Doxorubicin (DOX) as an effective chemotherapy drug has serious side effects such as dilated cardiomyopathy and congestive heart failure (1). Therefore, usage of this drug must be limited. The mechanisms of DOX-induced cardiotoxicity are not completely understood, but most evidences indicate that the generation of reactive oxygen species (ROS) is involved (2). Increased level of ROS leads to protein and lipid peroxidation, DNA damage and irreversible cell damage (3, 4). ROS can directly impair contractile function, activate hypertrophy signaling pathways, stimulate cardiac fibroblast proliferation and induce extracellular matrix remodeling (5, 6). Excessive ROS also cause accumulation of intracellular Ca²⁺ in cardiac cells, which increases the mitochondrial permeability and leads to the release of cytochrome c into the cytoplasm and the following apoptotic cascades (7). Interestingly, some natural foods have been reported to contain substantial amounts of antioxidants and free radical scavenging agents. These compounds diminish some side effects of chemotherapeutic agents on normal cells by reducing their genotoxicity (2). H9C2 myoblasts, a rat embryonic cell line, which has the ability to differentiate between a skeletal or cardiac muscle phenotype, can be instrumental in understanding DOX cytotoxicity in different stages (8). The recent studies have shown that some of the herbs act against oxidative injury-related cardiotoxicity.

*Lactuca serriola* L. (Compositae) is known by several names such as Prickly lettuce, jagged lettuce, Kahu and Khas (9). It is native to Himalaya, Siberia, and Atlantic areas (9) but cultivated also in temperate lands of Europe, India, Pakistan, and Iran (9). In traditional medicine, this plant is applied as a sedative, hypnotic, expectorant, coughs suppressant, purgative, diuretic, antiseptic, vasorelaxant, and antispasmodic (9). The plant contains vitamins, β-carotene, and iron. The obtained extract of this plant is composed of lactucone, lactucin, and lactucic acids (9). There are alkaloids, the bitter substance of lettuce, oxalic acid, lactucopicrin, and sesquiterpene esters in seeds. The antipyretic activity is related to alkaloid and lactucin, whereas isolated triterpenoid

### Abstract

The use of doxorubicin (DOX) is limited by its dose-dependency because of its cardiotoxicity. Reactive oxygen species (ROS) play an important role in the pathological process. The aim of this study is to evaluate the protective effect of *Lactuca serriola* against DOX-induced apoptosis and death in H9C2 cells. The cells were incubated with different concentrations of extract for 4 h which continued in the presence or absence of 5 µM doxorubicin for 24 h. Cell viability, apoptotic induction and the level of apoptotic proteins were determined by using MTT, PI and immunoblotting assays, respectively. The level of lipid peroxidation was measured by fluorimetric method. DOX significantly decreased cell viability which was accompanied by an increase in ROS production and lipid peroxidation. Pretreatment with *Lactuca serriola* increased the viability of cardiomyocytes and could decrease lipid peroxidation. Also, *Lactuca serriola* inhibited the reduction of anti-apoptotic Bcl-2 protein and elevation of apoptotic Bax and caspase-3 proteins. In conclusion, *Lactuca serriola* exerts protective effect against oxidative stress-induced cardiomyocytes damage. Therefore, it has the potential to be used as cardioprotective agent by the patients with cardiovascular diseases.

### Keywords

*Lactuca serriola*, H9c2, doxorubicin, apoptosis
saponin possesses antibacterial activity (9). The studies have shown that plant has analgesic, anti-inflammatory, and antioxidant activities due to totally high phenolic contents. The antioxidant activity scavenges the free radicals and reduces oxidative stress (9). In this study, the protective effect of hydro-alcoholic extract of *Lactuca serriola* on DOX-induced cardiotoxicity was evaluated.

**MATERIALS AND METHODS**

**Reagents**

*Lactuca serriola* was obtained from local market, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium (MTT), thiozetatic acid (TBA), bicinchoninic acid protein assay kit and protease inhibitor cocktail, propidium iodide (PI), sodium citrate and Triton X-100 were purchased from Sigma (St. Louis, MO, USA). High-glucose Dulbecco’s modified Eagles medium (DMEM), penicillin-streptomycin and fetal bovine serum were purchased from Gibco. Trichloroacetic acid (TCA) and malondialdehyde bis-(dimethyl acetal) (MDA) were obtained from Merck (Darmstadt, Germany). H9C2 cells were obtained from Pasteur Institute (Tehran, Iran). The Bax, Bcl-2 and caspase 3 antibodies were purchased from Cell Signaling Technology Inc. Doxorubicin, manufactured by EBWE company, was purchased from a pharmacy.

**Preparation of extracts**

The aerial parts of *Lactuca Serriola* were collected from the garden of Ferdowsi University of Mashhad, Mashhad, Iran. The plant sample was identified at the herbarium of School of Pharmacy (Mashhad University of Medical Sciences, Mashhad, Iran) and a specimen voucher (12829) was deposited in this institute. The aerial parts of lettuce were dried, powdered and 50 g of this powder was subjected to extraction with 70% ethanol in a Soxhlet apparatus for 48 h. The hydro-alcoholic extract was then dried on a water bath and kept frozen in less than -18°C for the following use. The yield of extract was 19% w/w. The extract was dissolved in dimethyl sulfoxide (DMSO) to a final concentration of 50 mg/mL before being used in cytotoxicity and apoptosis assays.

**Cell culture**

H9c2 cells were maintained at 37°C in a humidified atmosphere containing 5% CO₂. The cells were cultured in DMEM supplemented with 10% fetal bovine serum and 100 units/mL penicillin and 100 µg/mL streptomycin. For the experiments, they were seeded in 96-well and 24-well culture plates for MTT and MDA assays, respectively. For apoptosis assay, cells were seeded at 100,000/well in a 24-well plate. All treatments were carried out in triplicate. The cells were pretreated with extract (6-200 µg/mL) for 4 h and then incubation was continued in the presence of the extract with 5 µM doxorubicin for 24 h.

**Cell viability**

The cell viability was determined using a modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) assay as described previously (10, 11). Briefly, MTT solution in phosphate-buffered saline (5 mg/mL), was added to each well at final concentration of 0.05%. After 3 h, the formazan precipitate was dissolved in DMSO. The absorbance at 570 and 620 nm (background) was measured using a StatFAX303 plate reader.

**Apoptosis**

Apoptotic cells were detected by using PI staining of the treated cells followed by flow cytometry to detect the so-called sub-G1 peak (12). Briefly, H9C2 cells were cultured overnight in a 24-well plate and pretreated by *Lactuca serriola* for 4 h and then treated with DOX for 24 h. Floating and adherent cells were then harvested and incubated at 4°C overnight in the dark with 500 µL of a hypotonic buffer (50 µg/mL PI in 0.1% sodium citrate plus 0.1% Triton X-100).

**Lipid peroxidation assay**

The level of lipid peroxidation was estimated by measuring MDA which is the end product of lipid peroxidation. At the end of incubation, the cells were scraped and centrifuged for 30 min. Then, 400 µL of TCA (15%) and 800 µL of TBA (0.7%) were added to 500 µL of cell samples. The mixture was vortexed and then heated for 40 min in a boiling water bath. Then, 200 µL of the sample was transferred to 96-well plate and the fluorescence intensity was read with excitation/emission of 480/530 nm. The experiment was carried out in triplicate.

**Western blot analysis**

The cells were lysed using lysis buffer containing 50 mM Tris-HCl, 2 mM EDTA, 2 mM EGTA, 10 mM NaF, 1 mM sodium orthovanadate, 10 mM β-glycerophosphate, 0.2% w/v sodium deoxycholate, 1 mM phenylmethylsulfonyl fluoride and protease inhibitor cocktail. After centrifuging, the protein concentration of the supernatants was measured and equal amounts of protein from samples were mixed with loading buffer. Samples were sep-
arated by electrophoresis in 12% SDS-PAGE gels and were transferred to polyvinylidene fluoride membranes. The blots were blocked with 5% skim milk for an overnight in a refrigerator. After blocking, blots were incubated with Bax, Bcl-2 and caspase 3 antibodies for 2 h at room temperature. The membrane was washed with 0.1% Tween 20 and the bound antibody was made visible using horse radish peroxidase-conjugated goat anti-rabbit secondary antibody and an enhanced chemiluminescence system. Bands were analyzed using Gel Pro Analyzer Software (Media Cybernetics).

Characterization of the extract by HPLC

The quality of hydro-alcoholic extract of *Lactuca Serriola* was characterized by HPLC-UV fingerprint. The chromatographic separation was carried out with a reverse-phase Waters C18 analytical column (250 × 4.6 mm, 5 µm particle size). An isocratic elution was carried out by the mobile phase of methanol : acetonitrile : water (60 : 20 : 20% v/v/v, pH 5.9 adjusted with phosphoric acid) at the flow rate of 1 mL/min. The UV detector wavelength was set at 330 nm. A sample of the extract was dissolved in distilled water and passed through 0.45 µm membrane filter. Then, 20 µL of the sample (500 µg/L) was injected into the HPLC column.

Statistical analysis

All the results were expressed as the mean ± SEM. The significance of difference was evaluated with ANOVA and Bonferroni’s test. A probability level of p < 0.05 was considered statistically significant.

RESULTS

Effect of *Lactuca serriola* extract on cell viability

Incubation with DOX significantly decreased cell viability to 51.3 ± 0.95% of control (p < 0.001). Pretreatment with 25, 50, 100 and 200 µg/mL of *Lactuca serriola* could increase the viability of H9C2 cells to 71 ± 0.43% (p < 0.01), 79 ± 4% (p < 0.001), 87 ± 3.6% (p < 0.001) and 69 ± 2% (p < 0.01) of control, respectively (Fig. 1). At the dose of 6 and 12 µg/mL, however, *Lactuca serriola* was not able to protect H9C2 cells against DOX-induced cytotoxicity.

Effect of *Lactuca serriola* on apoptotic induction

Apoptosis in H9C2 cell line was detected by flow cytometry using PI staining. Cells were pretreated for 4 h with various concentrations of the extract and exposed to DOX for 24 h. Analysis of the subG1 peak in flow cytometry histograms revealed the induction of apoptosis in cells which were treated by DOX (p < 0.001). *Lactuca serriola* decreased apoptotic induction significantly, at the doses of 50 µg/mL (p < 0.01), 100 µg/mL (p < 0.001) and 200 µg/mL (p < 0.05) (Fig. 2). The most reduction of apoptotic rate was at the dose of 100 µg/mL (control 2 ± 1, DOX 72 ± 2 and 100 µg/mL 28 ± 1.5).

![Figure 1. Effect of *Lactuca serriola* on cell viability of H9C2 cells. The cells were pretreated with different concentrations of extract for 4 h then exposed to doxorubicin (DOX) for 24 h. Viability was quantitated by MTT assay. Results are the mean ± SEM (n = 3). ***p < 0.001 versus control, "p < 0.01 and ""p < 0.001 versus DOX. Cont = Control](image)
Effect of *Lactuca serriola* extract on MDA production

The level of lipid peroxidation was evaluated by measuring the level of MDA, which is the end product of lipid peroxidation. As shown in Figure 3, exposure of the cells to DOX resulted in a significant increase of MDA level (236 ± 7.9%, p < 0.001) as compared to control cells cultured in the absence of DOX (100 ± 1.3%). The content of MDA was significantly decreased in the cells pretreated with 25 µg/mL (181 ± 3.48%, p < 0.01), 50 µg/mL (168 ± 11.7%, p < 0.001), 100 µg/mL (144 ± 3.45%, p < 0.001), or 200 µg/mL (179 ± 5.6%, p < 0.01) of *Lactuca serriola*.

Effect of *Lactuca serriola* on pro-apoptotic and anti-apoptotic proteins

Incubation of H9C2 cells with DOX significantly up-regulated the expression of pro-apoptotic proteins (Bax and caspase 3) and down-regulated expression of anti-apoptotic protein (Bcl-2). Results showed that DOX increased Bax/Bcl-2 (p < 0.01)
Protective effect of *Lactuca serriola* on doxorubicin-induced toxicity

and caspase 3 (p < 0.01) in comparison with the control group (Fig. 4). Pretreatment with both 50 and 100 µg/mL of *Lactuca serriola* decreased the level of Bax/Bcl-2 (50 µg/mL, p < 0.05 and 100 µg/mL, p < 0.01) and caspase 3 (p < 0.01) proteins in comparison with DOX whereas the concentration of 200 µg/mL did not decrease Bax/Bcl-2 and caspase 3 significantly.

**HPLC profile of *Lactuca serriola***

A simple and reliable HPLC fingerprint had been developed for the standardization of the hydro-alcoholic extract. HPLC profile of *Lactuca serriola* was recorded under UV 330 nm. The corresponding HPLC chromatogram is presented in Figure 5. The extract revealed 5 major peaks with retention time (t_R) values in the range of 1.9 to 9.7 min for 20 µL application volume (Fig. 5).

**DISCUSSION**

Doxorubicin is widely used as antineoplastic agent in the treatment of a variety of solid malignancies, such as leukemias, bladder, lung, breast and ovarian cancers. It leads to cardiotoxicity, as a result, its clinical uses are limited. Studies have shown that doxorubicin induces cardiotoxicity via elevation of ROS. Doxorubicin leads to elevation of ROS via NADPH-dependent enzymatic and a non-enzymatic pathway to generate Fe²⁺ doxorubicin free radicals (13). The generated doxorubicin releases radicals that cause DNA breaks and lipid peroxidation (14-16). Cardiac tissue is sensitive to oxidative damage because of its high oxidative metabolism and low antioxidant defenses in this organ compared with others (17, 18).

In the present study H9C2 cells were used as a pharmacological model to evaluate the potential cardioprotective effect of *Lactuca serriola* against doxorubicin. The results showed that *Lactuca serriola* has protective effect on H9C2 cells against DOX-induced oxidative stress. H9C2 cells are morphologically similar to immature embryonic cardiomyocytes. Considering that these cells preserve electrical and hormonal signal pathways found in adult cardiac cells (19), they are a useful model for studying oxidative stress-induced cardiomyocyte damage (20). In this model, DOX significantly increased lipid peroxidation and induced the apoptotic rate. These changes are similar to the DOX-induced deleterious effects on normal cardiac cells, which lead to the loss of cardiomyocytes viability. These changes lead to a down-regulation of anti-apoptotic protein, Bcl-2, and an up-regulation of apoptotic proteins Bax and caspase 3 which finally are accompanied by the loss of viability of cardiomyocytes. In agreement with these observations, it was demonstrated that the exposure of H9C2 cells to DOX lead to a significant increase in caspase 3 and Bax, and a considerable decrease in Bcl-2 level and cell viability (21-23). In

![Figure 3](image-url)
this very study, pretreatment with *Lactuca serriola* could decrease the lipid peroxidation and apoptotic rate. The antioxidant actions of *Lactuca serriola* inhibited cardiac cell death by suppression of pro-apoptotic proteins levels and increasing the level of anti-apoptotic protein.

The studies have shown that *Lactuca serriola* includes alkaloids, the bitter substance of the lettuce, oxalic acid, lactucopicrin (24) and sesquiterpene esters. It has analgesic, anti-inflammatory (25), and antioxidant activities. Antioxidant activities due to high total phenolic contents are proved to be efficient to release radical scavenging potential like quercetin (26, 27). Recent studies have indicated that *Lactuca serriola* has Ca²⁺ channel blocker activity, and as a result, it could act similarly to Ca²⁺ channel blockers and it can be used in cardiovascular disorders (28, 29). Diltiazem is a calcium channel blocker (30). Studies have shown that doxorubicin cytotoxicity is due to induction of calcium cycling in cardiac mitochondria. However, doxorubicin reduces the amount of Ca²⁺ accumulation in the mitochondria (31, 32). Furthermore, elevation of Ca²⁺ influx in mitochondria could lead to the loss of mitochondrial membrane potential, causing cytochrome-c to be released and subsequent caspase to be activated (33). Recent studies have shown that the elevation of Ca²⁺ levels and decrease in mitochondrial membrane potential are associated with the production of ROS and that the both steps occur prior to the induction of mitochondrial permeability transition and subsequent cell death (34). However,
diltiazem via blocking the calcium channels of H9C2 cells significantly reduce the influx of extracellular Ca²⁺, which decreases the detrimental effects of doxorubicin (35). As a result, protective effect of Lactuca serriola can be related to antioxidant activity and Ca²⁺ channel blocker. It needs more investigation to understand the accuracy of this mechanism.

In the present chromatographic technique, in order to obtain a good resolution within a short analysis time, the composition of mobile phase was optimized. Acidic mobile phase was used in order to suppress the ionization of phenolic hydroxyl groups. This acidification was beneficial, leading to good peak separation and better peak shape. Various mobile phase compositions were evaluated. Methanol, acetonitrile and water containing little amount of phosphoric acid were chosen as the mobile phase, because all the peak components could be resolved under this condition. The HPLC fingerprint showed high stability and reproducibility, and thus, it could be used for the quality control of the hydro-alcoholic extract and Lactuca products.

CONCLUSION

In conclusion, our data demonstrated that Lactuca serriola exerts protective effect against DOX induced toxicity in cardiomyocytes. This effect is mediated by reducing oxidative stress and inhibiting of apoptotic pathways. Therefore, Lactuca serriola has the potential to be administrated as a cardioprotective agent to the patients with cardiovascular diseases.

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REFERENCES


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