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Effect of Piperine in Combination with Gamma Radiation on A549 Cells

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Abstract

Background Lung cancer is a major constrain that increases mortality globally. Radiotherapy is one of the treatment modalities against lung cancer. A high dose of targeted radiation is required to achieve the treatment efficacy of cell killing. After radiotherapy, eventual tumor progression and therapy resistance are still a consequence of patient who undertakes nonsurgical radiation therapy. Piperine, a plant alkaloid, has been known to enhance the action of the anticancer drugs in various drug-resistant cancer cells. The aim of the current in vitro study was to study the effect of piperine on radiosensitizing property against A549 cells.

Methods In vitro radiosensitizing activity of piperine was elucidated on A549 cells using MTT (3-(4, 5-dimethylthiazol-2-yl)-25-diphenyltetrazolium bromide) assay. CompuSyn analysis was used to compute the combination index values to analyze the combinatory effect of piperine and radiation

Results and Conclusion We observed that piperine increased tumor cell killing in combination with the γ-radiation in vitro. However, further studies are warranted to understand the molecular mechanism of the radiosensitizing action of piperine.

Keywords
- lung cancer
- piperine
- radiosensitization
- CompuSyn analysis

Introduction

The poor prognosis of patients with lung cancer is the major cause of cancer-related deaths.[1] According to GLOBOCAN 2018, lung cancer has the highest incidence rate and mortality rate.[2] Though the radiotherapy is a main treatment modality against lung cancer, gradual development of therapy resistance and cancer recurrence is a major constrain.[3] Intensive attempts to improve the outcome of radiotherapy treatment have been a remarkable challenge. One of the few achievable therapeutic strategies of lung cancer to increase the treatment efficacy demonstrated that the combination of radiosensitizer with radiation produces a significant decrease in mortality compared with irradiation group.[4] Because these studies use a relatively low dose of radiation and demonstrated a reduced effect on the occurrence of caner metastases and secondary cancer, the improvement realized by the use of radiosensitizer in therapy probably due to radiosensitization.[4-6] Thus, the discovery of a potent radiosensitizer against lung cancer could improve the outcome of treatment of this disease.

A most important concern about radiotherapy is that it acquires resistance by activating several alternating signaling pathways that elicit cancer and/or enhanced DNA repair pathways. Radiotherapy resistance, defined as a poor prognosis in the effectiveness of radio therapy, is a major hindrance in cancer treatment. In such cases, combinatorial approach is an effective way to augment treatment efficacy. Combinatory approach often follows three main strategies:

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(1) inhibition of possible alternative pathways, (2) targeting of the single pathway to accomplish downregulation or inhibition, or (3) targeting and downregulation or inhibition of two different pathways will lead to synergistic action on radiosensitization.[7]

Phytocompounds or natural based combinatorial approach may serve in the development of anticancer agents with minimal side effects and better efficacy.[8-11] In addition, natural compounds because of their antioxidant and anti-inflammatory effects have better effects as radiation protectors for healthy cells.[12] Some natural radiosensitizers are danshensu, curcumin, wortmannin, genistein, and quercetin.[13-18] *Piper nigrum* Linn commonly known as black pepper belongs to the spices widely consumed by a great number of people worldwide. Piperine is a bioactive compound and key alkaloid, present in *Piper nigrum* Linn and *Piper longum* Linn (long pepper). It has been found that piperine enhances the action of the anticancer drugs in various drug-resistant cancer cells.[19-23] Studies regarding the radiosensitizing effect of piperine on lung cancer are yet to be done. Combination index (CI) is a theorem of Chou-Talalay that defines quantitative explanation for additive effect ("CI" = 1), synergism ("CI" < 1), and antagonism ("CI" > 1) in drug combinations studies. This theory also explains algorithms for computer-based model for synergistic and/or antagonistic mechanism at any dose level and effect through isobologram and CI plot respectively.[24] As there is a need to develop a potent therapy to treat lung cancer, our study aimed to explore the effect of piperine pretreatment and to improve radiotherapy treatment on lung cancer cells and interpret its mechanism of action.

Materials and Methods

The phytocompound piperine (>97%) and dimethyl sulfoxide (DMSO) were procured from Sigma-Aldrich, India. Cell culture reagents such as Dulbecco's Modified Eagles Medium (DMEM), fetal bovine serum (FBS), penicillin and streptomycin solution, L-glutamine, Trypan blue dye, 3-(4, 5-dimethylthiazol-2-yl)-25-diphenyltetrazolium bromide (MTT), and other chemicals (analytical or molecular biology grade) were procured from HiMedia, India.

Cell Culture

Human lung adenocarcinoma cell line-A549 (NCCS, Pune, India) was cultured in culture media (DMEM), supplemented with FBS (10%), penicillin, and streptomycin (1%) and 2 mM L-glutamine. The cells were maintained at 37°C in a 5% CO_2 humidified atmospheric conditions.

Dose Optimization of Piperine on A549 Cells

Dose optimization of piperine was done using MTT assay.[25,26] Briefly, A549 cells ($3x10^3$cells/well) were seeded into 96-well plate and incubated 24 hours at normal culture conditions. Piperine dissolved in DMSO was taken at concentrations of 10 to 100 µg/mL and was added into the culture plates and incubated for 48 hours and then MTT assay was performed. The optical density readings at 570 nm were taken using the multimode plate reader (FLUOstar Omega, Mumbai, India). The experiment was performed in triplicates. The final DMSO concentration in the treatment was kept within 0.1%.

Dose Optimization of Ionizing Gamma Radiation on A549 Cells

A549 cells were exposed to ionizing gamma (γ)-radiation (IR) at a dose ranging from 1.25 to 10 Gy, using a low-dose gamma irradiator-2000 (BRIT, Mumbai, India), with ^{60} Co source as irradiator and 10.3 Gy/min deliverable dose rate. MTT cell proliferation assay was performed after 48 hours of incubation.[25,26]

Cytotoxicity Assessment Using MTT Cell Proliferation Assay

The two doses of piperine (low dose: 12.5 and high dose: 25 µg/ mL) were added to A549 cells in individual flasks, 2 hours before to γ-radiation treatment (1.25 Gy) and incubated at normal culture condition for 48 hours as described previously.[26] Cells (vehicle control) kept in the chamber but not irradiated were considered as sham control. After 48 hours of incubation, the cytotoxic effect of piperine and γ-radiation on A549 cells was evaluated by MTT cell proliferation assay as mentioned earlier.[25]

$$Percentage of\ cell\ viability = \frac{(No.\ of\ viable\ cells\ in\ control - No.\ viable\ cell\ in\ test)}{No.\ of\ viable\ cells\ in\ control} \times 100$$

CompuSyn Analysis to Check the Synergistic Effect of the Combination Treatment

CompuSyn software (ComboSyn, Inc., Paramus, NJ, United States) was used to quantitatively depict the mechanistic effect of the combination treatment. The data from the cytotoxicity studies were taken to compute CI values, CI plot, dose response curve, and normalized isobologram. CI values were used to analyze the synergism ("CI" < 1), additive effect ("CI" = 1) and/or antagonism ("CI" < 1) of the co-treatment.[24,27]

The CI is calculated by using the formula:

$$CI = \frac{d1}{DX1} + \frac{d2}{DX2} CI$$

Where Dx1 indicates, the dose of test agent 1 (γ-radiation) needed to decrease "x" percentage of proliferation alone, and d1 indicates the dose of test agent 1 needed to decrease "x" percentage of proliferation along with d2 treatment. Similarly, Dx2 indicates the dose of test agent 2 (piperine) needed to decrease "x" percentage of proliferation alone, and d2 indicates the dose of test agent 2 needed to decrease "x" percentage of proliferation along with d1.

Morphological Analysis

A549 cells at a cell density of 0.35×10^6 cells/mL were seeded into 35 mm dishes. After 24 hours of incubation, the cells were pretreated with piperine for 2 hours and then irradiated with γ-radiation. The cells that are untreated with piperine and

nonirradiated were taken as control. The changes in the cell morphology were observed using inverted light microscope (Zeiss Primo Vert, Mumbai, India) at a magnification of 40×.

Statistical Analysis

Data were represented as mean ± standard deviation. Data analysis was done using one-way analysis of variance using GraphPad PRISM version 7.0. A *p*-value < 0.0001 was scored significant.

Results

Effect of Piperine and Gamma Radiation on Cell Viability or Cell Proliferation

MTT cell proliferation assay is used to check the effect of piperine and γ-radiation on A549 cell proliferation or cell viability indirectly that measures mitochondrial succinate dehydrogenase activity spectrophotometrically. Inhibition of A549 cell proliferation against piperine was in a dose-dependent manner (►**Fig. 1A**) and A549 cells show high resistance toward γ-radiation of selected dose ranging from 1.25 to 10 Gy. Cell killing was less than 40% (33–37%) even at 10 Gy (►**Fig. 1B**).

Piperine Synergistically Enhances Radiation-Induced Cell Death on A549 Cells

To analyze piperine could sensitize A549 cells to γ-radiation treatment (IR), two doses of piperine (low dose: 12.5 and high dose: 25 µg/mL) were added to A549 cells in individual flasks 2 hours before to γ-radiation treatment (1.25 Gy) and incubated at normal culture conditions for 48 hours. We found that piperine treatment combined with γ-radiation exhibited enhanced inhibition of cell proliferation (~55%) compared with individual γ-radiation/piperine treatment alone (►**Fig. 1C**). Significant difference among various treatment groups in the observed cytotoxicity is given in ►**Table 1**. To study whether the radiosensitization finding of the combination treatment action is synergistic

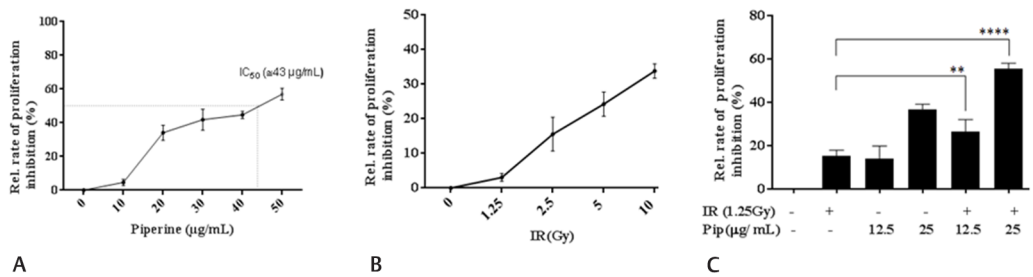

Fig. 1 Radiosensitization effect of piperine on A549 cells. (**A**) Optimization of piperine dose. Piperine was taken at different concentrations 10 to 100 µg/mL and 3-(4, 5-dimethylthiazol-2-yl)-25-diphenyltetrazolium bromide (MTT) assay was performed on A549 cells. DMSO treated cells were used as vehicle control. (**B**) Optimization of γ-radiation dose. (**C**) Relative cell proliferation inhibition analysis after combination treatment with piperine and γ-radiation by MTT assay on A549 cells. Data was represented as mean ± standard deviation. IR, ionizing gamma radiation; Pip, piperine.

Table 1 Multiple comparison analysis of MTT cell proliferation assay of combination experiment by post-ANOVA Bonferroni's multiple comparisons test

Comparisons among groups	Significance	Adjusted *p*-Value
Sham versus IR (γ)	***	0.0002
Sham versus Pip(12.5 µg/mL)	***	0.0007
Sham versus Pip(25 µg/mL)	****	<0.0001
Sham versus IR(γ) +Pip (12.5 µg/mL)	****	<0.0001
Sham versus IR(γ)+Pip (25 µg/mL)	****	<0.0001
IR (γ) versus Pip(12.5 µg/mL)	ns	>0.9999
IR (γ) versus Pip(25 µg/mL)	****	<0.0001
IR (γ) versus IR(γ) +Pip (12.5 µg/mL)	**	0.0066
IR (γ) versus IR(γ)+Pip (25 µg/mL)	****	<0.0001
Pip(12.5 µg/mL) versus Pip (25 µg/mL)	****	<0.0001
Pip(12.5 µg/mL) versus IR(γ) +Pip (12.5 µg/mL)	**	0.0019
Pip(12.5 µg/mL) versus IR(γ)+Pip (25 µg/mL)	****	<0.0001
Pip(25 µg/mL) versus IR(γ) +Pip (12.5 µg/mL)	****	<0.0001
Pip(25 µg/mL) versus IR(γ)+Pip (25 µg/mL)	NS	0.0801
IR(γ) +Pip (12.5 µg/mL) versus IR(γ)+Pip (25 µg/mL)	****	<0.0001

Abbreviations: ANOVA, analysis of variance; CI, confidence interval; IR(γ), ionizing gamma radiation; MTT, 3-(4, 5-dimethylthiazol-2-yl)-25-diphenylte trazolium bromide; Pip, piperine.

or additive, we computed the CI values and plotted CI plot isobologram for the two selected concentrations of piperine (low dose: 12.5 and high dose: 25 µg/mL) against exposed 1.25 Gy of γ-radiation using freely available CompuSyn software. This study was designed to analyze the nature of the effects of combination treatment.

A dose response curve for piperine and γ-radiation was generated as given in ►**Fig. 2A**, **D**. Synergism effect is higher than an additive and antagonism effect. As represented in the CI plots (►**Fig. 2B**, **E**) and isobologram (►**Fig. 2C**, **F**), the calculated CI values were less than 1 for the combination treatments (IR + Pip 12.5 µg/mL and IR + Pip 25 µg/mL). This clearly demonstrates a synergistic effect. Surprisingly, all the values (values computed from triplicate experiments) in the isobologram (►**Fig. 2C**, **F**) generated for the two selected concentrations of piperine (low dose: 12.5 and high dose: 25 µg/mL) with γ-radiation 1.25 Gy showed well within the stipulated region of synergism. CI points of each combination study are shown in ►**Tables 2** and **3**, respectively. Our findings from these results show that pretreatment with piperine efficiently sensitized A549 cells toward cell killing effects of γ-radiation, compared with a single regime treatment.

Cellular Morphological Analysis

Morphological assessment showed that piperine pretreatment in combination with γ-radiation increased the cell death considerably when compared with A549 cells treated with piperine/γ-radiation alone (►**Fig. 3**).

As given in ►**Fig. 3**, the control **(A)** exhibited colony features of A549 lung cancer morphology, while cells pretreated with piperine followed by γ-radiation (►**Fig. 3E**, **F**) showed

Table 2 CI data for combination of IR+Pip 12.5 µg/mL

IR(γ) (Gy)	Piperine (µg/mL)	Effect	CI
1.25	12.5	0.79	0.97832
1.25	12.5	0.75	0.84384
1.25	12.5	0.71	0.73908
1.25	12.5	0.67	0.65425

Abbreviations: CI, combination index; IR(γ), ionizing gamma radiation; Pip, piperine.

Table 3 CI data for combination of IR+Pip25 µg/mL

IR(γ) (Gy)	Piperine (µg/mL)	Effect	CI
1.25	25.0	0.44	0.63135
1.25	25.0	0.45	0.64766
1.25	25.0	0.41	0.58435
1.25	25.0	0.47	0.68139

Abbreviations: CI, combination index; IR(γ), ionizing gamma radiation; Pip, piperine.

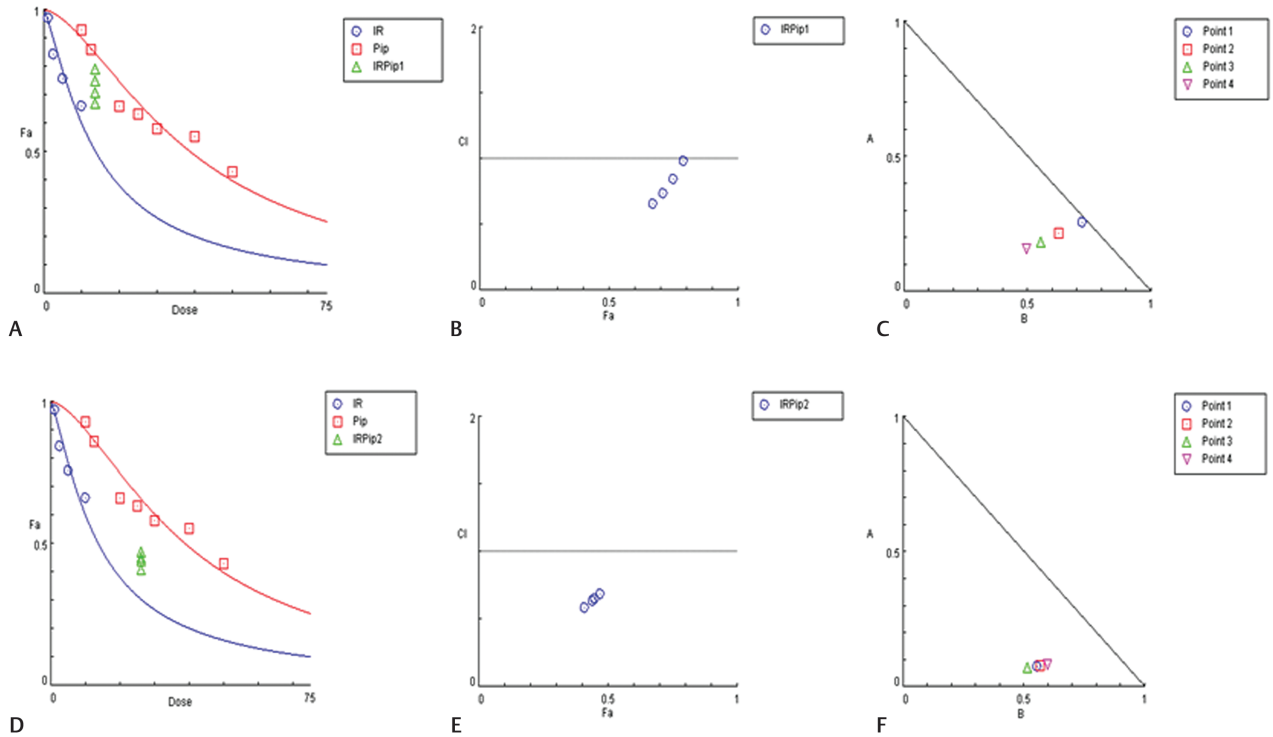

Fig. 2 CompuSyn analysis to determine the synergistic effect of the piperine and γ-radiation on A549 cells. (**A**) Dose response curve of piperine (Pip) and IR (ionizing γ-radiation). IRPip1 indicates combination of IR (ionizing γ-radiation) and piperine 12.5 µg/mL. (**B**) Combination index (CI) plot and CI table depict that CI value for the chosen combination treatment (IR+ Pip 12.5 µg/mL) is <1. (**C**) Normalized isobologram of the combination treatment (IR+ Pip 12.5 µg/mL). (**D**) Dose response curve of piperine (Pip) and ionizing γ-radiation (IR), IRPip2 indicates combination of IR (ionizing γ-radiation) and piperine 25 µg/mL. (**E**) CI plot and CI table show that CI value for the chosen combination treatment (ionizing γ-radiation (IR)+ Pip 12.5 µg/mL) is <1. (**F**) Normalized isobologram of the combination treatment (ionizing γ-radiation (IR)+ Pip 25 µg/mL).

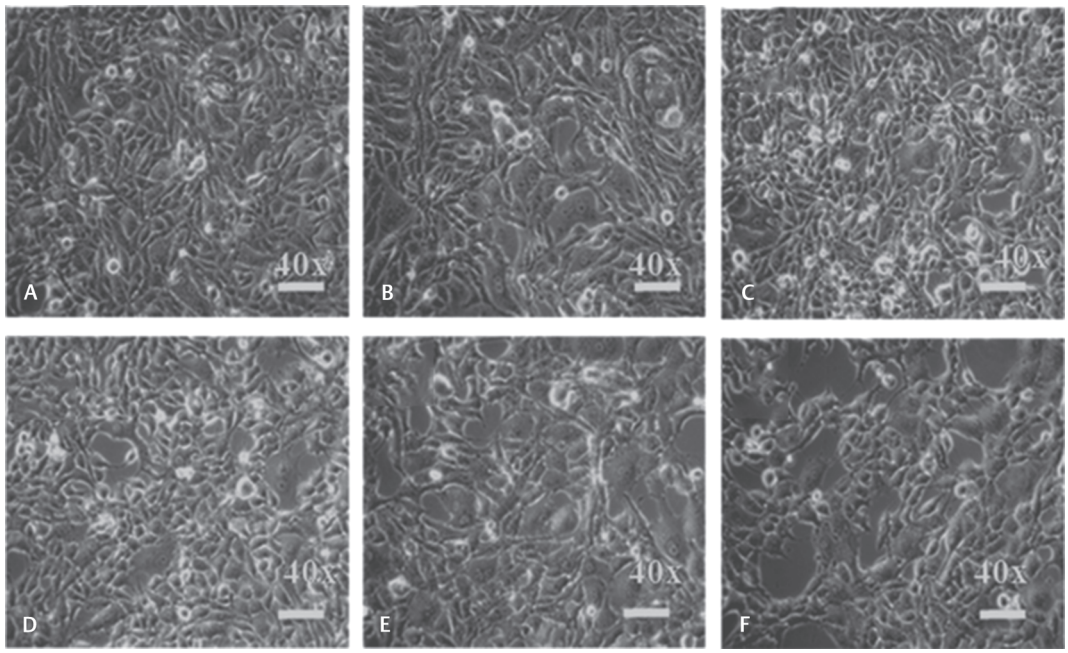

Fig. 3 Morphological analysis of A549 cells after combination treatment with piperine (Pip) and ionizing γ-radiation (IR), where (**A**) Sham control, (**B**) IR, (**C**) Pip 12.5 μg/mL, (**D**) Pip 25 μg/mL, (**E**) IR+ Pip 12.5 μg/mL, and (**F**) IR+ Pip 25 μg/mL.

characteristic features of apoptosis, including disappearance colony formation and appearance of cell shrinkage. The low-dose radiation along with piperine treatment strategy results suggests that piperine pretreatment may improve the treatment strategy by decreasing the dose of radiation treatment that is necessary to suppress the augmentation of lung cancer cells.

Discussion

Accumulating literature data on in vitro and in vivo activities of piperine show that piperine has immunomodulatory and antiallergic, anti-inflammatory, enhanced drug bioavailability potential, antimutagenic on healthy cells.[28,29] Cytotoxic effect is selective toward cancer cells.[30-32] In the present study, we observe piperine as a new compound for reducing the lung cancer proliferation and reveal a novel radiosensitization method of lung cancer via increased inhibition of cell proliferation after piperine pretreatment prior to the radiation treatment. Furthermore, we identify the role of piperine in tumor inhibition with radiation treatment being synergistic in nature. Our results provide new strategic insight into the radiosensitization of lung cancer and suggest that piperine may be an ideal tumor suppressor compound and can be used in radiosensitization in lung cancer treatment.

Programmed cell death or apoptosis is a target of antitumor therapy. Chemo/radiotherapy and phytocompounds like piperine induce the generation reactive oxygen species (ROS) leading to DNA damage and cell cycle arrest. DNA damage may lead to mitochondria-mediated intrinsic pathway of apoptosis.[26,33] Accumulating evidence has suggested that mechanisms significance to radiosensitivity include programmed cell death or apoptosis through inhibition of cell proliferation with characteristic morphological changes, alteration of cell cycle, inducing DNA damage and inhibition of repair pathways, and alteration of tumor immune microenvironment.[26,34,35] Studies have shown the targeting DNA damage response, double-strand break repair, and other molecular responses induced cell inactivation by radiation could hold a great approach for radiosensitization.[36] The experimental data indicate that the combination treatments augmented the cell death compared with individual regime. To study whether the combination effect is synergistic or addictive, CI analysis was performed. It clearly demonstrates that the combinatorial effect is synergistic in nature for the selected dose of individual regime. The CI theorem is based on the physical, chemical, and mathematical principles of the mass-action law[37,38] and the CI equation.[37,39] Although the mechanisms of each drug are valuable to know, it is not essential to know the mechanism of each drug for studying the synergism or antagonism.[37] The mechanism of synergistic action after the treatment with piperine and radiation may be due to enhanced DNA damage and cell cycle arrest through induction of ROS that may alter mitochondrial membrane potential leading to apoptosis as observed in our earlier studies with colon cancer cells[26] and other cancer cell line studies.[40] These findings have unique significance, as piperine may have the potential to be developed as a radiosensitizing against lung cancer cells.

Conclusion

The in vitro radiosensitization potential of piperine was elucidated on A549 lung cancer cells in combination with γ-radiation. Compusyn analysis shows that the combination treatment is synergistic in nature. This investigation on piperine revealed a basic knowledge on combinatory effect of piperine and γ-radiation on A549 cells. More studies are

warranted to understand the molecular mechanism of the radiosensitizing action of piperine.

Conflict of Interest

D.L.M. is supported with research grant/065–2018, Yenepoya (deemed to be University). S.K. is supported by ICMR-SRF, Govt. of India.

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