

SHORT COMMUNICATION

## Antioxidant and Radioprotective Effects of *Ocimum* Flavonoids Orientin and Vicenin in *Escherichia coli*

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### ABSTRACT

Antioxidant effect of the *Ocimum* flavonoids, orientin and vicenin (25-500  $\mu\text{M}$ ), was evaluated by the kat-sod assay in *Escherichia coli* mutants (DSH56, superoxide dismutase-deficient and DSH19, catalase-deficient) treated with 50 mM menadione or  $\text{H}_2\text{O}_2$  (1mM). Protection by orientin (200  $\mu\text{M}$ ) and vicenin (200  $\mu\text{M}$ ) against  $\text{H}_2\text{O}_2$ -induced DNA damage in DSH19 cells ( $\beta$ -galactosidase test) and against radiation lethality in wild-type (DSH7) and DSH19 cells exposed to 0-150 Gy gamma radiation was also studied. Menadione and  $\text{H}_2\text{O}_2$  reduced the surviving fraction to 0.2 and 0.4 in DSH56 and DSH19 cells, respectively. Even 25  $\mu\text{M}$  of either flavonoid significantly increased the surviving fraction, with maximum protection at 200  $\mu\text{M}$ .  $\text{H}_2\text{O}_2$  increased the  $\beta$ -galactosidase activity in a concentration-dependent manner, which was significantly ( $P < 0.050-0.001$ ) reduced by orientin and vicenin (200  $\mu\text{M}$ ). Radiation produced a dose-dependent decrease in the surviving fraction of both DSH7 and DSH19 cells. Pretreatment with 200  $\mu\text{M}$  orientin or vicenin significantly increased the survival (DRF: DSH7 = 2.2; DSH19 = 1.8). Both compounds were equally effective in reducing the cytotoxicity of radiation and the chemical oxidants. The cytoprotective action of these plant flavonoids could be ascribed to their free radical scavenging activity.

**Keywords:** *Escherichia coli*, *Escherichia coli* mutants DSH19 and DSH56, orientin, vicenin, radioprotection, antioxidant effect, *Ocimum* flavonoids, reactive oxygen species

### 1. INTRODUCTION

Many agents such as ionising radiation, certain drugs, and environmental pollutants, produce reactive oxygen species, which interact with cellular DNA

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and other macromolecules, leading to damage and cell death<sup>1,2</sup>. Normal cells are equipped with an inherent defence system; catalase, and superoxide dismutase (SOD) are important members of this

defence system. Superoxide dismutase dismutates superoxide anions to hydrogen peroxide ( $H_2O_2$ ) and catalase converts the latter to water and oxygen<sup>3</sup>. Cells deficient in these enzymes lack the natural defence against the reactive oxygen species. Since such cells could be protected by administration of exogenous antioxidants, these are suitable models for studying the antioxidant activity of protective molecules.

Orientin and vicenin are two water-soluble flavonoids, isolated from the medicinal plant *Ocimum sanctum* (The Indian holy basil or *Tulasi*; Family Labiatae). Both these compounds have shown significant protection against radiation-induced lethality<sup>4</sup> and chromosomal aberrations in mouse bone marrow<sup>5</sup> and cultured human lymphocytes<sup>6</sup>. Based on *OH* radical inhibition in a chemical system, free radical scavenging has been suggested as the principal mechanism of protection by these flavonoids<sup>7</sup>. The present study evaluates the radical scavenging property of these flavonoids in a cell system and examines its possible role in cytoprotective effect.

Two mutant strains of *Escherichia coli*, deficient in the enzymes catalase and superoxide dismutase, have been developed by Yonezawa<sup>8</sup>, *et al.* Nishioka and Hayashi<sup>9</sup> have developed a method (the kat-sod assay) to study the reactive oxygen species generation in these strains. These bacterial strains were used to study the radioprotection by orientin and vicenin, and their antioxidant effect was studied by the kat-sod assay.

## 2. MATERIALS & METHODS

### 2.1 Bacteria

The *Escherichia coli* strains DSH7 (wild-type), DSH19 (*kat* EG, deficient in catalase) and DSH56 (*sod* AB, deficient in SOD)<sup>8</sup> were used.

### 2.2 Chemicals

Luria Bertini (LB) broth, menadione, mercaptoethanol and *O*-nitrophenyl- $\beta$ -D-galactopyranoside were bought from Sigma (USA), agar powder from Hi-media (India). All the other chemicals were of analytical grade, supplied by SD Fine Chemicals, India.

#### 2.2.1 Preparation of Solutions

- Luria Bertini medium was prepared by dissolving LB broth (15.5 g) in double-distilled water (1 litre) and autoclaved. The pH was adjusted to 7.2 using 1N NaOH. Luria Bertini plate was prepared by dissolving LB broth (15.5 g) and agar powder (15 g) in double-distilled water (1 litre) (pH 7.2) and pouring into petriplates (Nunc, Germany).
- Menadione solution was prepared in double-distilled water to give a concentration of 1 mM.
- Required concentrations of  $H_2O_2$  were also prepared in double-distilled water.
- Orientin (Mol. Wt. 448) and vicenin (Mol. Wt. 594) were isolated from an aqueous extract of the *Ocimum sanctum* leaf powder<sup>4</sup> and dissolved in double-distilled water to give a concentration of 1mM.
- Composition of M9 and Z buffers are as given by Yonezawa<sup>8</sup>, *et al.*

## 3. EXPERIMENTAL PROCEDURE

### 3.1 Effect of Orientin & Vicenin on Survival of DSH56 after Exposure to Menadione

The concentration of menadione was selected on the basis of a preliminary test in which the bacterial survival was studied by clonogenic assay after treating with different concentrations of menadione (25-125 mM). Concentrations higher than 50 mM resulted in very few colonies (Fig. 1). Therefore, 50 mM menadione was selected for further study. DSH56 (approx.  $2 \times 10^8$  cells, 2 ml titer) was incubated with different concentrations of orientin or vicenin (25  $\mu$ M, 50  $\mu$ M, 100  $\mu$ M, 200  $\mu$ M, 250  $\mu$ M, 500  $\mu$ M) for 30 min at 37 °C in a shaking water bath (30 rpm), followed by 0.125 ml of 1 mM menadione (final concentration 50 mM) for 1 h. The cells were then washed twice and resuspended in M9 buffer. Survival of the bacteria was determined by clonogenic assay<sup>8</sup> by plating cell suspensions on Luria bertini plates (in triplicate) after proper dilution and incubating at 37 °C.

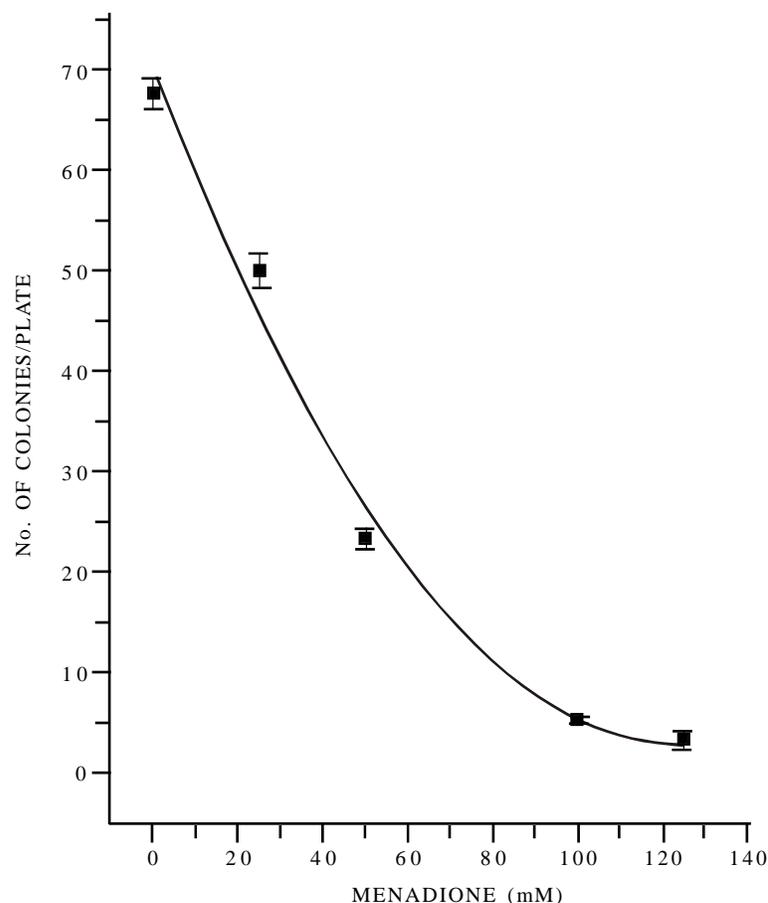


Figure 1. Effect of different concentrations of menadione on survival of DSH56 cells

### 3.2 Effect of Orientin & Vicenin on Survival of DSH19 Cells after Exposure to $H_2O_2$

A preliminary study on bacterial survival after treatment with different concentrations of  $H_2O_2$  (0.5–3.0 mM) was carried out to select the optimal concentration. A concentration-dependent decrease in cell survival, similar to that of menadione on DSH56 cells, was observed in the DSH19 cells treated with  $H_2O_2$  (data not shown). On the basis of this study,  $H_2O_2$  (1 mM) was selected for determining the protective effect of orientin and vicenin. Cell suspensions containing approximately  $2 \times 10^8$  cells (2 ml titer) were treated with different concentrations of orientin or vicenin (25–500  $\mu$ M) and incubated at 37 °C in a shaking water bath (30 rpm) for 30 min. Then the suspensions were incubated with 75  $\mu$ l of  $H_2O_2$  (100 mM) for 1 h (final concentration 1 mM), followed by survival assay.

On the basis of the above experiments, 200  $\mu$ M orientin and vicenin were selected for the next experiment.

### 3.3 Effect of Orientin & Vicenin on Survival of Bacteria Exposed to Gamma Radiation

The DSH7 (wild-type) and DSH19 (catalase-deficient mutant) of *Escherichia coli* were used in this study. The bacterial cultures were incubated for 30 min with or without 200  $\mu$ M of orientin or vicenin and then exposed to 0–150 Gy of  $^{60}Co$  gamma radiation (Gammatron Teletherapy Unit, Siemens, Germany) at 3.6 Gy/min. Dosimetry was done using a beam therapy dosimeter (Atomic Energy Canada Ltd, Canada). Clonogenic assay was carried out and the surviving fraction was determined. Dose reduction factor was calculated as the ratio of radiation doses needed to reduce the survival to 50 per cent of untreated control in the presence and absence of orientin or vicenin.

### 3.4 Effect of Orientin & Vicenin on the $H_2O_2$ -induced $\beta$ -Galactosidase Activity in DSH19

Cell suspensions in M9 buffer were treated with different concentrations (0-10 mM) of  $H_2O_2$  with or without 200  $\mu$ M of orientin or vicenin (total volume = 2.5 ml) or double-distilled water and incubated for 30 min at 37 °C in a shaking water bath. The optical density of the cell suspension was measured at 600 nm ( $OD_{600}$ ) in a UV spectrophotometer (Shimazu, Japan). The  $\beta$ -galactosidase test was carried out according to the method of Yonezawa<sup>8</sup>, *et al.* Briefly, 0.1 ml of cell suspension and 0.02 ml of 10 per cent toluene were added to 1.9 ml of Z-buffer in a test tube and stirred vigorously to disrupt cell membranes. The test tube was then incubated at 37 °C for 40 min in a shaking water bath (100 rpm), and then at 28°C for 10 min. The enzyme reaction was started by adding 0.2 ml of *o*-nitrophenyl- $\beta$ -galactopyranoside, followed by incubation at 28 °C for 30 min. Then 1 M  $Na_2CO_3$  (1 ml) was added to stop the reaction and the optical densities were measured at 420 nm ( $OD_{420}$ ) and 550 nm ( $OD_{550}$ ). Enzyme activity was calculated by the method of Miller<sup>10</sup> and expressed as units/ $OD_{600}$ .

All the experiments were repeated twice and the mean  $\pm$  standard error (S.E.) was calculated. Statistical analysis was performed by Student's *t* test using GraphPAD software. The radiation dose-response curves were drawn on a P II computer using Microcal Origin, Version 6 software.

## 4. RESULTS

- *Effect of orientin & vicenin on survival of DSH56 exposed to menadione*

Neither orientin (Ot) nor vicenin (Vc) by itself had any noticeable effect on cell survival. Menadione (50 mM) alone reduced the surviving fraction to 0.2. Pretreatment with either flavonoid significantly increased the survival at all concentrations used. The lowest concentration (25  $\mu$ M) produced a very significant increase ( $P < 0.001$ ) in survival. Further increments in concentration of orientin or vicenin to 50  $\mu$ M and 100  $\mu$ M did not result in any significant

**Table 1. Effect of orientin and vicenin on the survival of DSH56 cells treated with menadione (50 mM)**

Orientin/vicenin concentration ( $\mu$ M)	Surviving fraction	
	Without menadione	With menadione
0	1.000 $\pm$ 0.000	0.202 $\pm$ 0.004
25,Ot	1.006 $\pm$ 0.019	0.366 $\pm$ 0.0001 <sup>c</sup>
25,Vc	0.979 $\pm$ 0.023	0.375 $\pm$ 0.002 <sup>c</sup>
50,Ot	1.028 $\pm$ 0.002	0.370 $\pm$ 0.015 <sup>b</sup>
50,Vc	0.993 $\pm$ 0.017	0.353 $\pm$ 0.016
100,Ot	1.017 $\pm$ 0.004	0.373 $\pm$ 0.008 <sup>b</sup>
100,Vc	1.028 $\pm$ 0.011	0.351 $\pm$ 0.008 <sup>b</sup>
200,Ot	1.011 $\pm$ 0.020	0.613 $\pm$ 0.019 <sup>c</sup>
200,Vc	1.004 $\pm$ 0.000	0.624 $\pm$ 0.002 <sup>c</sup>
250, Ot	1.008 $\pm$ 0.001	0.308 $\pm$ 0.006 <sup>b</sup>
250, Vc	1.013 $\pm$ 0.009	0.308 $\pm$ 0.001 <sup>b</sup>
500,Ot	0.968 $\pm$ 0.001	0.288 $\pm$ 0.002 <sup>b</sup>
500,Vc	0.990 $\pm$ 0.018	0.291 $\pm$ 0.006 <sup>b</sup>

<sup>b</sup> $P < 0.01$ , <sup>c</sup> $P < 0.001$ , compared to menadione alone

increase in surviving fraction above that given by 25  $\mu$ M. When the orientin/vicenin concentration was raised from 100  $\mu$ M to 200  $\mu$ M, there was a steep increase in the surviving fraction. But at higher concentrations (250  $\mu$ M and 500  $\mu$ M), both the flavonoids led to a decrease in the surviving fraction (Table 1).

- *Effect of orientin & vicenin on survival of DSH19 exposed to  $H_2O_2$*

Orientin or vicenin, individually, did not have any appreciable effect on the survival at any of the concentrations used. Treatment with  $H_2O_2$  (1 mM) alone decreased the surviving fraction to 0.43. Pretreatment with either of the flavonoids, at all concentrations, significantly enhanced the surviving fraction above that obtained with  $H_2O_2$  alone. As in the case of DSH56 after menadione treatment, 200  $\mu$ M of the flavonoids produced the maximum effect and further increase in concentration of orientin and vicenin decreased the protective effect (Table 2). The above experiments demonstrated an optimum effective concentration for both flavonoids, which was 200  $\mu$ M.

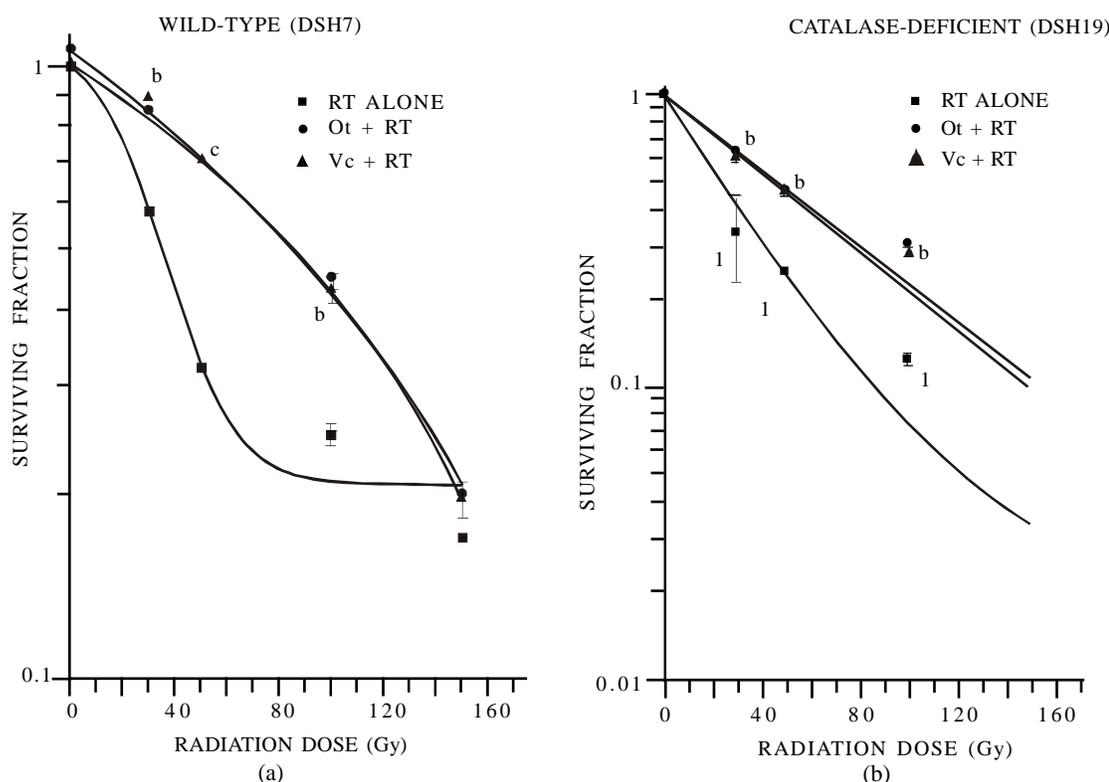
**Table 2. Effect of orientin and vicenin on the survival of DSH19 cells treated with  $H_2O_2$  (1 mM)**

Orientin/vicenin concentration ( $\mu$ M)	Surviving fraction	
	Without $H_2O_2$	With $H_2O_2$
0	1.000 $\pm$ 0.000	0.430 $\pm$ 0.009
25,Ot	0.964 $\pm$ 0.004	0.535 $\pm$ 0.002 <sup>b</sup>
25,Vc	0.973 $\pm$ 0.006	0.528 $\pm$ 0.013 <sup>a</sup>
50,Ot	0.984 $\pm$ 0.001	0.536 $\pm$ 0.002 <sup>b</sup>
50,Vc	0.969 $\pm$ 0.009	0.541 $\pm$ 0.010 <sup>a</sup>
100,Ot	0.971 $\pm$ 0.004	0.554 $\pm$ 0.008 <sup>b</sup>
100,Vc	0.951 $\pm$ 0.009	0.556 $\pm$ 0.009 <sup>a</sup>
200,Ot	0.980 $\pm$ 0.005	0.896 $\pm$ 0.019 <sup>c</sup>
200,Vc	0.958 $\pm$ 0.013	0.867 $\pm$ 0.008 <sup>c</sup>
250, Ot	0.975 $\pm$ 0.008	0.714 $\pm$ 0.004 <sup>b</sup>
250, Vc	0.962 $\pm$ 0.002	0.725 $\pm$ 0.004 <sup>b</sup>
500,Ot	0.977 $\pm$ 0.009	0.505 $\pm$ 0.008 <sup>a</sup>
500,Vc	0.973 $\pm$ 0.003	0.502 $\pm$ 0.010 <sup>a</sup>

<sup>a</sup>: P < 0.05, <sup>b</sup>: P < 0.01, <sup>c</sup>: P < 0.001 compared to  $H_2O_2$  alone.

- *Effect of orientin and vicenin on survival of DSH7 and DSH19 exposed to gamma radiation*

The surviving fraction decreased with radiation dose in both the bacterial strains studied. Irradiation resulted in a significantly higher (P < 0.01) cell death in the catalase-deficient mutant (DSH19) than in the wild-type bacteria. Survival fraction data for DSH7 and DSH19 cells fitted to a nonlinear model (Fig. 2). Pretreatment with orientin or vicenin resulted in a significant increase in the survival of both the bacterial strains and the effect of the two flavonoids was identical. But protection was more pronounced in the DSH7 (DRF = ~ 2.3) than in DSH19 (DRF = ~1.8). The surviving fraction of DSH19 was reduced to zero at 150 Gy, which was not increased by orientin/vicenin treatment [Fig. 2(b)].



**Figure 2. Radiation dose-response curves for survival of cells exposed to gamma radiation with or without orientin/vicenin pretreatment. Curve fitting, RT alone: Sigmoidal (Boltzmann model); Ot + RT and Vc + RT: linear regression. (a) DSH7,  $R^2 = 0.998$  (RT alone);  $R^2 = 0.964$  (Ot + RT);  $R^2 = 0.999$  (Vc + RT) and (b) DSH19,  $R^2=0.994$  (RT alone);  $R^2 = 0.967$  (Ot + RT);  $R^2 = 0.949$  (Vc + RT). b: P < 0.01 and c: P < 0.001 compared to respective RT alone groups. 1: P < 0.01 compared to RT alone in DSH7.**

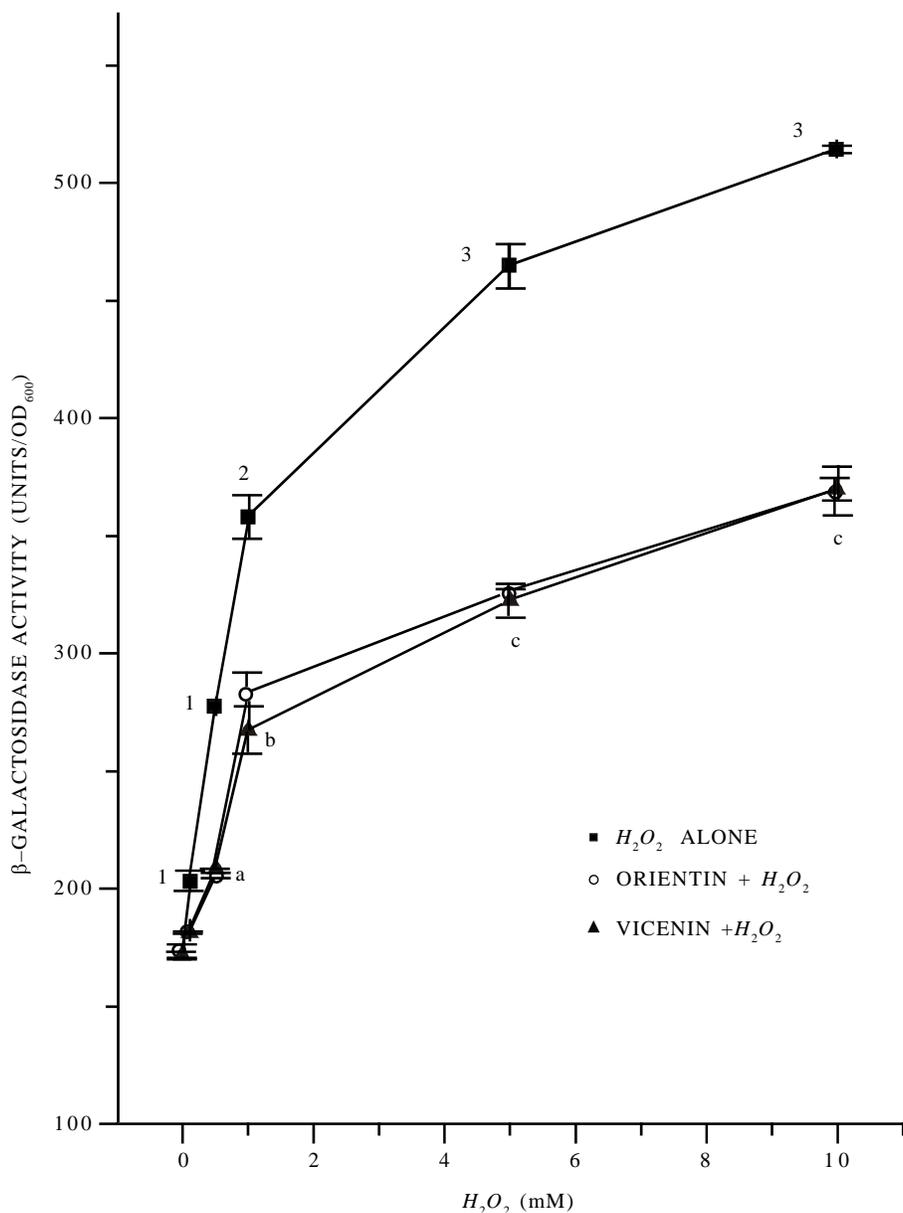
- *Effect of orientin/vicenin on the  $H_2O_2$ -induced  $\beta$ -galactosidase activity*

Treatment with  $H_2O_2$  significantly increased the  $\beta$ -galactosidase activity in the DSH19 cells in a concentration-dependent manner. The enzyme activity showed a steep rise at the lower concentrations, but the dose-response curve became shallower at higher concentrations above 5 mM. Both orientin and vicenin decreased the  $H_2O_2$ -induced  $\beta$ -galactosidase activity, which

was significant at all the concentrations of  $H_2O_2$  ( $P < 0.05-0.001$ ) (Fig. 3). Neither compound, when given alone, had any effect on the enzyme activity. The dose-response curves for  $H_2O_2$  alone and for the flavonoid-pretreated groups fitted well on the linear-quadratic model ( $R^2 = 0.9$ ).

## 5. DISCUSSIONS & CONCLUSIONS

The data from the experiments, where cells were treated with menadione or  $H_2O_2$ , demonstrate



**Figure 3.** Effect of orientin and vicenin on the  $H_2O_2$ -induced  $\beta$ -galactosidase activity (units/OD<sub>600</sub>) in DSH19 bacterial cells. 1:  $P < 0.05$ , 2:  $P < 0.01$ , 3:  $P < 0.001$ , compared to control. a:  $P < 0.05$ , b:  $P < 0.01$ , c:  $P < 0.001$ , compared to  $H_2O_2$  alone.

that both orientin and vicenin are good antioxidants. In the absence of superoxide dismutase, menadione-generated superoxide anions, through Fenton/Haber-Weiss reaction, are likely to cause oxidation of cellular macromolecules, consequently leading to cell death. This can explain the high lethality observed in the DSH56 mutants. Similarly, the mutants DSH19 cells, which lack the catalase, succumb to the toxicity of  $H_2O_2$ , as evident from the decline in surviving fraction observed in the study.

Both orientin and vicenin significantly increased the survival of DSH56 and DSH19 mutants cells treated with menadione and  $H_2O_2$ , respectively. This can be explained on the basis of radical scavenging by the externally administered flavonoids, in the absence of the corresponding cellular antioxidant enzymes. Radiation induces highly reactive oxygen-free radicals in the cells, causing cell death<sup>11</sup>. Normal cells possess an inherent defence system, containing the antioxidant enzymes such as superoxide dismutase, catalase, GSH-transferase, and peroxidase<sup>12</sup>, which are able to defend, to a great extent, against the free radical-induced damage. But cells that are deficient in the antioxidant enzymes succumb to the radiation-induced free-radical attack.

Both the wild-type strain (DSH7) and catalase-deficient mutant strain (DSH19) showed a radiation dose-dependent increase in cell death. But DSH19 cells, which lack catalase, were more sensitive to the lethal effect of radiation, as was evident from the significantly higher reduction in surviving fraction of these cells than in DSH7 at the same radiation doses. Orientin and vicenin pretreatment significantly increase the survival of both the cell types, indicating that these compounds are able to reduce the lethal effects of radiation on these cells, which could be attributed to the free radical scavenging by these flavonoids. However, the protection is more pronounced in the wild-type DSH7 than in the mutant strain DSH19. In the former, where the inherent antioxidant mechanism is not compromised, the flavonoids may act as complementary source of radical scavengers to protect against the radiation-induced free-radical attack. This could explain the higher protection in the DSH7 by orientin and vicenin. Even though

orientin and vicenin could compensate, to a great extent, for the catalase deficiency, these may not be able to completely substitute its function in the DSH19 cells, resulting in their lower survival compared to the wild-type DSH7 strain.

Many flavonoids have been reported to behave as potential antioxidants due to their ability to scavenge free radicals and to chelate metal ions<sup>13,14</sup>. Scavenging by the flavonoids, before the radicals can reach and interact with the cellular DNA, helps in preserving the reproductive integrity of the cells, as indicated by the higher clonogenicity of the flavonoid-pretreated and irradiated cultures. Protection against DNA damage by orientin and vicenin is also reflected in the decrease in the  $H_2O_2$ -induced  $\beta$ -galactosidase activity, as the latter is considered to be an index of DNA damage<sup>8</sup>. The protection was pronounced in the mutant strain deficient in the  $H_2O_2$  scavenging enzyme catalase.

Orientin and vicenin seem to be equally effective in protecting the bacteria against radiation lethality. This agrees with the earlier findings on mouse survival and chromosome protection<sup>4,6</sup>. Almost similar dose-modifying factors (DMFs) were obtained for mouse survival after lethal whole-body irradiation (orientin: 1.3; vicenin: 1.37)<sup>4</sup> and menadione induction in human lymphocytes irradiated *in vitro* (orientin: 2.6; vicenin: 2.5)<sup>6</sup> as well as against radiation-induced lipid peroxidation in the mouse liver<sup>7</sup>.

The present finding of an optimum protective concentration agrees with the earlier *in vitro*<sup>6</sup> and *in vivo*<sup>4</sup> results. A similar dose dependence has been reported for chromosome protection by DMSO<sup>15</sup>. The mutagenesis inhibitory activity of flavonoids has been reported to be concentration-dependent; at lowest doses, these exhibited competitive inhibition and at higher doses, exhibited a mixed inhibition pattern<sup>16</sup>. Laughton<sup>17</sup>, *et al.* have demonstrated that plant flavonoids like quercetin, gossypol, and myricetin, can accelerate oxidative damage to DNA by reducing  $Fe(III)$  ions to  $Fe^{2+}$ , or by oxidising to produce  $O_2^{\cdot-}$  and  $H_2O_2$ , or by both. Such an action could explain the decrease in protection observed at orientin and vicenin concentrations above 200  $\mu M$ .

Thus, using the *Escherichia coli* mutants deficient in superoxide dismutase and catalase genes, it was demonstrated that the *Ocimum* flavonoids, orientin and vicenin, are excellent reactive oxygen species scavengers. In the absence of the cellular antioxidants, these act as surrogate defence molecules and thereby protect these cells against the oxidative damage-inducing agents like menadione,  $H_2O_2$ , and gamma radiation.

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## Contributors

**Dr Vrinda Nayak** obtained her PhD (Radiation Biology) at the Manipal Academy of Higher Education (Deemed University) Manipal, India, in 2002 under the guidance of Dr P. Uma Devi. She has been working as Postdoctoral Research Assistant at the University of Bristol, UK, since June 2003. Areas of her research include: Preclinical aspects of radioprotective effects of two natural flavonoids against radiation-induced insult in biological system. Currently, she is working on the genomic instability caused by metal ions and its relation to telomeres/telomerase enzyme, genomic damage caused by various DNA damage causing agents and their relationship to DNA damage response, repair, malignant transformation, and other human diseases.

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