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Original Article

Ethanolic and water rhizome extracts of *Ligusticum chuanxiong* Hort alleviate hypoxia/reoxygenation-injured H9c2 cardiomyoblasts through up-regulated ERK1/2 and Bcl-2 expression

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Abstract

Ligusticum chuanxiong Hort (LC) is used in traditional medicine throughout Southeast Asia as a cardiovascular protective herb, antioxidant, vasorelaxant, and anti-inflammatory. To demonstrate the protective mechanisms of LC on hypoxia/reoxygenation (HR) induced injury in H9c2 cardiomyoblasts, intracellular reactive oxygen species (ROS) content was measured using dichlorodihydrofluorescein diacetate. The cardioprotective effects of LC ethanolic (E) and water (W) extracts were measured by the MTT assay. Changes in cell and nuclear morphology were observed under a phase-contrast and a fluorescent microscope. The mitochondrial membrane potential (MMP) was detected by Rhodamine123 staining. The expressions of pERK1/2, ERK1/2, and Bcl-2 were determined by immunoblotting. LCE and LCW extracts caused no cytotoxicity or ROS generation to H9c2 cardiomyoblasts. The incubation of LCE or LCW during hypoxia increased cell survival ($p < 0.05$) compared to HR without the extracts. HR-induced cells demonstrated cellular atrophy, cell membrane shrinkage, and DNA condensation. Healthy cells were found in the control, LCE and LCW-treated HR-induced cells. LC restored the reduction of MMP, while LCE and LCW increased the pERK/ERK ratio ($p < 0.001$). Meanwhile, LCW increased Bcl-2 expression ($p < 0.05$). LC extracts effectively protected H9c2 cardiomyoblasts from injury caused by HR exposure, suggesting a beneficial use of LC in alleviating damaged cardiomyoblasts from acute ischemia.

Keywords: ligusticum, ischemia/reperfusion, cardioprotection, ERK1/2, Bcl-2

1. Introduction

Acute myocardial infarction (AMI) is a disease encompassing both direct and indirect costs as well as diminished quality of life, and it is highly detrimental when compared to any other disease (Benjamin *et al*., 2017). Ischemia/reperfusion (IR) or hypoxia-reoxygenation (HR) injury is among the important causes of AMI, involving oxidative stress, inflammation, neurohumoral activation (Heusch & Gersh, 2017), and the loss of mitochondrial membrane potential (MMP), leading to cytochrome c release and the subsequent activation of the caspase family. This eventually leads to apoptotic cell death (Kang, Chen, Lin, Chilian, & Chen, 2017). In contrast to p38 and JNK signaling, ERK1/2 and Bcl-2 have been recognized as protective against cell damage in H9c2 cardiomyoblasts and anti-apoptotic pathways in heart ischemic preconditioning (Guo *et al*., 2018; Zhang *et al*., 2010). Herbal medicines, one of the treatment strategies used in folk medicine, have been used for the prevention, alleviation, and possible curing of AMI.

Herbal medicines are becoming increasingly attractive as complementary or alternative treatments for cardiac IR injury (Sedighi *et al.*, 2019), and this approach is

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supported by the study of *Ligusticum chuanxiong* Hort (Apiaceae) (LC) (Liu, Li, Ji, Cui, & Li, 2016). LC is also called *L. sinense* Oliv.Cv. *chuanxiong*. It is commonly called "Gao-ben" in China and "Kot Hua Bua" in Thailand. LC has been used extensively in traditional medicine for the treatment of common colds, headaches, and rheumatic pain (Cheng *et al*., 2018; Techadamrongsin *et al*., 2010). Additionally, LC also possesses anti-hypertension (Kim *et al*., 2015), anti-inflammatory and anti-apoptotic properties (Shi *et al*., 2020). Treatment using LC extracts reportedly possesses antioxidant effects and defends the human umbilical vein endothelial ECV304 cell line against damage induced by hydrogen peroxide-induced oxidative stress via increasing antioxidant enzymes (Nalinratana *et al*., 2014). It has been shown that the methanolic extract of LC rhizome inhibits myocardial ischemia injury induced by oxidative stress after left anterior descending coronary artery ligation in canines (Liu, Li, Ji, Cui, & Li, 2016). However, the mechanisms underlying the protective action of LC rhizome extract against HR injury remain unclear.

Considering the simultaneous functioning of multiple bioactive compounds in physiological conditions, studying the activity of LC crude extract could elucidate the folk wisdom behind using the entire plant or a part of the plant in traditional medicine. This study aimed to investigate the cardioprotective mechanism of LC rhizome extracts against HR injury, using H9c2 cardiomyoblasts as an experimental model system. The study elucidated cytotoxicity, cellular and nuclear morphological changes, the loss of MMP, and the expression of protective proteins, including phosphorylated (p) ERK1/2, ERK1/2, and Bcl-2. Since oxidative stress is involved in IR injury, LC-generated intracellular reactive oxygen species (ROS) on H9c2 cardiomyoblasts were also investigated.

2. Materials and Methods

2.1 Materials and chemicals

2-Deoxyglucose (2-DOG), sodium dithionite ($Na_2S_2O_4$), sodium lactate, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT), 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA), Rhodamine123 (Rh123), and dimethyl sulfoxide (DMSO) were provided by Sigma (St. Louis, MO, USA). 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) was purchased from Sigma-Aldrich. Fetal bovine serum (FBS) (HyClone®) was purchased from Perbio Science bvba (Belgium). Extracellular signal-regulated kinase (ERK1/2), pERK1/2 and Bcl-2 were obtained from Cell Signaling Technology (Beverly, MA, USA). Dulbecco's modified Eagle medium (DMEM) was obtained from Invitrogen (Carlsbad, CA, USA). All organic solvents and reagents were of analytical grade.

2.2 Preparation of extracts

The rhizomes of LC were acquired from the Oriental Medicine Drugstore in Bangkok, Thailand. The voucher specimens (NVK10-52) have been deposited at the Government Pharmaceutical Organization, Bangkok 10400, Thailand. Dr. Sanya Hokputsa identified the morphological characteristics of LC. LC was prepared according to the standard protocol by Dr. Sanya Hokputsa. Briefly, 1 kg of the dried plant materials was cleaned and extracted twice in 3 h, each by reflux in 20 L of 50% ethanol (E) or water (W). Each accumulated extract was then dried by spray drying. Meksuriyen, Hokputsa, Wiyakrutta, Nusuetrong, & Tirawanchai (2009) described a high-performance liquid chromatography of the LCE and LCW contents of ferulic acid (0.03% and 0.01 %w/w, respectively). All extracts were stored at -20°C until used. The crude extracts were dissolved in 100% DMSO and used as the stock solution.

2.3 Cell culture

H9c2, a permanent cell line derived from rat cardiac tissue, from the American Type Culture Collection (ATCC, CRL-1446) (Manassas, VA, USA), was cultivated in a complete medium (DMEM supplemented with 10% FBS and 100 IU/mL penicillin/100 µg/mL streptomycin) at 37°C in a controlled atmosphere with 5% CO_2. It was passaged using a 0.25% trypsin and 0.53 mM EDTA solution. The control and treated cells were cultivated at a final concentration of 0.5% DMSO in all treatment.

2.4 Measurement of intracellular ROS

Some plant extracts and their derived compounds can generate intracellular ROS, thus causing cell death (Farshori *et al*., 2023). The measurement of ROS content was used to investigate the impact of the extracts on intracellular oxidative stress. DCFH-DA can enter into cells, be hydrolyzed to nonfluorescent DCFH by esterases, and subsequently oxidized by intracellular ROS to the fluorescent compound dichlorofluorescein (DCF). Thus, DCF fluorescence intensity reflects the level of intracellular ROS.

Intracellular ROS accumulation was measured by using the fluorescent probe DCFH-DA. H9c2 cells (1×10^4 cells/well) were seeded in a 96-well plate and incubated in a 5% CO_2 atmosphere for 24 h. After 24 h of treatment with LCE or LCW (0.01-1 mg/mL), the cells were washed with ice-cold PBS and further incubated with DCFH-DA (1 µM) (Hervouet *et al*., 2008) for 4 h at 37°C in the dark. The ROS-associated DCF fluorescence intensity was detected using a fluorescent microplate reader (Microplate reader Synergy H1M, Agilent, USA).

2.5 Assessment of cell survival

The MTT colorimetric assay was used to measure cellular metabolic activity as an index of cell viability, proliferation, and cytoxicity, as well as mitochondrial activity. The H9c2 cells (5×10^4 cells/well) were cultured in 96-well plates and incubated for 24 h in a 5% CO_2 atmosphere. After incubation, cells were treated with various concentrations (0.01, 0.05, 0.1, 0.5, and 1 mg/mL) of LCE and LCW at the indicated time points (6, 12, 18, and 24 h). Subsequently, treated cells were assessed using standard MTT protocol. At the end-point, the medium was changed. An MTT solution (25 µL) was added to achieve the final concentration of 1 mg/mL in a 96-well plate, and incubated for 4 h at 37°C (Hansen, Nielsen, & Berg, 1989). Viable cells contain active mitochondria. The mitochondrial dehydrogenases of viable

cells cleaved tetrazolium rings, producing water-insoluble purple formazan crystals. The reaction was ended by adding a lysis buffer to each well, followed by overnight incubation at 37°C. The optical density (OD) was estimated with a microplate reader (Sunrise Classic, Tecan, Austria) at a wavelength of 595 nm. The percentage of cell viability was calculated using the following formula: (OD of treated cells/OD of control cells) ×100.

2.6 Simulated HR and treatment

The H9c2 cells were exposed to hypoxia for 6 h followed by 24 h of reoxygenation. The experimental protocol of simulated HR was established and modified based on the methods described by Gordon, Dusting, Woodman, and Ritchie (2003) and Hu *et al.* (2008). In summary, hypoxic conditions were induced in a hypoxic buffer solution. Control cells were incubated in a Krebs-Henseleit (KH) solution and maintained at pH 7.4. The cells were placed in a modular incubator chamber (Billups-Rothenberg, Inc., CA, USA), and the air in the chamber was replaced with pure N_2, incubating the cells at 37°C. After incubation for 6 h under hypoxic conditions, the cells were immediately reoxygenated by transferring them to a fresh complete medium. Subsequently, they were returned to a 5% CO_2 incubator with a temperature of 37°C for a reoxygenation period of 24 h.

The H9c2 cells were co-incubated for 6 h under hypoxia with LCE or LCW at a concentration ranging from 0.001-1 mg/mL, followed by a reoxygenation period of 24 h. At a concentration of 250 μM, Trolox served as the positive control for cardioprotection against HR injury, owing to its redox and anti-inflammatory activities (Ungurianu, Zanfirescu, Nițulescu, & Margină, 2021). The control cells were cultivated in a KH buffer with 0.5% DMSO (final concentration); the same concentration of DMSO was utilized with the treated cells.

2.7 Detection of changes in cell and nuclear morphology

Apoptosis was measured by the assessment of nuclear morphology. Chromatin condensation and nuclear fragmentation were observed by staining with acridine orange (AO) and detected by fluorescence microscopy. AO is a membrane-permeable dye that stains DNA in both live and dead cells, and detection was by fluorescence microscopy. During apoptosis, AO-stained cells demonstrated nuclear and cellular shrinkage, DNA condensation, and the loss of nuclear demarcation. In brief, cells were seeded on a 6-well plate (4×10^5 cells/well). After 24 h of incubation in a 5% CO_2 atmosphere, the cells were co-incubated under hypoxia with LCE (0.05 and 0.1 mg/mL) or LCW (0.1 and 0.5 mg/mL) for 6 h. Following reoxygenation, cells were incubated with AO (1 μg/mL) in a 5% CO_2 incubator for 5 min. Images were captured using an inverted microscope (Olympus FSX100 Bio Image navigator, Japan) at 400 × magnification.

Similar areas of each experimental condition were also photographed under a phase-contrast microscope to detect changes in cell morphology.

2.8 Detection of mitochondrial membrane potential (MMP)

A reduction in MMP levels represents depolarization of the mitochondrial membranes, which indicates mitochondrial dysfunction. We assessed changes in MMP using the cell-permeable cationic lipophilic fluorescence probe Rh123, which accumulates in the matrix of mitochondria based on the membrane potential. The presence of Rh123 staining indicates normal MMP. Membrane depolarization leads to the release of Rh123 into the medium, resulting in a decrease in fluorescence intensity in mitochondria, reflecting changes in MMP. H9c2 cells (4×10^5 cells/mL) were cultured in a 6-well plate for 24 h, after which they were co-incubated under hypoxia with LCE (0.05 and 0.1 mg/mL) or LCW (0.1 and 0.5 mg/mL) for 6 h. After reoxygenation, following the protocol described by Liu *et al.* (2018) with minor modifications, the treated cells were incubated with 4 μM Rh123 in PBS at 37°C for 10 min in a 5% CO_2 atmosphere. Excess Rh123 was removed, and fresh medium was added. The cells were immediately observed using an Olympus FSX100 Bio Image Navigator inverted fluorescence microscope.

2.9 Western blot analysis

The treated cells were washed with ice-cold PBS and harvested using a lysis buffer containing protease inhibitors. Protein concentrations were determined using the Bradford method. Equal amounts of proteins were resolved onto 10-12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis gels and transferred onto polyvinylidene fluoride membranes. Following incubation for 2 h with 5% non-fat milk in Tris-buffered saline with 0.1% Tween® 20 detergent (TBST), the membranes were incubated overnight at 4ºC with specific primary antibodies ERK1/2, pERK1/2, and Bcl-2 at a dilution of 1:1000. After washing with TBST, the membranes were incubated with the appropriate HRP-conjugated secondary antibodies. Protein bands were detected by enhanced chemiluminescence using a gel documentation system (GeneGnome5, Syngene, Cambridge, UK). The blots were also probed with β-actin antibody as a loading control for normalization.

2.10 Data analysis

Data are expressed as mean ± SEM and analyzed using one-way analysis of variance with Tukey's test (GraphPad Prism5, Boston, MA, USA). A p-value < 0.05 was considered statistically significant.

3. Results

3.1 Effect of LC extracts on ROS generation

Both LCE and LCW, at concentrations ranging from 0.01 to 1 mg/mL, showed no significant changes in DCF fluorescence intensity in H9c2 cardiomyoblasts (Figure 1). This result suggested that neither LC extract could induce ROS production in the cells.

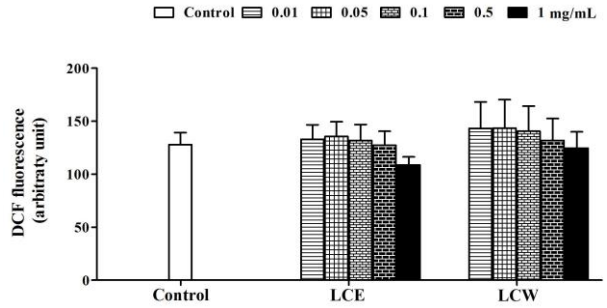

Figure 1. Effects of LC extracts on the ROS generation in H9c2 cardiomyoblasts. Cells were exposed to LCE (0.01-1 mg/mL) or LCW (0.01-1 mg/mL) for 24 h. Intracellular ROS contents were determined by using DCFH-DA and fluorescence intensity representing ROS content, measured by a fluorescence microplate reader. Neither LC extract could induce ROS production in the cells. Data are presented as mean ± SEM (n=4).

3.2 Cytotoxic effect of LC extracts

At concentrations up to 1 mg/mL, the extracts did not reveal toxicity to H9c2 cells, except at a high concentration (1 mg/mL) of LCE. Some concentrations of both LCE and LCW exerted a proliferative effect on the cells (Figure 2A, 2B). This suggests that LC extracts were likely non-toxic to H9c2 cardiomyoblasts, emphasizing their safety for tonic or therapeutic use on cardiac muscle cells.

3.3 Protective effect of LC extracts against HR-injured H9c2 cardiomyoblasts by MTT reduction assay

The H9c2 cells exposed to HR exhibited a significant decrease in cell viability compared to the normoxia control. Co-treatment for 6 h under hypoxia with LC extracts demonstrated that LCE (0.05 mg/mL and 0.1 mg/mL) and LCW (0.1 mg/mL and 0.5 mg/mL) significantly increased the survival of HR-injured cells (Figure 3). This suggests that LC may play a crucial role in protecting HR-injured cardiomyoblasts from cell death. The co-treatment with these extracts may partially protect HR-injured cells by supporting the function of mitochondrial succinate dehydrogenase.

3.4 Effect of LC extracts on cell and nuclear morphological changes

Cellular morphology, observed through phase-contrast imaging, confirmed the protective effects of LC extracts on HR-damaged cells. In the control, healthy cells were observed. HR induction caused an unhealthy appearance characterized by cellular atrophy and cell membrane shrinkage (Figure 4A). DNA condensation (Figure 4B), was observed after HR simulation, which implied apoptotic cell death. Notably, LCE (0.1 mg/mL) and LCW (0.5 mg/mL) substantially protected H9c2 cardiomyoblasts against HR damage. Co-incubation for 6 h under hypoxia with Trolox also protected cells from apoptosis caused by HR simulation. The cell morphology was substantially correlated with the survival percentage in the control, HR, LC- and Trolox-treated cells (Figure 2).

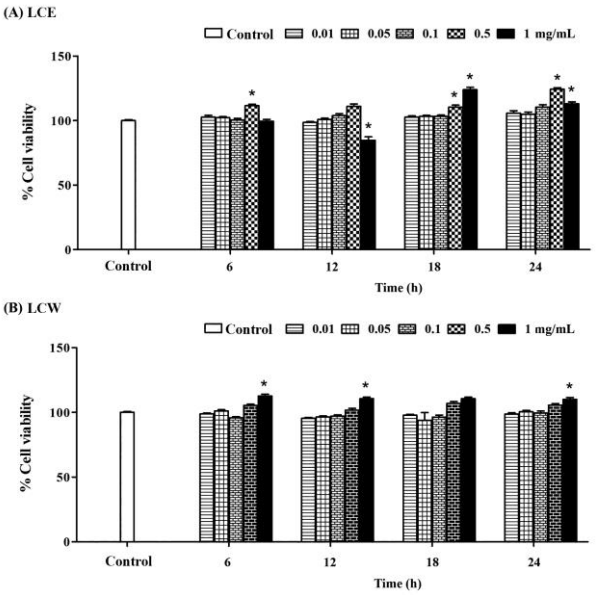

Figure 2. Cytotoxic effects of LC extracts on H9c2 cardiomyoblasts. The cells were treated with (A) LCE (0.01-1 mg/mL) and (B) LCW (0.01-1 mg/mL) at the indicated time points. Cell survival was measured by an MTT reduction assay. Some concentrations of both LCE and LCW exerted a proliferative effect on the H9c2 cells. Data are presented as mean ± SEM (n=4). *p < 0.05 in comparison with vehicle control group.

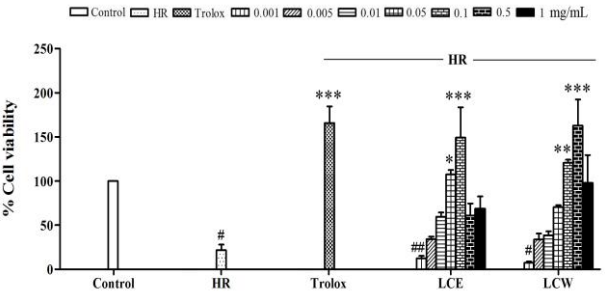

Figure 3. Protective effects of LC extracts on H9c2 cardiomyoblasts against HR injury. The cells were co-incubated under hypoxia with LCE (0.001-1 mg/mL) or LCW (0.001-1 mg/mL). After reoxygenation, cell survival was measured by the MTT reduction assay. Some concentrations of both LCE and LCW significantly increased the survival of HR-injured H9c2 cells. Values are expressed as a percentage of cell viability, compared to that in the normoxic control without the extracts, and are presented as mean ± SEM (n=4). #p < 0.05, ##p < 0.01 versus the control, *p < 0.05, **p < 0.01 and ***p < 0.001 versus HR without the extracts.

3.5 LC extracts restore MMP in HR-damaged H9c2 cardiomyoblasts

Considering the close association between the collapse of MMP and early mitochondrial damage followed by apoptotic cell death, we evaluated the loss of MMP using Rh123. Control cells showed Rh123 accumulation in mitochondria, suggesting live and healthy cells. Meanwhile, a

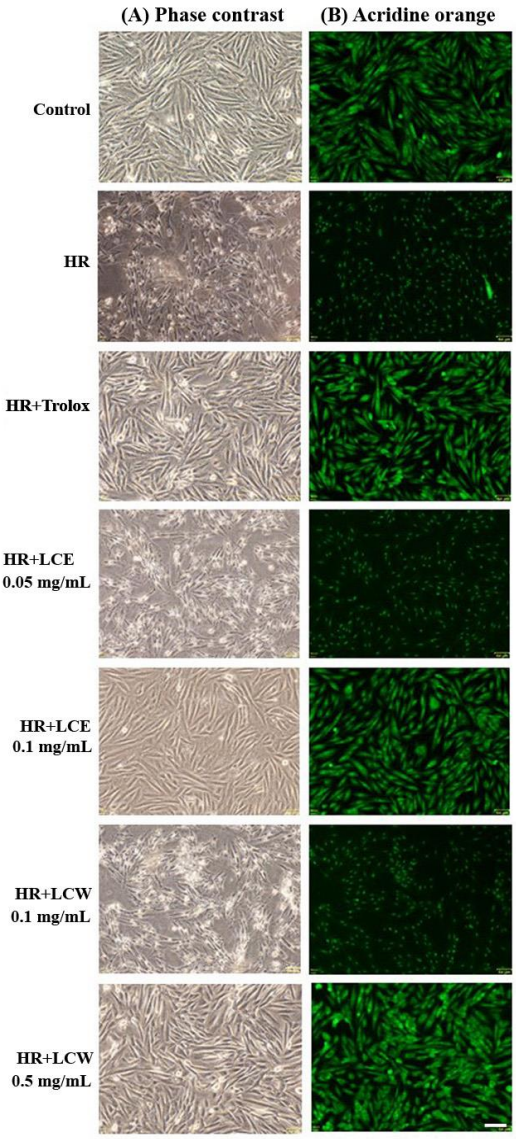

Figure 4. Protective effects of LC extracts on H9c2 cardiomyoblasts against nuclear morphological changes from HR injury. HR-stimulated cells were co-incubated under hypoxia with LCE (0.05 mg/mL and 0.1 mg/mL) or LCW (0.1 mg/mL and 0.5 mg/mL), followed by reoxygenation. The induction of apoptotic cell death by HR treatment was characterized by cell membrane shrinkage and DNA condensation in H9c2 cells. However, co-incubation with 0.1 mg/mL of LCE or 0.5 mg/mL of LCW substantially protected H9c2 cells from apoptotic cell death caused by HR damage. (A) Representative plasma membrane changes imaged using a phase-contrast microscope. (B) Representative cellular DNA changes after acridine orange staining, observed under a fluorescence microscope. The scale bar is for 100 microns (n=3).

collapse of MMP in the H9c2 cells exposed to HR conditions was observed (Figure 5). Co-incubation under hypoxia with LCE (0.1 mg/mL), LCW (0.5 mg/mL), or Trolox (250 μM) successfully prevented the loss of MMP in HR-injured H9c2 cardiomyoblasts.

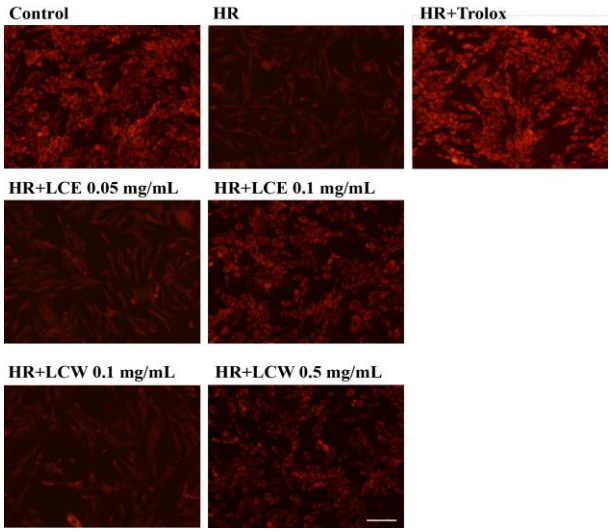

Figure 5. LC extracts restoring MMP in HR-simulated H9c2 cardiomyoblasts, as detected by Rh123 staining. Cells were co-incubated under hypoxia with LCE (0.05 mg/mL and 0.1 mg/mL) or LCW (0.1 mg/mL and 0.5 mg/mL). After reoxygenation, cells were imaged under a fluorescence microscope. Live and healthy cells showed Rh123 accumulation in mitochondria (bright red color) in H9c2 cells, while a collapse of MMP was observed in the H9c2 cells exposed to HR conditions (dull red color). Co-treatment with LCE (0.1 mg/mL), LCW (0.5 mg/mL), or Trolox (250 μM) during hypoxia prevented the loss of MMP in HR-injured H9c2 cells. The scale bar is for 100 microns (n=3).

3.6 Effects of LC extracts on protective and anti-apoptotic protein expression

To investigate whether the protective activity of LC extracts is mediated by protective and anti-apoptotic proteins, we investigated the expression of pERK1/2 and ERK1/2, as well as Bcl-2, respectively. The ratio of pERK1/2 to ERK1/2 increased significantly ($p < 0.001$) following co-incubation under hypoxia with LCE (0.1 mg/mL) or LCW (0.5 mg/mL) as well as Trolox, compared to HR treatment (Figure 6A, 6B). Meanwhile, Bcl-2 expression increased significantly ($p < 0.05$) following the treatment with LCW (0.5 mg/mL).

4. Discussion

The present study showed that LC extracts protected H9c2 cardiomyoblasts against HR-induced myocardial injury. The LC extracts (0.01-1 mg/mL) did not induce intracellular ROS generation in H9c2 cardiomyoblasts. Both LCE (0.1 mg/mL) and LCW (0.5 mg/mL) significantly increased cell viability and decreased apoptotic cell death caused by HR-induced H9c2 cell injury, which may be partially mediated through the inhibition of MMP loss, an increase in pERK/ERK ratio, and the up-regulation of Bcl-2 expression.

In the present study, LC extracts did not alter intracellular ROS production in H9c2 cells in a normal environment, suggesting that the extracts themselves did not cause ROS generation in H9c2 cells. An increase in ROS generation and a decrease in antioxidant defensive systems is known as a mechanism underlying myocardial ischemia (Zhao

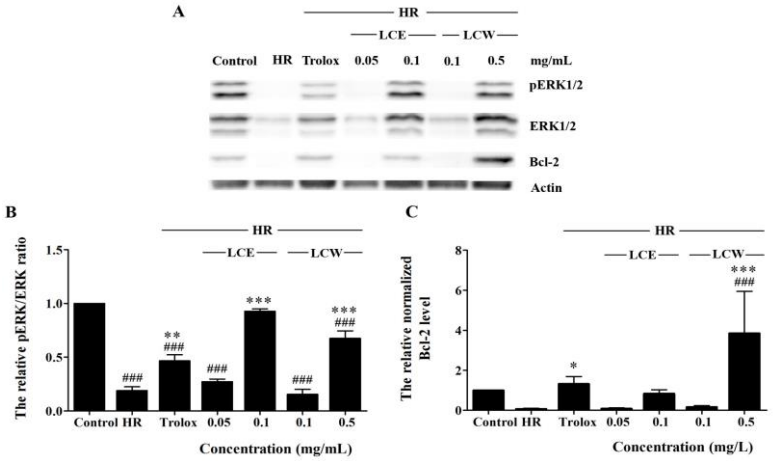

Figure 6. Western blot analysis of pERK1/2, ERK1/2, and Bcl-2 expressions in both LCE and LCW-treated HR-exposed H9c2 cardiomyoblasts. The cell lysate was subjected to electrophoresis on SDS–polyacrylamide gel electrophoresis, with a subsequent enzyme immune assay using enhanced chemiluminescence. (A) Shows representative bands, and (B) illustrates a quantitative analysis of protein expression. Each point represents mean ± SEM (n=4), ###p <0.001 versus the control untreated cells, *p < 0.05, **p < 0.01 and ***p < 0.001 versus the HR-treated cells.

& Zhao, 2010). MMP loss, usually via ROS generation, has been recognized as early initiator of apoptotic cell death by releasing cytochrome c, thereby leading to the activation of the caspase family (Zhao *et al*., 2022). This has shown the critical role of mitochondria in IR-damaged hearts of mice, by opening mitochondrial permeability transition pores, accompanied by a decrease in Bcl-2 expression, which could play a major role in leading to apoptotic cell death (Chen *et al*., 2015), and has displayed DNA condensation, as shown in the present study. An increase in Bcl-2 expression by LCW in the present study would serve as one of the key regulators to rescue H9c2 cells from myocardial IR injury (Korshunova *et al*., 2021), for which LCW has shown high amounts of phenolic compounds. The induction of Bcl-2 expression is a target of ERK1/2 phosphorylation in the reperfusion injury salvage kinase (RISK) pathway, which inhibits cardiomyocyte death in IR injury (Korshunova *et al*., 2021).

The protective mechanism of ERK1/2 expression during exposure to IR injury remains controversial. In contrast to the present study, curcumin reportedly rescues H9c2 cells from HR injury through the down-regulation of MAPK signaling pathways (ERK1/2, JNK, and p38) (Wei, Peng, & Li, 2019). Meanwhile, sufentanil was shown to be able to restore pERK1/2 expression in IR-injured rat hearts and also increased growth rates in H9c2 cells (Tao, Nuo, & Min, 2018). *Polygonum orientale* L. can, according to experimental evidence, attenuate myocardial ischemia by decreasing inflammation through activation of the MAPK/ERK signaling pathway (Fu *et al*., 2023).

LC extract (using 0.1 ml glacial acetic acid and 90% methanol), containing tetramethylpyrazine (TMP, chuanxiongize or ligustrazine), ferulic acid, cnidilide and ligustilide, could protect coronary artery ligation-induced acute myocardial ischemic injury in canines (Liu, Li, Ji, Cui, & Li, 2016). It has been demonstrated that both 50% ethanolic and water extracts of LC, which were also used in the present study, possess antioxidant activities against 2,2-diphenyl-1-picrylhydrazyl, superoxide anion, hydrogen peroxide, hydroxyl radical, and nitric oxide radical (Nalinratana *et al*., 2014). Water extract from LC exhibits excellent capacities to scavenge DPPH, •OH, and superoxide anion radicals *in vitro* as well as elevated antioxidant enzyme activities and gene expression including superoxide dismutase, catalase, and glutathione reductase in *Saccharomyces cerevisiae* (Yan *et al.*, 2023). However, the cardioprotective activities of LC should be cumulative results from its active ingredients, including ferulic acid, ligustrazine, and lactone component. In the present study, ferulic acid contents in 50% ethanolic and water extracts of LC were 0.03% and 0.01%, respectively. Li *et al.* (2012) demonstrated variations in the ferulic acid content of LC using different extraction solvents, such as 95% ethanol (0.9395 mg/g), 50% ethanolic extract (1.141 mg/g), and water extracts (1.87~2.17 mg/g). This suggests that the ferulic acid content could increase with the polarity of the solvent used (Ahmed *et al*., 2022). The relative polarities of ethanolic and water solvents were determined as 0.654 and 1.000 respectively. Moreover, water is acknowledged as an efficient solvent for extracting polar molecules (Plaskova & Mlcek, 2023). Study of 3 alternative ethanol concentrations (50%, 70%, and 96%) in extraction by cold maceration method revealed that 50% ethanol is the best solvent for extracting phenolic molecules (antioxidant compounds) with high power to inhibit free radicals by donating electrons from hydroxyl group to free radicals (Hikmawanti, Fatmawati & Asri, 2021) that are known as an important cause of severe cardiac injury from AMI. It has been shown that ferulic acid treatment during 2 h hypoxia can protect H9c2 cardiomyocytes against HR injury by reducing mitochondrial dysfunction and inhibiting apoptosis (Luo *et al*., 2020). Ligustrazine is one of the primary active ingredients, and its cardioprotective effects have been explored in various studies. A meta-analysis of 25 studies demonstrated the cardioprotective effects of ligustrazine against myocardial IR injury by significantly decreasing myocardial infarct size, cardiac enzymes, and troponin via myocardial metabolic disorders and structural remodeling. In addition, it involves possible mechanisms including antioxidant, anti-inflammation, and anti-apoptotic cell death, as well as improved coronary blood flow and

myocardial metabolism (Zheng *et al*., 2018). However, it has been suggested that a very low level of ligustrazine was detected in the *Chuanxiong* herb (Yi, Leung, Lu, Chan, & Zhang, 2006). Lactone component from LC also showed cardioprotective effects against isoproterenol-induced myocardial ischemia injury in rats and an ischemia model by oxygen-glucose deprivation in H9c2 cardiomyoblasts (Wang *et al*., 2018).

4. Conclusions

LC extracts did not cause intracellular ROS production in H9c2 cardiomyoblasts in normal situations. The results in this study first established the cardioprotective mechanisms of both 50% ethanolic and water extracts of LC against HR injury in H9c2 cells through the inhibition of MMP loss and an increase in pERK/ERK ratio. Additionally, only the water extract of LC increased Bcl-2 expression. The findings of this study suggest the tentative use of LC for alleviating or limiting IR damage to cardiomyoblasts in AMI.

Acknowledgements

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