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In-vitro evaluation of rutin and rutin hydrate as potential radiation countermeasure agents

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► ***Original article***

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ABSTRACT

Background: DNA damage is one of the major consequences of radiation exposure onto the biological systems. A series of compounds including flavanoids were found to render DNA protection against radiation damage. In this study we elucidated the potential of rutin and rutin hydrate to protect plasmid DNA against damage induced by irradiation. ***Materials and Methods:*** DPPH and hydroxyl radical scavenging assays were performed to assess the antiradical potential of rutin and rutin hydrate. Absorption measurements were performed to assess binding parameters of rutin and rutin hydrate with calf thymus (CT)-DNA. Plasmid relaxation assay was performed to compare the radio protective potential of rutin and rutin hydrate against gamma irradiation mediated oxidative damage of pET28 plasmid DNA. ***Results:*** DPPH assay indicated fast reaction kinetics for rutin and rutin hydrate. However antiradical parameter in terms of EC_{50} suggested better scavenging capacity for rutin hydrate as compared to rutin. Hydroxyl radical scavenging assay further suggested that both the compounds displayed significant reduction in hydroxyl radicals. Absorption binding study with CT-DNA suggested that rutin hydrate has better binding constant value (K_a = 8.257 x 10^4 M^{-1}) compared to K_a = 1.834 x 10^4 M^{-1} for rutin. Plasmid relaxation study demonstrated that plasmid DNA remains predominantly in super-coiled form in the presence of both rutin and rutin hydrate after exposure to 100 Gy of g-radiation. ***Conclusion:*** The mechanistic studies suggested that binding and scavenging capacity of rutin hydrate and rutin contributes towards DNA radioprotection. This study may be helpful in devising potent radioprotector molecules helpful for the radiotherapy treatment.

Keywords: *Rutin hydrate, DNA radioprotection, DPPH assay, flavonoids, binding constant.*

INTRODUCTION

Oxidative stress is causative agent for various pathological disorders and diseases like cancer, cardiovascular, ageing, neurological disorders etc. [1, 2]. Oxidative stress is state of imbalance between free radicals and antioxidants in favor of free radicals. In living system, there are enzymatic and non-enzymatic endogenous antioxidant systems which maintain the much required cellular redox homeostasis [3]. But in case of underperformance of intrinsic antioxidant machinery, these unregulated free radicals may cause damage to vital bio-macromolecules like DNA, protein, lipids etc., thereby leading to cellular dysfunction or death. One of the various environmental stresses is ionizing radiation exposure where low linear energy transfer ionizing radiations cause primarily biological damage through oxidative stress and DNA is one of the most critical targets of ionizing radiation in living system [4].

Rutin is a polyphenolic compound which is a glycoside of quercetin and found commonly in

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vegetables, fruits, herbs, leaves, seeds, red wine, tea and coffee [5]. Rutin is a bioflavonoid and antioxidant which is water-soluble and gets converted to quercetin once it enters into blood stream [6]. Several studies have reported various therapeutic usage of rutin such as anti-inflammatory [7], analgesic [8], inhibitor of platelet aggregation, reduces cytotoxicity of oxidized LDL, reduces heart risks and possess strong antioxidant properties as compared to many similar antioxidants like quercetin, acetin, morin, hispidulin, hesperidin, and naringin [9].

Polyphenols are potential protecting agents against the lethal effects of oxidative stress and offer DNA protection through more than one ways like chelation of redox-active transition metal ions, free radical scavenging, structural stability etc. which may acts solely or in conjunction with one another.

Londhe *et al.* [10] have reported that rutin conferred radioprotection to liver mitochondria owing to hydroxyl radicals scavenging in excised and homogenized liver sample exposed to 450 Gy of gamma radiation.

Similarly, rutin was shown to confer radioprotection in Swiss albino mice exposed to whole body irradiation [11]. In mammalian cell line (CHO10B2) Sunada *et al.* [12] have reported that monogluoscyl-rutin protected DNA against gamma irradiation in low concentration of monoglucosyl-rutin however, in higher concentration it decreased the plating efficiency of cells.

However, to the best of our best knowledge, no such radioprotective study is available for rutin hydrate in literature. However, Sana *et al.* [13] have used rutin hydrate as standard to determine antioxidant activity of root extracts of Spilanthes acmella in terms of DPPH values. It indicates that rutin hydrate has antioxidant potential. Besides literature suggests that rutin hydrate and rutin are poorly soluble in water and have good solubility in alcohols. In ethanol, maximum solubility for rutin is 0.072M [14] which is slightly better (0.075M) in ethanol from Sigma chemicals (R84042).

Therefore, the present study aimed to assess the radioprotective efficacy of rutin and rutin hydrate compounds using *in-vitro* DNA plasmid model and further mechanistic studies were performed to assess redox potential and binding capacity of rutin and rutin hydrate to explain the DNA protection effect of these compounds.

In brief, the investigations showed that both rutin and rutin hydrate significantly scavenge g-irradiation generated free radicals and bind significantly with CT-DNA through non-covalent interactions. The plasmid DNA relaxation assay further suggested that both rutin and rutin hydrate showed significant DNA radioprotection. Therefore, both rutin and rutin hydrate may be considered for radioprotection.

MATERIALS AND METHODS

Rutin, rutin hydrate, calf thymus DNA, 1,1-diphenyl-2-picryl-hydrazil (DPPH), agarose (low LEE), tris acetate buffer (TAE) and ethidium bromide were purchased from Sigma, USA. PET-28a plasmid was a kind gift from Dr. Sunil Lal, ICGEB, New Delhi, India. Plasmid DNA isolation kit was purchased from Qiagen. Dimethyl sulphoxide (DMSO), methanol (Spectroscopic grade) monobasic and dibasic phosphate salts, sodium hydroxide, 2-Deoxy ribose, trichloroacetic acid (TCA), 2-thiobarbituric acid (TBA) were obtained from E. Merck, Germany. The water used for the preparation of solutions was 18 Mega ohm MQ grade obtained from Millipore water system (Elix 3, Millipore Corp, USA).

Stock Solution preparation for rutin and rutin hydrate

Rutin and rutin hydrate were dissolved in methanol to prepare stock solutions of strength 10 mM. Working solutions for specific study were prepared by dilution of these stock solutions.

DPPH assay

The scavenging capacity of rutin and rutin hydrate were determined using DPPH· free radical scavenging assay. A fresh solution of DPPH· was prepared in methanol and the exact initial concentration of DPPH· solution in methanol was calculated spectrophotomerically

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using molar extinction coefficient 10,500 M^{-1} cm^{-1} [15]. The strength of working solution of DPPH· was determined as 57 µM. Working solutions of rutin hydrate and rutin (10, 30, 40, 50 and 100 µM) were prepared by diluting the respective stock solutions in methanol. The kinetic measurements were performed at 515 nm using a microplate reader (Model spectramax M2, Molecular Devices, USA) and steady state method of Brand-Williams *et al.* [16] was followed. The decrease in absorbance was monitored till steady state was reached for decrease in absorbance of [$DPPH^{\cdot}$]. The antiradical parameter EC_{50}, that is the efficient concentration required to decrease the initial DPPH· concentration by 50% was determined for rutin and rutin hydrate using calculation as discussed elsewhere [15].

Hydroxyl radical scavenging by rutin and rutin hydrate

Hydroxyl radical estimation was performed using 2-deoxyriobose assay [17]. 2 µM solution of 2-deoxyribose was prepared in 0.1 M phosphate buffer (pH 7.4). Different concentrations of rutin and rutin hydrate (10µM and 100µM) were mixed and solutions were exposed to 500 Gy. 1 ml each of TBA (1% prepared in 0.5 N NaOH) and TCA (10%) was added to 1 ml of the sample. The samples were heated at 95°C for 30 min in water bath. The samples were cooled to room temperature and absorbance was measured at 531 nm. Extinction co-efficient of MDA (1.56×10^5 $M^{-1}cm^{-1}$) was used to calculate the MDA concentration in each sample.

Absorption measurements

The stock solution of DNA was prepared by dissolving DNA in 10 µM of the phosphate buffer at pH 7.4 and dialyzing exhaustively against the same buffer for 24 h. A solution of CT-DNA gave a ratio of UV absorbance at 260 and 280 nm more than 1.8, indicating that DNA was sufficiently free from protein [18]. The DNA concentration of the stock solution (1000 µM) was determined by UV spectrophotometry in properly diluted samples using a molar absorption coefficient of 6600 $M^{-1}cm^{-1}$ at 260 nm [19]. Absorbance measurements were performed using a multimodal plate reader (Spectramax M2, Molecular Devices, USA) at room temperature. Absorption titration experiments were carried out by successive addition of CT-DNA stock solution to 2000 µl solution of 50 µM rutin/ rutin hydrate prepared in methanol. The effective concentrations of CT-DNA were ranging from 0–24.39 µM.

Plasmid DNA isolation

Plasmid DNA (pET 28a) for further plasmid relaxation assay was isolated using Qiagen Columns as per the manufacturer's instruction (Qiagen, Limburg, Netherlands) from a bacterial culture harboring the same plasmid.

Plasmid DNA protection assay

Plasmid DNA relaxation assay was performed using super coiled pET28-a plasmid DNA as described earlier [20]. Plasmid DNA (500 ng) was incubated with varying drug concentrations for 30 mins before exposing to radiation. Plasmid DNA in 0.2% methanol was used as vehicle control. The concentrations of rutin or rutin hydrate in methanol used were 0.75µM, 1.5µM, 3µM. The samples were exposed to ^{60}Co gamma-radiation from γ-chamber (Model GC-5000 unit, BRIT Mumbai, India) at a dose rate of 1.23 kGy/ hr to achieve the absorbed dose of 100Gy.

Gel electrophoresis and plasmid DNA analysis

Plasmid DNA samples were ran on a 1% agarose gel in TAE running buffer containing 4µl of Ethidium bromide (0.5 µg/ml) per 100 ml of buffer using a Biorad electrophoresis apparatus (Bio-Rad Laboratories, Inc., CA, USA). Various forms of plasmid DNA were observed and quantified using Biorad UV Gel Documentation system (Bio-Rad Laboratories, Inc., CA, USA).

Statistical analysis

Origin 7.0 statistical software was used for statistical analysis and plotting the data. The significance of data in 2-deoxy ribose assay was obtained by comparing the mean values using unpaired *t-test* and a *P* value of < 0.05 was considered as significant.

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RESULTS AND DISCUSSION

DPPH radical scavenging assay

Figure 1 represents the reaction kinetic profile for rutin or rutin hydrate with DPPH which reached the steady state within few minutes from the beginning of the reaction between DPPH and antioxidant. On the basis of this classification of Mishra *et al.* [15], both rutin and rutin hydrate displayed fast reaction kinetic with DPPH. The reaction kinetic profile of rutin and rutin hydrate at equimolar concentrations (30 µM) indicated that rutin hydrate decreased DPPH· absorbance at 515 nm much rapidly than rutin. Subsequently, the percentage of DPPH remaining at steady state was plotted against the moles of antioxidant/moles of DPPH to calculate the EC_{50} values (figure 1b). The plot was fitted with first order exponential decay function and EC_{50} values for rutin and rutin hydrate and EC_{50} values were determined as 0.098 and 0.081 respectively (or 5.82 µM and 4.81 µM for rutin and rutin hydrate respectively). Silva *et al.* [21] have reported the DPPH value of rutin around 9 mg/ml or approximately 14.73 µM. The difference in the value compared to our observed value could be attributed to the choice of experimental conditions. Silva *et al.* [21] had used a concentration of DPPH· as 300 µM while in the present study the concentration of DPPH· was 57 µM. Bhatt and Sharma [22] have reported DPPH concentration from 25 µM to 70 µM which falls in spectroscopic accuracy range from 0.221 –0.698 as optimal range and the concentration of DPPH used in the present study falls well within the recommended range. Further, Silva *et al.* [21] have used ethanolic solution of DPPH· and as demonstrated by Sharma and Bhat [22], DPPH· displayed better sensitivity in methanol than in ethanolic medium. Kurin *et al.* [23] have reported the DPPH· value of quercetin as 4.36 µM which is much close to 5.82 µM as obtained for rutin in the current study. Quercetin has hydroxyl group at position C-3 and higher radical scavenging activity than its glycoside derivatives including rutin. The data clearly demonstrate that the presence of rutinose at position C-3 in rutin (glucose in isoquercetin at position C-3) may block of its C-3 hydroxyl group which plays an important role in antioxidant activity such as radical scavenging and (or) transition metal chelating which is in agreement with previous study by Heim *et al.* [24]. Thus, the DPPH· value of rutin 5.36 µM obtained in the current study appears more correct and acceptable. The DPPH· value for rutin hydrate, however, was reported first time to our best knowledge. The DPPH value of rutin hydrate was 4.81 µM which was slightly lower than 5.82 µM for rutin.

Hydroxyl radical scavenging assay

Hydroxyl radicals are the most reactive free radicals causing oxidative damage. Hydroxyl radical scavenging potential of rutin and rutin hydrate was determined using 2-deoxy ribose

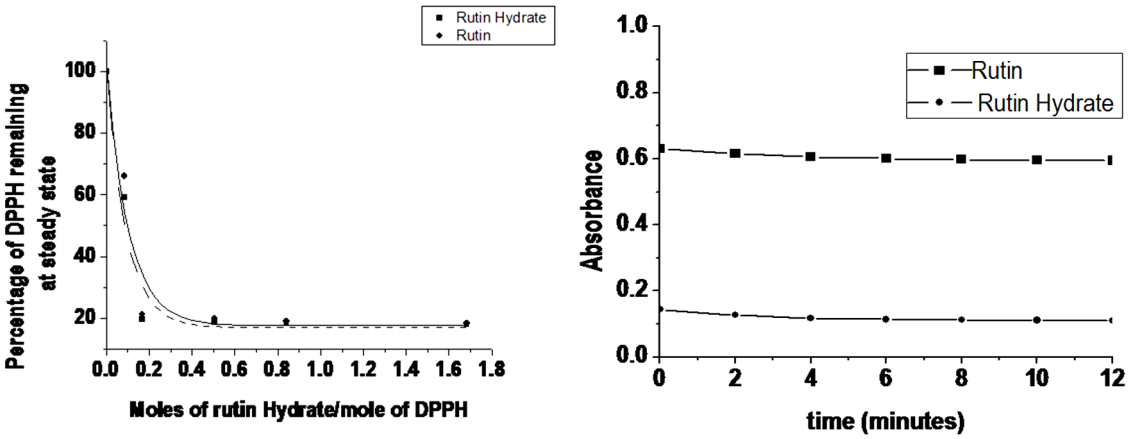

Figure 1. a) kinetic profile of reaction between (i) rutin (30 µM) and (ii) rutin hydrate (30 µM) with DPPH·. b) Representation of percentage of DPPH remaining at steady state *versus* mole of antioxidant/moles of DPPH.

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degradation assay. As seen in figure 2, after gamma irradiation exposure to 500 Gy, malondialdehyde (MDA) concentration was increased significantly from 0.583 mM in control to 6.3 mM in irradiated sample ($P \leq 0.005$). Treatment with 10 mM rutin hydrate caused significant decrease in MDA concentration reducing it to 0.417 which is comparable to the control level. However, no further decrease in MDA was observed at 100 mM. Similarly, 10 mM rutin decreased MDA concentration to 4.45 mM while 100 mM rutin caused no further reduction in MDA concentration. The study showed that rutin hydrate and rutin both significantly reduced hydroxyl radicals mediated increase in MDA.

Absorption measurements

UV-Visible absorption spectra of rutin (50 µM) and rutin hydrate (50 µM) in the presence of different concentration of CT-DNA are shown in figure 3. Both the rutin and rutin hydrate exhibit two major absorption bands in the ultraviolet/visible region. The absorption around 260 nm corresponds to the benzoyl system and the absorption around 355 nm corresponds to the cinnamoyl system [25]. The spectra are related to the π→π* transitions within the aromatic ring of the ligand molecules [26]. CT-DNA absorbs near 260 nm and may cause interference with the absorption band of rutin/rutin hydrate around 260 nm. Therefore, in the present study absorption band around 355 nm of rutin/rutin hydrate was chosen to investigate the binding of ligand to CT-DNA. With subsequent addition of CT-DNA to ligand solution, the absorbance around 355 nm in the rutin and rutin hydrate spectrum showed hypochromicity with red shift in the wavelength maxima from 355 nm to 361 nm. This typical hypochromic effect suggested that rutin and rutin hydrate binds with CT-DNA.

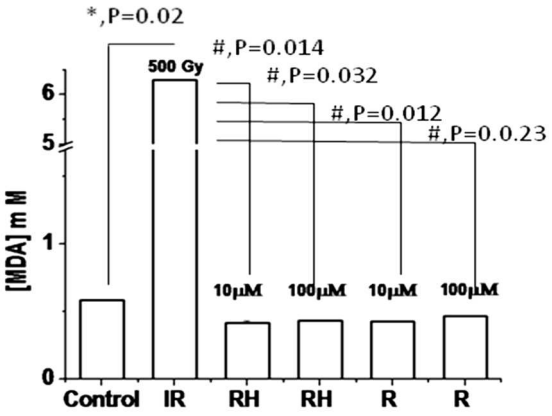

Figure 2. Radiation induced generation of hydroxyl radical and scavenging by rutin hydrate (RH) and rutin (R). Rutin hydrate and rutin decreased the radiation induced generation of hydroxyl radical in concentration dependent manner. 2-deoxyribose degradation assay was used to estimate the hydroxyl radical scavenging by rutin hydrate and rutin. Data represents mean ± SD of three independent experiments. *control vs radiation, # radiation vs rutin hydrate/ rutin treated group. The data was considered significant at the $p < 0.05$.

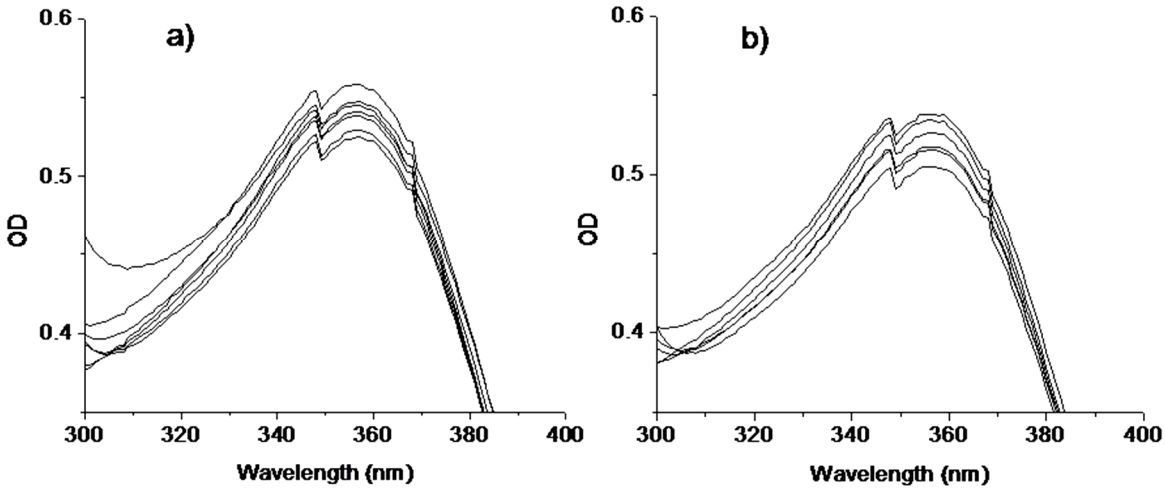

Figure 3. Absorption spectra of a) Rutin (50 µM) and b) Rutin hydtrate (50 µM) in the presence of CT-DNA (0 µM to 24.39 µM).

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Further the absorbance data of the absorption titration were fitted in the Scatchard equation:

$$r/c = nK_a - rK_a \quad (1)$$

where *r* is the ratio of the concentration of bound ligand to total available binding sites, *c* is the concentration of free ligand, and *n* is the number of binding sites per biomacromolecule. The binding constant values for rutin and rutin hydrate were $1.834 \times 10^4\ M^{-1}$ and $8.257 \times 10^4\ M^{-1}$ with CT-DNA. The magnitude of binding constant ($10^4\ M^{-1}$) represents the major role of non-covalent binding interaction in ligand-CT-DNA complexation [27]. Stalin *et al.* [25] reported the binding interaction of rutin with CT -DNA with binding constant value was $8.69 \times 10^4\ M^{-1}$. Mode of binding of rutin was purposed to be intercalation. Hypochromicity in the absorbance and red shift in the absorbance spectra which indicated the binding of rutin and rutin hydrate with CT-DNA with distortion of double helix structure of DNA due to stacking in between rutin and DNA [25, 28]. Rutin hydrate binding to CT-DNA has been reported for the first time in the present study to our best knowledge. Earlier reports suggest that binding of a ligand to CT-DNA increases the structural stability of CT-DNA [29]. Therefore, binding of rutin and rutin hydrate increased the structural stability of CT-DNA. Since rutin hydrate has higher binding constant value than that of rutin which may implicate in more structural stabilization of DNA in presence of rutin hydrate as compared to rutin. Therefore, rutin hydrate may confer more DNA stability than rutin which may be proved beneficial for DNA radioprotection.

Plasmid DNA relaxation assay

Exposure to γ-radiation led to DNA strand breaks resulting into relaxation of plasmid DNA from super coiled form to open circle form. Figure 4a shows that pET 28 plasmid DNA upon γ- irradiation at 100 Gy was converted to nicked circular plasmid DNA as a result of single strand breaks. It was observed that rutin and rutin hydrate reduced ionizing radiation-induced conversion of super coiled form of plasmid DNA to nicked circular form. The effect of different concentrations of rutin and rutin hydrate on plasmid DNA against 100 Gy γ-irradiation induced strand breaks is depicted in figure 4b. The percentage of super coiled form and open form after different treatments were plotted and it was observed that both rutin and rutin hydrate could retain the super coiled form of the DNA to around 80% in a range of 1.5 μM to 3.0 μM in a dose dependent manner. The DNA radioprotection induced by rutin and rutin hydrate was possibly due to the scavenging of radiation-derived primary as well as secondary reactive oxygen species and also due to direct binding of these antioxidants with the DNA as mentioned earlier.

The DPPH· assay and 2-deoxy ribose suggested that both rutin and rutin hydrate posses significant scavenging properties. DPPH· assay further suggested the fast reaction kinetics for both the compounds. Rutin hydrate was found to be slightly better than rutin in its scavenging capacity. However, hydroxyl radical scavenging assay showed scavenging action for both rutin and rutin hydrate. The assay does not indicate any such difference between hydroxyl radical scavenging capacity of rutin and rutin hydrate. Since the binding of ligand to CT-DNA increases structural stability of DNA and rutin hydrate displaying higher binding constant values as compared to rutin, rutin hydrate may be assumed to confer more structural stability to DNA than rutin. However, in DNA plasmid relaxation assay, both rutin and rutin hydrate showed DNA radioprotection at par with each other. Therefore, both the rutin and rutin hydrate may be potential candidate for radioprotection applications. The present study would be useful to explore potential of rutin and rutin hydrate *in-vivo* studies of similar nature.

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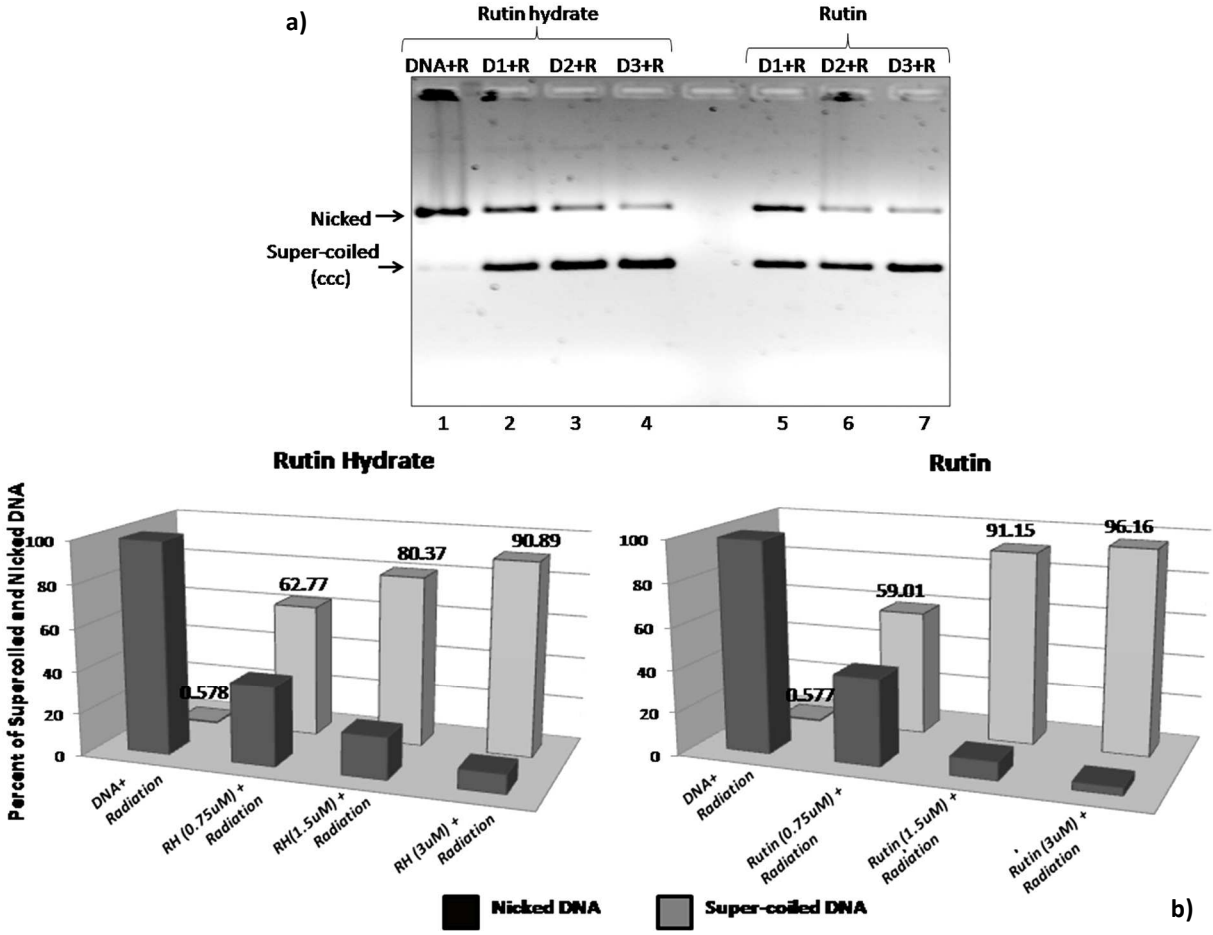

Figure 4. a) Agarose gel electrophoresis to show various forms of plasmid DNA. Lane 1: supercoiled form dominates in normal conditions, Lane 2-4 and 5-7 shows the protection ability of rutin hydrate and rutin in radiation exposed condition respectively. b) Quantitaive analysis showing percent of various forms of plasmid DNA after irradiation.

Conflicts of interest: *none to declare.*

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