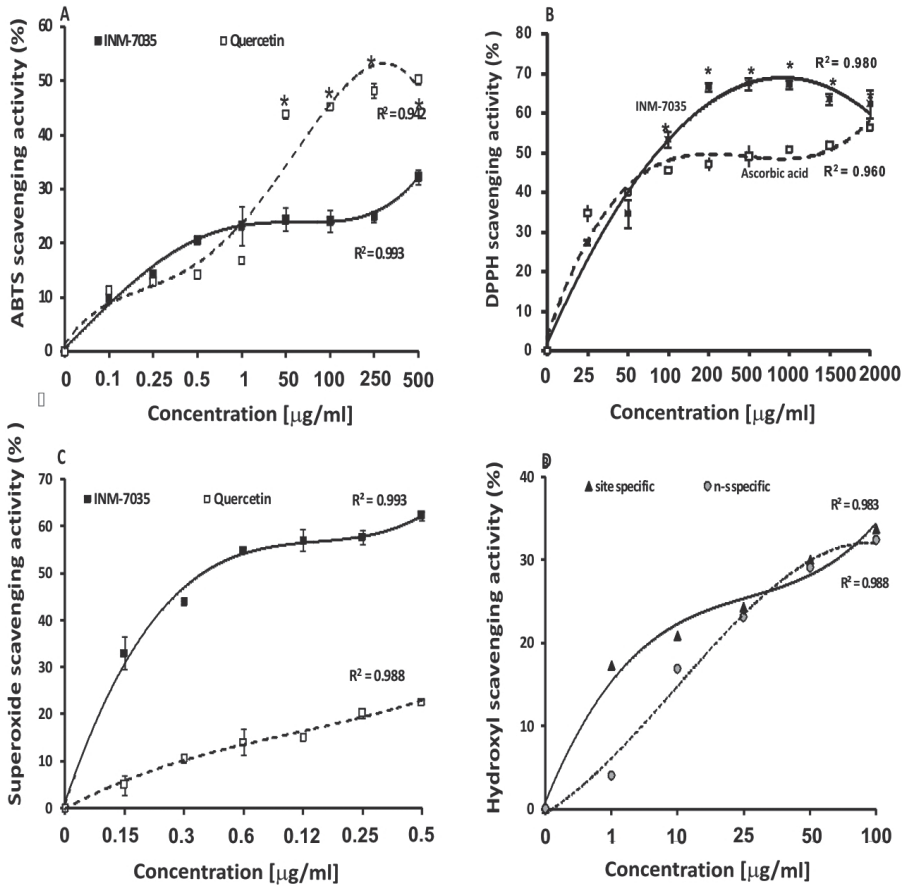

Fig. 2. Free radical scavenging potential of INM-7035, with illustrated functional trends as $f \sim x^n$, where n is the degree of equation. (A) Percentage of ABTS scavenging activity in comparison to the standard quercetin ($f = x^4$). (B) Percentage of DPPH scavenging activity in comparison to the standard ascorbic acid (INM-7035: $f = x^2$, ascorbic acid: $f = x^3$). (C) Percentage of superoxide ion scavenging activity in comparison to quercetin ($f = x^3$). (D) Comparative analysis of site-specific and non-site-specific percentage of hydroxyl scavenging activity ($f = x^3$). * Significant difference in the activity of INM-7035 with respect to the corresponding standard.

and increased in a concentration-dependent manner ($R^2 = 0.993$), while in the latter case, the activity was found to be 67.2% ($R^2 = 0.980$). Conclusing the DPPH scavenging activity, INM-7035 exhibited significantly ($p < 0.05$) higher activity as compared to ascorbic acid ($R^2 = 0.960$), which was used as standard (Figs. 2A, B). A difference of more than 30% activity in free radical modulation was observed in both models with higher activity in the DPPH model revealing its broad spectrum action against various free radicals. A broad-spectrum free radical scavenger is beneficial to reduce the side effects of chemotherapies and irradiation during cancer treatment (Kaji *et al.*, 2009). Further studies using the 2-deoxy-D-ribose degradation assay revealed a significant ($p < 0.05$) hydroxyl scavenging potential (>33%), thereby protecting biomolecules by termination of the free radical-induced chain reaction. All these free radical modulatory activities of INM-7035 suggested its probable role in protecting the physiological machinery against radiation-induced oxidative stress.

Another critical factor is the different action of antioxidant molecules in various phases, *i.e.*, aqueous, lipid, and intermediary phases. It has been postulated that an ability to act in multiphases provides holistic mode to control the free radical flux. Antioxidant activity in the aqueous phase was evaluated using the potassium ferricya-

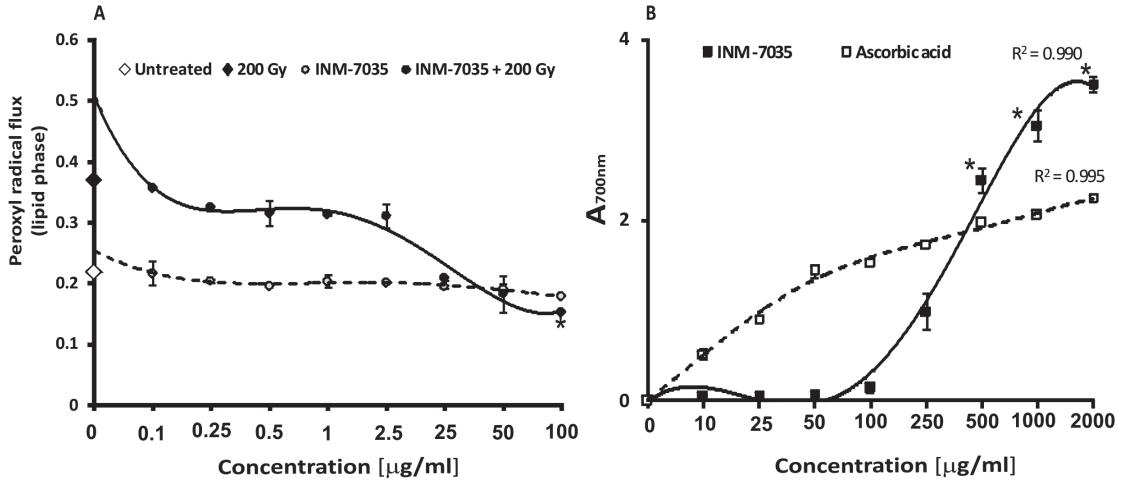

Fig. 3. Antioxidant activity in the aqueous and lipid phase, with illustrated functional trends as $f \sim x^n$, where n is the degree of equation). (A) Modulation of peroxyl radical flux in the presence of supralethal radiation stress (200 Gy) by INM-7035 ($f = x^4$) in the lipid phase developed using linoleic acid pre-emulsion; the optimal dose was found to be 100 μg/ml. (B) Reducing power in terms of increasing absorbance at 700 nm compared with ascorbic acid ($f = x^5$) in the aqueous phase. * Significant difference in the activity of INM-7035 with respect to corresponding controls.

nide assay. The observations revealed significantly ($p < 0.05$) higher antioxidant activity in the higher concentration range (500–2000 μg/ml) as compared to ascorbic acid, however, at lower concentration range, the activity was comparable. It exhibited a dose-dependent increase ($R^2 = 0.9951$) in the activity. On the other hand, the antioxidant activity in the lipid phase was evaluated using the linoleic acid degradation assay in the presence of supralethal radiation stress (200 Gy). Comparison of the INM-7035 only group with the INM-7035 + 200 Gy group on a scale of untreated to radiation only groups revealed that 50 μg/ml is an optimal dose with least self-autooxidation potential of lipids and maximal peroxyl radical scavenging activity (Figs. 3A, B). INM-7035 exhibited a signifi-

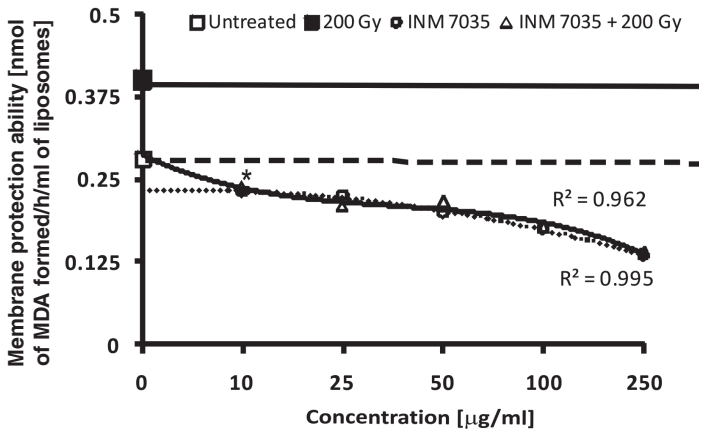

Fig. 4. Membrane protection ability of INM-7035 in the presence of supralethal radiation stress in the liposomal system with illustrated functional trends as $f \sim x^n$, where n is the degree of equation. INM-7035 + 200 Gy exhibited $f = x^2$, while INM-7035 exhibited $f = x^3$, wherein both exhibited significant protection from initiation of lipid peroxidation [negative trend in formation of malondialdehyde (MDA), a byproduct of lipid peroxidation]. Minimal concentration with optimal effect was 10 μg/ml.

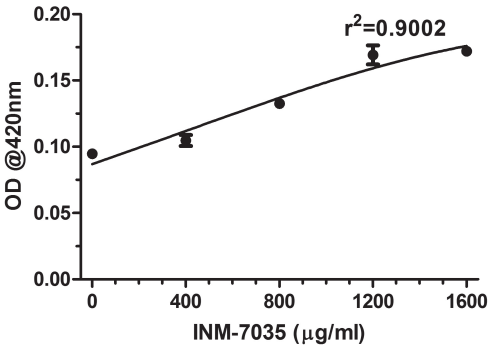

Fig. 5. Reporter gene assay of INM-7035 taken at 420 nm in the range 0–1600 μg/ml to evaluate the modulatory action on NF-κB.

cantly higher peroxyl radical scavenging potential at 50 μg/ml (>50% reduction in flux as compared to the control) in the lipid phase (Fig. 3A) with significant ($p < 0.05$) concentration-dependent reducing power in the aqueous phase (Fig. 3B). These observations explained its potential to decompose peroxides generated due to irradiation (200 Gy) as well as its ability to prevent chain initiation by donating electrons to free radical species.

Membrane protection is an important characteristic of radioprotective agents as observed in case of disulfiram which was reported to protect mice liver against 4 Gy-induced lipid peroxidation (Gandhi *et al.*, 2003). The membrane protection of INM-7035 was evaluated using a liposomal system at a radiation dose of 200 Gy (dose rate = 1.52 kGy/h). INM-7035 exhibited a significantly higher membrane protection potential at all concentrations (10–250 μg/ml) with a significant ($p < 0.05$) decrease in the level of malondialdehyde in both the INM-7035 only and INM-7035 + 200 Gy group (Fig. 4).

NF-κB is a transcription factor and is central to the oxidative stress cascade involved in pathogenesis of various diseases (Berg *et al.*, 2001). Its modulation is critical to radiation protection. The reporter gene assay depicts the concentration-specific range of action with respect to NF-κB revealing its radiomodulatory potential in 293T cells. Treatment of cells with INM-7035 showed a significant increase in activation of NF-κB. INM-7035 (0 μg/ml–1600 μg/ml) showed an increase in activity, even though 1600 μg/ml showed higher activity. Treatment of cells with higher INM-7035 concentrations (over 2 mg/ml) showed no change in activation of NF-κB with respect to 1.6 mg/ml (Fig. 5).

The results indicate that INM-7035 contains a significant amount of polyphenolics as well as silibins A and B having a significant potential in safeguarding against various induced oxidative stresses (Quan *et al.*, 2009). Phenolics have been well known in hydroxyl radical scavenging (Yildirim and Mavi, 2000), indicating their role in radiation protection. On this basis, it can be concluded that INM-7035 appears to be a promising radioprotector. Since silymarin is fairly non-toxic, its use in humans as one of the bioactive constituents in radioprotective formulations is possible.

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