Research Article **Biotechnology**

International Journal of Pharma and Bio Sciences **ISSN 0975-6299**

EFFECTS OF *AQUILARIA CRASSNA* CRUDE EXTRACT ON SIMULATED ISCHEMIA INDUCED CARDIAC CELL DEATH

PANADDA JERMSRI [1,2] ARUNYA JIRAVIRIYAKUL [2], SASIMANAS UNAJAK [3] AND SARAWUT KUMPHUNE [*1,2]

[1] Biomedical Research Unit in Cardiovascular Sciences (BRUCS), Faculty of Allied Health Sciences, Naresuan University, Phitsanulok 65000, Thailand
[2] Department of Medical Technology, Faculty of Allied Health Sciences, Naresuan University, Phitsanulok 65000, Thailand
[3] Department of Biochemistry, Faculty of Science, Kasetsart University, Bangkok 10900 Thailand

ABSTRACT

To investigate the effect of ethylacetate extract of *Aquilaria crassna* Pierre ex Lecomte on simulated ischemia-induced cardiac cell injury and its mechanism on p38 MAPK activation. Rat cardiac myoblast cell line (H9c2), were subjected to 2 hours simulated ischemia (sI) and 24 hours of reperfusion, in the presence and absence of the ethyl acetate extract of *Aquilaria crassna* (A.Q.). The cellular injury and viability were determined. The result found that A.Q. at concentration 5 mg/ml was the most effective concentration to reduce cell death and injury. Then, cells were pre-treated with or without 5 mg/ml of A.Q. for 1 hour before, or at the beginning of 40 minutes-sI. Activation of p38 MAPK was measured by Western blot analysis. Treatment of A.Q. significantly reduced ischemia-induced p38 MAPK phosphorylation (p <0.05). In conclusion, treatment of 5 mg/ml of the ethylacetate extract of *A. crassna* reduced ischemia induced cell death by attenuating p38 MAPK phosphorylation.

KEYWORDS: ***Aquilaria crassna;*** **Ischemic heart disease; Myocardial Ischemia; cardiac cell; cell death; p38 MAPK**

SARAWUT KUMPHUNE
Biomedical Research Unit in Cardiovascular Sciences (BRUCS), Faculty of Allied Health Sciences, Naresuan University, Phitsanulok 65000, Thailand
Department of Medical Technology, Faculty of Allied Health Sciences, Naresuan University, Phitsanulok 65000, Thailand

INTRODUCTION

Ischemic heart disease is considered as the leading cause of death worldwide and predicted to be the majority cause of motility in the near future [24]. Myocardial ischemia occurs when the coronary flow is severely block, which results in the inadequacy of the oxygen supply and the oxygen demands of the heart tissue and finally progresses to cellular necrosis [6]. Currently, the most efficient way to reduce the mortality in such patients is to achieve rapid reperfusion by thrombolysis [2]. Different intracellular signaling pathways are conceived of playing an important role in the myocardial response to ischemia/reperfusion injury. In particular, p38 MAPK has been widely studied in this issue. A growing body of evidence from preclinical investigations indicated that inhibition of p38 activation could reduce myocardial injury [1,2,5,9,10,15,20,22,25], suggesting the therapeutic potential of p38 inhibitors in ischemic heart disease.

Aquilaria crassna Pierre ex Lecomte or agarwood is heartwood of tropical tree belongs to the family *Thymelaeaceae* and class *Magnoliosida* [4], which can be found in many countries in oriental region [4,8,16]. It has been used as folk medical treatment for arthritis, gout, and headache [16]. In addition, it was reported the benefit of this medicinal plant in traditional treatment for vomiting, cough, asthma, cardiac disorders, leprosy, and anorexia [16]. Recently, our previous experiments on the ethylacetate extract of *A. crassna* suggested the potent anti-inflammatory effect inhibiting tumor necrosis factor alpha (TNF-α) expression by attenuating p38 MAPK activation [11]. In Thailand, *A. crassna* extract known as one of the composition in Ya-hom, a traditional Thai herbal formulation for the treatment of fainting by increasing blood pressure [21]. However, there have been no evidences on the cardioprotective effect of *A. crassna* until present. Therefore, in the present study, we aim to investigate if the crude extract of this medicinal plant could possibly inhibit p38 MAPK activation and reduce ischemic-induced myocardial injury.

MATERIALS AND METHODS

(i) Plant Material and extraction

The heartwood of *Aquilaria crassna* Pierre ex Lecomte used in this experiment were supplied from Mr. Choosak Rerngrattanabhume. The plant was identified by Dr. Pranee Nangngam, Department of Biology, Faculty of Science, Naresuan University. The specimen voucher number 002540 was kept at Department of Biology Herbarium, Faculty of Science, Naresuan University. The heartwood was sliced into small pieces and left air died. Briefly, the dried plant (1 Kg) was consecutively extracted with ethyl acetate (EtOAc) (800 ml reflux) for two days each. The resulting EtOAc solution was concentrated under reduced pressure to yield EtOAc extract (950mg). The crude extract of *A. crassna* (A.Q.) was reconstituted in dimethylsulfoxide (DMSO) as 1 mg/ml stock solution. Vehicle control addressed in this manuscript was equal to 0.001% DMSO (Ameresco, Solon, Ohio, USA).

(ii) Chemicals and Reagents

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (Gibco BRL; Life Technologies Inc., New York, NY, USA) and Trypsin-EDTA, Lactate dehydrogenase (LDH) liquiUV test (Human, Wiesbaden, Germany), 3-(4,5-dimethyl-2-thiazol)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) (Ameresco, Solon, Ohio, USA), total-p38 (T-p38) and diphospho-p38 (P-p38), (Santa cruz biotech, California, USA). Other chemicals were purchased from Sigma.

(iii)Cell Culture

The rat cardiac myoblast cell line, H9c2, was maintained in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum, 100

units/ml of penicillin and 100 μg/ml of streptomycin. Cell was maintained at 37°C, 5% CO_2 at sub-confluent densities. Sub-confluent cells (70–80%) were subcultured 1:3. The cells were trypsinized, plated ($1x10^5$ cells/ml) in 6-wells plate and incubated in humidified CO_2 incubator for 24-48 hours, or until reached 80% confluence, before performing experiments.

(iv) Simulated Ischemia (sI)

Simulated ischemia (sI) was induced by incubating H9c2 cell with specified modified Krebs-Henseleit buffer (137 mM NaCl, 3.8 mM KCl, 0.49 mM $MgCl_2$, 0.9 mM $CaCl_2$, and 4.0 mM HEPES) with 20 mM 2-deoxyglucose, 20 mM sodium lactate, and 1 mM sodium dithionite at pH 6.5. Control buffer composed of Krebs-Henseleit buffer (137 mM NaCl, 3.8 mM KCl, 0.49 mM $MgCl_2$, 0.9 mM $CaCl_2$, and 4.0 mM HEPES), supplemented with 20 mM D-glucose, 1 mM sodium pyruvate. After simulate ischemia was achieved, the ischemic buffer or control buffer were removed and the cells were subjected to reperfusion by the addition of 2 ml complete medium before further incubating at 37°C, 5% CO_2 for 24 hours.

(v) Measurement of cell viability Assay

The measurement of H9c2 cardiomyoblast viability was performed by the reduction of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) by mitochondrial reductases. At the end of reperfusion period, the medium was collected for lactate dehydrogenase (LDH) activity. Cells were incubated with 0.01 g/ml MTT for 2 hours at 37°C. Then, 1 ml of 0.04 M HCl in isopropanol was added to each well. The converted dye was collected and the optical density was determined spectrophotometrically at λ 570 nm with background subtraction at λ 650 nm. Cell viability was calculated as a percentage of control.

(vi) Measurements of cellular injury

The cellular injury of H9c2 cardiomyoblast cell line was measured based on the extracellular release of lactate dehydrogenase (LDH), according to the loss of plasma membrane integrity. The enzyme-kinetic measurement of LDH activity [LDH liquiUV test (Human, Wiesbaden, Germany)] was performed in the supernatant of collected culture medium, after simulated ischemia/reperfusion, using a commercially available kit. Ten microlitter of collected culture medium was added to 1 ml of reaction reagent, and incubated at 37°C for 1 minute. The absorbance was measured at λ 340 nm exactly after 1, 2, and 3 minutes. The mean absorbance change per minute (ΔA/min) was used to calculate LDH activity.

(vii) Measurement of p38 MAPK activation

The H9c2 cells were incubated with EtOAc extract of *A. crassna*, or vehicle control for 60 minute prior treatment with simulated ischemic buffer, or at the onset of simulated ischemia, or both pre-treatment and during simulated ischemia for 40 minutes. The protein lysates were analyzed for p38 MAPK activation by western blot analysis using primary antibody recognizing p38 protein (T-p38), phosphorylated form of p38 (P-p38). Bands corresponding to the detected protein of interest were developed by autoradiographic method. The films were scanned and all band densities were quantified and compared providing information on relative abundance of the protein of interest.

(viii) Statistical analysis

Results are expressed as mean± S.E. Data sets were analyzed by one-way analysis of variance followed by Tukey's posthoc test. A value of $p < 0.05$ was considered statistically significant.

RESULTS

1. Simulate ischemia induce cell death and injury

The optimization of an *in vitro* simulated ischemia protocol, to induce cellular injury

and cell death, was performed in rat cardiac myoblast cell line, H9c2. The simulated ischemia protocol in this study was adapted from Esumi *et al*. [3]. Cells were exposed to ischemic buffer from 10 minutes to 180 minutes. The percentages of cell viability and LDH release were measured to assess the optimal condition for simulated ischemia protocol. The results showed that incubation of H9c2 cell line with ischemic buffer for 10 minutes to 180 minutes significantly reduced cell viability (Figure 1a). The cell viability was reduced to about 40% when cells were exposed to ischemic buffer for 10 minutes. The longer period of simulated ischemic led to decreased in cell viability as well as the increasing in released LDH activity (Figure 1b). The duration of simulated ischemia for 120 minutes caused highest percentage of cell death, which was higher than 90%, and also gave a significant released LDH activity. Therefore, simulated ischemia for 120 minutes was an optimal condition used in all simulated ischemia experiments for determining cell viability.

Optimization of simulated ischemia model in H9c2 cell line

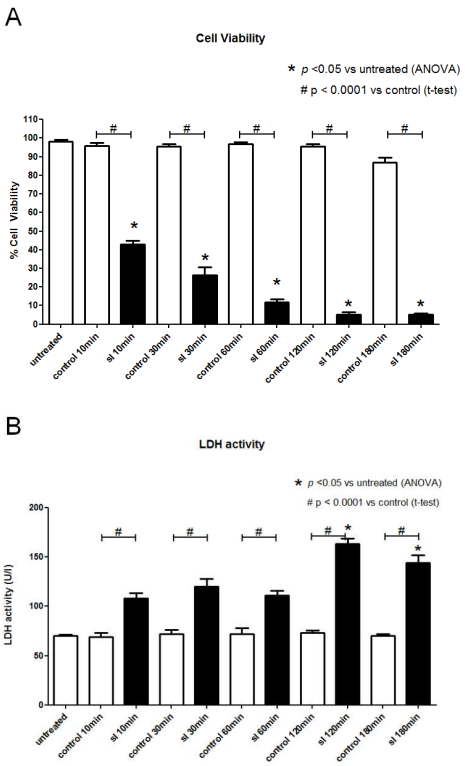

Figure 1

Optimization of simulated ischemia protocol. Cells were incubated with ischemic buffer for various periods of incubation times, e.g, 10 minutes, 30 minutes, 60 minutes, 120 minutes, and 180 minutes. (A) Percentage of cell viability by MTT assay. (B) The released lactate dehydrogenase activity (U/L). Each bar graph represents means ± S.E. for 3 experiments. * p < 0.05 vs untreated group (ANOVA), # p < 0.001 vs control of each sets (t-test).

2. Effect of the ethyl acetate extract of A. crassna on cardiac cell death

The effect of EtOAc extract of *A. crassna* to reduce ischemia induced cardiac cell death was performed by pre-incubation of various concentrations, 1-10 mg/ml, of the EtOAc extract of *A. crassna* prior to simulated ischemia. Pre-treatment with 2-10 mg/ml of the EtOAc extract of *A. crassna* prior to simulated ischemia significantly reduced simulated ischemia induced cell death in dose dependent manner (Figure

2a). The results showed that 5 mg/ml of the EtOAc extract of *A. crassna* gave highest percentage of cell viability up to approximately 80%. However, the concentration of the extract greater than 6 mg/ml significantly reduced cell viability. Moreover, pre-treatment of H9c2 cell with 5 mg/ml of EtOAc extract of *A. crassna* prior to simulate ischemia significantly reduced released LDH activity (Figure 2b), however, increasing of the concentration of the extract greater than 5 mg/ml also gave higher LDH activity. We then tested whether the reduction of cell death and cell necrosis, when treated with the extracts, observed in the previous results, was not due to the toxicity of the extract (Figure 3). In non simulate ischemic condition, treatment of H9c2 cells with various concentrations of the EtOAc extract of *A. crassna* did not reduce cell viability, whereas the concentration at 10 mg/ml showed slightly reduced in cell viability (Figure 3).

Pre-treatment of *A. crassna* extract reduced simulated ischemia induced cell death

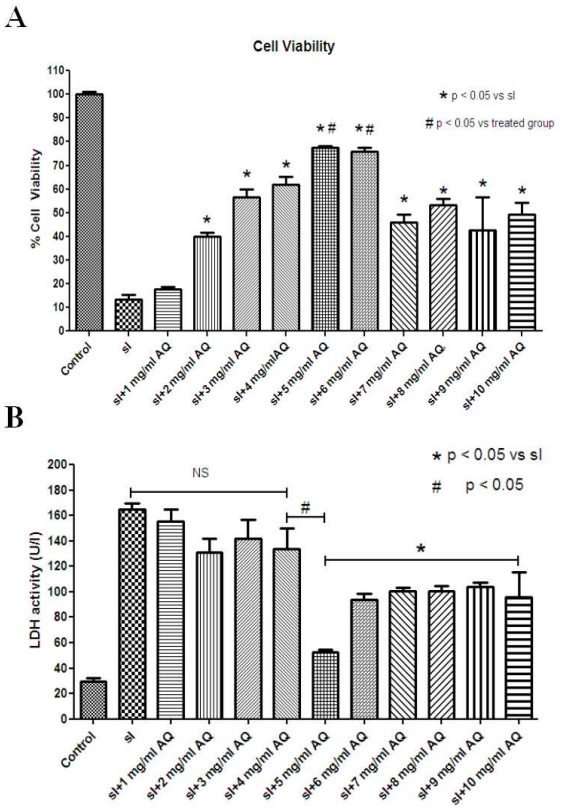

Figure 2

Effect of Aquilaria extract on cell viability and cell injury. Cells were subjected to 120 minutes simulated ischemia in the presence and absences of 1-10 mg/ml ethyl acetate extract of Aquilaria, pre-treatment. (A) Percentage of cell viability by MTT assay. (B) The released lactate dehydrogenase activity (U/L). Each bar graph represents means ± S.E. for 3 experiments. * p < 0.05 vs simulated ischemic group (ANOVA), # p < 0.05 vs among treated groups (ANOVA).

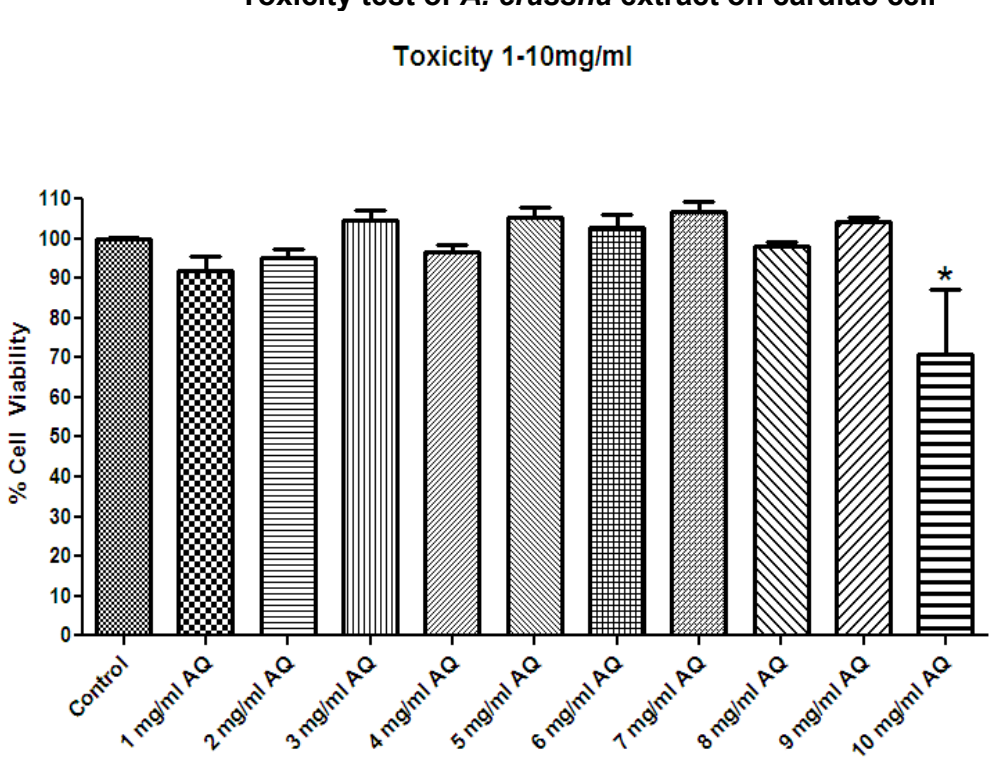

Figure 3

Effect of A. crassna extract (A.Q.) on cell viability. Cells were cultured in the presence of 1-10 mg/ml A.Q. for 24 hours. Each bar graph represents means ± S.E. of % viability by MTT assay. * $p < 0.05$ vs control group (ANOVA, n= 3).

3. *The ethyl acetate extract of Aquilaria crassna reduced p38 MAPK activation during simulate ischemia/reperfusion*

Myocardial ischemia is a potent stimulant of p38 MAPK activation, which accelerates injury [5,7,10,12,13,14,15,25]. Therefore, we hypothesized that the reduction of cell death, according to simulated ischemia, by the EtOAc extract of *A. crassna*, possibly resulted from an attenuation of p38 MAPK activation. To facilitate this hypothesis, cells were subjected to simulated ischemia, in the presence and absence of 5 mg/ml of the EtOAc extract of *A. crassna*. The results showed that simulated ischemia significantly enhanced p38 MAPK phosphorylation. Treatment of 5 mg/ml of the EtOAc extract of *A. crassna*, either 1 hour pre-treatment prior to 40 minutes simulated ischemia, at the onset of 40 minutes simulated ischemia, and for the whole period of experiment (pre-treatment + during simulated ischemia), significantly inhibited p38 MAPK phosphorylation (Figure 4). Interestingly, treatment of the extract at the onset of simulated ischemia and the whole experimental period, significantly reduced p38 MAPK phosphorylation than pre-treatment.

The *A. crassna* extract reduced simulated ischemia induced p38 MAPK phosphorylation

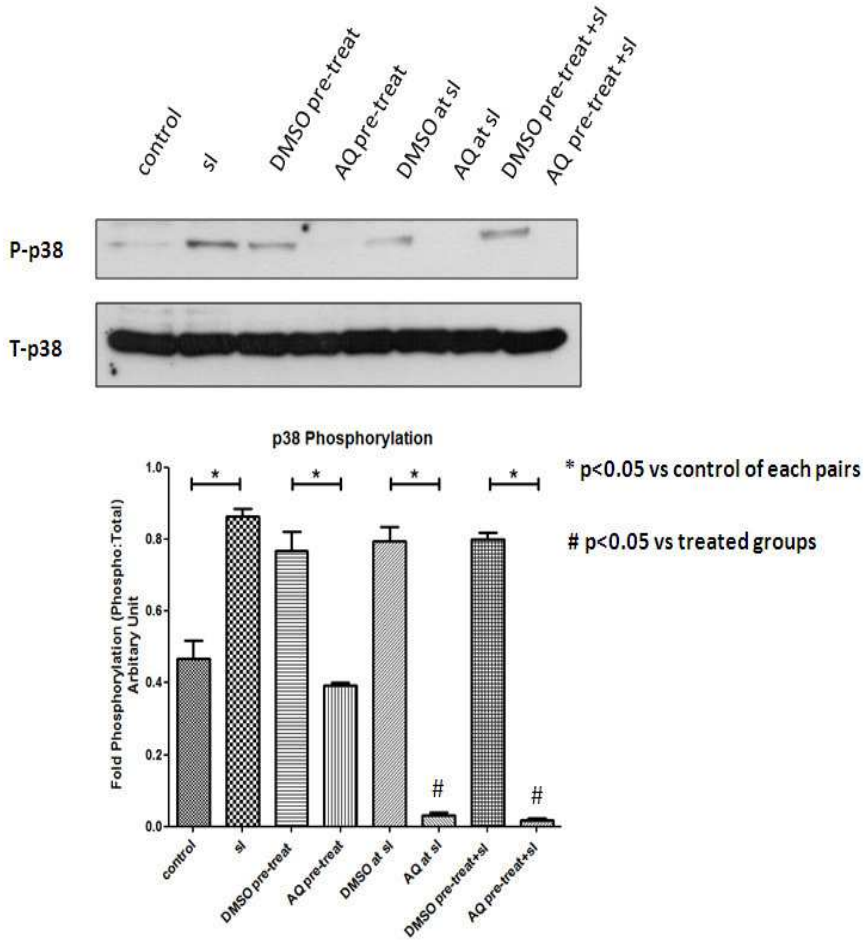

Figure 4

Effect of Aquilaria extract on ischemia-induced p38 MAPK activation. Western blot analysis of phosphorylated p38 MAPK from H9c2 cell subjected to 40 minutes simulated ischemia, in the presence and absence of 5 mg/ml Aquilaria extract. Each bar graph represents fold phosphorylation of p38 MAPK. * p < 0.05 vs vehicle control of each group (ANOVA, n= 3).

DISCUSSION

Aquilaria crassna has been used in many traditional therapeutic purposes such as treatment of inflammations, cancer, and found to be one of major composition in traditional Thai herbal formulation that targeting cardiovascular system. Recently, we demonstrated the underline mechanism of anti-inflammatory effect of the EtOAc extract of *A. crassna* that inhibited tumor necrosis factor-alpha (TNF-α) expression by attenuating p38 MAPK activation [11]. Taken together, the EtOAc extract of *A crassna* seem to have multi-activities, e.g. anti-inflammation, inhibition of p38 MAPK activation, and used as drug for heart treatment. Therefore, the EtOAc extract of *A. crassna* could possibly use as a drug in some diseases, which have been known that p38 is activated and/or mediated cellular pathology, such as ischemia/reperfusion injury.

In the present study, we demonstrated *in vitro* anti-ischemic effect of the EtOAc extract of *A. crassna* in cardiomyoblast cell line, H9c2, subjected to simulated ischemia. *In vitro* treatment with 5 mg/ml of the EtOAc extract of *A.*

crassna, either prior to ischemia, at the onset of ischemia, or both pre-treat and during ischemia, was found to protect the cardiac cells from ischemic injury. However, the results also showed that treatment of the EtOAc extract of *A. crassna* in control cardiac cell, without simulated ischemia, especially at the concentration of 1-9 mg/ml, did not cause cell toxicity. These results suggested the anti-ischemic effect of the EtOAc extract of *A. crassna* , and the plant extract itself could possibly be used without causing adverse effects to the heart cells. However, the sensitivity and toxicity of the extracts, in other different tissues or organs, need to be further investigated.

It has been known that p38 MAPK is activated as a result of myocardial ischemia and reperfusion, which predominantly lead to cellular injury and necrosis. Inhibition of p38 MAPK, by pharmacological inhibitors SB203580, reduced cellular injury, infarct size, and improved cardiac functions [5,7,10,12,13,14,15,25]. Interestingly, the anti-ischemic effect of SB203580 in H9c2 cell line subjected to simulate ischemia, the similar model in this study, was also showed to reduce cellular injury [17]. Recently, we showed that the EtOAc extract of *A. crassna* could inhibit TNF-α expression by inhibiting p38 MAPK activation [11]. In the present study, we hypothesized if the inhibitory effect of the extract on p38 MAPK activation could possibly be reduced ischemia-induced cardiac cell injury and cell death. The results showed that ischemia-induced p38 MAPK phosphorylation was inhibited by treatment of the EtOAc extract of *A. crassna*. The inhibitory effect of the extract on p38 activation was clearly seen either prior to ischemia, at the onset of ischemia, or both pre-treat or during ischemia. However, the anti-ischemic effect of this plant extract on other MAPKs need to be further investigated, in attempt to avoid the non-specificity or so called “off-target” effect.

In our hands, this is the first evidence showing the anti-ischemic effect of this plant extract, which was demonstrated in an *in vitro* model of cardiac H9c2 cell line. However, the experiments in this cell line have some limitations and weak points, as it may not closely be related to real physiological settings in the intact heart. Therefore, the more relevant models, such as an *in vitro* experiment in primary culture of isolated ventricular myocytes, an *ex vivo* experiment in isolated heart perfusion, or an *in vivo* experiment in animal model, will provide some functional data, which is close to the real physiological event in the heart and could be lead to more reliable interpretation. Moreover, this report was performed using the crude extract, so identification of active compounds, together with its therapeutic applications, is a challenge and needs to be further investigated.

CONCLUSION

In conclusion, treatment of the EtOAc extract of *Aquilaria crassna* exerted significant cardioprotective effect in simulated ischemia model. The EtOAc extract of *Aquilaria crassna* was found to reduced cell injury and cell death, by attenuating p38 MAPK phosphorylation.

REFERENCES

1. Barancik M., Htun P., Strohm C., Kilian S., Schaper W., Inhibition of the cardiac p38-MAPK pathway by SB203580 delays ischemic cell death. J. Cardiovasc. Pharmacol, 3:474-483, (2000).
2. Clark JE., Sarafraz N., Marber MS., Potential of p38-MAPK inhibitors in the treatment of ischaemic heart disease. Pharmacol. Ther, 2:192-206, (2007).
3. Esumi K., Nishida M., Shaw D., Smith TW., Marsh JD., NADH measurements

in adult rat myocytes during simulated ischemia. Am. J Physiol, 6 Pt 2:H1743-H1752, (1991).
4. Dash M., Patra JK., Panda PP., Phytochemical and antimicrobial screening of extracts of *Aquilaria agallocha Roxb*. African Journal of Biotechnology, 20:3531-3534, (2008).
5. Gorog DA., Tanno M., Cao X., Bellahcene M., Bassi R., Kabir AM., Dighe K., Quinlan RA., Marber MS., Inhibition of p38 MAPK activity fails to attenuate contractile dysfunction in a mouse model of low-flow ischemia. Cardiovasc. Res, 1:123-131, (2004).
6. Jennings RB., Reimer KA., The cell biology of acute myocardial ischemia. Annu. Rev. Med, Pp 225-246, (1991).
7. Kaiser RA., Lyons JM., Duffy JY., Wagner CJ., McLean KM., O'Neill TP., Pearl JM., Molkentin JD., Inhibition of p38 reduces myocardial infarction injury in the mouse but not pig after ischemia-reperfusion. Am. J. Physiol Heart Circ. Physiol, 6:H2747-H2751, (2005).
8. Kim YC., Lee EH., Lee YM., Kim HK., Song BK., Lee EJ., Kim HM., Effect of the aqueous extract of *Aquilaria agallocha* stems on the immediate hypersensitivity reactions. J. Ethnopharmacol, 1:31-38, (1997).
9. Kompa AR., See F., Lewis DA., Adrahtas A., Cantwell DM., Wang BH., Krum H., Long-term but not short-term p38 mitogen-activated protein kinase inhibition improves cardiac function and reduces cardiac remodeling post-myocardial infarction. J Pharmacol. Exp. Ther, 3:741-750, (2008).
10. Kumphune S., Bassi R., Jacquet S., Sicard P., Clark JE., Verma S., Avkiran M., O'Keefe SJ., Marber MS., A chemical genetic approach reveals that p38alpha MAPK activation by diphosphorylation aggravates myocardial infarction and is prevented by the direct binding of SB203580. J. Biol. Chem, 5:2968-2975, (2010).
11. Kumphune H., Prompun E., Phaebuaw K., Sriudwong P., Pankla R., Thongyoo P., Anti-inflammatory effects of the ethyl acetate extract of *Aquilaria crassna* inhibits LPS-induced tumour necrosis factor-alpha production by attenuating P38 MAPK activation. International journal of green pharmacy. A.D, 1:43-48, (2011).
12. Lee JC., Kumar S., Griswold DE., Underwood DC., Votta BJ., Adams JL., Inhibition of p38 MAP kinase as a therapeutic strategy. Immunopharmacology, 2-3:185-201, (2000).
13. Ma XL., Kumar S., Gao F., Louden CS., Lopez BL., Christopher TA., Wang C., Lee JC., Feuerstein GZ., Yue TL., Inhibition of p38 mitogen-activated protein kinase decreases cardiomyocyte apoptosis and improves cardiac function after myocardial ischemia and reperfusion. Circulation, 13:1685-1691, (1999).
14. Mackay K., Mochly-Rosen D., An inhibitor of p38 mitogen-activated protein kinase protects neonatal cardiac myocytes from ischemia. J. Biol. Chem, 10:6272-6279, (1999).
15. Martin JL., Avkiran M., Quinlan RA., Cohen P., Marber MS., Antiischemic effects of SB203580 are mediated through the inhibition of p38alpha mitogen-activated protein kinase: Evidence from ectopic expression of an inhibition-resistant kinase. Circ. Res, 9:750-752, (2001).
16. Miniyar PB., Chitre TS., Karve SS., Deuskar HJ., Jain KS., Anti-oxidant activity of ethyl acetate extract of *Aquilaria agallocha* on nitrite-induced methemoglobin formation. International Journal of Green Pharmacy, 1:43-44, (2008).
17. Nagarkatti DS., Sha'afi RI., Role of p38 MAP kinase in myocardial stress. J Mol Cell Cardiol, 30(8):1651–1664, (1998).
18. Okada T., Otani H., Wu Y., Kyoi S., Enoki C., Fujiwara H., Sumida T., Hattori R., Imamura H., Role of F-actin organization in p38 MAP kinase-mediated apoptosis and necrosis in neonatal rat cardiomyocytes subjected to simulated ischemia and

reoxygenation. Am. J Physiol Heart Circ. Physiol, 6:H2310-H2318, (2005).
19. Rouse J., Cohen P., Trigon S., Morange M., onso-Llamazares A., Zamanillo D., Hunt T., Nebreda AR., A novel kinase cascade triggered by stress and heat shock that stimulates MAPKAP kinase-2 and phosphorylation of the small heat shock proteins. Cell., 6:1027-1037, (1994).
20. Sumida T., Otani H., Kyoi S., Okada T., Fujiwara H., Nakao Y., Kido M., Imamura H., Temporary blockade of contractility during reperfusion elicits a cardioprotective effect of the p38 MAP kinase inhibitor SB-203580. Am. J. Physiol Heart Circ. Physiol, 6:H2726-H2734, (2005).
21. Suvitayavat W., Kodchawongs J., Thirawarapan SS., Bunyapraphatsara N., Effects of Ya-hom on the gastric secretion in rats. J. Ethnopharmacol, 2-3:331-338, (2004).
22. Tanno M., Bassi R., Gorog DA., Saurin AT., Jiang J., Heads RJ., Martin JL., Davis RJ., Flavell RA., Marber MS., Diverse mechanisms of myocardial p38 mitogen-activated protein kinase activation: evidence for MKK-independent activation by a TAB1- associated mechanism contributing to injury during myocardial ischemia. Circ. Res, 3:254-261, (2003).
23. Wachtel M., Frei K., Ehler E., Bauer C., Gassmann M., Gloor SM., Extracellular signal-regulated protein kinase activation during reoxygenation is required to restore ischaemia-induced endothelial barrier failure. Biochem J, pt 3:873-879, (2002).
24. World Heatlh Organization. World Heath Statistics, (2008). http://www.who.int/whosis/whostat/EN_WHS08_Full.pdf.2009;
25. Yada M., Shimamoto A., Hampton CR., Chong AJ., Takayama H., Rothnie CL., Spring DJ., Shimpo H., Yada I., Pohlman TH., Verrier ED., FR167653 diminishes infarct size in a murine model of myocardial ischemia-reperfusion injury. J. Thorac. Cardiovasc. Surg, 4:588-594, (2004).