Acetylcholinesterase Inhibitory Activity of Thai Traditional Nootropic Remedy and Its Herbal Ingredients

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The incidence of Alzheimer disease (AD) is increasing every year in accordance with the increasing of elderly population and could pose significant health problems in the future. The use of medicinal plants as an alternative prevention or even for a possible treatment of the AD is, therefore, becoming an interesting research issue. Acetylcholinesterase (AChE) inhibitors are well-known drugs commonly used in the treatment of AD. The aim of the present study was to screen for AChE inhibitory activity of the Thai traditional nootropic recipe and its herbal ingredients. The results showed that ethanolic extracts of four out of twenty-five herbs i.e. Stephania pierrei Diels. Kaempfera parviflora Wall.ex Baker, Stephania venosa (Blume) Spreng, Piper nigrum L at 0.1 mg/mL showed % AChE inhibition of 89, 64, 59, 50; the IC_{50} were 6, 21, 29, 30 µg/mL respectively. The other herbs as well as combination of the whole recipe had no synergistic inhibitory effect on AChE activity. However, some plants revealed antioxidant activity. More research should have be performed on this local wisdom remedy to verify the uses in scientific term.

Keywords: *Acetylcholinesterase inhibitor, Nootropic recipe*

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The incidence of Alzheimer disease (AD) is increasing every year in accordance with the increasing of elderly population and could pose significant health problems in the future. In 2010, the ratio of AD patients to Thai population with more than 65 years of age was 1 in 20. This was up to 1 in 4 among the group of 80 years old[1]. AZD is a chronic neurological disorder characterized by memory impairment, cognitive dysfunction, behavioral disturbances and deficits in activities of daily living. It was well-known that a deficiency of neurotransmitter, acetylcholine, in cerebral cortex is one of the major features seen in AD's patients and this was due to the increase of acetylcholinesterase (AChE), an enzyme that converts acetylcholine into inactive choline and acetate. Increasing acetylcholine level through AChE enzyme inhibition has been accepted as the effective treatment of AD[2-5]. At present, AChE inhibitors such as tacrine, donepezil and rivastigmine are synthetic drugs commonly used for management strategy against AD. However, these drugs are limited in use due to their adverse side-effects and are effective only against mild type of AD[6]. Nowadays, research issues focus on medicinal plants as natural sources of effective acetylcholinesterase inhibitor with little or no side effects which could be used as dietary intervention in the management of the disease[7].

Nootropic drugs, drugs and natural remedies acting on brain, which are today popular due to their proven effective qualities, have been described to enhance the memory and protects the memory functioning in dementia people. One of the acceptable mechanisms of action of these natural memory enhancing drugs is to control the activity of acetylcholinesterase by inhibiting the excessive AChE activity and protect the dementia progression. According to Thai traditional medicine, one of the popular nootropic recipes was claimed for analgesic, bitter tonic, energetic tonic, laxative, long living, rejuvenating and neurotonic purposes. This remedy

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was composes of 21 plants as shown in Table 1[8]. As a local wisdom, 30 grams of each plant was milled to fine powder then they were well-mixed and ready for use. Some plants in this recipe have the synonym names in Thai. For example, *Aegel marmelos* (L) Correa ex Roxb or ma-tum in Thai might referred to ma-tum (specimen number 3.1 in Table 1) or ma-tum-nim which its fruit pulp tasted differently (specimen number 3.2). Kra-chai might referred to *Boesenbergia rotunda* (L) Mansf (specimen number 4.1) or *Kaempfera parviflora* Wall.ex Baker, kra-chai-dam (specimen number 4.2). *Piper nigrum* L might referred to prick-thai (specimen number 11.1) or prick-thai-dum (specimen number 11.2). Hua-buo might be either *Stephania pierrei* Diels, *S. venosa* (Blume) Spreng. *S. suberosa* Forman, or Chinese herb named *Conioselenium univitatum* Turcz (specimen number 15.1-15.4)[9]. Also, there are two varieties of gingers *Zingiber officinale* L. var. *officinale,* the white ginger and the red one, var *rubrum Theilade*. Therefore, all of the plants sharing synonym names indicated in the recipe should be screened.

The objectives of the present study are to evaluate the potential of each plant and the whole recipe used for nootropic by focusing on acetylcholinesterase inhibitors. The effect of combination of herbs as nootropic recipe that might exhibit synergistic in their enzyme inhibition activity is also studied. Moreover, some synonym plants in Thai name should also be investigated in order to select the best AChE inhibitory activity for a more effective recipe.

Material and Method

Plant Materials

Three fresh plants, specimen number 15.1, 15.2 and 15.3 in Table 1 were purchased from Jatujak market, Bangkok, propagated until they flowered and were identified according to their characters of taxonomic importance[10]. Plant specimen number 4.2 was collected in Petchaboon province and was identified by its rhizomes and flower[11]. Other 21 dried plant materials were purchased from herbal drug store in Bangkok and identified by Associate Professor Arunporn Itharat, Faculty of Medicine, Thammasat University. All voucher specimens were kept at Department of Thai traditional Medicine, Faculty of Medicine, Thammasat University.

Chemicals

Acethylthiocholine Iodide (ACTI), 5,5'-dithiobis-(2-nitro-benzoic acid (DTNB), galantamine, AChE (from electric eel ,type VI-S lyophilized powder, 480 U/mg solid, 530 mg protein), bovine serum albumin (BSA) and Tris-HCl were purchased from Sigma (Thailand). 50 mM Tris-HCl, pH 8.0 was used as a buffer for all experiments. AChE was dissolved in buffer to obtain 1,130 U/mL stock solution and kept at -80°C and was further diluted in 0.1% BSA in buffer. DTNB and ACTI were dissolved in buffer and Millipore water respectively.

Extraction

Each plants was separately washed, dried in hot air incubator at 50°C and then grinded to fine powder. Each 10 gm of powder plant was twice macerated in 100 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-frozen (LyoLab LT, USA) to dryness. Ten grams of powder from each plant was mixed together and extracted to obtain crude extract of the whole recipe.

Standardization of the extract

Every crude extract was calculated for percentage of yield and also, the moisture content was analyzed by moisture analyzer (SCALTEC model SMO 01). Thin layer chromatography was performed to demonstrate fingerprints of each extract.

Thin layer chromatographic analysis

Silica gel GF_{254} was used as stationary phase and mobile phase was chloroform: methanol 95:5. Fifty microgram of each plant extract and the whole recipe extract were separately loaded on TLC plates. The chromatograms were detected by 3 spraying reagents such as 10% of suphuric acid in ethanol, anisaldehyde reagent and 10% of diphenyl-1-picrylhydrazyl (DPPH) in ethanol[12].

Microplate assay for AChE activity

The AChE activity was measured by following the increase of yellow color produced from thiocholine when it reacts with DTNB ion (Dithiobisnitrobenzoate) It is based on coupling of these reactions:

$$\text{ACTI} + H_2O \xrightarrow{\text{AChE}} \text{Acetate} + \text{Thiocholine}$$

$$\text{Thiocholine} + \text{Dithiobisnitrobenzoate} \rightarrow \text{Thiocholine-thionitrobenzoate disulfide} + \text{Thionitrobenzoate.}$$

The increase of spectrophotometer absorbance measured at 405nm was reversed to the amount of enzyme inhibitor and also, was linear for more than 2 min. The AChE activity assay were performed according to Elman et al 1961[13] and modified by Ingkaninan et al 2003[14]. Briefly, 125 µl of 3 mM

Table 1. The inhibitory activity on acetylcholinesterase of ethanolic extracts from plants used in Thai traditional nootropic recipe

No.	Plant name	Family	Part used	Used	% yield	% inhibition (SD)
1	*Albizia procera* (Roxb.) Benth.	Mimosaceae	root	nootropic	2.27	15.20 (1.98)
2	*Alpinia conchigera* Griff	Zingiberaceae	rhizome	Carminative, tonic	3.40	9.55 (1.63)
3.1*	*Aegel marmelos* (L.) Correa ex Roxb.	Rutaceae	fruit pulp	Tonic, nootropic	6.98	41.20 (2.34)
3.2*	*Aegel marmelos* var. ma-tum-nim	Rutaceae	fruit pulp	Tonic, nootropic	7.93	48.36 (1.87)
4.1*	*Boesenbergia rotunda* (L.) Mansf.	Zingiberaceae	rhizome	Antispasmodic, energetic tonic	10.57	30.69 (3.96)
4.2*	*Kaempfera parviflora* Wall.ex Baker	Zingiberaceae	rhizome	Rejuvenating, tonic	3.17	64.08 (0.64)
5	*Cinnamomum iners* Bl.	Lauraceae	stem bark	carminative	15.83	22.79 (4.58)
6	*Cyrtosperma johnstonii*	Araceae	zhizome	-	2.03	14.69 (2.25)
7	*Cyperus rotundus* L.	Cyperaceae	rhizome	Nootropic, cardiotonic	2.03	14.69 (2.25)
8	*Diospyros rhodocalyx* Kurz.	Ebenaceae	stem bark	Nootropic , energetic tonic	5.17	0.80 (3.13)
9	*Levisticum* officinale Koch.	Umbelliferae	root	carminative	7.90	5.25 (1.18)
10	*Maerua siamensis* Pax	Capparidaceae	root	tonic	7.57	27.64 (2.84)
11.1*	*Piper nigrum* L.	Piperaceae	fruit	tonic, brain tonic	4.16	46.50 (1.43)
11.2*	*Piper nigrum* L. (prick-thai-dum)	Piperaceae	fruit	Rejuvenating, tonic	3.83	55.93 (0.18)
12	*Piper retrofractum* Vahl.	Piperaceae	fruit	tonic, carminative	1.73	24.75 (1.69)
13	*Phyllanthus emblica* L.	Euphorbiaceae	fruit	Anti-oxidant , tonic	5.93	18.29 (3.04)
14	*Streblus asper* Lour.	Moraceae	fruit	nootropic	2.27	15.20 (1.98)
15.1*	*Stephania pierrei* Diels.	Menispermaceae	tuber	Nootropic, energetic tonic	8.07	89.20 (0.31)
15.2*	*Stephania venosa* (Blume) Spreng.	Menispermaceae	tuber	Energetic tonic, anticancer	3.80	59.25 (1.22)
15.3*	*Stephania suberosa* Forman.	Menispermaceae	tuber	tonic	2.97	26.67 (1.46)
15.4*	*Conioselenium univitatum* Turcz.	Umbelliferae	root	Pain relief , blood tonic	6.50	27.39 (1.53)
16	*Terminalia bellirica* Roxb.	Combretaceae	fruit pulp	Laxative, tonic	13.73	11.41 (2.32)
17	*Terminalia citrina* Roxb. Ex Fleming	Combretaceae	fruit	Laxative, tonic	15.40	12.72 (4.45)
18	*Terminalia chebula* Retz. var. *chebula*	Combretaceae	fruit pulp	Antipyretic, laxative, astringent, tonic	12.03	7.95 (4.15)
19	*Terminalia* sp.	Combretaceae	fruit	laxative	6.40	8.16 (1.62)
20	*Tinospora spinosa* (L.) Meiers ex Hook.f.& Thomson	Menispermaceae	stem	Antipretic, bitter tonic, tonic	3.07	22.09 (2.11)
21	*Zingiber officinale* L. (white ginger)	Zingiberaceae	rhizome	Carminative, tonic	2.07	26.33 (2.17)
22	The whole recipe.			Nootropic, brain tonic	8.09	26.93 (1.28)

*Synonym in Thai name

DTNB, 25 µl of 15 mM ATCI and 50 µl of buffer, 25 µl of sample dissolved in buffer containing not more than 10% methanol were added to the wells followed by 25 µl of 0.28 U/ml AChE. The microplate was then 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 the 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. Every experiment was done in triplicate.

Results

The percentage yields of the extracts were shown in Table1 and the moisture content of all extracts were less than 10%. The TLC chromatograms of each plant extract were shown in Fig. 1-3; demonstrated the compositions in each plant extract and the chromatogram positively stained with DPPH represents the anti-oxidation activity of each component in the extract.

The inhibitory activities on acetylcholinesterase of ethanolic extracts (0.1 mg/mL) from each plant and whole recipe were shown in Table 1. Plants with high activity were *Stephania pierrei* Diels, *Kaempfera parviflora* Wall.ex Baker, *Stephania venosa* (Blume) Spreng and *Piper nigrum* L (prick-thai-dum) showed the IC_{50} (SD) of 5.68 (0.74), 20.64 (1.95), 29.82 (2.30) and 30.67 (0.13) µg/mL, respectively. The IC_{50} of galantamine as positive control was 0.30 µg/mL or 0.816 µM. The whole recipe extract had no synergistic effect with % inhibition of 26.93. TLC fingerprints showed the anti-oxidation activity of some plants corresponded to the

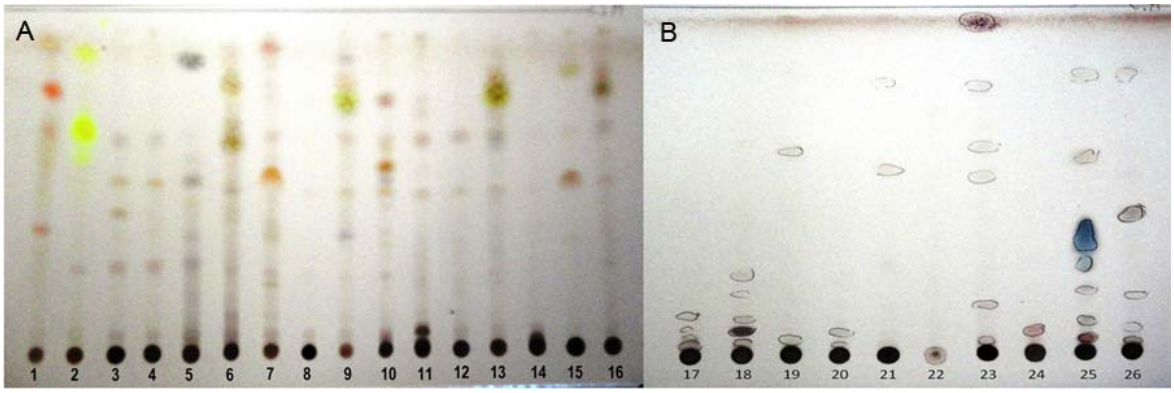

Fig. 1 TLC chromatogram of plant extract**s** sprayed with 10% H_2SO_4 in ethanol, heat at 100 °C for10 min. A = sample number 1-16; B = sample number 17-26

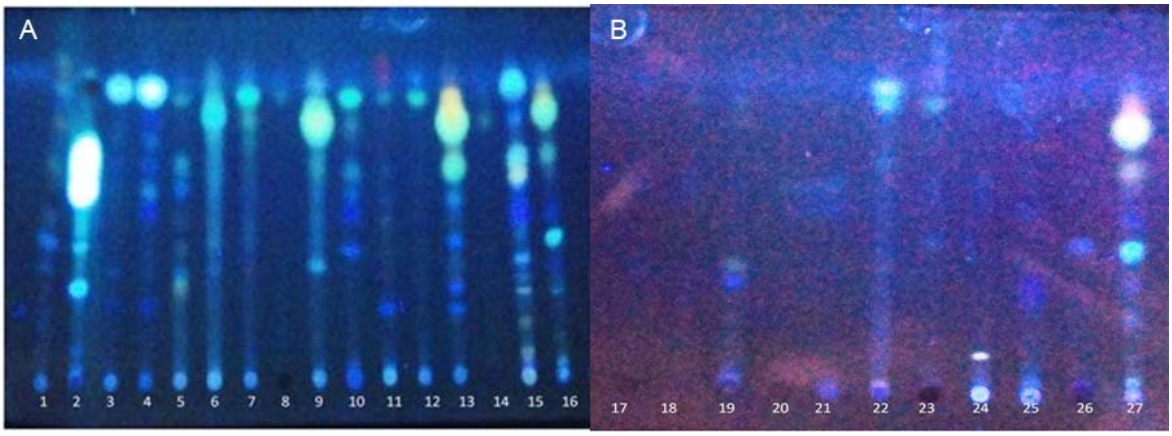

Fig. 2 TLC chromatogram of plant extracts sprayed with anisaldehyde reagent, UV 365 nm. A= sample number 1-16; B = sample number 17-27 as described in Fig. 1. The positive markers were characterized as blue, greenish, yellow or red fluorescent zones

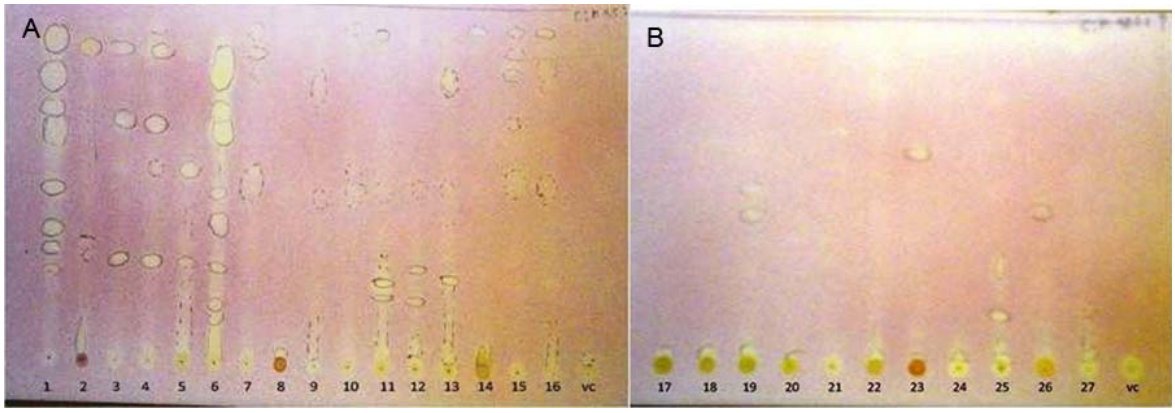

Fig. 3 TLC chromatogram of plant extracts sprayed with DPPH reagent. A = sample number 1-16, B = sample number 17-27. Lane number 1-27 as described in Fig. 1. The positive spots were characterized as yellow zones. The positive marker, VC = ascorbic acid, was yellow spot at base line

Fig. 1-3 TLC chromatogram lanes1-27 represented extracts from plants as followed:-

1. Boesenbergia rotunda, *2. Kaempfera parviflora,* *3. Levisticum officinale,*
4.Conioselenium univitatum, *5. Alpinia conchigera,* *6. Zingiber officinale,*
7. Streblus asper, *8. Maerua siamensis* *9. Piper retrofractum,*
10. Diospyros rhodocalyx, *11. Tinospora spinosa,* *12. Albizia procera,*
13. Piper nigrum, *14. Phyllanthus emblica ,* *15. Alpinia conchigera,*
16. The whole recipe *17. Terminalia citrine,* *18. Terminalia chebula,*
19. Terminalia sp., *20. Terminalia bellirica,* *21. Cyrtosperma johnstonii,*
22. Cyperus rotundus, *23. Cinnamomum iners,* *24. Stephania pierrei,*
25. Stephania venosa, *26. Stephania suberosa,* *27. The whole recipe.*

enzyme inhibitory activity.

Discussion

Eleven kinds of plants in this recipe had already been proven to inhibit acetylcholinesterase activity. Plants that showed inhibition activities less than 70% at the concentration of 0.1 mg/mL were *Albizia procera* (Ting-tong), *Aegel marmelos (matum)*, *Cyperus rotundus (hael-mou)*, *Diospyros rhodocalyx* (ta-ko-na), *Piper nigrum* (prick-thai), *Streblus asper (koi)*, *Terminalia bellirica (sa-moh-pi-pek)*, *Tinospora spinosa* (bo-ra-pet) and *Zingiber officinale* (Khing)[7,14,15]. Two plants that possessed inhibitory activities more than 70% inhibition were *Kaempfera parviflora* (kra-chai-dum) and *Stephania venosa* (Bua-bok)[16,17]. In the present study, only four plants exhibiting AChE enzyme inhibitor were *Stephania pierrei* Diels, *Kaempfera parviflora* Wall.ex Baker, *Stephania venosa* (Blume) Spreng and *Piper nigrum* L. The other plants as well as the whole recipe had no inhibitory effect on AChE activity.

From the results, it is noted that the plants sharing the same local name showed different activity on AChE. *Stephania pierrei* Diels (hua-boua) showed high AChE inhibitory activity whereas *Conioselenium univitatum* Turcz (Koh-hua-boua) had no inhibition effect on the enzyme. *Boesenbergia rotunda* (L) Mansf, named kra-chai had lower inhibitory activity than that of *Kaempfera parviflora* Wall.ex Baker or kra-chai-dum. *Piper nigrum* L, using whole fruits called prick-thai-dum, was more effective than fruits without peel (prick-thai). Two varieties of *Aegel marmelos*, which fruits pulp are totally different in taste, had nearly the same inhibitory activity. Hence, *Stephania pierrei* Diels, *Kaempfera parviflora* Wall.ex Baker, *Piper nigrum* L. (prick-thai-dum) and *Aegel marmelos* var. ma-tum-nim should be the right components in this nootropic remedy.

Zingiber officinale L (gingers) was reported to had both acetylcholinesterase and butyrylcholinesterase inhibitory activity. The water extracts of both white and red ginger inhibited AChE in a dose dependence manner in the range of 0-6.76 mg/mL and the IC_{50} were 2.86 and 3.03 mg/mL[7]. This dosage was estimated to be 30 folds of ethanol extract dose in our experiments. Moreover, the aqueous extract of ginger

decreased malondialdehyde content in rat brain[18]. Ginger's active principles possessed anti-oxidant action which protected cell against free radical damage[19], inhibited the β-amyloid peptide accumulation and protected neurotoxicity from oxidative stress[20], indicated its benefits in AZD treatment. Hence, more dosage of ginger should be added to this recipe.

Plants that showed moderate enzyme inhibitory activity might exhibit other mechanisms in AZD management such as anti-oxidation as seen in TLC fingerprints, promotion of neurite outgrowth, inhibition of β-amyloid accumulation. So, more experiments should be investigated both *in vitro* and *in vivo* to verify the nootropic remedy.

Potential conflicts of interest

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ผลต้านเอนไซม์ acetylcholinesterase ในสมุนไพรเดี่ยวและตำรับยาอายุวัฒนะ

พิมลวรรณ ทัพยุทธพิจารณ์, อรุณพร อิฐรัตน์, ศุณิตา มากชูชิต

อุบัติการณ์โรคสมองเสื่อมเพิ่มขึ้นตามสถิติประชากรผู้สูงอายุที่เพิ่มขึ้น การใช้ยาสมุนไพรจึงเป็นอีกทางเลือกหนึ่งในการดูแลตนเองเพื่อปกป้องและรักษาโรคดังกล่าว ยาอายุวัฒนะตามตำราการแพทย์แผนไทยมีตัวยาสมุนไพรที่น่าจะมีประโยชน์สอดคล้องกับการรักษาได้ทั้งในแบบแพทย์แผนปัจจุบันและแผนไทย กลไกการออกฤทธิ์อย่างหนึ่งของยาที่นำมาใช้รักษาโรคสมองเสื่อมในปัจจุบันคือฤทธิ์ต้านเอนไซม์ acetylcholinesterase

วัตถุประสงค์การวิจัยนี้ เพื่อศึกษาฤทธิ์ต้านเอนไซม์ดังกล่าวของตัวยาสมุนไพร ในตำรับยาอายุวัฒนะตำรับหนึ่งที่บันทึกไว้ในตำราการแพทย์แผนไทย ผลการทดลองพบว่าสารสกัดด้วยเอทธานอลของสมุนไพรในตำรับที่มีฤทธิ์ต้านเอนไซม์ดังกล่าวได้มี 4 ชนิดได้แก่ หัวบัวบก กระชายดำ บอระเพ็ดพุงช้างและพริกไทยดำ โดยสามารถยับยั้งการทำงานเอนไซม์ที่ความเข้มข้น 0.1 มก./มล.ได้เป็นร้อยละ 89.20, 64.08, 59.25, 49.96 และมีค่าความเข้มข้นที่ยับยั้งได้ร้อยละ 50 (IC_{50}) เป็น 5.68, 20.64, 29.82, 30.67 ไมโครกรัม/มล.ตามลำดับ ส่วนสมุนไพรชนิดอื่นและตำรับรวมไม่มีผลต้านเอนไซม์ดังกล่าวแต่มีฤทธิ์ต้านอนุมูลอิสระได้ ควรมีการศึกษาถึงกลไกการออกฤทธิ์อื่นเพิ่มเติมเพื่อพิสูจน์ตำรับยานี้