

Cholinesterase Inhibitory and Antioxidant Activities of Thai Traditional Remedies Potentially Used for Alzheimer's Disease

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Abstract

Background: At present, cholinesterase inhibition and neuronal protection from oxidative stress are well-known mechanisms that widely used for the treatment of Alzheimer's disease (AD) (1,2). According to Thai traditional medicine, brain hypo-function, which has similar symptoms to that of AD, was due to declining in wind-element, blood circulation and neurological system. Trikatuk is herbal remedy, strongly increases wind-element, had been used as nootropic remedy for body balance in rainy season. It's composed of 3 plants: *Zingiber officinale* rhizomes (Zo), *Piper nigrum* fruits (Pn) and *Piper retrofractum* fruits (Pr) in either ratio of 1:1:1(Tk1), 3:2:1(Tk2), 1:3:2(Tk3) or 2:1:3(Tk4).

Aims: This study focused on acetylcholinesterase (AChE) and human butyrylcholinesterase (BuChE) inhibitory activities by the method of Elman (3, 4) and antioxidant activity by DPPH method(5) of the 4 remedies and ingredients.

Methods: Sixty grams of each plants and recipes were extracted by 95 %ethanol (250 ml x 3), then evaporated under reduced pressure and dried in vacuum-dry at 45°C for 24 hour. Piperine and 6-gingerol of each extracts were also determined by HPLC.

Results: The results showed that the ethanolic extract of the 4 remedies at dose of 0.1 mg/ml had BuChE inhibitory effect more than 70%. The IC_{50} of AChE inhibitory activity of Zo, Pn, Pr, Tk1, Tk2, Tk3, Tk4, galantamine were >300, 25.46, 51.60, 45.6, 51.97, 41.19, 42.93, 0.1 µg/ml and that of BuChE were 60.82, 42.94, 34.58, 28.74, 25.39, 33.01, 33.28, 0.37 µg/ml respectively. All Trikatuk remedies exhibited higher BuChE inhibitory activity than that of AChE. The Tk1 and Tk2 exhibited higher BuChE inhibitory activity than single herb. The antioxidant activity of Zo and Tk2 were 16.59 and 48.80 µg/ml which was higher activity than the others. The percentage of piperine and 6-gingerol in Tk2 extract (w/w) were 18.57 ± 0.22 and 1.05 ± 0.01 mg/g.

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Conclusion: *The combined recipes had synergistic effect on BuChE inhibitory activity more than single herb. In addition, the Tk2 might protect neuronal cell from oxidative stress toxicity due to its antioxidant effect. These results revealed that Tk2 was the potential use for AD treatment.*

Keyword: Cholinesterase inhibitor, trikatuk remedy

Introduction

Thailand is an aged country that over 10% of population is now over 60. As predicted by Institute of Population and Social Research, Mahidol University, the population of older persons will exceed 12.9 million in 2025 and 20 million in 2050(1, 2). The incidence of Alzheimer disease (AD) is increasing every year in accordance with the increasing of elderly population and could be pose significant health problems in the future. AD is a chronic neurological disorder characterized by memory impairment, cognitive dysfunction, behavioral disturbances, and deficits in daily living. At present, acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) inhibitors are well-known drugs used in the treatment of AD (3). However, these drugs are limited in use due to their adverse effects and are effective only against mild type of AD (4, 5). In addition, oxidative stress is one of the major mechanisms causing neuron and astrocyte death in AD (6,7,8.). Dual effects of antioxidants in neurodegeneration were direct neuroprotection against oxidative stress and indirect protection via suppression of glia-mediated inflammation (9). Plants possessing profound central nervous system effects and antioxidant activity have received much attention as food supplement to improve cognitive function against cognitive deficit condition such as Alzheimer's disease.

According to Thai traditional medicine, brain hypo-function, which had similar symptoms to that of AD, was due to declining in wind-element, blood circulation and neurological system. Trikatuk was herbal remedy, strongly increases wind-element, had been traditionally used as nootropic remedies for body balance in rainy season. It composed of 3 plants: *Zingiber officinale* (Zo) Roscoe. rhizomes, *Piper nigrum* L.(Pn) fruits and *Piper retrofractum* Vahl (Pr) fruits in either ratio of 1:1:1 (Tk1), 3:2:1(Tk2), 1:3:2 (Tk3) or 2:1:3 (Tk4). The Tk 1 was used in general treatment whereas the Tk2, Tk3 or Tk4 were used to treat illness due to impairment of fire-element, wind-element or water-element respectively (10). Pn and Pr, had been reported to be AChE inhibitor at concentration of 0.1mg/ml with % inhibition of 58.02 and 65.16 µg/ml(11). Piperine, the major active principle in Pn and Pr, significantly improved memory impairment and neurodegeneration in animal model (12), beneficially influence antioxidant molecules and antioxidant enzymes (13). Moreover, piperine assessed bioavailability enhancer of others substance (14). 6-Gingerol and shogaol, two major substance in Zo, possessed high antioxidation and anti-inflammatory activities (15) whereas 6-Shogaol was found to modulate neuroinflammation (16). Crude extract of Zo showed specific inhibition of BuChE rather than AChE enzyme (17). The combinations of these plants might have synergistic effects on AD treatments.

This study focused on AChE and BuChE inhibitory activities and also, antioxidant activity of the four remedies and individual ingredients.

Materials and methods

Plant Materials

Zo rhizome was purchased from Petchaboon province, Pn fruits and Pr fruits were purchased from Chantaburi province, Thailand. The crude drugs were identified according to their characters of taxonomic importance. All voucher specimens were kept at Department of Thai Traditional Medicine, Faculty of Medicine, Thammasat University.

Chemicals

Acetylcholine iodide (ACTI), 5,5'-dithio-bis-(2-nitro-benzoic acid ,DTNB), galantamine, AChE (from electric eel, type VI-S lyophilized powder, 844 units/mg protein), butyrylcholine iodide (BuCTI), human BuChE (lyophilized powder, 122 units/mg protein), bovine serum albumin (BSA), Tris-HCL, butylated hydroxy-toluene (BHT), piperine and 6-gingerol were purchased from Sigma (Thailand). 50mM Tris-HCL, pH 8.0 was used as a buffer for all experiments. AChE / BuChE was separately dissolved in buffer to obtain 1130 U/ml stock solution, kept at -80°C and was further diluted in 0.1% BSA in buffer. DTBN and ACTI / BuCTI were dissolved in buffer and millipore water respectively.

Extraction

Crude drug was separately washed, dried in hot air incubator at 50 °C and then grinded to fine powder. Sixty grams of each

drug Zo, Pn, Pr and each remedy Zo: Pn : Pr in ratio of 1:1:1(Tk1), 3:2:1(Tk2), 1:3:2(Tk3) or 2:1:3(Tk4) was 3 times macerated in 250 ml of ethanol for 3 days and filtered. The combined filtrates were evaporated under reduced pressure (Rota evapor R-205, Germany) until nearly dry and further vacuum-dry (vacucell, Germany) to dryness.

Determination of active constituents in crude extracts

By means of HPLC using Agilent® LC 1100/1200 system, with photodiode array (PDA), detector (model G1315D), automatic injector (model G1329A), ZORBAX Eclipse XDB-C18 column (Agilent®), water and acetonitrile as mobile phase, gradient elution as 0 min: 40% B, 30 min: 50% B, 50 min: 90% B, 60 min: 100% B, elution rate of 1.0ml/min, the piperine and 6-gingerol were detected at wavelength 254 and 282 nm (18). Data were analyzed by ChemStation® software.

Microplate assay for AChE/BuChE activities

The AChE/BuChE activity was measured by following the increase of yellow color produced from thiocholine when reacts with DTNB ion (Dithiobisnitrobenzoate). The increase of spectrophotometer absorbance measured at 405 nm was reversed to the amount of enzyme inhibitor and, also, was linear for more than 2 min. The AChE/BuChE activity assay were performed according to Elman et al, 1961(19) and modified by Ingkani-nan et al, 2003 (11). Briefly, 125 µl of 3mM DTNB, 25 µl of 15 mM ATCI or BTCl, 50 µl of

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buffer and 25 µl of sample dissolved in buffer containing not more than 10% ethanol were added to the wells followed by 25 µl of 0.28 U/ml AChE or BuChE. The microplate was read at 405 nm every 5 second for 2 minutes by microplate reader (BioTex model Power Wave XS). The velocities of the reaction were automatically measured. Enzyme activity was calculated as a percentage of velocities compared to that of the assay using buffer without any inhibitor. The inhibitory activity was calculated from 100 subtracted by the percentage of enzyme activity.

Microplate assay for DPPH radical scavenging activity

DPPH radical scavenging assay (20) appears as a deep violet color and shows a strong absorption band at 520 nm. Briefly, pipette sample solution in each concentration 100 µl in 96-well plate. Add DPPH solution 100 µl in each sample and mixed (Final conc. of

sample 100, 50, 10, 1 µg/ml). The absorbance (A) was measured at 520 nm. Calculated by formula %inhibition = [(A control-A sample)/A control] X100 and EC₅₀ value calculated by linear regression analysis. BHT was used as positive control.

Results

Table 1 showed the percentage yield of each extract, the amount of active principles, piperine and/or 6-gingerol in each extract. Table 2 showed that the ethanolic extract of the four remedies at dose of 0.1 mg/ml had BuChE inhibitory effect were nearly 70%. The IC₅₀ of AChE and BuChE inhibitory activity of the all extracts were shown. The IC₅₀ values of BuChE were lower than that of AChE. The Tk1 and Tk2 exhibited the lower IC₅₀ of BuChE inhibitory activity than single herb. The IC₅₀ of DPPH activity of Zo and Tk2 were 16.59 and 48.80 µg/ml which were lower than the other extracts.

Table 1 showed the percentage yield and the amount of piperine or/and 6-gingerol in each extract (% w/w of extract).

extract	% yield	The amount of active principles (%w/w of extract) mg/g.	
		Piperine	6-Gingerol
Zo	5.754	-	1.59 ± 0.06
Pn	6.667	40.38 ± 0.13	-
Pr	15.045	21.15 ± 0.25	-
Tk1 (Zo:Pn:Pr 1:1:1)	9.613	20.07 ± 0.03	0.46 ± 0.00
Tk2 (Zo:Pn:Pr 3:2:1)	8.088	18.57 ± 0.22	1.05 ± 0.01
Tk3 (Zo:Pn:Pr 1:3:2)	9.374	20.94 ± 0.17	0.30 ± 0.01
Tk4 (Zo:Pn:Pr 2:1:3)	10.796	19.23 ± 0.42	0.42 ± 0.01

Table 2 showed activities of each extract as % inhibition of AChE and BuChE at dose of 0.1 mg/ml (means (sd)), the IC₅₀ of AChE, BuChE and IC₅₀ of DPPH (µg/ml).

Extract code	% inhibition of AChE	% inhibition of BuChE	IC ₅₀ of AChE (µg/ml)	IC ₅₀ of BuChE (µg/ml)	IC ₅₀ of DPPH (µg/ml)
Zo	22.63 (2.0)	40.42 (2.0)	> 300	60.82	16.59
Pn	57.98 (2.2)	50.04 (2.9)	25.46	42.94	>100
Pr	45.68 (3.4)	60.48 (2.0)	51.60	34.58	>100
Tk1	49.86 (2.2)	67.58 (2.2)	45.6	28.74	79.31
Tk2	48.84 (1.8)	71.84 (1.9)	51.97	25.39	48.80
Tk3	51.38 (1.3)	63.15 (2.5)	41.19	33.02	>100
Tk4	50.41 (3.1)	70.35 (3.4)	42.93	33.28	87.18
positive control	93.04 (0.9) *	72.50 (0.9)*	0.1 *	0.37 *	11.52 **

* galantamine as positive control, ** BHT as positive control

Discussion

This study focused on anticholinesterase and antioxidation effects of trikatuk, Thai traditional remedies, and their ingredients *in vitro* level. Acetylcholinesterase inhibitors have been a proper therapeutic approach to alleviate the cognitive symptoms of AD (21). Within particular condition such as moderate or advanced stages of AD, BuChE may replace AChE in hydrolyzing brain acetylcholine. Therefore, selective BuChE inhibitor or mixed double function inhibitor is the treatment of advanced cases (22). This study resulted that the combined recipes exhibited more BuChE inhibitory activity than individual herbs. The most AChE inhibitory activity was found in *Piper nigrum* extract as this plant had been claimed for the AChE inhibitory

activity (11). Piperine, the main active principle in *Piper nigrum* and *Piper retrofractum* and also in trikatuk extracts, had been revealed to protect against neurodegeneration and cognitive impairment in animal model of cognitive deficit like condition of AD(12). Trikatuk recipes were the mixtures of *Piper nigrum* and *Piper retrofractum* in four difference proportions, so the recipes beneficially acted as the enzymes inhibitors, especially Tk2 which showed the specific anti-BuChE effect.

It is known that oxidative stress is one of the major mechanisms causing neuron and astrocyte death in AD. Therefore, any antioxidants with anti-inflammatory action may be more beneficial in the prevention of this neurodegenerative disease (9). *Zingiber officinale*

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was another ingredient in trikatuk remedies. 6-gingerol and 6-shogaol, two main active principles in Zo, were clearly known as potent anti-oxidants which had been proved to protect neuroinflammation (15, 16). This study showed that the extract of Zo and Trikatuk remedy in the ratio of Zo: Pn : Pr 3:2:1 (Tk2) had the antioxidation activity with IC_{50} of DPPH activity of 16.59 and 48.80 $\mu\text{g/ml}$. This antioxidation activity were related to the 6-Gingerol content. The Tk2 might protect neuronal cell from oxidative stress toxicity due to its antioxidant effect of 6-gingerol. In addition, the Zo extract also showed specific inhibition of BuCh rather than ACh enzyme. This specific anti-BuChE effect was also due to 6-Gingerol content (17).

According to basic principle of Thai traditional pharmacy, components in drug compounding were divided into four categories as main ingredient, auxillary drug, controlling drug and coloring or flavoring agents (23). Usage of drug remedy should be more reasonable than single drug for the ingredients may have synergistic effects or reduce any toxic effect. In this study, the percentage of piperine and 6-gingerol in trikatuk remedy (Tk2) extract (w/w) were 18.57 and 1.05 mg/g. Besides piperine and 6-gingerol, in trikatuk remedies, there were other substances such as volatile

oils, oleoresins and theirs derivatives which also had synergic effects (24). Moreover, the trikatuk remedies were considered to be efficacious and safe. Subacute toxicity studies of water extracts of the three formulae of Trikatuk showed no differences in pathological between the extract-treated groups and the control groups (25). Based on this study, the use of trikatuk remedy extract in the ratio of Zo:Pn:Pr 3:2:1 was reasonable and potential for using as co-treatment for prevention or treatment of AD.

Conclusion

The combined recipes, trikatuk remedy in the ratio of Zo:Pn:Pr 3:2:1 (Tk2) showed specific anti-BuChE effect more than single herb. This recipe also had antioxidant activity than others and might protect neuronal cell from oxidative stress toxicity due to its antioxidant effect of 6-gingerol. These results suggested the potential use of trikatuk remedy, Tk2, as prevention or additional treatment of AD.

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