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Polyphenols, Luteolin and Pelargonidin, Modulate Radio- and Chemo-Sensitivity on Breast Cancer

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THE present study aims at elucidating the role of luteolin (LUT) in modifying the sensitivity of gamma radiation and cisplatin toward breast cancer. Likewise, pelargonidin (PEL) has been tested for its capability to enhance radiosensitivity. Human breast (MCF-7) cancer cell lines were incubated at different doses of LUT and PEL. Cells were exposed to gamma radiation combined with different LUT and PEL doses. The MTT test and calcein staining were used to evaluate the cell viability. For evaluation of LUT biocompatibility with normal mammary tissues, female Wistar rats were divided into three experimental groups (n= 6): control, irradiated (6 Gy), Irradiated (6 Gy) + LUT (dose 30 mg/kg, i.p.). Mammary tissues were collected for further determination of oxidative stress and lactated dehydrogenase (LDH). Either LUT or PEL could increase the sensitivity of gamma radiation applied on MCF-7 cells. In parallel, LUT modified the cisplatin effects on the same cell line. LUT exerted its cytotoxic impact through the GSK-3β-dependent effect, which was elucidated through the combined downregulation effect of GSK-3β inhibitor on MCF-7 cells. Moreover, LUT showed a protective effect on normal mammary tissues in the irradiated rats marked by balanced oxidative status and reduced LDH level. In sum, polyphenols (LUT or PEL) could be used as adjuvant therapeutic agents in the treatment of breast cancer with a further protective effect of normal healthy tissues against cytotoxic therapies.

Keywords: Cisplatin, Luteolin, MCF-7, Pelargonidin, Radiation.

Introduction

Many epidemiological studies emerge the prevalence of breast cancer as a prevalent reason of mortality among women aged 35 to 55 years old (Parkin et al., 2005; Ronckers et al., 2005). Only 5% of genotypic abnormalities inherited for breast cancer cases are known, whereas other sporadic occurrences remain unclear (Osborne et al., 2004). Different treatment strategies are currently accounted for various breast malignancies, ranging from chemotherapies, radiotherapies, immunotherapies, and surgical inclination. Clinically, no validated diagnostic tools and prominent biomarkers are reliable, reflecting the disease progression and the hereafter successful therapy. The major challenge facing clinicians is predisposed resistance toward the applied treatment regimen.

Polyphenols are chemically known components comprising in many plants and representing the antioxidant value in the human diet. Numerous studies revealed the therapeutic benefit of polyphenols, such as flavonoids and anthocyanins, against many pathological conditions (Martin & Appel, 2010; D'Archivio et al., 2008). Luteolin (LUT), a flavone subclass, is commonly constituted in flowering plants such as chamomile tea and green peppers (Lin et al., 2008). Previous literature elucidated the medical applications of LUT in the treatment of pathological disorders icluding but not limited to neurological and cardiovascular diseases,

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diabetes, pancreatitis, and cancers (Griffiths et al., 2016). Its pleiotropic antineoplastic activity has been examined on various cancer cell types, concluding several cellular pathways. These mechanisms include stimulation of apoptosis, cell cycle arrest, inhibition of cancer cell viability and proliferation, suppression of angiogenesis, and overwhelming metastasis tendency (Ruan et al., 2012; Ding et al., 2014). Evidence indicates that LUT could modulate multidrug resistance as well as stimulate chemosensitization on several cancers cell lines (Rao et al., 2012; Dia & Pangloli, 2017).

Anthocyanins are highly found as pigments responsible for the colors in fruits and vegetables. Through previous findings, anthocyanins are implicated in health benefits through the antioxidants-rich chemical structure (Miyazawa et al., 1999). In addition, anthocyanins have shown a dual-action; they have been revealed to protect against DNA damage (Lazzé et al., 2003) and to induce cellular death (apoptosis) of many human cancers *in* vitro; such as leukemia and colorectal cancer cells (Katsube et al., 2003). Pelargonidin (PEL) [2-(4-hydroxyphenyl) chromenylium-3,5,7- triol], an anthocyanidin, has been reported to possess a strong antioxidant effect via reactive oxygen species (ROS) scavenging ability and an anti-inflammatory role through its master inhibition of nuclear factor kappa B (NF-κB) activity (Hämäläinen et al., 2007). PEL has been investigated in numerous preclinical models, such as diabetes, cardiovascular disorders, and cancers (Noda et al., 2002; Min et al., 2018).

One of the causes that underline the incidence of breast carcinogenesis is exposure to ionizing radiation (Laden & Hunter, 1998; Ronckers et al., 2005). The molecular mechanisms that define the etiology of radiation-stimulated breast cancer are dominated by genetic and epigenetic alterations (Shackney & Silverman 2003, Simpson et al., 2005).

Despite the plethora of findings with the antineoplastic efficacy of LUT and PEL on cancer cells, the present study investigates whether these natural ingredients can sensitize human breast cancer (MCF-7) towards the conventional therapeutic approaches (radiation or alkylating agents), suggesting their roles as adjuvant treatment of breast cancers. Furthermore, the underline biocompatibility of LUT have been tested with healthy mammary tissue under acute radiation exposure.

Materials and Methods

Chemicals and drugs

Cisplatin (CIS, cis-diammine platinum (II) dichloride was obtained from Mylan, Canonsburg, PA, USA). All other chemicals and reagents were purchased from Sigma-Aldrich (Saint Louis, Missouri, USA) or the purest analytical grade available. The Luteolin (LUT, Purity > 98%) was supplied by AXENIC, UK, while Pelargonidin (PEL) was purchased from Sigma-Aldrich (Saint Louis, Missouri, USA).

In vitro experiments

Cell culture

Cryopreserved human breast cancer (MCF-7) cells were purchased from the Cell Bank of VACSERA (Cairo, Egypt). Cells were cultured in a humidified atmosphere (5% CO2, 37°C) with high glucose Dulbecco's Modified Eagle Medium (DMEM) medium supplemented with 10% Fetal Bovine Serum (FBS) and 1% penicillin-streptomycin at cells were passaged at 80–90% confluency after trypsinization with a pre-warmed trypsin-EDTA solution.

Cells treatment and gamma irradiation protocol

Cells were treated with different LUT concentrations (serial dilution, from 6.25 to 200 μM), 2 h before incubation with CIS (5 μM). In other experiments, MCF-7 cells were incubated with different LUT or PEL doses (serial dilution, from 25 to 300 μM) before being exposed to gamma radiation (2 Gy). The irradiation of process was carried out at the National Center for Radiation Research and Technology (NCRRT), Egyptian Atomic Energy Authority (EAEA), Nasr City, Cairo, Egypt, using the Gamma cell®-40 biological irradiator with Caesium137 source (Atomic Energy of Canada Limited; Sheridan Science and Technology Park, Mississauga, Ontario, Canada) at a 0.643 Gy/min dose rate. To elucidate the possible glycogen synthase kinase-3β (GSK-3β)-dependent mechanism, cells were pretreated with GSK-3β inhibitor (AR-A 014418, 1 μM) before being treated with LUT (El-Hamoly et al., 2017).

Cell viability and proliferation

The cell viability of all samples was investigated on MCF-7 cell lines using MTT assay. The selected

samples were two-fold diluted in culture media. Cells were treated with 100 µl of each sample and incubated for 24 hrs further. Then, 20µl of MTT yellow solution was added to each well and incubated for 1-5 h. Finally, the produced formazan (purple product) was dissolved in 200 µl DMSO, and the mean absorbance of three replicates was measured at 570 nm. The calcein-AM stock solution was added to each well with a final concentration 1 µM for 30 min at 37°C. Fluorescent intensity was quantified in the fluorometry plate reader at 480 and 530.

Cell proliferation was monitored using a colony formation assay. Briefly, 300 cells/well were grown in 6-cm plates and maintained in Dulbecco's modified Eagle's medium (Thermo Fisher Scientific, Inc.) containing 10% FBS with or without various concentrations of LUT. After 3 days, the number of viable cells was determined using MTT assay (El-Hamoly et al., 2017).

In vivo experiments

Animals

Animals used in the present study were adult female Wistar rats, weighing 200 ± 20 g, obtained from the National Research Center, Giza, Egypt. Prior to the experiment, rats were acclimatized for one week at the animal facility of the NCRRT-EAEA, with free access to a standard chow diet, tap water, and a 12 h day-light cycle. The study was conducted following the Environment-European Commission's (EEC) regulations (Revised Directive 86/609/EEC) guidelines.

Irradiation protocol

Whole-body irradiation of animals was carried out at the NCRRT-EAEA using the Gamma cell®-40 biological irradiator with Caesium137 source. In the plastic sample tray, non-anesthetized rats were placed and irradiated at 6 Gy as a single exposure delivered at a dose rate of 0.643 Gy/min.

Experimental protocol

Animals were allocated into three experimental groups (6 rats/group). Group I (control): Rats received oral saline solution. Group II (Irradiated, IRR): Rats were exposed to a single-radiation dose level of 6 Gy. Group III: Rats were exposed to 6 Gy, and 1 h later, they were treated with LUT, dissolved in saline, for 3 successive days at a dosage of 30 mg/kg intraperitoneal, according to Lodhi et al. (2019) and Zhang et al. (2021).

Twenty-four hours later, rats were anesthetized by ether, and blood was withdrawn from the heart for serum separation. Mammary gland pairs from the abdominal sector were carefully dissected and homogenized in various media according to the parameter to be assessed (20% w/v homogenate) using a Glass-Col homogenizer (Terre Haute, Indiana, USA) and kept at -80 °C for subsequent analysis.

Determination of thiobarbituric acid reactive substances (TBARS) and reduced glutathione contents

The mammary gland was homogenized in a buffer containing 1.5% potassium chloride to determine the lipid peroxidation. According to Uchiyama & Mihara (1978), the concentration of lipid peroxidation was calculated as thiobarbituric acid reactive substances (TBARS) and measured colorimetrically. Briefly, 0.5 ml of tissue homogenate was mixed with 3.0 ml of 1% ortho-phosphoric acid and 1 ml of 0.6% thiobarbituric acid aqueous solution. The mixture was left for 45 min in a boiling water bath. Then, samples were cooled in an ice bath, and 4 ml of n-butanol were added. The contents were mixed for 40 sec with a vortex mixer and then centrifuged at 3500 rpm for 10 min using a cooling centrifuge (Hettich® Zentrifugen, Mikro 22/22R, Germany). The organic layer was separated and read at 535 nm against blank using a Unicam 8625 UV/V spectrophotometer (Cambridge, UK). On the other side, the dissected mammary gland was homogenized in meta-phosphoric acid to assess the concentration of reduced glutathione (GSH), according to Beutler et al. (1963). The supernatant was separated at 3000 rpm for 15min using a cooling centrifuge, where 5,5`-dithio-bis (2-nitrobenzoic acid (DTNB) was added to the supernatant to give a yellow derivative that was spectrophotometrically measured at 412 nm.

Determination of total nitrate/nitrite

The mammary gland was homogenized in normal saline, and total nitrate/nitrite (NOx) content was measured following the method of Miranda et al. (2001). NOx protein content was precipitated by ethanol for 48 hrs, and then the homogenates were centrifugated at 15000 rpm/ 1 h. The separated supernatants were incubated with vanadium trichloride and Griess reagent [0.1% N-(1-naphthyl) ethylenediamine dihydrochloride, 1% sulfanilamide] at 37 oC for 45 min. Finally, the nitrite contents were measured at 540 nm.

Determination of lactate dehydrogenase activity

As a marker of cellular integrity, serum LDH activity was assessed using the kinetic ultraviolet (UV) kit purchased from BioSystems®, Barcelona, Spain.

Statistical analysis

All values are presented as mean ± standard error of the mean (SEM). Statistical analysis of experimental data was performed with one-way analysis of variance (ANOVA), followed by Tukey-Kramer Multiple Comparison Test. Results were considered statistically significant when at least $P< 0.05$. Graph pad software instant (version 8) was used to carry out these statistical tests.

Results

Pelargonidin and Luteolin downregulates the viability of MCF-7 cells in dose-dependent effect

The cytotoxicity was assessed using MTT assay. As shown in Fig. 1A, PEL significantly inhibited the viability of MCF-7 cells at a 300 µM dose level compared to untreated cells. On the other hand, cytotoxicity of LUT on MCF-7 cells was concluded in dose-dependent effect (Fig. 1B).

Pelargonidin and Luteolin enhances the sensitivity of gamma irradiation on MCF-7 cells

As for 2 Gy irradiated MCF-7 cells, a marked decrease in cell viability was observed upon incubation with either PEL or LUT compared to the untreated irradiated cells (Fig. 2 A, B). Such findings were demonstrated in a concentration-dependent manner.

Luteolin modulates proliferation of MCF-7 in a dose- and time-dependent effect

As shown in Fig. 3 A, LUT inhibited MCF-7 cells proliferation in concentration-dependent effect as indicated by colony formation test. To further assess the long-term effect of LUT treatment on cell proliferation, LUT inhibited the viability of MCF-7 cells in a time-dependent manner (Fig. 3B).

Luteolin promotes the cytotoxicity of cisplatin on MCF-7 cells

As indicated by calcein stain uptake, the viability of MCF-7 cells treated with CIS+LUT was significantly decreased compared with the 5 µg/ml CIS alone, amounting about 25% cytotoxicity. The inhibition was significant when cells were incubated with 50 and 100 µM LUT concentrations (Fig. 4).

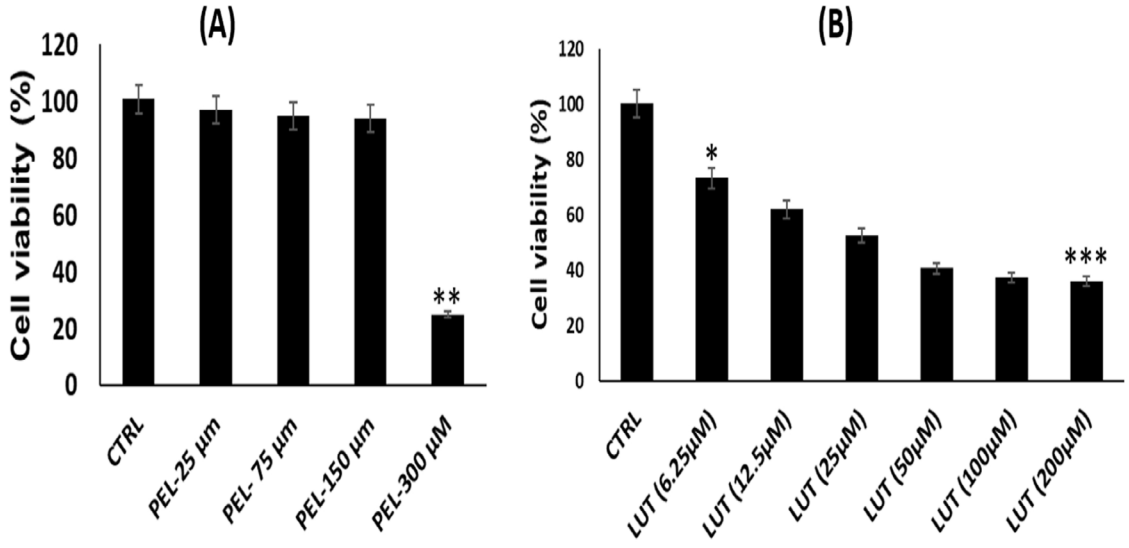

Fig. 1. Pelargonidin and Luteolin downregulate the viability of MCF-7 cells in dose-dependent effect, (A) Dose-dependent anti-tumor effect of Pelargonidin (PEL) on MCF-7 cells after 24 h; (B) Luteolin (LUT) exhibited a marked anti-tumor effect on the MCF-7 cell line determined by the MTT test [Vehicle-treated cells are served as control. Data are expressed as the means ± SEM. (n= 3). *P<0.05, ***P <0.001 vs. untreated irradiated cells]

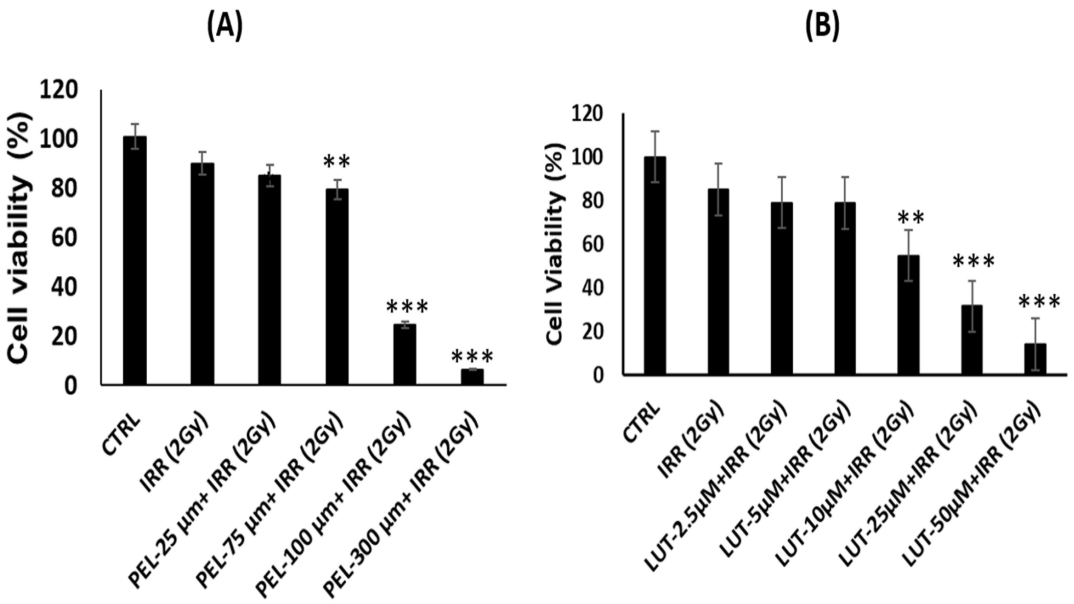

Fig. 2. Pelargonidin and Luteolin enhance the sensitivity of gamma irradiation on MCF-7 cells. Cells were exposed to gamma-radiation (2 Gy) 2 h after incubation with different treatment agents. MCF-7 cells were treated with dose-dependent of (A) Pelargonidin (PEL) or (B) Luteolin (LUT). Twenty-four-hour later, cell viability was determined via MTT assay [Data are presented as the means ± SEM (n= 3). *P<0.05, **P <0.01, ***P<0.001 vs. untreated irradiated cells]

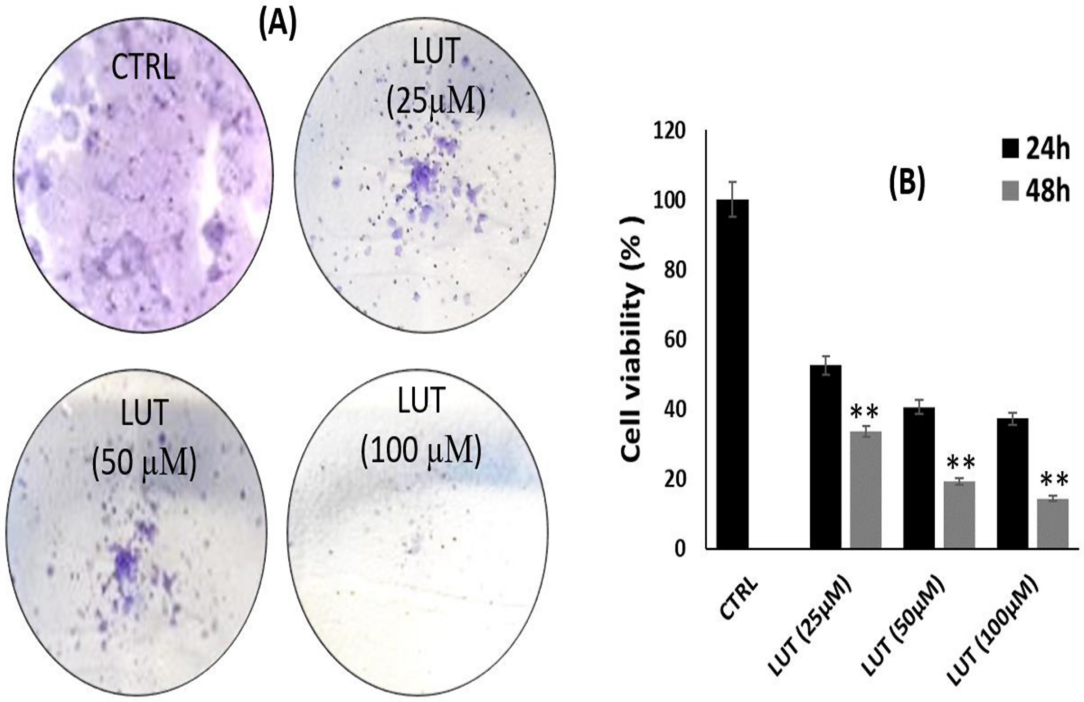

Fig. 3. Luteolin modulates proliferation of MCF-7 in a dose- and time-dependent effect. (A) Luteolin (LUT) showed a significant anti-tumor effect on the MCF-7 cell line determined by colony formation assay. (B) The proliferation of MCF-7 cells was inhibited by the dose-dependent effect of LUT [Data are presented as the means ± SEM. (n= 3). **P <0.01 vs. untreated control]

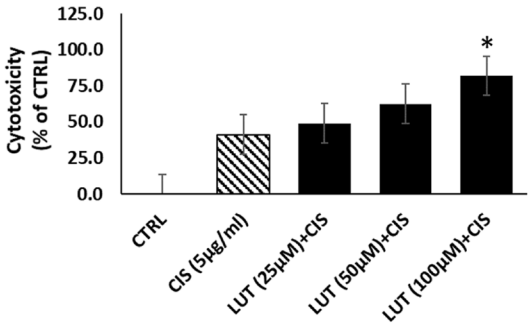

Fig. 4. Luteolin stimulates the cytotoxicity of cisplatin on MCF-7 cells. Cells were treated with cisplatin (CIS, 5 µM) 2 h after incubation with different doses of Luteolin (LUT). Twenty-four-hour later, cytotoxicity was determined via MTT assay [Data are presented as the means ± SEM. (n=3). *P<0.05 vs. cisplatin-treated cells]

Inhibition of glycogen synthase kinase-3β (GSK-3β) attenuates the cytotoxic effect of Luteolin on MCF-7

As shown in Fig. 5, GSK-3β inhibitor (AR-A 014418) at a dose level of 1 µM did not exert any significant effect on the viability of MCF-7 cells compared to the untreated control. MCF-7 cells pretreated with GSK-3β inhibitor for 2hrs before being incubated with LUT for 24 hrs exhibiting a promotion of cell viability compared to GSK-3β inhibitor-untreated cells.

Luteolin protects normal mammary tissues of female rats against oxidative stress and cellular damage induced by gamma irradiation

Excessive production of ROS and reactive nitrogen species (RNS) through irradiation is a checkpoint for tissue damage. Exposure to gamma radiation revealed about 1.5-fold increases in TBARS, a marker for lipid peroxidation (Fig. 6 A) associated with a more than 45% decrease in reduced GSH (a marker of antioxidant capacity) contents (Fig. 6 B), compared to the normal control. Furthermore, about 2.5-fold increases in NOx content have been recorded upon irradiation comparable to the normal control (Fig. 6 C). Additionally, the serum level of LDH of irradiated rats has been significantly increased, reaching a 2.7-fold increase compared to the normal control (Fig. 6 D). Treatment with LUT largely prevented the development of these changes as it tends to normalize the TBARS, GSH, and NOx contents in the mammary gland. In parallel, upregulation of serum LDH level was corrected upon LUT administration (Fig. 6).

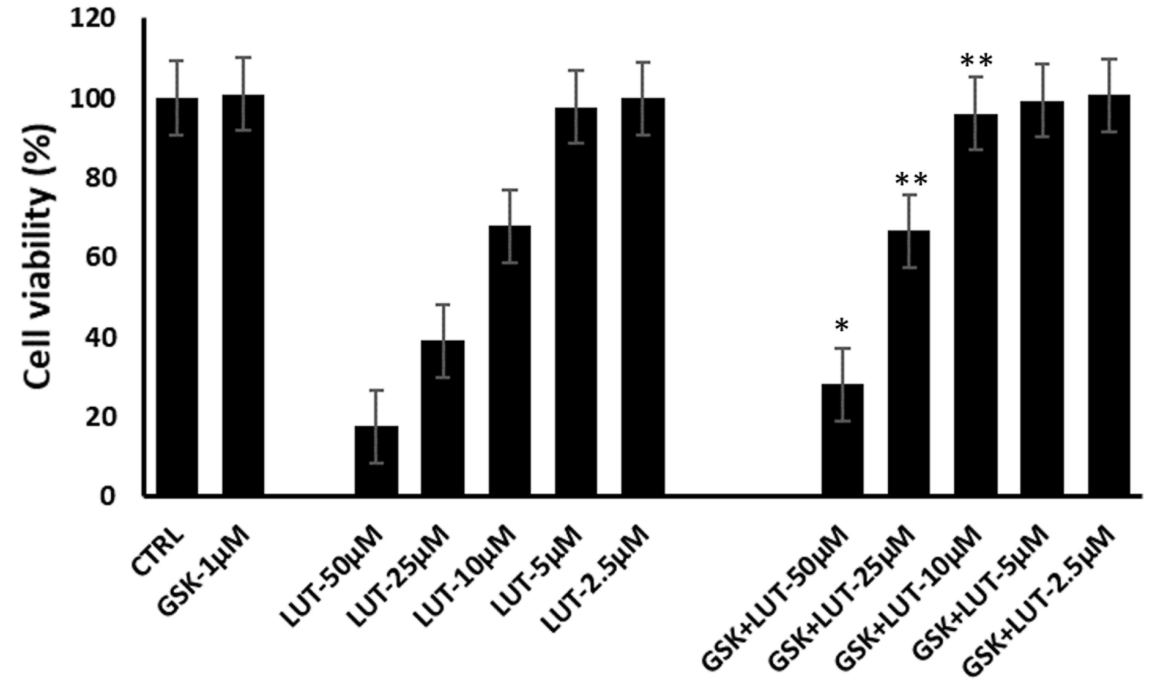

Fig. 5. Inhibition of GSK-3β attenuates the cytotoxic effect of Luteolin on MCF-7. Cells were pretreated with GSK-3β inhibitor (AR-A 014418, 1 µM) for 1 h. Then, cells were incubated with Luteolin (LUT) in different doses [Data are presented as the means ± SEM. (n= 3). *P<0.05 , **P <0.01 vs. LUT-treated cells]

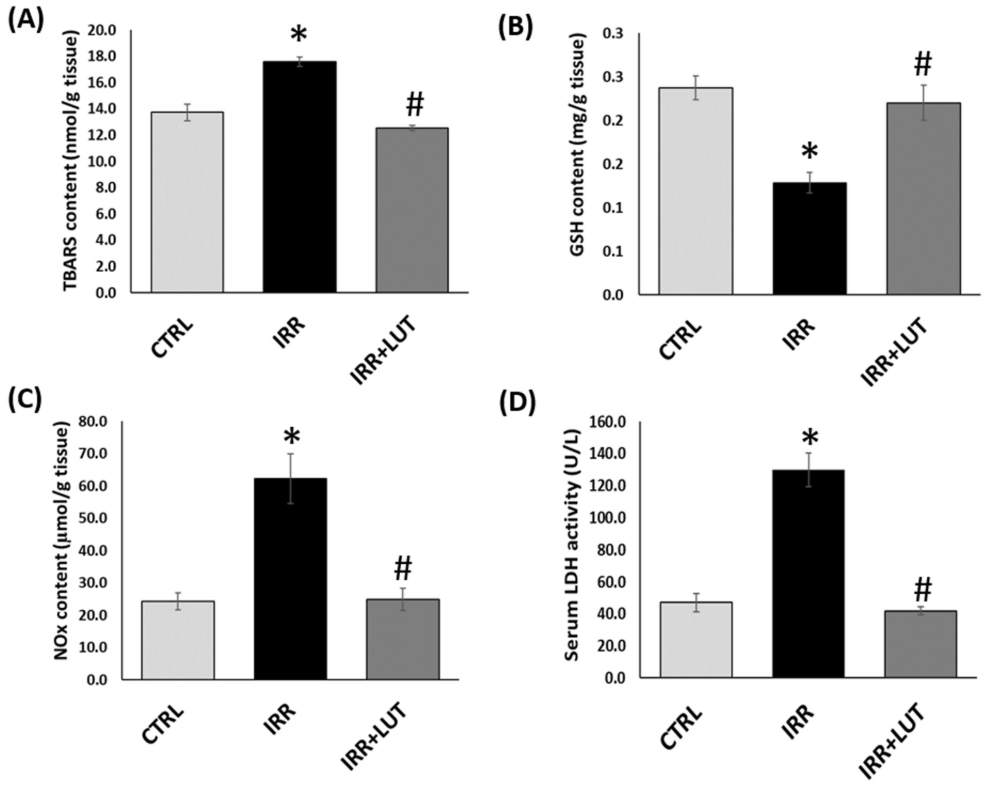

Fig. 6. Luteolin protects normal mammary tissue upon gamma irradiation in an *in vivo* model. Female Rats were subjected to whole-body gamma radiation at 6 Gy, 1 h later, the rats were treated with either saline or Luteolin (LUT, dose 30 mg/kg, i.p.) for three consecutive days. Oxidative stress was evaluated by (A) thiobarbituric acid reactive substances (TBARS), (B) reduced glutathione (GSH), and (C) total nitrate/ nitrite (NOx) contents in mammary glands

Discussion

Cancer resistance to commonly used therapeutic protocols and the incidence of metastatic response henceforward arises the awareness toward exploring new treatment approaches. Based on recent demographic studies, breast cancer represents the vastest cause of cancer-associated mortality in women (Ronckers et al., 2005). In the current study, the authors tried to find out therapeutic agents that were successfully recruited to increase the efficiency of commonly used anti-cancer chemotherapy and radiation therapy. Moreover, it could exert a certain protective effect for normal intact tissue that is probably affected thereafter.

In the present study, LUT and PEL significantly inhibited the viability of the human breast cancer cells (MCF-7) *in vitro*. It was identified that the two agents could increase the sensitivity of the IR-inhibitory effect of cell viability in dose-dependent effect. These outcomes provided evidence for their compatible anti-breast cancer effects. Kamenickova et al. (2013) revealed the anti-tumor effect of anthocyanins (PEL) for human hepatic cancer cells via AhR-CYP1A1 activation-dependent signal pathways. A previous study indicated that LUT exerts anti-tumor activities on different cancer cell lines (Cook et al., 2016; Ding et al., 2014). LUT was selected for further assays concerning the dose adequacy versus generated efficacy. Notably, LUT significantly inhibited the MCF-7 cell proliferation in a dose- and time-dependent manner, as indicated by MTT and clonogenic assays. These findings were parallel with a previous study suggested that high concentrations of LUT exhibited cytotoxic effects on MDAMB231 cells (Huang et al., 2019).

In parallel, LUT stimulated the cytotoxic effect of CIS on MCF-7 cells. In the present study, LUT could exert its anti-tumor effects on MCF-7 cells by interfering with essential cell cycle regulators such as cyclin D1 and hence causing abnormal cell

cycle arrest. These possible mechanisms may be explained by Huang et al. (2019) on MDAMB231 cells. On different cancer models, LUT upregulated the pro-apoptotic factor Bax and downregulated the survival factor BCL2, suggesting that LUT modulated mitochondrial function to mediate cell death (Park et al., 2014).

It has been indicated from the present results that LUT could exert its cytotoxic properties on MCF-7 through the GSK-3β-dependent effect. As revealed by the viability test, the selective inhibition of GSK-3β significantly decreased the anti-tumor effects of LUT, suggesting the role of GSK-3β, and further investigations in the future are needed to emphasize this concept. Based on previous results, either LUT or PEL may be suitable for treating the resistance of human breast cancer cells toward chemotherapy and radiotherapy.

On an *in* vivo model of gamma irradiation, LUT administration significantly enhanced antioxidant capacity, characterized by elevation of reduced GSH accompanied with a reduction in nitric oxide and lipid peroxides formation revealed by TBARS contents. Similar LUT effects were reported by Ahlenstiel et al. (2006), who showed that administration of LUT protected against ischemia/reperfusion-induced renal tubular cells injury; the downregulation of TBARS contents connected to activating the antioxidant enzyme and lowering free radical production. Moreover, LUT has been reported to protect against the cellular damage indicated by the elevation of LDH levels. Such finding was parallel with the study of Ahlenstiel et al. (2006).

Conclusion

In conclusion, the present findings imply that polyphenols, such as LUT or PEL, might be employed as adjuvant chemo-preventive agents used to treat breast cancer with other currently deployed treatment regimens such as alkylating agents and radiotherapy. Moreover, LUT demonstrated its inhibitory effect on MCF-7 viability and proliferation via probable upregulation of the GSK-3β activity arc. In addition to its antitumorigenic effects, LUT has been shown to protect normal tissue from the detrimental effects of radiation.

Declaration of interest: The authors report no conflicts of interest.

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