Isolation and Characterisation of Acetylcholinesterase Inhibitors from *Aquilaria subintegra* for the Treatment of Alzheimer’s Disease (AD)

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Abstract: *Aquilaria subintegra*, locally known as “Gaharu”, belongs to the Thymelaeaceae family. This plant’s leaves have been claimed to be effective for the treatment of Alzheimer’s disease (AD) by Malay traditional practitioner in Malaysia. In this research, the chloroform extracts of the leaves and stem of *A. subintegra* were tested for acetylcholinesterase (AChE) inhibitory activity. The Thin Layer Chromatography (TLC) results indicated the presence of phenols, flavonoids, terpenoids, and alkaloids compounds in the extracts. Analysis of the stem chloroform extracts with LCMS/MS displayed that it contains kaempferol 3,4,7-trimethyl ether. The AChE inhibitory activity of leaves and stem chloroform extracts and kaempferol were 80%, 93% and 85.8%, respectively. The Brine Shrimp Lethality Assay (BSLA) exhibited low to moderate toxicity of the chloroform extract from leaves (LC₅₀=531.18 ± 49.53 μg/ml), the stem chloroform extract (LC₅₀=407.34 ± 68.05 μg/ml) and kaempferol (LC₅₀=762.41 ± 45.09 μg/ml). The extracts and kaempferol were not cytotoxic to human umbilical vein endothelial cells (HUVEC), human normal gastric epithelial cell line (GES-1) and human normal hepatic cell line (WRL-68). The effect of leaf and stem chloroform extracts and kaempferol were determined in the Radial Arm Maze (RAM) after administration by oral gavage to ICR male and female mice with valium-impaired memory. Administration of kaempferol to the mice significantly reduced the number of repeated entries into the arms of maze in males and females. In conclusion, the inhibition of AChE by leaf and stem chloroform extracts of *A. subintegra* could be due to the presence of kaempferol. This extract is safe for use as a natural AChE inhibitor as an alternative to berberine for the treatment of AD.

Keywords: Acetylcholinesterase, kaempferol, Alzheimer’s disease, *Aquilaria subintegra*.

1. INTRODUCTION

Alzheimer’s disease (AD) is one of the most common neurodegenerative disorders that cause dementia with aging. Brookmeyer *et al.* [1] predicted that AD would affect 1 in 85 people globally by 2050. AChE inhibitors are chemical agents used for symptomatic treatment of AD. AChE hydrolyses the neurotransmitter acetylcholine due to its protease activity. Golan *et al.* [2] found that AChE has high catalytic activity, and it is able to hydrolyse 420 molecules of acetylcholine in one minute. AChE inhibitors are thought to be effective in treating AD by increasing acetylcholine-mediated neuron to neuron transmission. However, Tabet [3] demonstrate that AChE inhibitors could have an anti-inflammatory role and protect cells from free radical toxicity and amyloid beta peptide injury. McGleenon *et al.* [4] proposed that Aβ and abnormal tau protein could be main factors in the development of AD. An influence of hyperphosphorylated tau protein on the expression of AChE was observed in a study by Garcia-Ayllon *et al.* [5]. They also found an important role of AChE in AD via decreasing acetylcholine levels. Later, Anand *et al.* [6] reported the activity of AChE inhibitors in AD management through decreasing Aβ production and aggregation or by increasing the removal of Aβ.

*Aquilaria subintegra* is locally known in Malaysia as “Gaharu”. It is a plant that belongs to the Thymelaeaceae family. Recently, Malay traditional practitioners claimed that *A. subintegra* leaves are effective in the treatment of AD. In traditional medicine in south-east Asia, agarwood is a component in Kampo formulae, such as ‘kiogan’ and ‘rokushingan’, which are used as sedatives. However, there is no available scientific evidence to support these claims. Ueda *et al.* [7] considered that the effects of agarwood (*Aquilaria*) as a traditional sedative are due to its induction on the central nervous system. Additionally, their study in cultured neuronal cells from rats showed that a new compound, (4R, 5R, 7R)-1(10)-spirovetiven-11-o1-2-one, and a 2-(2-phenylethyl) chromone derivative isolated from the methanol extract of agarwood had a significant reduction effect on the mRNA level of brain-derived neurotrophic factor (BDNF). Furthermore, studies have identified natural AChE inhibitors in other plants. Yang *et al.* [8] reported strong AChE inhibitory activity of the alkaloid skimmianine extracted from *Zanthoxylum nitidum* roots. They suggested that the anti-AChE activity of skimmianine may be used in the treatment of AD. Pereira *et al.* [9] found that the water extract of alkaloids from leaves of *Catharanthus roseus* strongly inhibited AChE.
Kim et al. [10] suggested that kaempferol has anti-inflammatory properties similar to other flavonoids. Likewise, Serhan et al. [11] illustrated that chronic inflammation occurs when acute inflammation is not resolved. Kaempferol has a positive effect in numerous diseases including atherosclerosis, cancer, asthma and some neurological disorders such as AD. Song et al. [12] demonstrated that kaempferol isolated from butterbur (Petasites japonicus) leaves might prevent the effects of Aβ, which can cause AD by inducing neurotoxicity and by producing free radicals that lead to cellular death.

Using animal models (ICR mice) to study the effects of compounds might be beneficial to improve AChE inhibitors used in the treatment of AD. Dong et al. [13] investigated two AChE inhibitors, physostigmine and donepezil, on memory-related behaviours of mice. They suggested that AChE inhibitors could improve memory deficits in mice. Ikarashi et al. [14] examined mice memory disturbance by measuring AChE concentrations in their brains. They found that memory impairment in the cholinergic system of the mice is due to Aβ accumulation, which occurs when AChE increased. Figueiró et al. [15] extracted AChE inhibitors from Ptychopetalum olacoides and orally administered these compounds to mice. They found significant AChE inhibitor activity of these extracts, which is useful for AD treatment.

The Radial Arm Maze (RAM) is a practicable memory test to determine the effect of drugs. Foti et al. [16] found that most aspects of spatial function can be analysed by movement in the RAM, in addition to analytical memory and appropriate working memory.

In this study, the determination of the AChE inhibitory activity of the chloroform extract of A. subintegra chloroform leaves and stem were evaluated in an enzymatic bioassay system. The AChE inhibitory activity of the leaf and stem extracts and kaempferol detected in the chloroform extract of A. subintegra stems was determined using the Radial Arm Maze (RAM) after administration to ICR male and female mice with valium-impaired memory by oral gavage.

2. MATERIALS AND METHODS

2.1. Plant Materials

The leaves and stem of A. subintegra were collected from the RAL plantations farm in Kuala Kangsar, Perak, Malaysia. The authenticity of the plant was verified by the Institute of Biological morphology and taxonomy classification by plant taxonomist Professor Dr. Ong Heau hoi from the Institute of Biological Sciences, Faculty of Science, University of Malaya.

2.2. Chemicals

Acetylcholinesterase (EC3.1.1.7, Sigma product no C2888), acetylthiocholine iodide (ATCI), 5,5'-dithiobis [2-nitrobenzoic acid] (DTNB), and Berberine were purchased from Sigma (St. Louis, MO, USA). Standard kaempferol purchased from Calbiochem (Japan). TLC Silica gel 60 F254 was purchased from Merck KGaA (Germany).

2.3. Extraction and Isolation of Chemical

2.3.1. Compounds

The leaves and stem of A. subintegra were powdered and extracted with chloroform. One litre of chloroform and methanol was used to extract the leaves and powdered stem for 2 hours in a water bath at 40°C. Each sample extract was filtered, and the filtrate was concentrated to 10 ml using a rotary evaporator at medium speed at 40°C. One millilitre of each sample was evaporated to dryness for the preparation of the crude extract.

2.4. Determination of Bioactive Compounds by Thin Layer Chromatography (TLC) and Liquid Chromatography-tandem Mass Spectrometry (LCMS/MS)

TLC plates with 20 cm height and width were used. The crude extract was spotted as a thin line on the sample line using a tapered capillary tube. The TLC plate was transferred to a well-covered tank with chloroform or 10% methanol in chloroform as a solvent. When the sample travelled up the plate, separated bands appeared on each plate were identified under visible light and UV light. The Rf value of each band was calculated. Finally, Dragendorff, Vanillin-H2SO4 and Folin-Ciocalteu reagents were used to detect alkaloids, terpenoids and phenols, respectively. LCMS/MS (Perkin Elmer Flexar FX-15 ultra-high performance liquid chromatography (USA) with AB Sciex 3200Qtrap tandem mass spectrometry (Singapore) used to detect the presence of various chemicals in the leaves and stem of the crude chloroform extract at 5 mg/ml. The conditions were as follows: column: Phenomenex C18 50 mm x 2.0 mm x 5 μm; Buffer A: water with 0.1% formic acid and 5 mM ammonium formate, buffer B: acetonitrile with 0.1% formic acid and 5 mM ammonium formate; gradient run program: 10% A to 90% B from 0.01 min to 8.0 min, hold for 2 min and back to 10% B in 0.1 min and re-equilibrated for 5 min. The sample extracts were diluted two times with appropriate solvents and filtered with a 0.2 μm nylon filter prior to analyses. The MS/MS was performed with negative ESI, - 4500kV, at 500°C, with purified nitrogen gas as the nebulisation and collision gas (99.9995% purity); fragmentation energy: collision energy spread -35eV +/- 15eV. The data analysis was performed using AB Sciex Analyst 1.5 software with an internal MS/MS library search and further interpretation and processing with ACD/Labs MS fragmenter software.

2.5. Determination of Total Phenol Contents

The total phenolic content was measured according to the method described by Thind et al. [22]. Briefly, 500 μl of each crude extract (5 mg/ml) was mixed with 5 ml Folin Ciocalteu (1:10, V/V diluted with distilled water) and 4 ml 1 M sodium carbonate. The mixture was incubated in a water bath at 45°C for 15 min, and its absorbance was determined at 765 nm with a spectrophotometer. The standard calibration curve was prepared with 0, 50, 100, 150, 200, 250 and 500 mg/L solutions of gallic acid in 50:50 (v/v) methanol: water. The experiments were performed in triplicate.

2.6. Determination of Total Flavonoid Contents

The total flavonoid content was measured according to the method described by Zhang et al. [18]. Briefly, 500 μl of each crude extract (5 mg/ml) was added to 0.3 ml 5% sodium nitrate. The mixture was incubated at room temperature for 5 min. Then, 0.3 ml of 10% aluminium chloride was mixed into the solution before incubation at room tempera-
ture for 6 min. Subsequently, the mixture was added to 2 ml 1 M sodium hydroxide and 10 ml distilled water. The absorbance was read at 510 nm using a spectrophotometer. The standard calibration curve was generated with 0, 200, 400, 600, 800, 1000 and 2000 mg/L quercetin solution in methanol. The experiments were performed in triplicate.

2.7. Brine Shrimp Lethality Assay (BSLA)

The toxicity of the crude extract was determined by the brine shrimp lethality assay (BSLA). Artificial sea water was prepared by dissolving 38 g sea salt in 1 L distilled water. After filtering, brine shrimp (Artemia salina) eggs were placed in the water and kept in a dark environment. They were allowed to hatch after incubation at room temperature for 48 h. Three concentrations of each crude extract (1000 µg/ml, 100 µg/ml, 10 µg/ml) were transferred to each vial of a microplate with 5 ml of sea water and 10 brine shrimp in each vial. The control was performed simultaneously and prepared by replacing the crude extract with sea water [19]. After 24 h, the number of surviving shrimp was counted, and the data were analysed using a probit analysis program to determine the median lethal concentration (LC50). The experiments were performed in triplicate.

2.8. MTT Assay

2.8.1. Cell Culture

A Human umbilical vein endothelial cells (HUVEC), human normal gastric epithelial cell line (GES-1) and human normal hepatic cell line (WRL-68) were purchased from American Type Culture Collection (ATCC, Manassas, VA). HUVEC cells were grown in Endothelial Cell Medium (ECM, ScienCell Research Laboratories, Carlsbad, CA) supplemented with 5% heat-inactivated fetal bovine serum (FBS, ScienCell Research Laboratories, Carlsbad, CA), 1% (ECGS, ScienCell Research Laboratories, Carlsbad, CA) and 1% penicillin and streptomycin (P/S solution, ScienCell Research Laboratories, Carlsbad, CA). GES-1 cells were grown in Dulbecco’s Modified Eagle Medium (DMEM, Life Technologies, Inc, Rockville, MD) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Sigma-Aldrich, USA), 1% penicillin and streptomycin. WRL-68 cells were grown in Dulbecco’s Modified Eagle Medium (DMEM, Life Technologies, Inc, Rockville, MD) supplemented with 15% heat-inactivated fetal bovine serum (FBS, Sigma-Aldrich, USA), 1% penicillin and streptomycin. The cells were kept in an incubator at 37°C in a humidified atmosphere with 5% CO2. For experimental purposes, cells in the exponential growth phase (approximately 70-80% confluency) were used.

2.8.2. MTT Cell Viability Assay

The cytotoxic effect of the extract was assessed by the MTT cell viability assay (Mosmann et al., 1983). Briefly, 1.0 x 10^4 cells were seeded in a 96-well plate and incubated overnight at 37 °C in 5% CO2. On the next day, the cells were treated with a two-fold dilution series of six concentrations of the leaf and stem extract and kaempferol and incubated at 37 °C in 5% CO2 for 48 hours. MTT solution (4,5-dimethylthiazol-2-yl-2,5-diphenyltetrazolium bromide) was added at 2 mg/mL, and, after 2 hours of incubation at 37 °C in 5% CO2, DMSO was added to dissolve the formazan crystals. The absorbance was then read in a Chameleon multitechnology microplate reader (Hidex, Turku, Finland) at 570 nm. The cell viability percentage after exposure to the extract for 48 hours was evaluated based on the previously described method (Looi et al., 2011). The IC50 value was defined as the concentration of the compounds required to reduce the absorbance of treated cells to 50% of the DMSO-treated control cells. The experiment was carried out in triplicate.

2.9. Toxicity Test in Mice

2.9.1. Mean Body Weight

Mice were randomly divided into 8 groups. Each group had 3 male and female mice. Group 1: control; Crude extracts of the leaves and stems were administered to groups 2, 3 and 4 by oral gavage at 0.1 ml/g body weight, 0.5 ml/g body weight and 1 ml/g body weight, respectively, for a period of at least 28 days [22]. The mean body weight of each group was measured each day during the study. The mean body weight of mice was analysed by ANOVA, and p values of <0.05 were considered to indicate a significant difference between groups.

2.9.2. Behavioural and Physical Observations

Mice were observed once each day for mortality and evaluation of general condition, including appearance, activity, behavior, respiration, and the skin, fur, eyes, nose, oral activity, abdomen and external genitalia were observed for any sign of toxicity. During the study, once each week mice were removed from their cages for observation of mortality and general condition following the method described by DeMerlis et al. [23].

2.10. AChE Inhibitory Activity Assay

Ellman’s method, as described by Yang et al. [8], was used to determine the AChE inhibitory activity of the crude extract and kaempferol 3,4,7-trimethyl ether isolated from the chloroform extract of the stem. Briefly, 140 µl of 0.1 M sodium phosphate buffer, pH 8, and 20 µl 1 mg/ml sample solution were mixed with enzyme (AChE). The amount of AChE used was 15 µl, with an activity of 0.25 U/ml. The mixtures were pre-incubated at 4°C for 20 min. The reaction was started by adding 10 µl 0.01 M DTNB and 10 µl 0.075 M ATCI. The extract was incubated at room temperature for 20 min. The absorbance at 405 nm was measured with an ELISA reader. The percentage of inhibitory activity was calculated using the following formula: % AChE inhibition = \( A_c - A_t / A_c \times 100 \). Berberine was used as positive control. The experiments were performed in triplicate.

2.11. Animal Study

Male and female adult (2 months old, 35–45 g) imprinting control regions (ICR) mice were obtained from the University of Malaya Animal House. The mice were housed under controlled environmental conditions with a temperature of 22±1 °C, 12-h light/dark cycle and food and water ad libitum. During the 3 months of the study, the mice were divided to 12 groups. Each group had 3 males and 3 females. Group 1: control; Group 2: stem extract; Group 3: leaf extract; Group 4: leaf and stem extract; Group 5: kaempferol
and Group 6: berberine. Each extract or standard was given to the mice by oral gavage. Valium was used to impair the memory of the mice. All drugs were given at 0.1 ml/g body weight. The animal test was conducted in an eight-arm radial maze apparatus. The central hub is consisted of a white polypropylene octagonal base (28.6 cm in diameter, 11.4 cm sides). The arms radiated from the centre hub with equal spacing 8.9 cm wide with clear polycarbonate walls (17.5 cm high). Each baited arm had only one pellet. The working memory was assessed by measuring the number of repeat entries into arms of the maze already visited (NRE). For instance, if an NRE of 5 means that the mouse ate all 8 pellets and returned to the arms of the maze in which the pellet had already been eaten 5 times (5 errors). The number of entries until the first error occurs (NEF) is commonly used as an additional measure of performance in radial arm maze. For instance, an NEF of 6 means that the mouse ate 6 pellets without any repeated entries to the arms of the maze in which the mouse had already eaten the pellet. However, the mouse ate the 7th pellet only after repeated entries to empty arms. Each group was given valium before the test, and 30 min later it was placed in the radial arm maze. One hour after the mouse was given the test sample, it was placed in the radial arm maze again. Each mouse was kept in the radial arm maze for 10 min. The results of the memory testing data were analysed with ANOVA, and p values of <0.05 were considered to indicate a significant difference among groups in all analyses. The study was conducted in accordance with the principles outlined in the guide for Animal Use Protocol prepared by the University Of Malaya Animal Care and Use Committee (Ethic No: ISB/23/05/2013/AA(R)).

3. RESULTS AND DISCUSSION

The AChE inhibitory activity of the chloroform extract of A. subintegra leaves (LCAS), chloroform extract of A. subintegra stem (SCAS) and kaempferol were 80%, 93% and 85.8%, respectively (Fig. 1). Between each arm. Each arm was 76.2 cm long and Acetylcholine (ACh) is hydrolysed by AChE through a reaction between the thiocholine and DTNB to generate the yellow anion of 5-thio-2-nitrobanzoic acid [19]. The inhibition activity of AChE was measured by this reaction. LCAS and SCAS displayed high inhibitory activity at low concentrations, comparable to kaempferol, whereas the inhibitory activity of berberine was only observed at higher doses. Brine shrimp lethality assay demonstrated he LC50 of LCAS, SCAS, and kaempferol were 531.18 ± 49.53, 407.34 ± 68.05 and 762.41 ± 45.09 µg/ml (Table 1). The cytotoxic effect of LCAS, SCAS and kaempferol was evaluated in human umbilical vein endothelial cells (HUVEC), human normal gastric epithelial cell line (GES-1) and human normal hepatic cell line (WRL-68) using MTT assay, where no significant cytotoxicity and cell inhibitory effect was observed in the tested cells after 48 hours of treatment with the extracts and kaempferol. The IC50 values after 48 hours of treatment are shown in Table 2. The acute toxicity test of the extracts and kaempferol on ICR mice indicated no mortality during the study. Daily observations showed that administration of the articles had no side effects in mice. Additionally, there were no statistically significant differences among the 3 groups of mice given doses of 0.1, 0.5 and 1 ml/g body weight of LCAS and SCAS by oral gavage compared with the control group (Fig. 2). These results displayed insignificant toxicity of LCAS and SCAS, kaempferol and the standard drug. For LCAS and SCAS, the total flavonoid contents were 414 mg QE/g dw and 645 mg QE/g dw (Fig. 3A), and the total phenol contents were 164 mg GAE/g dw and 210 mg GAE/g (Fig. 3B), respectively.

The analysis of the LCAS with LCMS/MS indicated that it contains delphinidin-3-glucoside, 2(3,4-Dihydroxy-phenyl)-7-hydroxy-5-benzene propanoic acid and benzene-propanoic acid conjugates. The same analysis of SCAS indicated the presence of 5-hydroxy-7,4-dimethoxyflavone, kaempferol 3,4,7-trimethyl ether and 5,7-dihydroxyl-4’-dimethoxyflavone.

Foti et al. [16] suggested that the Radial Arm Maze (RAM) can assess most aspects of the spatial function, such as the analytical memory and the appropriate working memory, via movement. RAM is a practicable memory test for the effect of drugs. ICR mice (male and female) were used to evaluate the effectiveness of LCAS and SCAS. Working
memory was assessed by measuring the number of repeat entries to arms of the maze that were already visited (NRE). Comparing the results of the RAM assessment of all the different treatments including kaempferol and berberine, the lowest NRE was exhibited by the control group (Fig. 4).

Table 1. Brine Shrimp Lethality Assay (BSLA) demonstrating low to moderate toxicity of the extracts and kaempferol. Berberine was used as positive control.

<table>
<thead>
<tr>
<th>Extract</th>
<th>Sample</th>
<th>LC₅₀ (µg/ml) ±SD</th>
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<tr>
<td>Chloroform</td>
<td>Stem</td>
<td>407.34 ± 68.05</td>
</tr>
<tr>
<td></td>
<td>Leaves</td>
<td>531.18 ± 49.53</td>
</tr>
<tr>
<td></td>
<td>Fruit</td>
<td>683.81 ± 76.18</td>
</tr>
<tr>
<td>Test sample</td>
<td>Kaempferol</td>
<td>762.41 ± 45.09</td>
</tr>
<tr>
<td>Standard</td>
<td>Berberine</td>
<td>502.82 ± 39.81</td>
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Valium intensifies the effect of the neurotransmitter gamma-aminobutyric acid (GABA) leading to Central Nervous System (CNS) depression, so it is utilised to cause memory impairment in mice. In 2012, taking valium caused side effects in 18583 people, of which 23 (69.57% in females and 30.43% in males) experienced the development of AD. Regularly taking valium caused AD in 77.27% of people older than 60 and 69.57% of females. The NRE was significantly increased when the mice were given valium, to 11.2 and 13.1 in male and female mice, respectively (Fig. 4).

Plant extracts and kaempferol were administered to valium-treated mice. The lowest NRE, 3.3 and 4.1 in female and male mice, respectively, was observed in mice given kaempferol (Fig. 4). Serhan et al. [11] reported that chronic inflammation has a detrimental effect on AD. Kim et al. [24] reported that administration of kaempferol in ICR mice significantly reverse the effect of Aβ. They utilised the Y-maze test to prove the ability of kaempferol to ameliorate memory impairment in mice caused by Aβ. One of the primary features of AD patients is a deficiency of ACh, a neurotransmitter in the synapses of the cerebral cortex. AChE is an ACh hydrolysing enzyme that can promote the assembly of Aβ into fibrils. Tabet [3] suggested that AChE inhibitors could be effective in the treatment of AD by increasing the acetylcholine-mediated neuron-to-neuron transmission. García-Ayllón et al. [5] reported that AChE-mediated decreasing in acetylcholine has an important role in AD treatment. Because AChE inhibitors have anti-inflammatory activity, they could protect neuronal cells from injuries caused by free radicals and Aβ. The significant activity of kaempferol as a natural AChE inhibitor (Fig. 1) confirms its potential an alternative drug in the treatment of AD.

Berberine is an alkaloid with anti-inflammatory activity that is utilised as a drug to treat AD. Some investigations suggested that berberine may have AChE inhibitory effects, and it could be useful for the prevention of AD as well [25, 26]. The results of the RAM obtained from the mice given berberine, LCAS and SCAS, were approximately the same (Fig. 4). The decline in NRE might be due to the presence of delphinidin in LCAS and kaempferol in SCAS. Kaempferol is a flavonoid with an anti-inflammatory effect, as suggested by Takasawa et al. [27]. Inoue et al. [28] found that delphinidin decreased the amount of epigallocatechin-3-gallate (EGGG), which causes cytotoxicity and enhanced endoplasmic reticulum (ER) stress. ER stress is a marker of AD pathogenesis due to the generation of Aβ [29]. Mice administered LCAS had a higher NRE compare to those administered SCAS. Mice given valium exhibited a remarkable increase in NRE. In contrast, LCAS, SCAS and kaempferol-treated animals in the presence of valium exhibited a drastic decrease in NRE (Fig. 4).

As another measurement of memory in mice, the number of entries until the first error occurs (NEF) was counted. Fig. 5 shows that the control group had the highest NEF. The mice given valium had the lowest NEF (1.94 in male and 1.68 in female mice). The mice given kaempferol had NEFs of 5.4 and 5.9 in male and female mice, respectively. The NEF significantly decreased when the mice were treated with valium. The group of mice given kaempferol had the highest NEF, while the NEF of the mice given both LCAS and SCAS was higher than that of the mice given LCAS or SCAS individually. The lowest NEF was observed in the group of mice given LCAS.

Mice given LCAS, SCAS or kaempferol without valium showed statistically insignificant NRE and NEF in all groups, male and female compared to the control, illustrating that LCAS, SCAS and kaempferol were effective after the mice were given valium.

Table 2. The chloroform extracts and kaempferol exhibited no significant inhibitory effect on the proliferation of human umbilical vein endothelial cells (HUVEC), human normal gastric epithelial cells (GES-1) and human normal hepatic cells (WRL-68). The cells were treated with various concentrations of the extracts or kaempferol for 48 hours. The standard drug, doxorubicin was used as positive control. The IC₅₀ values were analyzed by non-linear regression analysis.

<table>
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<tr>
<th>Extract</th>
<th>Sample</th>
<th>IC₅₀ (µg/ml) ±SD</th>
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<tbody>
<tr>
<td>Cloroform stem extract</td>
<td></td>
<td>261.17 ± 12.42</td>
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<tr>
<td>Cloroform leaf extract</td>
<td></td>
<td>318.53 ± 27.39</td>
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<tr>
<td>Kaempferol</td>
<td></td>
<td>364.78 ± 23.17</td>
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<tr>
<td>Doxorubicin</td>
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<td>7.42 ± 0.15</td>
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Table 2. The chloroform extracts and kaempferol exhibited no significant inhibitory effect on the proliferation of human umbilical vein endothelial cells (HUVEC), human normal gastric epithelial cells (GES-1) and human normal hepatic cells (WRL-68). The cells were treated with various concentrations of the extracts or kaempferol for 48 hours. The standard drug, doxorubicin was used as positive control. The IC₅₀ values were analyzed by non-linear regression analysis.
Fig. (2). Mean body weights in male (A) and female (B) mice.

Fig. (3). The total flavonoid (A) and phenol (B) contents of LCAS and SCAS chloroform, methanol and water extracts.
Fig. (4). The number of repeat entries to arms of the maze (NRE) A. in male mice B. in female mice.

Fig. (5). The number of entries to arms of maze until the first error occurs (NEF) A. in male mice B. in female mice.
Administration of LCAS, SCAS and kaempferol after valium caused a marked reduction in NRE. This finding indicates that in the absence of memory impairment in mice, LCAS, SCAS and kaempferol exhibited insignificant effects.

**CONCLUSIONS**

The results from TLC of chloroform extracts of *A. subin-tegra* leaves and stem displayed the existence of alkaloids and phenols. The analysis of the stem chloroform extract with LCMS/MS indicated that it contains kaempferol. Kaempferol is a flavonoid with high AChE inhibitory activity. The leaf and stem chloroform extracts significantly reduced the NRE in male and female ICR mice with valium-impaired memory in the RAM. The decline in the NRE might be due to the presence of kaempferol. Furthermore, the highest NEF was observed in kaempferol-treated mice. This extract could improve memory impairment in mice. Additionally, the extract may be a potent natural AChE in-
hibitor and could be used as an alternative to berberine in the treatment of AD. The insight mechanism of action of the AChE is still not known. However, there is a possibility the kaempferol act at the ionic site or esteratic subsite of AChE. Thus, further mechanistic investigations are required to confirm and validate kaempferol’s effects for the treatment of AD.

CONFLICT OF INTEREST
The author(s) confirm that this article content has no conflicts of interest.

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REFERENCES


