



Antimutagenic Effect of *Phyllanthus orbicularis* Against γ -Radiation

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SUMMARY. The present work evaluated the protective effect of aqueous extract of *Phyllanthus orbicularis* against the mutagenicity of γ -radiation. The extract activity was determined in pre- and post-treatment procedures using *Salmonella* reversion assay. In either case, the plant extract protected bacterial cells against the mutations induced by γ -radiation, suggesting that it contains antimutagenic compounds which confer protection by at least two different mechanisms: *i*) as antioxidants targeting the oxidative mutagens induced by γ -radiation and *ii*) by modulation of the cellular repair enzymes acting on damaged DNA. The results are discussed in relation to the chemopreventive and radioprotective potential of *P. orbicularis*.

INTRODUCTION

Radioprotectors are administered to patients undergoing cancer radiotherapy to reduce the toxic, mutagenic, and carcinogenic effects of ionizing radiation on normal cells surrounding the tumor tissue. In light of the growing interest in identifying plant and natural compounds with radioprotective properties, phytochemicals are of particular interest due to their antiemetic, anti-inflammatory, antimicrobial, antioxidant, hematopoietic, immunostimulant, metal-chelating, and wound-healing activities ^{1,2}; all of which are relevant to the mitigation of ionizing-radiation-induced damage in mammalian systems. Moreover, these plant-derived compounds are of particular interest because they are less toxic than synthetic radioprotectors. As natural antioxidants, they exhibit a large window of protection, both pre- and post-irradiation, against lethality and mutagenesis.

Aqueous extract of the Cuban endemic plant *Phyllanthus orbicularis* has antiviral ³⁻⁵ and, as we have reported, antimutagenic properties. Thus, in Chinese hamster ovary (CHO) cells, the extract protects against hydrogen-peroxide-induced clastogenicity ⁶. In the *Salmonella* assay, it protects against mutagenicity by hydrogen peroxide ⁷ and exhibit antimutagenic effects against promutagenic aromatic amines ^{8,9}. Using the SOS chromotest and other SOS transcriptional-fusion-based assays, such as *recA* and *umuC* tests, we have shown that aqueous *P. orbicularis* extract ameliorates γ -radiation-induced genotoxicity in *Escherichia coli* cells, most likely through antioxidant and DNA protection mechanisms, the latter involving DNA repair systems ^{10,11}. Here, we show that the extract protects *Salmonella* cells against the mutagenic effect of γ -radiation, both in pre- and post-irradiation treatments. Our results provide additional evi-

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dence for the above-mentioned mechanisms in *P. orbicularis* mediated radioprotection.

MATERIALS AND METHODS

Bacterial strain and culture

Salmonella enterica Typhimurium strain TA102¹² was used in this study. Cells were grown for 16 h at 37 °C with shaking (100 rpm) in nutrient broth medium (17 g bacteriological peptone/l, 1 g nutrient powder/l, 2 g yeast extract/l, 5 g sodium chloride/l). The culture was then diluted five-fold in fresh medium and grown under similar conditions until the optical density at 600 nm (OD_{600nm}) was between 0.9 and 1.2, corresponding to 1–2 × 10⁹ colony-forming units (cfu)/ml.

P. orbicularis aqueous extract

P. orbicularis plants were collected from Cajalbana, Pinar del Río, Cuba. The identity of the specimens was verified and the plants were stored at the Cuban National Botanical Garden (No. 7/220). The *P. orbicularis* aqueous extract (POE) was obtained from leaves and stems as described by del Barrio and Parra⁴. The quality and biological stability of the lyophilized extract were determined as indicated previously¹³.

Survival of *Salmonella* cells after γ irradiation

Samples of cells grown to an OD₆₀₀ of 0.9 were centrifuged and re-suspended in phosphate buffer (0.2 M Na₂HPO₄/NaH₂PO₄) at a final concentration of 2 × 10⁸ cells/ml, distributed into glass test tubes, and stored in a refrigerator until irradiated. Irradiation was carried out at a temperature of 2 ± 0.5 °C using a Co⁶⁰ PX- γ -30M irradiator (Duhna, Russia), with a dose of 20–400 Gy administered at a rate of 33–42 Gy/min (calculated using Fricke's dosimeter). Irradiated cells were diluted in phosphate buffer; the dilution range depended on the radiation dose. Cell dilution samples of 0.1 ml were inoculated on plates containing nutrient broth medium, and colony-forming units were counted after 48 h of incubation at 37 °C.

Antimutagenicity assays

The antimutagenicity of POE against γ -radiation was studied by pre- and post-treatment of bacterial cells with the extract. In the pre-treatment procedure, bacterial cells were incubated with POE for 4 h at 8 °C without shaking, after which it was removed by washing the cells with distilled water. The cells were then suspended

in phosphate buffer, irradiated with a dose of 100 Gy as indicated above, and assayed by the standard plate incorporation, carried out as described by Maron and Ames¹⁴. In the post-treatment procedure, bacterial cells in phosphate buffer were irradiated, mixed with POE in top agar, and then assayed with the standard plate incorporation test. The POE dose in the assay was between 0.1 and 2 mg/ml. Controls to exposure with γ -irradiation (100 Gy) and solvent (distilled water) alone were always included in each experiment. The number of revertants following each treatment was calculated by averaging a minimum of three independent experiments, with three replicates per experiment. The antimutagenicity of POE was measured as a significant reduction in the number of revertants in combined treatments (POE + γ -irradiation) and expressed as percentage of mutagenesis inhibition:

$$\%MI = 1 - \frac{NR_{CO} - NR_E}{NR_I - NR_E} \times 100$$

where, NR_{CO} is the number of revertants in co-treated cells (POE + γ -irradiation), NR_E is the number of spontaneous revertants and NR_I is the number of revertants in irradiated cells.

Statistical Analysis

The average values for percent survival and plate revertants, and the corresponding standard errors were calculated. The normality of the data was tested using the Kolmogorov-Smirnov test. Variance homogeneity and analysis of variance (ANOVA) tests were also conducted. Mean values were compared using Student's *t*-test. For all statistical analyses, $p < 0.05$ was considered significant. The Statistica v.6 software package¹⁵ was used for all analyses.

RESULTS

First, the survival of strain TA102 following γ -radiation was determined (Table 1). The dose-response curve showed that survival decreased with increasing radiation dose. This decrease was significant beginning at a dose of 40 Gy. The median lethal dose (DL₅₀) was 152 Gy.

The mutagenic effect of γ -radiation on strain TA102 was then studied to select the optimal radiation dose for the antimutagenesis experiments (Table 1). The doses studied were lower than the DL₅₀ value and were chosen to avoid a decrease in the number of revertants because of

Dose (Gy)	Survival (%) †	Number of revertants per plate †
0	100 ± 0	353 ± 40
20	89 ± 11 n.s.	617 ± 47 *
30	86 ± 14 n.s.	709 ± 31*
40	80 ± 9 *	779 ± 58 *
50	70 ± 12 *	831 ± 70 *
100	62 ± 7 *	848 ± 97 *
200	33 ± 10 *	ND
300	22 ± 6 *	ND
400	7 ± 3 *	ND

Table 1. Survival and mutagenicity of *Salmonella* strain TA102 treated with γ -radiation †, Average values from at least three independent assays with three replicates each and the corresponding standard error are given. *, Significant differences with respect to non-irradiated cell ($p < 0.05$) were founded using Student's *t*-test. n.s., Not significant. ND, not determined.

an increase in cell death. The number of induced revertants was found to increase with radiation dose and was significant beginning at a dose of 20 Gy. Based on these results, a dose of 100 Gy, which yielded double the number of spontaneous revertants per plate (848 ± 97 vs. 353 ± 40) with a 62% of survival cells, was chosen for the antimutagenic study.

The number of revertants in POE-treated cells was very similar to that spontaneous revertants number in non-treated cells (Table 2), indicating that concentrations between 0.1 and 2 mg/ml of POE did not lead to mutagenesis in TA102 strain. The antimutagenic properties of POE in γ -irradiated cells are also shown in Table

2. In the pre-treatment procedure, POE produced a significant decrease in the number of induced revertants at doses of 0.1 and 0.5 mg/ml (%MI = 87 and 54%, respectively) but not at higher doses. When the extract was added after irradiation of the cells (post-treatment procedure), the number of induced revertants decreased significantly at a dose of 0.1 mg POE/plate (% MI = 50%).

DISCUSSION

This study focused on the antimutagenic effect of POE in γ -irradiated cells. Two different incubation procedures, pre- and post-irradiation, were tested. The basis of the pre-incubation assay was that oxygen radicals produced by γ -radiation have a very short lifetime; therefore, the antimutagenic effect of POE against oxidative mutation should be evident if the extract is already present during the irradiation. Under this experimental condition, doses of POE ≤ 0.5 mg/ml significantly decreased the mutagenic effects induced by γ -radiation. In previously reported studies with similar experimental conditions, a protective effect was also observed with mutagenic aromatic amines ⁸ and with hydrogen peroxide ⁷. The results obtained with the pre-treatment procedure indicated that the extract's antioxidant activity is important in its antimutagenic mechanism of action against γ -radiation, as was previously shown for hydrogen peroxide ^{6,7}. Antioxidant activity against the oxidative DNA damage produced by γ -radiation has also been demonstrated for other species of the genus *Phyllanthus*, e.g., *P. emblica* ^{16,17}.

Dose of POE (mg/ml)	Dose of γ -rays (Gy)	Number of revertants/plate † (Percentage of Mutagenesis Inhibition)	
		Pre-incubation procedure	Post-incubation procedure
0	0	286 ± 44	256 ± 43
0.1	0	225 ± 31	225 ± 31
0.1	100	255 ± 65 (87) *	430 ± 43 (50) *
0.5	0	252 ± 53	252 ± 53
0.5	100	400 ± 78 (54) *	610 ± 56 (0) n.s.
1.0	0	258 ± 48	258 ± 48
1.0	100	539 ± 124 (0) n.s.	541 ± 66 (18) n.s.
2.0	0	261 ± 30	261 ± 30
2.0	100	519 ± 91 (0) n.s.	532 ± 55 (21) n.s.
0	100	533 ± 74	605 ± 47

Table 2. Antimutagenicity of *Phyllanthus orbicularis* extracts (POE) against γ -radiation, as measured in *Salmonella* strain TA102. †, Average values from at least three independent assays with three replicates each and the corresponding standard error are given. The percentage of mutagenesis inhibition (%MI) in combined treatments (POE + γ -irradiation) is given in parentheses. *, A significant difference respect to irradiated cells ($p < 0.05$) was founded using Student's *t*-test. n.s., Not significant.

However, interestingly, POE was also antimutagenic in the post-treatment procedure, at a dose of 0.1 mg/plate (% MI = 50%). This finding suggests a mechanism of action other than antioxidant activity. In previous studies ¹¹, we showed post-irradiation DNA protection by POE in a SOS chromotest assay. The antigenotoxic effect detected in that experiment was explained by assuming that some component of the plant extract stimulates or facilitates the action of DNA repair enzymes, since diminished *E. coli* SOS induction following γ -irradiation may well be interpreted as a reduction in cellular DNA damage through DNA repair activity. The antimutagenic effect of POE detected in the post-irradiation experiments described here supports the hypothesis that the extract contains bioantimutagenic compounds that facilitate the repair of DNA damage induced by γ -radiation. Although there are clear differences between the endpoints of the SOS chromotest and the *Salmonella* reversion assay ¹⁸, our results and those previously reported ¹¹ point to similar modes of action in DNA radioprotection by POE in *Escherichia coli* and *Salmonella typhimurium* cells.

A preliminary phytochemical characterization of POE detected flavonoids, alkaloids, coumarins, saponins, aminoacids, anthocyanidins, mucilages, triterpenes and/or steroids, reducing substances, pyrocatecholic tannins, bitter and astringent agents, glycosides, and quinones ¹³. Several of these classes of compounds may have antimutagenic properties. In a bioassay-assisted fractionation of POE, we identified the antimutagenic compound (bis-2,4-di-tert-butylphenol), active against hydrogen peroxide (Ferrer *et al.*, results not published). The compound may also be effective against the oxidative DNA damage induced by γ -radiation; but this remains to be determined.

Different species of the genus *Phyllanthus*, including *P. amarus*, *P. emblica*, and *P. urinaria*, have been shown to exert chemopreventive, anticarcinogenic, and antitumor effects in animal models ¹⁹⁻²⁹. Based on its negative genotoxicity data ³⁰ and antigenotoxic properties against aromatic amines ^{8,9}, hydrogen peroxide ^{6,7} and γ -radiation ¹¹, and present work, *P. orbicularis* clearly has broad-ranging therapeutic potential as a source of chemopreventive and radioprotective compounds.

In conclusion, the present work presented evidence of the ability of POE to protect against

γ -radiation-induced mutagenesis both in pre- and post-irradiation treatments. Demonstration of post-irradiation DNA protection against DNA damage conducive to mutation by POE supports the idea that compounds of this plant extract could be used as a chemopreventive or therapeutic agent after radiotherapy in order to minimize the development of secondary tumors. Thus, the information derived from this and similar studies could be applied to optimize the use of radioprotective agents in cancer therapy.

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