Original Article

Antioxidative and neuroprotective effects of *Aquilaria crassna* leaf on SK-N-SH cell line

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Abstract:

Introduction: *Aquilaria crassna Pierre ex Lecomte (agarwood) has been used as Ayurvedic medicine and Thai traditional medicine. Its leaf powder is currently available in a form of tea. Although the agarwood leaf tea has been consumed for a period of time, there have been limited scientific data regarding this plant extract.* ***Objectives:*** *The purpose of this research was to evaluate antioxidant and neuroprotective effects against reactive oxygen species (ROS) in SK-N-SH cell line. In addition, toxicity was determined by using SK-N-SH, HEK 293 and HepaRG cell lines.* ***Methods:*** *Aquilaria crassna leave crude extraction was prepared by maceration with ethanol then partition with acetate and water. Aqueous fraction was used for all experiments. Toxicity was determined by MTT assay in 3 cell lines; SK-N-SH, HEK 293, and HepaRG. Anti-oxidative effects were investigated by using anti-lipid peroxidation assay, hydrogen peroxide scavenging activity, and intracellular ROS assay in SK-N-SH cell line induced by glutamate and dexamethasone.* ***Results:*** *MTT assay revealed the viability in 3 cell lines with the extract concentration ranging from 0 to 4 mg/mL. The intracellular ROS level in extract-treated group increased slower than that of non-pretreated group. The pretreated cells showed a slight change in cell morphology after exposure to dexamethasone. In addition, the extract exhibited superior anti-lipid peroxidation compared to vitamin E.* ***Conclusion:*** *Aquilaria crassna leaf extract possessed cytoprotective and antioxidant properties. It also exhibited a relatively safe property. These results encourage further development of Aquilaria crassna leaf extract as natural-derived medicine or nutraceuticals for treatment of neurodegenerative or oxidative-related diseases.*

Keywords: ● *Aquilaria crassna leaf extract* ● *Neuroprotective effect* ● *Antioxidant*

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นิพนธ์ต้นฉบับ

ฤทธิ์ต้านอนุมูลอิสระและปกป้องเซลล์ประสาทของสารสกัดหยาบจากใบกฤษณาในเซลล์ประสาทเพาะเลี้ยง SK-N-SH

ศราวุธ จินดารัตน์ และ ณัฐพล ใจสุภา*
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บทคัดย่อ

บทนำ *กฤษณาใช้ในตำรายาอายุรเวชและเป็นส่วนประกอบของยาแผนไทยมาเป็นเวลานาน ในปัจจุบันมีผลิตภัณฑ์ใบกฤษณาในรูปแบบของชาเพื่อบริโภคอย่างแพร่หลาย แม้ว่าจะมีการบริโภคชาใบกฤษณามาเป็นช่วงเวลาหนึ่งแล้ว ข้อมูลทางวิทยาศาสตร์เกี่ยวกับสารสกัดนี้ยังค่อนข้างมีอยู่อย่างจำกัด* ***วัตถุประสงค์*** *เพื่อศึกษาผลการต้านอนุมูลอิสระ และฤทธิ์ปกป้องเซลล์ที่เกิดจากสารอนุมูลอิสระโดยใช้เซลล์ประสาทเพาะลี้ยง (SK-N-SH) รวมถึงผลความเป็นพิษต่อเซลล์เพาะเลี้ยง 3 ชนิด คือ เซลล์ประสาท (SK-N-SH) เซลล์ไต (HEK 293) และเซลล์ตับ (HepaRG)* ***วิธีการ*** *สารสกัดใบกฤษณาชั้นน้ำ ได้จากการหมักและการแยกชั้นด้วยอะซีเตทและน้ำ และนำมาใช้กับการทดลองทั้งหมด การศึกษาความเป็นพิษของสารสกัดต่อเซลล์ด้วยวิธี MTT ในเซลล์เพาะเลี้ยง 3 ชนิด ได้แก่ เซลล์ประสาท (SK-N-SH) เซลล์ไต (HEK 293) และเซลล์ตับ (HepaRG) การประเมินฤทธิ์การต้านปฏิกิริยาออกซิเดชั่นโดย การวัดออกซิเดชั่นของกรดไขมันไลโนเลอิก ความสามารถในการกำจัดไฮโดรเจนเปอร์ออกไซด์ และการวัดค่า ROS ในเซลล์ประสาทเพาะเลี้ยงที่กระตุ้นด้วยกลูตาเมต และเด็กซาเมทาโซน* ***ผลการศึกษา*** *ความเข้มข้นของสารสกัดใบกฤษณาที่ 0 ถึง 4 มิลลิกรัมต่อมิลลิลิตรมีความปลอดภัยต่อเซลล์เพาะเลี้ยงทั้ง 3 ชนิด ค่าการเกิดอนุมูลอิสระภายในเซลล์ประสาทที่ได้รับสารสกัดใบกฤษณาก่อนกลูตาเมตและเดกซาเมทาโซนนั้นมีค่าต่ำกว่ากลุ่มที่ไม่ได้รับสารสกัดมาก่อน และมีรูปร่างเปลี่ยนแปลงเล็กน้อยกว่าหลังได้รับเดกซาเมทาโซน นอกจากนี้สารสกัดนี้ยังมีฤทธิ์ต้านการเกิดออกซิเดชั่นของกรดไขมันได้ดีกว่าวิตามินอี* ***สรุป*** *สารสกัดจากใบกฤษณามีฤทธิ์ในการปกป้องเซลล์ และฤทธิ์ต้านปฏิกิริยาออกซิเดชั่นที่ดี รวมถึงมีข้อมูลที่ค่อนข้างปลอดภัย จากผลลัพธ์ที่ได้นี้แสดงให้เห็นว่าสารสกัดใบกฤษณานี้มีศักยภาพในการพัฒนาเป็นยาที่ได้จากสมุนไพรหรือผลิตภัณฑ์เสริมอาหารได้ เพื่อใช้ในการรักษาโรคที่เกี่ยวข้องกับการเสื่อมสลายหรือการเกิดออกซิเดชั่นของเซลล์ประสาทได้*

คำสำคัญ: ● *สารสกัดใบกฤษณา* ● *ฤทธิ์ปกป้องเซลล์ประสาท* ● *ต้านอนุมูลอิสระ*

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Introduction

Aquilaria crassna Pierre ex Lecomte belongs to the family Thymelaeaceae. It is also called agarwood or aloewood. Agarwood is abundantly found in South East Asia including Thailand[1]. It is an economical plant of Thailand[2]. Agarwood has been traditionally used as Ayurvedic medicine for years.[3] This plant has been used as one ingredient of Ya-Hom, Thai traditional medicine, for treatment of fainting[4]. In Chinese folk medicine, it has been used for gastric symptoms, coughs, rheumatism, and fever[1]. Currently, *Aquilaria crassna* leaf is available as herbal tea[5]. The pharmacological activity of the leaf extract has been increasingly reported. For instance, water extract of its leaves showed an ability to inhibit *Staphylococcus epidermidis* and *Staphylococcus aureus*[2]. It also possessed anti-hyperglycemic, antipyretic, and analgesic effects[3]. In addition, *Aquilaria crassna* extract was claim to promote cognitive function in experimental animals[6]. Although tea from *Aquilaria crassna* leaf has been consumed, there has not been enough scientific data regarding this plant extract. This study, therefore, demonstrated antioxidant and neuroprotective effects with the safety profile of crude aqueous extract of *Aquilaria crassna* leaf.

Materials and methods

Preparation of *Aquilaria crassna* leaf extract

The extract was prepared according to the slightly modified method from other study[7]. Briefly, *Aquilaria crassna* leaves were dried at 45 °C in the oven for 2 days, then grinded into powder. The leaf powder was macerated in ethanol for 2 days, filtered through filter paper, and evaporated under reduced pressure using rotary evaporator. The obtained gummy syrup was further partitioned with ethyl acetate and water. Derived aqueous fraction was then filtered and evaporated under reduced pressure to yield crude gummy syrup.

Cell culture

Three cell lines [human neuroblastoma (SK-N-SH), human embryonic kidney (HEK-293), and human hepatocellular carcinoma cells (HepaRG)] were grown according to the previous study[7]. Briefly, the cells were cultured in Dulbecco's Modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS), penicillin G (100 u/mL), streptomycin (100 mg/mL), and L-glutamine (4 mM), then incubated at 37°C under 5% CO_2 until confluence.

Cell Viability assay

MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay was performed to investigate cells viability after exposure to various concentrations of *Aquilaria crassna* leaf extract as previously experimented[7]. Briefly, 10^4 cells were seeded into 96-well plate and incubated overnight. The cells were further incubated with extract solutions ranged from 500 to 8,000 μg/mL (weigh of gummy syrup) for 24 h and then changed to 1 mg/mL of MTT solution for 3 hours. Formazan crystal formed in the cell was solubilized by isopropanol and the optical density was read at 570 nm. The data were obtained from 3 independent experiments. Cell viability was presented as the relative percentage against the untreated group.

Protective effect against glutamate-induced intracellular reactive oxygen species (ROS)

This experiment was conducted to investigate the effect of *Aquilaria crassna* leaf extract against intracellular ROS induced by glutamate and dexamethasone according to the slightly modified method[8]. Briefly, 10^4 of SK-N-SH cells were seeded into 96-well plate and incubated overnight. The cells were further incubated with 2,000 μg/mL (gummy syrup) of the fractioned crude extract of *Aquilaria crassna* leaf extract for 24 h, then the medium was discarded. The cells were subsequently exposed to 2 μM of 2',7'-dichlorfluorescein-diacetate (DCFH-DA) for 15 min. A solution of monosodium glutamate at con-

centration of 500 mM was then added. The fluorescent intensity was recorded at the excitation and emission wavelengths of 485 and 530 nm, at 1, 2, 3 and 4 hours. The experiment was run independently in quadruplicate.

Protective effect against dexamethasone-induced intracellular reactive oxygen species (ROS)

This experiment was performed by following the aforementioned procedure[8], with a slight modification by using 1,000 μM of dexamethasone. The fluorescent intensity was measured every 2 h within 8 h duration. The experiment was run in quardruplicate independently. In addition, the morphology of the cells that were prior pretreated and non-pretreated with 2,000 μg/mL of *Aquilaria crassna* leaf extract for 24 h followed by 1,000 μM of dexamethasone for 24 h was observed under inverted microscope.

Anti-lipid peroxidation assay using linoleic acid system

Scavenging of lipid peroxide formation using linoleic acid followed the previous report with slight modification[7,9]. Briefly, linoleic acid mixture was prepared by mixing 0.41 mL of 2.51% v/v in ethanol, 0.8 mL of 0.05 M phosphate buffer (PBS) pH 7, 0.39 mL of distilled water, and 0.4 mL of 100 μg/mL extract in ethanol. The mixture was incubated at 37°C in the dark. Lipid peroxide formation was determined on day 0, 1, 2, and 4 by mixing 30 μL of the mixture with 140 μL of 75% ethanol, 30 μL of 0.02 M ferrous sulfate in 0.2 M hydrochloric acid, and 30 μL of 30% w/v potassium thiocyanate. The solution was mixed thoroughly for 3 min and its absorbance was recorded at 500 nm. Vitamin E at concentration of 100 μg/mL was employed as a positive control. The test was independently run in triplicate. The inhibition was evaluated by comparing the treated group (blank subtracted) with the untreated group (blank subtracted).

Hydrogen peroxide scavenging activity

Scavenging of hydrogen peroxide (H_2O_2) was assayed according to the previous publication[7]. Briefly, 100 μL of various concentrations of the extract were mixed with 200 μL of 20 mM H_2O_2 in 0.1 M PBS (pH 7.4) and incubated for 10 min. The mixture was then spectophotometrically measured the absorbance at 230 nm. The scavenging activity was determined by comparing the absorbance of extract-treated groups with the untreated group. Vitamin C was applied as a positive control. The assay was run in triplicate. Blanks (replaced H_2O_2 by PBS) were prepared in parallel to subtract the background.

Statistical analysis

The measurement data in these experiments were compared to the correlated-control group. To compare the differences, T-test was applied to calculate the *p*-value. The *p*-value of less than 0.05 was considered statistically significant.

Results and Discussion

Cell Viability assay against concentration dependent *Aquilaria crassna* leaf extract

Three cell lines were used to investigate the toxicity of the extract. They could be a representative of cells in vital organ in the body. After incubation of an increasing dose of the extract from 0 to 8 mg/mL in 3 cell types, the absorbance of violet solution of formazan was recorded. Higher intensity indicates higher level of viability. Cell viability remains normal when using the extract concentration ranging from 0 to 4 mg/mL (Figure 1). This data suggested that the cells were well tolerant to high concentrations of the extract solution (> 100 μg/mL). On the other hand, this result indicated that the extract was not relatively toxic to the cells[10].

Protective effect against glutamate-induced intracellular reactive oxygen species (ROS)

In this study, DCFH-DA was used as a detective probe for ROS generation. After diffusing into the cell, DCFH-DA was hydrolyzed to DCFH and further reacted

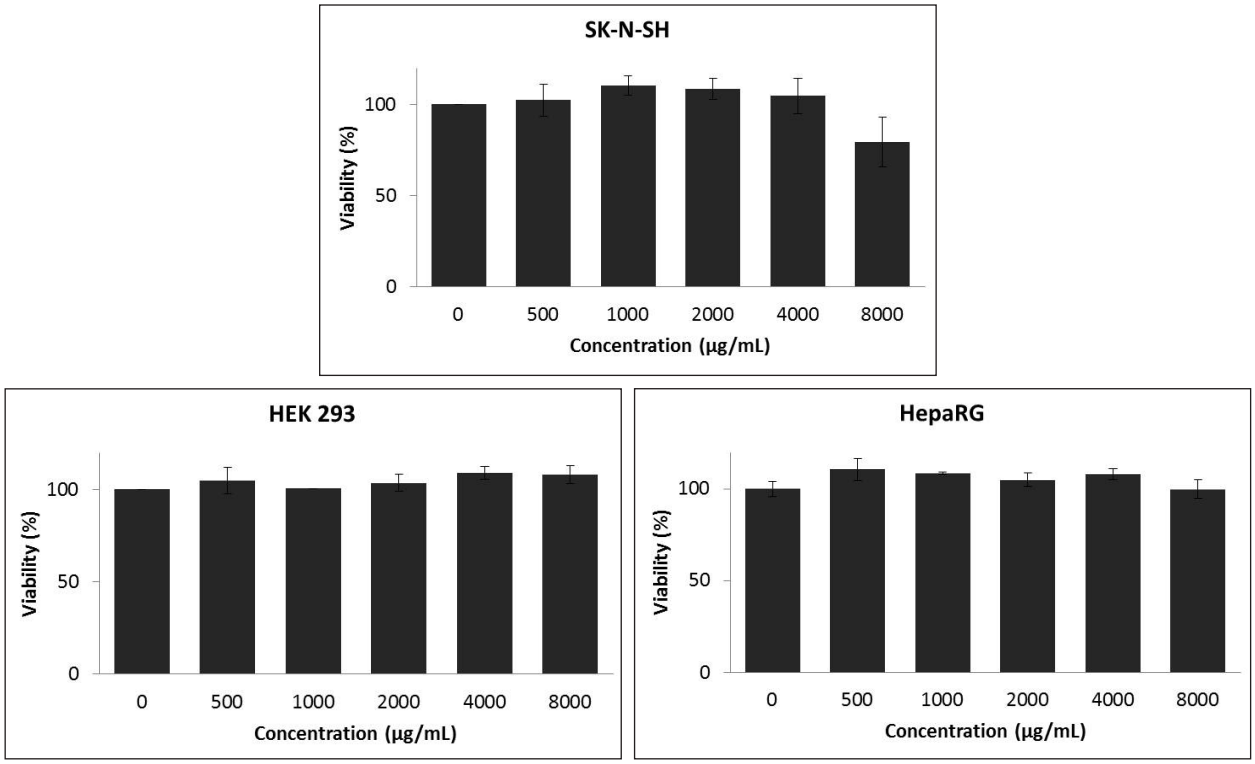

Figure 1 The percentage of cell viabilities after 24-h incubation with various concentrations of aqueous extract of *Aquilaria crassna* leaf (weigh of gummy syrup). The experiments were performed in triplicate. The data were presented as mean ± standard deviation (SD).

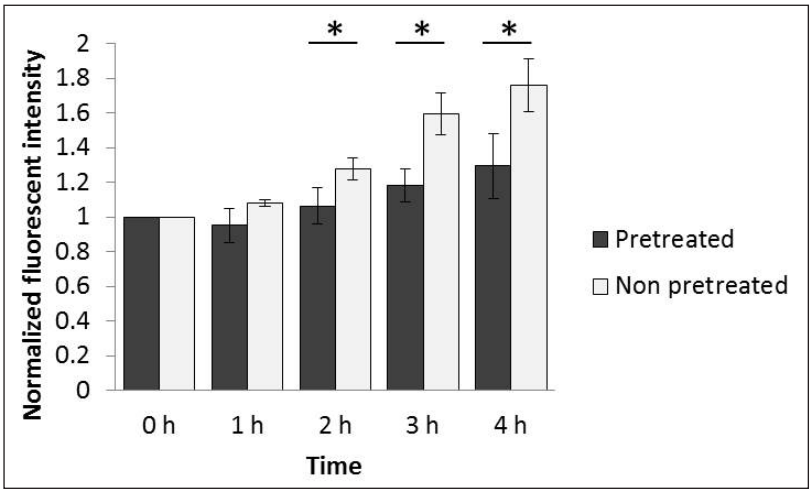

Figure 2 Normalized DCF fluorescent intensity obtained from 2 groups of the cells (pretreated and non-pretreated with the 2 mg/mL extract) exposed to 500 mM of monosodium glutamate. The data was presented as mean ± SD (n = 4). *indicates statistically significant with $p < 0.05$.

with ROS to form the fluorescent DCF. The level of ROS production was proportional to fluorescent intensity. As glutamate is a direct excitatory neurotransmitter, its effect can be observed early within hours. Therefore, the time course effects were recorded every hour from 0 to 4 hours. The data revealed lower ROS level in extract-treated groups compared to the non-pretreated group at 2, 3 and 4 hours (Figure 2). It has been well known that glutamate acts as an excitatory neurotransmitter abundantly found in central nervous system. Over activation of glutamate receptor results in excess intracellular ROS formation[11]. This might be one dominant property of the extract that could be further studied in the condition mimicking the disease involved in glutamate over activity such as in seizure and epilepsy.

Protective effect against dexamethasone-induced intracellular reactive oxygen species (ROS)

This study employed dexamethasone, representative of endogenous cortisol, at concentration of 1,000 μM to induce neuronal cell apoptosis. Lower level fluorescent signal was observed in extract-pretreated group compared to non-pretreated group (Figure 3). Because dexamethasone is not a direct excitatory neurotransmitter and has long term effect on cells hence the time course effects were monitored every 2 hours from 0 to 8 hours. The level of ROS production in extract-treated group was lower than non-pretreated group at 2, 4, 6 and 8 hours. The concentration of dexamethasone used to induce cell death in this study was consistent to the previous studies[12,13]. It has been well known that long duration exposure to high level cortisol leads to neuronal apoptosis[14]. Therefore any agents that attenuate this condition might be clinically useful and agarwood was one of the potential candidates.

Additionally, the pretreated cells showed less morphological change compared to the non-pretreated group (Figure 4). This might suggest neuroprotective effect of this extract against dexamethasone. The condition in this experiment mimicked the depressive condition that the level of endogenous cortisol has been maintained in high level for long period of time[15] as prescribed in previous reports[12,16]. Our study showed that *Aquilaria crassna* leaf extract could prevent cytotoxicity caused by dexamethasone.

Anti-lipid peroxidation assay using linoleic system

Anti-lipid peroxidation was determined using linoleic acid system. Oxidation of linoleic acid generates lipid peroxide that converts ferrous (Fe^{2+}) to ferric (Fe^{3+}). Ferric cation further form the complex with thiocyanate to generate crimson red, which can be measured at 500 nm optical density, reflecting proportion of lipid peroxide formation. The result revealed the superior scavenging effect of lipid peroxide formation over vitamin E at day 4. The intensity of the extract-treated group increased relatively slower compared to the intensity of the untreated and vitamin E groups (Figure 5). This suggested that the extract had a good antioxidant property and could be beneficial for health since lipid peroxidation truly occurs in the body.

Hydrogen peroxide scavenging activity

The extract was investigated for the ability to convert hydrogen peroxide to water. Vitamin C was applied as a positive control. The result indicated that vitamin C had a better potency than the extract. The inhibitory concentration at 50% maximum (IC_{50}) of vitamin C and the extract were approximately at 100 and 200 μg/mL, respectively. However, the extract provided an equal

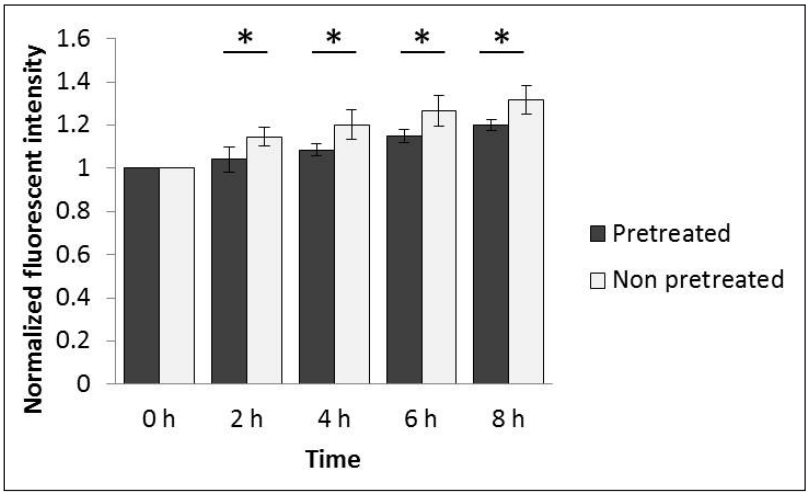

Figure 3 Normalized DCF fluorescent intensity obtained from of 2 groups of the cells (pretreated and non-pretreated with the extract) exposed to 1,000 μM of dexamethasone. The data was presented as mean ± SD (n = 4). *indicates statistically significant with $p < 0.05$.

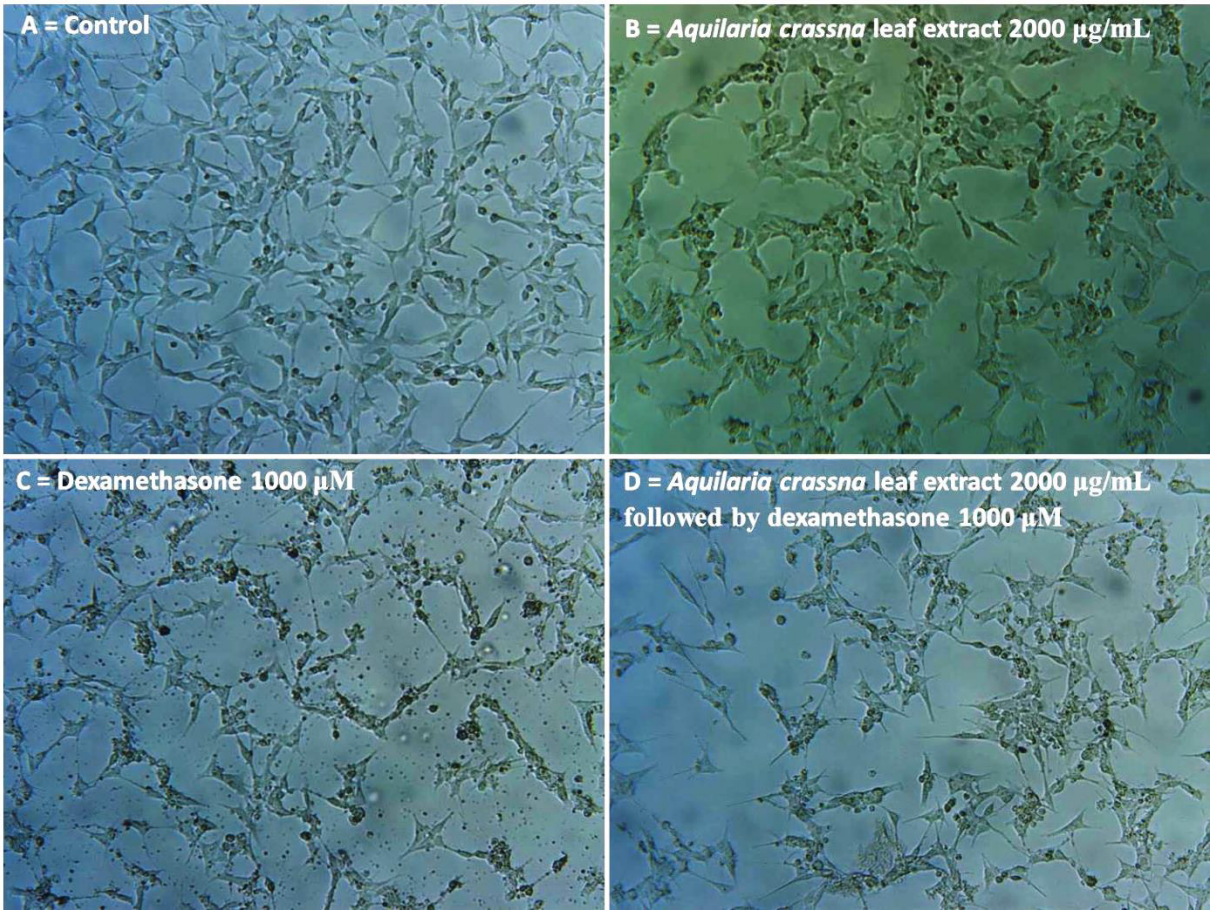

Figure 4 Morphological change of SK-N-SH cells in the pretreated and non-pretreated groups after 24 h exposure to dexamethasone under inverted microscope (20x). A = control cells grown in DMEM for 48 h, B = the cells incubated with extract for 48 h, C = the cell without pretreat for 24 h followed by dexamethasone for 24 h, D = the cell pretreated with the extract for 24 h followed by dexamethasone for 24 h

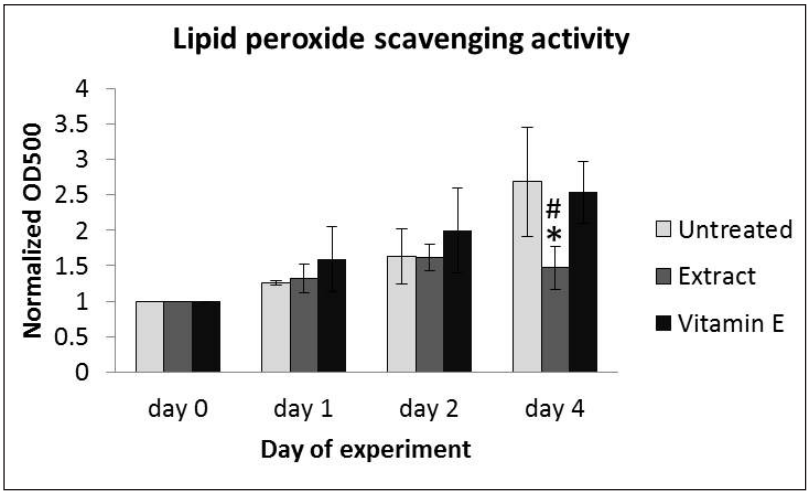

Figure 5 Lipid peroxide scavenging activity of 100 µg/mL *Aquilaria crassna* leaf extract compared to 100 µg/mL vitamin E. The data was shown as mean ± SD (n =3). *, # indicate *p-value* < 0.05 compared to control and vitamin E, respectively.

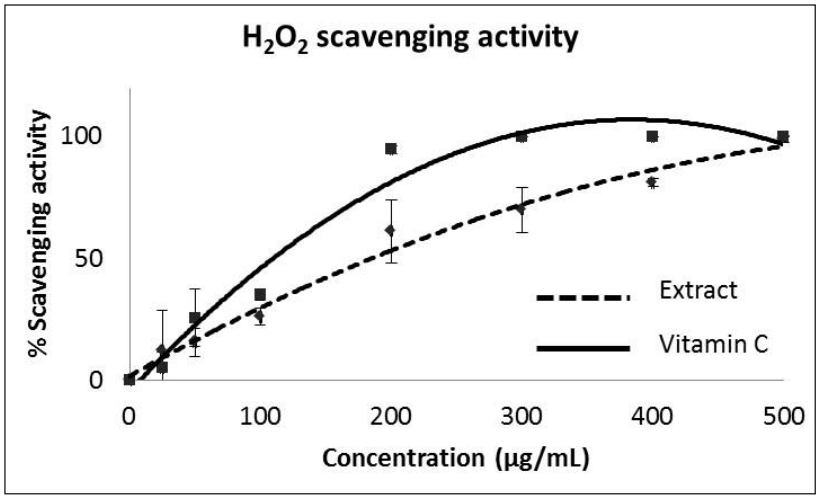

Figure 6 Hydrogen peroxide scavenging activity of *Aquilaria crassna* leaf extract compared to vitamin C. The IC_{50} for the extract and vitamin C were approximately at 200 and 100 µg/mL, respectively. These two compounds revealed the equal efficacy at concentration of 500 µg/mL.

maximum effect when the concentration was increased to 500 µg/mL (Figure 6). The data confirmed that the extract has antioxidant effect and could be beneficial for diseases related to hydrogen peroxide involved oxidative stress.

According to the antioxidant activities obtained from this study, we proposed that the phenolic compounds such as flavonoids possessed this effect. These valued ingredients included mangiferin, genkwanin, and other glycosides that have been identified from *Aquilaria crassna* leaf extract by the previous studies[3,4,17]. A compound with molecular weight around 421-422 corresponding to the mass of mangiferin was detected in the extract (data not shown). This compound was reported to exhibit a potent antioxidant and many pharmacological benefits including cytoprotective effect which was in agreement with this study[18].

Conclusion

In this study, we obtained 2 major parts of *Aquilaria crassna* leaf extract, ethyl acetate and water soluble fractions. We focused on the water soluble fraction as a partial purified crude ethanolic extract which could be the pioneer in the research of *Aquilaria crassna* leaf. The safety profile of the extract determined by MTT assay showed an equal cell viability at high concentration of the extract (4 mg/mL) compare to the control in three cell lines. It was also found that the extract exhibited neuroproctive effect against chemical-induced toxicity by using glutamate and dexamethasone induced intracellular ROS formation. Moreover, anti-lipid peroxidation and hydrogen peroxide scavenging activity were perform to confirm the antioxidant effect that could normally occur in human body. These data suggested a high potential of *Aquilaria crassna* leaf extract as a candidate for development of nutraceuticals or modified herbal medicines for the treatment of neuropsychiatric diseases. Further study should be employed for more information including safety, efficacy, and pharmacokinetic data.

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