Original article

Radioprotective properties of *Hippophae rhamnoides* (sea buckthorn) extract *in vitro*

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Abstract

Background - *Hippophae rhamnoides*, a high altitude habitat plant, has been extremely used in traditional medicinal practices for treating a variety of ailments. Recently, an extract (RH-3) prepared from berries of *Hippophae rhamnoides* has been reported to exhibit significant radioprotection against whole body lethal irradiation.

Objective - Present study was undertaken to elucidate the DNA binding ability of an extract (RH-3) prepared from berries of *Hippophae rhamnoides* and its role in modulating radiation induced frank and clustered DNA damage.

Method - Agarose gel electrophoresis was employed as method to understand DNA binding potential and DNA protective ability of RH-3.

Results - RH-3 in a dose dependent fashion interacted with plasmid DNA (*p*UC18) reducing the mobility of supercoiled form and increasing the amount of the complex in the well indicating its ability to interact with plasmid DNA. RH-3 at higher concentrations (> 0.4 mg/ml) almost completely prevented the migration of supercoiled form without interfering with mobility of open circular form indicating its ability to selectively interact with supercoiled form. Studies done with supercoiled or open circular form also revealed the binding specificity of RH-3 for supercoiled form of plasmid. Both inhibited radiation induced strand breaks and DNA interaction by RH-3 were found to be dependent upon pH and the order of efficacy was found to be acidic pH> neutral pH > alkaline pH. RH-3 in a dose dependent manner inhibited radiation induced frank single, double strand breaks as well as endonuclease IV detectable abasic sites (clusters) and maximum reduction was observed at a concentration of 200 µg/ml.

Conclusion - Results obtained in this study suggest that the ability of RH-3 to interact with DNA could be playing a significant role in preventing radiation induced DNA damage.

Key words: Clustered DNA damage, Hippophae rhamnoides, Radioprotection, pUC18, single strand breaks (ssb); double strand breaks (dsb).

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Introduction

Low LET (low energy transfer) gamma radiation causes damage to the cellular system mainly by generation of reactive oxygen species (ROS). The spectrum of the damage inflicted by the free radical to the cell architecture and function includes lipid peroxidation, oxidation of proteins, base modifications, adduct formation and strand breaks in DNA ultimately leading to apoptosis and cell death [(1)]. A number of synthetic and natural compounds have been evaluated to achieve protection against radiation induced injuries[(2)]. Among different modulators tried free radical scavengers [(3)], antioxidant enzymes [(4)], immunomodulators [(5,6)] are worth mentioning. DNA is a crucial molecular target for ionizing radiation induced lesions including base damage, single strand breaks (ssb) and double strand breaks (dsb) [(7)]. A significant feature of ionizing radiation is that, it introduces DNA damage clusters, two or more oxidized bases, abasic sites or strand breaks on opposite strands within a few helical turns, which are known to be difficult for cells to repair and thus represent potentially highly mutagenic lesions [8]. The damage can be appreciably mitigated if free radicals besides being scavenged are not permitted physically to interact with the target molecules like DNA. Thus a number of synthetic and natural compounds have been studied in the recent past for their ability to protect DNA from radiation induced strand break [(9)]. Among them most important compounds are polyamines [(10)], aminothiols [(11)], cysteine [(12)], Hoechst [(13)], archaebacterial DNA binding protein MC1[(14)] etc. *Hippophae rhamnoides* L. (F. Elaegnaceae), commonly known as *seabuckhorn* has been exploited extensively in Indian and Tibetan system of medicine for treatment of several ailments like circulatory disorders, ischemic heart disease, hepatic injury and neoplasia.[(15)] Recently an extract (RH-3) prepared from its berries has been shown to render significant protection against radiation-induced lethality in mice.[(16)] Free radical scavenging, modulations of antioxidant defense enzymes have been attributed towards its radioprotective manifestation. *Hippophae rhamnoides* has been reported to interact with cellular chromatin and stabilize it. [(16, 17)] Present study was undertaken to further unravel the RH-3: DNA interaction and its implications in radioprotective manifestation with special reference to radiation induced clustered DNA damage. In this study it is shown that interaction of RH-3 with plasmid DNA is

conformation and pH dependent. The interaction seems to be playing a significant role in countering radiation induced frank and clustered DNA damages.

Methods

1. Chemicals

Bovine serum albumin, proteinase-K, DTT, SDS, bromophenol blue, ethidium bromide from Sigma chemical Co, MO, USA. Endonuclease-IV, pUC18 was obtained from Genetix, India. Other chemicals of standard make and purity were used.

2. Plant material and preparation

Known quantity of fresh berries of *Hippophae rhamnoides* collected from Himalayan ranges (altitude 3000-4000 m) were washed and extracted using absolute alcohol and triple distilled water (50: 50, v/v; three changes) and the final extract was lyophilized, weighed and stored at 4 ^{o}C [18]. Henceforth the lyophilized extract will be called RH-3.

3. Plasmid

*p*UC18 DNA in TE (10 mM Tris, *p*H 8.0, 1 mM EDTA) was extensively dialyzed against potassium phosphate buffer (10 mM, *p*H 7.4), followed by dilution to a final concentration of 50 ng /µl.

4. Irradiation of Plasmid DNA & Endo IV treatment

Aliquots of pUC18 DNA solution in phosphate buffer with or without different concentrations of RH-3 at 4 ^{o}C were exposed to varied radiation doses using ^{60}Co source (Gamma Chamber-2000, BRIT, India). Fricke's dosimetry was done for gamma rays[19]. The dose rate during the course of experiments was about 5.7 k Gy/h. After irradiation the 10X TE (100 mM, 10 mM EDTA) was added at 1/10th of final volume of pUC18 DNA samples to stabilize the DNA from degradation and stored at 4 ^{o}C until further analysis. After irradiation (5, 10, 15 or 20 Gy), DNA samples were incubated with Endo IV enzyme method [20]. Briefly, the plasmid DNA was equilibrated with endonuclease reaction buffer (70 mM HEPES/KOH, *p*H 7.6, 100 mM KCl, 1 mM EDTA, 1 mM DTT,

100 ng/µl bovine serum albumin) in a final volume of 10 µl in duplicate for treatment with enzyme and one for mock treatment without enzyme. Optimal concentrations of Endo IV enzyme was determined by titration against control and gamma irradiated sample, so that there was significant non-specific cutting of non irradiated plasmid DNA while maximal cutting in irradiated DNA. The reaction mixtures were then incubated at 37 ^{o}C for 1 h for complete digestion and thereafter the traces of enzyme was removed by adding proteinase-K and EDTA to a final concentration of 1.33 µg/ml and 100 mM, respectively and incubation was continued overnight at 37 ^{o}C. A neutral stop mixture (0.125 % bromophenol blue, 0.5 % sodium lauryl sulfate in 50 % glycerol) was added to the samples to ensure dissociation of any persistent enzyme-DNA complexes.

5. DNA aggregation assay

The interaction between RH-3 and plasmid DNA was performed as follows. Samples containing 200 ng of pUC18 (4 µl in 10 mM phosphate buffer, *p*H 7.4), increasing concentration of RH-3 were mixed, brought to a final volume of 12 µl and incubated at 37 ^{o}C for 1 h. Thereafter, the samples were run on a 1.2 % agarose gels under neutral conditions as mentioned below.

6. Electrophoresis and determination of strand breaks

Plasmid DNA samples treated with lesion specific enzymes as described above were electrophoresed on neutral agarose gel (Tris-Acetate EDTA, *p*H 8.2, 1 % agarose gel) at 20V, for 22 h, at 9 ^{o}C. At the end of electrophoresis the gel was stained with ethidium bromide (0.5 µg/ml) in water. Thereafter, the gels were destained extensively in water to minimize background fluorescence and visualized on a UV transilluminator. A

quantitative electronic image was obtained and the area of each DNA band corresponding to supercoiled (form I), open circular (form II) and linear (form III)) topoisomer was determined using Imagequant software (Molecular Dynamics, Sunnyvale, CA). The value obtained for the supercoiled form was corrected by a factor of 1.4 for the reduced binding of ethidium ions into this form. The conversion of supercoiled DNA (form I) into open circular form (II) represents the single strand breaks and formation of linear form (form III) indicate the formation of double strand breaks. Non-double strand break clustered damage were obtained from the difference in the yields of double strand breaks in the absence or presence of endo IV.

Fraction of supercoiled (μ) without nicks was calculated using the formulae;

$$\mu = \frac{\text{Area (spc)} \times 1.4}{\{(\text{Area spc} \times 1.4) + \text{area of relaxed form}\}}$$

7. Statistical analysis

The data is presented as mean ± S.D. of three separate measurements; significance was determined by Student's t-test and probability level of < 5 % was considered significant.

Results

1. Interaction of RH-3 with plasmid DNA

RH-3 in a dose dependent manner (20-200 μg/ml) interacted with super coiled form of plasmid DNA and inhibited its migration on a 1.2 % neutral agarose gel (Fig-1A). At higher concentrations (400 μg/ml or more) it completely aggregated and prevented the migration of super coiled form from entering into the agarose gel. In the agarose gel (0.6%) having increased pore size, the RH-3: DNA complex which was retained in the loading wells moved into the gel and formed a band exactly parallel to the supercoiled

band of control DNA (Fig-1B). However, RH-3 did not interfere with open circular form of plasmid DNA at any of the concentration tried in the present study (10 - 1000 μg/ml) as can be evident from no change in the migration pattern of open circular form (fig-1A& B). The interaction studies of RH-3 with homogeneous open circular form indicated that RH-3 did not interfere with mobility of open circular form whereas it almost completely inhibited the migration of supercoiled form (Fig- 1 C).

2. Factors influencing the RH-3: DNA interaction

At acidic *p*H (3.0), RH-3 interacted strongly with plasmid DNA and non-specifically reduced the mobility of both supercoiled as well as open circular form and maximally at a concentration of 40 μg/ml or more. However, at neutral *p*H (7.0) RH-3 interacted with plasmid DNA in a specific manner and reduced the migration of supercoiled form without interfering with the migration of open circular form. Maximum inhibition of migration of DNA was found at a concentration of 100 μg/ml or more. At alkaline *p*H (9.0) RH-3 was observed to completely loose the DNA binding ability (Table-1).

3. RH-3: DNA interaction and Radiation-induced strand breaks

3.1. Qualitative estimation

Fig- 2 A & B show the effect of different *p*H on the ability of RH-3 to counter radiation induced strand breaks in plasmid DNA. Exposure of plasmid DNA (10 mM phosphate buffer *p*H 7.4) to a radiation dose of 20 Gy resulted in a significant amount of both single (formation of slow migrating open circular form) and frank double strand breaks (formation of linear form ; lane-2, fig-2A) in comparison to control DNA (lane- 1 ; Fig-2A). RH-3 at acidic *p*H (3.0) in a dose dependent fashion inhibited 20 Gy induced strand breaks and at concentration of 40 μg/ml or more it strongly countered radiation induced strand breaks (absence of linear form) and the DNA: drug complex was retained in the agarose well (Lane-4 -10; fig-2A). At neutral *p*H (7.0) also RH-3 inhibited radiation induced single as well as double strand breaks in a dose dependent manner and maximum amount of inhibition and retaining of supercoiled DNA complex was observed at concentration of more than 80 μg/ml (lane-6; fig-2B). At alkaline *p*H (9.0), RH-3 did not reveal any interaction with DNA and prevented radiation induced strand breaks less efficiently in comparison to acidic and neutral pH (Fig-2C). Significant amount of linear

as well as open circular form was evident even at the highest concentration of RH-3 used in this study (200 μg/ml).

3.2. Quantitative estimation of strand breaks

3.2.1. Single Strand Breaks (SSB)

Gamma rays induced significant amount of single strand breaks (Fig- 3 A) at the lowest dose (5 Gy) used in the present study. At higher doses a gradual decrease in the appearance of OC form (single strand breaks) was observed and a minimum was observed at highest dose used (20 Gy). Both 50 and 100 μg/ml of RH-3 significantly ($p < 0.05$) inhibited induction of single strand breaks by different doses of radiation and maximum amount of inhibition was observed at 100 μg/ml.

3.2.2. Double strand breaks (DSB)

Gamma irradiation induced double strand breaks in a dose dependent manner (Fig- 3B) and maximally at 20 Gy. RH-3 (50 or 100 μg/ml) significantly ($p < 0.05$) countered the formation of DSB by gamma rays (Fig: 3B) and maximum inhibition was observed at a concentration of 100 μg/ml.

3.2.3. DNA damage clusters

Gamma rays induced Endo IV (Nfo) detectable clusters in a dose dependent manner in comparison to control *p*UC18, which was around 95 % supercoiled and did not show any linear molecules with or without enzyme (Endo IV) treatment (Fig- 3C). RH-3 both at 50 and 100 μg/ml significantly inhibited gamma radiation induced DNA damage clusters and maximum inhibition was elicited at a concentration of 100 μg/ml.

Discussion

1. Significance of present study

RH-3, an aquoethanolic extract prepared from berries of *Hippophae rhamnoides*, has been reported to interact with cellular chromatin. [5, 16] Present study was undertaken to unravel the interaction of RH-3 with DNA in detail and its implications in radiation

protection. RH-3: DNA interactions were studied in simple plasmid DNA system by assessing the changes in the mobility of different forms (topoisomers) of plasmid DNA (closed circular, linear and open circular form) on neutral agarose gel. In fact similar methodology has been employed to characterize DNA aggregating proteins isolated from carp eggs.[21] RH-3 (up to 200 μg/ml) in a dose dependent fashion reduced the mobility of closed circular DNA while at high concentrations (> 0.4 mg/ml) it almost completely aggregated and retained the DNA in sample well (results not shown), clearly indicating its ability to interact with DNA. (Fig-1 A). It was interesting to ascertain whether the DNA which is retained in the well is supercoiled DNA or some other modified form. Since RH-3 is binding with DNA and increasing the molecular weight of the complex that is unable to pass through 1.2 % agarose gel, it is expected that by increasing the pore size of the agarose gel (0.6 % instead of 1.2 %) the DNA will migrate into the gel. It was observed that the DNA which is aggregated in the well entered the gel and formed intact band parallel to that of supercoiled band of plasmid DNA indicating strongly that the DNA retained in the well is intact supercoiled DNA.

2. Effect of pH on RH-3: DNA interaction and DNA protective ability

Intriguingly, at none of the concentrations RH-3 (up to 200 μg/ml) has shown any interaction with open circular form, evident from no change in its mobility pattern, suggesting RH-3 specificity towards supercoiled form (Fig-1A). Studies carried out with homogeneous preparation (by DNase treatment) of open circular form further supported the inability of RH-3 to interact with open circular form at neutral *p*H (Fig-1C). Since *p*H of the media is known to change the deprotonation properties of poly phenolic flavonoids and affect their antioxidant potential [22], the effect of *p*H on the RH-3: DNA interaction was evaluated. Interestingly, at acidic *p*H (3.0) RH-3 interacted with both supercoiled and open circular form and retained the DNA in the loading well at concentrations of 40 μg/ml or more (Table-1). At neutral *p*H, RH-3 interacted with plasmid DNA in a topoisomer specific manner and the concentration required for complete inhibition of supercoiled form migration was found to be 100 μg/ml (Table-I). However at alkaline *p*H, RH-3 completely lost the DNA binding ability and neither alteration nor retention of plasmid DNA in the sample well was observed. The loss of DNA binding ability of RH-3

at alkaline *p*H could partially be attributed to the degradation of polyphenols, as in alkaline solutions flavonoids are known to degrade and lose antioxidant activity. [23]

The ability of RH-3 to modulate radiation-induced strand breaks in plasmid DNA was studied under different *p*H conditions to evaluate the importance of RH-3: DNA interactions in ameliorating radiation-induced DNA damage. Under acidic conditions, RH-3 very efficiently countered 20 Gy induced single as well as double strand breaks. Complete reduction and retention of the drug: DNA complex in the well was observed at a concentration of 40 and 100 µg/ml or more in acidic and neutral *p*H conditions respectively (Fig- 3A & B). At alkaline *p*H, where RH-3 did not bind with DNA (Fig- 3C), ssb and dsb were still discernible at the highest concentration (200 µg/ml) suggesting that alkaline *p*H to be least effective. These results remarkably support the notion that the ability of RH-3 to bind with DNA is playing a significant role in preventing radiation-induced DNA damage.

3. RH-3: DNA interaction and radiation-induced clustered DNA damage

Radiation-induced DNA damage is generally accepted to play a crucial role in formation of chromosomal aberrations and cell killing.[24, 25] The results obtained from the qualitative studies clearly indicate that RH-3 protected DNA from radiation-induced strand breaks by virtue of its ability to bind with and stabilize the DNA. Hence it was considered essential to evaluate quantitatively the ability to modulate radiation-induced DNA damage including clustered damage. Studies with plasmid DNA (Fig- 3) with respect to SSB, revealed that beyond a radiation dose of 10 Gy the quantum of SSB started declining (Fig- 3A). The lowest radiation dose used in the present study (5 Gy) sufficiently induced high levels of SSB which converted supercoiled DNA into open circular form. At higher doses open circular form contributes to the formation of linear form (dsb) which explains a gradual decrease in the open circular form. RH-3 at both the concentrations (50 and 100 µg /ml) inhibited radiation-induced single as well as double strand breaks clearly suggesting its ability to protect the DNA. Among the spectrum of damages inflicted by ionizing radiation, clustered DNA damage is important lesion contributing significantly towards cell lethality. Clustered damages can be defined as two or more oxidized bases, abasic sites or frank strand breaks within one or few helical turns of double stranded DNA, which are often referred as signature lesions. [6, 17] The ability of

RH-3 to modulate radiation-induced clustered DNA damage was further probed in plasmid DNA using endonuclease IV as marker enzyme. Gamma radiation as anticipated induced a dose dependent increase in endo IV detectable damage clusters (Fig- 3C). RH-3 in a dose dependent fashion inhibited radiation-induced damage clusters which could be attributed to its free radical scavenging ability as well as its ability to bind with and stabilize the DNA.

The studies clearly reveal that RH-3 has the property of interacting with plasmid DNA system which evidently proved the significant role in alleviating radiation-induced DNA damage. Thereby, RH-3 to certain potency accounts its role in contributing towards overall radioprotective ability.

Implications:

1. The information obtained from the present study could effectively used to develop a bio assay to isolate the compound responsible for DNA binding and radioprotective abilities.
2. The interaction of RH-3 with plasmid DNA seems to be playing a significant role in countering radiation-induced frank and clustered DNA damages.
3. The DNA binding ability of RH-3 holds a prominent role in anti tumor/cancer properties.

Limitations:

1. *The high DNA binding ability shown by RH-3 cannot be considered as optimum level for its radiation protection.*
2. *The studies carried out in vitro are expected for similar in vivo.*

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Table 1. Effect of *p*H on RH-3: DNA interaction. ‾ indicates no DNA is evident in the well, + indicates DNA is available in the well, ++ indicate almost all of the plasmid DNA is retained in the well

RH-3 (μg/ml)	<	***p*H**	>
	3.0	***7.0***	***9.0***
0	_	_	_
10	+	+	_
20	+	+	_
40	++	+	_
60	++	+	_
80	++	+	_
100	++	++	_
150	++	++	_
200	++	++	_

Figure Legends:

Fig-1A: Effect of agarose concentration on mobility of RH-3: DNA complex where mixture was run on 1.2% neutral agarose gel. Lane-1 control DNA, lane-2-10, 200 ng DNA + 5, 10, 20, 40, 60, 80, 100, 150 or 200 μg/ml RH-3.

Fig 1B: Effect of agarose concentration on mobility of RH-3: DNA complex where mixture was run on 0.6% neutral agarose gel Lane-1 control DNA, lane-2-9, 200 ng DNA + 300, 400, 500, 600, 700, 800, 900 or 1000 μg/ml of RH-3.

Fig 1C: Effect of different topoisomers on the ability of RH-3 (400 μg/ml) to interact and aggregate DNA where mixture was run on 1.2% agarose gel. Lane-1 and 6, 100 bp marker, lane-2-3, supercoiled DNA without or with 400 μg/ml of RH-3, lane- 4- 5, open circular form DNA without or with 400 μg/ml of RH-3.

Fig 2 A, B & C. Effect of different *p*H on the ability of RH-3 where mixture was run on 1.2% agarose gel.2A: Lane-1 control DNA (*p*H 7.4), lane-2, DNA + 20 Gy, lane-3, control DNA (*p*H 3.0), lane- 4 - 10, DNA + 10, 20, 40, 60. 80, 100 or 150 μg/ml. 2B: Lane-1 control DNA, lane-2-8, DNA + 10, 20, 40, 60, 80, 100 or 150 μg/ml RH-3 in 10 mM phosphate buffer at pH 7.0. 2C: Lane-1 control, DNA, lane-2-9, DNA + 10, 20, 40, 60, 80, 100 or 150 μg/ml RH-3 in 10 mM phosphate buffer at *p*H 9.0.

Fig 3A. Effect of RH-3 on radiation (5, 10, 15 or 20 Gy) induced single strand breaks on open circular form where topoisomers were separated on 1% agarose gel. Each value is mean ± SD of 3 parallel experiments. All values are significant at $p < 0.05$ in comparison to radiation control.

Fig 3B. Effect of RH-3 on radiation (5, 10, 15 or 20 Gy) induced single strand breaks on linear form where topoisomers were separated on 1% agarose gel. Each value is mean ± SD of 3 parallel experiments. All values are significant at least at $p < 0.05$ in comparison to radiation control.

Fig 3C. Effect of different concentrations of RH-3 on radiation induced clustered DNA damage on in the presence or absence of Nfo protein where topoisomers were separated on 1% agarose gel. Each value is an average ± SD of 3 parallel experiments. All values are significant at $p < 0.05$ in comparison to radiation control.

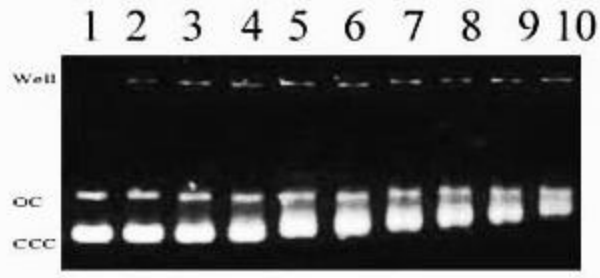


Fig-1A

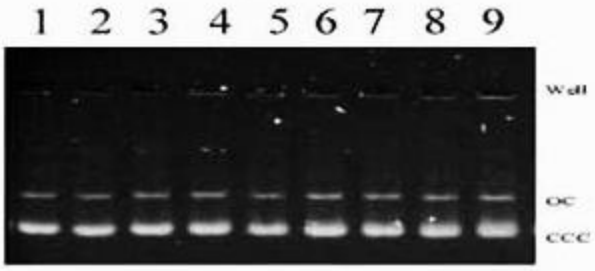


Fig-1 B

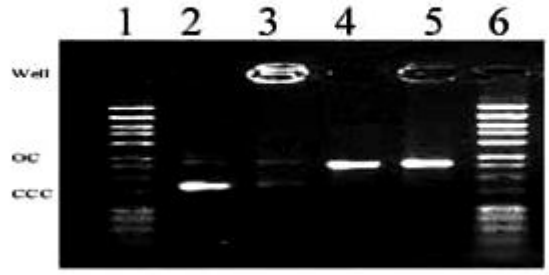


Fig-1C

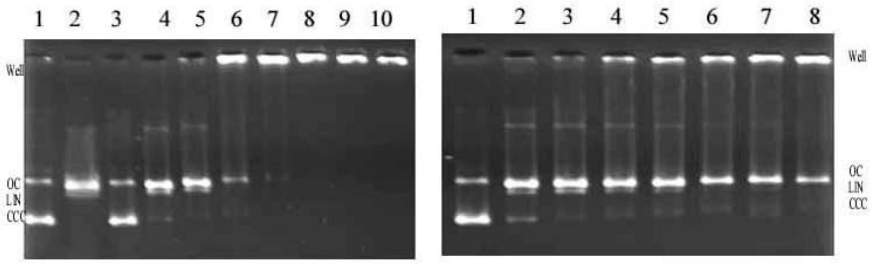


Fig-2A

Fig-2B

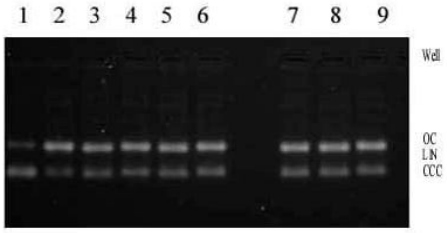


Fig-2C

Fig-3A

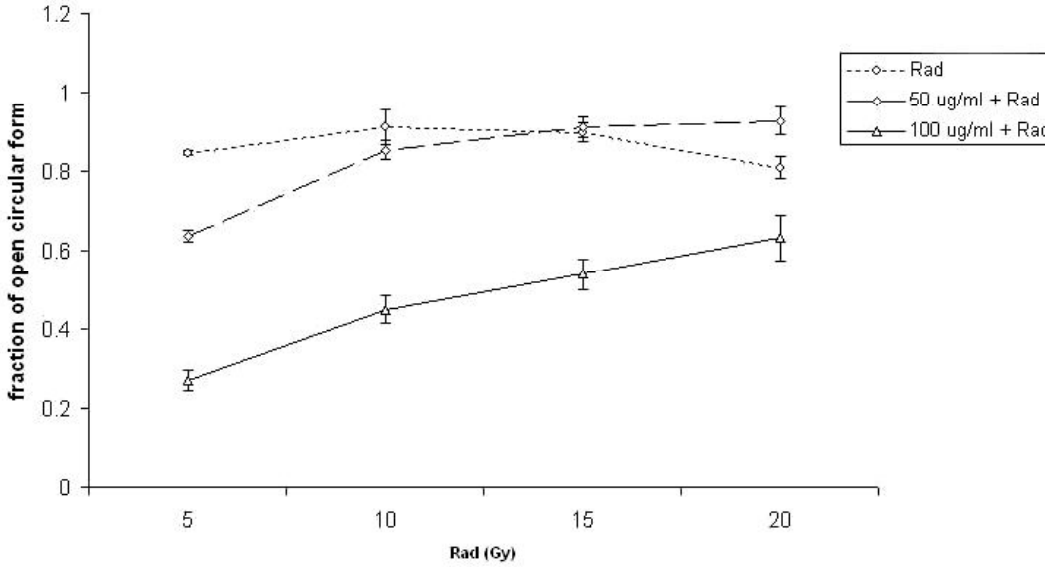


Fig-3B

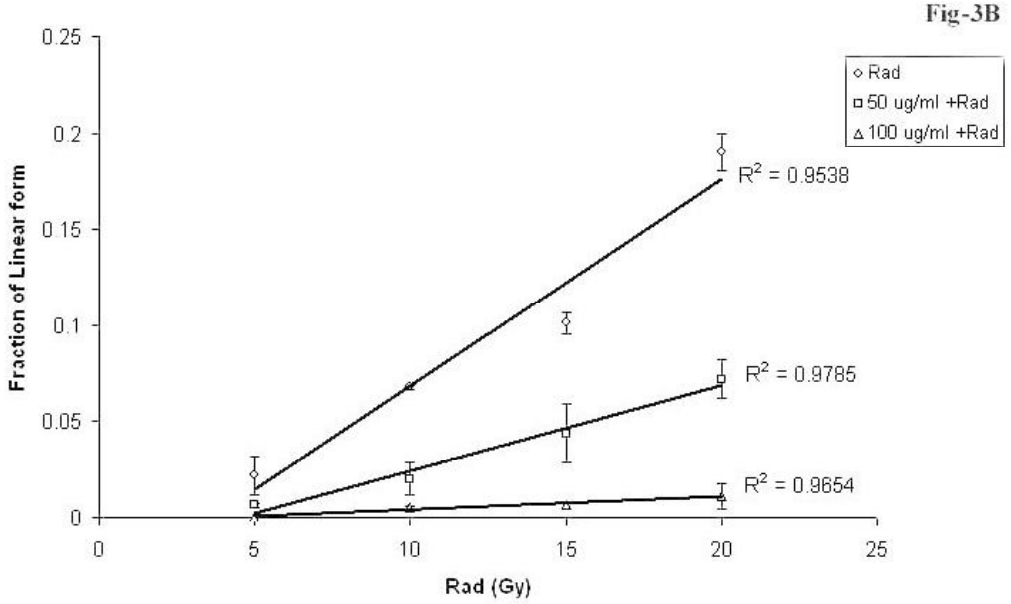
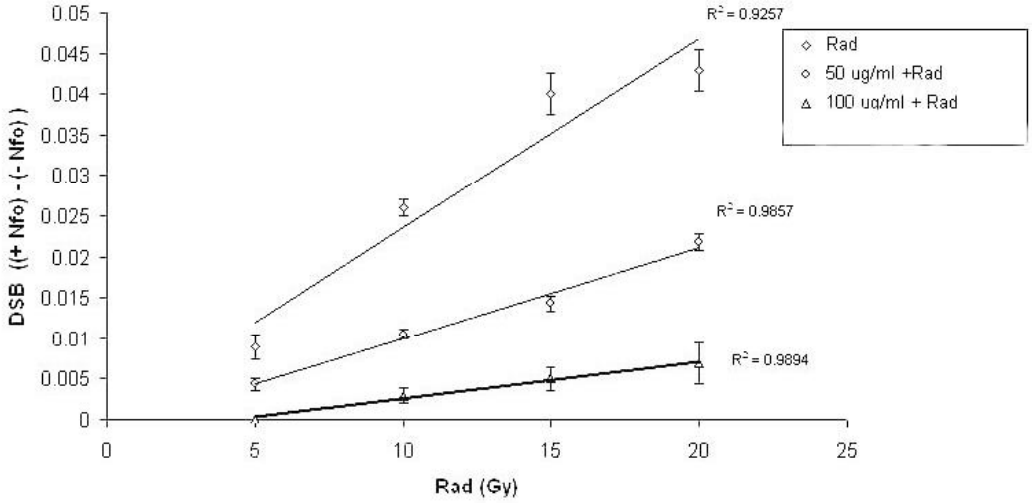

Rad
50 ug/ml +Rad
100 ug/ml +Rad
R^2 = 0.9538
R^2 = 0.9785
R^2 = 0.9654
Fraction of Linear form
Rad (Gy)
0.25
0.2
0.15
0.1
0.05
0
0
5
10
15
20
25

Fig-3C


R^2 = 0.9257
R^2 = 0.9857
R^2 = 0.9894
Rad
50 ug/ml +Rad
100 ug/ml + Rad
DSB ((+ Nfo) -(- Nfo))
Rad (Gy)
0.05
0.045
0.04
0.035
0.03
0.025
0.02
0.015
0.01
0.005
0
0
5
10
15
20
25