Cholinesterase Inhibitory Activities of Apai-sa-le Recipe and Its Ingredients

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Background: *Acetylcholinesterase and butyrylcholoinesterase inhibitors are well-known drugs commonly used in the treatment of Alzheimer's disease (AD) to improve cognitive function. These enzyme inhibitors were reported to be found in many plants. Apai-sa-le recipe was a Thai tradition used as nootropic recipe and formerly claimed to improve memory. Therefore, it is interesting to investigate cholinesterase inhibitory activity of the recipe and its ingredients.*
Objective: *To determine the whole recipe of Apai-sa-le and its ingredients for inhibitory effect on acetylcholinesterase (AChE) and human butyrylcholinesterase (BuChE) activities.*
Material and Method: *Thirty grams of each plant and 181 grams of the whole recipe were separately extracted by 95% ethanol, after filtered the filtrate were evaporated and vacuum-dried at 45°C. By Elman method, the inhibitory activities of both enzymes were assessed. The volatile constituents of each extract were determined by GCMS. The constituents in the non-volatile extract were examined by TLC and the antioxidant activity was determined.*
Results: *Four plants exhibited specific BuChE inhibitor were Lepidium sativum Linn. (Ls), Piper nigrum L. (Pn), Angelica dahurica Benth (Ad) and Atractylodes lancea DC. (Al), which shown the IC_{50} of 5.59, 24.52, 73.23, 96.25 μg/ml, respectively whereas galantamine and the whole recipe showed IC_{50} of 0.59 and 236 μg/ml. Only Pn extract inhibited AChE at IC_{50} of 25.46 μg/ml. By GCMS and TLC fingerprints revealed the main constituents in LS, Ad, Al and Pn as apiol, cumialdehyde, furanodiene and piperine. Moreover, nine plant extracts and the whole recipe showed antioxidant activity.*
Conclusion: *Lepidium sativum Linn. (Ls) extract showed the most potency on BuChE inhibitory effect. Three ingredients and the whole recipe exhibited mild activity. Only Piper nigrum L demonstrated inhibition effect on both AChE and BuChE.*

Keywords: *Cholinesterase inhibitor, Apai-sa-le recipe*

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Apai-sa-le is one of traditional recipes used for longevity or rejuvenating and claimed to improve blood circulation and memory[1]. It is believed that balancing of blood circulation can lead to improving memory. This recipe composes of 18 kinds of plants in difference weight ratio and each plant has its own functions. Three major ingredients in Apai-sa-le are *Micromelum minutum* (G.Forst.) Wight & Arn., *Piper nigrum* L., *Myristica fragrans* Houtt. while the other ingredients are *Lepidium sativum* L., *Cuminum cyminum* L., *Foeniculum valgare* L., *Anethum graveolens* L., *Atractylodes lancea* DC., *Angelica dahurica* Benth., *Acorus calamus* L., *Syzygium aromaticum* Merr. et Perry, *Amomum krervanh* Pierre., aril part and seed of *Myristica fragrans* Houtt., *Terminalia chebula* Retz., *Terminalia* sp. or Samothed, *Plumbago indica* L. In previous report it was found that *Syzygium aromaticum* can inhibit malonaldehyde formation[2] and inhibit platelet aggregation[3] while *Myristica fragrans* had hypnotic effect[1], anxiolytic effect[4] and improved cognitive function in animal model[5]. Especially, the previous studies revealed that *Piper nigrum and Plumbago indica*[6], *Foeniculum valgare* L.[7] and *Anethum graveolens* L.[8] showed cholinesterase inhibitory activity. Moreover, *Acorus calamus* L. showed memory enhancer in animal model[9].

According to Thai traditional medical concepts, the Apai-sa-le recipe enhances blood circulation through the wind element that might respond to improving brain function[10].

Acetylcholine is recognized as the important neurotransmitter involve for cognitive function. Marked reduction of acetylcholine level in hippocampus and oxidative stress are accepted to be the causes of

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Alzheimer disease (AD)[11]. At present, cholinesterase inhibition and neuronal protection from oxidative stress are well-known mechanisms that widely used for the treatment of AD[11,12]. However, these drugs are limited in use due to their adverse effects and are effective only against mild type of AD[13,14]. Therefore, it is interesting to investigate the acetyl cholinesterase (AChE) and butyrylcholinesterase (BuChE) inhibitory activities, and antioxidation activity of Apai-sa-le and its ingredients.

Material and Method

Plant materials

All crude drugs were purchased from herbal drug stores in Bangkok, Thailand and were authenticated by Associate Professor Arunporn Itharat and Assistance Professor Pimolvan Senavong, Department of Applied Thai Traditional Medicine, Faculty of Medicine, Thammasat University, where all the voucher specimens were deposited.

Chemicals

Acetylcholine Iodide (ACTI), 5,5'-dithiobis-(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 hydroxytoluene (BHT), piperine were purchased from Sigma (Germany). 50 mM Tris-HCL, pH 8.0 was used as a buffer for all experiments. AChE/BuChE was separately dissolved in buffer to obtain 1,130 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 drugs were separately washed, dried in hot air oven at 50°C and then ground to fine powder. Thirty grams of each drug and 181 grams of the whole recipe in proper proportion cited in Thai pharmacy scripture were macerated in 250 ml of ethanol for 3 days and filtered. After filtrating, the marc was repeatedly macerated two times. The combined filtrates were evaporated under reduced pressure (Rota evapor R-205, Germany) until nearly dry and further vacuum-dry (vacucell, Germany) to dryness.

Standardization of the extract

Each crude extract was calculated for percentage yield, and a moisture analyzer (SCALTEC model SMO 01, Germany) analyzed the moisture content. Volatile constituents in some extracts and the whole recipe were determined by gas chromatography-mass spectroscopy, GCMS (THERMO model Focus GC, Polaris Q, Italy). Thin layer chromatography was performed to demonstrate the fingerprints of the non-volatile extracts and the whole recipe.

Gas chromatographic-mass spectroscopic analysis

Each extract (5 mg) was dissolved in 2 ml of hexane: dichloromethane 1:1, sonicated for 15 min and filtered through 0.45 micron membrane. Sample of 0.5 µl was injected at T 60°C. GC conditions were set as following: -initial T 60°C, hold time 5 min; ramp 1- rate 5 ml/min, T 180°C; ramp 2-rate 10 ml/min, T 280°C; flow rate 1 ml/min (helium); split mode. MS conditions were set as following: -mass transfer line 275°C, ion source 200°C.

Thin layer chromatographic analysis

Silica gel GF_{254} was used as stationary phase and mobile phase was chloroform: methanol 95:5. Thirty micrograms of each extract was separately loaded on TLC plate. The chromatograms were displayed by using 10% sulphuric acid in ethanolas a general detector and 10% of diphenyl-1-picrylhydrazyl (DPPH) in ethanol was spray to detect antioxidant activity.

Microplate assay for AChE/ BuChE activities

The AChE/BuChE activity was measured by following the increase of yellow color produced from thiocholine when it reacts with DTNB ion (Dithiobisnitrobenzoate). The increase of spectrophotometer absorbance measured at 405 nm was reversed to the amount of enzyme inhibitor and was linear for more than 2 min. The AChE/BuChE activity assay were performed according to Elman et al, 1961[15] and modified by Ingkaninan et al, 2003[6]. Briefly, 125 µl of 3 mM DTNB, 25 µl of 15 mM ATCI or BTCI, 50 µl of 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. Three independent experiments were performed with every extracts. All the data were

expressed as means (SD).

Results

The percentage yields of the extracts were shown in Table 1 and the moisture content of all extracts were less than 10%. The percentage of inhibition of Apai-sa-le recipe and it ingredients at the concentration of 0.1 mg/ml on AChE and BuChE were showed in Table 1. Plant extracts that exhibited percentage inhibition more than 40 were further measured for IC_{50}. The GCMS fingerprints showed the main constituents in the volatile extracts and the whole recipe (Fig. 1,2). *Lepidium sativum* extract that showed the highest activity on BhChE inhibition composed of cumialdehyde 21.9%, apiol 20.2%, oleic acid ethyl ester 10%, beta-caryophyllene 6.2% isoanethol 5.6% and eugenol 2.8%. The Apai-sa-le extract composed of volatile substances as beta-caryophyllene 23.3%, eugenol 16.8%, 9-Octadecenoic acid ethyl ester 12%, cis-asarone 7.7%, apiol 4.8%, and estragole 3.3% (Fig. 2). The TLC chromatograms of non-volatile plant extracts were shown in Fig. 3.

Discussion

Only *Piper nigrum* L., one of the main ingredients in Apai-sa-le recipe, inhibited activities of AChE and BuChE at IC_{50} of 25.64 and 24.5 µg/ml, respectively. It is known that piperine, the major active principle in *Piper nigrum* L., beneficially influence antioxidant molecules and antioxidant enzymes[16], which significantly improved memory impairment and neurodegeneration in animal model[17].

Lepidium sativum L., *Atractylodes lancea* DC., *Angelica dahurica* Benth. *and Cuminum cyminum* L., showed specific inhibition only of BuChE activity. According to GC-MS, the main constituents of the extracts from these four plants were cumialdehyde, estragole, beta-caryophyllene, allomadendrene, furanodiene, apiol, isoanethol and thymoquinone, which were volatile terpenes. Some of these volatile substances were already known to have AChE activity[18] as well as those found in the Apai-sa-le extract. Only *Lepidium sativum* L. exhibited potent BuChE inhibitory activity at the least IC_{50} of 5.59 µg/ml. This anti-BuChE effect may benefit the treatment of

Table 1. Cholinesterases inhibitory activities of Apai-sa-le recipe and its ingredients. (n = 3, means (SD))

Plant name	Wt. in recipe (g)	% yield	% inhibition AChE	IC_{50} (µg/ml)	% inhibition BuChE	IC_{50} (µg/ml)
Acorus calamus L.	7	3.35	28.27 (3.8)	-	30.77 (3.3)	-
Amomum krervanh Pierre.	3	2.34	27.69 (2.5)	-	41.25 (1.0)	-
Amorphophallus paeoniifolius Nicolson.	15	5.03	19.04 (2.0)	-	8.81 (1.2)	-
Anethum graveolens L.	9	1.85	29.61 (1.8)	-	18.94 (1.2)	-
Angelica dahurica Benth.	8	1.34	25.66 (1.3)	-	56.64 (0.1)	73.23 (3.5)
Ardisia polycephala Wall.	6	8.95	24.24 (1.7)	-	5.74 (0.5)	-
Atractylodes lancea DC.	9	4.87	22.38 (2.0)	-	48.24 (0.5)	96.25 (2.4)
Cuminum cyminum L.	8	2.55	27.64 (1.2)	-	41.35 (0.7)	128.3 (5.3)
Foeniculum valgare L.	10	2.67	22.94 (0.6)	-	18.86 (1.2)	-
Lepidium sativum L.	11	2.38	32.63 (2.9)	-	91.45 (0.6)	5.59 (0.6)
Micromelum minutum (G.Forst.) Wight & Arn.	24	7.02	28.50 (1.6)	-	10.05 (1.1)	-
Myristica fragrans Houtt (woody part)	16	4.10	23.19 (1.4)	-	24.14 (0.9)	-
Myristica fragrans Houtt (aril part)	2	24.08	23.62 (2.4)	-	33.66 (1.1)	-
Myristica fragrans Houtt (seed)	1	24.38	20.33 (1.9)	-	32.0 (0.2)	-
Piper nigrum L.	16	8.66	58.96 (1.9)	25.46	62.14 (1.7)	24.5 (1.5)
Plumbago indica L.	12	5.62	29.94 (0.2)	-	38.28 (0.9)	-
Syzygiumaromaticum Merr. *et* Perry	4	17.13	36.01 (1.8)	>500	36.65 (0.8)	-
Terminalia chebula Retz.	13	13.73	11.41 (1.3)	-	23.14 (0.9)	-
Terminalia sp.	13	15.4	12.72 (1.4)	-	21.23 (1.2)	-
Api-sa-le recipe	181	4.41	33.16 (3.0)	359.25	40.15 (3.6)	236.33
Galantamine			93.68 (1.0)	0.3 (0.01)	76.50 (0.1)	0.59 (0.02)

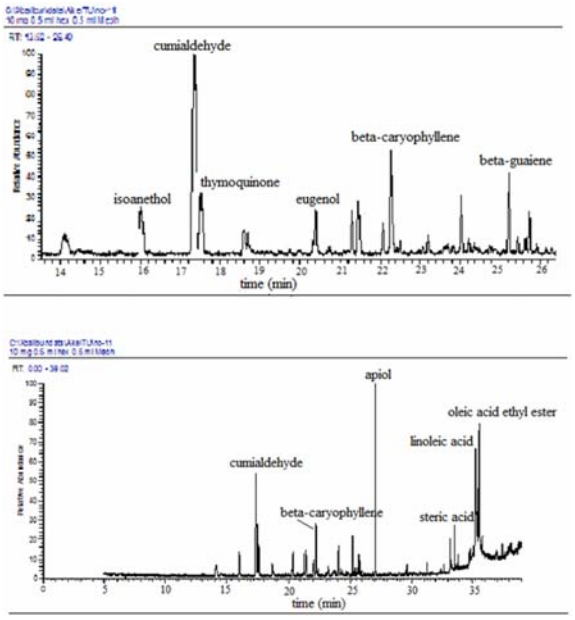

Fig. 1 GCMS fingerprints of *Lepidium sativum* L. extract revealed the main constituents.

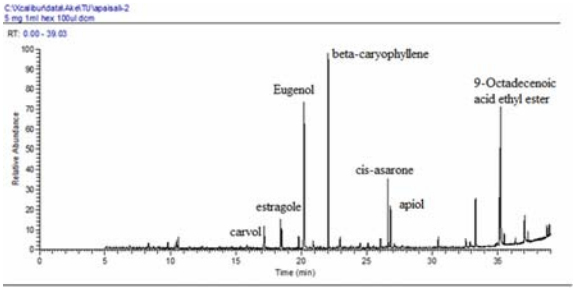

Fig. 2 GCMS fingerprint of Apai-sa-le extract revealed the main volatile constituents.

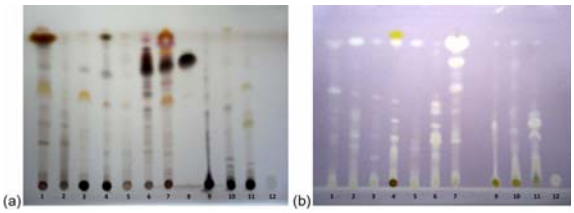

Fig. 3 TLC fingerprint of non-volatile extract (30 µg) sprayed with 10% sulphuric acid in ethanol, heat at 100°C for 10 min (a) and 10% DPPH in ethanol (b) 1 = *Acorus calamus*, 2 = *Micromelum minutum*, 3 = *Amorphophallus paeoniifolius*, 4 = *Plumbago indica*, 5 = *Myristica fragrans* (wood), 6 = *Piper nigrum* 3 µg, 7 = Apai-sa-le recipe, 8 = standard piperine 3 µg, 9 = *Terminalia chebula*, 10 = *Terminalia sp*., 11 = *Ardisia polycephala*, 12 = standard gallic acid 3 µg.

moderate stage of AD. Since AChE inhibitor has been a proper therapeutic approach to alleviate the cognitive symptoms of AD, but within moderate or advanced stages of AD, BuChE may replace AChE in hydrolyzing brain acetylcholine[19]. This study revealed that some plants in Apai-sa-le recipe could increase acetylcholine, neurotransmitter, at nerve ending by inhibited acetyl cholinesterase and butyrylcholinesterase activities.

It was also known that oxidative stress, toxic effect from beta-amyloid plague, was one of the major mechanisms causing neuron and asterocyte death in AD[20,21]. Therefore, any drug possessed antioxidant activity may be beneficial in the prevention of this neurodegenerative disease[22]. In this preliminary experiment, nine herbal ingredients in Apai-sa-le recipe and the whole recipe showed antioxidant activity by DPPH spraying on TLC chromatogram. Piperine, the main ingredient in *Piper nigrum* did not showed antioxidation activity by DPPH detection. This preliminary chemical-based assay is widely used to evaluate the ability of plant extracts to act as free radical scavengers or hydrogen donors. Actually, DPPH free stable radical is not a good scavenger for oxygen active species[23]. Piperine has been demonstrated in in vitro studies to protect against oxidative damage by inhibiting or quenching free radicals and reactive oxygen species[16]. In vivo study, supplement of piperine to high-fat-diet rat significantly decreased levels of thiobarbituric acid reactive substances (TBARS), conjugated dienes (CD) and maintained activities of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione-S-transferase (GST) and glutathione (GSH) to normal levels[24]. Therefore, lipid peroxidation assay, involving direct reaction of oxygen radicals with polyunsaturated fatty acids, together with dichlorofluorescin (DCF) assay, determining the power to prevent cell oxidative damages caused by free radicals, should also be used to test the capacity of plant extracts in Apai-sa-le recipe. These results would offer vital information since these two assays tended to more closely reflect antioxidant effects in vivo than the chemical assays.

Conclusion

Lepidium sativum Linn. extract showed the most potency on BuChE inhibitory effect. Three ingredients and the whole recipe exhibited mild activity. Only *Piper nigrum* L. demonstrated inhibition effect on both AChE and BuChE. Although the whole recipe had less potency of a cholinesterase inhibitory effect, the antioxidant activity of chemical constituents in

extract were revealed. Nine herbal ingredients in Apai-sa-le recipe and the whole recipe showed antioxidant activity by DPPH. Based on the present study, the use of Apai-sa-le recipe was reasonable and potential for using, at least as co-treatment for prevention of AD. Some plants from this recipe that revealed high potency in anti-butyrylcholinesterase activity may be developed as a group of drug or as food supplement for AD prevention or treatment.

What is already known on this topic?

Apai-sa-le recipe had been formerly used as drug nourishing wind element and formerly improving memory. No mechanism of actions has been proved for this used except anti-AChE activity of some individual herbals as *Piper nigrum* L.[16], *Anethum graveolens* L.[8], seed of *Myristica fragrans* Houtt[5]. Including, *Foeniculum valgare* L.[7] and *Acorus calamus* L.[9] were proved to enhance cognitive function in animal model.

What the present study adds?

The present study revealed that four plants in Apai-sa-le recipe inhibited BuChE activity. These plants were more selectively inhibited on BuChE than AChE activities. This enhancing acetylcholine effect at nerve ending and including antioxidant effect of some plants would correspondence to mechanisms of action of drugs used for AD.

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Potential conflicts of interest

None.

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ฤทธิ์ต้านเอนไซม์โคลีนเอสเตอเรสของสมุนไพรเดี่ยวและตำรับยาอภัยสาลี

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ภูมิหลัง: *ฤทธิ์ต้านเอนไซม์อะเซทธิลโคลีนเอสเตอเรสและบิวไทริลโคลีนเอสเตอเรสเป็นกลไกการออกฤทธิ์อย่างหนึ่ง ของยารักษาโรคสมองเสื่อมในปัจจุบัน ฤทธิ์ต้านเอนไซม์ดังกล่าวสามารถตรวจพบได้จากสมุนไพรหลายชนิด ตามตำราไทยดั้งเดิมได้ระบุว่าตำรับยาอภัยสาลีเป็นยาบำรุงร่างกาย ช่วยเจริญสติปัญญาดี จึงน่าจะมีสมุนไพรที่มีสรรพคุณสอดคล้องกับกลไกการรักษาของยาในปัจจุบันได้*

วัตถุประสงค์: *เพื่อศึกษาฤทธิ์ต้านเอนไซม์อะเซทธิลโคลีนเอสเตอเรสและบิวไทริลโคลีนเอสเตอเรสจากตัวยาสมุนไพรเดี่ยวและตำรับยาอภัยสาลี*

วัสดุและวิธีการ: *สกัดสารจากพืชแต่ละชนิด 30 กรัมและตำรับรวม 181 กรัมด้วยเอทธานอลระเหยแห้ง ทดสอบฤทธิ์ต้านเอนไซม์อะเซทธิลโคลีนเอสเตอเรสและบิวไทริลโคลีนเอสเตอเรสด้วยวิธีของ Elman ตรวจสอบส่วนประกอบของสารสกัดแต่ละชนิดที่มีน้ำมันหอมระเหยด้วยวิธี GCMS และตรวจสมุนไพรอื่นในตำรับด้วย TLC*

ผลการศึกษา: *สารสกัดด้วยเอทธานอลของสมุนไพรในตำรับที่มีฤทธิ์ต้านเอนไซม์บิวไทริลโคลีนเอสเตอเรสได้มี 4 ชนิด ได้แก่ เทียนแดง พริกไทย โกฐสอ และโกฐเขมา สามารถยับยั้งการทำงานเอนไซม์โดยมีค่าความเข้มข้นที่ยับยั้งได้ร้อยละ 50 (IC_{50}) เป็น 5.59, 24.52, 73.23, 96.25 ไมโครกรัม/มิลลิลิตรตามลำดับ สาร galantamine และตำรับรวมมีค่า IC_{50} เป็น 5.59 และ 236 ไมโครกรัม/มิลลิลิตร พริกไทยมีผลต้านเอนไซม์อะเซทธิลโคลีนเอสเตอเรสได้ด้วยโดยมีค่า IC_{50} เป็น 25.46 ไมโครกรัม/มิลลิลิตร สมุนไพรอื่นและตำรับรวมไม่มีผลต้านเอนไซม์โคลีนเอสเตอเรสทั้ง 2 ชนิดอย่างชัดเจน สารสำคัญตรวจพบในสมุนไพรที่มีฤทธิ์ดี เช่น apiol, cumialdehyde, furanodiene และ piperine สมุนไพรทุกชนิดที่ตรวจสอบมีฤทธิ์ต้านอนุมูลอิสระ*

สรุป: *เทียนแดงมีฤทธิ์ต้านเอนไซม์บิวไทริลโคลีนเอสเตอเรสได้ดีที่สุด พริกไทย โกฐสอ และโกฐเขมา ในตำรับอภัยสาลีและตำรับรวม มีผลยับยั้งเอนไซม์บิวไทริลโคลีนเอสเตอเรสได้บ้าง พริกไทยมีผลต้านเอนไซม์ได้ทั้ง 2 ชนิด*