

Luffa echinata Roxb. Induced Apoptosis in Human Colon Cancer Cell (SW-480) in the Caspase-dependent Manner and Through a Mitochondrial Apoptosis Pathway

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ABSTRACT

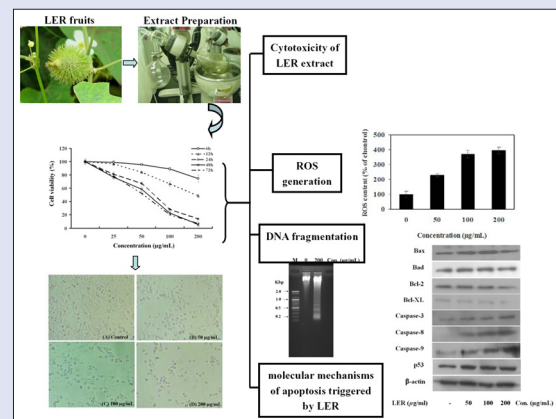
Background: *Luffa echinata* Roxb. (LER) (Cucurbitaceae) showed tremendous medicinal importance and are being used for the treatment of different ailments. **Objective:** In this study, the antiproliferative properties and cell death mechanism induced by the extract of the fruits of LER were investigated. **Materials and Methods:** MTT and LDH assay were used to test the antiproliferative and cytotoxicity of LER extract, respectively. The intracellular ROS were measured by a fluorometric assay. The expression of several apoptotic-related proteins in SW-480 cells treated by LER was evaluated by Western blot analysis. **Results:** The methanolic extract of LER fruits inhibited the proliferation of human colon cancer cells (SW-480) in both dose- and time-dependent manners. The LER-treated cells showed obvious characteristics of cell apoptosis, including cell shrinkage, destruction of the monolayer, and condensed chromatin. In addition, treatments of various concentrations of LER extracts caused the release of lactate dehydrogenase as a dose-dependent manner via stimulation of the intracellular metabolic system. LER induced apoptosis, DNA fragmentation, and cellular ROS accumulation in SW-480 cells. Treatment of LER on SW-480 cells promoted the expression of caspases, Bax, Bad, and p53 proteins and decreased the levels of Bcl-2 and Bcl-XL. **Conclusions:** These results indicated that treatment with LER-induced cell death in mitochondrial apoptosis pathway by regulating pro-apoptotic proteins via the up regulation of the p53 protein. These findings highlight the potentials of LER in the treatment of human colon cancer.

Key words: Apoptosis, caspases, colon cancer cells, *Luffa echinata* Roxb, p53

SUMMARY

- LER induced apoptosis, DNA fragmentation, and cellular ROS accumulation

in SW-480 cells. Treatment of LER on SW-480 cells promoted the expression of caspases, Bax, Bad, and p53 proteins and decreased the levels of Bcl-2 and Bcl-XL.



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INTRODUCTION

Colorectal cancer (CRC) is the third most common cancer in both males and females and the third leading cause of cancer death worldwide.^[1] CRC incidence is usually associated with obesity, a diet low in fruits and vegetables, physical inactivity, and smoking.^[2] So far, several strategies have been applied to cure this fatal disease, including surgical resection, chemotherapy protocol, and radiation therapy, which have several limitations.^[3-5] There is an urgent need for new therapeutic agents for CRC patients, such as some dietary phytochemicals, which can be used alone or in combination with other chemotherapeutic agents.^[6-8]

Luffa echinata Roxb. (LER) (Cucurbitaceae), a spreading climbing herb distributed throughout Pakistan, India, Bangladesh, and Northern Tropical Africa showed tremendous medicinal importance.^[9] Traditionally, various parts of the plant are being used for the treatment of different ailments such as jaundice, intestinal colic, leprosy, diabetes, bronchitis, nephritis, rheumatism, cirrhosis, anthelmintic, fever, diarrhea, and hemorrhoid disorder.^[9-13] Particularly, clinical studies revealed that the fruits of LER have a significant therapeutic effect on diabetes,^[14,15]

viral hepatitis,^[16] hypertension,^[17] and dropsy.^[18] In addition, it also has anthelmintic, diuretic, hepatoprotective, and anti-cancer activity.^[11,19-21] A number of biologically active compounds such as cucurbitacin-B, elatarin (cucurbitacin-E), elatarin-2-glucoside, isocucurbitacin B, β -sitosterol glucoside, chrysirol-7-glucoside, chrysirol-7-epiglucoside, echinatos A, echinatos B, and echinatin have been isolated from LER fruits.^[9,20,22,23]

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In the present study, the effects of LER on human colon cancer cells and the cellular and molecular mechanisms were further investigated to evaluate its potential as chemopreventive or chemotherapeutic agent against CRCs. The human colon cancer cell (SW-480) is a cell line widely used as an experimental model for *in vitro* studies of many normal and neoplastic processes. Thus, we investigated the effects of the methanolic extract of LER fruits on SW-480 cells.

MATERIALS AND METHODS

Plant material and extract preparation

LER fruits were dried in the shade at room temperature and powdered. Ten grams of sample were extracted with methanol (200 ml) for 72 h. The extract was filtered through filter paper (100 mm; Whatman, Maidstone, UK) and evaporated using a vacuum rotary evaporator (CCA-1110; Eyela, Tokyo, Japan) at 50°C to produce a crude extract. The dried sample was dissolved in dimethyl sulfoxide (DMSO), and the final concentration of DMSO in each sample is 0.1%. The control groups in each experiment were treated with 0.1% DMSO.

Cell lines and cell culture

The human colon cancer cell (SW-480) lines were maintained in Roswell Park Memorial Institute Medium 1640 (RPMI 1640). The media were supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 100 U/ml penicillin, and 100 µg/ml streptomycin. Cells were cultured in a humidified atmosphere and incubated at 37°C in 5% CO₂.

Anti-proliferation test

SW-480 cells were seeded in 96-well plates (1 × 10⁵ cells/well) and incubated in RPMI 1640, at 37°C in a humidified atmosphere of 5% CO₂ for 24 h. In addition, the SW-480 cells were incubated with 50, 100, and 200 µg/ml of LER extracts for 6, 12, 24, 48, 72 h, respectively. After removing supernatant of each well, a total of 20 µL of MTT solution (2 mg/ml in phosphate-buffered saline [PBS]) was added to each well at the time of incubation. After 4 h of incubation, the supernatant was discarded, and 200 µL of DMSO were added to each well to terminate the reaction. The absorbance was measured at 550 nm using an enzyme-linked immunosorbent assay (ELISA) plate reader (Bio-Tek, Winooski, VT, USA). For the treated cells, viability was calculated as the percentage of inhibition by the following formula:

$$\text{Inhibition (\%)} = (1 - [\text{OD}_s/\text{OD}_v]) \times 100\%$$

OD_s and OD_v indicated the optical density of cell lines incubated with sample and vehicle control, respectively.

Light microscopic examination

SW-480 cells were plated in 6-well plates (1 × 10⁵ cells/well) and incubated for 24 h. In addition, the cells were incubated with 50, 100, and 200 µg/ml of LER extracts for further 24 h and the cells were observed under a light microscope (Olympus, Japan).

Lactate dehydrogenase assay

The SW-480 cells were plated at a density of 1 × 10⁵ cells/well in a 96-well plate for 24 h, followed by treatment with various concentrations (25, 50, 100, and 200 µg/ml) of LER and incubated for 24 h. Four microliters (2% total volume) of Triton X-100 was added to the high control group 1 h before the testing. One hundred microliter of supernatant from the top of all the wells was mixed with the prepared detection kit reagent. After 30 min incubation, the absorbance was measured at 750 nm by an ELISA plate reader (Bio-Tek, Winooski, VT, USA).

DNA fragmentation assay

SW-480 cells (1 × 10⁶) were seeded in a 100 mm cell culture dish and incubated in RPMI 1640 for 24 h. After incubation, 200 µg/ml LER was added to the dish and kept for another 24 h. Then, cells were collected and washed with 1 × PBS, resuspended in 100 µl lysis buffer. Ten microliters of RNase (10 mg/ml) was added to the lysate and further incubated for 30 min at 37°C. The DNA samples were electrophoresed in a 1.5% agarose gel in Tris-acetate-EDTA buffer. The DNA bands were visualized by ethidium bromide staining and a Mini BIS image analysis system (DNR Bio-Imaging Systems Ltd., Kiryat Anavim, Israel).

Determination of reactive oxygen species level

The intracellular reactive oxygen species (ROS) were measured by a fluorometric assay using dichlorofluorescein diacetate (DCFH-DA) as the probe. The prepared SW480 cells (1 × 10⁶) were treated with 50, 100, and 200 µg/ml LER and incubated for 24 h, and then incubated with 10 nM of DCFH-DA for 30 min in the dark. After washed with PBS twice, the fluorescence was measured using an excitation of 485 nm and emission of 535 nm, in a SpectraMax[®] M2 Microplate Readers (Molecular Device, CA, USA) with an excitation filter of 485 nm and an emission filter 535 nm.

Western blot analysis

SW-480 cells were plated in 6-well plates at approximately 1 × 10⁶ cells/well and media were replaced with 10% FBS/RPMI. After 24 h incubation, the cells were treated with different concentrations of LER for 24 h. The harvested cells were washed with PBS twice and then lysed with cell lysis buffer (150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 50 mM Tris-HCl, pH 7.5, 2 mM storage) for 30 min at 4°C. The homogenates were centrifuged at 13,000 × g for 10 min to isolate the supernatants. Protein concentrations were determined using the pierce based on bicinchoninic acid protein assay. Thirty grams of the protein from the supernatants were then separated on 10% SDS-polyacrylamide gel and transferred to polyvinylidene difluoride (Bio-Rad) membranes. After transfer, the membrane was blocked at room temperature for 1 h with 5% BSA in Tris-buffered saline-Tween (TBST; 20 mM Tris, 500 mM NaCl, pH 7.5, 0.1% Tween 20). Then, the membranes were incubated with primary antibodies for 1 h, after three washes in TBST for 5 min, followed by incubation with secondary antibody for 1 h at room temperature. The immunoreactive protein bands were detected by using an enhanced chemiluminescence detection system. Blots were developed with a Mini BIS Image Analysis System (DNR Bio-Imaging Systems Ltd., Jerusalem, Israel).

Data analysis

All tests were carried out independently in triplicate (*n* = 3). The data are expressed as the mean ± standard derivation. One-way analysis of variance was performed to test the difference between the means; the mean values of biological activity between the experimental group and positive control were carried out by an independent samples *t*-test. All analyses were performed using SPSS 16 (SPSS Institute, Cary, NC, USA).

RESULTS

Luffa echinata Roxb. inhibits the proliferation of SW-480 cells

In this study, human colon cancer cell line (SW-480) was used to examine the anti-proliferative activity of LER extract by MTT assay [Figure 1]. Cultured SW-480 cells were treated with LER extract (25, 50,

100, and 200 $\mu\text{g/ml}$) for 6, 12, 24, 48, and 72 h, respectively [Figure 1]. LER dramatically inhibited SW-480 cell viability in both dose- and time-dependent manners. All concentrations of extracts exerted an inhibitory effect on the cells, and the proliferation of SW-480 cells was reduced to 50% following 24 h of exposure to LER at 70.2 $\mu\text{g/ml}$. However, after 24 h, the changes in the anti-proliferative effect of LER were not obviously different. Thus, the treatment with LER extract for 24 h was used as a model in the further study.

To examine the effect of the methanolic extract on cellular morphology during cell death, the morphological changes in untreated SW-480 cells and cells treated with LER extract at different concentrations for 24 h were analyzed by light microscopy [Figure 2]. A dose-dependent reduction in population size was noted in the LER-treated cells. Direct observation by microscopy revealed that the cellular morphology was severely disordered in the cells treated by 200 $\mu\text{g/ml}$ LER extract. Compared to the untreated cells, the LER-treated cells showed obvious cell shrinkage, destruction of the monolayer, and condensed chromatin, which was not seen in the untreated control cells.

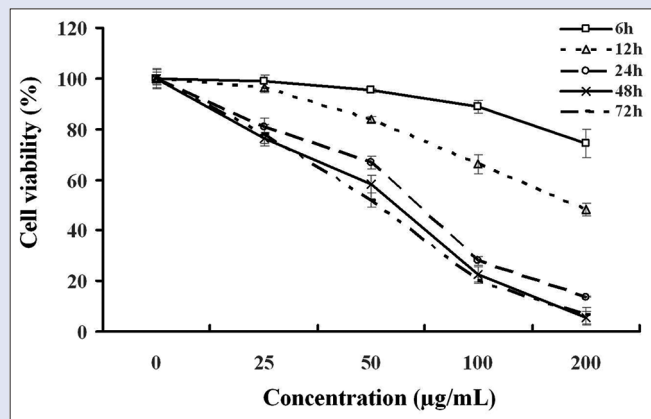


Figure 1: Anti-proliferative activity of *Luffa echinata* Roxb. Cultured SW-480 cells were treated with LER extract (25, 50, 100, and 200 $\mu\text{g/ml}$) for 6, 12, 24, 48, and 72 h, respectively. The cell viability of SW-480 cells treated by *Luffa echinata* Roxb. extract was measured by MTT assay

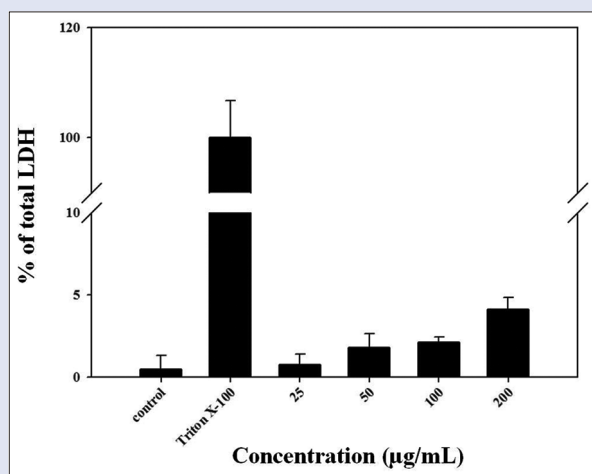


Figure 3: Cytotoxicity assays of SW-480 cells exposed to 25, 50, 100, and 200 $\mu\text{g/ml}$ of *Luffa echinata* Roxb. extracts for 24 h. The cytotoxicity of *Luffa echinata* Roxb. extract was analyzed by LDH assays (Triton X-100 as the positive control)

Cytotoxicity of *Luffa echinata* Roxb. extract

The cytotoxicity of LER extract was analyzed by lactate dehydrogenase (LDH) assay. As shown in Figure 3, LER treatment for 24 h caused the release of LDH in a dose-dependent manner in SW-480 cells [Figure 3]. In addition, treatments of various concentrations of LER extracts did not cause the release of LDH as much as the Triton X-100.

DNA fragmentation induced by *Luffa echinata* Roxb. extract

We next examined the effect of LER extract on chromosomal DNA fragmentation, a typical characteristic of cell death induced by ROS. In the absence of LER extract, the chromosomal DNA of cells remained intact for at least 24 h [Figure 4]. In contrast, in the presence of LER extract, chromosomal DNA was fragmented into giant DNA fragments and smaller

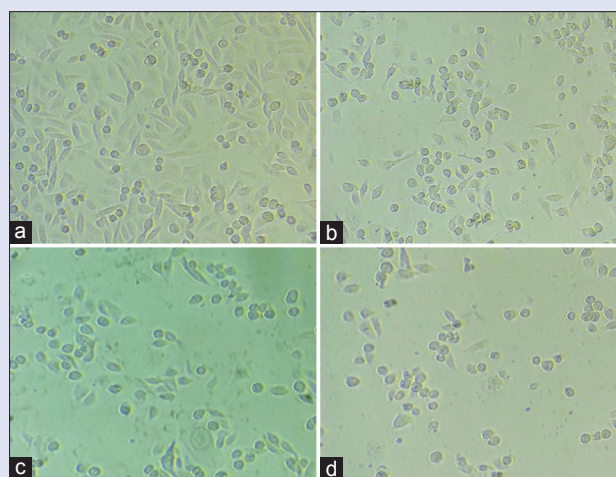


Figure 2: Morphological changes of SW-480 cells under a light microscope. SW-480 cells were incubated for 24 h with *Luffa echinata* Roxb. extracts. The medium was removed and the cells were rinsed and visualized under light microscope in control (a), 50 $\mu\text{g/ml}$ (b), 100 $\mu\text{g/ml}$ (c) and 200 $\mu\text{g/ml}$ (d) of methanolic extract of *Luffa echinata* Roxb. fruits

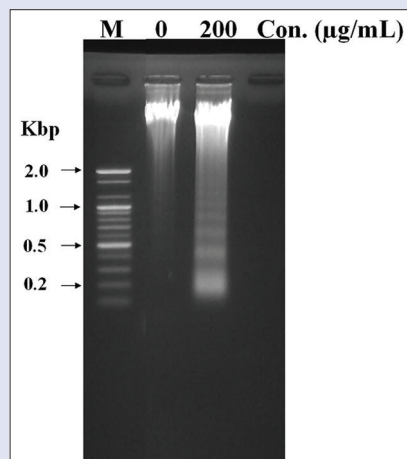


Figure 4: Effect of *Luffa echinata* Roxb. extracts on chromosomal DNA fragmentation. Samples were prepared from SW-480 cells incubated with or without 200 $\mu\text{g/ml}$ of *Luffa echinata* Roxb. extract for 24 h. The DNA samples were analyzed by agarose gel electrophoresis. Lanes M is marker

internucleosomal fragments of 100–1000 kbp revealed as DNA ladders. This modality of DNA fragmentation is typical of ROS-induced cell death, that is, giant DNA fragmentation followed by smaller internucleosomal DNA fragmentation shown as a ladder-like pattern.^[24] These data suggest that LER extract induced DNA fragmentation in an ROS-dependent manner.

ROS generation

To confirm whether induction of ROS was involved in LER-induced apoptosis in SW-480 cells, the ROS production after LER treatment for 24 h at various concentrations was measured [Figure 5]. Results showed that LER dramatically increased ROS production in a dose-dependent manner, indicating that LER-induced apoptotic cell death in SW-480 cells required the generation of ROS. Taken together, our findings indicated LER-induced apoptosis in SW-480 cells via a ROS-dependent mechanism.

Luffa echinata Roxb. induces cell death by triggering the mitochondrial apoptotic pathway

To investigate the possible molecular mechanism by which the LER extract triggered apoptosis in SW-480 cells, the expression of several apoptotic-related proteins in SW-480 cells treated by LER was evaluated by Western blot analysis with β -actin as an internal control [Figure 6]. Compared to untreated cells, expression of Bax and Bad were up-regulated after exposed to 50 and 100 $\mu\text{g/ml}$ of LER extract, while their expression was slightly decreased when LER concentration was 200 $\mu\text{g/ml}$. While the expression of Bcl-2 and Bcl-XL were down-regulated gradually after exposure to increasing concentrations of LER extract. In addition, the expression of caspase-3, -8, -9, and p53 were significantly increased with the treatment of 50, 100, and 200 $\mu\text{g/ml}$ of LER extract in a dose-dependent manner.

DISCUSSION

A certain agent that can selectively induce cell death in cancer cells with low cytotoxicity to the normal cells would be a potential chemotherapeutic agent against cancer since anti-tumor activity is usually estimated as the capacity of the test sample to selectively inhibit the growth of certain cancer cell lines.^[25] Previous study has shown that no obvious toxicity of high concentration of LER extract was observed to a normal cell line (CCD-986Sk).^[21] In our study, all concentrations of LER extract,

we tested, showed good inhibition effects on the proliferation of human colon cancer cell line SW-480 cells in a time- and dose-dependent manner [Figure 1]. These results demonstrated the potential anti-cancer effect on human colon cancer cells and the impetus for further studies on the anti-cancer mechanism of the LER extracts.

The cell death caused by the nanoparticles largely occurred through apoptosis or necrosis.^[26] LDH, a stable cytoplasmic enzyme present inside cells, is an important enzyme for energy metabolism.^[27] A large amount of LDH is released into the cell culture supernatant when the cell membrane is damaged, which can be used to evaluate the effect of a drug to cause cell death.^[28] In this research, we measured LDH release from LER-treated cells. As shown in Figure 3, LER treatment for 24 h caused the release of LDH in a dose-dependent manner in SW-480 cells [Figure 3]. In addition, treatments of various concentrations of LER extracts did not cause the release of LDH as much as the Triton X-100. These results clearly indicated that LER extract caused the release of LDH via stimulation of the intracellular metabolic system promotes cell death of SW-480 cells instead of a direct physical injury on cell membranes.

Apoptosis includes a highly complex cascade of cellular biochemical events characterized by blebbing, cell shrinkage, monolayer destruction, nuclear fragmentation, and chromatin condensation.^[29] The activity of apoptosis induction is necessary for cancer treatment, and several chemotherapeutic drugs have been shown to induce apoptosis *in vitro*.^[30] In this study, the treatment of LER extracts of 200 $\mu\text{g/ml}$ severely distorted the cellular morphology of SW-480 cells, and the morphological features typical of apoptotic cells were observed in these cells [Figure 2]. These results suggest that LER has anti-cancer properties in colon cancer cells via induction of apoptosis. We further observed nuclear and chromosomal DNA damage caused by LER extract. Some changes in nuclear shape such as nuclear condensation were observed after 24 h of treatment with LER extract. Nuclear condensation is a typical characteristic of apoptotic cell death.^[29] Furthermore, giant DNA fragments and high-molecular-weight chromosomal DNA fragments were further cleaved into smaller DNA ladder fragments after 24 h [Figure 4]. DNA is cleaved by caspase-activated DNase (CAD) during apoptosis, and the cleaved DNA is observed as a DNA ladder on electrophoresis.^[24] CAD cleaves DNA randomly between the nucleosome

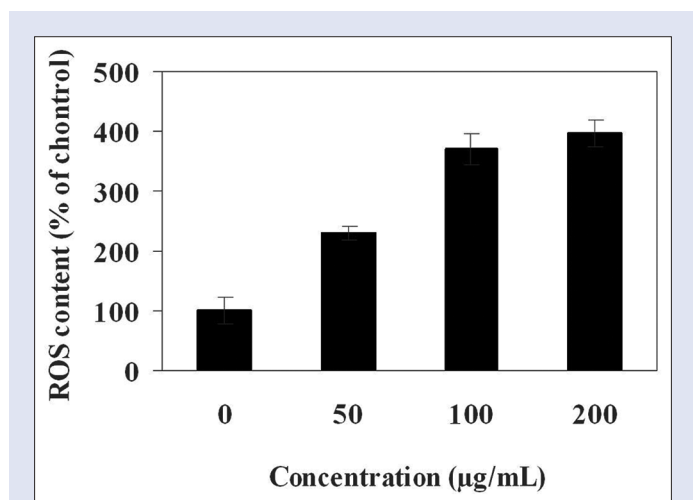


Figure 5: Reactive oxygen species content of SW-480 cells exposed to 50, 100, and 200 $\mu\text{g/ml}$ of *Luffa echinata* Roxb. extracts for 24 h

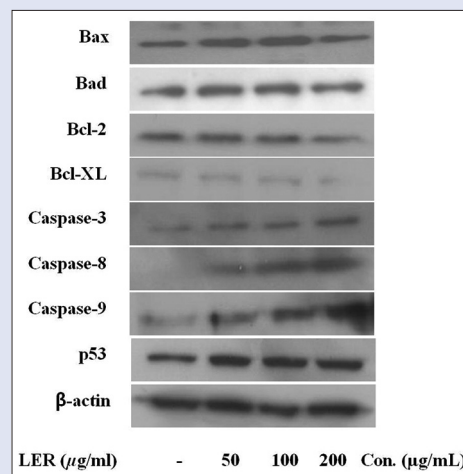


Figure 6: Effects of *Luffa echinata* Roxb. extracts on the expression of apoptosis-related proteins. Cells were treated with 25, 50, 100, and 200 $\mu\text{g/ml}$ of *Luffa echinata* Roxb. extracts for 24 h, and aliquots containing 30–50 μg of protein were subjected to sodium dodecyl sulfate-polyacrylamide gels followed by immunoblot analysis with specific antibodies. β -actin was used as an internal control for integrity and an equal amount of protein used in each lane

structures of chromosomes, in which the DNA twists around histones.^[31] Our observation of DNA ladder fragments after 24 h of LER treatment is consistent with the significant increase in caspase-3 activity after 24 h of LER treatment. These results suggest that LER extract induce chromosomal giant DNA fragmentation, followed by smaller DNA fragmentation through caspase-3 activation. Fragmentation producing giant DNA fragments of approximately 2 Mbp and 100–1000 kbp has been observed during cell death induced by some chemicals producing ROS.^[30]

The formation of ROS is a natural phenomenon of live cells, and it is a by-product of the cell metabolic activity of oxygen, however, excessive amounts of ROS can cause oxidative damage to cells, and also be toxic to cancer cells.^[32] ROS is a second messenger in multiple signaling pathways and plays important roles in apoptosis by regulating the activity of certain enzymes involved in the cell death pathway.^[33] One of the mechanisms of anti-cancer drugs-induced cellular death is the generation of ROS from mitochondria. ROS leads to oxidative stress and damage to the mitochondria, as well as a perturbation of mitochondrial activities which induces apoptosis.^[34] In this experiment, a fluorogenic probe DCFH-DA was used to study the oxidative stress potential induced by the LER treatment. DCFH-DA is able to penetrate the cell and convert into a highly fluorescent product (DCF) upon oxidation.^[35] The generated ROS level can be measured from the fluorescence intensity, which is proportionally correlated. Compared to untreated cells, the ROS level in SW-480 cells were increased by the treatment of LER extract as a dose-dependent manner [Figure 5], which indicated that the LER-induced apoptosis in SW-480 cancer cells require the generation of ROS and LER extract induced apoptotic cell death through a ROS-dependent mechanism.

To determine the possible molecular mechanisms underlying the apoptosis triggered by LER, we analyzed the expression of several apoptotic-related proteins in SW-480 cells after treatment with LER [Figure 6]. Apoptosis is a complex event, and many factors are involved in the apoptosis pathway.^[36,37] Caspases are a group of highly conserved cysteine-dependent aspartate-specific proteases that play pivotal roles in the execution of apoptotic signals.^[38] The apoptosis can be classified into caspase-dependent or caspase-independent mechanisms.^[39,40] While caspase-dependent pathways can be further divided into two major pathways: The death receptor-mediated pathway (extrinsic pathway) and the mitochondria-initiated apoptotic pathway (intrinsic pathway), as determined by involvement of caspase-8 or -9, respectively.^[41] Caspase-3 is the most commonly activated caspase during apoptosis, whose activation during apoptosis could be a direct consequence of enhanced oxidative stress (e.g., generation of ROS).^[42,43] In our study, ROS level was gradually increased accompanied with the increase of caspase-3 transcripts level [Figures 5 and 6], which supported this conclusion. So, we thought the increase of caspase-3 mRNA was caused by increased ROS generation during the process of apoptosis in SW-480 cells treated by LER extract of different concentrations. Furthermore, the expression of caspase-8 and -9 were also significantly increased with treatment of LER extract in a dose-dependent manner, which indicated that both extrinsic and intrinsic apoptotic pathways were involved in the apoptosis induction by LER treatment. Bcl-2 family proteins regulate cell death induced by caspases and contain two types of proteins, including pro-apoptotic proteins such as Bax, Bad, Bak, and Bcl-Xs and the other type is anti-apoptotic proteins such as Bcl-2 and Bcl-XL.^[44,45] Bcl-2, a caspase substrate, functions as an anti-oxidant to inhibit apoptosis in a wide variety of cell types and plays an important role in regulating the mitochondria-dependent pathway.^[46,47] Our results showed that LER promoted the expression of Bax and Bad (pro-apoptotic protein) and decreased the levels of Bcl-2 and Bcl-XL (anti-apoptotic proteins) in a dose-dependent manner. p53, a primary tumor suppressor,

plays a vital role in DNA repair and apoptotic pathway, whose gene is the most frequently mutated gene in cancers.^[47,48] Additional p53 phosphorylation enables p53 to activate the expression of proteins, which mediate apoptosis, in particular, pro-apoptotic Bax, and Bak.^[49] Therefore, these results indicated that treatment with LER-induced cell death in mitochondrial apoptosis pathway by regulating of pro-apoptotic proteins via up regulation p53 protein. Furthermore, apoptosis induction by LER was essentially dependent on caspase activation.

CONCLUSION

Our study indicates that the methanolic extract of LER exerts its anti-proliferative effects on human colon cancer cell line SW-480 by inducing apoptotic cell death. In addition, treatments of various concentrations of LER extracts caused the release of LDH as a dose-dependent manner via stimulation of the intracellular metabolic system. LER induced apoptosis, DNA fragmentation, and cellular ROS accumulation in SW-480 cells. Treatment of LER on SW-480 cells promoted the expression of caspases, Bax, Bad and p53 proteins and decreased the levels of Bcl-2 and Bcl-XL. These results indicated that treatment with LER-induced cell death in mitochondrial apoptosis pathway by regulating of pro-apoptotic proteins via the up regulation of the p53 protein. These findings highlight the potentials of LER in the treatment of human colon cancer. Its anti-cancer activities of LER fruits might be due to the synergistic actions of bioactive compounds present in them. Additional studies are currently underway to assess the *in vivo* biological activities and to identify more specific phytochemicals responsible for their anti-cancer activities.

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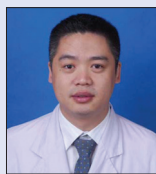
Conflicts of interest

There are no conflicts of interest.

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