

IF: 1.634

Asian Pacific Journal of Tropical Medicine

journal homepage: www.apjtm.org



doi: 10.4103/1995-7645.237185

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Attenuation of oxidative stress–induced neuronal cell death by *Hydnophytum formicarum* Jack.

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ARTICLE INFO

Article history:

Received 13 June 2017

Revision 10 May 2017

Accepted 8 June 2017

Available online 1 July 2017

Keywords:

Hydnophytum formicarum Jack.

Antioxidant enzymes

SIRT1

ADAM10

Neuroprotection

ABSTRACT

Objective: To investigate protective effects of *Hydnophytum formicarum* Jack. (*H. formicarum*) extracts *via* regulation of SIRT1-FOXO3a-ADAM10 signaling and antioxidant activity against H₂O₂-induced neurotoxicity in neuroblastoma SH-SY5Y cells. **Methods:** Cell viability and apoptosis of neuronal cells pretreated with *H. formicarum* Jack. extracts under oxidative stress were determined by MTT assay and flow cytometry. The intracellular reactive oxygen species (ROS) was performed using Carboxy-DCFDA assay. Additionally, a profile of protein expressions related to neuroprotection was detected by western blot analysis. **Results:** The plant extracts (methanol and ethyl acetate) elicited protective effects on the neuronal cell death as performed by the MTT assay and by apoptosis analysis *via* the activation of BCL-2. Both ethyl acetate and methanol extracts exerted inhibitory effects against H₂O₂-induced ROS generation in the SH-SY5Y cells. Furthermore, the possible mechanism of neuroprotection of *H. formicarum* Jack. was observed through its antioxidant properties by maintaining the levels of catalase and SOD2 proteins as well as activating SIRT1-FOXO3a pathway. Importantly, pretreatment of neuronal cells with *H. formicarum* Jack. significantly recovered the levels of ADAM10 protein compared with the H₂O₂ treatment alone. **Conclusions:** The recent findings suggest the protective effects of *H. formicarum* Jack. plant extracts on attenuating H₂O₂-induced neurotoxicity in human SH-SY5Y cells.

1. Introduction

Population aging is emerging as one of the most compelling impacts on nations worldwide. It is going to have many issues, for example, causing economic crisis and social problems. Specifically,

population aged 60 years or over is projected to increase to 1.6 billion by 2050. The occurrence of aging-related diseases including neurodegenerative disorders such as Alzheimer's disease (AD) has significantly increased[1,2]. This increasing trend in AD has become a public health concern that is a critical need to explore

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How to cite this article: Gay NH, Phopin K, Suwanjang W, Ruankham W, Wongchitrat P, Prachayasittikul S *et al.* Attenuation of oxidative stress-induced neuronal cell death by *Hydnophytum formicarum* Jack. Asian Pac J Trop Med 2018; 11(7):415-422.

new therapeutic agents to combat its progress. Aging is one of the biological processes associated with increased oxidative stress generating free radicals in the cells and tissues[3-5]. The exceptionally high metabolic activity in the brain is a key factor underlying brain aging leading to the vulnerability of the neuron to reactive species attack and ultimately neuronal cell loss[6,7].

AD is the most common aging-related progressive neurodegenerative diseases with impairment of memory and cognitive abilities, which are accompanied by neuronal cell loss in the forebrain. It is characterized by extracellular amyloid plaques and intracellular neurofibrillary tangles as neuropathological hallmarks[8,9]. The major risk factors of AD are aging and genetic mutations occurring in amyloid precursor protein (APP) including presenilin 1 and presenilin 2. Mutations in APP promote higher generation of toxic amyloid β (A β) by β -secretase (BACE1) and γ -secretase, whereas in the normal state APP is processed by α -secretase (ADAM10) that does not form the toxic A β . In addition, a number of neuron-damaging events include oxidative stress, imbalance between free radicals, and mitochondrial impairment constituted neurodegenerative conditions. Moreover, a selective regional susceptibility for neurodegeneration and oxidative damages in nervous tissue has been reported[4]. For example, AD brains and transgenic animal models of the disease have shown the increase of lipid peroxidation, protein and nucleic acid modifications as well as A β production, which are the markers of oxidative damages[7,10]. Subsequently, reactive oxygen species (ROS) mediated decline in cholinergic functions of neurons was found in basal forebrain[11]. Additionally, the mechanism of ROS can affect sirtuin expression and activity. Sirtuins consist of a family of nicotinamide adenine dinucleotide (NAD⁺) dependent enzymes, which are implicated as longevity and in the control of cell survival. In fact, SIRT1 deacetylation of the transcription factors FOXO in oxidative stress response is essentially associated with cell survival and controlling cellular ROS levels[12]. Taken together, oxidative stress impacts on a group of target genes involved in ROS detoxification, inflammatory responses, and apoptosis. Therefore, the regulation of intrinsic cellular antioxidant defenses is drawn considerable attention as therapeutic strategies for preventing the burden of oxidative stress or delaying the progression of neurodegenerative conditions.

Plants are considered as chemical sources that provide energy to human beings, and have been used since the ancient time for treatments of different diseases. Herbs, spices, and plant extracts are generally rich in bioactive compounds including phenolic compounds. The hydroxyl groups of phenols/polyphenols have been reported to scavenge free radicals and terminate redox reactions that cause cellular damage[13]. *Hydnophytum formicarum* Jack. (*H. formicarum*), a plant of the Rubiaceae family, is commonly

found in the East and South of Thailand and South-East Asia. It has been used as a traditional medicine to cure diabetes, as an anti-inflammatory remedy, and as a neurotonic[14,15]. The promising bioactive compounds including isoliquiritigenin, protocatechualdehyde, butin, and butein were isolated from the crude ethyl acetate extract of the plant species[14]. In addition, the plant extracts and isolated compounds were shown to exert a potent anti-proliferative, cardiovascular, anti-inflammatory, antiparasitic and antioxidative activities[16-18]. Recently, it has been shown that ethyl acetate extracts from the rhizome of *H. formicarum* Jack. exerts a potent antioxidative activity[19]. Additionally, *H. formicarum* Jack. from different growing areas was reported to possess comparable radical scavenging activity[20]. Although the number of studies on plant-derived anti-neurodegenerative effects is increasing, the precise mechanism of the compounds involved in neurodegeneration and aging remains to be elucidated. Herein, we examined the effects of the ant plant on the protection of neuroblastoma SH-SY5Y cells against oxidative stress *via* regulation of SIRT1-FOXO3a-ADAM10 signaling and antioxidant activity.

2. Materials and methods

2.1. Reagents and chemicals

Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), and 1% penicillin/streptomycin were received from Gibco BRL (Gaithersburg, MD, USA). MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) and carboxy-DCFDA assay kit were purchased from Invitrogen (Paisley PA4 9RF, UK). 7-Amino-actinomycin D (7-AAD) and annexin-V reagents for apoptosis assay were obtained from Merck (Billerica, MA, USA). For Western blot analysis, the following antibodies were purchased from Cell Signalling (Beverly, MA, USA): primary antibodies (anti-ADAM10, anti-FOXO3a, anti-SIRT1, anti-SOD2, anti-BCL-2, anti-catalase, and anti- β -actin), and secondary antibodies (horseradish peroxidase-conjugated anti-mouse IgG antibody and anti-rabbit IgG antibody). Human dopaminergic SH-SY5Y cell line was obtained from American Type Culture Collection (VA, USA). Western blotting and enhanced chemiluminescence (ECL) reagents for Western blot assay were purchased from Amersham Biosciences (Piscataway, NJ, USA). All other reagents were analytical grade and obtained from Sigma-Aldrich.

2.2. Plant extracts

H. formicarum Jack. methanol (MeOH) and ethyl acetate (EtOAc)

extracts were prepared as previously described[14].

2.3. Cell culture and treatments of *H. formicarum* plant extracts

SH-SY5Y cells were seeded in 75 cm² flask containing DMEM, 1% penicillin-streptomycin, and 10% heat inactivated FBS. The cells were incubated in humidified atmosphere at 37 °C and 5% CO₂. Every 3 days, the medium was refreshed and grown to 80% confluence. MeOH and EtOAc extracts of *H. formicarum* were dissolved in DMSO and diluted with DMEM containing 10% FBS for indicated concentration. After 24 h of seeding, the cells were treated with 1 µg/mL of MeOH and EtOAc extracts of *H. formicarum* for 3 h before exposure to 400 µM H₂O₂ for 24 h. Untreated cells were used as a control.

2.4. Cell viability assay

MTT assay based on the conversion of a blue formazan product by dehydrogenase enzymes found in metabolically active cells was performed to determine cell viability. Thus, the absorbance of formazan is directly proportional to the viable cells. SH-SY5Y cells (1.0 × 10⁵ cells/mL) were seeded in 96 well plates. MeOH and EtOAc extracts of *H. formicarum* were added to various final concentrations (0.1, 1, 5, 10, and 100 µg/mL), and the cells were incubated for 3 h prior to 24 h incubation with 400 µM H₂O₂. MTT solution (5 mg/mL) was loaded into each well and incubated at 37 °C for 3 h in the dark. The culture medium was discarded, and then the formazan crystals were dissolved by adding the extraction buffer (0.04 nmol/L in isopropanol). The absorbance was detected at 570 nm on a microplate reader (Bio Tek Instruments, Inc, Winooski, VT, USA). Cell viability was calculated as a percentage relative to the untreated cells.

2.5. Apoptosis analysis by flow cytometry

To characterize the apoptotic cell ratios, cells were stained with annexin V (annexin V-fluorescein isothiocyanate) using annexin V and dead cell assay kit. Annexin V was used to detect membrane phosphatidylserine of the apoptotic cells. On the other hand, the cells were stained with 7-AAD, a specific death cell marker. Briefly, SH-SY5Y cells (1.0 × 10⁵ cells/mL) were cultured in 6 well plates for 24 h. Following the incubation, the cells were exposed to 1 µg/mL of MeOH and EtOAc extracts of *H. formicarum* for 3 h prior to the incubation with 400 µM H₂O₂ for 24 h. Both floating and adherent cells were harvested and centrifuged at 1 000 rpm for 5 min. The fluorescent solution was mixed with 100 µL of cell suspension

for 20 min staining in the dark condition. The percentages of live, apoptotic, and dead cells were analysed by the Muse Cell Analyzer (Merck, Billerica, MA, USA).

2.6. Carboxy-DCFDA assay

The ROS-sensitive non-fluorescent probe (carboxy-DCFDA) was used for intracellular ROS production. In the presence of ROS, this reagent is converted to highly green fluorescent dichlorofluorescein (DCF). The cells (1.0 × 10⁵ cells/mL) were cultured in 96 well plates for 24 h, and incubated with 1 µg/mL of MeOH and EtOAc extracts of *H. formicarum* for 3 h prior to 24 h incubation with 400 µM H₂O₂. Afterward, the culture medium was discarded and washed with phosphate-buffered saline. A 10 µL of 25 µM carboxy-DCFDA was loaded into each well at 37 °C for 30 min in the dark. The fluorescence was immediately measured by fluorescence plate reader at an excitation and emission wavelength of 485 nm and 528 nm, respectively.

2.7. Protein detection by Western blot assay

SH-SY5Y cells (1.0 × 10⁵ cells/mL) were seeded in 6 well plates at 37 °C for 24 h. Then, the cells were pretreated with 1 µg/mL of MeOH and EtOAc extracts of *H. formicarum* for 3 h prior to 24 h incubation with 400 µM H₂O₂. Next, RIPA lysis buffer containing protease inhibitors was used to break the cells. The suspended cells were sonicated for 10 seconds and centrifuged at 10 000 *g* for 20 min at 4 °C. Protein concentration was measured using the Bradford protein assay (Bio-Rad Laboratories, Hercules, CA, USA). The protein lysates were resolved by 10% SDS-PAGE and blotted on polyvinylidene difluoride membrane. Then, the membrane was blocked by 5% skim milk in 1× Tris-buffered containing Tween-20 (TBST) at room temperature for 1 h, and rinsed with TBST. Subsequently, the membrane was probed with specific primary antibodies at 4 °C overnight followed by 1.3 h incubation with HRP-conjugated secondary antibody. Finally, the blotted membrane was developed with ECL before being captured chemiluminescent signals by ChemiDoc™ MP imager. The protein levels were quantitated using densitometry analysis with Image Lab software (Bio-Rad, Hercules, CA).

2.8. Statistical analysis

All data were revealed as mean ± S.E.M of the three independent experiments. Statistical analysis was assessed by a One-Way Analysis of Variance (ANOVA) with Tukey's test using GraphPad Prism 6 scientific software (Graph Pad Software, Inc., La Jolla, CA 92037 USA). Probability (*P*) values < 0.05 were determined as a

statistical significance.

3. Results

3.1. Effects of MeOH and EtOAc extracts of *H. formicarum* Jack. on cell viability induced by H₂O₂ in SH-SY5Y cells

To assess the effects of plant extracts (MeOH and EtOAc) of *H. formicarum* Jack. on SH-SY5Y cells viability, the viability of SH-SY5Y cells treated with the extracts was determined at various concentrations (0.1, 1, 5, 10, and 100 µg/mL) using the MTT assay. Percentage of cell viability from untreated sample was represented as 100%. Following 24 h incubation, the cells exposed to MeOH and EtOAc extracts did not elicit any significant cytotoxic effects at 0.1, 1, 5, and 10 µg/mL [(MeOH extract: (98.5±2.9)%, (101.0±1.6)%, (100.0±4.5)%, (106.6±2.0)%; EtOAc extract: (99.0±2.9)%, (102.0±4.4)%, (100.0±3.0)%, (106.0±2.8)%, respectively], whereas the cytotoxic effect was evidenced upon treatment of the cells with 100 µg/mL [(MeOH extract: (48.9±1.5)% and EtOAc extract: (49.0±2.5)%] (Figure 1A). From the results, 1 µg/mL of MeOH and EtOAc extracts was selected for the subsequent assays.

Neuroprotective effects of the plant extracts on neuronal cells induced by 400 µM H₂O₂ were investigated. Upon 24 h incubation with 400 µM H₂O₂, the cell viability was reduced to (65.3±0.3)% compared with the untreated control cells. This effect was reversed by pretreatment of the cells for 3 h with 1 µg/mL of MeOH and EtOAc extracts of *H. formicarum* Jack. [(96.8±0.8)% and (96.9±3.1)%, respectively). However, no significant difference was observed in cell viability between groups treated with MeOH and EtOAc extracts as indicated in Figure 1B. Furthermore, morphological changes were noted including cell shrinkage, rounding, and cell loss after the treatment with H₂O₂. Pretreatment with MeOH and EtOAc plant extracts did not reveal any cell alterations compared with the control cells, which showed extended neurites [21] and adequate confluence (Figure 1). These results indicated that the *H. formicarum* Jack. extracts exerted protective effects on the neuronal cells death.

3.2. Effects of MeOH and EtOAc extracts of *H. formicarum* Jack. on H₂O₂-induced intracellular ROS production in SH-SY5Y cells

To explore the antioxidative potential of plant extracts in H₂O₂-insulted oxidative stress model, the intracellular contents of ROS were measured using the ROS-sensitive fluorescence dye, DCFDA. The results showed that 24 h incubation of neuronal cells with

400 µM H₂O₂ caused the increased level of ROS accumulation [(129.8±1.0)%]. Interestingly, pretreatment of MeOH and EtOAc extracts of *H. formicarum* Jack. significantly reduced ROS levels to (104.7±6.4)% and (98.8±1.3)%, respectively. Moreover, the EtOAc extract showed better effects on reducing ROS accumulation than that of the MeOH extract (Figure 2).

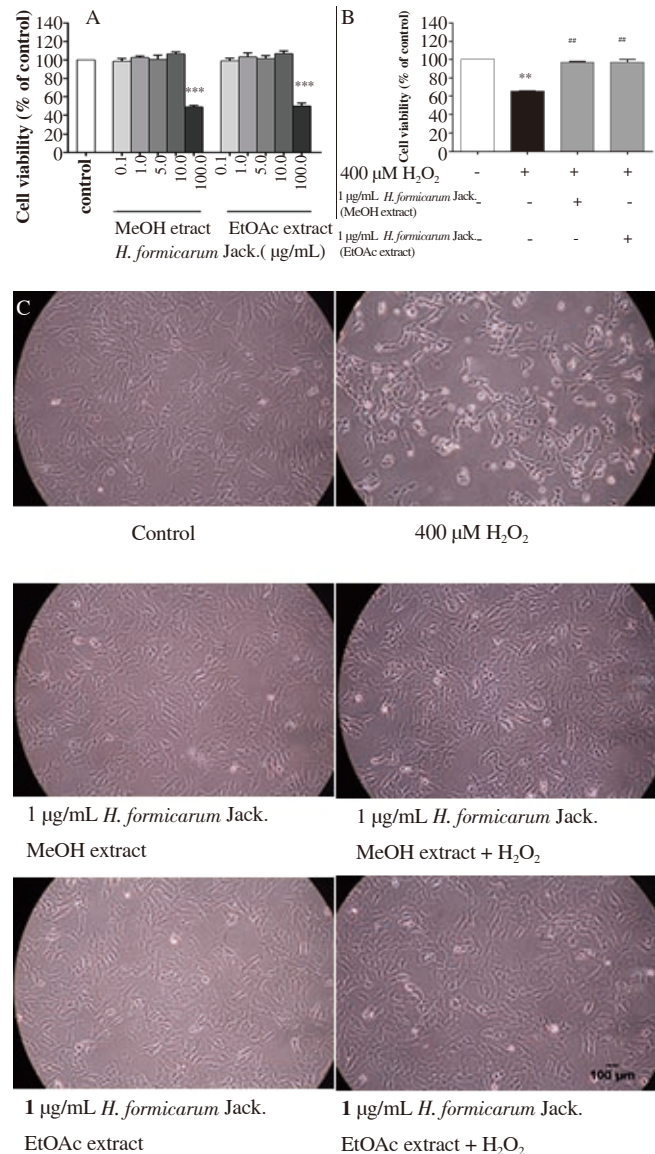


Figure 1. Effects of MeOH and EtOAc extracts of *H. formicarum* Jack. on cell viability induced by H₂O₂.

A. Effects of MeOH and EtOAc extracts of *H. formicarum* Jack. (0.1, 1, 5, 10, and 100 µg/mL) on SH-SY5Y cell viability for 24 h exposure; B. Effects of pretreatment of SH-SY5Y cells with MeOH and EtOAc extracts of *H. formicarum* Jack. on cell viability induced by H₂O₂. C. Light microscope observation in SH-SY5Y cell treated with MeOH and EtOAc extracts of *H. formicarum* Jack. in the presence or absence of H₂O₂ at 20 × magnification. The data are shown as mean±S.E.M. of three independent experiments. Statistical analysis was performed using ANOVA and Tukey–Kramer Post Hoc test, ** $P < 0.01$ vs. control, ### $P < 0.01$ vs. 400 µM H₂O₂-treated group).

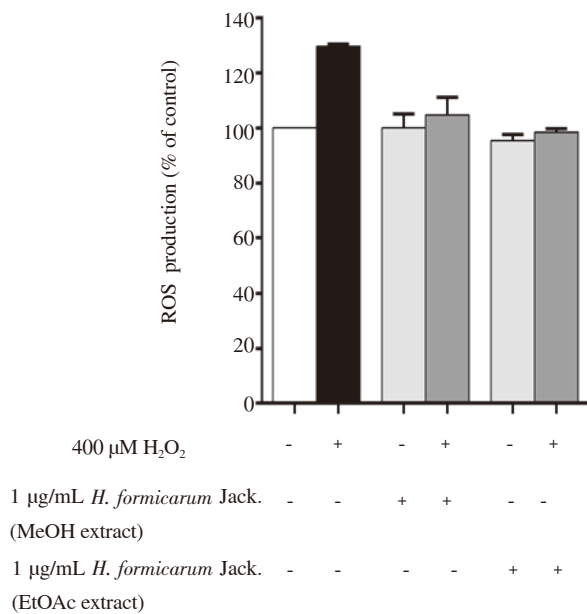


Figure 2. ROS production of SH-SY5Y cells treated with MeOH and EtOAc extracts of *H. formicarum* Jack. with and without H_2O_2 .

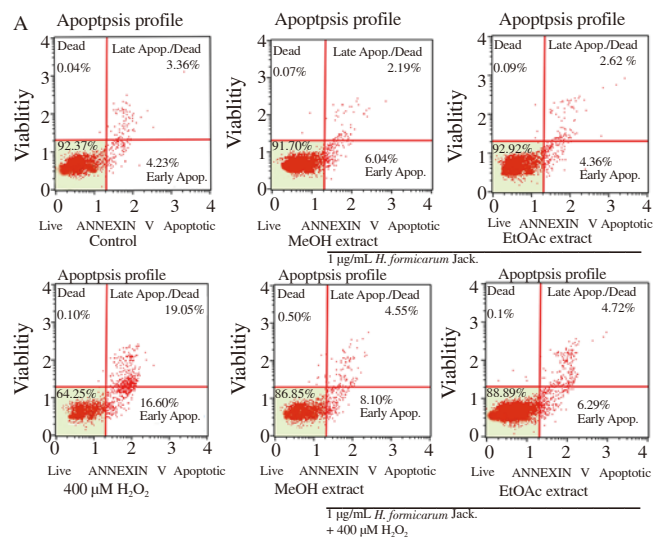
H. formicarum Jack. significantly reduced ROS levels induced by H_2O_2 . Data are shown as mean \pm S.E.M. of three independent experiments, ** $P < 0.01$ vs. control; ## $P < 0.01$ vs. 400 μM H_2O_2 -treated group.

3.3. Effects of MeOH and EtOAc extracts of *H. formicarum* Jack. against H_2O_2 -induced apoptosis in SH-SY5Y cells

The antiapoptotic potential of MeOH and EtOAc plant extracts against H_2O_2 -induced apoptosis in SH-SY5Y cells was tested using flow cytometry. As shown in Figure 3, the apoptosis was raised to (33.1 \pm 3.3)% of cells treated with 400 μM H_2O_2 for 24 h. Conversely, pretreatment of the cells with MeOH and EtOAc extracts of *H. formicarum* Jack. declined the percentages of cell apoptosis to (12.9 \pm 0.5)% and (11.0 \pm 0.6)%, respectively, compared with 400 μM H_2O_2 treatment alone. In particular, EtOAc extract-exposed cells revealed a slightly higher inhibitory effect on the apoptosis compared with the MeOH extract. Thus, the EtOAc extract was selected for further experiments. These findings were consistent with morphological features of cells prior exposure to H_2O_2 , and also supported the restoration of cell viability noted in the MTT assay.

3.4. Levels of antiapoptotic, antioxidant and FOXO3a proteins in SH-SY5Y cells treated with EtOAc extract of *H. formicarum* Jack.

Levels of H_2O_2 on BCL-2, SOD2, catalase, and FOXO3a proteins in SH-SY5Y cells were investigated. BCL-2 expression has been shown to enhance cell survival by inhibiting apoptosis under diverse conditions in a variety of cell types including neurons[22,23]; hence, the levels of BCL-2 were determined in this work. Moreover, FOXO3a is thought to participate in maintaining low levels of cellular ROS through the stimulation of mitochondrial enzymes including SOD2 and catalase[24]. Thus, protection of neuronal oxidative stress is achieved by the increase of these antioxidant enzymes.



B

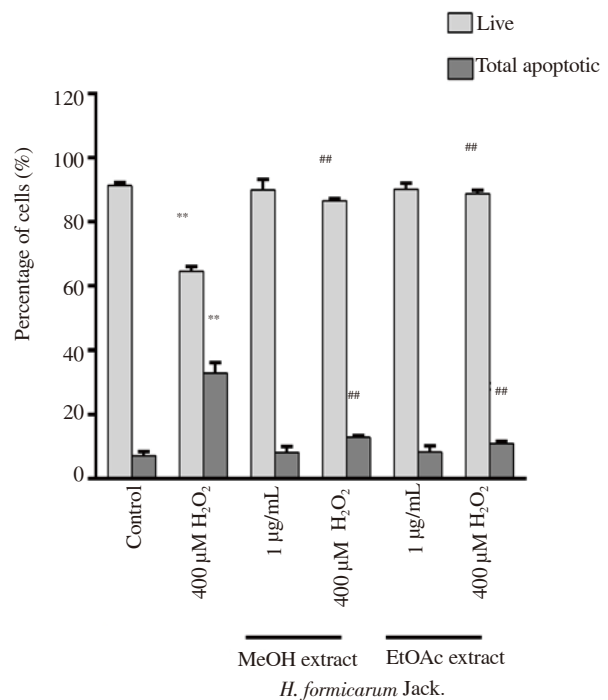


Figure 3. Flow cytometric analysis of live and apoptotic cells pretreated with MeOH and EtOAc extracts of *H. formicarum* Jack. followed by 400 μM H_2O_2 for 24 h.

A. Histograms of live and apoptotic cells stained with annexin V ; B. Bar diagram of live and total apoptotic cells. The data are shown as mean \pm S.E.M. of three independent experiments, ** $P < 0.001$ vs. control; ## $P < 0.01$ vs. 400 μM H_2O_2 -treated group.

The cells were incubated with 400 μM H_2O_2 for 24 h in the presence or absence of 1 $\mu\text{g/mL}$ *H. formicarum* Jack. (EtOAc extract). As expected, H_2O_2 downregulated BCL-2, SOD2, catalase, and FOXO3a proteins (56%-65%) in comparison with the control value. The effects were significantly reversed by pretreating the cells with the plant extracts resulting in the increases of BCL-2, SOD2, catalase, and FOXO3a proteins to (101.4 \pm 6.5)%, (100.2 \pm 4.0)%, (107.9 \pm 4.9)%, and (102.5 \pm 4.0)%, respectively (Figure 4). These findings suggested that the reduction of BCL-2, SOD2, catalase,

and FOXO3a levels during H₂O₂ treatment were recovered by the treatment of *H. formicarum* Jack. (EtOAc extract).

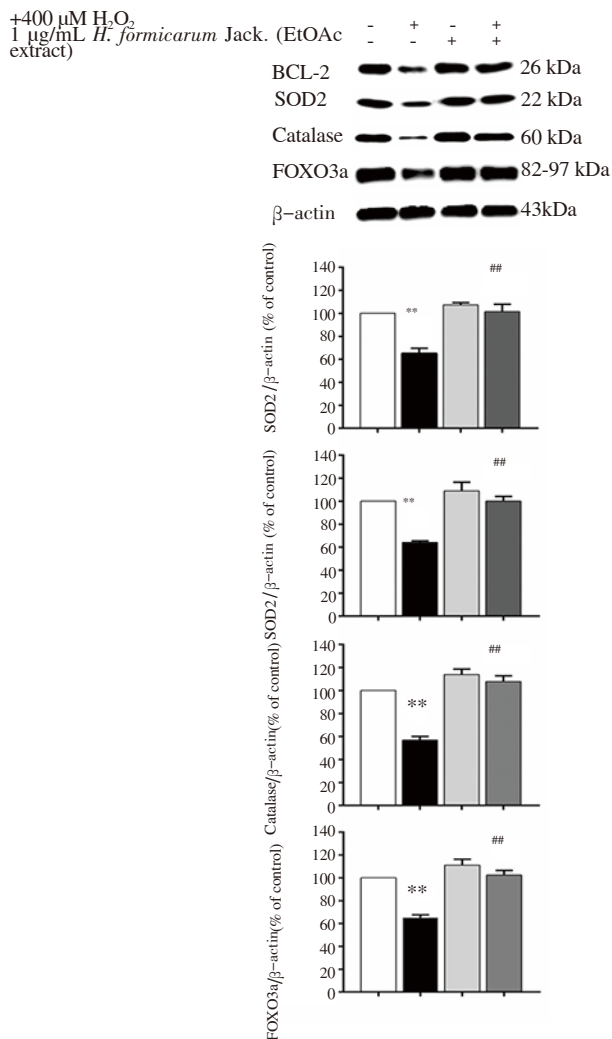


Figure 4. BCL-2, SOD2, catalase, and FOXO3a protein levels of cells pretreated with EtOAc extract of *H. formicarum* Jack. prior exposure to 400 μ M H₂O₂.

BCL-2, SOD2, catalase, and FOXO3a protein expressions in 400 μ M H₂O₂-induced cells were recovered by the pretreatment of EtOAc plant extract determined by Western blot analysis. Densitometric analysis of protein bands was presented in the graph as the ratio to β -actin. The results are expressed as mean \pm S.E.M. of three independent experiments, ** P <0.01 vs. control and ## P <0.01 vs. H₂O₂-treated group.

3.5. Effects of EtOAc extract of *H. formicarum* Jack. on SIRT1 and ADAM10 proteins in SH-SY5Y cells

One of the causes of AD is associated with increased generation of toxic A β peptide by favoring proteolytic processing of APP by BACE1 and γ -secretase. On the other hand, ADAM10, a putative α -secretase belonging to the ADAM family converts APP into nontoxic sAPP, which protects neuronal cell death under oxidative stress condition[25]. Additionally, SIRT1 typically acts as longevity factors associated with neuroprotection. Thus, SIRT1 and ADAM10 proteins were determined in this study to explore their roles in protecting human neuronal cells against oxidative stress. Obviously, H₂O₂ induced the reduction of SIRT1 and ADAM10 levels to (64.2 \pm 5.6)% and

(65.7 \pm 1.0)%, respectively. Dramatically, the pretreatment with 1 μ g/mL of EtOAc extract of *H. formicarum* Jack. recovered the protein levels of SIRT1 and ADAM10 to (112.5 \pm 5.6)% and (104.7 \pm 4.1)%, respectively compared with H₂O₂ exposure alone (Figure 5).

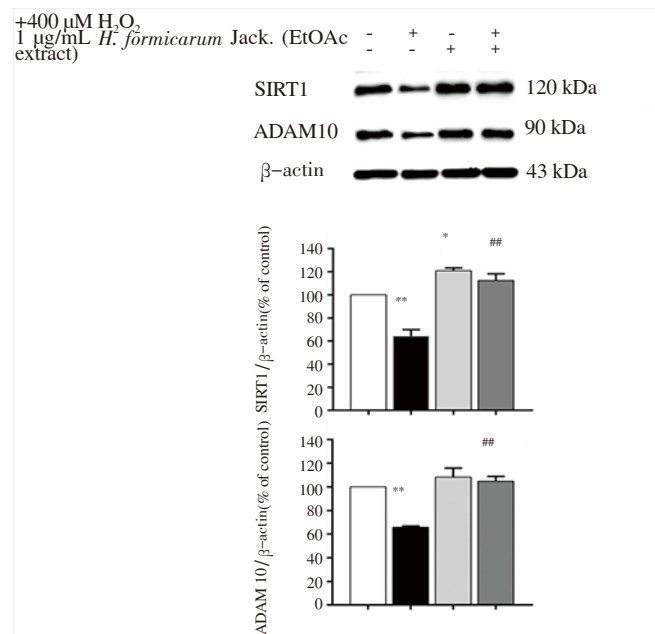


Figure 5. SIRT1 and ADAM10 protein levels of cells pretreated with EtOAc extract of *H. formicarum* Jack. prior exposure to 400 μ M H₂O₂.

SIRT1 and ADAM10 protein expressions in 400 μ M H₂O₂-induced cells were recovered by the pretreatment of EtOAc plant extract determined by Western blot analysis. Densitometric analysis of protein bands were presented in the graph as the ratio to β -actin. The results are expressed as mean \pm S.E.M. of three independent experiments, * P <0.05 and ** P <0.01 vs. control and ## P <0.01 vs. 400 μ M H₂O₂-treated group.

4. Discussion

Oxidative stress has been extensively involved in various chronic diseases including neurodegenerative diseases such as AD. The oxidative stress is one of the important risk factors causing AD with the appearance of senile plaques and neurofibrillary tangles in the brain. Different mechanisms are known for generation of ROS triggering the activation of signaling pathways and oxidative damages[7]. Consistently, the excess of OS reflects the disturbances between antioxidant defense system and prooxidant inducing ROS generation, cell apoptosis, neuronal damage, and ultimately resulting in neuronal pathology[26]. Despite numerous efforts in the development of drugs that target the specific markers of AD, presently, there is still no cure for the disease. Moreover, herbs with antioxidative and anti-amyloidogenic activities have been moved to the development as preventive and therapeutic drugs[27-29]. The search for the most potent novel plant compounds with neuroprotective benefits is continually explored.

Herbal drugs are going to replace the conventional treatments

of various types of cancer[17] and other diseases including neurodegenerative diseases[30]. In the current work, the cytotoxic effects of H₂O₂-treated neuronal cells and the protective roles of *H. formicarum* Jack. were revealed by *in vitro* models.

The oxidative products of H₂O₂ can stimulate reactive oxygen species to activate the release of Bax from mitochondria allowing cytochrome-c release into the cytosol, which activates downstream caspases and cellular apoptosis[31]. *H. formicarum* Jack. extracts have been found to have radical scavenging activity, so they may contain neuroprotective activities. As shown in our data, H₂O₂ significantly induced the cell death, compared with the untreated cells, and this H₂O₂-induced neurotoxicity was attenuated by 1 µg/mL of *H. formicarum* Jack., MeOH and EtOAc plant extracts. Moreover, cell death with morphological changes caused by H₂O₂ were observed. In this study, the upregulated levels of ROS involved in SH-SY5Y cell apoptosis were also investigated. The characterization of apoptotic profiles was carried out using annexin V assay. The flow cytometry revealed that the apoptotic cells were significantly upregulated following the treatment of SH-SY5Y cells with H₂O₂. BCL-2 is a major regulator of the anti-apoptotic BCL-2 family that plays a crucial role in modulating cell apoptosis mediated by mitochondria. Additionally, the activation of caspase-3 has been reduced by BCL-2, and over expression of BCL-2 can protect neuronal cell death caused by toxic substances[32]. Thus, the anti-apoptotic BCL-2 protein was assessed and the result exhibited that BCL-2 was downregulated in H₂O₂-induced SH-SY5Y cells. These deleterious effects on the neuronal cells can be recovered by 1 µg/mL of *H. formicarum* Jack. (EtOAc extract). The findings indicated that cellular apoptosis with increased ROS levels and decreased BCL-2 could be induced by H₂O₂. However, these effects were suppressed by exposure of the cells with *H. formicarum* Jack., EtOAc extract.

Recent studies have shown that SIRT1 increased catalase and SOD2 levels by promoting its antioxidant properties *via* deacetylation of FOXO4 and attenuating ROS production in astrocytes[33]. Additionally, expression of SIRT1 in neurons is related to neuroprotection[34,35]. The deacetylation ability of SIRT1 is important for controlling several transcription factors including FOXO3a. FOXO transcription factors activate a cascade of target genes associated with the cellular responses to stress stimuli comprising genes that control ROS detoxification and cell death[36]. Consistently, the present study has demonstrated that *H. formicarum* Jack. extracts play a crucial role in neuroprotection by maintaining antioxidant status through the modulation of antioxidant enzymes (SOD2 and catalase) involved in controlling cellular ROS levels. Subsequently, SIRT1-FOXO3a axis was also activated to counteract the excess of ROS by upregulating SIRT1 and FOXO3a proteins. This may critically control neuronal cell survival in H₂O₂-induced toxicity in the cells. Several studies have reported that the disintegrin and metalloprotease 10 (ADAM10) is an enzyme generating amino-terminal APP cleavage product (sAPP), which is considered as an important mechanism in preventing the generation of Aβ[37]. Another

study has revealed that ADAM10 and the corresponding sAPP are decreased in cerebrospinal fluid of AD patients[38]. Importantly, a stimulatory effect of *H. formicarum* Jack. on ADAM10 protein levels was investigated by *in vitro* models of the current study. This ADAM10 activation could be linked to the reduced toxicity of Aβ proteins in the neuronal cells under oxidative damage. Additionally, it could be hypothesized that the effects of *H. formicarum* Jack. ameliorated the induction of oxidative stress in SH-SY5Y cells.

Taken together, the upregulation of a SIRT1-FOXO3a-ADAM10 signaling pathway by the treatment of *H. formicarum* Jack. investigated herein might be involved in protecting neuronal cell death, and this plant could be a promising herb for preventing neurodegeneration caused by oxidative stress.

Conflicts of interest statement

The authors declare that there are no conflicts of interest.

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