Evaluation of cholinesterase inhibitory activity of six Indonesian *Cassia* species

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ABSTRACT: Alzheimer's disease (AD) is a neurodegenerative disorder, which is the most common cause of dementia. The aging population means that the number of people suffering from AD is expected to increase each year if there is no effective treatment found. One of the strategies for the treatment of AD is the use of cholinesterase inhibitors. Plants have been the source of many bioactive metabolites, including cholinesterase inhibitors. The objective of this study is to investigate the potency of several plant extracts from the genus *Cassia* as cholinesterase inhibitors. The cholinesterase inhibitory screening was carried out against two enzymes, acetylcholinesterase (AChE) and butyrylcholinesterase (BChE), according to the modified Ellman's method. The chemistry of the active fractions was studied by LC-MS/MS method. The results suggested that amongst six plant extracts from the genus *Cassia* investigated, the ethanolic extract of *Cassia spectabilis* showed the strongest inhibition against both AChE and BChE enzymes, with IC_{50} values of 39.5 and 36.9 µg/mL, respectively. Investigation on the *n*-hexane, ethyl acetate, and *n*-butanol fractions obtained from the *C. spectabilis* extract showed that the ethyl acetate and the *n*-butanol fractions gave better inhibitory activity compared to the *n*-hexane fraction. Based on the LC-MS/MS data, the two active fractions gave similar profile. Both fractions contained alkaloid cassine and spectaline, which may responsible for the cholinesterase inhibitory activity.

KEYWORDS: Cholinesterase inhibitor; Alzheimer's disease ; *Cassia* species; *Cassia spectabilis*.

1. INTRODUCTION

Alzheimer's disease (AD) is a progressive neurodegenerative disorder, which is the most common cause of dementia [1]. The manifestation of this disease includes irreversible memory and cognitive deficiency. The common feature of this disease is the low level of the neurotransmitter acetylcholine in the brain due to loss of cholinergic nerves as well as the presence of senile plaques and neurofibrillary tangles which can destroy the neurons [1,2]. This disease commonly occurs in elderly ages, and the onset mostly appears in the mid 60's population [1]. The increase in life expectancy means that the number of people suffering from AD is anticipated to increase each year if there is no effective treatment found.

As cholinergic deficiency is the common feature of AD, one of the treatments for this disease is the use of acetylcholinesterase (AChE) inhibitor, such as tacrine, donepezil, rivastigmine, and galantamine. Tacrine and donepezil are both synthetic compounds, while rivastigmine is a derivative developed from natural compound physostigmine [3]. Galantamine is an AChE inhibitor derived from natural sources. It is an alkaloid which first isolated from the snowdrop *Galanthus nivalis* (Amaryllidaceae) [4]. Studies have shown that these compounds gave benefits on cognitive, functional and behavioral effects on AD patients, however, several side effects and limited effectiveness have also been reported [5,6]. Another natural product that has

been studied thoroughly as AChE inhibitor is a sesquiterpene alkaloid, Huperzine A, obtained from *Huperzia* spp. The product containing extract of *Huperzia* spp has been commercialized as a food supplement for memory support in China [2].

Considering the two AChE inhibitors above are natural origin, research on finding either new or more effective compounds has been a target of many researchers. Recently we have reported the potency of several marine sponges as acetylcholinesterase inhibitor [7]. In the present study investigation of *Cassia* spp as cholinesterase inhibitor was carried out. The genus *Cassia* in the family Caesalpiniaceae have been known as flowering plants which comprising of more than 500 species, and are distributed widely in the tropical and subtropical regions [8-10]. *Cassia* spp are usually grown as ornamental plants, but many of these plants have great economic importance as well as its utilization in traditional medicine. The leave and the bark of *Cassia fistula* have been used to treat blackwater fever as well as a laxative [8]. Other traditional usages of *Cassia* spp have also been documented, such as for gastrointestinal problems, skin diseases, cough, bronchitis and cardiac disorders [10,11]. Metabolites reported from *Cassia* species include anthraquinones, terpenoids, xanthones, flavonoids as well as alkaloids. Many of these compounds posses promising biological activities, such as antioxidant, anti-inflammatory, anticancer, antiplasmodial, and hepatoprotective agents [11-14]. Anti-AD activity was reported from *Cassia obtusifolia*; the seeds extract of this plant can improve memory and learning in mice [15], as well as ameliorates amyloid β-induced synaptic dysfunction through anti-inflammatory and Akt/GSK-3β pathways [16]. *C. obtusifolia* extract was also found to inhibit AChE, BChE, and BACE1 (β-site amyloid precursor protein cleaving enzyme 1) [17]. It was suggested that the napthopyrone compounds may be responsible for these activities [18]. Freitosa *et al.* (2011) have reported that the extract of *Cassia alata* leaves has shown AChE inhibition [19]. Based on these reports, the potency of other *Cassia* species as cholinesterase inhibitors is worth to be investigated.

2. RESULTS AND DISCUSSION

2.1. Cholinesterase inhibitory assay

The ethanolic extracts were prepared from the leaves of six species of the genus *Cassia*, namely *Cassia spectabilis* L. (Syn. *Senna spectabilis* L.) , *Cassia javanica* L., *Cassia grandis* L.f, *Cassia moschata* Kunth, *Cassia fistula* L., and *Cassia siamea* Lam.(Syn. *Senna siamea* (Lam.) H.S. Irwin & Barneby. The samples were screened against acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) enzymes at a concentration of 100 µg/mL. The results as can be seen in Figure 1 suggested that the highest inhibition against both enzymes was given by *C. spectabilis* and the lowest inhibition was shown by *C. fistula* extract. The inhibitory activity of *C. spectabilis* extract was higher against BChE (85.2%) compared to that against AChE enzyme (53.8%). The *C. spectabilis* extract was further partitioned with, *n*-hexane, ethyl acetate, and *n*-butanol, sequentially. Each of the fractions was then subjected to cholinesterase inhibitory assay against both AChE and BChE enzymes. The results presented in Figure 2 indicated that the ethyl acetate and the *n*-butanol fractions gave higher inhibition against both enzymes, which suggested that the active compounds are possibly semipolar to polar compounds.
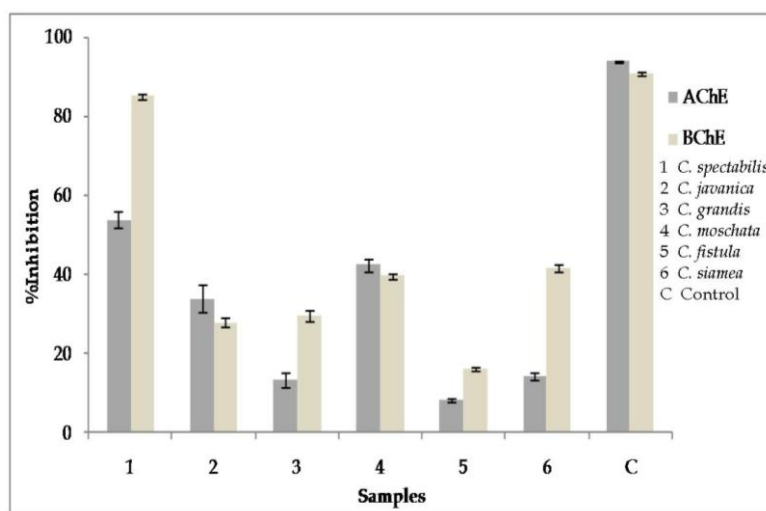

Figure 1. %Inhibition of *Cassia* spp extracts and control (galantamine) against AChE and BchE.
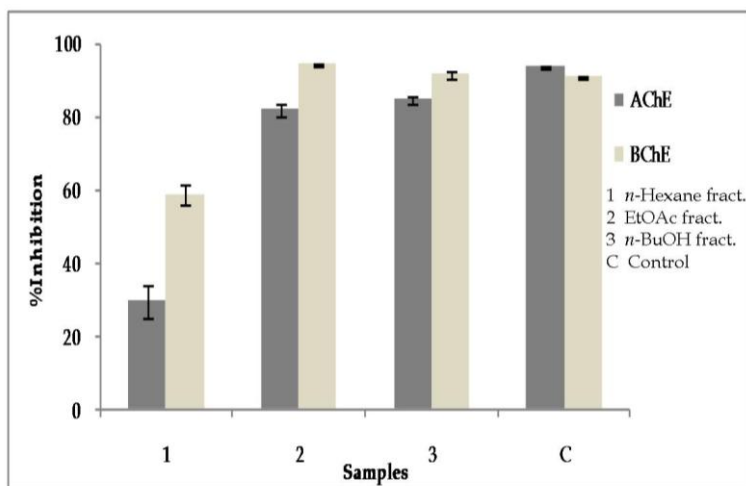
Figure 2. %Inhibition of *C. spectabilis* fractions and control (galantamine) against AChE and BChE.

The 50% inhibitory activities of the ethanolic extract as well as the ethyl acetate and *n*-butanol fractions of *C. spectabilis* were also investigated. The results given in Table 1 show that all samples tested except galantamine exhibited lower IC$_{50}$ values against BChE compare to AChE. The lowest IC$_{50}$ value was given by the ethyl acetate fraction of *C. spectabilis* at 10.8 and 4.7µg/mL against AChE and BChE, respectively.

Table 1. The IC$_{50}$ *C. spectabilis* extract and fractions.

Samples	IC$_{50}$ (µg/mL)[a]	
	AChE	**BChE**
Ethanol extract	39.5 ± 2.2	36.9 ± 5.1
Ethyl acetate fraction	10.8 ± 0.2	4.7 ± 0.1
***n*-Butanol fraction**	12.8 ± 0.9	10.2 ± 0.9
Galantamine	0.4 ± 0.1	2.2 ± 0.3

[a] Data presented as mean ± SD of three experiments, each done in triplicate.

The ethanolic extract of *C. spectabilis* was subjected to enzyme inhibiting kinetics study, and a Michaelis-Menten plot was determined (Figure 3). The Km value of the substrate ATCI (control) for AChE was 68.4 µM and *V*max was 99.1 µM/min. When the ethanolic extract of *C. spectabilis* leaves was added the Km and *V*max were 62.9 µM and 74.7 µM/min, respectively. There was no significant change in the Km values; indicated that the ethanolic extract of *C. spectabilis* inhibited AChE enzyme in a non-competitive way.
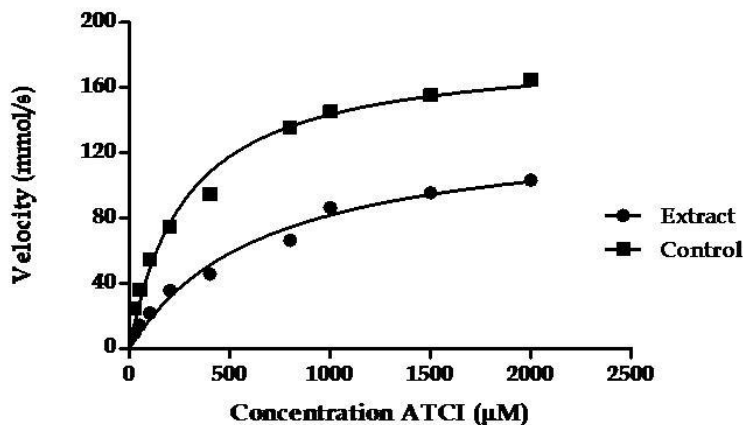

Figure 3. Michaelis-Menten plot of AChE inhibition of *C. spectabilis* extract.

2.2. Identification of metabolites by LC-MS/MS

The chemistry of the ethyl acetate and the *n*-butanol fractions were studied by using LC-MS/MS instruments. The total ion chromatograms (TIC) of the two fractions were compared (Figure 4), which showed a similar TIC profile. Both fractions exhibited 2 major peaks at RT 2.57 (**1**) and 6.14 (**2**) mins, however, the ethyl acetate fraction also gave several minor peaks at RT approximately 6.9 – 17.9 mins which was not seen in the *n*-butanol fraction. Based on the MS/MS data (Table 2) and comparison to the literature, the two major peaks were identified as cassine (**1**) and spectaline (**2**) (Figure 5) [20,21]. In the ethyl acetate fraction, a peak at RT 6.95 mins was observed, which was identified as 3-*O*-acetylspectaline (**3**). However, other minor peaks can not be identified unambiguously. Cassine and spectaline are two major piperidine alkaloids that have been reported from several parts of *C. spectabilis*, such as from the leaves and flowers [11, 22-24]. Piperidine alkaloids, such as iso-6-cassine and iso-6-spectaline, isolated from *C. spectabilis* have shown potency as depressant and anticonvulsant agents [25,26]. Viegas *et al.* (2005) and Castro *et al.* (2008) have developed semisynthetic derivatives from (-) spectaline isolated from the flower of *C. spectabilis*, the compounds exhibited inhibition against rat brain cholinesterase [27,28]. Freitas *et al.* (2018) have reported that the chloridrate form of cassine, spectaline, and 3-*O*-acetylspectaline demonstrated acetylcholinesterase inhibitory activity [21]. Based on the findings, it is possible that the cholinesterase inhibitory activity in the ethyl acetate and the *n*-butanol fractions of *C. spectabilis* corresponded to the presence of cassine, spectaline, and 3-*O*-acetylspectaline.
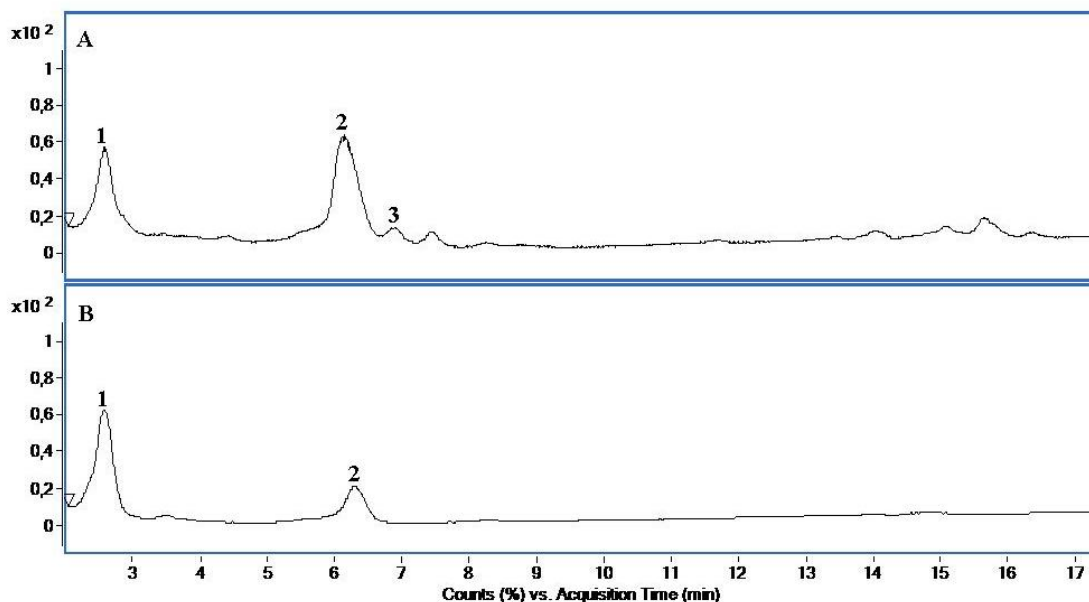

Figure 4. Total Ion Chromatograms (TIC) of the ethyl acetate (A) and *n*-butanol (B) fractions of *C. spectabilis*.

Table 2. LC-MS/MS data for identified alkaloids (**1-3**) from *C. spectabilis* extract.

Compounds	RT (mins)	[M+H]+ (*m/z*)	Product ions *m/z* (relative abundance)[a]	Formula	Exact mass	Mass error (ppm)
1	2.62	298.2730	280.2625 (100), 210.1841(3.2), 198.1848 (4.1), 123.1168 (5.3), 109.1014 (45.6)	$C_{18}H_{35}NO_2$	298.2741	-3.69
2	6.22	326.3050	308.2931 (100), 280.2631 (4.7), 240.2335 (1.0), 226.2181 (3.6), 123.1165 (5.4), 109.1012 (21.8)	$C_{20}H_{39}NO_2$	326.3054	-1.23
3	6.95	368.3155	308.2942 (100), 280.2630 (2.4), 240.2343 (2.6), 226.2149 (3.0), 123.1166 (3.3), 109.1005 (17.2)	$C_{22}H_{41}NO_3$	368.3159	-1.09

[a] Product ions at collision energy 40 eV

1. n = 7; R = H
2. n = 9; R = H
3. n = 9; R = COCH$_3$

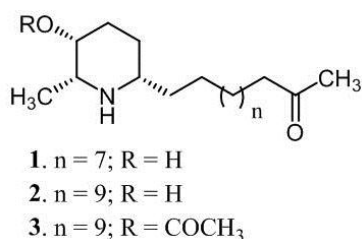**Figure 5.** Structures of identified compounds (**1-3**) from *C. spectabilis*.

3. CONCLUSION

Based on these results, the ethanolic extract of *Cassia spectabilis* showed the strongest inhibition against both AChE and BChE enzymes amongst *Cassia* species tested. Further fractionation on *C. spectabilis* extract suggested that the ethyl acetate and the *n*-butanol fractions gave better inhibitory activities compared to the *n*-hexane fraction. Piperidine alkaloids, cassine, spectaline and 3-*O*-acetylspectaline which were identified in the active fractions, may contribute to the cholinesterase inhibitory activity of *C. spectabilis*.

4. MATERIALS AND METHODS

4.1. Reagents

Acetylcholinesterase from electric eel (AChE type VI-S), human recombinant acetylcholinesterase (hrAChE), horse-serum butyrylcholinesterase (BChE), acetylthiocholine iodide (ATCI), butyrylthiocholine iodide (BTCI), 5,5'-dithiobis[2-nitrobenzoic acid] (DTNB), and bovine serum albumin (BSA), tris buffer, and galantamine were obtained from Sigma-Aldrich.

4.2. Plant collection, extraction and fractionation

Plant materials were collected from Purwodadi Botanic Garden, East Java, Indonesia on March 9th, 2019. Sample specimens (Voucher numbers: PWD 01 – PWD 06) were kept at the Institute of Tropical Diseases Universitas Airlangga. The identification of plant materials was conducted by Purwodadi Botanic Garden (Identification letter number: 0371/IPH.06/HM/III/2019).

The leaves were air-dried for seven days at room temperature followed by grinding to obtain powdered materials. Ten grams of each samples were extracted with ethanol by using ultrasonic extraction for 3 x 10 mins, then the residue and filtrate were separated by filtration. The residue was re-extracted with ethanol using the same procedure for 3 times. The collected filtrate was then concentrated in *vacuo* to yield the crude ethanolic extracts (0.5 - 2.0 g). The ethanolic extract of *C. spectabilis* (0.5 g) was dissolved with a mixture of ethanol and H$_2$O (1:1) (20 mL), then further separated by a liquid-liquid partition with *n*-hexane (15 mL), ethyl acetate (15 mL), and followed by *n*-butanol (15 mL). Fractionation with each solvent was conducted 3 times. Each of the fractions obtained was evaporated in *vacuo*, and yielded *n*-hexane (46.9 mg), ethyl acetate (184.4 mg), and *n*-butanol (97.2 mg) fractions.

4.3. Cholinesterase inhibitory assay

The assay was carried out according to the modified Ellman's method [7,29-31]. Plant extracts were dissolved in methanol to make 10 mg/mL concentration, and was serially diluted with 50 mM Tris buffer to obtain a 1 mg/mL concentration containing not more than 10% of methanol. These samples were further diluted in the microplate well to a final test concentration of 100 µg/mL. Sample solutions were added to a 96-well microplate, followed by the addition of 1.5 mM ATCI or 1.5 mM BTCI (25 µL), 3 mM DTNB (125 µL), and Tris buffer (50 µL). The substrate was then hydrolyzed by the addition of 25 µL of 0.22 U/mL of either *Ee*AChE, *hr*AChE or BChE. The solutions were shaken for 30 s in a microplate reader (Bio-Tek Instrument, USA) before measurement. The product, 5-thio-2-nitrobenzoate, indicated by a yellow color was measured at 405 nm every 5 s for 2 min. Every experiment was carried out in triplicates. Galantamine (100 µL) was used as a positive control, and 10% methanol was used as a negative control. For the measurement of IC$_{50}$, serial concentrations of the samples were prepared. The inhibitory activity was calculated as: % Inhibition =[(Mean velocity of control – Mean velocity of sample)/ Mean velocity of control]x100.

4.4. Identification of Metabolites by LC-MS/MS

Identification of metabolites was conducted by using Agilent 1260 Infinity Series HPLC system with an auto-sampler fitted with analytical C-18 column Agilent Poroshell 120 (Agilent Technologies, Santa, Clara, CA, USA). The HPLC was connected with an Agilent 6530 UHD Accurate-Mass Q-TOF LC/MS (Agilent Technologies), equipped with dual electrospray ionization (ESI). Samples were dissolved in methanol, and 10 µL of samples were injected into the system employing gradient elution of 40-100% methanol/H_2O containing 0.1% v/v formic acid for 30 mins, flowrate 0.25 mL/min. N_2 was employed as drying gas at a flowrate of 10 L/min. The temperature of the nebulizer was set at 325ºC, and the potential of the capillary was 3.5 kV. Positive mode was used and the scan mass range was 100–1000. The MS/MS collision energies were set at 10, 20, and 40 eV. The mass spectrometry data was analyzed using Agilent Mass Hunter Qualitative Analysis software version B06.00.

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