

Exploring the Enzymatic-Based Biological Activities of *Kratom* Leaves Extracts on Alpha-Amylase, Alpha-Glucosidase, Angiotensin-Converting Enzyme, and Cholinesterases

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Abstract

Introduction and Objective: *Kratom* leaves (*Mitragyna speciosa* Korth) have been an integral part of traditional Thai medicine and local folk medicine for a long time. In Thailand, it has recently been legalized for use as a medicinal plant or traditional Thai medicine. Consequently, *kratom* has gained popularity as a medicinal plant based on people's beliefs. This study aimed to investigate the enzymatic-based biological activities of *kratom* extracts, specifically their anti-diabetic, anti-hypertensive, and anti-Alzheimer's disease properties, in order to provide scientific information for further development of *kratom* for medical use.

Methods: The *kratom* extracts used in this study were 95%, 70%, and 50% ethanolic extracts obtained from maceration, as well as an aqueous extract obtained from decoction method. All extracts were evaluated for inhibitory activities on alpha-glucosidase and alpha-amylase for anti-diabetes while its effect on angiotensin-I converting enzyme was examined for anti-hypertension. For the anti-Alzheimer's disease property, the inhibitory effects on acetylcholinesterase and butyrylcholinesterase were investigated.

Results: The study found that *kratom* extracts exhibited inhibitory effects on acetylcholinesterase, butyrylcholinesterase, and alpha-glucosidase enzyme. The 95% ethanolic extract exhibited the highest activity on butyrylcholinesterase and acetylcholinesterase, with the half inhibitory concentration (IC_{50}) values of $64.40 \pm 16.74 \mu\text{g/mL}$ and $127.99 \pm 6.15 \mu\text{g/mL}$, respectively. The 70% and 50% ethanolic extracts demonstrated comparable effects on alpha-glucosidase enzyme, with IC_{50} values of $382.64 \pm 16.18 \mu\text{g/mL}$ and $409.43 \pm 19.14 \mu\text{g/mL}$, respectively.

However, all extracts were inactive on angiotensin-I converting enzyme and alpha-amylase enzyme. The content of mitragynine in the 95%, 70%, 50% ethanolic extracts and the aqueous extracts were 54.00 ± 1.17 , 35.14 ± 0.64 , 25.05 ± 0.05 , and 18.08 ± 0.30 mg/g, respectively.

Discussion: *Kratom* extracts showed the most potential as butyrylcholinesterase and acetylcholinesterase inhibitors, and exhibited moderate activity in inhibiting alpha-glucosidase. The 95% ethanolic extract exhibited the highest mitragynine content, which was correlated with its ability to inhibit acetylcholinesterase and butyrylcholinesterase enzymes. This study was limited to explore only the *in vitro* enzymatic-based assays which require additional *in vitro* and *in vivo* assay models to conclude the possibility of using kratom as a medicine for patients or as dietary supplement.

Conclusion and Recommendation: The findings of this study suggested the inhibitory effects of *kratom* extracts on acetylcholinesterase, butyrylcholinesterase and alpha-glucosidase. Moreover, further *in vitro* and *in vivo* assay models as well as clinical trials are necessary to confirm its potential for development as a traditional medicine, or dietary supplement.

Key words: *kratom*, *Mitragyna speciosa*, Alzheimer, alpha-glucosidase, butyrylcholinesterase

การศึกษาฤทธิ์ทางชีวภาพด้วยปฏิกิริยาเอนไซม์ของสารสกัดใบกระต่อมต่อ แอลฟา-อะไมเลส, แอลฟา-กลูโคซิเดส, แองจิโอเทนซิน-คอนเวอร์ติง เอนไซม์ และ โคลิโนเอสเตอเรส

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บทคัดย่อ

บทนำและวัตถุประสงค์: กระต่อม (*Mitragyna speciosa* Korth) เป็นพืชที่มีการใช้ในการแพทย์แผนไทยและการแพทย์พื้นบ้านมาอย่างยาวนาน ปัจจุบันในประเทศไทยกระต่อมเป็นพืชที่ถูกกฎหมายโดยมีจุดประสงค์เพื่อใช้ประโยชน์ทางการแพทย์เป็นพืชสมุนไพรและยาแผนไทย กระต่อมจึงได้รับความสนใจอย่างแพร่หลายและมีการนำมาใช้ในการรักษาอาการต่าง ๆ ตามความเชื่อของผู้ใช้ การศึกษานี้มีวัตถุประสงค์เพื่อศึกษาฤทธิ์ทางชีวภาพของสารสกัดจากใบกระต่อม โดยใช้ปฏิกิริยาเอนไซม์ที่เกี่ยวข้องกับการรักษาเบาหวาน ความดันโลหิตสูงและอัลไซเมอร์ เพื่อเป็นข้อมูลทางวิทยาศาสตร์ในการใช้กระต่อมเพื่อประโยชน์ทางการแพทย์

วิธีการศึกษา: สารสกัดใบกระต่อมที่ใช้ในการศึกษาประกอบด้วย สารสกัดจากการหมักด้วย 95%, 70%, 50% เอทานอล และสารสกัดจากการต้มด้วยน้ำ ศึกษาฤทธิ์ด้านเบาหวานโดยฤทธิ์ยับยั้งเอนไซม์แอลฟา-กลูโคซิเดส และแอลฟา-อะไมเลส ฤทธิ์ลดความดันโลหิตโดยการยับยั้ง แองจิโอเทนซิน 1-คอนเวอร์ติงเอนไซม์ (angiotensin-I converting enzyme) และฤทธิ์ต้านอัลไซเมอร์โดยการยับยั้งเอนไซม์อะเซทิลโคลีนเอสเตอเรสและบิวทิลโคลีนเอสเตอเรส

ผลการศึกษา: สารสกัดใบกระต่อมมีฤทธิ์ในการยับยั้งเอนไซม์อะเซทิลโคลีนเอสเตอเรส, บิวทิลโคลีน-

เอสเตอเรส และ แอลฟา-กลูโคไซด์ โดยสารสกัด 95% เอทานอล มีฤทธิ์สูงสุดในการยับยั้งเอนไซม์บิวทิลโคลีน-เอสเตอเรส และ อะเซทิลโคลีนเอสเตอเรส โดยมีค่าความเข้มข้นที่ยับยั้งได้ครึ่งหนึ่ง (IC_{50}) เท่ากับ 64.40 ± 16.74 และ 127.99 ± 6.15 ไมโครกรัมต่อมิลลิกรัม ตามลำดับ สารสกัด 70% และ 50% เอทานอล มีฤทธิ์ยับยั้งเอนไซม์แอลฟา-กลูโคไซด์สูงสุด มีค่า IC_{50} เท่ากับ 382.64 ± 16.18 และ 409.43 ± 19.14 ไมโครกรัมต่อมิลลิกรัม ตามลำดับ สารสกัดทุกชนิดไม่มีฤทธิ์ยับยั้งเอนจิน 1-คอนเวอร์ตเอนไซม์ และ เอนไซม์แอลฟา-อะไมเลส ปริมาณสารไมตราไจนีน (mitragynine) ในสารสกัดเอทานอล 95%, 70%, 50% และสารสกัดน้ำ มีค่าเท่ากับ 54.00 ± 1.17 , 35.14 ± 0.64 , 25.05 ± 0.05 , และ 18.08 ± 0.30 มิลลิกรัมต่อมิลลิกรัม ตามลำดับ

อภิปรายผล: สารสกัดกระท่อมมีฤทธิ์มากที่สุดในการยับยั้งเอนไซม์ บิวทิลโคลีนเอสเตอเรส ตามด้วย เอนไซม์อะเซทิลโคลีนเอสเตอเรส และมีฤทธิ์ปานกลางในการยับยั้งเอนไซม์แอลฟา-กลูโคไซด์ สารสกัดเอทานอล 95% มีปริมาณสารไมตราไจนีนสูงที่สุดสอดคล้องกับฤทธิ์ยับยั้งเอนไซม์อะเซทิลโคลีนเอสเตอเรส และ บิวทิลโคลีนเอสเตอเรส การศึกษานี้เป็นการศึกษาเฉพาะปฏิกิริยาเอนไซม์ในหลอดทดลองซึ่งจำเป็นต้องมีการศึกษาอื่นทั้งในหลอดทดลองและในสัตว์ทดลองเพื่อสรุปความเป็นไปได้ในการใช้กระท่อมเป็นยาสำหรับผู้ป่วยหรือผลิตภัณฑ์เสริมอาหาร

ข้อสรุปและข้อเสนอแนะ: การศึกษานี้บ่งชี้ว่าสารสกัดใบกระท่อมมีฤทธิ์ยังการทำงานของเอนไซม์อะเซทิลโคลีนเอสเตอเรส, บิวทิลโคลีนเอสเตอเรส และแอลฟา-กลูโคไซด์ แต่อย่างไรก็ตามจำเป็นต้องศึกษาฤทธิ์ทางชีวภาพอื่นๆ ทั้งในหลอดทดลองและในสัตว์ทดลอง รวมถึงงานวิจัยเชิงคลินิกเพื่อยืนยันศักยภาพของกระท่อมสำหรับพัฒนาเป็นผลิตภัณฑ์ยาแผนไทยหรือผลิตภัณฑ์เสริมอาหารต่อไป

คำสำคัญ: พืชกระท่อม, *Mitragyna speciosa*, อัลไซเมอร์, แอลฟา-กลูโคไซด์, บิวทิลโคลีนเอสเตอเรส

Introduction and Objectives

Mitragyna speciosa Korth or Thai name, Kratom, belongs to Rubiaceae family, is a deciduous or evergreen tree. It is native to Southeast Asia and can be found in countries such as Thailand, Malaysia, Indonesia, the Philippines and New Guinea, along the Peninsular Malaysia and Borneo.^[1] In Thailand, Kratom has first illegal since A.C.1943 under the Kratom Plant Act, B.E.2486^[2], and later classified as category V narcotic under the Narcotic Act, B.E.2522 (1979), prohibiting all consumption, cultivation, trade, and distribution.^[3] However, in the recent years, Kratom has become legal again in Thailand under the

Narcotic Act (No.8) B.E.2564, with the legislative intention of utilizing it as an herbal or traditional medicines.^[4]

In traditional Thai medicine (TTM), Kratom has long been utilized as an ingredient in several TTM remedies, but it has never been used as a single herb in TTM practice. The “A-Ti-Sa-Ra” (Thai word) scripture documented the use of Kratom as an ingredient in anti-diarrheal or anti-dysentery remedies, such as the “Ha-Nu-Man-Jong-Tha-Non” (Thai word) remedy. Kratom is also mentioned in the “Cha-Wa-Darn” (Thai word) scripture as an ingredient in the “Klom-Ar-Rom” (Thai word) remedy, which is utilized to treat

gastroesophageal reflux disease (GERD)-like symptoms.^[5] “Khun-So-Pit-Ban-Na-Ruk” (Thai name), a traditional Thai doctor, documented four Kratom-containing remedies for treating diarrheal or dysentery symptoms in a scripture titled “Paet-Thai-Pan-Bo-Ran” (Thai word).^[6] According to TTM theory, these anti-diarrheal or anti-dysentery remedies are utilized to treat rotten pus, rotten phlegm, and rotten blood conditions.^[6] In local folk medicine, Kratom leaf has been utilized as single herb and ingredient in remedy for various reasons, such as treating diarrhea or dysentery, muscle pain, hemorrhoids, acting as an antipsychotic, serving as a substitute medication for opium addicts, and even enhancing labor performance.^[7]

Kratom has been investigated for its various pharmacological properties, including analgesic activity^[8-9], anti-oxidant^[10], anti-diabetes^[11-12], anti-inflammation^[13] anti-depression^[14-15], antibacterial^[10], antiproliferation activity^[16] and anti-Alzheimer’s disease^[17]. Innok and colleagues demonstrated the molecular docking and in vitro experiments on mitragynine and Kratom extract to explore AChE inhibition. They found that methanolic Kratom extract and mitragynine exhibited significant in vitro anti-AChE activity^[17]. However, they did not explore BuChE inhibition. Limcharoen and co-workers reported the potential inhibitory effects of Kratom extracts on the alpha-glucosidase enzyme without

investigating alpha-amylase^[11]. Two other studies also examined the inhibitory effect of Kratom extract on alpha-glucosidase; nevertheless, all prior investigations missed the examination of alpha-amylase inhibition^[18-19]. Regarding phytochemicals constituents, previous researches have revealed the phytochemical constituents of Kratom leaves including alkaloids, flavonoids, triterpenoids, saponins, and glycosides. The most prominent of these compounds is the indole alkaloids, of which mitragynine, is the major component with other minors such as 7-hydroxymitragynine, paynantheine, speciogynine, speciociliatine^[20-21].

Since the restrictions of Kratom has unlocked in Thailand, it has become increasingly popular for people to use it as a medicine, based on their beliefs, such as for anti-diabetes, anti-hypertension. To clarify the scientific validity of Kratom, this study aimed to investigate the enzymatic based biological activities of Kratom leaves extracts in relation to diabetes, hypertension, and Alzheimer’s disease, using enzymatic assays, including alpha-glucosidase, alpha-amylase, angiotensin-I converting enzyme (ACE), acetylcholinesterase (AChE), and butyrylcholinesterase (BuChE). The findings of this study demonstrated the potential of Kratom in scientific manner and provided information for the development of Kratom as a traditional medicine, dietary supplement or

functional food. However, additional in-depth *in vitro*, *in vivo* and clinical studies on efficacy of Kratom should be conducted to confirm its potential.

Methodology

1. Plant materials and preparations

Kratom leaves were collected from Trang province, Thailand, in December 2021 and authenticated by the Medical Plant Research Institute, Department of Medical Science, Thailand. The plant specimen (voucher number DMSC 5290) was deposited at the herbarium of Medicinal Plant Research Institute, Department of Medical Sciences, Ministry of Public Health, Nonthaburi, Thailand.

Kratom leaves were washed and then dried in a hot air oven at a temperature of 45°C. The dried Kratom leaves were ground into coarse powder and were extracted separately by maceration, and decoction methods. For maceration, the coarse powder of Kratom leaves were separately macerated with 95% ethanol, 70% ethanol, and 50% ethanol. Each extract was filtered and dried under reduced pressure at a temperature below 45°C. The residue was re-extracted by a similar process twice. For decoction, Kratom leaves were boiled in distilled water for 15 minutes. The extract was filtered with filter paper. The residue was re-extracted by a similar process

twice. The extracts were combined and dried by using a freeze dryer. All extracts were stored at -20°C until used.

2. Chemicals and reagents

Donepezil and captopril were purchased from USP (Rockville, MD, USA). and acarbose were purchased from Sigma (Darmstadt, Germany). They were utilized as positive control in their corresponded enzymatic assays. In anti-Alzheimer's assay, acetylcholinesterase enzyme (AChE), butyrylcholinesterase enzyme (BuChE), acetylthiocholine iodide (ACTI), butyrylthiocholine iodide (BuTI), 5,5'-dithiobis-2-nitrobenzoic acid (DNTB), bovine serum albumin (BSA) and tris-(hydroxymethyl) aminomethane (Tris-base) were purchased from Sigma (Darmstadt, Germany). For antihypertension, angiotensin-converting enzyme (ACE) from rabbit lung, Hippuryl-His-Leu (HHL) and Tris-HCl were purchased from Sigma (Darmstadt, Germany). Purified water was prepared by the Milli Q® system from Millipore (Bedford, MA, USA). Acetonitrile (HPLC grade) was purchased from Labscan (Bangkok, Thailand). In anti-diabetes assay, alpha-glucosidase and alpha-amylase enzymes, p-nitrophenyl alpha-D-glucopyranoside (*p*-NPG), starch, potassium sodium tartrate, tetrahydrate, 3,5-dinitrosalicylic acid were purchased from sigma (Darmstadt, Germany).

3. *In vitro* inhibitory effect on alpha-glucosidase

The inhibitory activity of Kratom extracts on alpha-glucosidase was investigated *in vitro* using a method from Gowri et al.^[22]. The hydro-alcoholic extracts were prepared in DMSO at a concentration of 20 mg/mL, the aqueous extract was prepared in water. Working sample solutions were prepared at concentrations of 100, 1,000, 2,000, 5,000, and 10,000 $\mu\text{g/mL}$ by diluting the stock solution with phosphate buffer pH 6.8. DMSO in buffer at concentrations at 0.5, 5, 10, 25 and 50%v/v were utilized as control solvent at their responsive concentration, whereas water was utilized as the control for the aqueous extract. The enzymatic reaction was initiated by adding 80 μL of phosphate buffer pH 6.8, 20 μL of sample solution, and 50 μL of alpha-glucosidase (0.15 unit/mL) to each well of a 96-well plate. After shaking, the plate was incubated at 37°C for 15 minutes. Next, 50 μL of the substrate, 5 mM p-nitrophenyl-D-glucopyranoside (p-NPG) in water, was added to the wells and incubated at 37°C for another 15 minutes. The final concentrations of the extracts in enzymatic reaction were 10, 100, 200, 500, and 1,000 $\mu\text{g/mL}$. To terminate of the enzymatic reaction, 100 μL of 1M sodium carbonate was added. The reaction product (p-nitrophenol) was determined by measuring the absorbance at a wavelength of 405 nm using a microplate reader. The inhibitory activity

was calculated using the following equation, and the IC_{50} of the extract was determined using curve fitting regression analysis with the Prism program (GraphPad Prism 5.0, CA, USA).

$$\text{Inhibition (\%)} = \frac{\text{OD}_{\text{control}} - \text{OD}_{\text{sample}}}{\text{OD}_{\text{control}}} \times 100$$

Where, $\text{OD}_{\text{control}}$ represented the optical density of the control sample and $\text{OD}_{\text{sample}}$ represented the optical density of the tested sample.

4. *In vitro* inhibitory activity on alpha-amylase

The alpha-amylase inhibitory effect of Kratom extracts was investigated using the method described by Ademiluyi and colleagues.^[23] The stock solutions of hydro-alcoholic extracts were prepared in DMSO, while aqueous solutions were prepared in water at a concentration of 10 mg/mL. Working sample solutions were then prepared by diluting the stock solution with phosphate buffer pH 6.9 to achieve concentrations of 2,500 and 5,000 $\mu\text{g/mL}$. DMSO in buffer at a concentration of 50% was used as control solvent for hydro-alcoholic extracts and water was used for aqueous extract. Next, in a reaction microtube, 50 μL of the sample solution was mixed with 50 μL of alpha-amylase (0.5 mg/mL in pH 6.9 phosphate buffer) and 100 μL of 0.02 M sodium phosphate buffer pH 6.9 containing 0.006 M sodium chloride. The mixture was then incubated in

an oven at 37°C for 10 minutes. After that, 50 µL of 1% starch solution in pH 6.9 buffer was added to the reaction tube and the mixture was incubated again at 37°C for 20 minutes. The final concentrations of the extracts in the enzymatic reaction were 500 and 1,000 µg/mL. The enzyme-substrate reaction was stopped by adding 100 µL of DNS reagent (96 mM 3,5-dinitrosalicylic acid, 3.5 M sodium potassium tartrate in 2 M NaOH) to the reaction microtube. The reducing sugar produced during the reaction reacted with the DNS reagent by placing the reaction tube in a heat box at a temperature of 90-100°C for 5 minutes. After that, the reaction microtube was immediately placed on ice for 5 minutes and 650 µL of water was added and mixed. The mixture solution (200 µL) was transferred to a 96-well plate and the optical density of the reaction was determined by a microplate reader at a wavelength of 540 nm. The percentage inhibition was calculated using the following equation. The IC₅₀ of the extract was calculated by curve-fitting regression analysis using the Prism program.

$$\text{Inhibition (\%)} = \frac{\text{OD}_{\text{control}} - \text{OD}_{\text{sample}}}{\text{OD}_{\text{control}}} \times 100$$

Where, OD_{control} represented the optical density of the control sample and OD_{sample} represented the optical density of the tested sample.

5. *In vitro* inhibitory activity on angiotensin-I converting enzyme

The effect Kratom extracts on ACE inhibition was investigated using method of Lahogue and co-workers with slight modification.^[24] Briefly, a reaction mixture containing 40 µL of 0.1 M sodium borate buffer pH 8.3 was mixed with 20 µL of 0.1 unit/mL of ACE in buffer and incubated at 37°C for 5 minutes. Next, 20 µL of the sample solution and 40 µL of the substrate (7 mM HHL) were added into the reaction mixture, which was then incubated at 37°C for 30 minutes. The reaction was stopped by adding 100 µL of 0.1M HCl and determined the reaction product using HPLC. Stock sample solutions of hydro-alcoholic and aqueous extracts were prepared at a concentration of 50 mg/mL in DMSO and water, respectively. Working solutions of sample were prepared by diluting the stock solution with buffer to produce solution of 6 mg/mL (final concentration 1000 µg/mL). For IC₅₀ evaluation, working solutions in buffer were prepared at concentration, 0.6, 3, 6 and 12 mg/mL (final concentration 100, 500, 1000 and 2000 µg/mL). Captopril was utilized as positive control and the HPLC analysis was performed on a C18 column (4.6 mm i.d. × 150 mm length), particle size 5 µm column using a Dionex® Ultimate 3,000 HPLC system. The isocratic mobile phase consisted of 0.1% trifluoroacetic acid and acetonitrile at ratio of

75 : 25 and the flow rate was set at 1.0 mL/min. The reaction product (hippuric acid) and the remained substrate (HHL) were detected at a wavelength of 228 nm. The peak area of hippuric acid was utilized to calculate the %inhibition as the following equation and the IC_{50} was calculate by Prism software.

$$\text{Inhibition (\%)} = \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \times 100$$

Where, A_{control} represented the peak area of the control solvent and A_{sample} represented the peak area of the tested sample.

6. *In vitro* inhibitory activity on acetylcholinesterase and butyrylcholinesterase

The inhibitory activity of AChE/BuChE enzymes was evaluated using Ellman's method with slight modification.^[25-26] Sample stock solutions of the extracts were prepared at a concentration of 50 mg/mL in DMSO for the hydro-alcoholic extract and in water for the aqueous extract. A stock solution of donepezil in DMSO (2 mg/mL) was utilized as positive control. The working sample solutions at concentrations of 12.5, 25, 125, 325, 1,250 and 2,500 $\mu\text{g/mL}$ were prepared by sequential dilution of the stock sample solution with buffer I (50 mM TRIS buffer pH 8.0). DMSO in buffer I at concentration of 0.025, 0.25, 1.25, 2.5, and 5%v/v were utilized as control solvent. The final concentrations of sample in reaction were 1, 10, 50, 100, and 200 $\mu\text{g/mL}$. The assay was

initiated by mixing 70 μL of buffer I with 20 μL of substrate (1.5 mM ACTI for AChE or BuTI for BuChE) in a well of a 96-well plate. Then, 20 μL of sample, 120 μL of freshly prepared 3 mM DNTB, and 20 μL of AChE (for AChE inhibitory activity) or BuChE (for BuChE inhibitory activity) were added to the mixture. The plate was incubated in room temperature for 30 minutes and the enzyme activity was measured using a microplate reader at 405 nm. Percentage of inhibition was calculated using the following equation.

$$\text{Inhibition (\%)} = \frac{OD_{\text{control}} - OD_{\text{sample}}}{OD_{\text{control}}} \times 100$$

Where, OD_{control} represented the optical density of the control sample and OD_{sample} represented the optical density of the tested sample.

7. Content of mitragynine in the extracts

Mitragynine, the major compound in Kratom leaves, was determined using a high-performance liquid chromatography (HPLC) method following the procedure described by Sharma and colleagues with modification^[27]. The chromatographic system consisted of C18 column (4.6 mm i.d. $\times$ 150 mm length), particle size 5 μm column using a Dionex[®] Ultimate 3,000 HPLC system. The mobile phase consisted of acetonitrile (A) and 0.1%v/v formic acid in 10 mM ammonium formate. The gradient elution of mobile phase was programmed

as following: 0-5 min, 5%A : 95%B; 5-20 min, 5%A : 95%B - 20%A : 80%B; 20-30 min, 20%A : 80%B - 70%A : 30%B; 30-35 min, 70%A : 30%B; 35-40 min 5%A : 95%B. The flow rate was set at 1.0 mL/min, and the analysis was conducted at room temperature. Mitragynine was detected at a wavelength of 245 nm. Standard solutions were prepared with concentrations ranging from 10 to 250 $\mu\text{g/mL}$. To ensure the quality of the analysis, quality control (QC) samples were prepared by spiking standard solution into sample solution at concentrations 15, 75 and 150 $\mu\text{g/mL}$. The extracts were prepared by diluting with methanol and filtered through a 0.45 μm membrane filter prior to injection into the HPLC system.

8. Statistical analysis

The experiments were conducted in triplicate and the results were reported as mean $\pm$ standard deviation (SD). The IC_{50} values were calculated by using a regression analysis. The differences between groups were statistically analyzed using one-way ANOVA with post hoc analysis. GraphPad Prism software (CA, USA) was utilized for statistical analysis.

Results

The extraction yields of the Kratom extracts are shown in Table 1. The 70% ethanol extract (KE70) exhibited the highest extraction yield, whereas the 95% ethanol extract (KE95) demonstrated the lowest.

Table 1 Extraction yield of Kratom leaves extracts

Extraction solvent	Extraction method	Code	Extraction Yield (%)
95% Ethanol	maceration	KE95	18.8
70% Ethanol	maceration	KE70	36.2
50% Ethanol	maceration	KE50	34.5
Water	decoction	KDec	31.5

1. *In vitro* inhibitory effect on alpha-glucosidase and alpha-amylase

The anti-diabetes activity of the Kratom extracts were evaluated using alpha-glucosidase and alpha-amylase enzymes. Prior to determining IC_{50} , the % inhibition of the Kratom extracts was screened, as shown

in Figure 1. All extracts at a concentration of 1,000 $\mu\text{g/mL}$ demonstrated activity of less than 50%, indicating that they were ineffective in inhibiting the alpha-amylase enzyme. However, for alpha-glucosidase, KE70 and the 50%etahnolic extract (KE50) exhibited inhibitory activity greater than 50%, and were thus

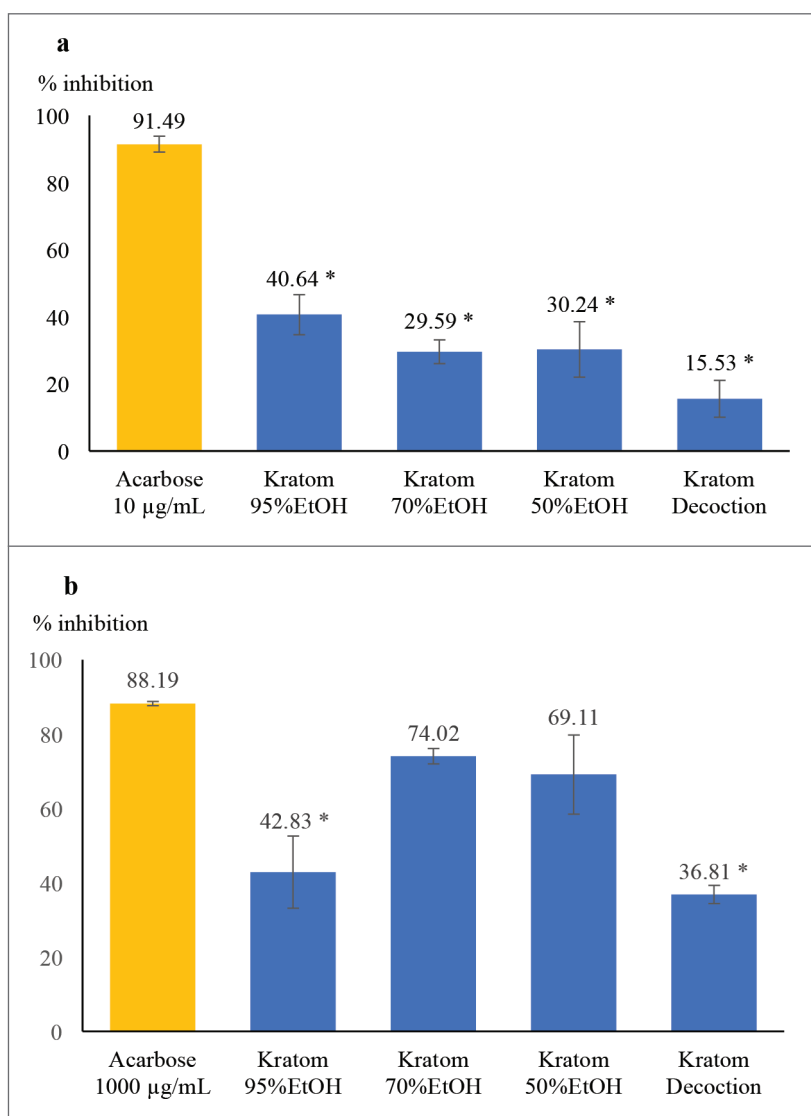


Figure 1 Inhibitory activity (% inhibition) of Kratom extracts (1,000 µg/mL) and acarbose on alpha-amylase (a) and alpha-glucosidase (b). *Significant difference from the positive control, Acarbose, analyzing by one-way ANOVA with Dunnett's test.

evaluated for IC_{50} . As demonstrated in Table 2, KE70 and KE50 exhibited comparable inhibitory effects on alpha-glucosidase with IC_{50} values of 382.64 ± 16.18 and 409.43 ± 19.14 µg/

mL, respectively. Nonetheless, their activity was less than acarbose, which was used as a positive control ($IC_{50} = 115.96 \pm 15.20$ µg/mL).

Table 2 Inhibitory activity of Kratom extracts on alpha-glucosidase and alpha-amylase enzymes.

Kratom Extracts	Inhibitory activity (IC ₅₀ ; µg/mL)	
	alpha-amylase	alpha-glucosidase
KE95	> 1000*	> 1000*
KE70	> 1000*	382.64 ± 16.18*
KE50	> 1000*	409.43 ± 19.14*
KDec	> 1000*	> 1000*
Acarbose	7.43 ± 0.71	115.96 ± 15.20

*Significant difference from the positive control, Acarbose, analyzing by one-way ANOVA with Dunnett's test.

2. *In vitro* inhibitory effect on angiotensin-I converting enzyme (ACE)

The inhibitory activity of Kratom extracts on ACE was investigated to determine their potential as an anti-hypertensive medicine. The extracts were screened at a concentration of 1,000 µg/mL. All extract demonstrated the %inhibition significantly less than 50%, except for the aqueous extract (KDec), which exhib-

ited the inhibition level of nearly 50% (data not shown). Consequently, KDec was later evaluated to determine its IC₅₀ value. However, the IC₅₀ value of KDec was found to be 1056.08 ± 3.4 µg/mL. Captopril was utilized as a positive control and demonstrated potent effects with an IC₅₀ value of 0.007 ± 0.0004 µg/mL (Table 3).

Table 3 Inhibitory activity of Kratom extracts on angiotensin-I converting enzyme (ACE).

Kratom Extract	Inhibitory activity (IC ₅₀ ; µg/mL)
KE95	> 1000*
KE70	> 1000*
KE50	> 1000*
KDec	1051.19 ± 8.80*
Captopril	0.007 ± 0.0004

*Significant difference from the positive control, Captopril, analyzing by one-way ANOVA with Dunnett's test.

3. *In vitro* inhibitory activity on acetylcholinesterase and butyrylcholinesterase

The inhibitory effects of Kratom extracts on AChE and BuChE were investigated as a model of anti-Alzheimer's disease. All extracts tended to have inhibitory activity on both AChE and BuChE enzymes. Consequently, the IC₅₀ of all extracts were evaluated, as shown in Figure 2. Of all the extracts, KE95 exerted the

highest inhibitory activity on BuChE, similar to AChE, with IC₅₀ of $64.40 \pm 16.74 \mu\text{g/mL}$ and $127.99 \pm 6.15 \mu\text{g/mL}$, respectively. However, all extracts exhibited lower inhibitory effects than the positive control, donepezil. Additionally, Kratom extracts were found to have better inhibitory activity on BuChE as compared to AChE.

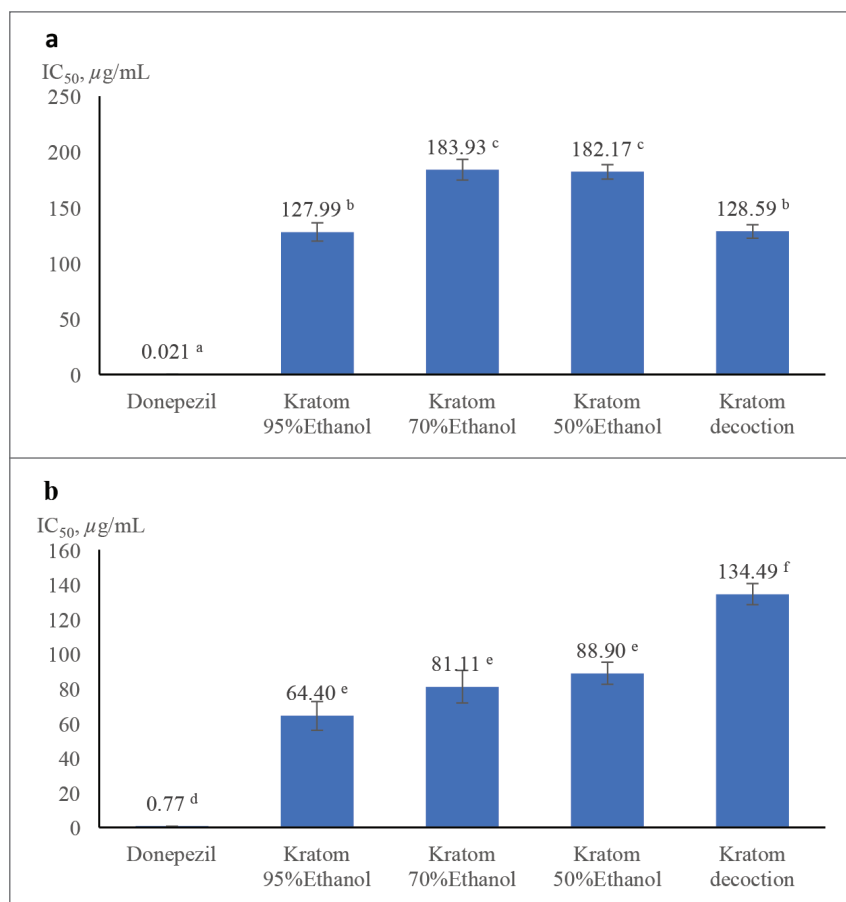


Figure 2 Inhibitory activity (IC₅₀, $\mu\text{g/mL}$) of Kratom extracts on acetylcholinesterase (a) and butyrylcholinesterase (b). The different alphabets above the bar graph show statistically significant differences (p -value < 0.05) analyzing by one way ANOVA with Tukey's multiple comparison test. (AChE: a-c; BuChE: d-f).

In summary, all extracts exhibited inhibitory effects on AChE and BuChE enzymes, with KE95 demonstrating the most significant effect on BuChE. The KE70 and KE50 also exerted moderate inhibition of alpha-glucosidase, while aqueous extract (KDec) exhibited low inhibitory activity on ACE. All extracts were found to be ineffective in inhibiting alpha-amylase and ACE enzymes.

4. Content of mitragynine

The retention time of mitragynine in

sample solution was 26.3 minute (Figure 3) and UV spectrum of mitragynine peak in sample solution corresponded to the peak of standard solution (Figure 4a). The linearity of calibration curve determining by the coefficient of determination (r^2) was 0.9997 (Figure 4b) and the recovery of the QC samples ranged from 97.26% to 102.34%. The content of mitragynine in KE95, KE70, KE50 and KDec samples was determined to be 54.00 ± 1.17 , 35.14 ± 0.64 , 25.05 ± 0.05 , and 18.08 ± 0.30 mg/g, respectively.

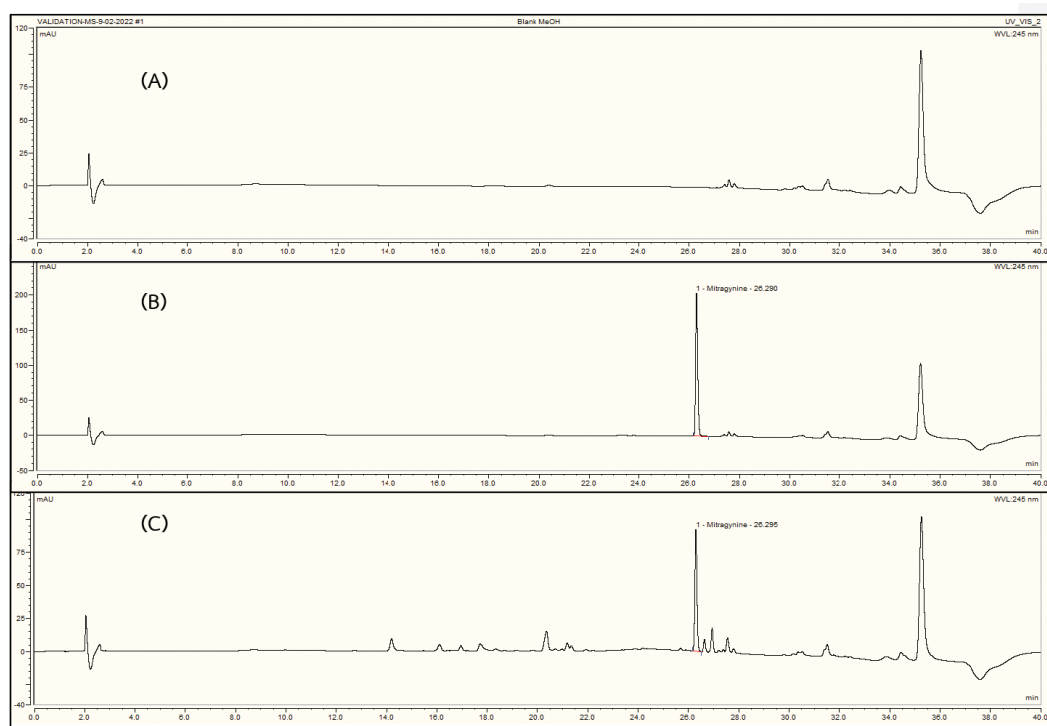


Figure 3 Chromatogram of diluent (methanol) (A), mitragynine standard solution (B) and sample solution (C).

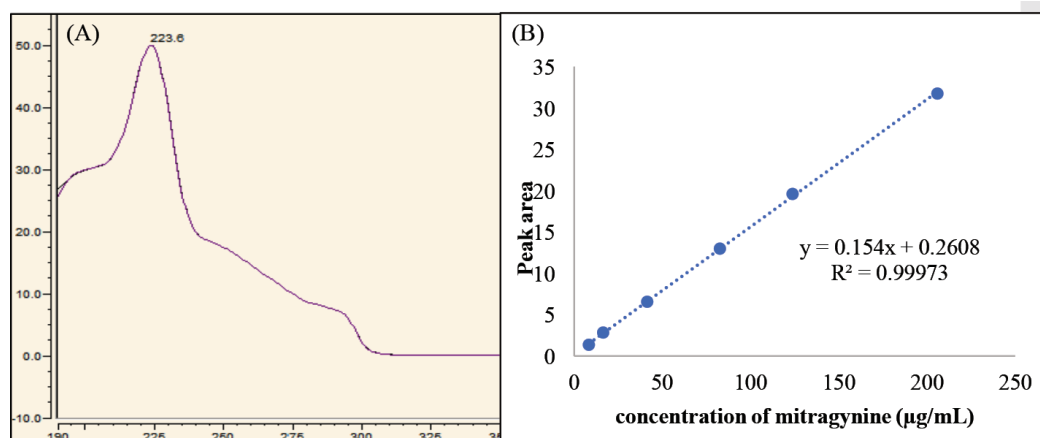


Figure 4 Overlay spectrum of mitragynine peak in sample solution and standard solution (A) and calibration curve of mitragynine at various concentrations (B).

Discussion

Kratom has a long and rich history of use in Thailand particularly as an ingredient in TTM remedies, but it has never been utilized as single herb in TTM practice. However, local folk medicine, especially in southern Thailand, has a tradition of chewing Kratom leaves for variety of reasons, including the treatment of diabetes, hypertension, and even to boost work performance^[7]. The objective of this study was to investigate the enzymatic-based biological activities related to anti-diabetes, anti-hypertension, and anti-Alzheimer's disease based on both traditional medicine and the folk usage of Kratom leaves, in order to support the legislative intention of unlocking its use as an herbal or traditional medicine.

In this study, the inhibitory effects of Kra-

tom ethanolic and aqueous extracts on AChE and BuChE were reported for the first time, indicating that Kratom potentially be used in Alzheimer's disease. AChE and BuChE inhibitors are crucial targets for drugs used in treating Alzheimer's patients since they inhibit acetylcholine turnover, leading to restore the synaptic level of acetylcholine in patients.^[28] Innok and their colleagues have conducted a study on the inhibitory activity of AChE using molecular docking and confirmed with an *in vitro* experiment. They found that mitragynine, which is a main alkaloid in Kratom leaves extract exhibited a good binding affinity towards AChE. Their experiment also revealed that the methanolic Kratom extract and mitragynine exerted *in vitro* anti-AChE activities with IC_{50} values of 252.68 µg/mL and 105.12

$\mu\text{g/mL}$, respectively. However, in our study, all Kratom extracts exhibited higher anti-AChE activity compared to their methanolic Kratom extract, although mitragynine still showed superior activity. This finding confirmed the effect of Kratom on AChE inhibition^[17]. Interestingly, Kratom extract demonstrated the most significant inhibitory effects on BuChE, suggesting that it may provide substantial benefits for patients with chronic Alzheimer's disease.^[29] Additionally, the relationship between BuChE and the formation of β -amyloid plaques has been reported in the previous study^[30]. Other studies demonstrated that BuChE provided better benefits for late phase Alzheimer's patients due to their tolerance to AChE inhibitors.^[31-32] With regard to phytochemicals, we suggested that mitragynine and other indole alkaloids, which are prominent constituents of Kratom may play an important role in inhibition of AChE^[17] and BuChE. Furthermore, the high anti-oxidant activity of Kratom have been reported by several previous studies^[10,16,29] which may encourage the benefit of Kratom in Alzheimer's disease. Based on these reasons, we suggest that Kratom extract may have potential as AChE and BuChE inhibitors in Alzheimer's disease. Nevertheless, further investigations on activities associated with anti-Alzheimer's disease both *in vitro* and *in vivo*, including neuroprotective activity,

anti-inflammation, inhibition of tau protein aggregation and accumulation of amyloid β , as well as clinical trials, are necessary.

Regarding anti-diabetes, alpha-glucosidase inhibitors act by inhibiting the degradation of disaccharide to monosaccharide as the absorbable sugar formation causing the reduction of blood sugar level in diabetes patients^[33]. The KE70 and KE50 exhibited moderate inhibitory activity on alpha-glucosidase which was approximately three times lower than the positive drug, acarbose. These results were consistent with a previous study conducted by Limcharoen and colleagues, which demonstrated the potential inhibitory effects of Kratom extracts on the alpha-glucosidase enzyme.^[11] However, our study conducted different extraction solvent and method from their study. Another study by Purintrapiban and colleagues demonstrated that aqueous Kratom extract increased glucose transport to muscle cells by enhancing the activities of the key enzymes that depend on insulin-stimulated glucose transport for their acute action *in vitro*. Additionally, the extract increased the glucose transporters (GLUT1) in long-term effect.^[12] These suggested the potential of Kratom as an anti-diabetes, however, further investigation is required to identify the responsive constituents, and conduct *in vivo* studies and clinical trials.

For alpha-amylase, Kratom extracts were not effective on this enzyme indicating that Kratom was unable to inhibit the conversion of starch into soluble polysaccharides or disaccharides. As well as ACE, Kratom extracts did not exert inhibition of ACE, suggesting that Kratom is unlikely to be effective in reducing hypertension through this mechanism. In contrast, there has been report of Kratom intake leading to cardiovascular side effects, including tachycardia and hypertension.^[34] Patients should be advised for caution when considering Kratom intake.

Regarding the content of mitragynine, KE95 exhibited the highest concentration of mitragynine, while KDec displayed the lowest. The mitragynine content varies depending on the polarity of the extraction solvent. The inhibitory activity on BuChE tended to correlate with the mitragynine content. A higher concentration of mitragynine demonstrated greater potency in inhibiting BuChE, whereas a lower concentration of mitragynine tended to result in decreased inhibitory activity. These findings suggest that mitragynine may be associated with BuChE inhibition, however, other indole alkaloids should also be determined. In contrast to α -glucosidase, KE70 and KE50 exhibited the highest inhibitory activity. However, these results were not linked to the mitragynine content, indicating that other

compounds may play a significant role in this activity. Therefore, further research, such as bioassay-guided fractionation, is necessary to identify additional responsive markers for quality control purposes.

This study was limited to explore only the in vitro enzymatic-based assays which require additional in vitro and in vivo assay models to conclude the possibility of using Kratom as a medicine for patients or as dietary supplement.

Conclusion

Kratom leaves have long been utilized as traditional and folk medicines in Thailand. After it became legal, the widespread use of Kratom based on personal belief raised caution among certain patients. This study aimed to investigate the pharmacological potential of Kratom using enzymatic-based assays. The results of this study revealed that Kratom extracts demonstrated the most potential as BuChE inhibitors, which is relevant to anti-Alzheimer's disease treatment. As anti-diabetes, Kratom exhibited moderate activity in inhibiting alpha-glucosidase but did not inhibit alpha-amylase. Additionally, Kratom extracts did not demonstrate anti-hypertension effects as ACE inhibitor, instead, its side effects on tachycardia and hypertension should be cautioned. This study is the first to report on the inhibitory effects of Kratom extracts on BuChE,

ACE, and alpha-amylase. The findings from this study indicated the potential of Kratom extract as Alzheimer's disease and anti-diabetes. However, additional in vitro and in vivo assay models, as well as clinical trials are required to establish conclusively the potential of Kratom for use as a traditional medicine or dietary supplement. Furthermore, it is important to conduct further phytochemical studies, not limited to mitragynine, to identify bioactive markers which can be used for quality control during product development.

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Conflict of interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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