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Anti-ischemic effect of ethyl acetate extract of *Aquilaria crassna* by attenuation of p38-MAPK activation

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ABSTRACT

Aquilaria crassna has been traditionally used in traditional Thai herbal formulation for treatment of fainting by targeting the cardiovascular system. This study was aimed to investigate the *ex vivo* effect of ethyl acetate extract of *Aquilaria crassna* (A.E) in isolated mouse heart, subjected to ischemia/reperfusion, and its mechanism on p38 MAPK activation. The hearts from ICR mouse, age 6-8 weeks, were retrograde-perfused on Langendorff perfusion system. The hearts were randomized to 5-mg/ml A.E for 30 min prior to 30 min of global ischemia, followed by 2 h of reperfusion. After reperfusion, all hearts were stained with 1% triphenyltetrazolium chloride (TTC) and sliced. The TTC-negative infarction volume was expressed as a percentage of heart volume. The p38-MAPK activation was performed by Western blot analysis. The results showed that global ischemia was significantly increased the infarct volume. Pre-treatment with 5-mg/ml of A.E. for 30 min prior to global ischemia significantly reduced infarct volume (47.61±3.68 % versus 21.08± 8.16 %, $p < 0.01$). In addition, ischemia-induced p38 MAPK phosphorylation was inhibited by pre-treatment with 5-mg/ml of A.E. In conclusion, the ethyl acetate extract of *Aquilaria crassna* could protect the heart from myocardial ischemia/reperfusion injury by, at least in part, attenuating p38 MAPK phosphorylation.

INTRODUCTION

Myocardial ischemia exists when the reduction of the coronary flow is so severe that the supply of oxygen to the myocardium is inadequate for the oxygen demands of the tissue, resulting in the accumulation of metabolites in the ischemic region. Severe and prolonged ischemia ultimately results in cellular necrosis (Jennings & Reimer, 1991). Currently, the most efficient way to reduce aggravation of the disease is to achieve rapid reperfusion (Braunwald, 1996). Numerous intracellular signaling pathways play an important role in the myocardial response to ischemia/reperfusion injury.

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In particular, p38 MAPK has been widely investigated in this issue. Pre-clinical investigation indicated that inhibition of p38 MAPK activation could reduce myocardial injury, suggesting the therapeutic potential of p38 MAPK inhibitors in ischemic heart disease (Kumphune *et al*., 2012[a]) . *Aquilaria crassna* Pierre ex Lecomte or agarwood is heartwood of tropical tree belongs to the family *Thymelaeaceae* and class *Magnoliosida* (Dash *et al*., 2008), which can be found in many countries throughout the oriental region. It has been used as folk medical treatment for treatment of cardiac disorders (Miniyar *et al*., 2008). Interestingly, in Thailand, *A. crassna* extract has been using as the ingredient of Ya-hom, a traditional Thai herbal formulation for the treatment of fainting (Suvitayavat *et al*., 2005), by targeting the cardiovascular system. Our previous finding demonstrated the anti-inflammatory

effect of ethyl acetate extract of *A. crassna* on tumor necrosis factor alpha (TNF-α) expression by attenuating p38 MAPK activation (Kumphune *et al*., 2011).

Recently, we reported that 5 mg/ml of *A. crassna* extract could reduce simulated ischemia induced cell death in cardiac myoblast cell line, H9c2 (Jermsri *et al*., 2012[a]), as well as isolated adult rat ventricular myocytes (ARVMs) (Kumphune *et al*., 2012[b]). The findings from these previous studies showed that the *A. crassna* extract could inhibit ischemia-induced p38 MAPK activation.

Moreover, Jermsri *et al.* reported the cytoprotective effect of *A. crassna* extract on actin cytoskeleton organization, in cardiac cell subjected to simulated ischemia (Jermsri *et al*., 2012[b]). However, the effect of this plant extract on infarct size still needs to be investigated. Therefore, the present work is designed to study the effect of *A. crassna* crude extract on infarct volume and its mechanism on p38 MAPK activation.

MATERIALS AND METHODS

Plant Material and extraction

Aquilaria crassna Pierre ex Lecomte was obtained from Mr. Choosak Rearngrattanabhume. The plant was originally cultivated at the area in Pong Nam Ron district, Chantaburi province, Thailand. The specimens were collected on September 2010 and subsequently 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, left air died, and extracted by the methods described in the previous study (Kumphune *et al*., 2011).

Animals

Adult ICR mice (6-8 weeks or body weight greater than 25 g) were purchased from National Laboratory Animal Center, Mahidol University, Bangkok, Thailand. The animals were housed in a room maintained at 23 ± 2 ℃ and a relative humidity of 50% with 12h:12h of dark: light cycle, at center for animal research, Naresuan University, Phitsanulok, Thailand for at least 2 weeks before performing the experiments. The study protocol was approved by Naresuan University Animal Ethics committee (protocol license No.55 04 0005).

Retrograde perfusion of isolated murine heart

All male mice were anesthetized by intraperitoneal injection (IP) with pentobarbital (300 mg/kg) and heparin (150 units). The hearts were rapidly isolated and placed in ice cold modified Krebs-Henseleit (K-H) buffer (18.5 mM of NaCl, 25.0 mM of $NaHCO_3$, 4.75 mM of KCl, 1.18 mM of KH_2PO_4, 1.19 mM of $MgSO_4$, 11.0 mM of D-glucose, and 1.4 mM of $CaCl_2$). The aorta was cannulated on a Langendorff apparatus and retrograde-perfused at a constant pressure with K-H buffer equilibrated with 95% O_2 and 5% CO_2 at 37 °C. The hearts were randomized to 5-mg/ml ethyl acetate extract of *Aquilaria crassna* (A.E) for 30 min prior to ischemia with blinding to the corresponding vehicle (0.001% Dimethysulfoxide). Infarction was caused by 30 min of global ischemia followed by 2 h of reperfusion and delineated by 1% triphenyltetrazolium chloride (TTC) (Sigma, St. Louis, MO, USA) (Fig.1A).

Infarct volume assessment in isolated murine heart

The infarction assessment in Isolated Murine Hearts was performed as previously described (Kumphune *et al*., 2010). After 2 hours of reperfusion, hearts were perfused for 1 min with 5 ml of 1% (w/v) TTC, then removed and placed in 1% TTC solution at 37°C for 10 min. The hearts were incubated in 2.5% glutaraldehyde for 1 minute, and set in 5% agarose before sectioning in 750μm thick slices. All slices were incubated in 10% (v/v) formaldehyde overnight at room temperature before re-hydration overnight phosphate buffer saline (PBS) at 4°C.

The heart sections were scanned and planimetry was carried out and surface area of the whole, and TTC-negative, myocardium was transformed to volume. The TTC-negative infarction volume was expressed as a percentage of heart volume. All analyses of infarct size were done by an investigator, who was blinded with regard to the group assignments.

Measurement of p38 MAPK MAPK activation by western blot analysis

The hearts were subjected to 30 min of stabilization by perfusion with modified K-H buffer and then randomly exposed to 5-mg/ml the extract or vehicle control (0.001% DMSO) for 30 min prior to 10 min of global ischemia.

Then the hearts were rapidly snap frozen (Fig.1B). The heart tissues were homogenized and solubilized in SDS-Polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer as described previously (Kumphune, 2010).

The heart proteins was separated on 10 % SDS-polyacrylamide gels; transferred to polyvinylidene difluoride (PVDF) membranes, and probed overnight at 4 °C with 1:1000 total p38 MAPK or diphospho-p38 MAPK antibody (Santa Cruz Biotechnology Inc., CA, USA). Bands corresponding to the detected protein of interest were developed by autoradiographic method. Band densities were quantified and express as fold phosphorylation (Phospho:Total p38 MAPK).

Statistical analysis

All values are expressed as Mean ± S.D. All comparisons involving more than one group were assessed for significance using one-way analysis of variance (ANOVA) with Tukey post hoc test. A statistical value of less than 0.05 was considered significant.

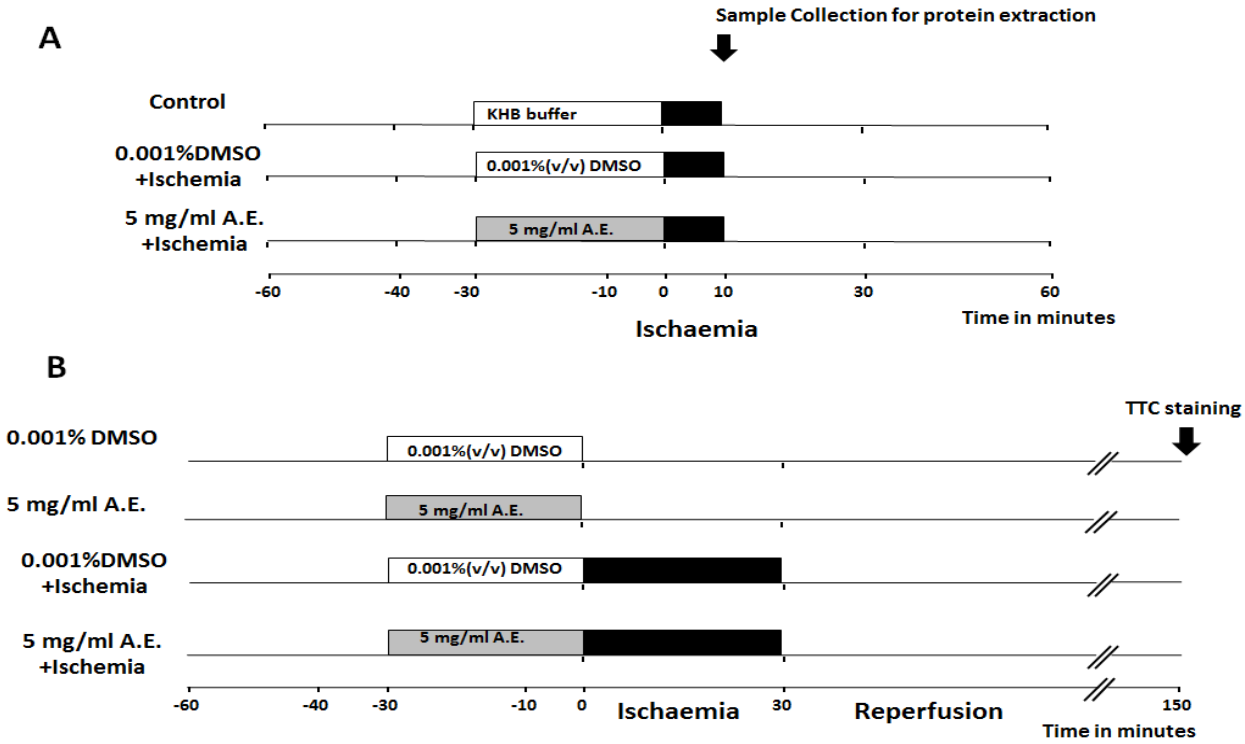

Fig. 1: Schematical representation of isolated mouse heart perfusion protocol used to assess the effect of ethyl acetate extract of *A. crassna* (A.E.) on p38 MAPK MAPK phosphorylation and the sensitivity to infarction. (A) Isolated mouse hearts were subjected 40 min stabilization with K-H buffer before exposing to 10 min ischemia, in the presence and absence of pre-treatment with 5-mg/ml A.E., or vehicle control (0.01% DMSO), for 30 min. (B) Infarct size determination, The heart samples were isolated mouse hearts were stabilized with K-H buffer for 30 min, before subjected to 30 min global ischemia, in the presence and absence of 5-mg/ml A.E., or vehicle control (0.001% DMSO). At the end of reperfusion, the hearts were perfused with 1% TTC for 1 min, and the samples were collected from infarct size analysis.

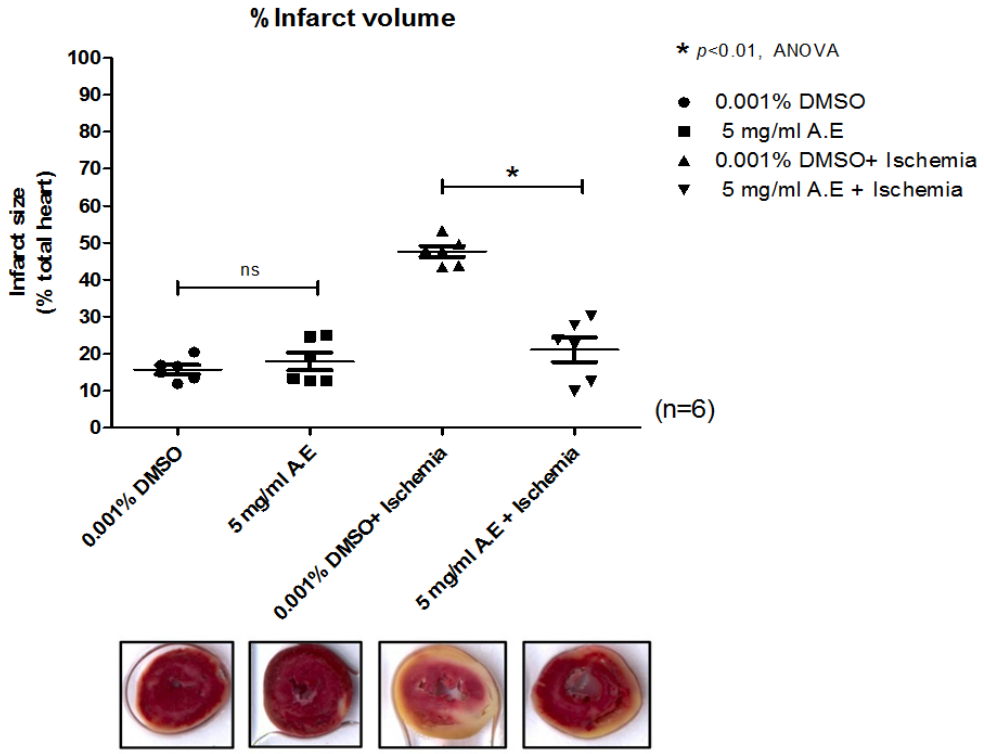

Fig. 2: The sensitivity to myocardial infarction in murine hearts subjected to global ischemia/reperfusion, in the presence and absence of *A. crassna* extract.The data shown is the normalized infarction volume of isolated murine hearts in the presence of 5-mg/ml A.E., or vehicle (0.001% DMSO). *ns*, not significant; * $p<0.01$ vs group, (one way ANOVA, n =6).

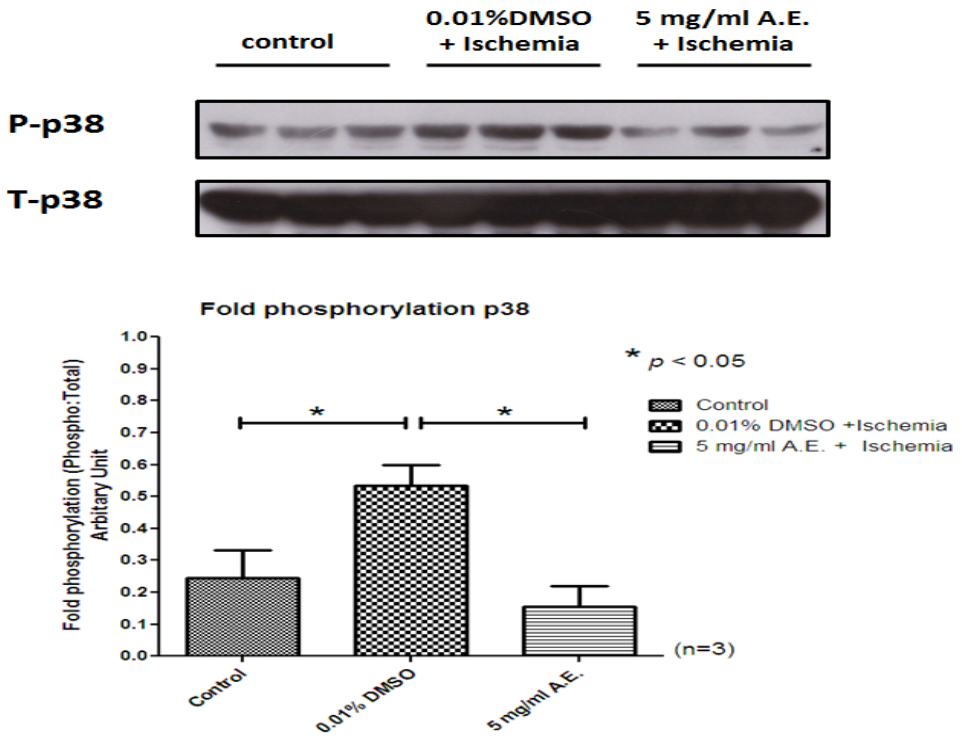

Fig. 3: p38 MAPK MAPK activation during myocardial Ischemiain hearts exposed to pre-treatment of *A. crassna* extract. Isolated hearts were subjected to 10 min of ischemia in the presence and absence of 5-mg/ml A.E., or vehicle (0.001% DMSO). The quantification of band density was expressed as Mean ± S.D. * p<0.05 vs group, (one way ANOVA, n =3).

RESULTS

The effect of ethyl acetate extract of Aquilaria crassna on sensitivity to myocardial infarction in isolated murine heart

We examined the sensitivity to infarction of isolated murine hearts in response to 30 min of global ischemia, in the presence and absence of 5-mg/ml ethyl acetate extract of *Aquilaria crassna* (A.E.; *Aquilaria* Extract). The concentration of 5-mg/ml used in the whole experiments is the concentration that previously showed cardioprotective in *in vitro* model of H9c2 (Jermsri *et al*., 2012[a]) and isolated adult rat ventricular myocytes (Kumphune *et al*., 2012[b]).The results showed that isolated murine hearts perfused with 5-mg/ml of A.E. or vehicle control (0.001% DMSO) for 30 min on Langendorff perfusion system caused small and non-significant infarct volume (15.73 ± 2.99 % *versus* 17.93 ± 5.866 %, respectively). However, 30 min of global ischemia caused greater in infarct volume. Pre-treatment of the heart with 5-mg/ml of A.E. for 30 min prior to global ischemia significantly reduced infarct volume (47.61 ± 3.68 % *versus* 21.08 ± 8.16 %, p< 0.01) (Fig. 2)

The effect of ethyl acetate extract of Aquilaria crassna on p38 MAPK Dual Phosphorylation during Myocardial Ischemia

To determine whether the reduction of infarct size in heart pre-treated with the extract involving the inhibition of p38 MAPK as the dominant mechanism, we examined p38 MAPK phosphorylation in the presence and absence of 5-mg/ml A.E. The results showed that p38 MAPK was dual-phosphorylated during ischemia (Fig. 3) and was inhibited by pre-treatment with 5-mg/ml *Aquilaria* extract. The results suggested that p38 MAPK is activated during myocardial ischemia and could be inhibited by 5-mg/ml *Aquilaria* extract.

DISCUSSION

Aquilaria crassna has been used in many traditional therapeutic purposes and known to be a major composition in traditional Thai herbal formulation (Suvitayavat *et al*., 2005). Recently, we demonstrated the underline mechanism of anti-inflammatory effect of the ethyl acetate extract of *A. crassna* by attenuating p38 MAPK activation (Kumphune *et al*., 2011). Avariety of studies have demonstrated that the dual phosphorylation of p38 MAPK occurring during myocardial ischemia is reduced in the presence of p38 MAPK inhibitor (Kumphune *et al*., 2010), which pointed the cardioprotective effect of p38 MAPK inhibition. Therefore, we hypothesized that the ethyl acetate extract of *A. crassna* could possibly have cardioprotective effect.

The major finding in this manuscript is that the ethyl acetate extract of *A. crassna* protect the heart from myocardial ischemia/reperfusion injury in isolated murine heart model, and this cardioprotective effect of the extract is ,at least in part, inhibited p38 MAPK phosphorylation, which is the major

signaling known to aggravate myocardial cell injury and death. Pre-treatment with 5-mg/ml ethyl acetate extract of *A. crassna* for 30 min prior to global ischemia/reperfusion significantly reduced the infarct volume. In addition, the same concentration of the extract could also inhibit p38 MAPK dual-phosphorylation.

In our hands, this is ***the first evidence*** showing the *ex vivo* anti-ischemic effect of this *A. crassna* extract, on isolated murine hearts. The results were consistent with our findings in an *in vitro* model of cardiac cell, H9c2 (Jermsri *et al*., 2012[a]), as well as isolated adult rat ventricular myocytes (ARVMs) (Kumphune *et al*., 2012[b]) , that 5 mg/ml of the extract could reduce simulated ischemia-induced cellular injury and death. Moreover, the same concentration of the extract also inhibited ischemia induced-p38 MAPK activation. However, the experiments in model still have some limitations and weak points, as it may not closely related to real physiological settings in the intact heart. Therefore, the more relevant models, such as an *in vivo* experiment in animal model, will provide some more beneficial and reliable functional data. The intensive study in large animal model such as rabbit, pig, dog, or etc., should be performed and could provide more functional data related to human heart, in term of heart size, heart volume, hemodynamic parameters. Moreover, this report was performed using the crude extract, so identification of active compounds is still needs to be further investigated.

CONCLUSIONS

The ethyl acetate extract of *A. crassna* contain anti-ischemic activity against myocardial ischemia/reperfusion injury. The cardioprotective mechanism of the extract could, at least in part, explained by attenuation of p38 MAPK phosphorylation, which is the major signaling pathway involve in cardiac cell injury and death.

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