Acetylcholinesterase inhibition activity and phytochemical screening of red betel leaf (*Piper crocatum* Ruiz & Pav) as anti-dementia agents

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Abstract. Alzheimer's Disease (AD) is a neurodegenerative condition that results in progressive cognitive deterioration. The AD therapeutic approach with acetylcholinesterase inhibitors aims to boost cognitive function by raising acetylcholine levels in synaptic neurons. Piper crocatum Rui & Pav has antioxidant and anti-inflammatory properties, suggesting its potential to alleviate AD symptoms. This research aims to investigate the inhibitory activity of acetylcholinesterase (AChE) *in vitro* and identify the active compounds present in the fractions of red betel leaf. The methods of this research are Ellman's colorimetric method and Liquid Chromatography-Mass Spectrometry (LC-MS). The red betel leaf fractions demonstrated effective AChE inhibition, as reflected by their IC_{50} values: 11.0965 µg/ml (ethanol extract), 16.7908 µg/ml (ethyl acetate fraction), 23.7390 µg/ml (n-hexane fraction), and 41.0044 µg/ml (water fraction). The ethanol extract with the lowest IC_{50} value was analyzed by LC-MS. The result showed 200 active compounds, 28 of which had concentrations exceeding 0.5%. The predominant active compounds include flavonoids, steroids, polyphenols, alkaloids, phenolics, vitamins, and carboxylic acids. In conclusion, the ethanol extract of red betel leaf shows promising potential as an AChE inhibitor, suggesting its use as a therapeutic agent to enhance cognitive function in patients with Alzheimer's Disease.

1 Introduction

Dementia encompasses a range of brain conditions marked by cognitive decline, making it challenging for individuals to carry out daily tasks independently [1]. This cognitive impairment is persistent and worsening, going beyond typical aging effects. Dementia is the world's seventh most common cause of death [2]. Presently, about 55 million people have dementia worldwide and expected to rise untill 152 million by 2050 [3, 4]. Apart from its societal and health repercussions, dementia also brings a substantial financial burden. In 2019, it cost the world approximately US$ 1.3 trillion [5]. Given its growing prevalence and economic impact, research into dementia is vital.

Dementia is an umbrella term that includes various illnesses [6]. Every form of dementia presents distinct characteristics and pathological processes, yet they exhibit comparable clinical symptoms that typically deteriorate over time. Individuals afflicted with dementia

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commonly manifest various clinical symptoms, including a decline in memory retention, cognitive function, problem-solving skills, alterations in behaviour and mood, and challenges in communication [7, 8].

Alzheimer's disease (AD) is the predominant type present in 80% of dementia instances. The characteristic histopathological features of AD are the accumulation of β-amyloid plaques in the extracellular space and the walls of blood vessels (outside neurons), as well as the presence of abnormal tau protein inside neurons, result in the deterioration and demise of nerve cells [9]. Among those affected by AD, the cholinergic system's function is diminished, notably in brain regions crucial for cognitive processes. Cholinergic neurons, which release the neurotransmitter acetylcholine (ACh), suffer impairment and loss as AD advances, resulting in decreased ACh levels [10]. This decline significantly contributes to the clinical symptoms responsible for cognitive impairment in AD patients.

Therapy for AD is symptomatic and aims to enhance the well-being of those affected. Several medications classified as acetylcholinesterase inhibitors have been allowed by the FDA (Food and Drug Administration) to treat AD symptoms [11]. The main purpose of these AChE inhibitors, especially when used to treat AD, is to boost and maintain the presence of acetylcholine at synapses by blocking the activity of the enzyme AchE [12]. This process enhances signal transmission, leading to improved cognitive function in AD patients [13, 14]. At present, there is a growing interest in utilizing herbal plants as a therapeutic approach. These plants have been utilized for a considerable time to address symptoms or relieve discomfort linked to dementia or other cognitive challenges [15]. Herbal plants are regarded as therapeutic agents possessing multi-target characteristics, allowing them to engage with various mechanisms implicated in disease progression [16].

Compounds found in plants or derivatives thereof are the source of AChE inhibitors like donepezil, rivastigmine, and galantamine [17-19]. compounds typically sourced from the alkaloid group have demonstrated effectiveness in managing symptoms of AD. Additionally, AChE inhibitor compounds derived from natural origins can also be identified within various other phytochemical compound categories, including terpenes, sterols, and flavonoids [20]. This suggests that there is considerable potential for further development of plant-based treatments for Alzheimer's disease.

Piper crocatum, commonly known as red betel, has a longstanding history in traditional medicine. Numerous studies have investigated the potential benefits of red betel leaf and extracts across various health contexts. Multiple research findings suggest that red betel extract exhibits antioxidant [21-24], antibacterial [25], anticancer [26], anti-inflammatory [27], and antidiabetic properties [28, 29]. The antioxidant and anti-inflammatory properties of *Piper crocatum* may protect nerve cells from oxidative stress and inflammation, factors that causing to the onset and progression of neurodegenerative disorders [30]. This study aims to assess the in vitro activity of acetylcholinesterase inhibition and to identify active compounds present in the *Piper crocatum* extract fraction.

2 Research Methodology

2.1 Preparation of Red Betel Leaf Extract

The main material utilized in this research is red betel leaf (Piper crocatum) simplicia from the garden of the Tropical Biopharmaca Study Center at LPPM-IPB, Bogor, Indonesia. Moisture Content of Red Betel Leaf Simplicia. Moisture content analysis was performed to determine the quality of the simplicia [30].

2.1.1 Extraction of Red Betel Leaf Simplicia

Red betel leaf simplicia was extracted by 70% ethanol using maceration method for 24 hours at room temperature. The filtrate was concentrated using a rotary evaporator to obtain a crude extract [31]. The extract yield was expressed as a percentage using the following equation:

$$\text{Yield} = \{\text{extract weights}/[\text{simplicia weights} \times (1\text{-moisture content})]\} \times 100\% \quad (1)$$

2.1.2 Fractionation of Red Betel Leaf Simplicia

Fractionation was carried out using the separating funnel method in stages with 3 types of solvents, namely n-hexane, ethyl acetate and water. Each fraction was then concentrated using rotary evaporator [33]. The yield of each fraction, expressed as a percentage, was calculated using the following equation:

$$\text{Yield} = \frac{[\,(\frac{\text{fractionation weights}}{\text{extract weights}}) \times \text{total extract weights}\,]}{\text{total weight of simplicia} \times (1\text{-water content})} \times 100\% \quad (2)$$

2.2 Acetylcholinesterase Inhibitory Activity of Red Betel Leaf Extracts

The inhibition of acetylcholinesterase activity of red betel leaf extract was conducted using varying concentrations (10, 20, 30, and 50 ppm). Each treatment, including sample, positive control, negative control, and blank was added to the microplate according to the volume in Table 1. This measurement was carried out using the Elman's method which was measured at 408 nm.

Table 1. Volume of Reagents in AChE Inhibitory Activity Assay

Treatment / Reagents	Blank	Negative Control	Sampel
ACTh Mix (µL)	50	50	50
AChE (µL)	50	-	50
ddH_2O (µL)	50	50	-
Buffer (µL)	-	50	-
Samples (µL)	-	-	50

Enzyme activity and percentage inhibition are calculated using the following equation:

$$\text{Inhibition \%} = [(\text{negative control activity} - \text{sample activity})/\text{negative control activity}] \times 100\% \quad (3)$$

2.3 Identification of Active Compound Content using Liquid Chromatography–Mass Spectrometry

In this study, 10 mg of red betel leaf extract was dissolved in 10 mL of methanol and filtered with a 0.2 um sieve. Analysis of the active compound content was then carried out using Liquid Chromatography-Mass Spectrometry (LC-MS) with a flow rate of 0.20 mL/min. The mobile phases used are formic acid and acetonitrile. LC-separated components were identified by MS in Tof MSE mode with Positive Electrospray Ionization (P ESI), producing spectra showing the mass-to-charge ratio (m/z) [34].

2.4 Data Analysis

Statistical methods were used to analyze the impact of red betel leaf extract on acetylcholinesterase inhibition. The analysis was carried out using Minitab 17 software. One-Way ANOVA was applied at a 95% confidence level, with significant differences marked by a p-value < 0.05.

3 Results

The moisture content of red betel leaf simplicia was measured and found to be 8.38%. The simplicia was then extracted using 70% ethanol, followed by fractionation using 3 different solvents. The results showed that yield of ethanol extract, n-hexane, ethyl acetate and water are 13.27±0.24%, 1.00±0.06%, 3,93±0.56%, dan 7.80±0.62% respectively (Figure 1).

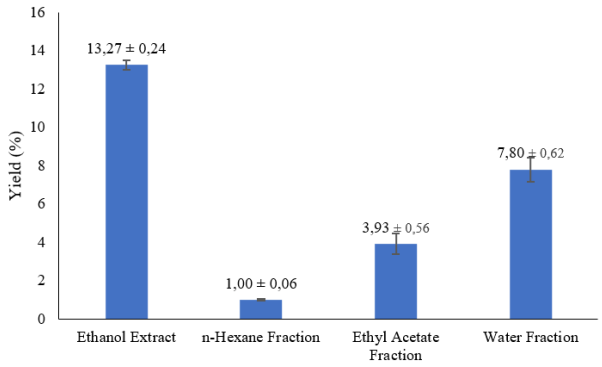

Fig. 1. Yield of red betel leaf extracts

These four red betel leaf extracts were tested for their ability to inhibit acetylcholinesterase activity using the Ellman's method. The test results showed that the ethanol extract had the best ability with an IC_{50} value of 11%, while the n-hexane, ethyl acetate, and water fractions had IC_{50} values of 23%, 16%, and 41%, respectively (Figure 2).

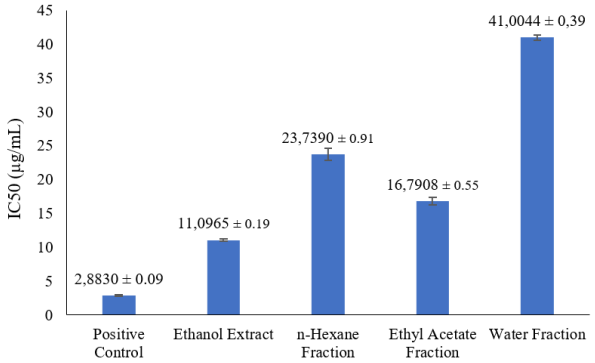

Fig 2. IC_{50} of red betel leaf extracts against acetylcholinesterase enzyme

The ethanol extract with the best inhibitory ability was then analyzed using LC-MS to identify the active compound components responsible for inhibiting acetylcholinesterase enzyme activity. Based on the LC-MS results, the ethanol extract of red betel leaf was found

to contain 200 compounds (Figure 3), with only those with high or significant concentrations analyzed further. From this analysis, a total of 28 compounds were identified, including 2 unknown compounds.

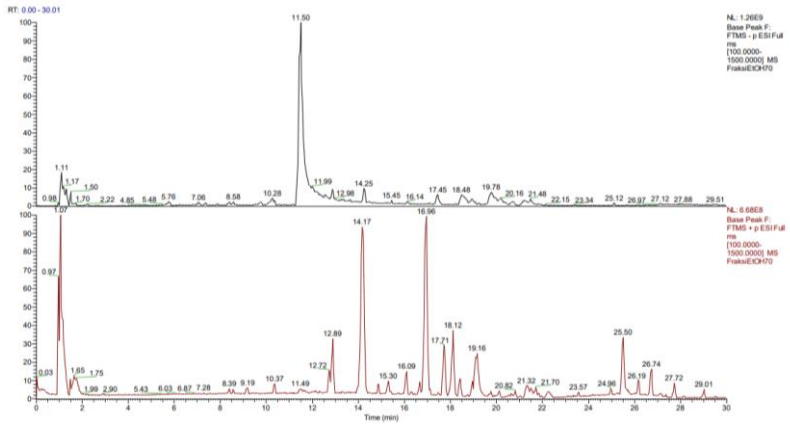

Fig. 3. Liquid Chromatography-Mass Spectrometry Result

4 Discussion

4.1 Moisture Content of Red Betel Leaf Simplicia

Five leaf from the top of the red betel plant were selected. This selection is based on the understanding that these top leaf are younger and actively growing, which may result in higher active compound content [34]. Drying is a vital step in herbal processing as it influences the quality, stability, and shelf life of the simplicia [35]. Simplicia drying can be achieved through sun drying, air drying, or using an oven at temperatures not exceeding 60°C [36]. For this study, the red betel leaf simplicia was dried for 3 days at 50°C [37], aiming to reduce moisture content without damaging the active compounds.

The moisture content was assessed by drying the simplicia in an oven at 105°C until a consistent weight was achieved. The weight difference between the initial and final measurements indicates the amount of water lost. Moisture content is the percentage ratio of the weight of water lost to the initial weight of the simplicia. The moisture content in the red betel leaf simplicia is 8.38% ± 0.0294. This is lower compared to a previous study by Safithri et al. in 2012, which recorded a moisture content of 9.27%. Moisture content may vary due to different weather conditions during harvest, affecting the water availability to the plant [38].

The result of moisture content measured complies with the Indonesian Herbal Pharmacopoeia's requirement of being less than 10% [36]. Drying serves as a preservation technique for simplicia by preventing the growth of microorganisms and enzymatic degradation, which could harm the active compounds in natural products [39, 40]. Maintaining appropriate moisture content in simplicia is essential for preserving the integrity and quality of its active compounds.

4.2 Yield of Red Betel Leaf Extracts

Extraction is the initial step to separate the active compounds in red betel leaf. Grind the leaves to a 100 mesh size to enhance the contact surface area between the simplicia and the solvent [41]. The powder is then extracted using the maceration method in 70% ethanol for 24 hours. Maceration is a simple and effective method for screening active compounds, especially thermolabile ones [42]. The choice of solvent is crucial, with a mixture of water and 70% ethanol providing better extraction results [43]. Water dissolves polar compounds, while ethanol can dissolve a broad range of polarities [44].

The maceration process was done at room temperature with a shaker at 130 rpm to enhance contact between the simplicia and solvent, making the extraction more effective [45]. The filtrate was then separated and concentrated by vacuum evaporation at 45°C to maintain the stability of the plant extract and reduce the risk of damage to the active compounds [46]. The extraction yield, expressed as the percentage ratio of the extract weight to the simplicia weight, was 13.27 ± 0.2365% (Figure 1), higher than the 6.43 ± 0.1% found by Ramdhani (2019). Yield indicates the efficiency of the extraction process, with higher values showing more effective extraction [47].

The 70% ethanol extract was then separated using a multi-step fractionation process with a separating funnel. This technique involves using a separating funnel to divide the compounds in a mixture into more purified fractions [48]. The process relies on the solubility of the compounds in specific solvents or their varying solubility in two or more different solvents. The fractionation starts with a non-polar alkane solvent (n-hexane), followed by a semi-polar solvent (ethyl acetate), and finally a polar solvent (water) [49].

The yields of the red betel leaf water fraction, in descending order, were 7.80 ± 0.62%, ethyl acetate fraction 3.93 ± 0.56%, and n-hexane fraction 1.00 ± 0.06% (Figure 1). These yields are higher than those reported by Chairunisa et al. (2022) [50], which were 3.85% for the water fraction, 2.87% for the ethyl acetate fraction, and 1.72% for the n-hexane fraction. The higher yields of the red betel leaf fractions suggest that the dominant compounds in the sample are polar. This observation is consistent with the Indonesian Herbal Pharmacopoeia, which states that ethanol extract from red betel leaf contains a significant amount of flavonoids [36]. Flavonoids can include compounds that are either polar or semi-polar, depending on their chemical structure.

4.3 Inhibitory Activity of Red Betel Leaf Extracts

A deficiency of the neurotransmitter ACh in the cholinergic system significantly contributes to AD symptoms. ACh is crucial for nerve impulse transmission in the central nervous system [51]. AD treatments aim to boost ACh availability by inhibiting acetylcholinesterase (AChE), the enzyme that breaks down ACh after nerve signal transmission [52]. Inhibiting AChE activity increases ACh at synapses, helping reduce cognitive symptoms in AD patients.

Evaluating the AChE inhibition potential of red betel leaf extract is essential for assessing its cognitive benefits. The Ellman method measures AChE inhibition using DTNB reagent, which reacts with thiocholine from AChE's hydrolysis of acetylthiocholine [53]. The standard curve in this test shows a direct correlation between AChE activity and absorbance, with a regression value near 1 (0.9875) (Figure 4), indicating reliable and consistent measurement results [54].

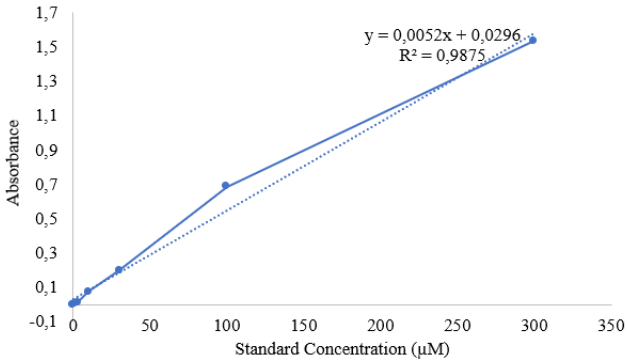

Fig. 4. Standard curve of AChE inhibitory activity.

The IC_{50} value (Inhibitor Concentration 50) is used to measure the effectiveness of enzyme activity inhibition. IC_{50} represents the concentration of a compound or substance needed to inhibit 50% of enzyme activity [55]. A lower IC_{50} value shows a stronger ability of the sample to inhibit enzyme activity. The inhibition potential of red betel leaf extract on AChE activity is assessed by calculating the IC_{50} value at different concentrations of the extract, ranging from 10 to 50 ppm.

The AChE activity inhibition test results indicated that all extracts and fractions of red betel leaf have potential as AChE inhibitors, with IC_{50} values ranging from 11.09 to 41.00 µg/mL (Figure 2). The ethanol extract of red betel leaf demonstrated the highest AChE inhibition with an IC_{50} of 11.0965 µg/mL, suggesting that the polar active compounds in the red betel leaf have strong inhibitory potential against AChE. One-Way ANOVA analysis revealed a significant difference between the average IC_{50} values of the ethanol extract, water fraction, ethyl acetate fraction, and n-hexane fraction on AChE activity, with a p-value of 0.000. This very low p-value (less than the significance level of 0.05) confirms a significant difference. The red betel leaf extract with the highest inhibition activity will be further analyzed to identify its active compounds.

4.4 Active compound screening of ethanol extract from red betel leaf

Phytochemical screening is a technique used to analyze the active compounds present in plants [56]. Analysis of ethanol extracts from red betel leaf was carried out to identify their active compounds using LC-MS technology. Compound identification involved assessing peak area and retention time on the LC-MS chromatogram. Peak area indicates the quantity of detected compounds, while retention time reflects the time each compound takes to travel through the column and reach the mass detector [57]. However, LC-MS identification results are preliminary due to the possibility of compounds existing in isomeric or isobaric forms with identical molecular weights [58].

The ethanol extract of red betel leaf was found to harbor 200 compounds, with only those exhibiting sufficiently high or notable concentrations undergoing further analysis. Out of these, a total of 28 compounds were identified, including 2 unknown ones. Among the identified compounds, the 10 most abundant include Hydrocinnamic acid, d-Colin, 2-(4-Allyl-2,6-dimethoxyphenoxy)-1-(3,4,5-trimethoxyphenyl)-1-propanol, (2S)-4-Methyl-2-({[(3S,4S,5R)-2,3,4-trihydroxy-5-(hydroxymethyl)tetrahydrofuran-2yl]methoxy}(methyl) amino)pentanoic acid, Xanthohumol, L-Valine, Choline, Benzyl succinate, 4,4-Bis[4-(acetyloxy)phenyl]3-hexanone, and DL-Malic acid (Table 2).

Red betel leaf have been recognized for containing diverse bioactive compounds such as alkaloids, flavonoids, tannins, saponins, steroids, and terpenoids [23, 59, 60]. These

phytochemicals in red betel leaf have potential as AChE inhibitors, which are relevant in Alzheimer's disease treatment. The range of compounds present provides opportunities for developing herbal formulations that could offer comprehensive benefits in maintaining neurological balance and safeguarding the brain from AD-associated damage.

Table 2. Volume of Reagents in AChE Inhibitory Activity Assay

No	Name	Chemical formula	Observation (m/z)	RT (min)	%
1	Hydrocinnamic acid	$C_9H_{10}O_2$	149,05975	11,431	21,89
2	d-Corlin	$C_{23}H_{28}O_6$	401,19363	16,965	12,51
3	2-(4-Allyl-2,6-dimethoxyphenoxy)-1-(3,4,5-trimethoxyphenyl)-1-propanol	$C_{23}H_{30}O_7$	419,20447	14,221	11,94
4	(2S)-4-Methyl-2-({[(3S,4S,5R)-2,3,4-trihydroxy-5-(hydroxymethyl)	$C_{12}H_{23}NO_7$	294,15347	1,570	2,19
5	Xanthohumol	$C_{21}H_{22}O_5$	355,15247	18,151	1,81
6	L-Valine	$C_5H_{11}NO_2$	118,08601	1,148	1,78
7	Choline	$C_5H_{13}NO$	104,10693	1,071	1,57
8	Benzyl succinate	$C_{18}H_{18}O_4$	297,11292	14,272	1,31
9	4,4-Bis[4-(acetyloxy)phenyl]3-hexanone	$C_{22}H_{24}O_5$	369,16809	17,772	1,15
10	DL-Malic acid	$C_4H_6O_5$	133,01314	1,161	1,11

5 Conclusion

Red betel leaf show promise in treating neurodegenerative diseases linked to declining cholinergic function. Testing of ethanol extract, n-hexane fraction, ethyl acetate fraction, and water fraction from red betel leaf revealed inhibition of acetylcholinesterase enzyme activity. The ethanol extract exhibited the most effective IC_{50} value at 11.09 µg/mL. Moreover, the ethanol extract contained 10 predominant active compounds categorized as phenolic compounds, flavonoids, steroids, vitamins, and amino acids. These compound categories possess potential for inhibiting acetylcholinesterase, which is pivotal in managing neurodegenerative diseases, particularly Alzheimer's.

References

1. J. Arvanitakis, R.-C. Shah, D.-A, Bennett. Diagnosis and management of dementia: a review. JAMA. **322**, 1589-1599 (2019)
2. E.-J. Tettevi, D.-N.-O. Kuevi, B.-K. Sumabe, D.-L. Simpong, M.-B. Maina, J.-T. Dongdem, M.-Y. Osei-Atweneboana, A Ocloo. In silico identification of a potential tnf-alpha binder using a structural similarity: a potential drug repurposing approach to the management of alzheimer's disease. Bio. Med. Res. Int. **19,** 1598-1695 (2024)
3. [ADI] Alzheimer's Disease International. Dementia statistics [accessed October 20th, 2022]
4. E. Nichols, J.-D. Steinmetz, S.-E. Vollset, K. Fukutaki, J. Chalek, F. Abd-Allah, A. Abdoli, A. Abualhasan, E. Abu-Gharbieh, T.-T. Akram, *et al*. Estimation of the global

prevalence of dementia in 2019 and forecasted prevalence in 2050: an analysis for the Global Burden of Disease Study 2019. The Lancet Public Health. **7**, E105-E125 (2022)

5. A. Wimo, K. Seeher, R. Cataldi, E. Cyhlarova, J.-L. Dielemann, O. Frisell, M. Guerchet, L. Jönsson, A.-K. Malaha, E. Nichols, *et al.* The worldwide costs of dementia in 2019. Alzheimer's. Dement. **19,** 2865-2873 (2023)
6. S. Duong, T. Patel, F. Chang. Dementia: what pharmacists need to know. Can. Pharm. J. **250**, 118-129 (2017)
7. H. Chertkow, H. H. Feldman, C. Jacova, F. Massoud. Definitions of dementia and predementia states in Alzheimer's disease and vascular cognitive impairment: consensus from the Canadian conference on diagnosis of dementia. Alzheimer's Res. Ther. **5**, S2 (2013)
8. [APA] American Psychiatric Association. Supplement to diagnostic and statistical manual of mental disorders, fifth edition (American Psychiatric Association Publishing, Washington DC, 2018)
9. Z. R. Chen, J. B. Huang, S. H. Yang, F. F. Hong. Role of cholinergic signaling in Alzheimer's disease. Molecules. **27**, 1816 (2022)
10. B. Yegla, V. Parikh. Developmental suppression of forebrain trkA receptors and attentional capacities in aging rats: A longitudinal study. Behav. Brain Res. **335**, 111-121 (2017)
11. M. Bond, G. Rogers, J. Peters, R. Anderson, M. Hoyle, A. Miners, T. Moxham, S. Davis, P. Thokala, A. Wailoo, M. Jeffreys, C. Hyde. The effectiveness and cost-effectiveness of donepezil galantamine rivastigmine and memantine for the treatment of Alzheimer's disease (review of technology appraisal no. 111): A systematic review and economic model. Health Tech. Assess. **16**, 1-469 (2012)
12. G. Carney, K. Bassett, J. M. Wright, M. Maclure, N. McGuire, C. R. Dormuth. Comparison of cholinesterase inhibitor safety in real-world practice. Alzheimer's Dement.: Transl. Res. Clin. Interventions. **5**, 732-739 (2019)
13. J. S. Kreutzer, J. DeLuca, B. Caplan. Encyclopedia of clinical neuropsychology (Springer, New York, 2011)
14. T. H. Ferreira-Vieira, I. M. Guimaraes, F. R. Silva, F. M. Ribeiro. Alzheimer's disease: targeting the cholinergic system. Curr. Neuropharmacol. **14**, 101-115 (2016)
15. J. Liu, L. N. Wang, J. Z. Tian. Recognition of dementia in ancient China. Neurobiol Aging. **33**, 2948.e11-13 (2012)
16. M.-J. R. Howes, J. Houghton. Etnobotanical treatment strategies againts Alzheimer's disease. Curr. Alzheimer Res. **9**, 67-85 (2012)
17. S. Sepehri, M. Saeedi, B. Larijani, M. Mahdavi. Recent developments in the design and synthesis of benzylpyridinium salts: mimicking donepezil hydrochloride in the treatment of Alzheimer's disease. Frontiers in Chemistry. **10** (2022)
18. Suciati, D. Poerwantoro, A. Widyawaruyanti, K. Ingkaninan. Acetylcholinesterase inhibitory activity of extract and fractions from the root of Rauvolfia serpentina (L.) Bth.ex Kurz. J. Basic Clin. Phys. Pharmacol. **32**, 313-317 (2021)
19. J. A. Lima, L. Hamerski. Chapter 8 - alkaloids as potential multi-target drugs to treat Alzheimer's disease. Stud. Nat. Prod. Chem. **61**, 301-334 (2019)
20. F. Ahmed, R. M. Ghalib, P. Sasikala, K. K. Ahmed. Cholinesterase inhibitors from botanicals. Pharmacogn. Rev. 7, 121-130 (2013)
21. H. O. Tayeb, H. D. Yang, B. H. Price, F. I. Tarazi. Pharmacotherapies for Alzheimer's disease: beyond cholinesterase inhibitors. Pharmacol. Ther. **134**, 8-25 (2012)

22. F. Ramadani. Aktivitas antioksidan, total tanin ekstrak dan fraksi daun sirih merah (Piper crocatum) dan identifikasi dengan LC-MS, Bachelor thesis, Institut Pertanian Bogor, Indonesia (2018)
23. R. Septiani. Ekstral dan fraksi daun sirih merah (Piper crocatum Ruiz & Pav) sebagai antioksidan dengan metode 2,2-difenil-1-pikrilhidrazil, Bachelor thesis, Institut Pertanian Bogor, Indonesia (2017)
24. A. B. S. Lestari. Y. Dwiatmaka. Aktivitas antioksidan ekstrak daun sirih merah (Piper crocatum) hasil optimasi pelarut etanol-air. JIFI. **12**, 75-79 (2014)
25. Krisdianty. Aktivitas antioksidan ekstrak dan fraksi daun sirih merah (Piper crocatum) dengan metode ferric reducing antioxidant power, Bachelor thesis, Institut Pertanian Bogor, Indonesia (2019)
26. P.J. Puspita, M. Safithri, N. P. Sugiharti. Antibacterial activities of sirih merah (Piper crocatum) leaf extracts. Curr. Biochem. **5**, 1-10 (2018)
27. D. A. P. Suci. Potensi antikanker ekstrak daun sirih merah (Piper crocatum) terhadap sel HeLa, Bachelor thesis, Institut Pertanian Bogor, Indonesia (2014)
28. A. Fitriyani, L. Winarti, S. Muslichah, Nuri. Uji antiinflamasi ekstrak metanol daun sirih merah (Piper crocatum Ruiz & Pav) pada tikus putih. Majalah Obat Tradisional. **16**, 34-42 (2011)
29. M. Safithri, S. Yasni, M. Bintang, A. S. Ranti. Toxicity study of antidiabetics functional drink of Piper crocatum and Cinnamomum burmannii. HAYATI J. Biosci. **19**, 31-36 (2012)
30. M. Soheili, M. Karimian, G. Hamidi, M. Salami. Alzheimer's disease treatment: the share of herbal medicines. Iran J. Basic Med. Sci. **24**, 123-135
31. M. Alfarabi, M. Bintang, Suryani, M. Safithri. The comparative ability of antioxidant activity of Piper crocatum in inhibiting fatty acid oxidation and free radical scavenging. HAYATI J. Biosci. **17**, 201-204 (2010)
32. [BSN] Badan Standardisasi Nasional. SNI 01-3182-1992 tentang Penentuan Kadar Air (Badan Standardisasi Nasional, Jakarta, 1992)
33. I. Firdausi, R. Retnowati, Sutrisno. Fraksinasi ekstrak metanol daun mangga kasturi (Mangifera casturi Kosterm) dengan pelarut n-butanol. Kimia Stud. J. 1, 785-790 (2015)
34. E. S. Sulasmi, S. E. Indriwati, E. Suarsini. Preparation of various type of medicinal plants simplicia as material of jamu herbal, in International Conference on Education, Universitas Negeri Malang, Malang, Indonesia, November (2016).
35. R. M. Yasi, R. S. Harsanti, T. T. Larasati. The effect of simplicia drying method on the scquisition of active compound levels of grinting grass simplicia extract (Cynodon dactylon(L.) Pers.). Berkala Sainstek. **10**, 147-154 (2022)
36. [Kemenkes] Kementerian Kesehatan Republik Indonesia. Farmakope Herbal Indonesia (Kementerian Kesehatan Republik Indonesia, Jakarta, 2017)
37. R. F. Rosmi. The effect of drying method on Turmeric Rhizome simplicia quality. **1**, 248-256 (2021)
38. M. Salim, N. Sulistyaningrum, A. Isnawati, H. Sitorus, Yahya, T. Ni'mah. Karakterisasi simplicia dan ekstrak kulit buah duku (Lansium domesticum Corr) dari Provinsi Sumatera Selatan dan Jambi. Jurnal Kefarmasian Indonesia. **6**, 117-128 (2016)
39. D. Singh, V. Singh. Isolation and characterization of flavonoids in urena lobata leaves. European Journal of Medicinal Plants. **11**, 1-6 (2016)
40. S. M. Kamel. H. A. Thabet, E. A. Algadi. Influence of drying process on the functional properties of some plants. Chem. Mater. Res. **3**, 1–9 (2013)

41. R. Y. Asworo. H. Widwiastuti. Pengaruh ukuran serbuk simplicia dan waktu maserasi terhadap aktivitas antioksidan ekstrak kulit sirsak. Indonesian Journal of Pharmaceutical Education. **3**, 256-263 (2023)
42. A. N. Adham. Comparative extraction methods, phytochemical constituents, fluorescence analysis and HPLC validation of rosmarinic acid content in Mentha piperita, Mentha longifolia and Osimum basilicum. J. Pharmacogn. Phytochem. **3**, 130-139 (2015)
43. Q. W. Zhang, L. G. Lin, W. C. Ye. Techniques for extraction and isolation of natural products: a comprehensive review. Chin. Med. **13** (2018)
44. B. Rahmanto, W. Halwany, F. Lestari, K. Anwar, L. Triyasmono, M. I. Rizki, M. Turjaman. Characterization of ethanol extract from agarwood (Aquilaria microcarpa Baill.) Leaf. J. Jamu Indonesia. **3**, 68-74 (2018)
45. S. Kumowal, F. Fatimawali, I. Jayanto. Uji aktivitas antibakteri nanopartikel ekstrak lengkuas putih (Alpinia galanga (L.) Willd) terhadap bakteri Klebsiella pneumoniae. PHARMACON. **8**, 781–790 (2019)
46. C. Y. Leong, L. S. Chua. Optimization of concentrating process using rotary vacuum evaporation for pineapple Juice. Chem. Eng. Transact. **78**, 7-12 (2012)
47. E. S. Syamsul, O. Anugerah, R. Supriningrum. Penetapan rendemen ekstrak daun jambu mawar (Syzygium jambos L. Alston) berdasarkan variasi konsentrasi etanol dengan metode maserasi. JRKI. **2**, 147-157 (2020)
48. A. V. Rao. Phytochemicals - A Global Perspective of Their Role in Nutrition and Health (IntechOpen, Rijeka, 2012)
49. A. Gori, B. Boucherle, A. Rey, M. Rome, N. Fuzzati, M. Peuchmaur. Development of an innovative maceration technique to optimize extraction and phase partition of natural products. Fitoterapia. **148,** 1-28 (2021)
50. F. Chairunisa, M. Safithri, M. Bintang. Antibacterial Activity of Ethanol Extract of Red Betel Leaves (Piper crocatum) and Its Fractions against Escherichia coli pBR322. Curr. Biochem. **9** (2022)
51. M. Johannsson, J. Snaedal, G. H. Johannesson, T. E. Gudmundsson, K. Johnsen. The acetylcholine index: an electroencephalographic marker of cholinergic activity in the living human brain applied to Alzheimer's disease and other dementias. Dement. Geriatric Cognitive Disord. **39**, 132–142 (2015)
52. S. F. Cavalcante, A. B. C. Simas, M. C. Barcellos, V. G. M. Oliveira, R. B. Sousa, P. A. Cabral, K. Kuca, T. C. C. Franca. Acetylcholinesterase: the "hub" for neurodegenerative diseases and chemical weapons convention. Biomolec. **10**, 414 (2020)
53. G. L. Ellman, D. Courtney, V. Andreas, R. M. Featherston. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem. Pharmacol. **7**, 88-89 (1961)
54. L. J. Saunders, R. A. Russell, D. P. Crabb. The coefficient of determination: what determines a userful R2 statistic?. Investigative Ophthalmology & Vis. Sci. **53**, 6830-6832 (2012)
55. S. Aykul, E. Martinez-Hackert. Determination of half-maximal inhibitory concentration using biosensor-based protein interaction analysis. Analyt. Biochem. **508**, 97-103 (2017)
56. N. A. Pratima, R. Gadikar. Liquid chromatography-mass spectrometry and its applications: A brief review. Arch. Org. Inorg. Chem. Sci. **1** (2018)
57. W. Zhang, R. Sharma, M. W. Li, X. Fan. Retention time trajectory matching for peak identification in chromatographic analysis. Sensors. **23**, 6029 (2023)

58. J. Kaur, V. Dhiman, S. Bhadada, O. P. Katare, G. Ghoshal. LC/MS guided identification of metabolites of different extracts of Cissus quadrangularis. Food Chem. Adv. **1** (2022)
59. M. Anugrahwati, T. Purwaningsih, Rustina, J. A. Manggalarini, N.B. Alnavis, D. N. Wulandari, H. D. Pranowo. Extraction of ethanolic extract of red betel leaves and its cytotoxicity test on HeLa cells. Procedia Eng. **148**, 1402-1407 (2022)
60. Weni M, Safithri M, Seni DSH. 2020. Molecular docking of active compounds Piper crocatum on the alpha-glucosidase enzyme as antidiabetic. Indonesian J. Pharm. Sci. Tech. **7**, 64- 72 (2020)