

# Dichloromethane Extract of Giloe (*Tinospora Cordifolia*, Wild) Increases the Radiosensitivity of Cultured Hela Cells Exposed to Different Doses of $\Gamma$ -Radiation

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Research Article

Volume 1 Issue 2

Received Date: November 15, 2017

Published Date: December 07, 2017

## Abstract

Ionizing radiations are in frequent clinical use to treat cancer either alone or in combination with surgery or chemotherapy. The radioresistance of tumors is a stumbling block to realize the full potential of radiotherapy. Therefore, pharmacophores that reduce the radioresistance of tumors may be of great importance during tumor therapy. In the present study an attempt has been made to evaluate the potential of dichloromethane extract of giloe *i.e.* *Tinospora cordifolia* (TCE) in cultured HeLa cells. Exposure of HeLa cells to TCE for 4 h before exposure to 2 Gy  $\gamma$ -radiation led to a significant decrease in the cell viability (approximately 50 %) accompanied by a reduction in the surviving fraction (SF) up to 0.52 after 4 h of TCE treatment. Thereafter, clonogenicity of HeLa cells declined negligibly with increase in treatment duration up to 6 h post-treatment. There has been a dose dependent attrition in the cell viability of HeLa cells exposed to 1-4 Gy  $\gamma$ -irradiation, whereas treatment of HeLa cells with various doses of TCE further decreased the cell viability depending not only on the irradiation dose but also on the concentration of TCE. The irradiation of HeLa cells resulted in a radiation dose dependent decline in the SF and increasing TCE concentration before irradiation caused a further TCE concentration dependent reduction in SF, and a lowest SF was observed for 4  $\mu$ g/ml TCE for all irradiation doses. Treatment of HeLa cells with different concentrations of TCE before 3 Gy irradiation resulted in a concentration dependent depletion in glutathione-S-transferase activity until 12 h post-irradiation, whereas lactate dehydrogenase and lipid peroxidation increased up to 4 h post-irradiation and declined gradually thereafter up to 12 h post-irradiation. The TCE treatment increased the radiosensitivity of HeLa cells by reducing glutathione-S-transferase activity and increasing the activity of lactate dehydrogenase and lipid peroxidation.

**Keywords:** Giloe; Radiation; Hela Cells; Glutathione-S-Transferase; Lactate Dehydrogenase; Lipid Peroxidation

**Abbreviations:** TCE: *Tinospora Cordifolia*; SF: Surviving Fraction; MEM: Minimum Essential Medium; GST: Glutathione-S-Transferase; CDNB: 1-Chloro,2,4-Dinitrobenzene; LOO: Lipid Peroxidation; MDA: Malondialdehyde; LDH: Lactate Dehydrogenase

## Introduction

With the discovery of X-rays, ionizing radiations found their immediate use in cancer therapy and diagnosis. Radiotherapy has emerged as one of the important modalities of cancer treatment and approximately 50% of all cancer patients receive radiotherapy either alone or in combination with chemotherapy or surgery [1,2]. Radiotherapy causes secondary cancers after the remission of primary cancer, which typically occurs several years later [3,4]. Apart from the induction of secondary cancers, radiotherapy is associated with several disorders including, neurological, cardiac, liver, pulmonary, and skin diseases [5-9]. Radiation therapy is associated with both acute and delayed disturbances in nutritional status and impairment of tissues [10]. Despite the fact that radiotherapy is an important cancer treatment modality approximately half of the cancer patients receive radiotherapy either as curative treatment or as a palliative care [11]. During radiotherapy the normal tissues also get irradiated and it is also well known that irradiation does not distinguish between neoplastic and normal healthy cells surrounding tumor tissue as a result, the latter gets a significant amount of damage during cancer cure leading to various abnormalities and also limiting the radiation dose to the tumor tissue [12].

The irradiation dose to normal tissues can be reduced by combining agents that can increase the radiosensitivity of tumors thereby sparing normal tissues from the adverse effect of radiation. This paradigm has been tried in cancer therapy where chemotherapeutic agents have been combined to reduce the radiation dose in the hope of therapeutic gains. This combination proved beneficial in solid neoplastic disorders in randomized clinical trials. The most frequently used chemotherapeutic agents in conjunction with radiotherapy include cis-dichlorodiammine-platinum (II), 5-fluorouracil, docetaxel, mitomycin C, paclitaxel, gemcitabine, topotecan, irinotecan, crytophycins, camptothecin and combretastatin A-4 [13-21]. The combination of these agents with radiation led to improvement in the therapy of several solid tumors. However, the toxicity has been severe and there has been development of second malignancies in the survivors [22,23]. Therefore, search for new treatment modalities shall continue to reduce the adverse toxic effects of combined treatment modalities and also alleviate the risk of second malignancies in the

survivors. The plants have been used for healthcare since the advent of human history and many of the modern chemotherapeutic molecules have been isolated from medicinal plants before their chemical synthesis began [24]. The plants also form a vast reservoir of innumerable medicines/new molecules, which still need to be explored.

*Tinospora cordifolia* (Willd.) Miers ex Hook. F. & Thoms. (Family: Menispermaceae), is commonly known as giloe (Giloy), guduchi, or amrita [25]. Giloe is a non-toxic herbal Ayurvedic medicine. Ayurveda describes its pleotropic properties in various texts and categorized it as a rasayana drugs. It has been credited to arrest aging, used as a general tonic and boost immune functions [26,27]. Giloe possesses antiinflammatory, antiarthritic, antiallergic, antimalarial, antidiabetic, antibacterial, antiviral, antileprotic, anti-periodic, anti-spasmodic, antistress, hypoglycemic, hepatoprotective and aphrodisiac properties [26-29]. It has also been reported to treat throat cancer in man [30]. Methanol and dichloromethane extracts of giloe have been reported to be a non-toxic in preclinical studies up to a dose of 3.5 and 1.2 g/kg in mice and rats [28,31]. Our earlier studies have shown that giloe extract effectively killed cancer cells in preclinical models in vivo and in vitro [25,32]. The giloe has also been reported to increase the effect of ionizing radiations in HeLa cells and Ehrlich ascites carcinoma transplanted mice [28,33]. It has also been reported to induce DNA damage *per se* and also increase the amount of radiation induced DNA damage at the molecular and genomic level [26,34,35]. The alcoholic extract of giloe has been reported to attenuate the cyclophosphamide-induced urotoxicity in vivo [36]. The crude extract of another species of giloe, *Tinospora crispa* has been also found to exert cytotoxic effect on HeLa, MCF-7, MDAMB-231 and 3T3 cells in vitro [37]. However, detailed and systematic studies on the radiomodulatory effect of dichloromethane extract of guduchi need to be undertaken. Therefore, the present investigation was undertaken to study the radiosensitizing effect of different concentrations of dichloromethane extract of giloe in HeLa cells exposed to different doses of  $\gamma$ -radiation.

## Materials and Methods

### Drugs and Chemicals

Methylene chloride stem extract of Giloe, *Tinospora cordifolia* (TCE) was provided by Krüger Pharmaceuticals (Bombay, India). Minimum Essential Medium (MEM), L-glutamine, gentamicin sulfate, fetal calf serum, 1-chloro,2,4-dinitrobenzene (CDNB), Tris, ethylenediaminetetraacetic acid, glutathione (reduced), nicotinamide adenine dinucleotide (reduced), thiobarbituric, trichloroacetic

acid and malondialdehyde were procured from Sigma Chemical Co., St. Louis, USA). The other routine chemicals were supplied by Merck India, Mumbai.

### Preparation of Drug

TCE was dissolved in dimethyl sulfoxide at a concentration of 5 mg/ml and diluted in sterile MEM in such a way so as to obtain required concentrations. The TCE was filter sterilized using a 0.22  $\mu$ m filter (Millipore, India, Bangalore). The concentration of DMSO never exceeded 0.02%. All drug solutions were prepared afresh immediately before use.

### Cell Line and Culture

HeLa S3 cells with a doubling time of  $20 \pm 2$  h were procured from National Centre for Cell Science, Pune, India and grown in 25 cm<sup>2</sup> culture flasks (Techno Plastic Products, Trasadingen, Switzerland) containing 5 ml Eagle's minimum essential medium (MEM) supplemented with 10% fetal calf serum, 1% L-glutamine and 50  $\mu$ g/ml gentamicin sulfate at 37°C in an atmosphere of 5% CO<sub>2</sub> in 95% humidified air in a CO<sub>2</sub> incubator (NuAir, Plymouth, USA) with loosened caps.

### Experimental Design

A fixed number ( $5 \times 10^5$ ) of exponentially growing cells were seeded into several culture flasks (Techno Plastic Products, Trasadingen, Switzerland) and were allowed to reach plateau phase and were divided into the following groups according to the treatment: -

**MEM + irradiation:** The cells of this group were treated with 0 or 10  $\mu$ l of sterile MEM.

**TCE + irradiation:** The cell cultures of this group were inoculated with 0, 1, 2 or 4  $\mu$ g/ml of TCE before exposure to various doses of  $\gamma$  - radiation.

### Irradiation

The HeLa cells were exposed to 0, 0.5, 1, 2, 3 or 4 Gy  $\gamma$ -radiation from a tele-cobalt therapy source (Theratron Atomic Energy Agency, Ontario, Canada) at a dose rate of 1 Gy/min at a distance (SSD) of 91 cm after treating them with TCE or MEM for different times.

### Optimum Treatment Duration Selection

**Pratt and Willis assay:** The optimum duration for TCE treatment before irradiation was evaluated by Pratt and Willis test [38]. Usually  $1 \times 10^5$  HeLa cells were seeded into 25 cm<sup>2</sup> culture dishes (Cellstar, Greiner, Germany). They were allowed to grow for 24 h before addition of 1, 2 or 4  $\mu$ g/ml TCE. After 0, 1, 2, 4 or 6 h of TCE treatment, the cells were exposed to 2 Gy and the drug-containing medium was

replaced with a fresh drug-free medium. After 72 h of drug inoculation, the cultures were harvested and the cells were counted using a hemocytometer, under an inverted microscope (Labovert microscope, Ernst Leitz, Wetzlar GmbH, Germany). The viability of cells was determined using trypan blue dye-exclusion test.

**Clonogenic assay:** The results obtained from Pratt and Willis assay were confirmed by clonogenic assay, where 200 cells were plated on to several individual culture dishes (Cellstar, Greiner, Germany) containing 5 ml drug free medium in triplicate for each drug dose for each group [39]. The cells were exposed to 1, 2 or 4  $\mu$ g/ml TCE for 0, 1, 2, 4 or 6 h, respectively and exposed to 2 Gy  $\gamma$ -radiation. The cells were allowed to grow for 11 days. The resultant colonies were stained with 1% crystal violet in methanol and clusters containing 50 or more cells were scored as a colony. The plating efficiency of cells was determined and the surviving fraction was fitted on to a linear quadratic model,  $SF = \exp - (\alpha D + \beta D^2)$ .

**Radiosensitizing activity:** A separate experiment was conducted to determine the optimum concentration of TCE, where experimental design was essentially similar to that described above except that cells were treated with 0, 1, 2 and 4  $\mu$ g/ml before exposure to 0, 0.25, 0.5, 1, 2, 3 or 4 Gy of  $\gamma$ -radiation.

**Pratt and Willis assay:** The cytotoxicity of various treatments was measured by Pratt and Willis test [38] as described earlier, except that HeLa cells were treated with different concentrations of TCE for 4 h before exposure to various doses of  $\gamma$ -radiation.

**Clonogenic assay:** The clonogenic assay was carried out as described above, except that HeLa cells were treated with different concentrations of TCE for 4 h before exposure to various doses of  $\gamma$ -radiation [39].

### Biochemical Analyses

A separate experiment was carried out to examine the effect of various concentrations of TCE (0, 1, 2 or 4  $\mu$ g/ml) on the enzyme activities in the cell homogenates (glutathione-S-transferase and lipid peroxidation) or medium (lactate dehydrogenase), after 3 Gy  $\gamma$ -irradiation at 0, 0.5, 1, 2, 4, 8 and 12 h post-irradiation. The grouping and other conditions were essentially similar to that described above.

### Glutathione-S-Transferase (GST)

The cytosolic glutathione-S-transferase (GST) activity was determined spectrophotometrically at 37°C according to the procedure of Habig et al. [40]. Briefly, the reaction mixture containing 2.7 ml of 100 mM phosphate buffer (pH 6.5) and 0.1 ml of 30 mM CDNB was pre-incubated at 37°C for 5 min, 0.5 ml of 20 mM GSH and the reaction was initiated by the addition of 0.1 ml cell

homogenate. The absorbance was recorded for 5 min at 340 nm in a UV-Visible double beam spectrophotometer (UV-260, Shimadzu Corporation, Tokyo, Japan). Reaction mixture sans enzyme (cell homogenate) was used as blank. The GST activity has been expressed as Units/mg protein.

### Lipid Peroxidation (LOO)

LOO (TBARS) was measured by the method of Beuge and Aust, [41]. Briefly, the cell homogenate was mixed with TCA-TBA-HCl and heated for 15 min in a boiling water bath. After centrifugation the absorbance was recorded at 535 nm using a UV-Visible double beam spectrophotometer (UV-260, Shimadzu Corp, Tokyo, Japan). Lipid peroxidation in the samples has been determined against the standard curve of MDA (malondialdehyde). The lipid peroxidation has been expressed as TBARS nmol/mg protein.

### Lactate Dehydrogenase (LDH)

The activity of LDH was estimated at 0, 0.5, 1, 2, 4, 8 and 12 h post-drug treatment or post-irradiation as the case may be in the culture medium of all the three groups simultaneously. The estimation of LDH release in the culture medium of above groups was carried out by the method described by Decker and Lohman-Matthes with minor modifications [42]. The whole medium from each cell culture of each group was removed and collected separately immediately after irradiation (within 5 min after irradiation) and was considered 0 h after treatment. The cells were fed with a fresh 5 ml medium and the above procedure (removal of media) was successively repeated at each assay period (i.e., 0.5, 1, 2, 4, 8 and 12 h) until the termination of the experiment. Briefly, the tubes containing media were centrifuged and 50  $\mu$ l of the medium was transferred into individual tubes containing Tris-EDTA-NADH buffer followed by 10 min incubation at 37°C and the addition of pyruvate solution. The absorbance was read at 339 nm on a UV-Vis spectrophotometer (UV-260, Shimadzu Corp, Tokyo, Japan) and the data have been expressed as units/litre (U/L).

### Statistical Analysis

The statistical analyses were performed using GraphPad Prism 2.01 statistical software (GraphPad Software, San Diego, CA, USA). The significance among all groups was determined by one-way ANOVA and Bonferroni's post-hoc test was applied for multiple comparisons. The experiments were repeated for confirmation of results. The results are average of five individual experiments. The test of homogeneity was

applied to determine variation among each experiment. The data of each experiment did not differ significantly from one another and hence, all the data have been combined and means calculated. A p value of <0.05 was considered statistically significant.

## Results

The results are expressed as % viability for Pratt and Willis assay and surviving fraction (SF) for clonogenic assay in Figures 1 - 4. The results of biochemical analyses are expressed as GST (Units/mg protein), lipid peroxidation (TBARS nmol/mg); and LDH (units/L) in Figures 5 to 7.

### Optimum Treatment Duration Selection

**Pratt and Willis assay:** MEM treatment did not alter the spontaneous viability of HeLa cells significantly with time (Figure 1). Exposure of HeLa cells to 2 Gy resulted in an approximate 12 % decline in the cell viability. Treatment of HeLa cells with different concentrations of TCE before exposure to 2 Gy  $\gamma$ -radiation caused a further concentration dependent decline in the cell viability at all post-TCE treatment times, when compared to 2 Gy alone. However, the difference between 4 and 6 h treatment durations was statistically non-significant (Figure 1).

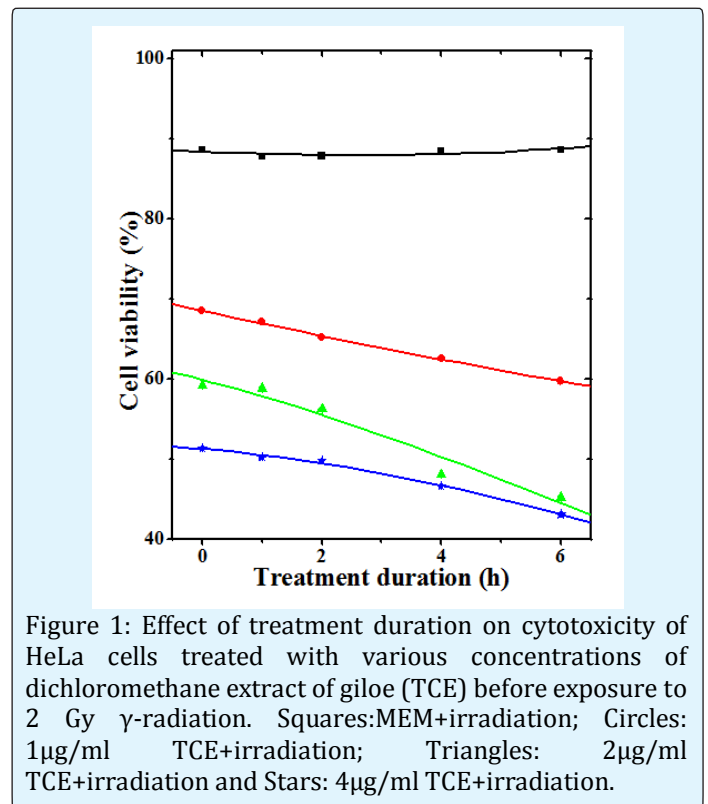


Figure 1: Effect of treatment duration on cytotoxicity of HeLa cells treated with various concentrations of dichloromethane extract of giloe (TCE) before exposure to 2 Gy  $\gamma$ -radiation. Squares: MEM+irradiation; Circles: 1  $\mu$ g/ml TCE+irradiation; Triangles: 2  $\mu$ g/ml TCE+irradiation and Stars: 4  $\mu$ g/ml TCE+irradiation.

**Clonogenic assay:** The reproductive integrity of HeLa cells remained unaffected by MEM treatment before exposure to

2 Gy  $\gamma$ -radiation as evidenced by non-significant change in the survival of HeLa cells (Figure 2). Treatment of different concentrations of TCE for various time periods before exposure to 2 Gy  $\gamma$ -radiation, exhibited a concentration dependent decrease in the surviving fraction (SF) that reduced to almost 50 % (0.5) in cells treated with 1  $\mu\text{g}/\text{ml}$  TCE for 4 h. Thereafter, the clonogenicity of HeLa cells declined negligibly with treatment time up to 6 h post-treatment, the last exposure time evaluated (Figure 2). Therefore 4 h TCE treatment duration was considered as the optimum time of drug treatment and further experiments were carried out using this TCE treatment duration.

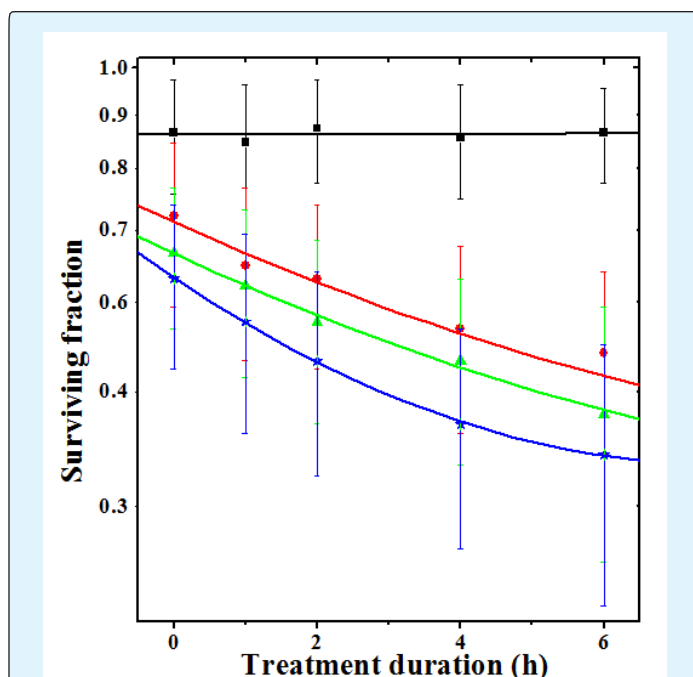


Figure 2: Effect of treatment duration on clonogenicity of HeLa cells treated with different concentrations of dichloromethane extract of giloe (TCE) before exposure to 2 Gy  $\gamma$ -radiation. Squares: MEM+irradiation; Circles: 1  $\mu\text{g}/\text{ml}$  TCE+irradiation; Triangles: 2  $\mu\text{g}/\text{ml}$  TCE+irradiation and Stars: 4  $\mu\text{g}/\text{ml}$  TCE+irradiation.

### Radiosensitizing Activity

**Pratt and Willis assay:** MEM treatment did not alter the spontaneous viability of HeLa cells significantly (Figure 3). When HeLa cells were treated with different concentrations of TCE, the cell viability declined in a concentration dependent manner and lowest cell viability was observed for 4  $\mu\text{g}/\text{ml}$ , the highest concentration of TCE evaluated. Irradiation of HeLa cells with different doses of  $\gamma$ -rays resulted in a dose dependent decline in the viability of HeLa cells, whereas treatment of HeLa cells with various concentrations of TCE before irradiation further decreased

the cell viability depending not only on the irradiation dose but also on the TCE concentration (Figure 3). Treatment of HeLa cells with various concentrations of TCE caused a significant decline in cell viability after exposure to 1 to 4 Gy  $\gamma$ - radiation. The lowest concentration of 1  $\mu\text{g}/\text{ml}$  TCE increased cytotoxic effect of  $\gamma$ - radiation significantly when compared with the non-drug treated control. Exposure of HeLa cells to 2  $\mu\text{g}/\text{ml}$  TCE further reduced the cell viability at all irradiation doses in comparison with MEM+irradiation and an approximate 2-fold decline in cell viability was observed for 2 and 3 Gy, respectively. A further increase in irradiation dose to 4 Gy caused a 3-fold decline in cell viability. Increase in TCE concentration to 4  $\mu\text{g}/\text{ml}$ , before exposure to different doses of  $\gamma$ - radiation further reduced cell viability of HeLa cells, which was lowest among all TCE concentrations. This decline was approximately 1.5 and 1.7 folds when compared with 1  $\mu\text{g}/\text{ml}$  TCE after exposure to 3 or 4 Gy (Figure 3).

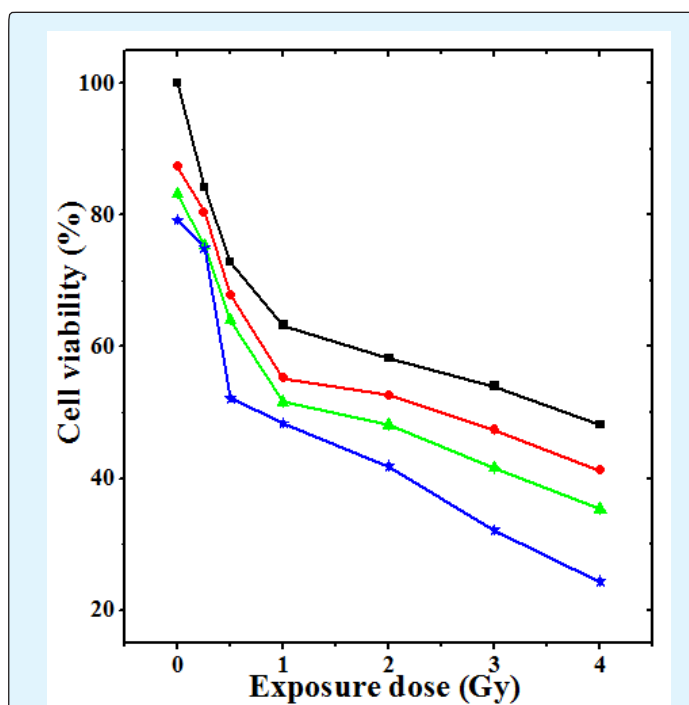


Figure 3: Effect of various concentrations of dichloromethane extract of giloe (TCE) on the cytotoxicity in cultured HeLa cells exposed to different doses of  $\gamma$ - radiation. Squares: MEM+irradiation; Circles: 1  $\mu\text{g}/\text{ml}$  TCE+irradiation; Triangles: 2  $\mu\text{g}/\text{ml}$  TCE+irradiation and Stars: 4  $\mu\text{g}/\text{ml}$  TCE+irradiation.

**Clonogenic assay:** Irradiation of HeLa cells to 0 to 4 Gy  $\gamma$ - radiation resulted in a radiation-dose dependent decline in the cell survival (Figure 4). Treatment of HeLa cells with different concentrations of TCE before exposure to various doses of  $\gamma$ - radiation resulted in a further decline in the cell survival, which was significantly lower than MEM +

irradiation group. This reduction in surviving fraction of cells was also dependent on the TCE concentration. Greater was the TCE concentration used before irradiation, higher was the reduction in cell survival (Figure 4). The greatest reduction in SF was observed for 4  $\mu\text{g}/\text{ml}$  TCE at all irradiation doses, where surviving fraction reduced to 0.24 after 4 Gy irradiation (Figure 4).

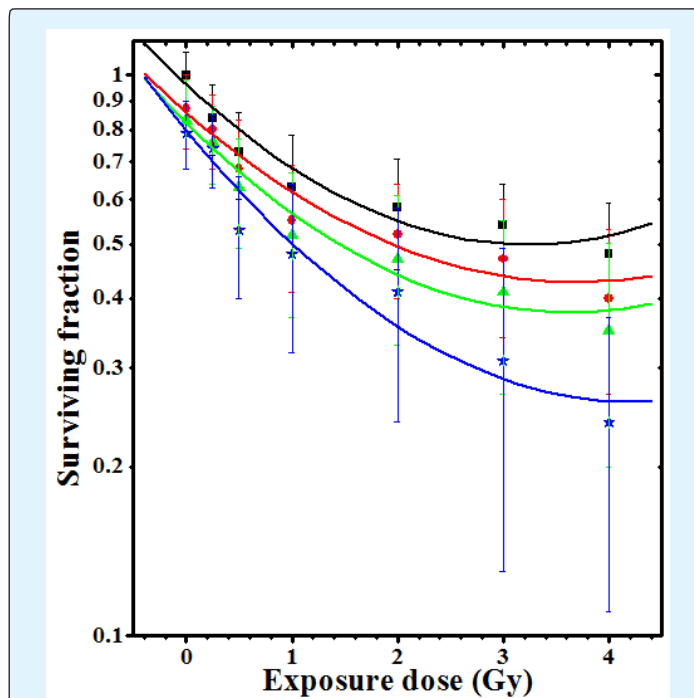


Figure 4: Alteration in the clonogenic potential of HeLa cells treated with various concentrations of dichloromethane extract of giloe (TCE) before exposure to different doses of  $\gamma$ -radiation. Squares: MEM+irradiation; Circles: 1  $\mu\text{g}/\text{ml}$  TCE+irradiation; Triangles: 2  $\mu\text{g}/\text{ml}$  TCE+irradiation and Stars: 4  $\mu\text{g}/\text{ml}$  TCE+irradiation.

### Biochemical Analyses

The radiosensitizing activity of TCE was also confirmed by determining various biochemical parameters listed below.

**Glutathione-S-transferase (GST):** The spontaneous

activity of cytosolic GST remained unaltered with assay time, whereas treatment of HeLa cells with different concentrations of TCE resulted in a significant decline ( $p < 0.001$ ) in the GST activity (Table 1). Exposure of HeLa with 3 Gy caused a significant decline in the GST activity at all post-irradiation times and treatment of HeLa cells with 1, 2 and 4  $\mu\text{g}/\text{ml}$  TCE before exposure to different doses of  $\gamma$ -radiation led to a further decline in the GST activity at all post-irradiation times (Figure 5). The decline in GST activity was gradual and a maximum decline was observed at 4 h post-irradiation and thereafter the GST activity remained almost unaltered thereafter (Figure 5). The lowest GST activity was detected at 12 h post-irradiation (Table 1).

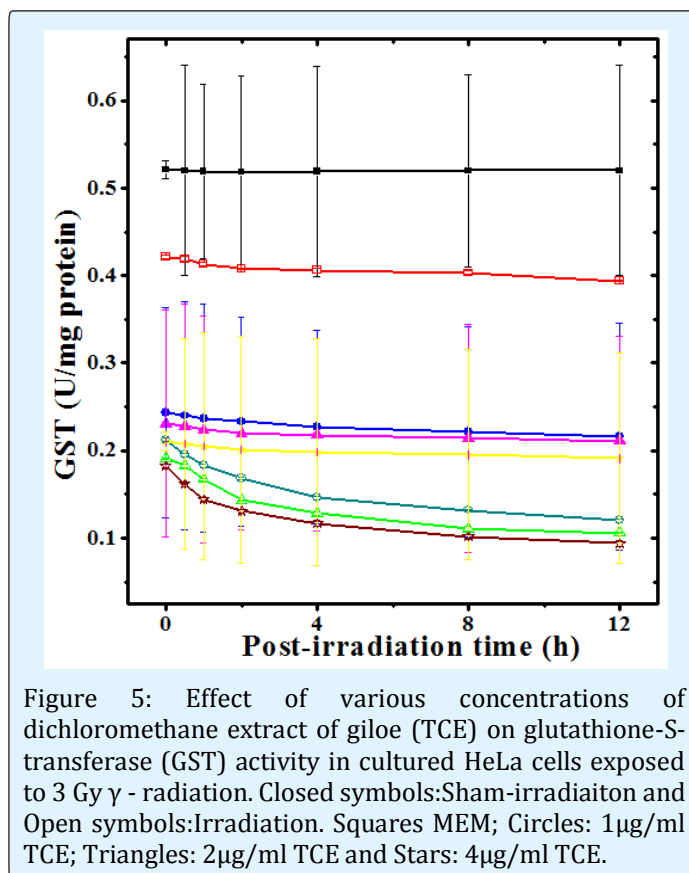


Figure 5: Effect of various concentrations of dichloromethane extract of giloe (TCE) on glutathione-S-transferase (GST) activity in cultured HeLa cells exposed to 3 Gy  $\gamma$ -radiation. Closed symbols: Sham-irradiation and Open symbols: Irradiation. Squares MEM; Circles: 1  $\mu\text{g}/\text{ml}$  TCE; Triangles: 2  $\mu\text{g}/\text{ml}$  TCE and Stars: 4  $\mu\text{g}/\text{ml}$  TCE.

| Post-IR time(h) | Glutathione-S-transferase (GST) $\pm$ SEM (U/mg protein) |                   |                                 |                   |                  |                   |                  |                   |
|-----------------|--|-------------------|---------------------------------|-------------------|------------------|-------------------|------------------|-------------------|
|                 | MEM  |                   | TCE ( $\mu\text{g}/\text{ml}$ ) |                   |                  |                   |                  |                   |
|                 |  |                   | 1                               |                   | 2                |                   | 4                |                   |
|                 | SIR  | IR                | SIR                             | IR                | SIR              | IR                | SIR              | IR                |
| 0               | 0.521 $\pm$ 0.12   | 0.421 $\pm$ 0.002 | 0.243 $\pm$ 0.12                | 0.212 $\pm$ 0.002 | 0.231 $\pm$ 0.13 | 0.191 $\pm$ 0.001 | 0.210 $\pm$ 0.11 | 0.183 $\pm$ 0.002 |
| 0.5             | 0.520 $\pm$ 0.12   | 0.419 $\pm$ 0.003 | 0.240 $\pm$ 0.13                | 0.195 $\pm$ 0.002 | 0.228 $\pm$ 0.14 | 0.183 $\pm$ 0.002 | 0.208 $\pm$ 0.12 | 0.162 $\pm$ 0.001 |
| 1               | 0.519 $\pm$ 0.10   | 0.413 $\pm$ 0.002 | 0.237 $\pm$ 0.13                | 0.183 $\pm$ 0.002 | 0.224 $\pm$ 0.13 | 0.167 $\pm$ 0.001 | 0.205 $\pm$ 0.13 | 0.144 $\pm$ 0.002 |
| 2               | 0.518 $\pm$ 0.11   | 0.408 $\pm$ 0.001 | 0.233 $\pm$ 0.12                | 0.168 $\pm$ 0.001 | 0.220 $\pm$ 0.11 | 0.143 $\pm$ 0.001 | 0.201 $\pm$ 0.13 | 0.131 $\pm$ 0.002 |

|    |            |             |            |             |            |             |            |             |
|----|------------|-------------|------------|-------------|------------|-------------|------------|-------------|
| 4  | 0.519±0.12 | 0.406±0.003 | 0.227±0.11 | 0.146±0.002 | 0.218±0.11 | 0.128±0.002 | 0.198±0.13 | 0.117±0.003 |
| 8  | 0.520±0.11 | 0.403±0.002 | 0.221±0.12 | 0.132±0.001 | 0.214±0.13 | 0.111±0.003 | 0.195±0.12 | 0.102±0.002 |
| 12 | 0.520±0.12 | 0.394±0.001 | 0.216±0.13 | 0.120±0.002 | 0.211±0.12 | 0.106±0.001 | 0.191±0.12 | 0.094±0.003 |

Table 1: Alteration in the glutathione-S-transferase activity in HeLa cells treated with various concentrations of dichloromethane extract of giloe (TCE) before exposure to 3 Gy  $\gamma$ -radiation at different post-irradiation times.

a = p < 0.001 (Comparison of TCE concentrations with MEM)

TCE: Dichloromethane extract of giloe (*Tinospora cordifolia*); MEM: Minimum Essential Medium, SIR: Sham-irradiation and IR: Irradiation.

**Lipid peroxidation (LOO):** The baseline lipid peroxidation remained unchanged with assay time (Figure 6). Treatment of HeLa cells with various concentrations of TCE caused a significant elevation in the lipid peroxidation, which was approximately 3 folds greater than 3 Gy irradiation at 4 h post-irradiation (Table 2). The maximum lipid peroxidation was observed at 4 h post-irradiation and depletion thereafter for all TCE concentrations (Figure 6). The lipid peroxidation increased with increasing concentration of TCE and the maximum LOO was observed for 4  $\mu$ g/ml TCE alone or in conjunction 3 Gy irradiation (Figure 6).

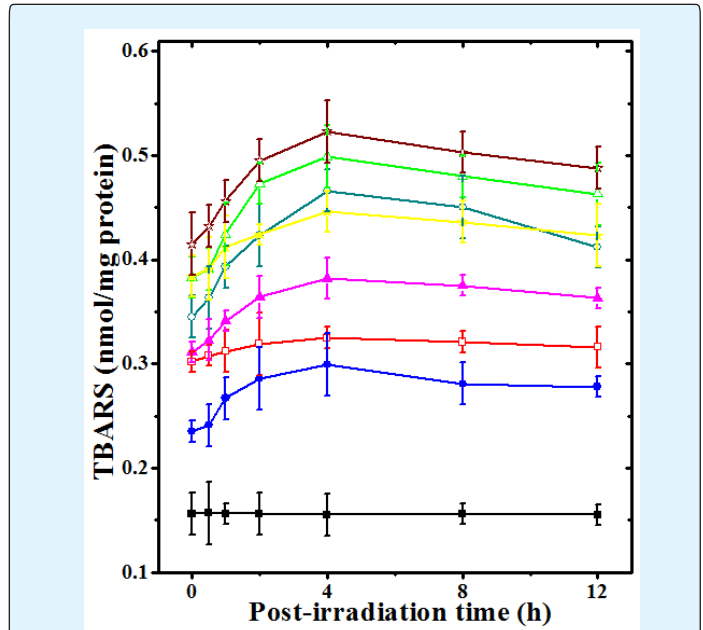


Figure 6: Effect of various concentrations of dichloromethane extract of giloe (TCE) on the radiation-induced lipid peroxidation in cultured HeLa cells exposed to 3 Gy  $\gamma$  - radiation. Closed symbols: Sham-irradiation and Open symbols: Irradiation. Squares MEM; Circles: 1 $\mu$ g/ml TCE; Triangles: 2 $\mu$ g/ml TCE and Stars: 4 $\mu$ g/ml TCE.

| Post-IR time(h) | TBARS (nmol/mg protein) $\pm$ SEM |            |                   |            |            |            |            |            |
|-----------------|-----------------------------------|------------|-------------------|------------|------------|------------|------------|------------|
|                 | MEM                               |            | TCE ( $\mu$ g/ml) |            |            |            |            |            |
|                 |                                   |            | 1                 |            | 2          |            | 4          |            |
|                 | SIR                               | IR         | SIR               | IR         | SIR        | IR         | SIR        | IR         |
| 0               | 0.156±0.02                        | 0.302±0.01 | 0.235±0.01        | 0.345±0.02 | 0.311±0.01 | 0.383±0.02 | 0.384±0.02 | 0.415±0.03 |
| 0.5             | 0.157±0.03                        | 0.308±0.01 | 0.241±0.02        | 0.363±0.03 | 0.323±0.02 | 0.391±0.02 | 0.391±0.03 | 0.432±0.02 |
| 1               | 0.156±0.01                        | 0.312±0.02 | 0.267±0.02        | 0.393±0.02 | 0.341±0.01 | 0.424±0.03 | 0.412±0.03 | 0.456±0.02 |
| 2               | 0.156±0.02                        | 0.319±0.03 | 0.286±0.03        | 0.423±0.03 | 0.364±0.02 | 0.473±0.02 | 0.424±0.01 | 0.495±0.02 |
| 4               | 0.155±0.02                        | 0.325±0.01 | 0.299±0.03        | 0.466±0.02 | 0.382±0.02 | 0.499±0.03 | 0.446±0.02 | 0.523±0.03 |
| 8               | 0.156±0.01                        | 0.321±0.01 | 0.281±0.02        | 0.450±0.03 | 0.375±0.01 | 0.480±0.02 | 0.436±0.02 | 0.503±0.02 |
| 12              | 0.155±0.01                        | 0.316±0.02 | 0.278±0.01        | 0.412±0.02 | 0.363±0.01 | 0.463±0.03 | 0.423±0.03 | 0.488±0.02 |

Table 2: Alteration in radiation-induced lipid peroxidation in HeLa cells treated with various concentrations of dichloromethane extract of giloe (TCE) before exposure to 3 Gy  $\gamma$ -radiation at different post-irradiation times.

a = p < 0.001 (Comparison of TCE concentrations with MEM)

TCE: Dichloromethane extract of giloe (*Tinospora cordifolia*); MEM: Minimum Essential Medium, SIR: Sham-irradiation and IR: Irradiation

**Lactate Dehydrogenase (LDH):** Irradiation of HeLa cells with 3 Gy  $\gamma$ -radiation caused an elevation in LDH released in the medium when compared to sham-irradiated controls. Treatment of HeLa cells with various concentrations of TCE before irradiation elevated LDH levels significantly when compared to 3 Gy irradiation (Table 3). The LDH activity was maximum immediately after irradiation (0 h) in all the groups. However, this elevation was 2 folds greater at other post-irradiation assay time in TCE + irradiation group (Table 3). The LDH activity declined with assay time (since the whole media was removed at each time, the values in tables and figures are lower) reaching a nadir at 8 h post-irradiation (Figure 7), however, the LDH contents were significantly higher (p < 0.0001) than the sham-irradiated control (MEM + 0 Gy) as well as MEM + 3 Gy irradiation group for all TCE concentrations (Table 3).

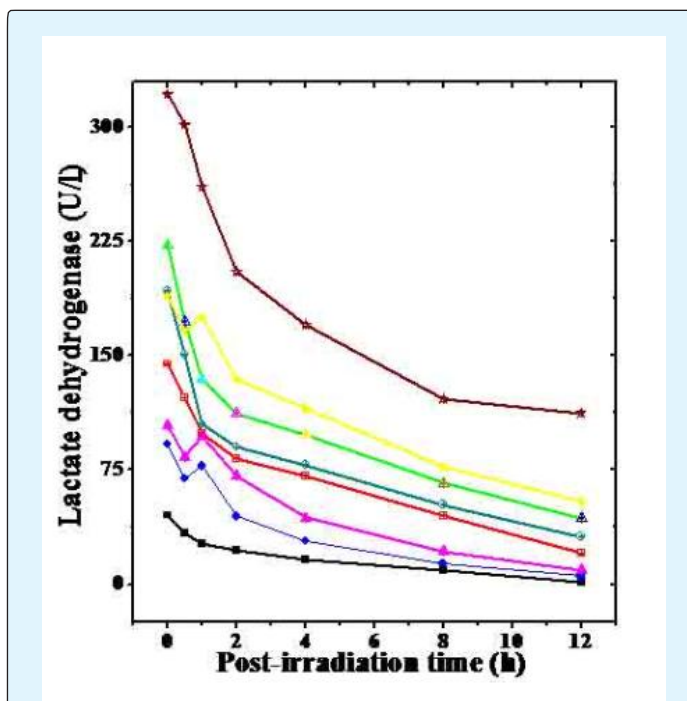


Figure 7: Effect of various concentrations of dichloromethane extract of giloe (TCE) on lactate dehydrogenase (LDH) release in cultured HeLa cells exposed to 3 Gy  $\gamma$  - radiation. Closed symbols: Sham-irradiation and Open symbols: Irradiation. Squares MEM; Circles: 1 $\mu$ g/ml TCE; Triangles: 2 $\mu$ g/ml TCE and Stars: 4 $\mu$ g/ml TCE.

| Post-IR time(h) | Lactate dehydrogenase (LDH) $\pm$ SEM (U/L) |                   |                   |                   |                   |                   |                   |                   |
|-----------------|---|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|
|                 | MEM   |                   | TCE ( $\mu$ g/ml) |                   |                   |                   |                   |                   |
|                 |   |                   | 1                 |                   | 2                 |                   | 4                 |                   |
|                 | SIR   | IR                | SIR               | IR                | SIR               | IR                | SIR               | IR                |
| 0               | 45.31 $\pm$ 0.33                            | 144.57 $\pm$ 0.35 | 92.04 $\pm$ 0.41  | 192.34 $\pm$ 0.34 | 104.35 $\pm$ 0.34 | 222.35 $\pm$ 0.43 | 189.05 $\pm$ 0.32 | 321.51 $\pm$ 0.50 |
| 0.5             | 33.21 $\pm$ 0.34                            | 122.34 $\pm$ 0.41 | 69.26 $\pm$ 0.43  | 150.57 $\pm$ 0.42 | 83.64 $\pm$ 0.35  | 172.35 $\pm$ 0.45 | 166.24 $\pm$ 0.29 | 301.44 $\pm$ 0.41 |
| 1               | 26.24 $\pm$ 0.33                            | 99.25 $\pm$ 0.35  | 77.82 $\pm$ 0.41  | 105.06 $\pm$ 0.41 | 97.52 $\pm$ 0.31  | 134.41 $\pm$ 0.52 | 175.15 $\pm$ 0.31 | 261.03 $\pm$ 0.43 |
| 2               | 22.41 $\pm$ 0.36                            | 82.41 $\pm$ 0.39  | 44.58 $\pm$ 0.45  | 90.23 $\pm$ 0.38  | 71.05 $\pm$ 0.24  | 112.04 $\pm$ 0.41 | 134.64 $\pm$ 0.29 | 205.15 $\pm$ 0.50 |
| 4               | 16.34 $\pm$ 0.34                            | 71.04 $\pm$ 0.44  | 28.24 $\pm$ 0.36  | 78.33 $\pm$ 0.42  | 43.58 $\pm$ 0.35  | 98.39 $\pm$ 0.55  | 115.43 $\pm$ 0.34 | 170.36 $\pm$ 0.52 |
| 8               | 9.23 $\pm$ 0.36                             | 45.22 $\pm$ 0.42  | 13.45 $\pm$ 0.37  | 52.11 $\pm$ 0.39  | 21.54 $\pm$ 0.31  | 66.41 $\pm$ 0.40  | 76.84 $\pm$ 0.41  | 121.56 $\pm$ 0.29 |
| 12              | 1.05 $\pm$ 0.34                             | 20.24 $\pm$ 0.36  | 5.38 $\pm$ 0.37   | 31.12 $\pm$ 0.35  | 9.35 $\pm$ 0.36   | 43.33 $\pm$ 0.38  | 54.43 $\pm$ 0.42  | 112.33 $\pm$ 0.34 |

Table 3: Alteration in the radiation-induced LDH release by HeLa cells treated with various concentrations of dichloromethane extract of giloe (TCE) before exposure to 3 Gy  $\gamma$ -radiation at different post-irradiation times.

a = p < 0.001 (Comparison of TCE concentrations with MEM)

TCE: Dichloromethane extract of giloe (*Tinospora cordifolia*); MEM: Minimum Essential Medium, SIR: Sham-irradiation and IR: Irradiation.

## Discussion

Plants by virtue of their wide usage in the traditional medicine and less toxic implications have been drawing the

attention of researchers around the world in the recent past. Plants also played a key role in the isolation of several useful drugs including many modern common cancer chemotherapeutic agents [24]. The use of



chemotherapeutic agents in combination with radiation has facilitated the treatment of unamenable neoplasia. Concurrent application of chemotherapeutic agents with radiation has resulted in an increased survival of patients receiving combinations treatment but at the cost of development of second malignancies [23]. Therefore, there is a need to find novel agents, which could enhance the effect of radiation with no adverse toxic side effects or a negligible toxicity. The present study was attempted to evaluate the radiation sensitizing activity of low doses of dichloromethane extract of giloe (*Tinospora cordifolia*) in cultured HeLa cells.

The Pratt and Willis assay provides a gross indication of the cytotoxicity of any pharmacophore [43]. Treatment of HeLa cells with different concentrations of TCE before irradiation caused radiation dose-dependent decline in the cell viability. An identical effect has been observed earlier where, various extracts of giloe were found to increase the effect of  $\gamma$ -radiation [44]. Similarly, berberine, an alkaloid present in the giloe has been found to increase the effect of ionizing radiation in HeLa cells by Pratt and Willis test [45]. The clonogenic assay is the gold standard and it provides the precise information of the cytotoxicity of any chemical or physical agent as it is a long-term assay [46]. Treatment of HeLa cells with various concentrations of TCE caused a significant decline in their reproductive potential after exposure to 1 to 4 Gy  $\gamma$ -radiation and a greatest reduction in surviving fraction (0.24 after 4 Gy) was observed for 4  $\mu\text{g/ml}$  TCE at all irradiation doses. The lowest concentration of 1  $\mu\text{g/ml}$  TCE increased cytotoxic effect of  $\gamma$ -radiation significantly when compared with the non-drug treated control. The different extracts of giloe were found to increase the effect of radiation by clonogenic assay [28]. Phytochemicals like beberine, taxol, podophyllotoxins, vindesine, derived from *Taxus brevifolia*, *Podophyllum hexandrum*, and *Catharanthus roseus*, respectively have been reported to increase the effect of radiation in vitro and in vivo [44,45,47,48]. Similar results were obtained with topotecan, a topoisomerase I inhibitor in irradiated U1-Mel cells and also aclacinomycin [49,50]. A natural product ellagic acid has been found to increase the radiosensitivity of HepG2 cells in an earlier study [51].

Glutathione-S-transferases are phase-II detoxification enzymes and they are known for their ability to conjugate glutathione with electrophiles. Apart from detoxification GSTs play important role in the cell survival and death [52,53]. The over expression of these enzymes is responsible for higher survival and resistance to chemotherapy and radiotherapy, whereas their downregulation has been found to be effective in cancer chemotherapy [54,55]. Treatment of HeLa cells with

different concentrations of TCE led to a concentration dependent decline in the GST activity and irradiation of TCE treated cells caused a further decline in the GST activity which may be one of the reasons of reduced cell survival in the TCE+irradiation group, when compared to MEM+irradiation group. The expression of GST has been correlated with high cell proliferation and reduced radiosensitivity in HeLa cells [56]. Hematoporphyrin dimethyl ether has been reported to increase the radiosensitivity of Ehrlich ascites carcinoma by reducing GST activity earlier [57].

Lipid peroxidation and LDH are hallmarks of membrane damage. Lipid peroxidation is an important event related to cell death and it has been reported to cause severe impairment of membrane function through increased membrane permeability and membrane protein oxidation that eventually leads to cell death by damaging the cellular DNA [58,59]. TCE has increased the radiation-induced lipid peroxidation at all post-irradiation estimation times. LDH is a cytosolic enzyme, which is released by the cells whose membranes are damaged and serves as an indirect indicator of cytotoxicity of any physical or chemical agent [60]. TCE treatment increased the radiation-induced LDH release significantly at all evaluation times. Earlier berberine present in TCE has been reported to increase the LDH activity in irradiated HeLa cells [45]. An increase in LDH contents after paclitaxel and VM-26 treatment has been reported [47,61]. The increased LDH activity is closely related to the reduced surviving fraction. A direct correlation between increase in LDH and a consequent decline in cell survival has also been reported [47,61,62].

The exact mechanism of action of TCE is not well known. However, the increased cytotoxicity of TCE may be due to its pleotropic nature. TCE has been reported to induce molecular DNA damage and also enhance the radiation-induced DNA damage that is finally converted into the genomic damage in HeLa cells [26,34,35]. This may be one of the most important mechanisms of reduced clonogenicity in the present study as severe DNA damage is hallmark of cell mortality. The reduced GST activity and increased lipid peroxidation and LDH are known to trigger DNA damage that subsequently causes cell death [58-60]. Our recent studies have shown that TCE contains berberine and the reduced reproductive potential by TCE in irradiated cells may be due to the action of berberine which has been found to suppress the activity of DNA topoisomerase-II that might have increased the DNA damage and consequently reduced the clonogenicity of HeLa cells in the present study. Inhibition of NF $\kappa$ B, COX-II and Nrf2 may have also played a crucial role in increasing radiation-induced cell death by TCE. Since berberine has been reported to repress the expression of NF $\kappa$ B, COX-II

and Nrf2 [55,63,64]. The radiosensitizing activity may be due to berberine and other phytochemicals individually or in concert with one another.

## Conclusions

Pretreatment of HeLa cells with different concentrations of TCE increased their radiosensitivity in a concentration dependent manner as indicated by a constant decline in the clonogenicity of cells with a maximum effect at 4 µg/ml TCE. This effect may be due to the reduced glutathione – S-transferase activity, accompanied by elevated levels of lipid peroxidation and LDH release that may have resulted in increasing DNA damage in HeLa cells at molecular level. The inhibition of topoisomerases by TCE may have played an important role in increasing the radiosensitivity by damaging the cellular DNA. Suppression of the transcriptional activation of NFκB, COX-II and Nrf2 may have played a major role in the reduction in the clonogenicity of HeLa cells. Presence of berberine and other alkaloids in giloe may have been responsible for its varied actions on HeLa cells. The application of giloe may offer an alternative treatment strategy for cancer in combination with γ-radiation.

## Acknowledgements

This work was supported by grants from the Council of Scientific and Industrial Research, and Indian Council of Medical Research, Govt. of India, New Delhi, India.

## Conflict of Interest Statement

Authors do not have conflict of interest statement to declare.

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