

# Acetylcholinesterase inhibitory activity of extract and fractions from the root of Rauvolfia serpentina(L.) Bth.ex Kurz

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**Submission date:** 28-Dec-2021 02:28PM (UTC+0800)

**Submission ID:** 1736036177

**File name:** actions\_from\_the\_root\_of\_Rauvolfia\_serpentina\_L.\_Bth.ex\_Kurz.pdf (476.75K)

**Word count:** 3121

**Character count:** 17418

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# Acetylcholinesterase inhibitory activity of extract and fractions from the root of *Rauvolfia serpentina*(L.) Bth.ex Kurz

<https://doi.org/10.1515/jbcpp-2020-0401>

Received November 26, 2020; accepted January 26, 2021

## Abstract

**Objectives:** Alzheimer's disease (AD) is a degenerative brain disease characterized by confusion, behavior changes, decline in memory and cognitive skills. One of the strategies in the treatment of AD is to use acetylcholinesterase (AChE) inhibitors. The current study aims to determine the AChE inhibitory activities of the extract and fractions of the root of *Rauvolfia serpentina*.

**Methods:** Extraction was carried out by maceration method using ethanol, followed by liquid–liquid partition using *n*-hexane, ethyl acetate and *n*-butanol. Further fractionation was conducted by using vacuum liquid chromatography (VLC). The AChE inhibitory assays were performed by using Ellmann's method. Phytochemical screening was carried out by TLC method.

**Results:** The ethanolic extract of *R. serpentina* showed inhibition against AChE enzyme with an IC<sub>50</sub> value of 7.46 µg/mL. The extract and fractions showed higher inhibition against butyrylcholinesterase (BChE) compared to AChE. Amongst three fractions obtained, the *n*-butanol fraction showed the strongest inhibition with an IC<sub>50</sub> value of 5.99 µg/mL against AChE. VLC fractionation of the *n*-butanol fraction yielded 13 subfractions (VLC 1–VLC 13). Four out of 13 subfractions gave more than 80% inhibition against

AChE, namely subfractions 4–7, with IC<sub>50</sub> values ranging from 4.87 to 47.22 µg/mL. The phytochemical screening of these subfractions suggested the presence of alkaloids.

**Conclusions:** The ethanolic extract, as well as fractions of *R. serpentina* root, are potential for AChE inhibitor. The alkaloid compound may be responsible for this activity.

**Keywords:** acetylcholinesterase inhibitor; Alzheimer's disease; *Rauvolfia serpentina*.

## Introduction

Alzheimer's disease (AD) is a degenerative brain disease characterized by confusion, behavior changes, decline in memory and cognitive skills. This disease has been recorded as the most common cause of dementia in the elderly population [1, 2]. AD often begin with minor symptoms and grow into severe brain damage or even death. The onset of AD usually occurs in population at the age of 65 years or above [3]. 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 medication found. The pathophysiology of AD is credited to several factors including the acetylcholine (ACh) deficiency due to neuronal loss in the brain. ACh is a neurotransmitter, produced in the nerve ending of the presynaptic nerve, which is associated with memory and cognitive functions. Therefore one of the target treatment of AD is the use of acetylcholinesterase (AChE) inhibitors, which can prevent the hydrolysis of ACh to choline and ethanoic acid [4]. There are three AChE inhibitors approved to be used by FDA for AD, namely galantamine, rivastigmine and donepezil. Donepezil is of a synthetic origin, while rivastigmine is a derivative developed from the natural compound physostigmine [5]. Galantamine is an alkaloid isolated from natural sources, *Galanthus nivalis* (Amaryllidaceae) [6]. The AChE inhibitors are beneficial to improve cognitive, functional and behavioral effects on AD patients. However, studies have shown the presence of several side effects and limited effectiveness of these medications [7, 8]. A sesquiterpenoid alkaloid Huperzine A, isolated from *Huperzia* spp., has also been reported as a potential

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therapy for AD. The product containing extract of *Huperzia* spp. has been commercialized as a food supplement for memory improvement in China [1].

Plants containing alkaloids have been the target of screening for AChE inhibitors. Recently, we have reported the potency of several *Cassia* spp. as well as several marine sponges as AChE inhibitor [9, 10]. In the current study, investigation on the AChE inhibitory activities of extract and fractions from the root of *Rauvolfia serpentina* was conducted. The root of *R. serpentina* possesses high therapeutic properties. It is traditionally used as a tranquilizer for nervous and mental disorders [11]. The root also provides high protein, starch and micronutrient which is useful for treating malnutrition [12–14]. More than 60 indole alkaloids have been reported from the root of *R. serpentina* [11, 15]. These alkaloids, especially reserpine and rescinnamine have an important role in the well-known hypotensive activity of this plant [11]. The presence of alkaloid compounds in this plant made this worth to be investigated for AChE inhibitory assay.

## Materials and methods

### Reagents

AChE from electric eel (AChE type VI-S), 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 purchased from Sigma–Aldrich.

### Plant collection

The dried root of *R. serpentina* was obtained from the local market in Surabaya, East Java, Indonesia. The sample specimen (voucher number: PSR 06) was kept at the Institute of Tropical Diseases Universitas Airlangga. The identification of the plant material was conducted by Purwodadi Botanic Garden (Identification letter number: 814/IPH.06/HM/VIII/2019).

### Extraction and fractionation

The dried root was grinded to obtain powdered material. One kilogram of the powdered root was extracted with ethanol 96% by using the maceration method. The sample was submerged in the solvent (2 L) for 24 h, followed by filtration. The residue was re-extracted with ethanol using the same procedure three times. The filtrate was then evaporated in the rotary evaporator to obtain a crude ethanolic extract (18.23 g). A portion of the ethanolic extract (18.0 g) was dissolved with a mixture of ethanol and H<sub>2</sub>O (1:1) (220 mL), then further separated by a liquid–liquid partition with *n*-hexane (250 mL), ethyl acetate (250 mL), and followed by *n*-butanol (50 mL). Fractionation with each solvent was conducted

three times. Each of the fractions obtained was evaporated *in vacuo*, and yielded *n*-hexane (3.61 g), ethyl acetate (6.80 g), and *n*-butanol (2.63 g) fractions. Further fractionation of the *n*-butanol fraction was conducted by using vacuum liquid chromatography (VLC). Sample (2.6 g) was mixed with silica gel (2.6 g) to obtain a dried powdered sample, which was then applied to the VLC. The sample was eluted with a combination of dichloromethane and methanol in order of increasing polarity, and yielded 13 subfractions namely VLC 1–VLC 13.

### Phytochemical screening

Samples were dissolved in ethanol. The solutions were applied on the TLC plate (Silical gel F<sub>254</sub>), which was then developed with a combination of dichloromethane:methanol (1:3). The plate was visualized under UV 254 and 366 nm, followed by Dragendorff spray to indicate the presence of alkaloid.

### Cholinesterase inhibitory assay

The assay was performed according to the modified Ellman's method [9, 10, 16]. The solution of plant extract and fractions were made in methanol at a concentration of 10 mg/mL, which was then diluted to 1 mg/mL with 50 mM Tris buffer. The final test concentration is 100 µg/mL after the dilution of samples in the microplate well. 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 EeAChE 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 was used as a positive control, and 10% methanol was used as a negative control. For the IC<sub>50</sub>, measurement serial concentrations of the samples were prepared ranging from 0.2–200 µg/mL. The enzyme activity was calculated as a percentage of the velocity of the test sample, compared with that of the nontreated control. The inhibitory activity was calculated as:

$$\% \text{inhibition} = \frac{(\text{mean velocity of control} - \text{mean velocity of sample})}{\text{mean velocity of control}} \times 100$$

### Data analysis

The 50% inhibitory concentration (IC<sub>50</sub>) was determined using GraphPad Prism 7.04 software by plotting log concentrations as axis and % inhibition as ordinate. The inhibition data of the samples against AChE and BChE were analyzed using unpaired *t*-tests in GraphPad Prism 7.04.

## Results

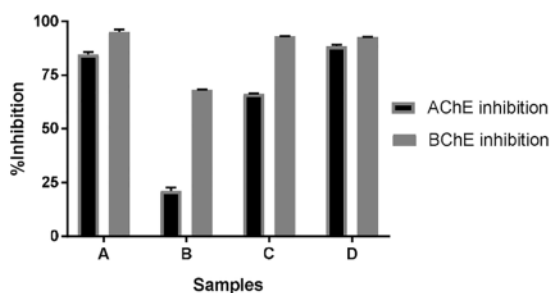
The ethanolic extract of the root of *R. serpentina* showed inhibition against the AChE enzyme with an IC<sub>50</sub> value of 7.46 µg/mL. To investigate the selectivity against cholinesterase enzyme, the sample was also tested against both

AChE and BChE at a concentration of 100  $\mu\text{g/mL}$ . The results shown in Figure 1 indicated that the extract inhibited both enzymes; however, the inhibition was higher against BChE compared to AChE. Likewise, the *n*-hexane, ethyl acetate, and *n*-butanol fractions also gave higher inhibition against BChE than against AChE enzyme (Figure 1). Amongst the three fractions, the *n*-butanol fraction gave the strongest inhibition against AChE with an  $\text{IC}_{50}$  value of 5.99  $\mu\text{g/mL}$  (Table 1). The determination of the  $\text{IC}_{50}$  value was not conducted on the *n*-hexane fraction since it only gave low inhibition against the AChE enzyme.

Subfractionation of the *n*-butanol fraction by using VLC yielded 13 subfractions. All subfractions were subjected to AChE inhibitory assay. The results presented in Figure 2 showed that four subfractions, namely subfractions 4–7 gave more than 80% inhibition against the tested enzyme. The  $\text{IC}_{50}$  values of these subfractions were also determined. The results (Table 2) showed that subfraction 5 gave the strongest inhibition against the AChE with  $\text{IC}_{50}$  value of 4.87  $\mu\text{g/mL}$ . Subfractions 4–7 were subjected to phytochemical screening by using TLC method. The results showed that after spraying with Dragendorff dye, dark orange color spots on a yellowish-orange background were observed. This suggested that subfractions 4–7 of *R. serpentina* roots contain alkaloid.

## Discussion

ACh is a neurotransmitter, produced in the nerve ending of the presynaptic nerve, that has been discovered to be involved in the pathogenesis of AD. This neurotransmitter plays an important role in memory and learning function [17]. In AD patients, the ACh that is released has a very short half-life due to the presence of large amounts of the cholinesterase enzymes, namely AChE and BChE [4, 18].

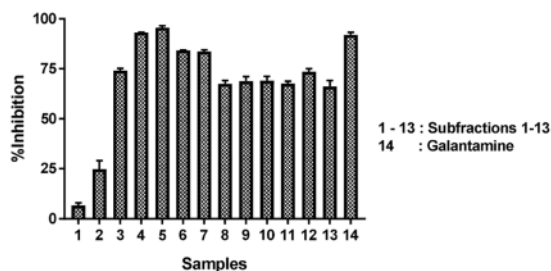


**Figure 1:** Inhibition of ethanolic extract (A), *n*-hexane (B), ethyl acetate (C), and *n*-butanol (D) fractions of *R. serpentina* root against AChE and BChE at 100  $\mu\text{g/mL}$ .

**Table 1:**  $\text{IC}_{50}$  values of extract and fractions of the root of *R. serpentina* against AChE.

Samples	$\text{IC}_{50}$ , $\mu\text{g/mL}$
Ethanol extract	7.46 $\pm$ 0.28
Ethyl acetate fraction	23.62 $\pm$ 2.97
<i>n</i> -Butanol fraction	5.99 $\pm$ 0.86
Galantamine	0.63 $\pm$ 0.05

Data presented as mean  $\pm$  standard deviation of three independent experiments, each done in triplicate.



**Figure 2:** Inhibition of subfractions 1–13 of *R. serpentina* (100  $\mu\text{g/mL}$ ) and galantamine (100  $\mu\text{M}$ ) against AChE.

**Table 2:**  $\text{IC}_{50}$  values of selected VLC subfractions of the root of *R. serpentina* against AChE.

Samples	$\text{IC}_{50}$ , $\mu\text{g/mL}$
Subfraction VLC 4	7.08 $\pm$ 0.53
Subfraction VLC 5	4.87 $\pm$ 0.45
Subfraction VLC 6	19.88 $\pm$ 1.58
Subfraction VLC 7	47.22 $\pm$ 1.08

Data presented as mean  $\pm$  standard deviation of three independent experiments, each done in triplicate.

These enzymes hydrolyze the ACh molecule into choline and ethanoic acid. In the healthy brain, AChE predominates, while BChE plays a minor role. However, in AD patients, the activity of BChE is increased progressively, while the activity of AChE remains unchanged [19]. The cholinergic strategy in the therapy for AD patients included stimulation of cholinergic receptors or increasing the availability of ACh by inhibiting AChE and BChE enzymes [8].

The current AChE inhibitors approved by FDA as medication for AD patients are donepezil, rivastigmine, and galantamine. Considering that all the three drugs are alkaloids that are synthetic or natural origins, searching on the new AChE inhibitor from natural sources has focused on the alkaloids, although other class of compounds, such terpenes, sterols, and flavonoids have also shown potency as AChE inhibitor [20].

Plant from the genus *Rauwolfia* has been known to contain alkaloids. The well-known compound reserpine and rescinnamine have been used as antihypertensive agents. In the present studies, we evaluated the anti-AD activity of the ethanolic extract as well as fractions of *R. serpentina* by measuring the inhibition against AChE and BChE. The extract as well as several fractions have shown strong inhibitions against both enzymes. The *n*-hexane fraction gave the lowest inhibition compared to the ethanolic extract, the ethyl acetate and the *n*-butanol fractions. These results indicated that the active compounds are semipolar to polar compounds. At the concentration of 100 µg/mL, the samples tested demonstrated higher inhibition against BChE compared to AChE enzymes. Analysis by using unpaired t-test, suggested that there were significant differences observed between the inhibition of extracts as well as *n*-hexane, ethyl acetate, and *n*-butanol fractions against AChE and BChE enzymes with *p*-value < 0.001 for *n*-hexane and ethyl acetate fractions and *p*-value = 0.003 and 0.004 for the ethanolic extract and *n*-butanol fraction, respectively.

Since the highest enzyme inhibition was given by the *n*-butanol fraction, further fractionation was conducted on this sample by using VLC. Thirteen subfractions were yielded and subjected to the AChE inhibitory assay. The results showed that subfractions 4–7 gave higher potency compared to other subfractions. These subfractions were eluted in a solvent combination of dichloromethane:methanol (85:15) to dichloromethane:methanol (60:40), which suggested that the active compounds are semipolar.

The phytochemical screening of subfractions 4–7 indicated the presence of alkaloid. *R. serpentina* has been known to contain more than 60 alkaloids in the root [11, 15]. Another plant from the same genus, *Rauwolfia reflexa*, has been reported to show inhibition against AChE enzyme. The methanol extract as well as the isolated indole alkaloids from *R. reflexa* demonstrated inhibition against both AChE and BChE enzymes [21]. Several indole alkaloids were found to be more selective against BChE compared to AChE [21, 22]. This suggested that indole alkaloids present in the root of *R. serpentina* may responsible for the cholinesterase inhibitory activity.

## Conclusions

The ethanolic extract as well as fractions from the root of *R. serpentina* inhibited cholinesterase enzymes. The alkaloid compounds in the extract and fractions may contributed to the AChE and BChE inhibition.

**Research funding:** This research was funded by the Ministry of Research, Technology, and Higher Education of the Republic of Indonesia through PDUPT 2020 research grant.

**Author contributions:** All authors have accepted responsibility for the entire content of this manuscript and approved its submission.

**Competing interests:** Authors state no conflict of interest.

**Informed consent:** Not applicable.

**Ethical approval:** Not applicable.

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