

Screening for Acetylcholinesterase Inhibitory Activity from the Piperaceae การตรวจกรองฤทธิ์ยับยั้งเอนไซม์อะเซทิลโคลีนเอสเทอเรสจากพืชวงศ์พริกไทย

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บทคัดย่อ

การยับยั้งการทำงานของเอนไซม์อะเซทิลโคลีนเอสเทอเรสที่ใช้ทำลายอะเซทิลโคลีนมีบทบาทสำคัญในการรักษาโรคอัลไซเมอร์ พืชสมุนไพรนับเป็นแหล่งสำคัญของสารที่มีฤทธิ์ยับยั้งการทำงานของเอนไซม์อะเซทิลโคลีนเอสเทอเรส ในการวิจัยนี้ผู้วิจัยสนใจศึกษาฤทธิ์ยับยั้งการทำงานของเอนไซม์อะเซทิลโคลีนเอสเทอเรสในหลอดทดลองของสารสกัดหยาบในตัวทำละลายเมธานอลจากพืชสมุนไพรวงศ์พริกไทยพบว่า สารสกัดหยาบส่วนของผลพริกไทย ลำต้นชะพลู ผลติป्ली ใบชะพลู ใบพริกไทย สามารถยับยั้งการทำงานของเอนไซม์อะเซทิลโคลีนเอสเทอเรสได้ดีมาก (ที่ความเข้มข้น 100 ไมโครกรัม/มิลลิลิตร ยับยั้งเอนไซม์ได้ 65.97 - 76.39%) โดยมีค่าความเข้มข้นของสารสกัดหยาบที่สามารถยับยั้งการทำงานของเอนไซม์ได้ 50 เปอร์เซ็นต์ เท่ากับ 11.13, 13.59, 14.08, 26.74, และ 36.25 ไมโครกรัม/มิลลิลิตร ตามลำดับ และสารสกัดหยาบส่วนของใบพลูและใบติป्ली สามารถยับยั้งเอนไซม์อะเซทิลโคลีนเอสเทอเรสได้ดีปานกลาง (ที่ความเข้มข้น 100 ไมโครกรัม/มิลลิลิตร ยับยั้งเอนไซม์ได้ 40.28%) งานวิจัยนี้สนับสนุนข้อมูลการใช้ส่วนของพืชสมุนไพรเหล่านี้ในตำรายาพื้นบ้านที่มีสรรพคุณช่วยบำรุงธาตุ บำรุงสมอง

คำสำคัญ : สารยับยั้งอะเซทิลโคลีนเอสเทอเรส อัลไซเมอร์ พืชวงศ์พริกไทย พริกไทย ชะพลู ติป्ली

Abstract

Inhibition of acetylcholinesterase (AChE), the key enzyme in the breakdown of acetylcholine, is considered as a promising strategy for the treatment of Alzheimer's disease. A potential source of AChE inhibitors is provided by the abundance of plants in nature. This research aimed to investigate the methanolic extracts of medicinal plants in the family Piperaceae for *in vitro* acetylcholinesterase (AChE) enzyme inhibitory activity. The potent AChE inhibiting methanolic plant extracts included the fruits of *Piper nigrum*, stems of *P. sarmentosum*, fruits of *P. retrofractum*, leaves of *P. sarmentosum*, and leaves of *P. nigrum* (65.97 to 76.39% inhibition at the concentration of 100 $\mu\text{g/ml}$). The IC_{50} values obtained for these extracts were 11.13, 13.59, 14.08, 26.74, and 36.25 $\mu\text{g/ml}$ respectively. The moderate AChE inhibiting methanolic plant extracts included the leaves of *P. betle*, and *P. retrofractum* (40.28% inhibition at the concentration of 100 $\mu\text{g/ml}$). These results partly substantiate the traditional use of these herbs for the improvement of cognition.

Keywords: Acetylcholinesterase inhibitor; Alzheimer's disease; Piperaceae; *Piper nigrum*;

Piper sarmentosum; *Piper retrofractum*

Introduction

Alzheimer's disease (AD), a progressive neuro-degenerative brain disorder, is characterized by dementia, cognitive impairment, and memory loss. In 2015, AD affected 46.8 million people worldwide and is estimated to rise to 74.7 million in 2030, and to 131.5 million in 2050. This disease severely influences the patient's family life and social activities. The global costs of dementia-related diseases have increased 35% from US\$ 604 billion in 2010 to US\$ 818 billion in 2015 [1].

The cholinergic hypothesis postulates that memory impairment in patients with AD is mainly characterized by the decrease in levels of the neurotransmitter acetylcholine (ACh) due to damage of cholinergic neurons in some special parts of the brain. One of the rational and effective approaches to alleviate the AD's symptoms is an increase the ACh through inhibiting acetylcholinesterase (AChE), the key enzyme which hydrolyses ACh [2]. Thus, several acetylcholinesterase inhibitors have been used for the clinical treatment of AD patients. Along with the prototype inhibitor of AChE physostigmine obtained from the plant *Physostigma venenosum*, other molecules with highly significant anti-cholinesterase activity are galantamine, huperzine-A obtained from *Galanthus nivalis* and *Huperzia serrata* [3]. Therefore, plants can serve as a potential source of AChE inhibitors. Galantamine and other AChE inhibitors have been used for the symptomatic treatment of AD. However, many recent studies reported several limitations of AChE inhibitors related to their non-selectivity, limited efficacy, poor bioavailability, and adverse effects, such as nausea, vomiting, diarrhea, dizziness, and hepatotoxicity [2]. Consequently, there is still a great demand to find new drug candidates for AD treatment.

The Piperaceae family, represented by plants such as black pepper (*Piper nigrum* L.) and Kava (*Piper methysticum* G. Forst.), has been a significant source of alternative medicine for individuals afflicted with mental illness and may enhance cognitive performance in Thai traditional medicine [4,5]. Ingkaninan et al (2007) screened acetylcholinesterase inhibitory activity of the plants used in the rejuvenating remedies and reported that the methanolic extract from the seeds of *P. nigrum* showed $58.02 \pm 3.83\%$ inhibition at the concentration of 100 $\mu\text{g/ml}$ [6]. To date, there are a few scientific data to support the acetylcholinesterase inhibitory activity of Piperaceae plants.

The objectives of the present study were to investigate *in vitro* possible AChE inhibitors from plants in the Piperaceae which enhanced cognitive performance, and to point out the role of these plants as potential sources for the development of novel potent natural therapeutic agents of AD.

Materials and Methods

Chemicals

Acetylcholinesterase from electric eel (Type V-S lyophilized powder) 480 U/mg solid, 530 U/mg protein, acetylthiocholine iodide (ATCI), bovine serum albumin (BSA), 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB), eserine hemisulfate, hydrochloric acid, magnesium chloride, sodium chloride and Tris (hydroxymethyl) methylamine were obtained from Sigma (USA). Methanol analytical grade and sodium hydroxide were purchased from Carlo Erba Reagent (Italy).

Fifty millimolar Tris-HCl pH 8.0 was used as a buffer. The lyophilized acetylcholinesterase enzyme was prepared in the buffer to obtain 1130 U/ml stock solution. The enzyme stock solution was kept at -80°C until used. The further enzyme dilution

was dissolved in 0.1% BSA in buffer. DTNB was dissolved in the buffer containing 0.1 M NaCl and 0.02 M MgCl₂. ATCI was dissolved in de-ionized water.

Preparation of the plant extracts

Piperaceae plants including the leaves of *Piper betle*, *P.nigrum*, *P.retrofractum*, and *P.sarmentosum*, fruits of *P.nigrum* and *P. retrofractum*, and stems of *P. sarmentosum* were collected in Warinchamrap, Ubon Ratchathani, Thailand. The plant materials were cut into small

pieces and dried in a hot air oven at 40°C. The dried materials were macerated in methanol for 3 days. This was repeated twice with fresh solvent each time. The filtrates were pooled and evaporated under reduced pressure until dry. All dried extracts were weighed and stored at 4°C in light-protected containers until used.

Determination of plant yield

The percentage of extract yield was calculated by the following equation:

$$\text{Extract yield (\%)} = \frac{(\text{weight of the dried extract} \times 100)}{(\text{weight of the dried plant})}$$

Inhibition of acetylcholinesterase activity

The acetylcholinesterase inhibition was determined by spectrophotometrically using Ellman's colorimetric method [7] as modified by Ingkaninan and et al [6]. The acetylcholinesterase enzyme hydrolyzes the substrate ATCI to thiocholine and acetic acid. Thiocholine is allowed to react with DTNB, and this reaction resulted in the development of a yellow color. The color intensity of the product is measured at 405 nm, and it is proportional to the enzyme activity.

The acetylcholinesterase inhibition of Piperaceae plant extracts were carried out in 96-well plates. Briefly, 25 µl of 15 mM ATCI, 125 µl of 3 mM DTNB, 50 µl of 50 mM Tris-HCl pH 8.0, and 25 µl of the plant extract dissolved in buffer containing not more than 10% methanol were added to the wells. Thereafter, 25 µl of AChE solution (0.28 U/ml) was added and the absorbance was measured by using a microplate reader (Dyner MRX Microplate Reader, Dyner Technologies Revelation Program Version 4.25, USA) at a wavelength of 405 nm and monitored every 15 seconds over period of 5 minutes. Eserine hemisulfate was used as a

standard drug. The velocities of the reactions were measured. Enzyme activity was calculated as a percentage of the velocities compared to that of the assay using buffer without any inhibitor. Inhibitory activity was calculated from 100 subtracted by the percentage of enzyme activity.

Statistical analysis

All determinations were carried out in triplicate. The results of the percentage of acetylcholinesterase enzyme inhibition were reported as mean ± standard error of mean (SEM). The extracts that showed potency of acetylcholinesterase inhibition (> 50% inhibition) determined the effects of potent extracts in different concentrations on acetylcholinesterase inhibition assay. The concentration of extract that inhibited hydrolysis of substrate by 50% (IC₅₀) and 95% confidence interval (95%CI) values were calculated by using GraphPad Prism Version 5.0 for Windows (GraphPad Software Inc.)

Results and Discussion

Extract yield of plant in Piperaceae

In Table 1 shows that the methanolic extract from the leaves of *P. betle* gave the highest yield (18.05%) while the extract from the fruits of *P. nigrum* gave the lowest yield (1.73%).

Effect of Piperaceae plant extracts on acetylcholinesterase activity (*in vitro*)

Most of the extracts showed acetylcholinesterase inhibitory activity to a different degree (40.28 - 76.39%) as shown in Figure 1. Vinutha and et al (2007) reported that the acetylcholinesterase inhibitory activity of crude extract at 100 µg/ml with

inhibition rate of >50% inhibition, 30–50% inhibition, and <30% inhibition may be defined as potent, moderate, and low activity in acetylcholinesterase inhibition respectively [8]. At the concentration of 100 µg/ml, the methanolic extracts of the leaves of *P. nigrum*, fruits of *P. nigrum*, fruits of *P. retrofractum*, leaves of *P. sarmentosum*, and stems of *P. sarmentosum* showed potent acetylcholinesterase inhibition (65.97 to 76.39% inhibition). The methanolic extract of the leaves of *P. betle* and *P. retrofractum* showed moderate acetylcholinesterase inhibition (40.28% inhibition at the concentration of 100 µg/ml).

Table 1 Percentage yield of plant extracts

| Plant name | Parts used | Weight of the original | Weight of the crude | Extract yield (%) |
|------------------------|------------|------------------------|---------------------|-------------------|
| | | sample (g) | extract (g) | |
| <i>P. betle</i> | Leaf | 268.15 | 48.41 | 18.05 |
| <i>P. nigrum</i> | Leaf | 108.75 | 15.74 | 14.48 |
| | Fruit | 518.68 | 8.99 | 1.73 |
| <i>P. retrofractum</i> | Leaf | 407.7 | 39.52 | 9.69 |
| | Fruit | 299.74 | 35.68 | 11.90 |
| <i>P. sarmentosum</i> | Leaf | 212.5 | 25.35 | 11.93 |
| | Stem | 112.23 | 4.71 | 4.20 |

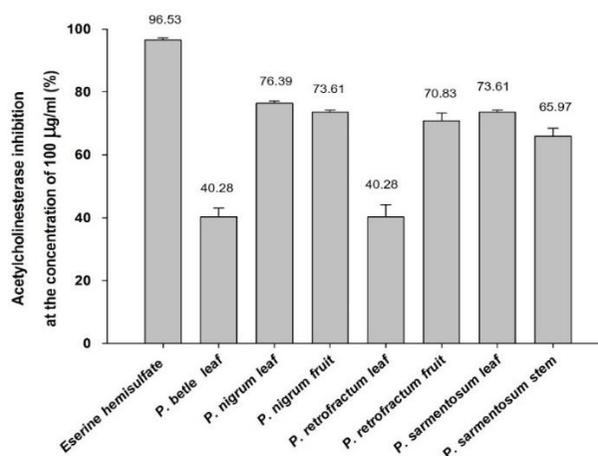


Figure 1 Percentage of acetylcholinesterase inhibition of the extracts at the concentration of 100 µg/ml

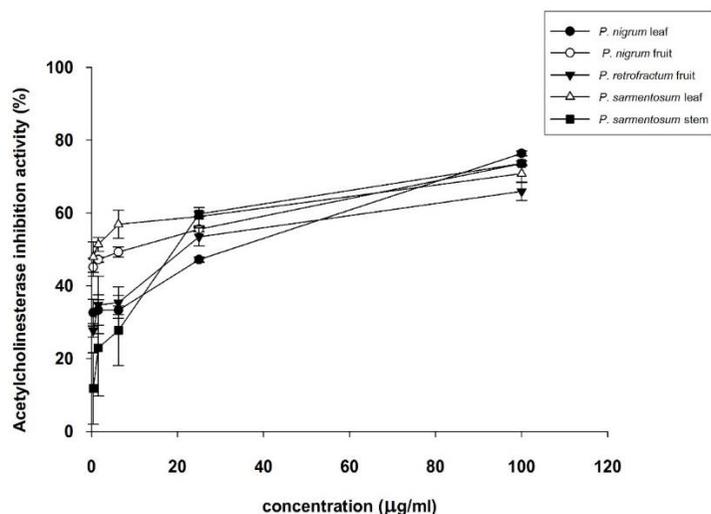


Figure 2 Acetylcholinesterase inhibitory property of the potent extracts at the concentrations of 0.39, 1.56, 6.25, 25, and 100 µg/ml. Determinations were done in triplicate. Each point represents the mean ± standard error of mean.

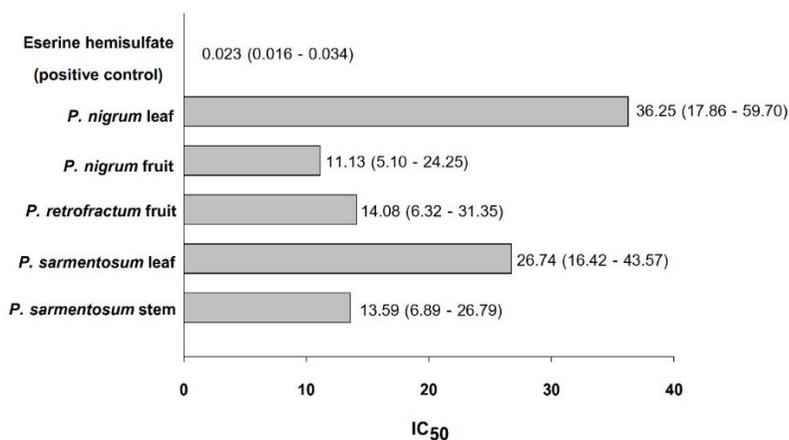


Figure 3 IC₅₀ values of positive control and the potent extracts in acetylcholinesterase inhibition assay. 95% confidence interval in parentheses

In this experiment, eserine hemisulfate was used as the standard in acetylcholinesterase inhibition assay. Only the five extracts that showed potent acetylcholinesterase inhibition were subjected to determine the effects of potent extract in different concentrations on acetylcholinesterase inhibition and the IC₅₀ test.

Acetylcholinesterase inhibitory properties of the potent extracts at the concentrations of 0.39, 1.56, 6.25, 25, and 100 µg/ml are shown in Figure 2. The IC₅₀ values of eserine hemisulfate and the potent extracts in acetylcholinesterase inhibition assay are shown in Figure 3. Eserine hemisulfate inhibited the activity of acetylcholinesterase with an

IC₅₀ of 0.023 µg/ml. The potent extracts were capable of inhibiting the enzyme in a concentration-dependent manner. The IC₅₀ values obtained for the methanolic extracts from the fruits of *P. nigrum*, stems of *P. sarmentosum*, fruits of *P. retrofractum*, leaves of *P. sarmentosum*, and leaves of *P. nigrum*, were 11.13, 13.59, 14.08, 26.74, and 36.25 µg/ml respectively.

From the results, the acetylcholinesterase inhibitory activity of the methanolic extract from the fruits of *P. nigrum* at the concentration of 100 µg/ml was $73.61 \pm 0.69\%$. Ingkaninan et al reported that the acetylcholinesterase inhibitory activity of the methanolic extract from the seeds of *P. nigrum* at the concentration of 100 µg/ml was $58.02 \pm 3.83\%$ [6]. The seeds of *P. nigrum* in the study of Ingkaninan et al may have been ripe fruit seeds (white pepper) and in our experiments, the fruits of *P. nigrum* were unripe fruit form (black pepper). Therefore there was some difference in the part of the plant that was used in acetylcholinesterase inhibitory activity. However, these results indicated that the methanolic extract from the seeds and the fruits of *P. nigrum* showed very strong action against AChE activity (> 50% inhibition). The IC₅₀ study indicated that the extracts from the fruits of *P. nigrum* showed the most potency in acetylcholinesterase inhibition (IC₅₀ = 11.13 µg/ml) and had a greater potency than the methanolic extract from the leaves of *P. nigrum*. The extract yield of the fruits of *P. nigrum* was the lowest yield (1.73%), indicating that the preparation of the extract required more plant materials to get more yield. In Thai traditional medicine, *P. nigrum* is a very popular plant used for the improvement of memory and cognition enhancement. Recently, Tu et al (2015) isolated piperine, piperettine, and

piperettyline from the ethyl acetate extract from the fruits of *P. nigrum* and reported their anti-cholinesterase and antioxidant properties [9]. Hence, the acetylcholinesterase inhibitory activity of *P. nigrum* may have been affected by a main active piperine alkaloid [5]. In addition, the presence of other polar compounds in the methanolic extract may have also contributed to anticholinesterase activity. Therefore, the phytochemical method is still necessary to analyze the bioactive compounds in the methanolic extract from the fruits of *P. nigrum*.

P. retrofractum Vahl., long pepper, is a plant widely distributed throughout Thailand. This plant has a "hot" flavor and is used to aid food digestion, blood circulation, asthma, and overall health. In Malaysia, the aromatic spice is taken as a tonic for languidness and after childbirth, and administered internally for degenerative organs, cramps, congestion of the liver, and ulceration of the bones. *Piper retrofractum* was found to contain piperine, piperlidine, retrofractamide-D, retrofractamide A [10]. In our study, the methanolic extract from the fruits of *P. retrofractum* showed good activity in the inhibition of acetylcholinesterase activity. It is possible that piperine and other chemical constituents of the *P. retrofractum* essential oil are the active compounds in acetylcholinesterase inhibition.

P. sarmentosum Roxb., commonly known as "Cha Plu" in Thai, is used as important ingredients for various medicinal purposes in traditional medicine in many Asian countries. It was reported that the components in the *P. sarmentosum* are sarmentamide A, sarmentamide B, sarmentamide C, β-sitosterol, aromatic alkene compounds, and antioxidant naringenin [11, 12]. From our results, the methanolic extract from the stems of *P. sarmentosum* and the leaves of *P. sarmentosum*

possessed acetylcholinesterase inhibitory activity. Hence, accumulating evidence suggested that brain tissues in AD patients were exposed to oxidative stress during the development of the disease [13]. The antioxidant property of chemical constituents in *P. sarmentosum* may also have some roles in the treatment of AD.

P. betle L. is a widely distributed plant in tropical regions. It is recognized as a medicinal species in India, Sri Lanka, Malaysia, Thailand, Taiwan, and East Africa because of its anti-inflammatory, anti-allergic, hepato-protective, antioxidant, anti-mutagenic, anti-carcinogenic, anti-helminthic and chemopreventive properties. Ferreres et al (2014) reported that the aqueous extract and ethanolic extract from the leaves of *P. betle* showed a capacity to inhibit acetylcholinesterase and the ethanolic extract was more effective against acetylcholinesterase inhibition assay. Their acetylcholinesterase inhibition assay of the ethanolic extract from the leaves of *P. betle* was done in the range of concentration between 0-3 mg/ml which was higher than our assay. The IC₅₀ value of the ethanolic extract from the leaves of *P. betle* in acetylcholinesterase inhibition assay was 0.722 mg/ml [14]. However, it was consistent with our study that the methanolic extract from the leaves of *P. betle* did not show the highest activity in acetylcholinesterase inhibition (40.28% inhibition at the concentration of 0.1 mg/ml).

Our results demonstrated that the methanolic extracts from the leaves of *P. nigrum*, fruits of *P. nigrum*, fruits of *P. retrofractum*, leaves of *P. sarmentosum* and stems of *P. sarmentosum* possessed a high anti-acetylcholinesterase activity (65.97 to 76.39%). These findings suggested that these extracts may function as acetylcholinesterase inhibitors and have potential

application in AD therapeutics. The studies supported the traditional use of the plants for the enhancement of cognition performance. Further investigations of new compounds of Piperaceae extracts are necessary for the development of drugs against AD.

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