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Enhancement of Radiation-induced Cytotoxicity in Hepatocellular Carcinoma Cells by Some Plant Extracts *In vitro*

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RADIOSENSITIZERS are introduced to boost cytotoxicity against tumor cells while having much less toxicity on normal cells. The radio sensitizing properties of methanolic extracts of *Euphorbia pulcherrima* and *Euphorbia heterophylla* against a hepatocellular cancer cell line were investigated in this work (HepG2). The cytotoxic effect of *E. pulcherrima* and *E. heterophylla* combined with ionizing radiation (IR) were detected. Caspase-3 levels, Bcl_2, Bax & P53 gene expression and Annexin V-FITC apoptosis test were determined. The combination of these extracts and IR showed a considerably higher cytotoxic effect than the one using either extracts or IR alone. There is a substantial increase of Bax & P53 a combined with a decrease in Bcl2. In comparison to the control, there is a 12 to 9 fold increase in total active caspase-3. Both extracts increased the proportion of annexin-positive apoptotic cells from 1.69 percent to 17.22 percent and 14.07 percent, respectively. The combination of extracts and IR showed 17 and 11 folds higher p53 level compared to the control in the nucleus, respectively. These results showed that combined *E. Pulcherrima* and *E. Heterophylla* and IR contribute to increased cytotoxic effects on the HPG2. It can be suggested that these extracts can be used as radio-sensitizers.

Keywords: *Euphorbia pulcherrim, Euphorbia heterophylla,* Gamma radiation, Hepatocellular carcinoma**.**

Introduction

The (HCC) Hepatocellular carcinoma is the world's most prevalent malignancy causing mortality due to cancer-illness (Dertli et al., 2020; Mak & Kramvis, 2021). There are many curative alternatives accessible for HCC comprising curative surgery, liver transplantation, trans-arterial chemo-embolization, radio-embolization radiofrequency ablation, and Sorafenib as a targeted systemic agent. Management of HCC depends on the stage of tumor, status of patient success and function of the liver. Nexavar (Sorafenib) is an approved therapy in the progressive phases of the disease, however it is a new systemic selective molecule and its combinations were established (Raza & Sood, 2014). To enhance the cure rate in HCC patients, new treatments are required. Radiation therapy has a little influence on the liver, so it has been disregarded conventionally to control HCC by radiotherapy (Yu et al., 2019). Advances in imaging techniques allowed a high dose of radiation to be delivered to a specific tumor volume, while avoiding the normal liver parenchyma surrounding it (Boas et al., 2017).However, after radiotherapy, the curative rate remains restricted as a result of the high incidence of hepatic recurrences (Krause et al., 2017).

It was established that the use of the radio sensitizer's agents to increase the efficacy of RT is a way to resolve these difficulties by increasing

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tumor tissue damage and hastening DNA-damage and generating free radicals (Wang et al., 2018).

Poinsettia (*Euphorbia pulcherrima*) is a blooming plant. Despite safety concerns, people use poinsettia to treat fevers, encourage the production of breast milk, and cause abortions. In rats, the antinociceptive effectiveness of three *Euphorbia heterophylla* root preparations was examined (Hamill et al., 2000). In traditional African medicine, infusions of the stems and fresh or dried leaves are used as a purgative and laxative to alleviate stomach discomfort and constipation, and to expel intestinal worms. It was reported that *E. heterophylla* has wound healing activity, anti-inflammatory, antimicrobial and anticancer activity (Manikandarajan et al.,2018).

E. pulcherrima and *E. heterophylla* (Family: Euphorbiaceae) showed a low to moderate cytotoxic activity in vitro on HepG2 (El Manawaty et al., 2013).

As far as we know, there is no previous study concerning *Euphorbia pulcherrima* and *Euphorbia heterophylla* and they were not assessed as radiosensitizers against hepatocellular carcinoma cell line (HepG2). The authors aim at evaluating the in vitro radio sensitizing efficacy of *E. pulcherrima* and *E. heterophylla* on HepG2 cell line.

Materials and Methods

Extracts preparation

The Voucher specimens of the plants were obtained from the Herbarium of the Pharmacognosy Department, National Research Center (NRC), Dokki, Giza, Egypt. The plant organs were separated, dried at temperature ≤ 45˚C in solar ovens and powdered.

Every plant's powder (30-50g) was exhaustively extracted with 80% methanol-GR (CH_3OH) for analysis, obtained from Merck. The aqueous methanolic extracts were filtered, evaporated under vacuum at 40˚C using BüchiRotatory evaporator-114 (Swither land), freeze dried using Virtis Freeze dryer (USA) and stored at -20˚C. The extraction and drying procedures were conducted thoughtfully to guard against denaturation and/ or artefact formation. The freezing dried plant extracts were deposited at the plants extract bank of the In vitro Bioassay Laboratory, NRC, Dokki, Giza, Egypt.

Cell line and culture media

The HepG2 were obtained from the Department of Oncology-Pathology, Cancer Center Karolinska Institute, Sweden. The Stock was grown in Roswell Park Memorial Institute medium (RPMI), Biowhittaker, supplemented with 10% foetal bovine serum, Biowhittaker, Germany and 1% penicillin-streptomycin solution (penicillin 10,000 IU/mL; streptomycin 10,000 µg/mL, Biowhittaker)

Subculture of cell line

Cultures were viewed using an inverted microscope (CKX41; Olympus, Japan) to assess the degree of confluence and to confirm the absence of the bacterial and fungal contaminants. Cell monolayer was washed with phosphate-buffered saline (PBS) free of Ca2þ/Mg2þ using a volume equivalent half of the volume of culture medium. Trypsin/EDTA was added on to the washed cell monolayer using 1 ml/ 25 cm^2 of surface area. The flask was rotated to merge the monolayer with Trypsin/EDTA.The flask was returned to the incubator and left for 10 min. The cells were examined using an inverted microscope to ensure that all the cells were detached and floated.

Cytotoxicity assay

For the estimation of the *in vitro* cytotoxic potency of the extracts, the MTT assay was conducted. The cell line was cultured at 37˚C in 95% air humidity, and 5% CO_2. The cells were seeded into flat bottom 6-well plates (Gholm, Reiner, Vienna) at a density of 300.000 cells per well. After 24 h, the plates were divided into groups.

Culture groups

The first group, HPG2 was treated with each of the two extracts of *E. Pulcherrima* and *E. Heterophylla* (100, 50, 25 and 12.5 ppm).

The second group, HPG2 was exposed to extract before radiation (5, 10 and 15Gy).

The third group, HPG2 was exposed to extract after radiation (5, 10 and 15 Gy). Moreover, *E. pulcherrima*& *E. heterophylla* combined with 10 Gy γ radiation was found to enhance cytotoxicity in addition to being safer than 15 Gy. Thus, the dose of 10 Gy was selected to fulfill the subsequent experiments.

The forth group, Doxorubicin was used as a positive control, and 0.5% dimethyl sulfoxide (DMSO) was used as a negative control.

Radiation exposure

Cells were exposed to gamma-radiation using ^{137}cesium as a source of gamma radiation; (Gamma cell-40 Canadian, Activity 3032 Curies, purchased on April, 1977) the dose Rate: 0.42Gy/min at NCRRT, Egyptian Atomic Energy Authority, Cairo, Egypt.

Cell proliferation assay

Triplicates of each treatment were established. Forty-eight hours later each well was treated with 40 µl of MTT (Sigma-Aldrich) saline solution, and after 4 h of incubation 200µl Sodium dodecyl sulphate (SDS) 10% and after 20 min the color was measured at 495nm with reference 690 nm. Then, the inhibition percentage was calculated according to [1-(av(S)/ (av (NC))] x 100 where av (NC) is the average absorbance of the three negative control wells and av(S) is the average absorbance of the three sample wells. 50% inhibition concentration (IC_{50}) values were calculated by non-linear regression (Mak & Kramvis., 2021).

The Cell Counting Kit-8 (CCK-8) kit (Dojindo Laboratories, Kumamoto, Japan) was used to determine cell viability and proliferation. Briefly, cells were seeded in 96-well plates (3000 cells/ well, four replicates) and incubated with different final concentrations of SYB (0, 50 nmol/l, 100 nmol/l, 150 nmol/l, 200 nmol/l) for different periods of time (12 h, 24 h, 36 h, and 48 h). The cells were replenished with medium containing CCK-8 solution (10 µl CCK-8 in 100 µl medium) and incubated for 2 h, after which the absorbance at 450 nm was measured using a microplate reader (Bio-Tek Instruments, Winooski, VT, USA). The survival rate of the cells was calculated as the ratio of the normalized absorbance to that of the non-irradiated controls.

Extraction of RNA and quantitative RT-PCR analysis

HepG2 cells were treated with IC_{50} concentration of promising methanol extract of *E. Pulcherrima* and *E. heterophylla* for one hour followed by exposure to 10 Gy gamma rays. Then after incubation for 48hours in 95% air humidity, and 5% CO_2 at 37˚C.Total RNA was isolated using Qiagen tissue extraction kit (Qiagen to assess gene-expression of apoptotic (Bax) and anti-apoptotic (Bcl2) markers., USA) according to the instructions of the kit. The total RNA was reversetranscripted into cDNA using the high capacity cDNA reverse transcription kit (Fermentas, USA). Quantitative real-time polymerase chain reaction amplification and analysis were performed using an Applied Biosystem with software version 3.1 (StepOne™, USA). The reaction contained SYBR Green Master Mix (Applied Biosystems), gene-specific forward and reverse primers were as follows:

Oligonucleotide primers

p53 F	5’- CCCCTCCTGGCCCCTGTCATCTTC-3’,
p53 R	5’-GCAGCGCCTCACAACCTCCGTCAT-3’
Bax F	5’-GTTTCA TCC AGG ATC GAG CAG-3’
Bax R	5’-CATCTT CTT CCA GAT GGT GA-3’
bcl-2 F	5’-CCTGTG GAT GAC TGA GTA CC-3’
bcl-2 R	5’-GAGACA GCC AGG AGA AAT CA-3’
β-actin F	5’-GTGACATCCACACCCAGAGG-3’
β-actin R	5’-ACAGGATGTCAAAACTGCCC-3’

The relative expression of the studied genes was calculated according to Applied Bio- system software. All values were normalized to the expression of b-actin as an endogenous control (reference gene) (El -Kady et al., 2011).

Annexin V-FITC apoptosis by flow cytometry

Annexin V-FITC Apoptosis Detection Kit cat: 556547 was used in, one-step staining procedure to detect apoptosis by staining phosphatidylserine molecules which have translocated to the outside of the cell membrane. Analysis was conducted by flow cytometry.The cells (HepG2) were treated with 19.71 and 34.3 µg/ml of *E. Pulcherrima and E. heterophylla*, respectively for 1 hour then exposed to 10Gy gamma rays. Then after incubation for 48 hours at 37˚C in 95% air humidity, and 5% CO_2, the cells were washed twice with ice-cold phosphate buffered saline (PBS). Subsequently, the treated cells were collected by centrifugation, fixed in ice-cold 70% (v/v) ethanol, washed with PBS, re-suspended with 0.1 mg/ml RNase, stained with 40 mg/ml PI, and analysed by flow cytometry using FACS Calibre (Becton Dickinson, BD, USA). The cell cycle distributions were calculated using Cell Quest software (Becton Dickinson).

One of the well-recognized hallmarks of cells going into apoptosis is the externalization of the phospholipid phosphatidylserine at the cell membrane (Kavanagh et al., 2016).

In the present study, the apoptotic effect of *E. pulcherrima* and *E. heterophylla* was further assessed by Annexin V-FITC/PI (AV/PI) dual staining assay to examine the occurrence of phosphatidylserine externalization and also to comprehend whether cell death is due to physiological apoptosis or nonspecific necrosis.

Assay of the apoptosis marker caspase-3 level

Human Caspase-3 ELISA Kit (ab285337, K4221) is a sandwich ELISA assay for the quantitative measurement of human Caspase-3 in serum, plasma and cell culture supernatants. The density of color is proportional to the amount of Caspase-3 captured from the samples.

Statistics

Statistical analysis was performed by one-way analysis of variance (ANOVA) using statistical package of social science (SPSS) version 15.0, for windows $P<0.05$ were considered as a level of significance.

Results

Proliferation of HepG2 cells after irradiation

The viability was checked 48 h after exposure of the cells to different doses of radiation (5, 10 and 15 Gy). Cell inhibition increased to 70.15 ± 5.69% after 48h of exposure to 15 Gy of irradiation. Radiation at a dose of 10 Gy increased the inhibition to51.69±4.99%. IR inhibited cell viability in HepG2 cells in a dose-dependent manner. (Table 1).

TABLE 1. Effect of gamma radiation on HepG2 cell inhibition at 5, 10 and 15 Gy

Gamma radiation dose (Gy)	% Inhibition
Control (0)	09.23±1.89
5	19.87±2.57
10	51.69±4.99
15	70.15±5.69

Effect of E. pulcherrima & E. heterophylla and IR on cancer cell viability

To determine the effects of *E. pulcherrima & E. heterophylla* in combination with IR on hepatocellular carcinoma, MTT bioassay on HepG2 cell line was performed. A significant enhancement of the radiation response was found after treatment with *E. pulcherrima& E. heterophylla* (Table 2) ($P<0.05$). These results demonstrated that the inhibitory effect induced by the combination of each of *E. pulcherrima & E. heterophylla* and IR on cell inhibition was highly effective. Moreover, it was found that *E. pulcherrima & E. heterophylla* combined with 10 Gy γ radiation enhance the cytotoxicity in addition to being safer than 15 Gy. Thus, the dose of 10 Gy was selected to fulfill the subsequent experiments.

Effect of radiation exposure and (E. pulcherrima & E. heterophylla) treatment on Bcl-2, Bax and P53

The effect of radiation exposure and *E. pulcherrima* and *E. heterophylla* on the expression levels of Bcl-2 (apoptotic inhibitor) and Bax (apoptotic promoter) in HepG2 cells was investigated using RT-PCR. As shown in Fig. 1. Exposure of cells to the IC50 of *E. pulcherrima* and *E. heterophylla* for one hour, followed by exposure to 10 Gy gamma rays and incubation for 48 h, resulted in a significant decrease in anti-apoptotic Bcl-2 protein expression, which was associated with an increase in pro-apoptotic Bax protein expression. Radiation exposure and *E. pulcherrima& E. heterophylla* treatment also increased the p53 levels in HepG2 cells.

The combination of extracts and IR showed 17.45823 and 10.9479 folds higher p53 level ($P< 0.05$) with *E. pulcherrima& E. heterophylla*; respectively in the nucleus compared to the control (Table 3).

Effects on the levels of active caspase-3

The results in Table 4 show that the treatment of HepG-2 cells followed by exposure to 10 Gy γ rays resulted in a significant elevation in the activity of caspase-3 by about 11.97and 8.91folds; respectively compared to the control.

Annexin V-FITC apoptosis

Flow cytometric analysis revealed that HepG2 cells treated with *E. pulcherrima & E. heterophylla* followed by exposure to 10 Gy gamma rays showed a significant increase in the percentage of annexin V-FITC positive apoptotic cells (URþLR) from 1.69% to 17.22% and 14.07; respectively, which comprises about 10 and 8 folds compared to the control (Fig. 2).

TABLE 2. Cytotoxicity of the two extracts of *E. pulcherrima* & *E. heterophylla* on HepG2

Plant	IC_{50}	IC_{50} Exposure to extract before radiation (µg / ml)			IC_{50} Exposure to extract after radiation (µg / ml)		
		5 Gy	10 Gy	15 Gy	5 Gy	10 Gy	15 Gy
E. pulcherrima	>100	19.75±1.78	19.62±1.91	3.123±1.83	81.96±6.685	75.14±6.905	67.18±8.145
E. heterophylla	>100	38.06±4.23	34.3±5.57	17.53±0.86	>100	>100	>100
Doxorubicin**	20.6±1.2	20.5±1.7	20.7±1.8	19.2±2.1	19.9±2.2	21.6±1.3	20.9±0.9
-ve control	>100	>100	>100	>100	>100	>100	>100

*Results are expressed as means of three experiments (µg / ml). ** Doxorubicin is the positive control.

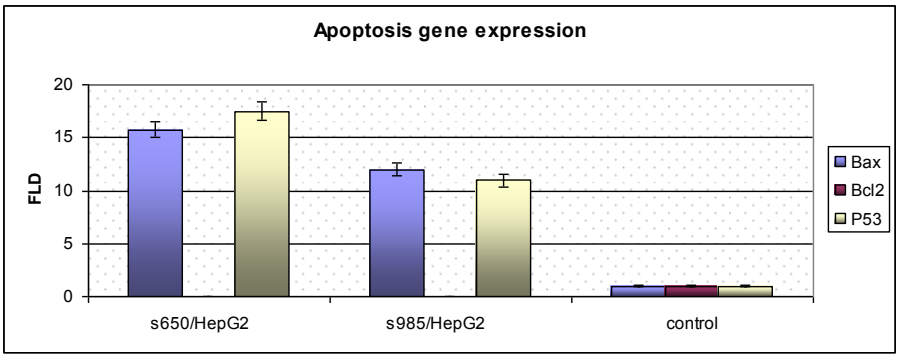

Fig. 1. Effect of *E. pulcherrima & E. heterophylla* and gamma radiation on apoptosis- related genes (Bcl-2, Bax and P53) on HepG2 treated with each extract at its IC_{50} concentration

TABLE 3. Effect of *E. pulcherrima & E. heterophylla* and gamma radiation on apoptosis- related genes (Bcl-2, Bax and P53) on HepG2 treated with each extract at its IC_{50} concentration

Sample	BAX	BCL2	P53
	Fold change	Fold change	Fold change
E. pulcherrima	15.76989	0.020964	17.45823
E. heterophylla	11.97583	0.05493	10.9479
Control	1	1	1

TABLE 4. Effect of *E. pulcherrima* & *E. heterophylla* on the active caspases-3 level in HepG2 cells treated with each extract at its IC_{50} concentration

Extract	Caspase-3 (pg / ml)
E. pulcherrima	642.79 (11.97)*
E. heterophylla	478.8 (8.91)*
Control	53.7

* Numbers given between parentheses are the number of folds of the control.

Discussion

The HCC is a fatal malignancy with a poor prognosis. Radiation therapy has no function in HCC treatment policy due to its exceedingly damaging effects on normal liver tissue. With radiotherapy evolving as a treatment option, it may become one of the non-surgical options for HCC. However, increasing the dose of radiation is impractical for most patients with advanced liver cancer who also have significant liver disorders such as cirrhosis and hepatitis (Schaub et al., 2018).

Many phytochemicals, including camptothecin, epigallocatechin gallate (EGCG), paclitaxel, etoposide, curcumin, and others, have significant growth inhibitory and apoptosis inducing effects on human and animal cancer cells in vitro by targeting multiple cellular signaling pathways in vitro. As a result, these chemicals may be useful in combination with standard chemotherapeutic agents/radiation for the treatment of cancer, with minor toxicity, but better efficacy. Furthermore, new in vivo pre-clinical investigations and clinical trials have offered more evidence in favor of multi-targeted medicines in combination with natural ingredients (Hazra et al., 2012).

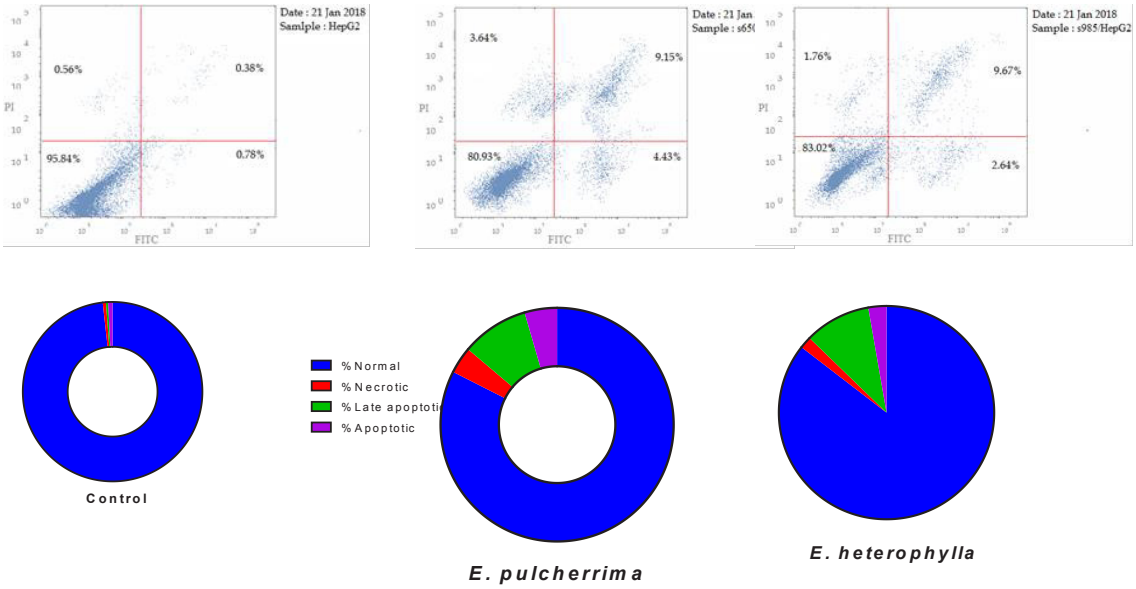

Fig. 2. Effect of *E. pulcherrima* & *E. heterophylla* followed by exposure to 10 Gyγ rays on the percentage of annexin V–FITC-positive staining in HepG2 cells

In the present results both *E. pulcherrima* and *E. Heterophylla* are promising when combined with γ-radiation. The underlying mechanisms however, by which both *E. pulcherrima and E. Heterophylla* are largely unknown for their effects with γ radiation. This effect may be due to alterations in DNA repair caused by inhibiting the DNA damage repair mechanism, which has not determined yet. Such result suggested that radiosensitivity to *E. Pulcherrima& E. heterophylla* may result from the increase in DNA damage caused by γ radiation**.** In addition, the treatment of HepG-2 cells by *E. pulcherrima* and *E. Heterophylla* followed by irradiation resulted in a significant elevation in the activity of caspase-3. It has been reported that Caspases, a family of aspartic cysteine proteases, are the critical apoptosis mediators that provide vital links in cell-regulating networks that regulate cell deaths. Caspase-3 is the main caspase executioner that ultimately modifies the proteins responsible for apoptosis (Van Opdenbosch & Lamkanfi, 2019).

As shown in the results, treatment of HepG-2 cells by *E. pulcherrima* and *E. Heterophylla* Following irradiation, induced a considerable increase in caspase-3 activity, which could be attributed to the radio-sensitizing action of *E. pulcherrima* and *E. heterophylla*.

Ionizing radiation is known to cause oxidative stress in cancer cells by producing reactive oxygen species (ROS), resulting in an imbalance in the cells' redox equilibrium, which is thought to lead to cell death (Das et al., 2017).

Molecular oxygen has long been recognized as a powerful radiosensitizer that enhances the cell-killing efficiency of ionizing radiation. Ionizing radiation produces hydroxyl free radicals upon encountering the abundant water molecules in biological systems. Subsequent interaction between hydroxyl free radicals and DNA strands results in the formation of DNA-derived free radicals. In the absence of O2, the DNA free radicals can be relatively easily restored through chemical reduction by sulfhydryl chemical reduction, which leads to "fixation" of DNA damages (Liu et al., 2015).

Apoptosis in cancer cells is investigated in this study. HepG2 cells treated with *E. pulcherrima*, *E. heterophylla*, and gamma radiation showed increased p53 and Bax with decreased Bcl2 overexpression of Bax and higher level of p53 which were observed in co-treated group indicated that p53 augmented Bax expression (Das et al., 2017). It was discovered that HepG2 cells in culture had a more cytotoxic response to radiation than that of the untreated cells. The increased pro-oxidant activity of the radiation group could be the cause of increased cytotoxicity in tumor cells

(Aleksandrov et al., 2019). An earlier study found that flavonoids, triterpenes, and polyphenols, such as those found in *Euphorbia* species, have pro-oxidant activity and operate as an inducer of intracellular oxidative stress in tumor cells, in addition to the generation of reactive oxygen species. The cytotoxic activity and metabolic profiling of Euphorbia species reveals cytotoxic activity against HepG2 and MCF-7 cells (El-Hawary, 2020).

Several researches have reported that several *Euphorbia* species may have cytotoxic action against various cell line models *(*Aleksandrov et al., 2019; Salehi et al., 2019). Moreover, treatment using *E. pulcherrima* and *E. heterophylla* followed by exposure to 10 Gy gamma rays showed a significant increase in the percentage of annexin V-FITC positive apoptotic cells (URþLR).

Radiation exposure would largely produce intracellular reactive oxygen species (ROS, such as superoxide and hydroxyl radicals), which would result in DNA strand breakage and biomolecule conformational changes (Deng et al., 2021). Furthermore, a combination regimen of diospyrin's diethtylether derivative (D7) and 5 Gy radiation applied in two fractionated doses (2.5 Gy each) could provide a considerable reduction of tumor growth and improved life span in experimental mice, restoring liver enzyme function to normal levels (Hazra et al., 2012).

Many various factors can alter cytokine profiles, including gene expression and cell death caused by radiation exposure, as well as radiation dose, tissue type, and tumor cell inborn features, which might influence the local response into a pro- or anti-neoplastic effect (Di Maggio et al., 2015).

In the case of solid tumors, ionizing radiation has been the treatment of choice in addition to surgery and chemotherapy (Kinsella, 2011). However, a significant proportion of such tumors would fail to respond well to radiation treatment and would require a very large dose to be eliminated, posing a significant limitation to radiotherapy. Furthermore, radiation injury to the surrounding normal tissues, as well as the cancer patient's skin, brain, heart, lung, kidney, liver, or gastrointestinal tract, would result in unfavorable effects. Moreover, symptoms such as tissue fibrosis, hair loss, xerostomia, xerophthalmia, and others significantly limit the use of a high dose radiation targeting the tumor-bearing organs (Dest, 2006; Issani, 2022).

The *E. pulcherrima* and *E. Heterophylla* extracts in combination with 10 Gy γ radiation show an obvious boost of cytotoxicity in the HepG2 cell line. Based on the results currently available, *E. pulcherrima* and *E. Heterophylla* increased the radiation response significantly, as the percentage inhibition of hepatocellular carcinoma HepG2 cells in combination therapy was significantly lower than that of the control group, suggesting that *E. pulcherrima* and *E. Heterophylla* are potential sensitizers to cancer radiation therapy. This is the first report of radiation sensitization of HepG2 cell by *E. pulcherrima* and *E.heterophylla,* along with the evidence-based results. The doses were tuned out combining the gamma radiation and *E. pulcherrima* and *E.heterophylla* on HepG2 cells.

Conclusion

The present study confirmed that *E. pulcherrima* & *E. heterophylla* have a radiosensitizing effect on HepG2 cells. The combined dose not only prevented the survival, but also enhanced the cell cycle arrest and apoptosis of HepG2. Future in vivo clinical studies in this combination may test the effectiveness of the current approach.

Author contributions: Salwa M. El-Hallout, Lamiaa H. Gohar & Eman M. Elgazzar contributed to the achievement of the present research, analysis of the results, and writing of the manuscript.

Data availability: The data and materials used in the present study are available from the corresponding author on reasonable request.

Ethical approval: This experiment was carried out according to the international guidelines of animal handling and care (NIH no. 85:23, 1996). Human participants, human data or human tissue are not applicable.

Competing Interests: The authors declare that they have no conflict of interest.

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