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Research article

Stichoneuron calcicola Inthachub, A Potential Source of Acetylcholinesterase Inhibitor and Anti-Inflammatory

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Abstract

A major target for the treatment of Alzheimer's disease (AD) is the inhibition of acetylcholinesterase (AChE) activity to reduce acetylcholine (ACh) hydrolysis. Inflammation is believed to play a role in AD progression. The present study is the first to report the anti-inflammatory and anti-AChE effects of ethyl acetate extracts from the leaves of *Stichoneuron calcicola* Inthachub, an endangered plant species. The anti-inflammatory activity was determined using nitric oxide (NO) inhibitory assay in LPS-activated RAW 264.7 macrophages. AChE inhibition activity was measured by using a modification of Ellman's method. The results revealed that the plant extract showed strong potential anti-inflammatory activity with an IC_{50} value of 4.48±0.04 μg/mL. The plant extract also presented anti-AChE capacity, with an IC_{50} value of 60.3±0.7 μg/mL. Furthermore, cell viability study using MTT assay revealed that the extract was not cytotoxic to RAW 264.7 cells at concentrations of 1-30 μg/mL. The findings indicate that *S. calcicola* Inthachub exhibited high potential effects on inflammation and AChE, which may be related to the high content of phenolic compounds and alkaloids in the plant. This endangered plant species could be a high-value medicinal plant and of great medical importance in the development of novel effective drugs for treating and preventing the progression of AD.

Keywords: Stemonaceae; anti-inflammatory; raw 264.7 macrophages; acetylcholinesterase; Alzheimer's Disease

1. Introduction

Alzheimer's disease (AD) is one of the most common progressive neurodegenerative disorders and is a common cause of age-related dementia. Patients with AD gradually lose their capacity for memory, thinking, and learning (Chen et al., 2022; Martins et al., 2023; Twarowski & Herbet, 2023). However, the mechanism of AD pathogenesis is still unclear (Chen et al., 2022). Amyloid-β plaque accumulation and neurofibrillary tangles have been investigated as major biomarkers of AD. Furthermore, inflammation, oxidative stress, and decreased levels of the neurotransmitter acetylcholine (ACh) have been investigated as a cause of AD pathology (DeTure & Dickson, 2019; Du et al., 2018; Sehar et al., 2022;

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Martins et al., 2023). ACh is a major neurotransmitter in the brain and is secreted by cholinergic neurons (Chen et al., 2022). It has also been known to influence the immune system through the cholinergic anti-inflammatory pathway (Rosas-Ballina & Tracey, 2009). Inflammation has been identified as an important factor in the progression of AD. However, the reduction of ACh by the catalytic activity of acetylcholinesterase (AChE) is believed to play a crucial role in cognitive impairment in AD patients and leads to a reduction in anti-inflammatory activity (Das, 2012; Sharma, 2019; Pascoal et al., 2021; Chen et al., 2022; Novoa et al., 2022;). AChE is able to promote the aggregation of amyloid-β-peptides and neurofibrillary tangles (NFTs) through fibril formation (Saini & Saxena, 2018). Therefore, inhibition of the biological activity of AChE leads to an increase in the concentration of ACh, which is one of the potential therapeutic targets for treating AD (Li et al., 2015; Sharma, 2019). Nowadays, drug development and clinical trials for AD can improve some cognitive symptoms of the disease but cannot stop its progression and can also produce some side effects (Cummings, 2018; Huang et al., 2020). The most promising therapy target for AD in recent years has been focused on finding AChE inhibitors from medicinal plants. Over the years, many plant-derived natural compounds, particularly alkaloids, have been investigated as potent AChE inhibitors (Murraya et al., 2013; Marucci et al., 2021). Furthermore, other natural non-alkaloid compounds have been investigated as AChE inhibitors, including terpenes, flavonoids, and other phenolic compounds (Murraya et al., 2013; Santos et al., 2018; Tamfu et al., 2021).

Stichoneuron calcicola Inthachub is an endangered plant species found exclusively in a specific region, growing on limestone rock in Khlong Phanom National Park and Khlong Saeng Wildlife Sanctuary, Surat Thani, Thailand (Inthachub et al., 2009). It is in the *Stichoneuron* genera of the small family Stemonaceae, which are mostly distributed in Peninsular Thailand and Malaysia (Rakarcha et al., 2020). The Stemonaceae family is composed of three genera, *Stemona*, *Croomia*, and *Stichoneuron*, that contain approximately 25 species (Ramli et al., 2013). The plants within the family Stemonaceae have been investigated for various potential applications including their use as insecticides, traditional medicines, anti-parasitic agents, AChE inhibitors, and anti-inflammatory agents (Inthachub et al., 2009; Majumdar & Datta, 2013; Ramli et al., 2013). However, the biological activity from the endangered species *S. calcicola* Inthachub has never been previously reported. Therefore, the aim of the present study was to investigate the inhibitory effects of *S. calcicola* Inthachub against inflammatory and AChE activities. In addition, medicinal plants are valuable sources of natural products, so the conservation of endangered plant species is very necessary to prevent the extinction of important sources of bioactive compounds, while exploring further the possibility of pharmaceutical applications.

2. Materials and Methods

Analytical grade solvent ethyl acetate was purchased from Merck. Lipopolysaccharide (LPS), caffeic acid phenethyl ester (CAPE), 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), RPMI-1640 medium and Griess reagent were purchased from Sigma-Aldrich (St. Louis, MO, USA). RAW264.7 cell line was purchased from CLS cell lines service. Acetylcholinesterase from electric eel, acetylthiocholine iodide (ATCI), 5,5-dithiobis-2-nitrobenzoic acid (DTNB) and galantamine hydrobromide were obtained from Sigma-Aldrich.

2.2 Plant materials

The leaves of *S. calcicola* Inthachub were harvested from Khlong Phanom National Park, Surat Thani province in southern Thailand. The plant species was identified by Dr. Piya Chalermglin, Institute of Scientific and Technological Research, Pathum Thani, Thailand.

2.3 Plant extraction

The leaves of *S. calcicola* Inthachub were washed and air dried under shade for 2 weeks. The dried leaves were ground and extracted with 95% ethanol by maceration at room temperature for 48 h. Subsequently, the 95% ethanol solvent was evaporated under reduced pressure using a vacuum rotary evaporator to obtain ethanol crude extract. The fraction of ethanol crude extract was separated by liquid-liquid partition with hexane: H_2O, ethyl acetate: H_2O, and *n*-butanol: H_2O, and then evaporated in a rotary evaporator to give the hexane, ethyl acetate, and butanol fractions, respectively. The ethyl acetate fraction was used to evaluate biological activities, including AChE inhibitory activity, anti-inflammatory activity, and cytotoxicity.

2.4 Determination of the total phenolic content

The total phenolic content from ethyl acetate extract of *S. calcicola* Inthachub leaves was determined using the Folin-Ciocalteu colorimetric method, with slight modifications as previously described (Chen et al., 2018; Carmona-Hernández et al., 2021). The reaction mixture was initially combined with 40 μL of the plant extract and 50 μL of 10% Folin-Cioculteu reagent in a 96-well plate. The mixture was left for 3 min at room temperature, and then 100 μL of 7.5% w/v of sodium carbonate solution was added to stop the reaction. The absorbance was measured at 765 nm in a microplate reader after 45 min of incubation at room temperature in the dark. The total phenolic content was expressed as mg of gallic acid equivalents (GAE) per g of the plant extract. Analysis was performed in triplicate.

2.5 Determination of the total alkaloid content

The analysis of the total alkaloid content was carried out according to the previously described method with some modifications (Lee et al., 2021). Briefly, the ethyl acetate extract of *S. calcicola* Inthachub leaves was mixed with 25 mM bromocresol green (BCG) solution and 10 mM citrate-phosphate buffer, pH 4.7, followed by agitation for 2 min. Subsequently, 5 mL of chloroform was added before vigorous shaking. The reaction mixture was placed at room temperature, and the chloroform layer was separated. Then, the absorbance of the yellow-colored complex at 470 nm was measured by a spectrophotometer. Atropine was used as a standard solution. The total alkaloid content was expressed as mg of atropine equivalents per g of the plant extract. All determinations were performed in triplicate.

2.6 Determination of AChE inhibitory activity

AChE inhibitory activity was performed based on an improved Ellman's method, as previously described, using acetylthiocholine iodide (ATCI) as a substrate (Ellman et al., 1961; de Torre et al., 2022). Briefly, the reaction mixture containing 50 mM phosphate buffer pH 8.0 and 25 μL of the ethyl acetate extract of *S. calcicola* Inthachub leaves at

various concentrations were mixed with 25 μL of AChE solution and incubated at 37°C for 15 min. After incubation, 1.5 mM of ATCI and 0.3 mM of thiol reagent 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) were added to give a final volume of 250 μL and then incubated at room temperature at 37°C for 30 min. The inhibitory activity of the plant extract on AChE was detected at 412 nm by using a microplate reader. Each reaction was performed in triplicates. Galantamine was used as a positive control. The control was also examined without an inhibitor. The inhibitory activity of AChE was calculated according to the following equation (1):

$$\text{Inhibitory activity (\%)} = (A_{control} - A_{sample})/(A_{control}) \times 100 \quad (1)$$

Where $A_{control}$ is the absorbance of the control reaction, and A_{sample} is the absorbance of the plant extract.

2.7 Anti-inflammatory effects assay

The inflammatory activation of the macrophage cell line (RAW 264.7) was performed by determining LPS-induced nitric oxide (NO) production using the Griess reagent system, following the method described previously by Tewtrakul et al. (2009). Briefly, RAW 264.7 cells were cultured in 96-well microplates (density of 1×10^5 cells/well) in RPMI-1640 containing 10% FBS, penicillin (100 units/mL) and streptomycin (100 μg/mL) by incubating cells in a 5% CO_2 humidified atmosphere (95%) at 37°C for 12 h. Then, the culture medium was replaced with fresh RPMI-1640 media containing lipopolysaccharide (LPS) at a concentration of 1 μg/mL. Subsequently, the ethyl acetate extract of *S. calcicola* Inthachub leaves was applied to the wells at different concentrations (3-100 μg/mL). After incubation at 37°C for 24 h, 100 μL of Griess reagent was added to each well to determine LPS-induced nitric oxide (NO) production. The absorbance was measured at 570 nm by a microplate reader, after color development. Caffeic acid phenethyl ester (CAPE) was used as a positive control. Analysis was performed in triplicate. The percentage of NO inhibition was calculated as per the following equation (2):

$$\text{\% Inhibition} = [(A\text{-}B) / (A\text{-}C)] \times 100 \quad (2)$$

Where A-C: nitrite concentration, A: LPS (+), sample (-), B: LPS (+), sample (+), C: LPS (-), sample (-).

2.8 Cell viability assay

Cell viability was performed by using 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) colorimetric method based on the conversion of yellow tetrazolium salt (MTT) to generate a purple formazan product by the mitochondrial dehydrogenases of the viable cells (Mosmann, 1983). In brief, RAW 264.7 macrophage cells were treated with different concentrations of the plant extract for 24 and 48 h. Then, the medium was replaced by MTT (5 mg/mL) solution in each well and incubated under 5% CO_2 at 37°C for 4 h. Subsequently, DMSO was added to each well to dissolve the formazan crystals before measuring the absorbance at 570 nm by a microplate reader. The extract has cytotoxic potential when cell viability is reduced to less than 80% in comparison with untreated cells. The percentage of viability was calculated as the following formula, as shown in equation (3) below.

% cell viability = (Absorbance of treated cells/Absorbance of untreated) x 100 (3)

2.9 Statistical analysis

The significant difference among the various treated groups and control groups was analyzed by one-way ANOVA followed by Duncan's test. Statistical significance was considered p value<0.05.

3. Results and Discussion

3.1 Total phenolic content

The total phenolic content of ethyl acetate extract of *S. calcicola* Inthachub leaves was determined by the Folin-Ciocalteu method. The amount of total phenolic of the plant extract was calculated from the calibration curve (y=0.0084x+0.0206, R^2 = 0.999) and reported as gallic acid equivalents. The result indicated that the plant extract contained the high total phenolic content of 285±1.1 mg GAE/g extract, as shown in Table 1.

3.2 Total alkaloid content

The content of alkaloids of the ethyl acetate extract of *S. calcicola* Inthachub leaves was measured following the method based on alkaloids and bromocresol green (BCG) reaction to produce a yellow-colored complex. The total alkaloid content of the plant extract was calculated from the calibration curve (y = 0.0035x + 0.1967, R^2 = 0.9998). The values obtained for the concentration of total alkaloid content were expressed as mg of AE/g of the plant extract. The results suggested that the plant extract showed a high concentration of total alkaloid content, which was 571±0.3 mg atropine equivalent (AE)/g extract (Table 1).

3.3 AChE inhibitory activity

Based on the measurement of thiocholine formation as a result of the AChE-catalyzed reaction using acetylthiocholine iodide (ATCI) as a substrate, the inhibition of the AChE study was determined using Ellman's colorimetric method with some modifications. The thiocholine product is combined with Ellman's reagent (DTNB) to form 2-nitrobenzoate 5-mercaptothiocholine and 5-thio-2-nitrobenzoate, yellow-colored substances, which can be detected at 412 nm. The presence of the AChE inhibitor can inhibit the AChE-catalyzed hydrolysis of ATCI, preventing the production of the yellow-colored product (Ellman et al., 1961; de Torre et al., 2022; Singh et al., 2023).

The AChE inhibitory activity of ethyl acetate extract from *S. calcicola* Inthachub leaves is presented in Figure 1. The results suggested that the inhibition activity gradually increased with increasing concentrations of the plant extract. The plant extract at the concentration of 100 µg/mL exhibited the highest AChE inhibitory activity with an inhibition of 88.1±0.3% (Figure 1). The IC_{50} value of the plant extract was 60.3±0.7 µg/mL, which showed significantly (p<0.05) lower AChE inhibitory activity than did the positive control, galanthamine (IC_{50} value of 0.65±0.02 µg/mL), as shown in Table 1. The present study revealed that the plant extract effectively reduced the AChE activity. This result is consistent with a previous report that the ethyl acetate extract from leaves inhibited AChE

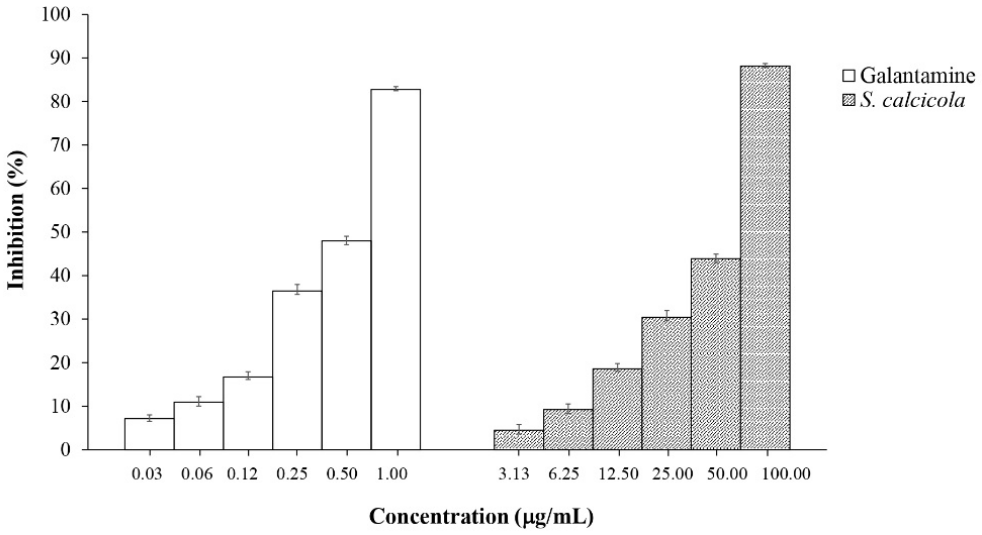

Figure 1. Inhibition of acetylcholinesterase activity at different concentrations of galanthamine (the positive control) and ethyl acetate extract of *S. calcicola* Inthachub leaves

Table 1. Total Phenolic content, total alkaloid content, IC_{50} values of inhibitory activities of AChE and NO of the ethyl acetate extract from *S. calcicola* Inthachub leaves

Samples	Total Phenolic content (mg GAE/g of the extract)	Total Alkaloid Content (mg AE/g of the extract)	NO Inhibitory Activity (μg/mL)	AChE Inhibitory Activity (μg/mL)
S. calcicola Inthachub	285.6±1.1	571.1±0.3	4.48±0.04[a]	60.3±0.7[a]
Galanthamine	-	-	-	0.65±0.02[b]
CAPE	-	-	1.35 ± 0.01[b]	-

Different letters (a, b) in the same column represent significant differences ($p < 0.05$); -: not detected

activity. Interestingly, the ethyl acetate extract of *S. calcicola* Inthachub leaves showed stronger inhibitory activity of AChE than the ethyl acetate fraction from *Croton sylvaticus* and *Ficus benghalensis* L. leaves (IC_{50} values of 235 μg/mL and 605±5.5 μg/mL, respectively) (Aderogba et al., 2013; Hassan et al., 2020). Moreover, this study gave a close result to the ethyl acetate extract from the whole plant of *Wedelia chinensis* (IC_{50} value of 57.8±0.4 μg/mL) (Islam et al., 2021). The potential effect of the extract from *S. calcicola* Inthachub on AChE inhibitory activity from this investigation could be useful for treating AD.

3.4 Anti-inflammatory effect

The effect of the ethyl acetate extract of *S. calcicola* Inthachub leaves on anti-inflammatory activity was determined by measuring NO production in RAW 264.7 macrophage cells stimulated by LPS. The results revealed that the plant extract showed potential anti-inflammatory activity through inhibiting NO production. The amount of NO production was decreased in the presence of the plant extract. The plant extract at concentrations of 1, 3, 10, 30, and 100 μg/mL showed nitric oxide inhibition values of 8.4±2.6%, 15.7±2.4%, 43.0±0.4%, 87.4±0.8%, and 101.3±0.3%, respectively. Furthermore, at 30 and 100 μg/mL, the plant extract exhibited no significant difference (p<0.05) in NO inhibitory activity compared with CAPE, the positive control, which is shown in Figure 2. However, the plant extract displayed a statistically significant difference (p<0.05) in the IC_{50} value of 4.48±0.04 μg/mL when compared with CAPE (IC_{50} value of 1.35±0.01 μg/mL), as shown in Table 1.

According to the findings, the plant extract exhibited a strong potential effect to reduce inflammation with an IC_{50} value of 4.48±0.04 μg/mL. This result was similar to that previously reported in the investigation of the ethyl acetate extracts of *Acokanthera oppositifolia*, *Artemisia vulgaris*, *Plantago lanceolata* and *Conyza canadensis*, which inhibited NO production with IC_{50} values of 32.57, 41.13, 44.05 and 17.69 μg/mL, respectively (Adebayo et. al., 2019). The results revealed that the *S. calcicola* Inthachub extract has the ability to prevent LPS-activated RAW 264.7 macrophage cells from producing nitric oxide (NO), which is associated with the inflammatory response. Therefore, the anti-inflammatory activity of the plant extract may have the potential for therapeutic AD treatment.

3.5 Cell viability

The cell viability measurement by MTT assay plays an important role as an indicator for evaluating the cytotoxic effect of the extracts. Based on the MTT results, the extract had no cytotoxicity effect on RAW 264.7 cells (cell viability>80%) after treating with the extract at concentrations of 1, 2, 10, and 30 μg/mL for 24 and 48 h, compared with the untreated control. Moreover, a reduction in cell viability was observed only at the maximum extract concentration. The cell viability decreased to 77.33%±1.03 and 66.67%±1.86 after treatment with the extract at a concentration of 100 μg/mL for 24 and 48 h, respectively (Figure 3), suggesting that high concentrations of the extract can lead to toxicity. This result is similar to the previous study of the EtOAc fractions from *Acokanthera oppositifolia, Artemisia vulgaris,* and *Conyza canadensis*, where it was noted that the viability of the treated cells decreased with increasing the extract concentrations (Adebayo et. al., 2019). In contrast, *Plantago lanceolata* ethyl acetate extract was not cytotoxic at the maximum concentration of 100 μg/mL (Adebayo et. al., 2019). In our study, the ethyl acetate extract of *S. calcicola* Inthachub at concentrations lower than or equal to 30 μg/mL had no toxicity on the macrophage cells. Furthermore, according to the anti-inflammatory assay, the extract concentrations of 10 and 30 μg/mL exhibited strong potential for anti-inflammatory effect by inhibiting LPS-stimulated NO production. This finding suggests that the reduction of NO production was not the result of cell death but was probably due to inhibition by the plant extract.

The potent inhibition of inflammation and AChE activities by the ethyl acetate extract from *S. calcicola* Inthachub leaves may be associated with the presence of high concentrations of phenolic compounds and alkaloids in the plant. Phenolic compounds are

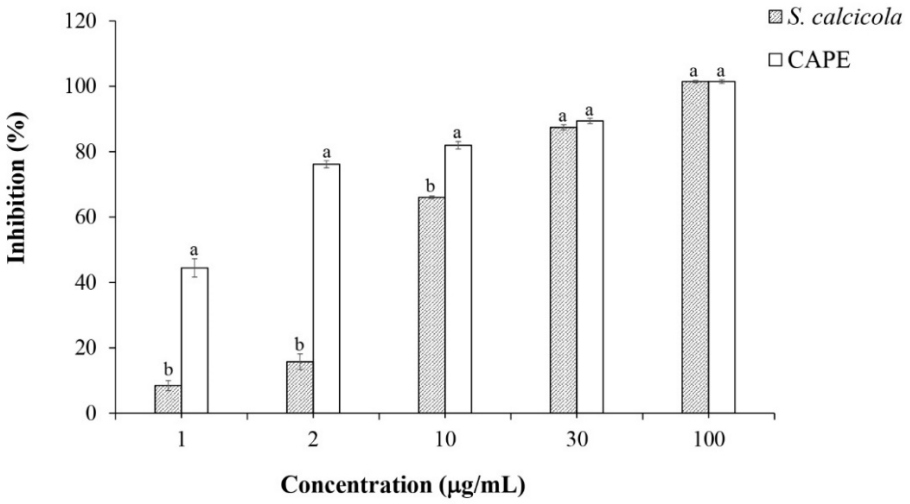

Figure 2. NO inhibitory activity of ethyl acetate extract from *S. calcicola* Inthachub leaves and CAPE, the positive control. Different alphabet labels indicate significant differences (p<0.05).

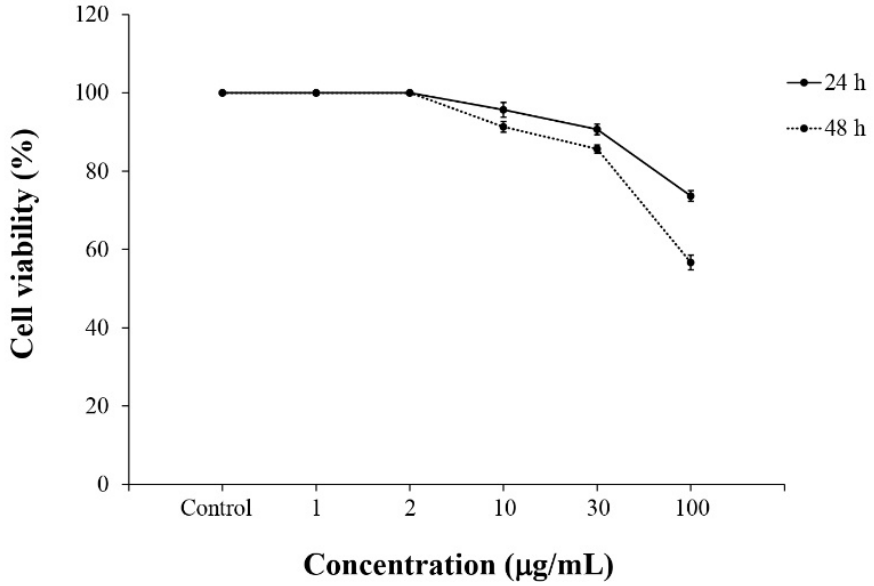

Figure 3. Effect of *S. calcicola* Inthachub leaf extract on cell viability based on MTT assay

secondary metabolites commonly found in plants that play an important role in various biological activities, especially anti-inflammatory (Shahidi & Yeo, 2018; Rahman et al., 2022). Moreover, phenolic compounds have been reported for potential AChE inhibitory activity (Yahia et al., 2019; Sundaramoorthy & Packiam, 2020). Phenolic compounds are believed to exhibit an anti-inflammatory effect by suppressing the binding of pro-inflammatory mediators, NO synthase, and COX-2 activity (Rahman et al., 2022).

Furthermore, the presence of multiple hydroxyl groups in the chemical structure of phenolic compounds is believed to enhance the inhibition of AChE activity by allowing for stronger interactions with the active site of AChE (Falé et al., 2012; Suciati et al., 2022). Alkaloids, one of the many families of natural compounds, are recognized for their inhibitory effect on acetylcholinesterase. The anti-AChE activity of alkaloids is involved in their complex nitrogen structures and the interaction of the positively charged nitrogen with the active site of AChE (Konrath et al., 2013; Suciati et al., 2022).

Additionally, alkaloids from plants in the *Stemona* genus which belong to the same family of the plant in this study, Stemonaceae, including *Stemona tuberosa* and *Stemona sessilifolia*, have been reported to have anti-inflammatory and anti-AChE effects, respectively (Lai et al., 2013; Xu et al., 2022).

However, several studies investigated that inflammation is involved in pathogenesis and contributes to the progression of AD (Kinney et. al., 2018; Sinyora et. al., 2020). Furthermore, some studies have also found that the accumulation of amyloid-β (Aβ) results in increased inflammatory responses and triggers brain cells to release pro-inflammatory by increasing the expression level of cytokines and chemokines (Kirkley et. al., 2017; Gao et. al., 2023). The continued release of pro-inflammatory leads to exacerbation of neuroinflammation, which induces neurotoxin and neuronal death (Kinney et. al., 2018; Sinyora et. al., 2020; Amelimojarad et. al., 2024). Additionally, previous studies have indicated that AChE has been found to colocalize with Aβ deposits in the brain of an Alzheimer's patient. *In vitro* and *in vivo* studies indicated that AChE binds with Aβ to form a complex by interaction throughout the peripherical anionic site on the enzyme leading to accelerating amyloid fibril formation and Aβ aggregation, which increases neurotoxicity (Ferrari et.al., 2001; Inestrosa et.al., 2008; Carvajal and Inestrosa, 2011; Sie et.al., 2023). Therefore, multitarget anti-alzheimer natural compounds that exhibit both AChE inhibitory and anti-inflammatory activities are of interest in drug development as they may have therapeutic potential in AD treatment and reduce progression of AD.

Interestingly, the endangered species, *S. calcicola* Inthachub, has strong inhibitory effects both on inflammatory and AchE activities. Consequently, it may have great medical importance in the development of new effective drugs for treating and preventing the progression of AD. However, the determination of the main bioactive compounds and evaluation of the anti-inflammatory and anti-AChE mechanisms, including mechanisms for inhibition of amyloid fibril formation and aggregation of Aβ in this plant, are required for further study.

4. Conclusions

The present study is the first report on the anti-inflammatory and anti-AChE effects of the ethyl acetate extract from *S. calcicola* Inthachub leaves, the endangered plant species. The study surprisingly shows that the extract of this endangered plant species has a strong ability to reduce inflammation through inhibition of NO production in RAW 264.7 macrophage cells, and is non-toxicity. Furthermore, it also inhibits the activity of AChE. The effectiveness of the extract of this endangered species in reducing inflammation and AChE action is probably related to the high content of phenolic compounds and alkaloids in the plant leaves. The findings indicated that this endangered species is a potential source of anti-inflammatory and anti-AChE compounds. Th plant may be a valuable medicinal herb and a potential target for the development of new drugs for treating and reducing the progression of AD. Thereby, conserving this endangered plant species is crucially important not only to prevent the extinction of the plant but also to protect the loss of

important sources of natural medicinal agents. However, further investigations are required to find out the specific phytochemical compounds and elucidate the mechanisms of the anti-inflammatory and anti-acetylcholinesterase activities.

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6. Conflicts of Interest

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

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