# Cardioprotection of *Atractylodes lancea* against Hypoxia/ Reoxygenation-Injured H9c2 Cardiomyoblasts

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***Background:*** *Atractylodes lancea (Thunb) DC has been widely used as traditional medicine in many countries including Thailand for the treatment of fever, common cold and sore throat.*
***Objective:*** *To evaluate the cardioprotective effects of Atractylodes lancea extracts against hypoxia/reoxygenation (HR)-injured H9c2 cardiomyoblasts.*
***Material and Method:*** *For cytotoxic determination, the H9c2 cells were incubated with the ethanolic extract of Atractylodes lancea (AL-E) and water extract (AL-W) at the concentrations between 0.01-1 mg/mL in normoxia for 6, 18, 24, and 48 h. Cell viability were determined by MTT assay and observed cellular morphology under phase contrast microscopy. In a time-course study of HR model on H9c2 cardiomyoblasts, the cells were exposed to hypoxic condition at various time points (0.5, 1, 2, 4, 6, and 8 h) before 24 h reoxygenation. According to more than 90% of cell death, 6 h exposure to hypoxia was used for cardioprotective evaluation throughout the present study. Cell viability, DNA condensation by Hoechst 33342, and protein expression of ERK1/2, p-ERK1/2 and HO-1 by western blot analysis were investigated.*
***Results:*** *Incubation of AL-E and AL-W at concentrations up to 1 mg/mL for 24 h showed no toxicity on H9c2 cells. Exposure of the H9c2 cells to HR showed a time-dependent decrease in cell viability. Treatment of both AL-E and AL-W (0.05 and 0.1 mg/mL) showed protection against cell death and cellular shrinkage as well as inhibited DNA condensation. AL at the same concentrations increased the expression of ERK1/2, p-ERK1/2 and HO-1, when compared to HR-injured cells.*
***Conclusion:*** *AL protected HR-damaged H9c2 cells by inhibiting cell death, cellular shrinkage and DNA condensation, which was partially through restoring ERK1/2, p-ERK1/2, and HO-1 expression. The present results are beneficial use of AL as alternative medicine.*

***Keywords:*** *Atractylodes lancea, Cardioprotection, Hypoxia/reoxygenation, DNA condensation, ERK, HO-1*

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*Atractylodes lancea* (Thunb) DC, which belongs to the Asteraceae family, has been widely used as traditional medicine in many countries including China, Japan, and Thailand. In Thai traditional medicine, the dried rhizome of *A. lancea* (called Kot-Khamao) has been used for treatment of fever, flu, sore throat, and common cold[1]. It also contained in Thai folk medicines including "Yahom Navakot" for treatment of cardiovascular symptoms including dizziness and fainting[2] as well as Pra-Sa-Prao-Yhai recipe for anti-emetic and analeptic[1]. *A. lancea* contains at least two major active ingredients including terpenoids and many sesquiterpenoid glycosides including beta-eudesmol and hinesol[3]. It contains antioxidant components including phenolics, flavonoids, and anthocyanins, and antioxidant properties both in cell-free system and on human endothelial cells[4]. It also showed an anti-inflammatory activity against lipopolysaccharides-stimulated murine RAW 264.7 macrophage cells[5]. Hence, it is interesting to study the possibility of *A. lancea* to protect and/or alleviate the disease causing from oxidative stress as well as ischemia/reperfusion.

Hypoxia/reoxygenation (HR) is used as a cell culture model to mimic ischemia-reperfusion in tissues and organs. Hypoxia causes a massive production of reactive oxygen species (ROS) at mitochondria, however, the generation of ROS showed augmented increase during reperfusion period[6]. HR has been known to be an etiological factor for the tissue damage associated with ischemic diseases including ischemic heart disease[7,8], finally leading to cardiac myocyte death[9]. ROS cause cell membrane lipid peroxidation,

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protein and DNA damage, mitochondrial permeability transition, and activate apoptotic signaling molecules or inactivate survival proteins leading to cell death[10]. An imbalance between the generation of ROS and the activities of antioxidant defenses, oxidative stress, is also one of the major causes of ischemic heart disease which is a worldwide health problem[11]. Herbal medicines have become increasing popular as alternative medicines for treatment of numerous diseases including myocardial ischemia as shown from previous studies[12,13]. However, little scientific evidence of *A. lancea* on HR-injured cardiomyoblasts has been demonstrated. The purpose of this study was to screen the cytotoxic and cardioprotective effects of *A. lancea* against HR-damaged cardiomyoblasts using H9c2 cells, a useful tool in elucidating the cellular mechanisms involved in ischemic heart diseases.

## Material and Method

### *Drugs 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) and dimethyl sulfoxide (DMSO), and Hoechst 33342 were purchased from Sigma (St. Louis, MO, USA). Fetal bovine serum (FBS) was from Hyclone UK Ltd., Northumberland, UK. Dulbecco's modified Eagle medium (DMEM), and penicillin G and streptomycin were obtained from Invitrogen (Carlsbad, CA, USA). Other organic solvents were all of analytical or reagent grade.

### *Preparation of extracts*

The rhizomes of *A. lancea* were purchased from the Oriental Medicine Drugstore in Bangkok, Thailand in October 2009. The morphological characteristics were identified by Dr. Sanya Hokputsa according to the quality control parameters in Thai Herbal Pharmacopoeia, and compared with the authentic specimens, generously provided by Associate Professor Dr. Noppamas Soonthornchareonnon, Faculty of Pharmacy, Mahidol University, Thailand. The Voucher specimens (NVK10-52) have been deposited at the Phytochemical Research Group, Research and Development Institute, Government Pharmaceutical Organization, Thailand. Briefly, the dried specimens were cleaned, and 1 kg of each plant or formula was extracted two times by reflux in 5 L of 50% ethanol or water for 3 h. After the extraction, each extract was accumulated and dried by freeze drying. To reduce the variation between each extraction, a massive single production of individual samples was prepared for the whole experiments. The ethanol extracts of *A. lancea* (AL-E) and water extract (AL-W) were kept in light-protected bottles or dissolved in 200 mg/mL DMSO, and stored at -20°C until the experiment was performed. The stocked solutions were further diluted in culture media to achieve the final concentration of 0.5% DMSO.

### *Cell culture*

H9c2, a rat cardiomyoblast cell line from the American Type Culture Collection (ATCC, CRL-1446; Manassas, VA, USA) was grown in 75-$cm^3$ tissue culture flasks in complete medium (DMEM supplemented with 10% FBS and 100 IU/mL penicillin/ 100 µg/mL streptomycin) in 5% $CO_2$ atmosphere at 37°C, and routinely subcultured using 0.25% trypsin when cells reached 70-80% confluence.

### *Assessment of cell viability*

The cells were seeded at a density of $5x10^4$ cells/well in 96-well plates and stood for 24 h in 5% $CO_2$ incubator. After incubation with various concentrations (0.01, 0.05, 0.1, 0.5 and 1 mg/mL) of both extracts of AL for 24 h, the cells were incubated with MTT reagent at a final concentration of 1 mg/mL for 4 h at 37°C. Viable cells contain active mitochondria, the tetrazolium ring is cleaved and a visible dark blue product is generated through the formazan reaction. In order to stop the reaction, lysis buffer (50% N, N-dimethyl formamide and 20% sodium dodecyl sulphate, pH 4.7) was added into each well[14]. The reaction mixture was left overnight at 37°C, and the optical density was measured by a microplate reader (Sunrise Classic, Tecan, Austria) using a wavelength of 595 nm. The percentage of cell viability was expressed as the relative formazan formation in treated cells when compared to the vehicle control using the following formula: (absorbance of treated cells/absorbance of control cells) x100.

### *H9c2 cardiomyoblast model of HR*

The experimental protocol of HR model in H9c2 cells was according to Hu et al (2008)[15]. Hypoxia was achieved by using hypoxic buffer solution consisted of 137 mM NaCl, 15.8 mM KCl, 0.49 mM $MgCl_2$, 0.9 mM $CaCl_2$, 4 mM HEPES supplemented with 20 mM 2-DOG (an inhibitor of glycolysis), 2.5 mM $Na_2S_2O_4$ (an oxygen scavenger), and 20 mM sodium lactate (to mimic local lactate accumulation), pH 6.5 (to mimic acidosis). The cells were placed in a modular incubator chamber (Billups-Rothenberg, Inc., CA,

USA), flushed with pure $N_2$, and incubated at 37°C for designated time points (0.5, 1, 2, 4, 6 and 8 h). Control cells were incubated in normoxia Krebs-Henseleit (KH) solution containing 118 mM NaCl, 5 mM KCl, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$, 1.2 mM $KH_2PO_4$, 1.25 mM $CaCl_2$, and 11 mM glucose, pH 7.4, and placed in 5% $CO_2$ incubator. When the time was over, hypoxic cells were immediately exposed to complete medium for extended 24 h. At the end of each experiment, cell viability was determined by MTT assay as described above.

***Determination of cell viability by MTT assay and cell morphology by phase contrast microscopy***

The H9c2 cells were exposed to various concentrations (0.01, 0.05, 0.1, 0.5 and 1 mg/mL) of AL-E and AL-W in hypoxic buffer (co-treatment), and incubated at 37°C in an ischemic chamber for 6 h. Treated cells were then reoxygenated by changing ischemic buffer to complete medium, and then returning to a normoxia environment at 37°C in 5% $CO_2$ atmosphere for 24 h. Trolox (250 μM) were used as positive control for protection of HR injury[16]. At the end point, cell survival was measured by MTT assay, as described above. Cell morphology was also observed under phase contrast microscopy using Bioimage FSX100 (Olympus, Tokyo, Japan).

***DNA condensation by Hoechst 33342 staining***

Treated cells were also stained by Hoechst 33342 with a final concentration of 1 mg/mL for 15 min in the dark at 37°C, as described previously[17]. The morphological change of DNA was visualized with a fluorescence microscope using Bioimage FSX100. The nuclear morphology with brightly blue stained condensed chromatin, nuclear fragmentation or apoptotic bodies were considered as apoptotic cells.

***Western blot analysis***

The HR-treated cells in 6-well plates were incubated with AL-E and AL-W (0.01, 0.05 and 0.1 mg/mL) during exposure to hypoxic buffer, meanwhile, the control cells were incubated with KH solution for 6 h before reoxygenation with complete medium for 24 h. HR-induced cells co-incubated with trolox was used as a positive control. At the end of the experiment, the cells were washed with ice-cold PBS and solubilized in lysis buffer containing 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 20 mM EDTA, 50 mM NaF, 1% NP-40, 0.02% $NaN_3$ with protease inhibitors (1 mM phenyl-methanesulfonyl fluoride, 2 μg/mL leupeptin, and 2 μg/mL aprotinin). Lysates were briefly sonicated, centrifuged, and protein concentrations of supernatant were determined by Bradford method. After mixed with loading buffer and boiled for 5 min, the equal amounts of protein (40 μg) were separated by SDS gel electrophoresis and then transferred onto PVDF membranes. The membranes were blocked with 5% skimmed milk in TBST for 1 h at room temperature, incubated with primary antibodies ERK1/2, p-ERK1/2 and HO-1 (1:1,000) for overnight, and then probed with secondary antibody for 1 h. Protein bands were visualized by ECL Detection under gel documentation (GeneGnome5, Syngene, Cambridge, UK).

***Data analysis***

Data were expressed as means ± SEM, and analyzed using one-way analysis of variance (ANOVA) with Tukey's test. A *p*-value of <0.05 was considered statistically significant.

**Results**

***Effect of AL on cell viability***

To evaluate the cytotoxicity of AL on H9c2 cardiomyoblasts, the cells were incubated with various concentrations (0.01-1 mg/mL) of both extracts in normoxic condition for 24 h. After incubation, AL-E and AL-W showed no severe cytotoxicity to H9c2 cells (Fig. 1). Meanwhile, 48 h incubation of AL-E or AL-W at a concentration of 1 mg/mL caused a significant decrease in cell viability (Fig. 1).

***HR-injured H9c2 cardiomyoblasts in time-dependency***

To test the H9c2 model for HR simulation, the survival rate of the HR cardiomyoblasts was tested at various time periods (0.5, 1, 2, 4, 6 and 8 h) of hypoxia followed by exposure to 24 h reoxygenation. The results showed that exposure of H9c2 cells to HR at various time points caused a time-dependent decrease in the percentage of cell survival (Fig. 2A, 2B). Since, 6 h exposure to hypoxia was the first time point showing more than 90% cell death, this time point has been entirely used to test the protective properties of the extracts throughout the present study.

***AL protected H9c2 cardiomyoblasts against HR injury***

To demonstrate whether AL was able to protect HR-injured H9c2 cells, co-incubation during hypoxia before the cells exposed to reoxygenation was performed. Exposure of H9c2 cells to HR significantly

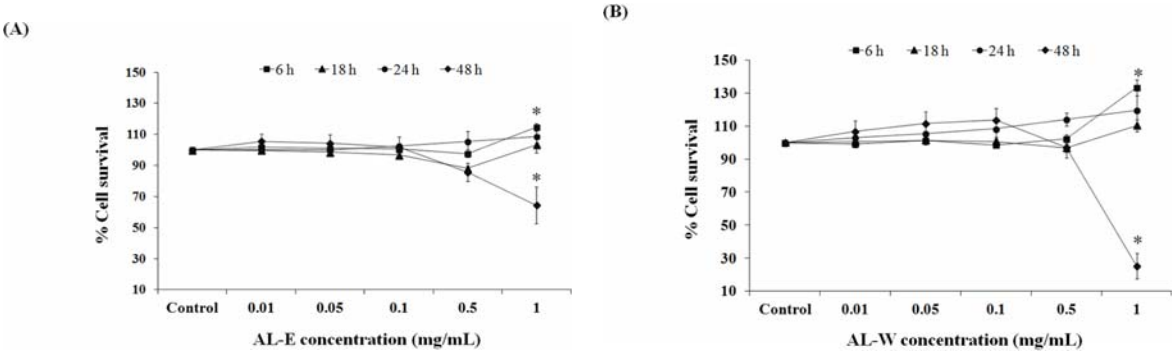


**Fig. 1** Cytotoxic effect of AL-E (A) and AL-W (B) in H9c2 cardiomyoblasts. The cells were treated with AL-E and AL-W at concentrations between 0.01-1 mg/mL in normoxia for 6, 18, 24 and 48 h. Cell viability measured by MTT assay was represented as the percentage relative absorbance compared to vehicle control. Data represent the mean ± SEM of four independent experiments, triplicate. * $p<0.05$ vs. vehicle control group. AL-E, ethanolic extract of *Atractylodes lancea*; AL-W, water extract of *Atractylodes lancea.*

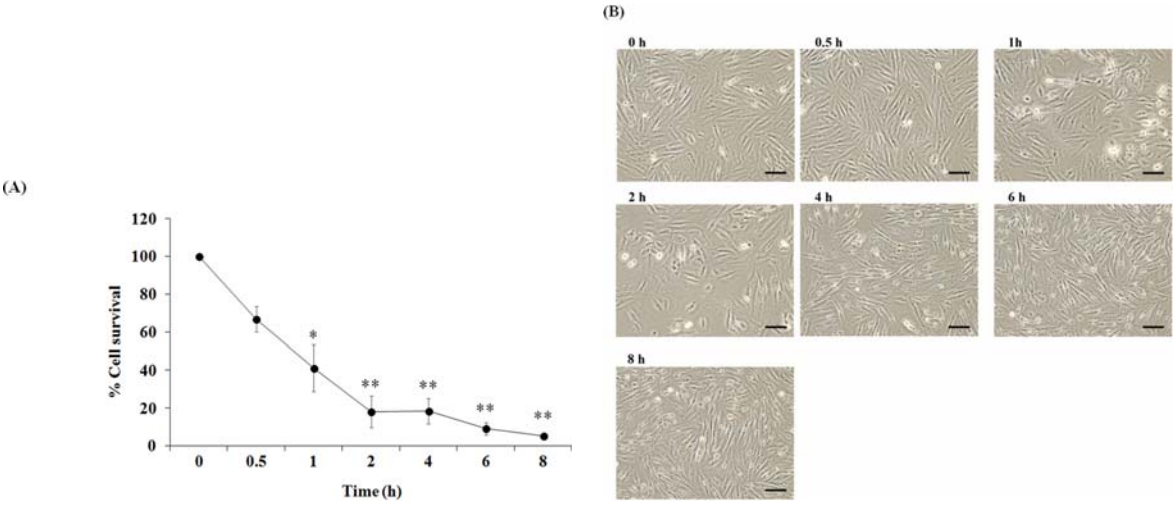


**Fig. 2** Time-course study of HR-injured H9c2 cardiomyoblasts. The cells were exposure to hypoxic buffer at various time points (0.5, 1, 2, 4, 6 and 8 h) followed by 24 h reoxygenation in complete medium. Cell viability determined by MTT reduction assay. (A) The percentage relative absorbance compared to vehicle control, and (B) the represented microscopy images were shown. Data represent the mean ± SEM of five independent experiments, triplicate. * $p<0.05$ vs. vehicle control group; ** $p<0.01$ vs. vehicle control group. Scale bar = 100 microns. HR = hypoxia/reoxygenation.

decreased ($p<0.05$) the percentage of cell survival when compared to normoxia control (Fig. 3A). Under phase contrast microscope, HR-exposed cells also presented with unhealthy appearance including cellular shrinkage (Fig. 3B) accompanied by DNA condensation (Fig. 3C). In addition, co-treatment of AL-E and AL-W (0.05 and 0.1 mg/mL) during hypoxia showed a significant increase ($p<0.05$) in the percentage of cell survival when compared to hypoxic cells (Fig. 3A), which were protected by AL-E and AL-W (Fig. 3B, C). Our results suggested that AL may play a partial role in protecting HR-injured cardiomyoblasts.

### *AL restored ERK1/2, p-ERK1/2 and HO-1 expression on HR-injured H9c2 cardiomyoblasts*

To elucidate the mechanism underlying AL-protected H9c2 cells against HR injury, the expression of survival proteins including ERK1/2, p-ERK1/2 and HO-1 were investigated. HR-exposed cells showed a decrease in the expression of ERK1/2, p-ERK1/2 and HO-1 when compared to the control (Fig. 4A, B). Treatment of AL-E and AL-W at concentrations of

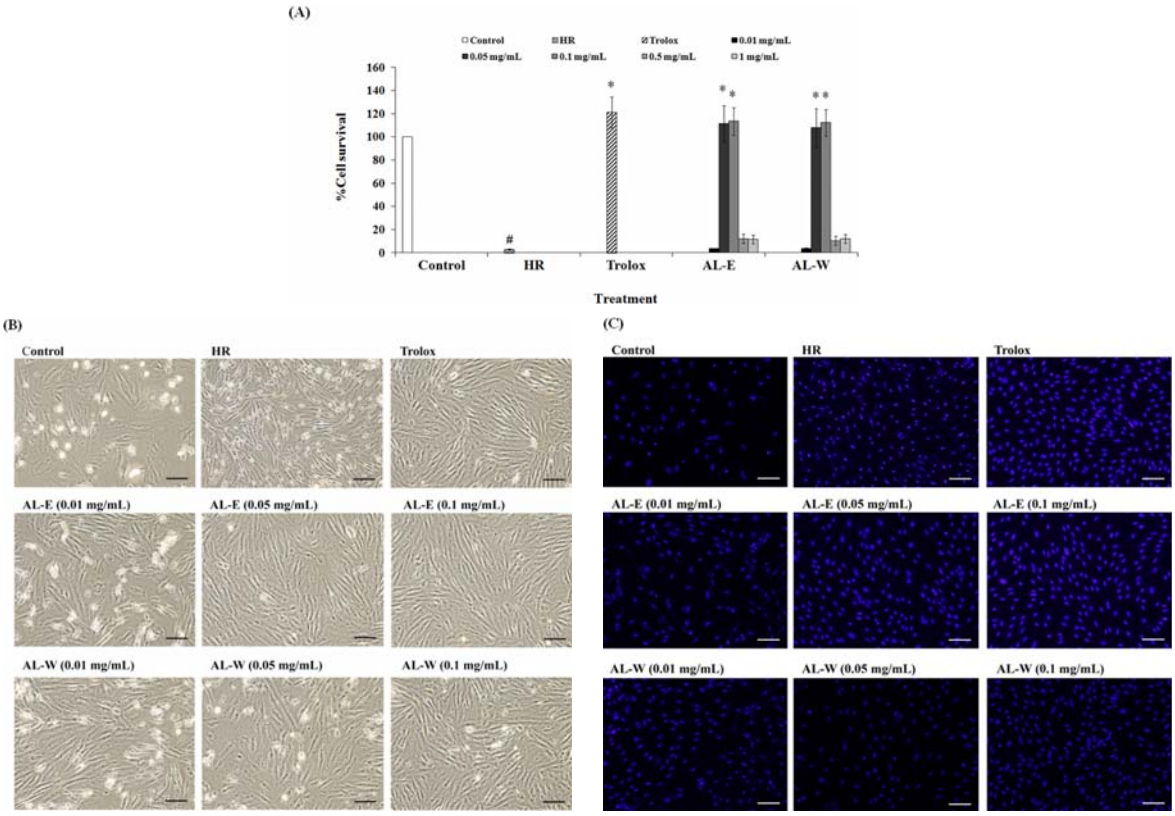


**Fig. 3** Protective effect of AL-E and AL-W against HR-injured H9c2 cardiomyoblasts. The cells were co-incubated with AL-E and AL-W (0.01, 0.05 and 0.1 mg/mL) in hypoxic buffer for 6 h before exposure to 24 h reoxygenation. (A) Cell viability was expressed as a percentage of cell viability compared with the control cells (normoxia without the extracts), (B) cell morphology was observed under phase contrast microscope, and (C) DNA stained by Hoechst 33342 was observed under Fluorescent microscope. Values are presented as the mean ± SEM of four independent experiments. # $p<0.05$ vs. control normoxia, * $p<0.05$ vs. HR without the extracts. Scale bar = 100 microns. AL-E, ethanolic extract of *Atractylodes lancea*; AL-W, water extract of *Atractylodes lancea*; HR, hypoxia/reoxygenation

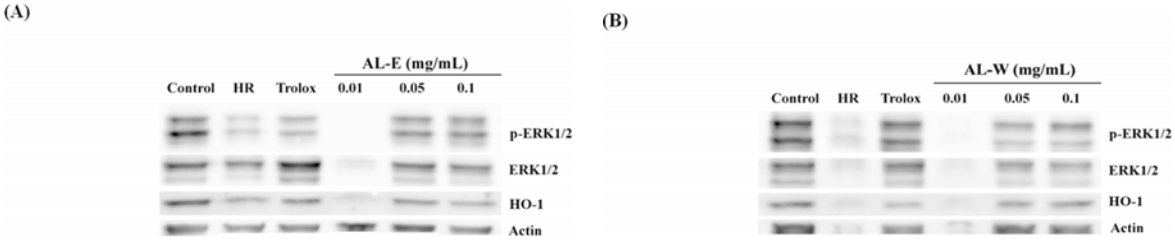


**Fig. 4** Protein expression of ERK1/2, p-ERK1/2 and HO-1 after the H9c2 cardiomyoblasts co-incubated with AL-E and AL-W during 6 h hypoxia followed by 24 h reoxygenation. Representative protein bands were shown, (A) AL-E treatment and (B) AL-W treatment at the concentrations between 0.01-0.1 mg/mL. AL-E, ethanolic extract of *Atractylodes lancea*; AL-W, water extract of *Atractylodes lancea.*

0.05 and 0.1 mg/mL showed augmented increase in band intensity of p-ERK1/2, ERK1/2, and HO-1 when compared to HR-exposed cells (Fig. 4A, B). The results suggested that a cardioprotective effect of AL may be at least partially acting through ERK1/2 and HO-1 signaling.

## Discussion

In the present study, we have demonstrated the cytotoxic and protective effects of the ethanolic

and aqueous extracts of AL against myocardial injury induced by HR.

In the present study, 24 h incubation of 50% ethanolic and water extract of *A. lancea* (AL-E and AL-W) showed no toxicity against H9c2 cardiomyoblasts and at the concentrations of 0.05 and 0.1 mg/mL were able to protect H9c2 cells injury from HR exposure. Meanwhile, previously it was demonstrated that 95% ethanolic extract of AL caused selective damage against anticholangiocarcinoma with an $IC_{50}$ value of 24.09 mg/mL[18]. *A. lancea* extract has showed potent anti-inflammatory activity against lipopolysaccharide-induced NO production in RAW 264.7 cells with an $IC_{50}$ value of 9.70 mg/mL[19]. However, hydroethanolic and water extracts of *A. lancea* were not cytotoxic ($IC_{50}$ >5,000 mg/mL) on human endothelial ECV304 cells[4]. The controversial results are usually from various factors including type of extract, the potency of the active compound, exposure time, conditions, and cell types as previously described[18,20] as well as the time course of each ischemia and reperfusion[9,21,22].

Ischemic heart disease, one of the major causes of cardiovascular diseases[6], which in part, has been from massive generation of ROS demonstrated in both primary rat cardiac cells[23] and H9c2 rat cardiomyoblast cell lines[24]. During myocardial ischemia, it has been demonstrated that an increase in ROS generation and a decrement in the antioxidant defensive systems[25] lead to apoptotic cell death[26]. Our results showed that AL was able to protect DNA condensation, which accompanied by a restoration of ERK1/2, p-ERK1/2 and HO-1. Both hydroethanolic and water extracts of *A. lancea* have been demonstrated to exhibit strong antioxidant potential against 2,2-diphenyl-1-picrylhydrazyl scavenging assay, superoxide anion, hydroxyl radical and nitric oxide radical in cell-free antioxidant assays[4]. The hydroethanolic extract (50-200 mg/mL) was able to decrease intracellular ROS generated from $H_2O_2$-treated human endothelial ECV304 cells[4]. It has been shown that pretreatment of *Atractylodes japonica* Koidzumi showed an increase in the phosphorylation of ERK in HMC-1 cells[27]. The activation of Nrf2/ERK mitogen activated protein kinase dependent HO-1 expression was stimulated by oxidative stress[28]. In addition, HO-1 inducer and activation of ERK/Nrf2 pathway were able to protect oxidative stress-induced apoptotic cell death[29].

In conclusion, the results first establish the cardioprotective effect of AL against HR injury in H9c2 cells by inhibiting cell death, cellular shrinkage and DNA condensation, and partly via the expression of ERK1/2, p-ERK1/2 and HO-1.

**What is already known on this topic?**

No document has been evaluated the effect of *A. lancea* on HR-injured cardiomyoblasts.

**What this study adds?**

The present study is the first study to demonstrate the cardioprotective effects of *A. lancea* against HR injury on H9c2 cardiomyoblasts which would be through the expression of ERK1/2 and HO-1.

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**Potential conflicts of interest**

None.

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## *ฤทธิ์ของโกฐเขมาในการปกป้องเซลล์กล้ามเนื้อหัวใจ H9c2 จากภาวะขาดออกซิเจนแบบชั่วคราว*

***พรรณี หนูซื่อตรง, อรพิน เกิดประเสริฐ***

***ภูมิหลัง:*** *โกฐเขมา (Atractylodes lancea (Thunb) DC) เป็นยาสมุนไพรที่ใช้กันอย่างแพร่หลายในหลายประเทศรวมทั้งประเทศไทยในการรักษาอาการไข้ ไข้หวัด และอาการเจ็บคอ*

***วัตถุประสงค์:*** *เพื่อศึกษาฤทธิ์ของสารสกัดโกฐเขมาในการปกป้องเซลล์กล้ามเนื้อหัวใจ H9c2 จากภาวะขาดออกซิเจนแบบชั่วคราว*

***วัสดุและวิธีการ:*** *การทดสอบพิษของสารสกัดโกฐเขมาในเอทานอล (AL-E) และในน้ำ (AL-W) โดยบ่มเซลล์กล้ามเนื้อหัวใจ H9c2 ด้วยสารสกัดที่ความเข้มข้นระหว่าง 0.01-1 มิลลิกรัมต่อมิลลิลิตร ในสภาวะอากาศปกติเป็นเวลา 6, 18, 24 และ 48 ชั่วโมง เมื่อครบกำหนดเวลาทำการวัดอัตราการอยู่รอดของเซลล์ด้วยวิธี MTT assay และสังเกตการเปลี่ยนแปลงรูปร่างของเซลล์โดย phase contrast microscopy การทดสอบเพื่อจำลองสภาวะขาดออกซิเจนแบบชั่วคราวในเซลล์ H9c2 โดยให้เซลล์สัมผัสกับสภาวะขาดออกซิเจนที่เวลาต่าง ๆ (0.5, 1, 2, 4, 6 และ 8 ชั่วโมง) ก่อนนำมาเลี้ยงต่อในสภาวะอากาศปกติเป็นเวลา 24 ชั่วโมง การขาดออกซิเจนเป็นเวลา 6 ชั่วโมงทำให้เซลล์ H9c2 ตายมากกว่า 90 เปอร์เซ็นต์ จึงใช้ระยะเวลาดังกล่าวในการประเมินฤทธิ์ปกป้องเซลล์หัวใจต่อไป การวัดอัตราการอยู่รอดของเซลล์ สังเกตลักษณะของดีเอ็นเอจากการย้อมเซลล์ด้วย Hoechst 33342 และศึกษาการแสดงออกของโปรตีน ERK1/2, p-ERK1/2 และ HO-1 โดยวิธี western blot*

***ผลการศึกษา:*** *การบ่ม AL-E และ AL-W ที่ความเข้มข้น 0.01-1 มิลลิกรัมต่อมิลลิลิตร เป็นเวลา 24 ชั่วโมง ไม่เป็นพิษต่อเซลล์ H9c2 ผลการทดสอบเพื่อจำลองสภาวะขาดออกซิเจนแบบชั่วคราวพบว่าอัตราการตายของเซลล์มากขึ้นแปรผันตามเวลาที่เซลล์สัมผัสกับสภาวะขาดออกซิเจน การบ่มสารสกัดที่ความเข้มข้น 0.05 และ 0.1 มิลลิกรัมต่อมิลลิลิตร ในสภาวะขาดออกซิเจนแบบชั่วคราวสามารถลดอัตราการตายของเซลล์ การเกิด cellular shrinkage และ DNA condensation และที่ความเข้มข้นเดียวกันมีผลเพิ่มการแสดงออกของโปรตีน ERK1/2, p-ERK1/2 และ HO-1 เมื่อเปรียบเทียบกับเซลล์ที่อยู่ในสภาวะขาดออกซิเจนแบบชั่วคราว*

***สรุป:*** *โกฐเขมาสามารถปกป้องการตายของเซลล์กล้ามเนื้อหัวใจจากภาวะขาดออกซิเจนแบบชั่วคราวได้ โดยยับยั้งการตายของเซลล์ การเกิด cellular shrinkage และ DNA condensation โดยกลไกบางส่วนผ่านทางการแสดงออกของโปรตีน ERK1/2, p-ERK1/2 และ HO-1 การศึกษาในครั้งนี้จะเป็นแนวทางในการนำโกฐเขมามาใช้ประโยชน์ทางการแพทย์ทางเลือก*