Enhancement of Radiation Effect by *Aphanamixis polystachya* in Mice Transplanted with Ehrlich Ascites Carcinoma

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The effect of radiation on tumor tissue can be optimized by adding radiosensitizing agents, in order to achieve a greater degree of tumor damage than expected from the use of either treatment alone. The ethanolic extract of *Aphanamixis polystachya* (APE) was tested in Swiss albino mice transplanted with Ehrlich ascites carcinoma (EAC) and exposed to various doses of γ-radiation. EAC mice received 0, 10, 25, 50, 75, 100, 150 or 200 mg/kg body wt APE before exposure to 6 Gy γ-radiation followed by once daily administration for another 8 consecutive days post-irradiation. The optimum radiosensitizing dose was found to be 50 mg/kg APE that was further tested in EAC mice exposed to 0, 1, 2, 4, 6 or 8 Gy hemi body γ-radiation. The best effect of APE and radiation was observed for 6 Gy γ-radiation. The splitting of 50 mg into two equal fractions of 25 mg and administering the split dose with a gap of 8 h on 1, 3, 5, 7 or 9 d of tumor inoculation resulted in an increased survival even when the drug was administered at late stages (day 5) of tumor development. The APE treatment before irradiation elevated lipid peroxidation followed by a reduction in the glutathione contents. Treatment of tumor bearing mice with APE before irradiation further reduced the activities of various antioxidant enzymes like glutathione peroxidase, glutathione-s-transferase, superoxide dismutase and catalase at different post last drug administration (PLDA) times.

Key words  *Aphanamixis polystachya*; mice; Ehrlich ascites carcinoma; radiation; survival; biochemical study

The biological effects of radiation affect both neoplastic and normal tissues. The nature and extent of such effects, however, depend on selected biological parameter (e.g., oxygen supply, cell cycle) and can be modified by chemical agents such as radiosensitizers, radioprotectors and chemotherapeutic agents. A precise control of the mode of action of the radiation is important in order to achieve the maximum effect on tumor tissue, while minimizing the effect on normal tissue. The concept of an optimal irradiation dose providing a maximally positive response of the target tissue and minimal toxicity to the perilesional area is the basis of various fractionation schedules for the radiotherapeutic treatment of tumors. The combination therapy of treatment of tumors with radiation and different anticancer drugs like 5-fluorouracil,1) mitomycin C,2–3) etoposide4) and carboplatin5) have been tried clinically. However, the side effects of such modes of treatment are severe and have resulted in the occurrence of secondary malignancies. Some agents under test are interferon beta that has cytotoxic synergism with radiation against MO59J cell line in vitro,4) and MDM2 inhibitors, such as antisense oligonucleotides as radiosensitizer against various neoplastic cell lines in vitro and different tumor xenografts in vivo.6) Nevertheless efforts to explore better radiosensitizers shall be continued to reduce toxic side effects of combination treatment.

The effect of radiation on tumor tissue can be optimized by adding radiosensitizing agents, in order to achieve a greater degree of tumor damage than expected from the additive effect of each modality. Most of the known and routinely used radiosensitizers are neither selective nor tumor specific. Such compounds are commonly toxic per se and thus lead to severe systemic side effects. In addition, local failure is still commonly observed even with the application of radiosensitizers and local tumor regrowth may occur in 60 to 80% of cancer patients at the time of death.7) Herbs offer a vast source of new chemicals and some of them are structurally so complex that they can not be synthesized even with the advanced technology at our disposal.8) The herbal drugs have gained attention and popularity because of their negligible toxicity and possibly with a ray of hope that they may replace some of the available antineoplastic drugs that are highly toxic. 7-Beta-hydroxysterol, a natural oxysterol has been shown to posses marked antiproliferative and cytotoxic activities and demonstrated enhanced radiosensitivity of RDM4 cells.9) Certain herbal preparations from plants like *Withania somnifera* have also been reported to show a marked tumor inhibitory activity in vitro, against cells derived from human carcinoma of the nasopharynx and experimental tumors.10) It has also been reported to enhance the effect of radiation in mice.11) Plumbagin, derived from the plant *Plumbago zeylanica*, has been reported to enhance the effect of radiation in mice bearing sarcoma S180 and Ehrlich ascites carcinoma.12)

*Aphanamixis polystachya* WALL & PARKER (*Amoora rohituka* (ROXB.) WIGHT & ARN.) is distributed throughout India in evergreen forests and is a member of the family Meliaceae. It has been reported to posses several medicinal properties against various ailments including spleen, liver tumors and Friend's leukemia.13–23) Our earlier experiments have shown that the alcoholic fraction of the stem bark of *Aphanamixis polystachya* (APE) possess anticancer activity against the Ehrlich ascites carcinoma transplanted in mice (data not shown). However, the radiosensitizing effect of APE has not been studied. Therefore, the present investigation was undertaken to study the radiosensitizing effect of APE in mice transplanted with Ehrlich ascites carcinoma and exposed to different doses of γ-radiation.
MATERIALS AND METHODS

**Chemicals** Glutathione (GSH), 5,5'-dithiobis-2-nitrobenzoic acid (DTNB), reduced nicotinamide adenine dinucleotide (NADPH) and Thiobarbituric acid (TBA) were purchased from Sigma Chemical Company, St. Louis, U.S.A., while all other chemicals were procured from Ranbaxy Fine Chemicals, Mumbai, India.

**Preparation of the Extract** The identification of the plant *Aphanamixis polystachya* Wall and Parker (Family—Meliaceae) was done by Dr. G. K. Bhat (a well known taxonomist of this area) Udupi, India and the herbarium specimen—RBAP01 is stored with us. The bark of the tree was carefully peeled off, shade dried, and coarsely powdered in a ball mill. The powdered material was extracted with petroleum ether in a Soxhlet apparatus at 40°C for thirty cycles and dried at 40°C overnight. The petroleum ether free powdered material was further extracted with 95% ethyl alcohol in a Soxhlet apparatus at 60°C extensively for 3 d. The cooled liquid extract was concentrated by evaporating its liquid contents under reduced pressure. The dried extract was subjected to freeze drying to obtain a fine powder of the extract. Henceforth the extract will be called as APE.

**Animal Care and Handling** The animal care and handling were done according to the guidelines issued by the World Health Organization, Geneva, Switzerland and the INSIA (Indian National Science Academy, New Delhi, India). Ten to twelve weeks old female Swiss albino mice weighing 30 to 36 g were selected from an inbred colony maintained in the INSA (Indian National Science Academy, New Delhi, India) and the World Health Organization, Geneva, Switzerland and the INSIA (Indian National Science Academy, New Delhi, India). The animal care and handling were done according to the guidelines issued by the World Health Organization, Geneva, Switzerland and the INSIA (Indian National Science Academy, New Delhi, India).

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**Tumor Model** Ehrlich ascites carcinoma (EAC) procured from the Cancer Research Institute (ACTREC), Mumbai, India, was maintained and propagated by serial intraperitoneal transplantation of EAC cells in an aseptic environment. 10^6 viable EAC cells were injected intraperitoneally into each animal in an aseptic condition and the day of tumor inoculation was considered as day zero. All the experiments of tumor bearing mice were conducted 24 h after the EAC transplantation and that day was considered as day one.

**Preparation of Drug and Mode of Administration** The APE was dissolved freshly in sterile physiological saline (SPS) containing 0.5% CMC (carboxy methyl cellulose) with constant trituration in a mortar, before use. Unless otherwise stated SPS or APE were administered intraperitoneally 24 h after transplantation of tumor cells into the mice.

**Selection of Optimum APE Dose** The drug dose was selected according to standard protocol recommended by the Drug Evaluation Branch, Drug Research and Development, NIH, U.S.A. Twenty-four hours after tumor inoculation, the animals were divided into the following groups:

- **SPS+Irradiation:** The animals of this group received 0.3 to 0.36 ml SPS 1 h before exposure to 6 Gy γ-radiation and then once daily for another eight consecutive days after irradiation.
- **APE+ Irradiation:** The animals of this group were injected with 10, 25, 50, 75, 100, 150 or 200 mg/kg body wt APE 1 h before exposure to 6 Gy γ-radiation and then once daily for another eight consecutive days after irradiation.
- **APE+ Irradiation:** Since a maximum tumor free survival was observed for the EAC mice receiving 50 mg/kg body wt of APE in conjunction with 6 Gy γ-radiation when compared with other doses of APE, further experiments were carried out with this dose.
- **Selection of Optimum Irradiation Dose** A separate experiment was conducted to establish the optimum dose of irradiation, which will give highest tumor cell killing effect in conjunction with 50 mg/kg APE, where the animals were divided into the following groups:
- **SPS+Irradiation:** The animals of this group received 0.3 to 0.36 ml of SPS 1 h before exposure to 6 Gy of hemi-body γ-irradiation.
- **APE+Irradiation:** The optimum drug dose (50 mg/kg) of APE was split into two equal fractions. Twenty-four hours after EAC inoculation, the animals were injected with a single dose of first fraction (25 mg/kg) 1 h before exposure to 6 Gy γ-radiation. The second dose of 25 mg/kg of APE was administered after an elapse of 8 h on day 0 (the day of irradiation of mice), 1, 3, 5, 7 or 9 d post-irradiation.
- **Irradiation** Prostate, immobilized (achieved by inserting cotton plugs in the restrainer) and unanesthetized tumor bearing animals of all experiments were restrained in a specially designed well-ventilated perspex box and their lower half of the body, below rib cage (hemi-body) was then exposed to different doses of gamma radiation. A batch of ten animals was irradiated at a dose rate of 2.0 Gy/min at a source to animal distance (midpoint) of 78.9 cm using ^60^Co γ-radiation from a Tele Cobalt Therapy source (Theratron, Atomic Energy Agency, Ontario, Canada).

**Survival Analysis** After treatment the animals were monitored regularly for body weight changes, signs of toxicity and mortality. The weight of each animal was recorded every third day up to 30 d after tumor inoculation in all the groups. A 33% of drug related deaths or a weight loss of 5 g per mouse was considered as an index of toxicity. The animal survival was monitored daily up to 120 d, since the survival of animals up to 120 d is roughly equivalent to 5 years survival in man. The optimum drug dose was selected on the basis of tumor response and survival. The tumor response was assessed on the basis of median survival time (MST) and the average survival time (AST). The MST and AST were calculated from the animals dying within 120 d and those surviving 120 d were excluded from it. The increase in mean life span (% IMLS) and the increase in average life span (% IALS) were calculated using the formulae:
Biochemical Estimations To understand the mechanism of action of APE in combination with radiation, a separate experiment was carried out to estimate various antioxidant parameters and lipid peroxidation in the EAC cells exposed to 6 Gy γ-radiation alone or APE in conjunction with radiation. The EAC animals were divided into the following groups:

S1: Irradiation: The animals of this group received 0.01 ml/g body wt. of SPS one hour before and 8 h after exposure to 0 or 6 Gy γ-radiation on day 1. SPS administration was continued up to day 6 post-irradiation.

S2: Irradiation: The optimum drug dose (50 mg/kg) of APE was split into two equal fractions. Twenty-four hours after EAC inoculation, the animals were injected with a single dose of first fraction (25 mg/kg) 1 h before exposure to 0 or 6 Gy γ-radiation. The second fraction was administered after a gap of 8 h from the first dose. The treatment of mice with only APE was continued for up to day 6 post-irradiation. Four animals from each group were sacrificed at 1, 2, 3, 6, 9, 12, 18 or 24 h post drug administration times (PLDA). The tumor cells were aspired in an aseptic condition and were washed thrice with SPS. The cells were counted under an inverted microscope (Ernst Leitz Wetzlar GmbH, Wetzlar, Germany) using a hemocytometer. 1×10⁶ cells from each EAC mice were suspended in 1 ml of 0.15 M Tris–HCl (pH 7.4) with 50 μl of Triton X100 and sonicated on ice for 20 s using a microprobe sonicator (Sonics Vibra-cell, CT, U.S.A.). The sonicated cell suspension was centrifuged at 13000 rpm (Sorvall Instruments, DuPont, Minnesota, U.S.A.) at 15000 rpm at 4 °C for 15 min in a high speed centrifuge and the supernatant (S1) free of proteins was collected. The entire supernatant (S1) was immediately mixed with 0.5 ml of NADPH (4 mg of reduced form was dissolved in 100 ml of 0.5% NaHCO₃), 0.5 ml of glutathione reductase (6 units/ml in 0.1 M phosphate buffer, pH 7.0) and 1 ml of 0.6 M DTNB [5,5′-dithio-bis-(2-nitrobenzoic acid), prepared in 0.2 M phosphate buffer (pH8)]. The formation of TNB [5-thio-(2-nitrobenzoic acid)] was read at 412 nm in a UV–Visible double beam spectrophotometer (Shimadzu UV-260, Shimadzu Corp, Tokyo, Japan). The GSH concentration has been expressed as nM/10⁶ cells. Standard curve was prepared from a stock solution of 10 mM GSH (30.7 mg GSH/10 ml) in 5% 5-sulfosalicylic acid diluted to 1—10 mM GSH/ml. A sample blank lacking GSH was used to determine the background rate and the resulting background rate of product formation was subtracted from the sample values prior to GSH quantification.

Glutathione Peroxidase The assay was carried out by the method of Ho et al. The following reagents were placed in a cuvette. The reaction was initiated by adding 1 ml of supernatant (S) to 1 ml of buffer containing 20 mM potassium phosphate (pH 7.0), 0.2 ml EDTA (0.6 mM), 0.1 ml NADPH (0.15 mM), 4 units of glutathione reductase, 2 mM GSH, 1 mM sodium azide, and 0.1 mM H₂O₂ at 25 °C. The rate of decrease in absorption of NADPH per minute was followed at 340 nm in a UV–Visible double beam spectrophotometer (Shimadzu UV-260, Shimadzu Corp, Tokyo, Japan). The GSHPx activity has been expressed as nm of NADPH consumption per min/10⁶ cells at 2 mM GSH. Consumption of NADPH was calculated using a nm extinction coefficient for NADPH of 6.22×10⁶ cm²/mol.

Glutathione-S-transferase The cytosolic glutathione-S-transferase activity was determined spectrophotometrically at 37 °C according to the procedure of Habig et al. The reaction mixture contained 2.7 ml of 100 mM phosphate buffer (pH6.5), 0.1 ml of 30 mM CDNB. After preincubating the reaction mixture at 37 °C for 5 min, the reaction was started by the addition of 0.1 ml of supernatant (S) and the absorbance was followed for 5 min at 340 nm. Reaction mixture without the enzyme was used as blank. The GST activity has been expressed as nm of GSH–CDNB conjugate formed/min/10⁶ cells. The activity measured was multiplied by 10 to get total activity per million cells.

Superoxide Dismutase (SOD) Assay The activity of superoxide dismutase was determined by the method of Misra and Fridovich based upon the ability of SOD to inhibit the auto-oxidation of epinephrine to adrenochrome at alkaline pH. The entire supernatant 1 ml (S) was taken in 0.1 m carbonate buffer (pH10.2). After addition of epinephrine, the increase in absorbance was measured at 480 nm using a UV–Visible double beam spectrophotometer (Shimadzu UV-260, Shimadzu Corp, Tokyo, Japan). The activity of the enzyme has been expressed as U/mg protein of 10⁶ cells, where 1 U of the enzyme is defined as the amount of enzyme required to inhibit the rate of epinephrine auto-oxidation by 50% under the conditions of the assay.

Catalase The activity of catalase was determined by the method of Aebi. The change in absorbance was followed spectrophotometrically at 240 nm after the addition of H₂O₂ (30 mM/l prepared by diluting 0.34 ml of 30% H₂O₂ with 50 mM phosphate buffer to 100 ml) to 1 ml supernatant (S), in
50 mM phosphate buffer (mixed 1 volume of 6.81 g KH$_2$PO$_4$ in 11 water and 1.5 volume of 8.9 g Na$_2$HPO$_4$ in 11, pH 7). The absorbance was read at 240 nm initially and then at an interval of every 15 s using a UV–Visible double beam spectrophotometer (Shimadzu UV-260, Shimadzu Corp, Tokyo, Japan). The activity of the enzyme has been expressed as U per 10$^6$ cells, where 1 U is equivalent to 1 mol of H$_2$O$_2$/µg/min/10$^6$ cells.

Calculation for H$_2$O$_2$ concentration was made using a molar extinction coefficient for H$_2$O$_2$ of 0.0394. The catalase activity was calculated by the following formula:

$$\text{enzyme activity} = \frac{V}{v} \times \frac{2.3}{10} \times \log \frac{A_1}{A_2} \text{ s}^{-1} \text{ per liter}$$

Where $A_1$ is absorbance at time $t=0$; $A_2$ is absorbance at $t=10$ s; $V$ is total assay volume=3 ml; $v$=sample volume=1 ml.

**Statistical Analysis** The statistical significance between the treatments for survival studies was determined using the “$Z$” test,$^{32}$ while the Student’s ‘$t$’ test was used for biochemical studies. All the data are expressed as mean±standard error of the mean (S.E.M.).

**RESULTS**

**Selection of Optimum APE Dose** Mice transplanted with Ehrlich ascites carcinoma (EAC) did not show spontaneous tumor regression. The tumor bearing mice showed constant weight gain and increase in tumor volume due to multiplication of EAC cells and growth of the tumor (Fig. 1). The MST and AST were 17 d, respectively (Table 1).

Exposure of tumor bearing mice to a single dose of 6 Gy hemi-body γ-irradiation resulted in an increase in the MST and AST approximately by 8 d. The animals did not survive beyond 28 d (Table 1). Treatment of EAC mice with various doses of APE before exposure to a single dose of 6 Gy gamma irradiation resulted in an APE dose dependent increase in the MST and AST. The greatest effect was observed for the animals receiving 50 or 75 mg/kg APE before 6 Gy irradiation, where the MST increased by approximately 23 and 22 d, respectively when compared with the SPS+irradiation group. The AST was also elevated by 22.5 and 21 d for 50 and 75 mg/kg APE+irradiation, respectively. The least effect was observed for 10 mg/kg body wt APE+irradiation, where MST and AST was found to be approximately 28 and 26.5 d respectively. This elevation in the MST and AST was non-significant when compared with SPS+irradiation group.

The highest radiomodifying effect was observed for 50 mg/kg body wt APE, that resulted in approximately 96% long-term survivors (LTS) up to 60 d ($p<0.0001$), followed by a 60% survival until 90 d ($p<0.05$). This dose also showed approximately 30% survivors beyond 120 d (Table 1). It did not induce any toxic effects in the form of debility, loss of body weight and also showed more number of LTS, therefore, 50 mg/kg APE was considered as an optimum dose and further studies were carried out using this dose.

Treatment of EAC mice with 25, 50, 75, 100 or 150 mg/kg body wt APE before exposure to 6 Gy hemi-body irradiation, arrested the tumor weight gain, indicating inhibition of tumor cell proliferation and growth (Fig. 1). The administration of 200 mg/kg APE before irradiation, was accompanied by toxic side effects like ruffling of hair, sluggishness, lacrimation followed by death, and none of the animals survived beyond day 13 post-tumor inoculation (Fig. 1).

**Selection of Optimum Irradiation Dose** Irradiation of EAC mice caused a dose dependent reduction in the tumor growth as evidenced by a dose dependent increase in MST, AST, IMLS and IALS. However, irradiation failed to prolong the life span of EAC mice beyond 30 d (Table 2). Administration of 50 mg/kg APE before irradiation resulted in long-term survivors of tumor up to 6 Gy as evidenced by an increase in AST and MST by 27 and 26 d, respectively over SPS+6 Gy irradiation. This was reflected in an increased IMLS and IALS of approximately 120% (Table 2). The combination of 50 mg/kg APE with 6 Gy resulted in LTS as well as 30% survivors up to 120 d, while this effect was not discernible for the combination treatment with other irradiation doses. An increase in irradiation dose up to 8 Gy, resulted in a decline in MST and AST indicating that this combination was not as effective in
Inoculation, (stage I, II, III, IV, V and VI, respectively) where carried out in EAC animals at 1, 2, 4, 6, 8 or 10 d post-tumor formation using this regimen. Therefore, combination of 50 mg/kg APE with 6 Gy irradiation was effective even at stage III and IV as is evidenced by an increased life span of EAC animals to 20 and 10% respectively when compared with the SPS irradiation treatment. The administration of APE in conjunction with 6 Gy γ-irradiation at stage I and II has lengthened the life span of 30 and 20% animals beyond 120 d. Combination of APE and radiation was found to be effective even at stage III and IV as is evidenced by an increased life span of EAC animals to 20 and 10% beyond 60 and 90 d, respectively.

Biochemical Estimations The spontaneous values of LPx, GSH concentration, GSHPx, GST, SOD and CAT activity did not change significantly in EAC cells with assay time (Figs. 2—4). LPx: The irradiation of EAC mice resulted in a time dependent increase in LPx that reached a highest level at 3 h PLDA and declined thereafter, without reaching to reducing the tumor growth as with 6 Gy irradiation (Table 2). Therefore, combination of 50 mg/kg APE with 6 Gy irradiation was considered the best and remaining studies were performed using this regimen.

Tumor Stage Specific Radiosensitization The effect of repeated administration of APE after radiation treatment at different stages of tumor development was also evaluated. The tumor stage specific radiosensitization of APE was carried out in EAC animals at 1, 2, 4, 6, 8 or 10 d post-tumor inoculation, (stage I, II, III, IV, V and VI, respectively) where a dose of 25 mg/kg of APE were administered twice daily for 9 d, consecutively at stage I, II, III, IV, V and VI. The APE was effective in increasing the life span of EAC mice up to stage IV where the animals did survive beyond 60 d, which is reflected in the body weight changes. The APE treatment of EAC mice in conjunction with 6 Gy γ-irradiation increased the survival and inhibited tumor weight gain during stage I, II, III and IV when compared with the concurrent SPS + irradiation group. However, combination of APE with radiation at stage V and VI did not increase the life span of EAC mice significantly when compared to SPS + irradiation treatment (Table 3).

Table 1. Effect of Various Doses of APE in Combination with 6 Gy of γ-Irradiation on the Survival of Tumor Bearing Mice

<table>
<thead>
<tr>
<th>APE dose (mg/kg)</th>
<th>Sham-irradiation</th>
<th>Irradiation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Survival (d)</td>
<td>Percent survivors (d)</td>
<td>Survival (d)</td>
</tr>
<tr>
<td>MST</td>
<td>IMLS</td>
<td>AST</td>
</tr>
<tr>
<td>SPS</td>
<td>17</td>
<td>16</td>
</tr>
<tr>
<td>APE (10)</td>
<td>18</td>
<td>5.88</td>
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<tr>
<td>APE (25)</td>
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<td>APE (50)</td>
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<td>29.4</td>
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<td>APE (75)</td>
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<td>29.4</td>
</tr>
<tr>
<td>APE (100)</td>
<td>21</td>
<td>23.53</td>
</tr>
<tr>
<td>APE (200)</td>
<td>21</td>
<td>23.53</td>
</tr>
</tbody>
</table>

a) Sham-irradiation=0 Gy.  
b) Toxicity due to highest drug dose in combination with 6 Gy γ-irradiation. * p<0.05, ** p<0.002, *** p<0.0001, when compared with SPS + irradiation group.

Table 2. Effect of 50 mg/kg of APE in Combination with Different Doses of γ-Irradiation on the Survival of Tumor Bearing Mice

<table>
<thead>
<tr>
<th>Exposure dose (Gy)</th>
<th>SPS + irradiation</th>
<th>APE + irradiation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Survival (d)</td>
<td>Percent survivors (d)</td>
<td>Survival (d)</td>
</tr>
<tr>
<td>MST</td>
<td>IMLS</td>
<td>AST</td>
</tr>
<tr>
<td>0</td>
<td>18.5</td>
<td>17.2</td>
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<tr>
<td>2</td>
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<td>19.1</td>
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<tr>
<td>4</td>
<td>21.2</td>
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<td>6</td>
<td>22.5</td>
<td>22.2</td>
</tr>
<tr>
<td>8</td>
<td>24</td>
<td>29.7</td>
</tr>
</tbody>
</table>

* p<0.05, ** p<0.002, *** p<0.0001, when compared with SPS + irradiation group.
Tumor aspiration time (h)

![Image](image-url)

**Table 3. Altered Treatment Regimen of APE and/or Radiation**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Tumor stage</th>
<th>Survival (d)</th>
<th>Percent survivors (d)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>MST</td>
<td>IMLS</td>
</tr>
<tr>
<td>SPS+irradiation</td>
<td>I</td>
<td>24.5</td>
<td>—</td>
</tr>
<tr>
<td>APE+irradiation</td>
<td>I</td>
<td>48.5</td>
<td>97.96</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>43.8</td>
<td>78.77</td>
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<tr>
<td></td>
<td>III</td>
<td>36.9</td>
<td>50.6</td>
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<td></td>
<td>IV</td>
<td>31.5</td>
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<tr>
<td></td>
<td>V</td>
<td>28</td>
<td>14.28</td>
</tr>
<tr>
<td></td>
<td>VI</td>
<td>26.5</td>
<td>8.16</td>
</tr>
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</table>

a) APE treatment was continued for 8 d beginning from irradiation day. *p<0.05, **p<0.002, ***p<0.0001 when compared with SPS+irradiation group.

GSH: The irradiation of EAC mice to 6 Gy caused a time dependent but significant decline in GSH concentration up to 3 h, where a maximum reduction (1.4 fold; *p<0.001) was observed (Fig. 2b). The GSH concentration showed steady elevation thereafter up to 24 h PLDA in SPS+irradiation group, where it was significantly higher when compared with 0 h (*p<0.001). APE treatment further reduced the GSH concentration with a maximum decline at 6 h PLDA when compared with the SPS+irradiation group. This decline was significant from 6 to 24 h PLDA (*p<0.001 to *p<0.001; Fig. 2b). The reduction in GSH concentration in APE+irradiation group was 1.2 fold higher when compared with SPS+irradiation group at 6 h PLDA.

GSHPx: Exposure of EAC mice to 6 Gy resulted in a time dependent decline in GSHPx activity and a lowest activity was recorded at 6 h (1.7 folds; *p<0.05) that continued up to 9 h PLDA (1.6 fold; *p<0.05). Thereafter the GSHPx levels rose steadily up to 24 h in SPS+irradiation group. The combination of APE and γ-radiation resulted in a further decline in the glutathione-peroxidase activity in EAC cells and a maximum reduction was observed at 6 h (2 fold; *p<0.01) that remained almost unaltered up to 12 h PLDA (2 fold; *p<0.05; Fig. 3a). The reduction in GSHPx activity in APE+irradiation group was 1.5 fold (*p<0.05) higher than that of concurrent untreated drug control group at 24 h PLDA (Fig. 3a).

GST: irradiation of EAC mice caused a time dependent decline in GST activity and a greatest reduction was observed at 3 h PLDA in SPS+irradiation group (*p<0.05). This decline was approximately 2 (*p<0.01) and 1.6 (*p<0.05) folds at 3 and 24 h PLDA, respectively when compared with the sham-irradiation group. APE pretreatment further reduced the GST activity and a maximum decline was observed at 6 h PLDA in APE+irradiation group (*p<0.01). This reduction in GST activity in APE+irradiation group was 1.3 and 1.4 folds higher when compared with SPS+irradiation group, while it was 2.5 and 2.2 folds higher when compared with the sham-irradiation group at 6 and 24 h PLDA, respectively. The GST activity did not reach to sham-irradiation level even up to 24 h PLDA (*p<0.001; Fig. 2a). Treatment of mice with APE before irradiation to 6 Gy caused a further elevation in LPx which was significantly greater than SPS+irradiation group. The combination treatment of APE and γ-radiation caused a drastic rise in the LPx levels after 2 h and a greatest lipid peroxidation was recorded at 6 h PLDA. Thereafter the LPx levels declined steadily up to 24 h PLDA (*p<0.0001 at 24 h; Fig. 2a), which was 1.3 and 1.6 folds higher for SPS+irradiation and APE+irradiation groups, respectively when compared with the SPS+sham-irradiation group.

SOD: irradiation of EAC mice to 6 Gy caused a steady decline in SOD activity up to 6 h PLDA where a maximum decline was observed (1.7 fold; *p<0.05; Fig. 4a). Treatment of EAC mice with APE before irradiation resulted in a further reduction in the SOD activity and the least activity was observed at 6 h PLDA (2.5 fold; *p<0.05). This decline in...
SOD activity was significant at 24 h PLDA for APE/H11001 irradiation treatment (p < 0.05) whereas it was non-significant for SPS/H11001 irradiation group. The reduction in SOD concentration in APE/H11001 irradiation group was 1.5 and 1.4 folds less when compared with SPS/H11001 irradiation group at 6 and 24 h PLDA, respectively. The SOD activity rose marginally after 6 h and remained unaltered in both the groups up to 24 h PLDA (p < 0.05 up to 12 h for SPS+irradiation group; p < 0.05 up to 24 h PLDA for APE+irradiation group; Fig. 4a).

CAT: Exposure of EAC mice with 6 Gy drastically reduced the CAT activity at all assay time periods. The maximum decline in CAT activity was observed at 6 h PLDA (p < 0.01) which remained almost unchanged up to 24 h PLDA (p < 0.01; Fig. 4b). The pattern of decline in CAT was similar to that of SPS+irradiation group after APE pretreatment except that the reduction in CAT activity was significantly higher than that of SPS+irradiation group at 6 h (2.8 folds; p < 0.01; Fig. 4b). APE treatment caused a significant decline in CAT activity up to 24 h (p < 0.01; Fig. 4b). The reduction in CAT activity in APE+irradiation group was 1.4 fold higher when compared with SPS+irradiation group at 6 and 24 h PLDA.

DISCUSSION

A benefit of ionizing radiation as a therapeutic tool is the possibility to apply it locoregionally thereby preventing systemic toxicity. However like chemotherapeutic agents ionizing radiation does not affect all target cells, which can lead to severe side effects in the surrounding tissue after the therapy. In addition there are large number of human malignant tumor cells that respond poorly to ionizing radiation. However, radiation dose to the tumor can not be increased as needed because of the normal tissue toxicity in the radiation field. Hence there is a need for chemical agents which upon contact with tumor cells increase their sensitivity to radiation thus minimizing large doses of radiation and also spare normal tissue from the combined toxic effects.

Attempts to develop clinically relevant radiosensitizers have traditionally used an empirical approach combining radiation with standard cytotoxic chemotherapeutic agents. Randomized trials have shown that combination treatment improves survival compared with radiation alone in the treatment of locally advanced cancers of the head and neck, lung, esophagus, stomach, pancreas and rectum. Various synthetic radiosensitizers including, metal complexes of 4 (5)-nitroimidazoles and indolocarbazoles (under investigation), which have been reported to enhance the radiation response in dif-
Tetrandrine (obtained from the roots of *Stephania tetrandra*), withaferin-A (from *Withania somnifera*), nicotinamide (Vitamin B3), echitamine chloride (obtained from the stem bark of *Alstonia scholaris*), berberine (obtained from *Tinospora cordifolia*) have also been shown to possess radiosensitizing activities *in vivo* and *in vitro*. A vast number of drugs including topotecan, oleandrin, resveratrol have been reported to augment radiation-induced tumor cell killing effect in various types of tumor cells.

Ehrlich ascites carcinoma (EAC) is an undifferentiated malignancy and is sensitive to radiation when compared with other tumor models. APE has been found to enhance the cell-killing effects of radiation at 1/20th dose that showed optimum anticancer effect in EAC mice. Our earlier studies have demonstrated that APE has been able to inhibit the proliferation of EAC cells significantly in mice at a dose of 1 g/kg body wt (data not shown). The radiosensitizing effect of APE in EAC cells is evidenced by an increased number of tumor free survivors (30%) beyond 120 d and a significant increase in the MST, AST, IMLS and IALS. The greater number of long term survivors in APE treated irradiated group when compared to non-APE treated irradiated group indicates that APE has got a synergistic effect in conjunction with radiation. From the clinical point, we have also carried out the study in mice at different stages of tumor development, where the APE treatment in combination with radiation caused an increase in the life span of 30 and 20% of tumor-free animals beyond 120 d at stage I and II, respectively. As far as authors are aware a similar type of regimen has not been used in an experimental set up for radiosensitization. However, treatment of EAC mice with alcoholic extract of *Alstonia scholaris* during late stages of development has been reported to be effective.

This is the first experimental finding where administration of alcoholic extract of the stem bark of *Aphanamixis poly-stachya* in tumor bearing mice has been found to enhance the radiation effect. Similarly, phytochemicals like withaferin and plumbagin, isolated from *Withania somnifera* and *Plumbago rosea* have been reported to enhance the antitumor effect of radiation in EAC bearing mice. The optimum dose of 50 mg/kg body wt APE in conjunction with 6 Gy irradiation has been found to be most effective while the other higher doses of APE did not enhance the radiation effect further. This effect may be attributed to the elevation of radiation-induced free radicals by APE. These free radicals in turn may have damaged the important biomolecules like DNA, proteins and lipids causing cell death. The combination of radiation and APE may have further enhanced the free radicals production up to a dose of 50 mg/kg where almost saturation levels are reached. A further escalation in APE dose could not enhance the production of free radicals as a result no further increase in the radiosensitizing activity of APE could be observed beyond 50 mg/kg. This contention is supported by the highest number of tumor-free survivor at 50 mg/kg APE + 6 Gy irradiation and also decline in GSH concentration in the APE treated irradiated group. A further increase in APE dose may have also enhanced the effect of radiation in the normal tissues of irradiated EAC mice leading to the reduction in the survival of EAC mice receiving higher doses of APE. A similar mechanism seems to be operational when the dose of irradiation is increased to 8 Gy in conjunction with 50 mg/kg APE treatment.

The exact mechanism of action of APE in regressing the tumor in conjunction with radiation is not well understood. However, the increased radiosensitization may be due to the operation of multiple mechanisms. APE treatment may have increased the production of radiation-induced free radicals causing increased tumor cell kill and increased survival. The reduction in the GSH concentration of tumor cells and increased lipid peroxidation (LPx) in the present study support this contention. The depletion of glutathione has been reported to enhance cell death and apoptosis of the tumor cells along with the loss of essential sulfhydryl groups that result in an alteration of calcium homeostasis and eventually loss of cell viability. The early and severe tumor GSH depletion may have increased the sensitivity of tumor to radiation and because of this reason the initial complete cell killing might have occurred at the early stages of tumor development. The agents that deplete GSH, such as N-ethylmaleimide, bromobenzene, allyl alcohol, buthionine sulfoxamine and diethyl maleate, increase the radiation effect. The lipid peroxidation is another important event related to cell death and has been reported to cause severe impairment of membrane function through increased membrane permeability and membrane protein oxidation, DNA damage, cytotoxicity and eventually cell death. The increased LPx may have also contributed to the higher tumor regression in the APE + irradiation group. The increase LPx and reduction in GSH by APE, may have damaged the DNA of EAC cells thereby killing tumor cells effectively. APE has also depleted the inventory levels of CAT, SOD, GSHPx and GST in EAC cells, and disturbed the redox status of tumor cells making them more vulnerable to death. It may also have caused a cell cycle arrest at G2 + M phase and thereby making EAC cells more vulnerable to radiation-induced death. From the present study it is clear that the life span of EAC mice is prolonged in APE + irradiation group when compared to other groups confirming the synergistic effect of APE with radiation. The involvement of several other unknown mechanisms, such as apoptosis and nuclear enzymes, cannot be ruled out.

The beneficial effect of APE in combination with irradiation in regressing the tumor cell growth may partially be attributed to the presence of alkaloids like rohitukin, amorarastatin and 12α-hydroxyamorastatin in APE, which have been reported to kill neoplastic cells. A semi-synthetic derivative of rohitukin, the flavopiridol has been found to be active against various cancers by inhibiting the cell proliferation via targeting the serine/threonine pathway.

The present study demonstrates that 50 mg/kg APE has a significant radiosensitizing activity in EAC mice and this may be due to the increased lipid peroxidation, reduced glutathione contents and depletion of important antioxidant enzymes like, CAT, SOD, GSHPx, GST in tumor cells. Fur-
ther experiments are underway to identify the molecule responsible for radiosensitizing activity ofAPE with special emphasis on potentiation of radiation-induced DNA double strand break induction or apoptosis in EAC cells.

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