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Title page

An *in vitro* anti-ischemic effect of *Aquilaria crassna* in isolated adult rat ventricular myocytes subjected to simulated ischemia

Short running title: An *in vitro* anti-ischemic effect of Agarwood

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Abstract

To investigate the effect of ethyl acetate of *Aquilaria crassana* crude extract on simulated ischemia-induced cardiac cell injury, mechanism on p38 MAPK activation, in isolated Adult Rat Ventricular Myocytes (ARVMs), so as to provide some evidence for its traditional use. The ARVMs were isolated from 6-8 weeks male Wistar rat by collagenase-based enzymatic digestion and maintained in cell culture system. ARVMs were subjected to 3 hrs simulated ischemia/reperfusion, in the presence and absence of various concentrations of the extract. The cellular injury and viability were determined. Cells were pre-treated with 5 mg/ml of *Aquilaria* extract for 1 hour before, or at the beginning of 40 minutes simulated ischemia. Activation of p38 MAPK was measured by Western blot analysis. The results showed that 3 hr of simulated ischemia was significantly produced cellular injury and cell death, which was significantly inhibited when treated with 5 mg/ml ethyl acetate extract of Aquilaria crassna ($p<0.05$). Treatment 5 mg/ml of *Aquilaria* extract significantly reduced ischemia-induced p38 MAPK phosphorylation ($p<0.05$). The results suggested the cardioprotective effect of *Aquilaria* crassna extract against myocardial ischemia/reperfusion injury.

1. Introduction

The pathophysiology of myocardial ischemia is happened when the coronary artery is occluded and lead to insufficiency of the oxygen supply to the heart, and finally progresses to cellular necrosis (Jennings and Reimer 1991) . Currently, the most efficient way to reduce aggravation of the disease is to achieve rapid reperfusion (Clark et al. 2007). However, the reperfusion also known to aggravate cardiac cell injury and finally result in cardiomyocytes necro-apoptosis (Gottlieb et al. 1994). The cellular damage according to ischemia and reperfusion is referred to ischemia/reperfusion injury. Therefore, any way capable of slow down the rate of ischemia/reperfusion injury are likely to save many lives(Braunwald 1996).It has been known that myocardial ischemia/reperfusion injury is a strong stimulant of some key signaling pathway, particularly p38 MAPK (Barancik et al. 2000;Cuenda and Rousseau 2007;Gorog et al. 2004;Nagarkatti and Sha'afi 1998;Tanno et al. 2003). Evidences in preclinical investigation indicated that inhibition of p38 activation could reduce myocardial injury, suggesting the therapeutic potential of p38 inhibitors in ischemic heart disease (Kumphune et al. 2012)

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 Asian countries (Dash, Patra, & Panda 2008;Kim et al. 1997;Miniyar et al. 2008). This plant is known to be useful in traditional medical treatment for many inflammatory diseases and also found to be used in treatment of cardiac disorders (Miniyar, Chitre, Karve, Deuskar, & Jain 2008). Interestingly, in Thailand, *A. crassna* extract has been using as one of the major ingredients in Ya-hom, a traditional Thai herbal formulation for the treatment of fainting by increasing blood pressure (Suvitayavat et al. 2005), suggested the cardiovascular targeting effect of this plant. Recently, our previous experiments on the ethyl acetate extract of *A. crassna* suggested the potent anti-inflammatory effect inhibiting tumor necrosis factor alpha (TNF-α) expression by attenuating p38 MAPK activation (Kumphune et al. 11 A.D.).Recently, we reported that *A. crassna* extract could reduce cell death in cardiac myoblast cell line, H9c2, induced simulated ischemia by inhibiting p38 MAPK activation (*Jermsri et.al.2012 Article In press*) and also preserve actin

cytoskeleton organization (*Jermsri et.al. 2012 Article In press*). Therefore, in the present study, we aim to investigate the anti-ischemic effect of *A. crassna* crude extract in more relevant *in vitro* model of isolated Adult Rat Ventricular Myocytes (ARVMs) in attempt to provide closer cardiac cell model.

2. Materials and methods

2.1 Plant Material and extraction

Aquilaria crassna Pierre ex Lecomte used in this experiment was obtained from Mr. Choosak Rerngrattanabhume. The plant was originally cultivated at the area in Pong Nam Ron district, Chantaburi province, Thailand 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. The dried plant (1Kg) was consecutively extracted with ethyl acetate (ethyl acetate) (800 ml reflux) for two days each. The resulting ethyl acetate solution was concentrated under reduced pressure to yield Ethyl acetate extract (950mg) (Kumphune, Prompun, Phaebuaw, Sriudwong, Pankla, & Thongyoo, 11 A.D.).

2.2 Chemicals and Reagents

All basic chemicals were purchased from Sigma (Sigma, St.Louis, MO, USA). M199 medium and Fetal bovine serum (FBS) (Gibco BRL, Life Technologies, Inc., New York, USA.); 3-(4,5-dimethyl-2-thiazol)-2,5- diphenyl-2*H*-tetrazolium bromide (MTT, Amereseo, USA.); For SDS-PAGE and Western blot analysis, the 30% polyacrylamide gel was from Biorad, polyvinylidenedifluoride (PVDF) membrane was from GE Healthcare Life science.. The Antibodies recognizing the dual phospho-Thr180/Tyr182 form of p38 MAPK and total p38 MAPK were from Santa Cruz Biotech. Enhanced Chemiluminsecence (ECL) and hyperfilm were from GE Healthcare Life science.

2.3 Isolation of Adult Rat Ventricular Myocytes(ARVM) and culture

Ventricular myocytes were isolated from the hearts of adult male Wistar rats (200-250 g) by collagenase-based enzymatic digestion using an adaptation of the method (Kumphune et al. 2010). Hearts were excised and initially perfused for 5 min with modified Krebs solution (solution A) containing 130 mM NaCl, 4.5 mM KCl, 1.4 mM $MgCl_2$, 0.4 mM NaH_2PO_4, 0.75 mM $CaCl_2$, 4.2 mM HEPES, 20 mM taurine, 10 mM creatine and 10 mM glucose, pH 7.3 at 37°C). Heart were then perfused with calcium-free solution containing 100 μM EGTA for 4 min, followed by perfusion with solution A containing 100 μM $CaCl_2$ and 1 mg/ml Worthington type II collagenase for 8 min. The ventricles were then cut into small pieces, which were incubated in 10 ml of collagenase solution gassed with 100% O_2 for a further 7 min at 37°C, with regular triturating. Isolated myocytes were separated from undigested ventricular tissue by filtering through cell strainer. Then isolated myocytes were allowed to settle into a loose pellet and the supernatant was removed and replaced with solution A containing 1% BSA and 500 μM $CaCl_2$. The isolated myocytes were then allowed to settle and the supernatant was removed and replaced with 10 ml of solution A containing 1 mM $CaCl_2$.The cell pellet was washed at room temperature with M199 culture medium containing 100 IU/ml penicillin/streptomycin. The myocytes were resuspended in modified M199 containing 2 mM creatine, 2 mM carnitine, and 5 mM taurine, and then seeded on pre-laminin coated 6-well plates (15 μg/ml laminin) and allowed to adhere for 1 hour in an incubator (37°C, 5% CO_2). The culture medium was replaced with fresh modified M199 medium, prior to further experiments.

2.4 Simulated Ischemia protocol

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.

2.5 Determination of Cell Viability

The measurement of ARVMs viability was performed by the reduction of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) by mitochondrial reductase. 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.

2.6 Determination of cellular injury

The cellular injury of ARVMs 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 min. The mean absorbance change per minute (ΔA/min) was used to calculate LDH activity.

2.7 Measurement of p38 MAPK activation by western blot analysis

Cells were washed twice in ice-cold PBS, before addition of 200 µl of 2x SDS-sample buffer, containing β-mercaptoethanol. Cells were scraped and the samples were taken and transferred to the new pre-cooled micro-centrifuge tubes. The samples were boiled for 10 min and stored at -80°C before analysis. The extract proteins were separated on 12% SDS-polyacrylamide gels; transferred to polyvinylidenedifluoride (PVDF) membranes, which were blocked for 1 h with 5% nonfat milk + 1% bovine serum albumin in Tris-buffered saline (pH 7.4) containing 0.1% Triton X-100; and probed overnight at 4 °C with the appropriate primary antibody as follows: total p38, diphospho-

p38, from Santa Cruz Biotechnology. After washing and exposure for 1 h at room temperature to horseradish peroxidase-conjugated secondary antibody, antibody-antigen complexes were visualized by enhanced chemiluminescence. 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.

2.8 Statistic 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), followed when appropriate by the Tukey post hoc. test. A statistical value of less than 0.05 was considered significant.

3. Results and discussion

3.1 Simulate ischemia induce cell death and injury

The isolated ARVMs were exposed to ischemic buffer from 10 min to 180 min. 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 ARVMs with ischemic buffer for first 30 min was not cause cell death, compared to control. Incubation with ischemic buffer for 60 min to 180 min significantly reduced cell viability. The results also showed that exposure to ischemic buffer for 180 min reduced cell viability greater than 50% (Figure 1a). The release LDH activity represented cellular injury according to simulate ischemia showed that the increasing in the period of simulated ischemia enhanced the released LDH activity (Figure 1b). The duration of simulated ischemia 30-180 min showed significantly increased in released LDH activity. Therefore, simulated ischemia for 180 min was an optimal condition used in all simulated ischemia experiments for determining cell viability.

3.2 The ethyl acetate extract of Aquilaria crassna extract reduced simulate ischemia induced cell injury and death in isolated Adult Rat Ventricular Myocytes.

The effect of ethyl acetate extract of *A. crassna* (A.E.; *Aquilaria* Extract) to reduce ischemia induced cardiac cell death was performed by pre-incubation of various concentrations, 1-10 mg/ml of the ethyl acetate extract of *A. crassna* prior to 180 min of simulated ischemia. Pre-treatment with vehicle (0.01% DMSO) for 1 hr did not increase the cell viability of the ARVMs. Pre-treatment with 1-4 mg/ml of the ethyl acetate extract of *A. crassna* prior to simulated ischemia slightly, but not significantly, increased cell viability. The ethyl acetate extract of *A. crassna* at 5-7 mg/ml significantly prevent ischemic induced cell death. The results showed that 5 mg/ml of the Ethyl acetate extract of *A. crassna* gave highest percentage of cell viability to 90.51 ± 6.056 %, while the extract at 6 mg/ml and 7 mg/ml gave the percentage of cell viability to 88.75 ± 5.709 % and 85.75 ± 2.918 %, respectively (Figure 2a). However, increasing in concentration of the extract 8-10 mg/ml failed to protect the ARVMs from cell death. Moreover, pre-treatment of H9c2 cell with 2-9 mg/ml of ethyl acetate extract of

A. crassna 1 hr prior to simulate ischemia significantly reduced released LDH activity (Figure 2b), However, the concentration of the extract greater at 5 mg/ml was the lowest concentration that gave the lowest released LDH activity. These results suggested that 5 mg/ml ethyl acetate extract of *A. crassna* was the optimal concentration to reduce cardiac cell injury and death.

We also tested whether the reduction of cell death and cell necrosis, when treated with the extracts, observed in our findings, was not due to the toxicity of the extract (Figure 3). In non simulate ischemic condition, pre-incubation of ARVMs with various concentrations, 1-10 mg/ml, of the *Aquilaria* extract did not significantly reduce cell viability, suggesting non toxic effect of the extract to the cells.

3.3 The ethyl acetate extract of Aquilaria crassna reduced Adult Rat Ventricular Myocytes cell injury and death by attenuating p38 MAPK activation

Myocardial ischemia is a potent stimulant of p38 MAPK activation, which accelerates injury. Therefore, we hypothesized that the reduction of cell death, according to simulated ischemia, by the ethyl acetate 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 ethyl acetate extract of *A. crassna*. The results showed that simulated ischemia significantly enhanced p38 MAPK phosphorylation. Treatment of 5 mg/ml of the ethyl acetate extract of *A. crassna*, either 1 hour pre-treatment prior to 40 min simulated ischemia and for the whole period of experiment (pre-treatment + during simulated ischemia), significantly inhibited p38 MAPK phosphorylation (Figure 4). These results suggested the effect of ethyl acetate extract of *A. crassna* could inhibit activation of p38 MAPK. Interestingly, treatment of the extract at the onset of simulated ischemia failed to reduced p38 MAPK phosphorylation.

4. Discussion

The major findings of this manuscript demonstrate the *in vitro* anti-ischemic effect of the ethyl acetate extract of *Aquilaria crassna* in isolated Adult Rat Ventricular Myocytes (ARVMs) subjected to simulated ischemia. According to *Aquilaria crassna* has been used in many traditional therapeutic purposes and found to be one of major composition in traditional Thai herbal formulation that targeting cardiovascular system. Recently, we demonstrated that the ethyl acetate extract of *A. crassna* seem to have dual-activities that found to be a major cause of cellular injury and death in response to myocardial ischemia/reperfusion, e.g. anti-inflammation, inhibition of p38 MAPK activation(Kumphune, Bassi, Jacquet, Sicard, Clark, Verma, Avkiran, O'Keefe, & Marber 2010). Therefore, we hypothesized that the ethyl acetate extract of *A. crassna* could possibly use as a drug for myocardial ischemia/reperfusion injury.

In the present study, we demonstrated that *in vitro* treatment of 5 mg/ml of the *Aquilaria* extract prior to ischemia could protect the ARVMs from ischemic injury. This result was consistent with our finding in other cardiac cell model of adult rat cardiac myoblast cell line, H9c2 (*Jermsri et al.* 2012 in press). In addition, with the similar concentration of the same extract also showed the cardiac protective effect in an ex vivo model of isolated murine heart perfused on Langendorff perfusion system (*Suwannasing et al.*, 2012 in press). The results from these studies were demonstrated the cardiac protective effect of *Aquilaria* extract in the similar mechanism, by which the *Aquilaria* extract, could inhibit myocardial ischemia-induced p38 MAPK phosphorylation. The inhibitory effect of the extract on p38 activation was clearly seen either prior to ischemia or both of pre-treat and 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.

Interestingly, it seem that the plant extract itself could possibly used without causing adverse effects to the heart cells according to exposure of the extract with most effective concentration was not cause cellular toxicity. However, the sensitivity and toxicity of the extracts, in other different tissues or organs, need to be further investigated.

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 primary culture of

isolated murine cardiomyocytes. However, the our experiments still have some limitations and weak points, as it may not closely related to real physiological response to myocardial ischemia in the intact heart. Therefore, the more relevant models, of 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.

5. Conclusion

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

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Figure legends

Figure 1. Optimization of simulated ischemia protocol. Cells were incubated with ischemic buffer for various periods of incubation times, e.g, 10 min, 30 min, 60 min, 120 min, and 180 min. (A) Percentage of cell viability by MTT assay. (B) The released lactate dehydrogenase activity (U/L). Each bar graph represents means ± S.D. for 3 experiments. * $p < 0.05$ vs untreated group (ANOVA).

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 crassna*, pre-treatment. (A) Percentage of cell viability by MTT assay. (B) The released lactate dehydrogenase activity (U/L). Each bar graph represents mean ± S.D. for 3 experiments. * $p < 0.05$ vs simulated ischemic group (ANOVA), # $p< 0.05$ vs among treated groups (ANOVA).

Figure 3. Effect of *Aquilaria* extract on cell viability. Cells were cultured in the presence of 1-10 mg/ml ethyl acetate extract of *Aquilaria* for 24 hours. Each bar graph represents mean ± S.D. of % viability by MTT assay.

Figure 4. Effect of *Aquilaria* extract on ischemia-induced p38 MAPK activation. Western blot analysis of phosphorylated p38 MAPK from Adult Rat Ventricular Myocytes (ARVMs) 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).

Figure 1.

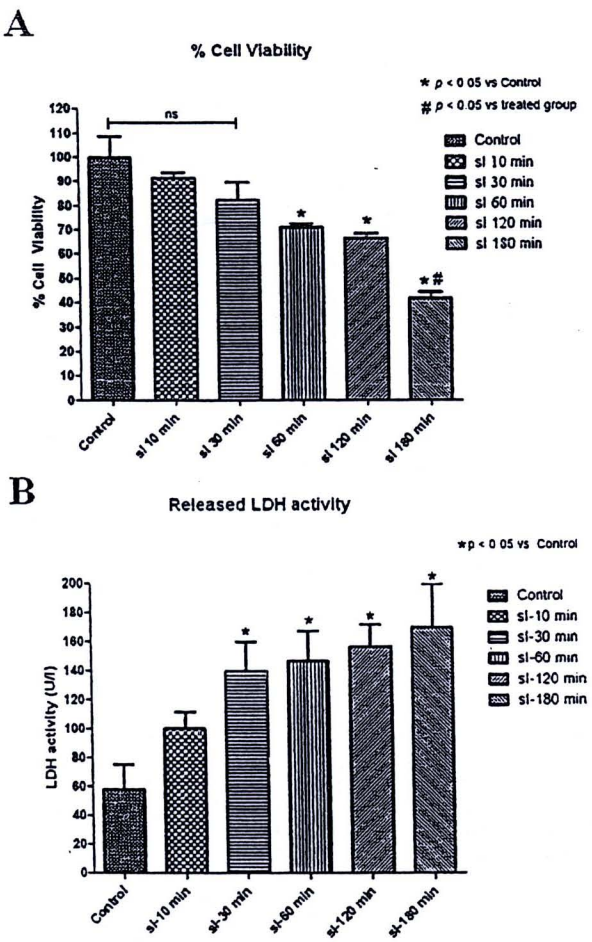
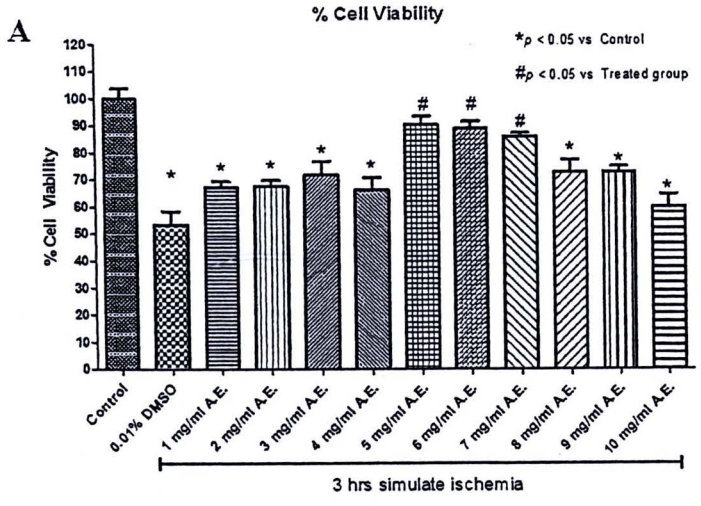


Figure 2.



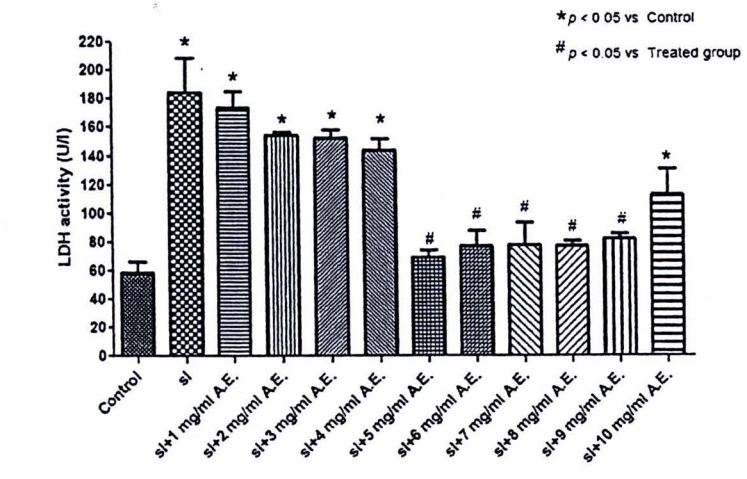


Figure 3.

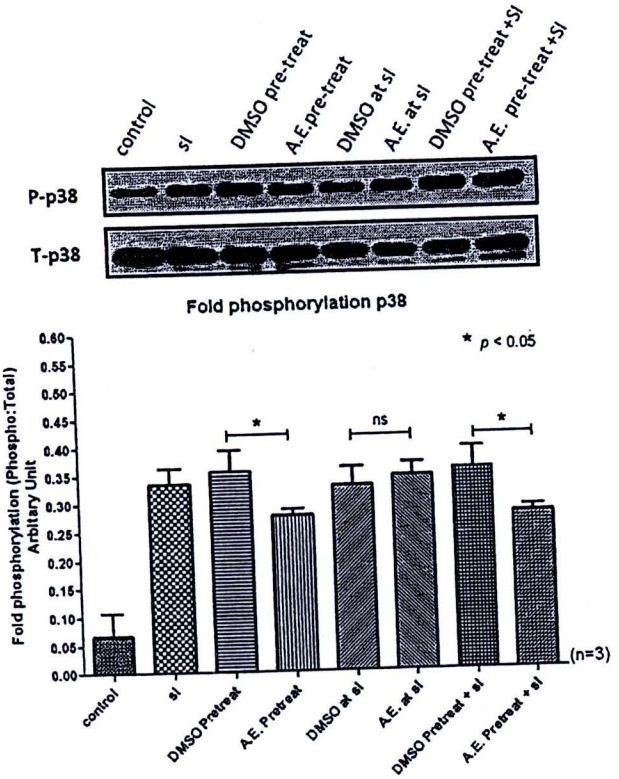


Figure 4.