The antiaging property of aqueous extract of *Millingtonia hortensis* flowers in aging neuron

Pichaya Jumnongprakhon,
Decha Pinkaew[1],
Pravaree Phuneerub[2,3]

Department of Anatomy, Faculty of Medical Science, Naresuan University, Phitsanulok, [1]Department of Physical Therapy, Faculty of Associated Medical Science, Chiang Mai University, Chiang Mai, [2]Department of Applied Thai Traditional Medicine, School of Integrative Medicine, Mae Fah Luang University, [3]Medicinal Plants Innovation Center of Mae Fah Luang University, Mae Fah Luang University, Chiang Rai, Thailand

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Abstract

Cellular senescence is the key mediator of cellular dysfunction before undergoing degenerative disease such as Alzheimer's disease. The aging process was mainly by the overactivation of senescence associated β-galactosidase (SA-β-gal) enzyme before mediated several negative responses, including intracellular reactive oxygen species (ROS) production, cellular senescence regulation, and death prior encourage synaptic loss. Thus, in the recent work, we evaluated the *in vitro* effects of aqueous extract of *Millingtonia hortensis L.* (MH) from flower in hydrogen peroxide (H_2O_2)-induced senescence in SK-N-SH cells. Herein, we demonstrated that MH significantly increased cell viability and decreased both of apoptotic cells and ROS production in a dose-dependent manner comparing to aging group ($P < 0.01$) using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, flow cytometry, and ROS assay. Furthermore, the number of SA-β-gal-positive cells was also reduced in MH treatment ($P < 0.01$) together with the promotion of Sirt-1 protein. Importantly, MH also promoted the synaptic plasticity by decreased acetylcholinesterase activity and increased synaptophysin expression in aging neurons comparing to aging group ($P < 0.01$). Hispidulin (the active ingredient in MH) was also revealed the similarly effects to MH. Therefore, we suggested that MH might be beneficially for neurodegenerative disease that caused by aging.

Key words: Aging neuron, cellular dysfunction, *Millingtonia hortensis L*, neurodegenerative disease, Sirt-1, Hispidulin

INTRODUCTION

Cellular senescence or aging is the cellular response implicated with various pathological conditions such as heart disease and Alzheimer's disease (AD).[1] Previous reports demonstrated that the aging process in brain tissue of AD was commonly found in several cell types such as neuron, glia cells, and brain endothelial cells before leading the cell stress, dysfunction, and death in finally.[2] The pathological hallmark in aging neuron was primarily mediated the production of reactive oxygen species (ROS) that caused protein degradation, intracellular antioxidant defense imbalance, and lipid peroxidation prior encourage various negative responses such as inflammation.[3] Simultaneously, the inflammatory response in neuronal cells also suppressed sirtuin (silent mating type information regulation 2 homolog)-1 (Sirt-1), the mediator of cellular defense system and cell division,

Address for correspondence:
Dr. Pravaree Phuneerub,
Department of Applied Thai Traditional Medicine, School of Integrative Medicine, Mae Fah Luang University, Chiang Rai 57100, Thailand.
E-mail: pravaree.phu@mfu.ac.th

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before underwent senescence and death.[4] Moreover, the reduction of Sirt-1 also promoted the overactivation of senescence-associated β-galactosidase enzyme (SA-β-gal) and telomerase enzyme leading telomere shortening and then the cells become aging.[5] Afterward, the cell death response or apoptosis will activate by firstly modulated the cleavage caspase-3 that caused cellular deformation and then affected to neuronal function, especially decreasing both of pre- and postsynaptic protein such as synaptophysin.[6] The degradation of synaptic protein was mainly by monoamine oxidase and acetylcholinesterase (AchE) that caused AD progression.[7] Several studies were showed that the reduction of AchE activity in neuronal tissue plays a key role in the reversing of synaptic protein degradation in the AD model.[8] Therefore, the reduction of aging-induced negative response in neurons might be helpful for promoting of AD or brain disease recovery.

Millingtonia hortensis (MH) belongs to family Bignoniaceae. The common name in English is "Indian cork tree" or "tree jasmine" and commonly known in Thai as "Peep."[9] In Thai traditional, the flower of MH has an efficacy for curing disease, especially in respiratory system disease such as asthma, sinusitis, cholagogue, and tonic.[10] Moreover, it has high antioxidant component such as flavonoid and hispidulin which were recorded in the reduction of negative response-induced cellular dysfunction and death in brain tissue.[11] Nevertheless, its effect has not been understood in aging neuronal cells. Based on the above data, we speculated that MH would reduce cell stress, senescence, death, and synaptic plasticity impairment on aging neuron. To investigate this hypothesis, the SK-N-SH cells were triggered by H_2O_2 to establish an aging neuronal model and then explored the effect of MH on the aging neuron.

MATERIALS AND METHODS

Reagent Hydrogen
Hydrogen peroxide, Hispidulin, were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO, USA). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 2′,7′-dichlorofluorescine diacetate, Muse®Annexin V and Dead Cell kit, and BetaRed™ β-Gal assay kit were purchased from Merck Millipore, (Millipore, MA, USA). The anti-Sirt1, anti-synaptophysin, and anti-actin were purchased from Abcam, Cambridge, UK.

Preparation of *Millingtonia hortensis*
Flowers of MH were collected at Chiang Rai Province in Thailand in October 2019. The plant material was authenticated by a botanist at the office of the QSNC, Thailand. Flowers were dried at 40°C. Then, drug was powdered by grinder (coarse powder) and macerated with water. The water extracts were lyophilized to dryness and stored at − 20°C.

Phytochemical screening
MH extract was evaluated by phytochemical which was divided into 10 groups: alkaloids, flavonoids, anthraquinones, terpenoids, phenolic, saponins, tannins, steroids, carbohydrate, and cardiac glycosides.[12]

Determination of hispidulin in the extract by high-performance liquid chromatography
High-performance liquid chromatography (HPLC) method was used for the determination of hispidulin in MH by comparing it with the standards of hispidulin. The stock solution of hispidulin and MH was freshly prepared at 1 mg/mL in methanol and aqueous, respectively. HPLC analysis was performed using Dionex Ultimate 3000, equipped with an auto sample, a column oven, and DAD detector. The separation was performed with Acclaim C18 (3 μm × 4.6 mm × 150 mm) using aqueous and acetonitrile as mobile phases. The flow rate was 0.6 ml/min. The column temperature was set at 35°C, and then injection volume was 1.5 and 10 μl of hispidulin and MH, respectively. The detection wavelength was determined at 280 nm.

Brine shrimp lethality assay
Brine shrimp lethality assay was a preliminary toxicity investigation. Ten shrimps per vial were added. Extract plants at 10, 100, and 1000 ug/ml were prepared on filtered paper. After 24 h, the number surviving were recorded and determined percentage LC_{50} from the graph. LC_{50} value lower than 1000 ug/ml is considered Toxic.[13]

Total phenolic content
The total phenolic content was investigated by using Folin–Ciocalteu reagent and external calibration with gallic acid. In brief, 800 μl of samples was added with 200 μl of Folin–Ciocalteu reagent. After 5 min, 1 ml of a 7.5% Na_2CO_3 solution was mixed in the dark for 60 min at room temperature. The absorbance was measured at 756 nm using a spectrophotometer. The results were averaged which expressed as micrograms of gallic acid equivalents (GAE) per 100 μg of extract sample.

DPPH scavenging activity
The 100 μl of various concentrations of MH extract dissolved in DMSO were added to 100 μL DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) radical solution 0.5 mM in ethanol. The absorbance is read at 517 nm after incubated 30 min with a microplate reader. The positive control was ascorbic acid. IC_{50} values were used for the expression of the activity.[14]

Antiaging activities in human neuroblastoma (SK-N-SH) cell lines
Cell culture
SK-N-SH cells were culture in 10% fetal bovine serum with minimum essential medium supplemented with penicillin/

streptomycin (GIBCO-BRL, Gainthersburg, MD) at 37°C in humidified 5% CO_2 incubator.

Cell viability assay

Briefly, 2×10^4 cells/mL of SK-N-SH cells were seeded in 96-well microplate, followed by treatment with H_2O_2 (0–100 μM) for 4 h, the both MH and hispidulin (0–1,000 μg/mL) for 24 h. Assessing of therapeutic effect of both MH and hispidulin on H_2O_2-induced aging, cells were treated with 10 μM H_2O_2 for 4 h prior treated with 0.1, 1, and 10 μg/mL of MH or hispidulin for 24 h. Thereafter, the (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay was performed. The wavelength was measured at 570/600 nm using spectrophotometer.

Flow cytometry

To determine the number of apoptosis, 5×10^5 cells/mL cells were incubated with Muse™Annexin-V. The dead cell reaction assay kit was measured using Muse Cell Analyzer (Merck Millipore, MA, USA), according to the manufacturer's protocol.

Reactive oxygen species assay

The 20 μM of H2DCF-DA in phosphate-buffered saline (PBS) was incubated with the cell treatment (2×10^4 cells/mL) for 2 h. The fluorescence values were then measured wavelength at 485/535 nm using a Synergy HT microplate reader (Biotek, VT, USA).

Senescence-associated β-galactosidase enzyme assay

According to the manufacturer's instruction, the cells (1×10^5 cells/mL) were incubated with fixing solution for 15 min at room temperature. After that, the assay was incubated with the SA-β-Gal detection solution for 24 h. Finally, the cells were investigated under phase contrast microscopy for counting the SA-β-gal-positive cells (blue) as a percentage of the total cell number.

Acetylcholinesterase activity (AChE) assay

The AChE activity in SK-N-SH cells was assessed following to Ellman's method. First, the cell lysate was preincubated with 3 mM DTNB. Then, the acetylthiocholine iodide was mixed in the reaction and immediately read the absorbance at 405 nm for a regular interval of 2 min using a spectrophotometer (Synergy HT microplate reader, Biotek, VT, USA).

Western blot analysis

The 50 μg proteins were electrophoresed to 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis, followed by transferred to a Polyvinylidene fluoride (PVDF) membrane and blocked with 5% skim milk at room temperature for 2 h. The membranes were then incubated overnight with anti Sirt1 (1:1000) and anti synaptophysin (1:1000). Thereafter, the membranes were probed with anti rabbit IgG peroxidase conjugated secondary antibodies

(1:2000). Finally, the HRP substrate was used to incubate the membrane and then detected with an X ray film. The ImageJ®software was used for analyzing the densitometry.

Statistical analysis

All data were expressed as mean ± standard error of the mean ($N = 3$) and were considered using one-way analysis of variance. After that, *post hoc* Dunnett's test was used comparing the significance between the groups. $P < 0.05$ was analyzed as statistically significant.

RESULTS

Phytochemical screening and determination of hispidulin in *Millingtonia hortensis*

From phytochemical screening tests of the MH extract, it was found that there were five types of phytochemicals: flavonoids, terpenoids, phenolic, cardiac glycosides, and carbohydrate [Table 1]. Hispidulin showed at retention time 18.67 min (1.09%) [Figure 1a]. HPLC chromatogram of MH extract revealed several chemical compounds containing in the extract [Figure 1b].

Brine shrimp lethality assay

The % lethality of MH extract at 10, 100, and 1000 μg/ml showed 0% ± 0%, 0% ± 0%, and 36% ± 1.73%, respectively. MH extracts had LC_{50} >1000 μg/ml which was considered as nontoxic.

Determination of antioxidant activities of *Millingtonia hortensis* extract

The total phenolic content in the aqueous extract was found to be 55.5 ± 0.03 μgGAE/100 μg extract [Figure 2a]. For DPPH assay, MH extract showed IC_{50} at 0.6 mg/ml [Figure 2b] and ascorbic acid showed IC_{50} at 0.03 mg/ml.

Antiaging activities
The cytotoxicity to SK-N-SH cells

The concentration of H_2O_2 at 10 μM significantly reduced cell viability (80.6% ± 3.7%) ($P < 0.001$) which previously report for aging model by Nopparat *et al.*,[15] [Figure 3a].

Table 1: Phytochemicals screening of MH extract

Test	MH extract
Alkaloids test	
[a] Dragendorff's Test	-
[b] Mayer's Test	-
Flavonoids Test	+
Terpenoids (Salkowski Test)	+
Phenolic compounds	+
Steroids (Liebermann-Burchard Test)	-
Cardiac Glycosides (Killer-Kallani Test)	+
Saponins (Foam Test)	-
Tannins (Ferric Chloride Test)	-
Anthraquinones (Borntragerts Test)	-
Carbohydrate (Benedicts Test)	+
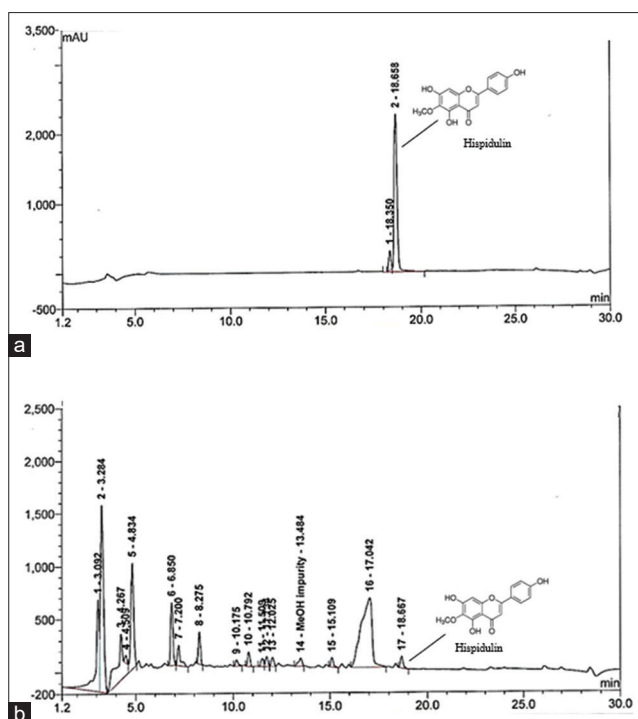
Figure 1: Chromatogram of hispidulin (a) and *Millingtonia hortensis* extract (b) (280 nm)

Figure 2: Graph showed standard curve of gallic acid equivalent. (a) DPPH activity of *Millingtonia hortensis* extract (b)

The results revealed that the MH and hispidulin at 50 μg/mL showed the cytotoxicity effect (95.0% ± 2.9% and 94.4% ± 2.8%) ($P < 0.01$) [Figure 3b and c]. According to the subsequent experiment, we used the concentration of MH at 0.1, 1, and 10 μg/mL, and hispidulin at 10 μg/mL (positive control).

Millingtonia hortensis alleviated cell apoptosis and inhibited reactive oxygen species production on H_2O_2-induced aging in SK-N-SH cells
The results demonstrated that H_2O_2 treatment significantly decreased cell viability ($P < 0.001$) [Figure 4a] and promoted apoptosis (21.7% ± 3.5%) ($P < 0.001$). On the other hand, MH significantly reversed these effects in dose-dependent manner [Figure 4a and b]. Importantly, 10 μg/mL MH has similarly affected hispidulin in the promotion of cell viability (95.4% ± 2.1%) and reduction of apoptosis (7.3% ± 2.5%).

For inhibited ROS production, MH treatment demonstrated the reduction of ROS production in dose-dependent manner comparing H_2O_2 treatment (231.8% ± 17.6%) [Figure 5]. Interestingly, the MH (133.7% ± 10.3%) has a similar effect comparing to hispidulin treatment (132.5% ± 10.6%). Taken together, the MH and hispidulin treatment alone did not show any negative effect in the neuronal cells.

Millingtonia hortensis reversed senescence on H_2O_2-induced aging in SK-N-SH cells
The result showed that H_2O_2 promoted a high number of SA-β-gal-positive cells (blue) approximately 50% [Figure 6a].
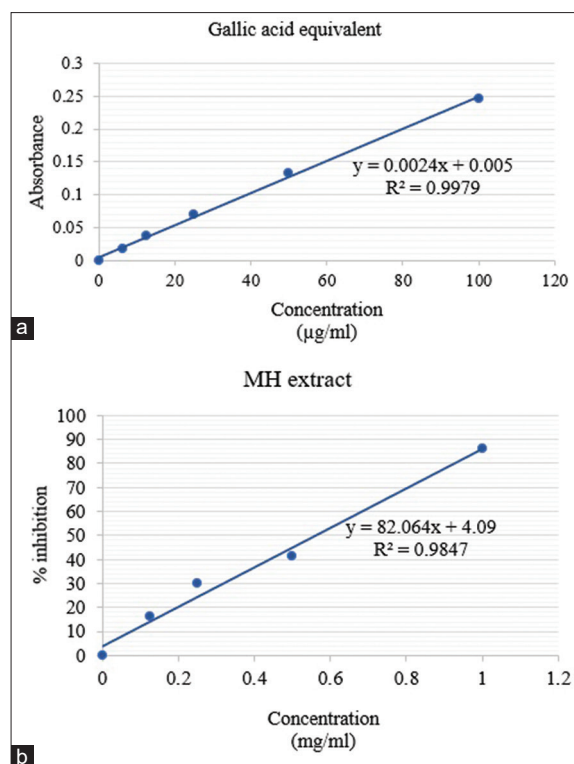
Treatment with MH significantly decreased of SA-β-gal-positive cells ($P < 0.001$). Importantly, we found that the highest concentration of MH treatment has higher potency in the inhibition of senescence (24.7% ± 7.4%) than hispidulin (35.1% ± 1.6%). Moreover, the Sirt-1 protein expression, which is the cellular senescent suppressor, was also investigated. The result showed that H_2O_2 treatment significantly decreased Sirt-1 expression (48.7% ± 7.0%) ($P < 0001$) [Figure 6b]. On the other hand, we found that both MH (86.4% ± 4.7%) and hispidulin (85.3% ± 5.1%) treatment could promote the expression of Sirt-1 in H_2O_2-induced aging in the cells.

Millingtonia hortensis promoted synaptic plasticity on H_2O_2-induced aging in SK-N-SH cells
The results indicated that H_2O_2 group significantly promoted the AChE activity (1.76 ± 0.07) ($P < 0001$) [Figure 7a] together with significantly decreased of synaptophysin expression (43.3% ± 12.1%) ($P < 0001$) [Figure 7b]. Treatment with MH significantly caused reduction of AChE activity (1.26 ± 0.21) and promotion of synaptophysin expression (82.97% ± 6.9%) ($P < 0.001$). Interestingly, at the highest dose of MH treatment (10 μg/mL) has similarly affect to hispidulin (84.8% ± 7.2%).

DISCUSSION

Brine shrimp lethality was used for investigation the preliminary cytotoxicity of MH extract. The result revealed that MH extract was nontoxic. The qualitative
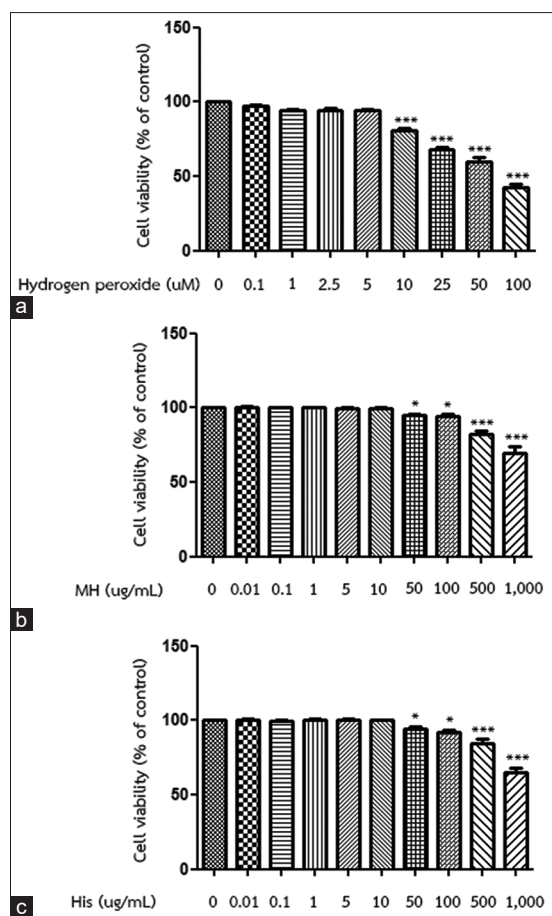
Figure 3: The cytotoxicity test in H_2O_2 treatment for 4 h (a), *Millingtonia hortensis* treatment for 24 h (b), and Hispidulin treatment for 24 h (c) were analyzed by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay in SK-N-SH cells. The values present the mean ± standard error of the mean from 3 independent experiments. **$P < 0.01$, ***$P < 0.001$, in comparison with the control treatment
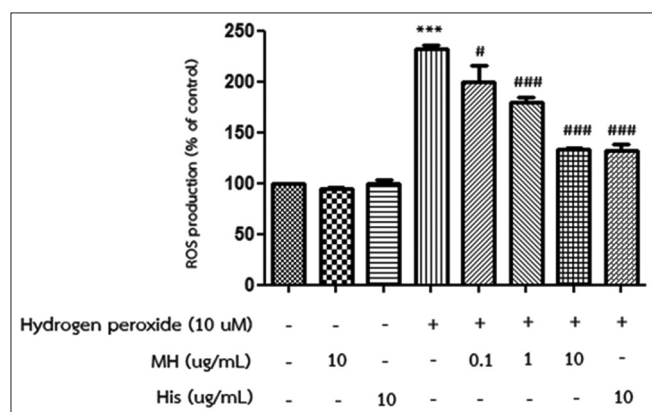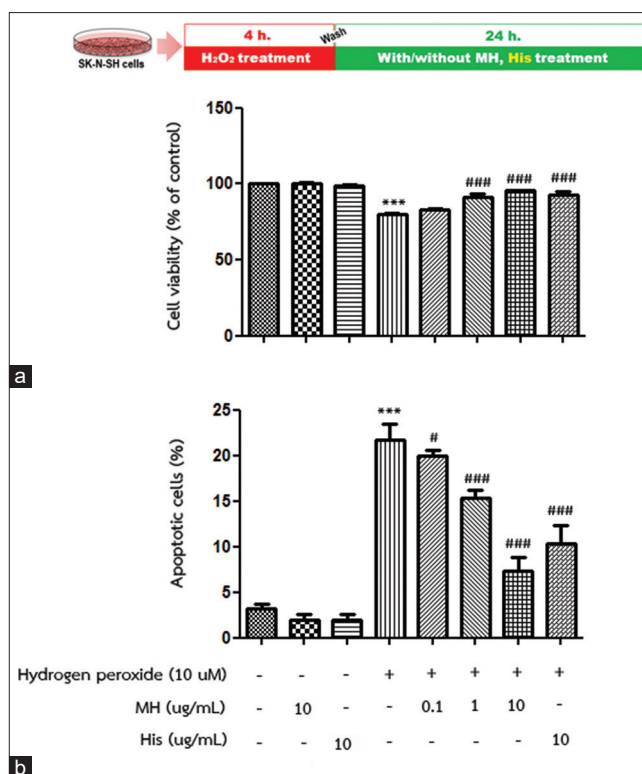

Figure 4: *Millingtonia hortensis* promoted cell survival and inhibited apoptosis in H_2O_2 treated SK-N-SH cells. The SK-N-SH cells were prior treated with H_2O_2 for 4 h and then discard the media and replaced with *Millingtonia hortensis* or His treatment for 24 h. The cell viability was analyzed by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay and the number of apoptotic cells was investigated by flow cytometry. The values present the mean ± standard error of the mean from 3 independent experiments. **$P < 0.01$, ***$P < 0.001$, in comparison with the control treatment; [#]$P < 0.05$, [##]$P < 0.01$, [###]$P < 0.001$, in comparison with the H_2O_2 treatment alone

Figure 5: *Millingtonia hortensis* scavenged intracellular free radical in SK-N-SH cell-treated with H_2O_2. The level of intracellular reactive oxygen species production was determined by reactive oxygen species assay. The values present the mean ± standard error of the mean from 3 independent experiments. **$P < 0.01$, ***$P < 0.001$, in comparison with the control treatment; [#]$P < 0.05$, [##]$P < 0.01$, [###]$P < 0.001$, in comparison with the H_2O_2 treatment alone

phytochemical analyses of extract showed the flavonoids, terpenoids, phenolic, cardiac glycosides, and carbohydrate. The obtained results are in concordance with reported previously.[16] Phenolic compounds are the key agent's incumbent and promote largely in the antioxidant activities of medicinal plants.[17] The result revealed that MH extract had high phenolic content and exhibited potential in scavenging free radical, the observed result agreed with previous studies.[18] One active ingredient of MH flower is hispidulin which is one of the polyphenols that has the ability in the scavenging of free radical-induced brain diseases.[19] Hispidulin was used as a positive control to compare the potential in antiaging with MH extract. From the results, the qualitative analysis demonstrated that MH extract was found to be less hispidulin. The results were related to the previous studies in 1986, which found hispidulin from the flowers of MH.[20]

This study showed the result of H_2O_2-induced aging in SK-N-SH cells that it significantly decreased in cell
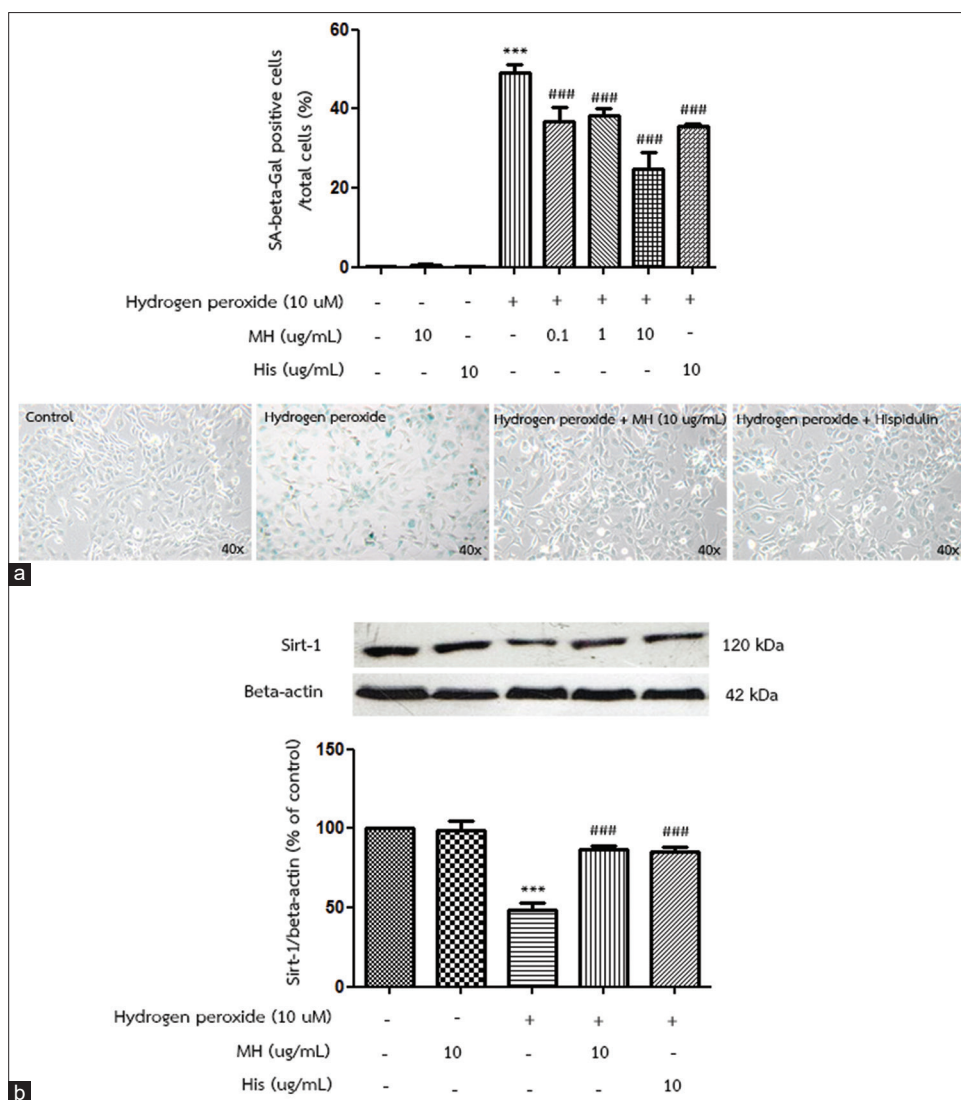
Figure 6: *Millingtonia hortensis* reversed H$_2$O$_2$-induced cellular senescence in SK-N-SH cells. The β-galatoxidase assay was used to investigate the percent of β-Galactosidase-positive cells in SK-N-SH cells (a). The western blot analysis was used for determining the expression of Sirt1 (b). The values present the mean ± standard error of the mean from 3 independent experiments. ***$P < 0.001$, in comparison with the control treatment; #$P < 0.05$, ##$P < 0.01$, ###$P < 0.001$, in comparison with the H$_2$O$_2$ treatment alone

viability at 10 μM comparing to control ($P < 0.001$) and it also affected to cellular formation (data not showed) and promoted SA-β-gal expression over than 50% of total cells. The reduction of cell survival in senescence neuron is one of the major events in aging response and it was commonly recorded with the high level of apoptosis.[21] This study found that the SK-N-SH cells exposing to H$_2$O$_2$ significantly decreased of cell survival ($P < 0.001$) that closely related to the significantly increased of apoptotic cells ($P < 0.001$). The high number of apoptosis might cause by the activation via intrinsic apoptotic pathway that correlated with the intracellular stress from ROS production.[22] From our result, we found that the decline of cell survival in SK-N-SH treated-H$_2$O$_2$ was closely associated with the overproduction of ROS content. Thus, we suggested that the ROS plays an important role in

cell survival and apoptosis response in aging neurons. Sirt-1 plays an important role in cellular division, cellular defense system, and autophagy induction. Previous study found that the high level of cellular stress from free radical could suppress both cellular defense system and Sirt-1 protein leading cell aging, dysfunction, and death.[23] From our results, we suggested that the Sirt-1 might be the target of free radical-induced aging which mostly reports in several evidences.[24,25] This study revealed that the treatment of H$_2$O$_2$ in SK-N-SH cells mediated the AchE activity ($P < 0.001$) and suppressed the synaptophysin protein expression ($P < 0.001$) comparing to control. In accordance to the results, we suggested that the loss of synaptic plasticity in neurons might be closely associated with the negative responses in aging. However, the treatment of brain disease was
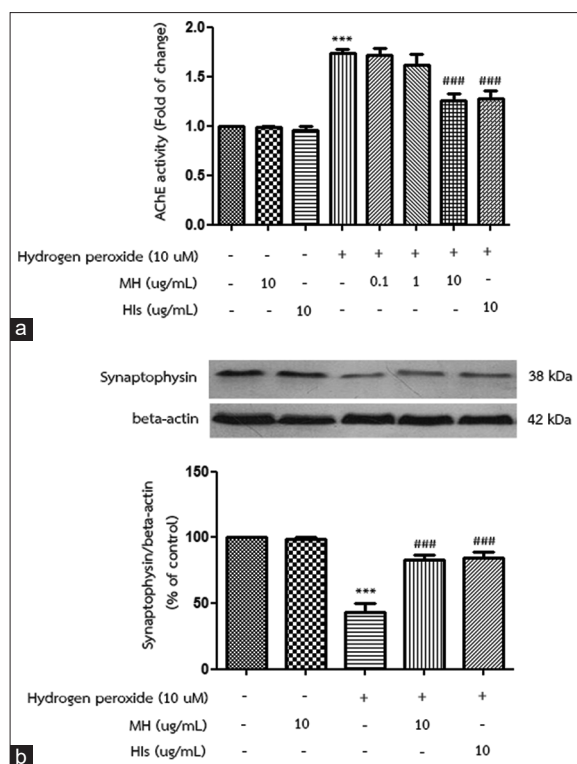
Figure 7: *Millingtonia hortensis* reversed H2O2-impaired synaptic plasticity in SK-N-SH cells. The SK-N-SH cells were prior treated with H2O2 for 4 h and then discard the media and replaced with *Millingtonia hortensis* or this treatment for 24 h. The activity of AChE in the cells was determined by AChE assay (a). The expression protein of synaptophysin (b) was determined by western blot analysis. The values present the mean ± standard error of the mean from three independent experiments. ***$P < 0.001$, in comparison with the control treatment; #$P < 0.05$, ##$P < 0.01$, ###$P < 0.001$, in comparison with the H2O2 treatment alone

highest concentration of MH has similarly effect comparing to hispidulin treatment, so the reduction of ROS by MH treatment might be by the hispidulin effect.

To confirm the indirect effect in scavenging of ROS by MH, the Sirt 1 was then evaluated. In this study we found that the both of MH and hispidulin treatment showed the significantly increased of Sirt 1 expression ($P < 0.001$) that correlated with the decline of ROS. Consequently, we suggested that the reduction of ROS in aging neurons by MH and hispidulin might be by both direct and indirect free radical scavengers. Moreover, Sirt-1 was also recorded as the cellular senescence inhibitor. Hence, we then evaluated the number of senescence cells in MH treatment whether involved with Sirt-1 expression or not. The result showed that MH treatment in SK-N-SH treated with H_2O_2 markedly decreased of SA-β-gal-positive cells ($P < 0.001$). Interestingly, the high concentration of MH has slightly better effect in the reduction of SA-β-gal-positive cells than hispidulin. We suggested that hispidulin in MH plays a pivotal role in the induction of Sirt-1, but the inhibition of senescence by MH might cause by hispidulin and other polyphenol.[28,29]

According to our present study, it was found that MH has potency in the reduction of negative responses in aging neuron; hence, we then investigated the role of MH on synapse in aging neuron. This study revealed that MH treatment in aging neuron markedly decreased AchE activity and promoted the synaptopysin expression ($P < 0.001$). We suggested that the MH could promote the synaptic plasticity in aging neurons via the reduction of negative responses and the positive effect might be by the hispidulin.

CONCLUSIONS

The therapeutic role of MH in diminishing the negative responses including cell stress response, apoptosis, cell senescence, and loss of synapse in H_2O_2 treated SK-N-SH cells was mainly by hispidulin. These present studies might be beneficial implications in the use of MH for alternative treatment in aging brain disease.

mainly focused on the promotion of synapse by using the drug-inhibited enzyme degraded neurotransmitter or drug influencing neurotransmitter, and the reduction of negative response-suppressed synapse did not recommend.[26] Therefore, the reduction of negative responses in aging neuron might be beneficial as the alternative treatment for promoting neuronal cell survival and its function.

Recently, studies found that MH treatment has high potency in the promotion of cell viability in dose-dependent manner in H_2O_2-treated SK-N-SH cells. Interestingly, the results showed that MH treatment also caused reduction of apoptotic cell number ($P < 0.001$) and the highest concentration of MH showed a slightly better effect than hispidulin. We suggested that the effect of MH on apoptosis in H_2O_2-treated SK-N-SH cells might be by hispidulin and other active ingredients such as flavonoid that has a high number of studies on anti-apoptosis in neuronal tissue.[27] We found that the MH could alleviate ROS production in SK-N-SH cell-treated with H_2O_2 in a dose-dependent manner ($P < 0.001$). Otherwise, the

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Conflicts of interest

There are no conflicts of interest.

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