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Antioxidant and acetylcholinesterase inhibitory activities *in vitro* of different fraction of *Huperzia squarrosa* (Forst.) Trevis extract and attenuation of scopolamine-induced cognitive impairment in mice

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ABSTRACT

Ethnopharmacological relevance: *Huperzia squarrosa* (Forst.) Trevis is used in traditional medicine for improving memory deficits. Alkaloids, triterpenoids, flavonoids are main bioactive compounds of *Huperzia squarrosa* (Forst.) Trevis.

Aim of the study: This study aimed to investigate the antioxidant, AChE inhibitory activities *in vitro* of differents fraction of *Huperzia squarrosa* (Forst.) Trevis extract and neuroprotective effects of EtOAc fraction on scopolamine-induced cognitive impairment in mice.

Materials and methods: Antioxidant activity was measured by DPPH assay. AChE inhibitory effect *in vitro* and detail kinetic inhibition mechanism was evaluated by Ellman's assay. For *in vivo* assay, mice were administrated orally EtOAc fraction (150 and 300 mg/kg) for fourteen days, and injected scopolamine at a dose of 1 mg/kg intraperitoneally for four days to induce memory injured. The memory behaviors were evaluated using the Morris water maze. ACh levels were measured in brain tissue. Superoxide dismutase (SOD), glutathione peroxidase (GPx) activities, malondialdehyde and protein thiol groups were also evaluated in the brains.

Results: Our data also demonstrated that EtOAc fraction had the strongest antioxidant with an IC_{50} value of 9.35 ± 1.68 μg/mL and AChE inhibitory activity with an IC_{50} value of 23.44 ± 3.14 μg/mL in a concentration-dependent manner. Kinetic inhibition analysis indicated that EtOAc fraction was mixed inhibition type with Ki (representing the affinity of the enzyme and inhibitor) was 34.75 ± 1.42 μg/mL. Scopolamine significantly increased the escape latency time, reduced the crossings number, and swimming time in the target quadrant, while EtOAc fraction reversed these scopolamine-induced effects. EtOAc fraction significantly increased levels of acetylcholine in the brain. EtOAc fraction also significantly decreased oxidative stress in mice.

Conclusion: Our data suggest that EtOAc fraction of *Huperzia squarrosa* extract exhibited a strong neuroprotective effect on cognitive impairment, and may be a potential candidate for the treatment of Alzheimer.

1. Introduction

Alzheimer's disease (AD) is a neurodegenerative disorder related to age, which leads to loss memory and cognitive function. The patient suffered the AD is increasing every day (Blennow et al., 2006). Cholinergic hypothesis is one of the most important hypotheses which have been used to explain the pathogenesis of the Alzheimer. This hypothesis explained that the deficit of the neurotransmitter acetylcholine (ACh) in the brain leads to AD. Many drugs for AD treatment are based on the cholinergic hypothesis (Francis et al., 1999). Many researchers showed a low level of neurotransmitters in cholinergic system is responsible for the cognitive decline and memory loss in Alzheimer patients (Pappas et al., 2000). Acetylcholinesterase enzyme (AChE) plays an important role in the central and peripheral nervous systems. AChE degrades the neurotransmitter ACh and subsequently reduces the Ach level in the brain. The AChE inhibitor can increase ACh level in cholinergic synapses which have been shown the alleviation of the disease. For that, many study conduct to find new potential AChE inhibitors which may be served as a drug for the treatment of AD (Upadhyaya et al., 2010). Drugs such as galantamine, tacrine, donepezil, metrifonate or rivastigmine are inhibitors of AChE, enhance the ACh level, and then improve cholinergic transmission. These drugs

Abbreviations: ACh, acetylcholine; AChE, acetylcholinesterase; AD, Alzheimer's disease; SCP, Scopolamine; ROS, reactive oxygen species; MDA, malondialdehyde; SH, Thiol; SOD, superoxide dismutase; GPx, glutathione peroxidase; CAT, catalase DPPH: 1,1-diphenyl –2-picrylhydrazyl; DTNB, 5,5´-dithio-bis-(2-nitro) benzoic acid;; ATCI, Acetylthiocholine iodide

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have been used to alleviate the symptoms of Alzheimer which caused by degeneration of cholinergic neurons and injured transmission. But these drugs have several adverse reactions, then are limited to use for treatment of AD (Williams et al., 2011).

Oxidative stress is characterized by the imbalance in the production of reactive oxygen species (ROS) and antioxidative defense system which are responsible for the removal of ROS. Oxidative stress leads to the age-related neurodegeneration and cognitive decline and is also a man caused to the development of Alzheimer (Harman, 1981). It has observed a high amount of protein oxidation, lipid oxidation, DNA oxidation and glycoxidation in Alzheimer patient (Lovell and Markesbery, 2007). The previous studies have shown the brain in AD patients demonstrated the lipid peroxidation amount increased and, activities of the antioxidant enzymes superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (CAT) decreased (Marcus et al., 1998). Protein thiol (SH) groups play important role in the metabolism as antioxidant protectors and in detoxification reactions. The free protein thiol groups are essential for the function of many enzymes, such as lactate dehydrogenases and other enzymes in the respiratory chain (Medina-Navarro et al., 2010). In the antioxidant enzyme system, the superoxide dismutase (SOD) is the first line of defense against oxidative stress, it detoxifies $O_2^{\bullet -}$ to O^2 and H_2O_2 (Fukai and Ushio-Fukai, 2011), which H_2O_2 is subsequently transformed into H_2O by catalase (Fukai and Ushio-Fukai, 2011). Glutathione peroxidase (GPx) is an enzyme which reduces both H_2O_2 and organic peroxides. They play important role in pathogenesis of AD. Decreasing lipid peroxidation and increasing antioxidant enzyme may inhibit disease-promoting of AD. Recently, antioxidants treatment is a promising strategy for inhibiting AD progression (Grundman and Delaney, 2002). Some studies showed the treatment with antioxidants such as vitamin E, vitamin C could inhibit the development of AD (Staehelin, 2005).

Many medicinal plants have been used to treat cognitive impairment and memory loss. *Huperzia squarrosa* (Forst.) Trevis have been used in traditional medicine to enhancing memory and alleviate brain disorders (Yumkham and Singh, 2011, 2013). This plant is also used to treat limb numbness, pyrexia, joint pains, adetumescence, and injuries from falls (Ma et al., 2006). *Huperzia squarrosa* also contains high amount of huperzine A, which is potent acetylcholinesterase (AChE) inhibitor (Sahidan et al., 2012). Some alkaloids have been isolated from *Huperzia squarrosa* (Forst.) Trevis such as lycosquarosine A, acetylaposerratinine, huperzine A, huperzine B, 8α-hydrophlemariurine B, huperzinine, squarrosine A, and pyrrolhuperzine A (Chuong et al., 2014; Nilsu et al., 2016). These alkaloids have been showed strong AChE inhibitory activity *in vitro* (Chuong et al., 2014; Nilsu et al., 2016). However, there are no reports about the effect of *Huperzia squarrosa* plants on Alzheimer model *in vivo*. In this study, we aimed to investigate the antioxidant, AChE inhibitory activities *in vitro* of different fractions of *Huperzia squarrosa* extract. Then, we evaluated the effect of EtOAc on scopolamine-induced cognitive deficits in mice by Morris water maze test and measured lipid peroxidation (MDA), protein thiol group (SH), the antioxidant enzyme such as SOD and GPx in mice brain tissue.

2. Material and methods

2.1. Reagents

1,1-diphenyl-2-picrylhydrazyl (DPPH, Sigma-Aldrich, Singapore), 5,5′-dithio-bis-(2-nitro) benzoic acid (DTNB) (Himedia, India), Scopolamine hydrobromide (Sigma, Singapore), Acetylthiocholine iodide (ATCI) (Sigma, Singapore), Acetylcholinesterase (Sigma, Singapore), Berberine chloride (Himedia, India). Others solvents *n*-hexane, ethyl acetate (EtOAc), *n*-butanol, ethanol (EtOH) were analytical grade (Shouguang, China).

2.2. Plant material

The aerial part of *Huperzia squarrosa (Forst.) Trevis* were collected in Ha Giang, Vietnam during 2015 and authenticated by Department of Pharmacognosy and Traditional Pharmacy, School of Medicine and Pharmacy, Vietnam National University, Hanoi, Vietnam (SMP-VNU). A voucher specimen (No. SMP-2015-0012) has been deposited in the SMP-VNU. Dried samples (3 kg) were extracted with 96% ethanol (10 L) by ultrasonic at 40 °C for three hours for three times. The extracts were filtered, combined and evaporated under low pressure to afford the EtOH extract (547.3 g). The EtOH extract was suspended in water and successively partitioned with *n*-hexane and ethyl acetate, *n*-butanol (3×1 L, each solvent). Combined solvent was then evaporated under low pressure to obtain the *n*-hexane fraction (89.2 g), and ethyl acetate fraction (171.6 g) and butanol (186.4 g).

2.3. Scavenging effect on 1,1-diphenyl-2-picryl hydrazyl radical (DPPH)

The radical scavenging ability was determined as described previously (Kedare and Singh, 2011). Briefly, 150 μL of 3.3 mM alcohol solution of DPPH was added to 100 μL from the samples with different concentrations of fractions of *Huperzia squarrosa* (Forst.) Trevis extract with 2.9 mL methanol. The samples were kept at room temperature in the dark and after 30 min the absorbance was measured at 517 nm. The antioxidant capacity was compared with the reference standard, ascorbic acid. The antioxidant activity (AOA) was determined by the following formula:

$$\%\mathrm{AOA} = \frac{\mathrm{Ac} - \mathrm{At}}{\mathrm{Ac} - \mathrm{Ao}} x100$$

where: AOA is antioxidant activity

A_c: Absorbance of Control (150 μL of 3.3 mM DPPH +3 mL methanol)
A_t: Absorbance of sample (100 μL sample+150 μL of 3.3 mM DPPH +2.9 mL methanol)
A_o: Absorbance of blank (using methanol as blank)

Value IC_{50} was calculated using the graph of log (dose) *vs*. % AOA.

2.4. AChE inhibitory assay

AChE inhibitory activity of fractions of *Huperzia squarrosa* (Forst.) Trevis was evaluated using Ellman's assay with minor modifications (Ellman et al., 1961). Samples were dissolved in dimethyl sulfoxide (DMSO). Reaction mixture were 140 μL of 0.1 M sodium phosphate buffer (pH 8.0), 20 μL of samples and 20 μL of AChE 0.25 IU/mL. Incubated the mixture for 15 min at 25 °C. Added 10 μL of 5-5′-dithiobis-2-nitrobenzoic acid (DTNB) 2.5 mM và 10 μL ATCI 2.0 mM and mixed well. Then incubate the mixture for 10 min at 25 °C. The absorbance was measured at 412 nm. Each assay was repeated three times. Berberine chloride was used as positive control.

Percentage of AChE inhibition (% I) was calculated by followed formula:

$$\%I = \frac{\mathrm{Ac} - \mathrm{At}}{\mathrm{Ac} - \mathrm{Ao}} x100$$

where: I% is the percentage of AChE inhibition

A_c: Absorbance of control (without 20 μL sample)
A_t: Absorbance of sample
A_o: Absorbance of blank (200 μL of 0.1 M sodium phosphate buffer)

Value IC_{50} was calculated using the graph of log (dose) *vs*. % I.