Potential of *Alpinia galanga* Rhizome and Pseudostem Extracts on Anti-free Radical and Anti-cholinesterase Activities

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Abstract

The objective of this research is to improve the potential of *Alpinia galanga* (L.) Willd. for prevention and rehabilitation of Alzheimer's disease. Ethanolic, ethyl acetate, dichloromethane, and hexane extracts of galangal pseudostem and rhizome were subjected to screen for phytochemicals, including carbohydrates, proteins, alkaloids, polyphenols, flavonoids, anthocyanins, and tannins. Furthermore, these extracts were assessed for their radical scavenging, anti-acetylcholinesterase, and anti-butyrylcholinesterase activities. The result of phytochemical screening indicated that polyphenols were observed in the ethanolic, ethyl acetate, and dichloromethane extracts of galangal pseudostem. For biological activity evaluations, the dichloromethane pseudostem extract at 0.1 mg/mL concentration exhibited the highest inhibitory activity against free radical (95.48%). Moreover, this extract at 0.2 mg/mL concentration also possessed the highest acetylcholinesterase (88.57%). The concentration providing 50% acetylcholinesterase inhibition (IC_{50}) of the extract was 0.158 mg/mL. The hexane rhizome extract at 0.2 mg/mL concentration presented the greatest butyrylcholinesterase inhibitory activity (57.84%), followed by the dichloromethane pseudostem extract at 0.2 mg/mL concentration (39.09%). These findings suggested the potential of using dichloromethane extract of galangal pseudostem for prevention and treatment of Alzheimer's disease. Consequently, further research focusing on extraction development and bioactive compounds identification from this extract should be carried out.

Keywords : Alpinia galanga, Alzheimer's disease, cholinesterase, free radical, galangal extract

1. Introduction

Prevalence and incidence of Alzheimer's disease is increasing, and the burden of mortality will more than double in the next 20 years (Javaid *et al.*, 2021). Signs of Alzheimer's disease include memory loss that disrupts daily life, difficulty completing familiar tasks, confusion with time or place, as well as trouble understanding visual images and spatial relationships. The brain changes of Alzheimer's disease are abnormal proteins beta-amyloid and phosphorylated tau accumulation, and neurons deterioration (Alzheimer's Association, 2022). The progression of Alzheimer's disease due to oxidative stress production from amyloid protein and tau hyperphosphorylation may be reduced when consuming antioxidants (Sinyor, Mineo and Ochner, 2020). Presently, Alzheimer's disease pharmacological treatments are cholinesterase inhibitors, which elevated cerebral acetylcholine levels by inhibiting cholinesterase activities. Acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE), the most important types of cholinesterase enzyme in human brain, are the focus (Adewusi, Moodley and Steenkamp, 2010).

Alpinia galanga (L.) Willd., referred to "Kha" in Thai, is one of a Thai herb with potential for anti-Alzheimer's. Previous studies have demonstrated that galangal rhizome contained several bioactive compounds such as 8–9' linked neolignans, galanganal, galanganols A, B and C, and 1'δ-1'-acetoxyeugenol acetate, which exhibited biological activity associated to Alzheimer's disease (Simon *et al.*, 2022). Moreover, some previous studies indicated that ethanolic, methanolic, aqueous extracts and essential oil obtained from galangal rhizome had potential to be employed as natural antioxidants due to their anti-free radical ability (Devi *et al.*, 2018; Mahae and Chaiseri, 2009; Tachakittirungrod and

Chowwanapoonpohn, 2007). Furthermore, some studies revealed the effects of galangal rhizome against AChE and BuChE. Galangal essential oil could inhibit both AChE and BuChE activities (Chaiyana, Sriyab and Okonogi, 2022). Ethanolic extract of galangal rhizome could inhibit BuChE (Khattak *et al.*, 2005), while methanolic galangal rhizome extract had AChE inhibitory activity (Adewusi, Moodley and Steenkamp, 2010). Moreover, 1'δ-1'-acetoxyeugenol acetate isolated from chloroform fraction of *A. galanga* rhizome had anti-amnesia effect by increasing Na^+/K^+-ATPase and antioxidant activity, as well as decreasing AChE level in mice (Jayasingh Chellammal *et al.*, 2019). Although several studies on galangal have been published, no data of agricultural waste as galangal pseudostem have been reported. Therefore, the pseudostem has been an initial investigation in addition to focusing on the rhizome with the attention to increasing its value. In order to assure the potential of galangal as a treatment of Alzheimer's disease, both rhizome and pseudostem of *A. galanga* were evaluated for phytochemical screening tests, anti-free radical and anti-cholinesterase activities.

2. Objectives

The purpose of this study is to explore the potential of *A. galanga* (L.) Willd. pseudostem and rhizome for Alzheimer's disease prevention and rehabilitation by screening phytochemicals and determining anti-free radical, anti-AChE, and anti-BuChE properties.

3. Materials and methods

3.1 Materials

Rhizomes and pseudostems of *A. galanga* were obtained from eight-month-old plants cultivated in Samut Prakan, Thailand. The plant specimens were preserved in Queen Sirikit Botanic Garden Herbarium (QBG), Ministry of Natural Resources and Environment (specimen number: QBG 108548). All reagents and solvents used were analytical grade. Chemicals used for preparation of extracts included hexane (J.T. Baker, USA), dichloromethane (Fisher Scientific, Belgium), ethyl acetate (Fisher Scientific, Belgium), and 95% ethanol (VWR BDH chemical, France). Reagents used for phytochemical screening tests were DMSO (Scharlau, Spain), α-napthol (Fluka, USA), ferric chloride (Ajax FineChem, Australia), concentrated HCl (Supelco, USA), ammonia solution (Panreac AppliChem, Germany), and tannic acid (Sigma-Aldrich, USA). Moreover, Fehling's, ninhydrin, Hager's, Mayer's, Wager's, Dragendorff's, and gelatin solutions were prepared by using reagents from Merck, Germany. The reagents used for biological activity tests, including quercetin, DPPH, donepezil HCl, polysorbate 20, acetylthiocholine iodide (ATCI), 5,5'-dithio-bis-[2-nitrobenzoic acid] (DTNB), AChE from electric eel, BuChE from equine serum, and butyrylthiocholine iodide were purchased from Sigma-Aldrich, USA. Bovine serum albumin was bought from Himedia, India. Tris(hydroxymethyl)aminomethane was bought from Fisher Scientific, Belgium. The activity tests were carried out using flat-bottomed 96-well microplates (Thermo Fisher Scientific, USA) and UV-visible spectrophotometer (EZ Read 2000 microplate reader, Biochrom, UK).

3.2 Methods

The extracts of rhizomes and pseudostems of *A. galanga* (L.) Willd. were screened for phytochemicals, including carbohydrates, proteins, alkaloids, polyphenols, flavonoids, anthocyanins, and tannins. Moreover, DPPH radical scavenging, AChE and BuChE inhibition assays were performed.

3.2.1 Preparation of extracts

The rhizomes and pseudostems were cleaned, cut into small pieces, and dried in a hot air oven at 40°C before grinding. The pulverized samples were then extracted by using soxhlet apparatus with four different organic solvents for 12 hours of each. Low to high polarity solvents, i.e., hexane, dichloromethane, ethyl acetate, and 95% ethanol, were utilized,

respectively. The solvents in the extracts were evaporated using rotary evaporator. Finally, the crude extracts were freeze-dried and stored in a fridge (4°C) until use. Yield of extracts were calculated using the following equation: Extraction yield (%w/w) = WHE/WDP × 100, where WHE is weight of herbal extract and WDP is weight of dry herbal powder.

3.2.2 Phytochemical screening test

All of galangal extracts were dissolved in 50% dimethyl sulfoxide (DMSO) in ethanol to obtain final concentration of 1 mg/mL. The extract solutions were then determined for the absence or presence of carbohydrates, proteins, alkaloids, polyphenols, flavonoids, anthocyanins, and tannins.

Test of carbohydrates

The presence of carbohydrates and reducing sugar were determined by using Molisch's and Fehling's tests, respectively. Molisch's test was done by adding 3 drops of 1% w/v of α-napthol into 1 mL of each extract solution. The mixture was mixed until homogenous. Next, 1 mL of concentrated sulfuric acid was gently added down into tube's side. If carbohydrates were found, reddish-purple color at the junction of the solution mixture would be observed. According to Fehling's test, 1 mL of Fehling solution was added into 1 mL of each extract solution before mixing until consistent. Then, the mixture was boiled in water bath for 5 minutes. If reducing sugar was observed, reddish-orange precipitate would appear.

Test of proteins

To a 1-mL solution of each extract, 1 mL of ninhydrin solution was added. The mixture was thoroughly combined before being boiled in a water bath for 5 minutes. When the extracts contained proteins, the color would change to blue-purple color.

Test of alkaloids

Five reagents, including Hager's, Mayer's, Wager's, Dragendorff's, and tannic acid solutions were used. The tests were carried out on mirror plates. One drop of each reagent was combined with two drops of each extraction solution. If alkaloids were found, precipitates in orange, yellow, white, reddish-brown, and white color would be observed when using Dragendorff's, Hager's, Mayer's, Wager's, and tannic acid reagents, respectively.

Test of polyphenols

Three drops of 1% w/v ferric chloride solution were mixed with 0.5 mL of each extract solution. If polyphenols were detected, the color would change to blue or dark-blue, green or greenish brown, with or without precipitate.

Test of flavonoids

Flavonoids were observed using Shinoda's and Pew's tests. For Shinoda's test, 0.5 mL of each extract solution was mixed with 0.05 g of magnesium powder before adding 5 drops of concentrated HCl. Orange-red color indicating the presence of gamma benzopyrone. For Pew's test, 0.5 mL of each extract solution was mixed with 0.25 g of zinc powder before adding 1 drop of 2 N HCl. After shaking the mixture, 5 drops of concentrated HCl were added and mixed well. The presence of flavanonol and flavonol-3-glycoside resulted in a dark-red color, whereas the presence of flavanone and flavonol resulted in a fade-red color.

Test of anthocyanins

One drop of 2 N HCl was added to 0.5 mL of each extract solution and mixed thoroughly. Then, drop by drop of 10% ammonia solution was added into the mixture. In the presence of anthocyanin, red and blue colors were produced in acid and base conditions, respectively.

Test of tannins

One mL of 1% w/v gelatin was mixed with 0.5 mL of each extract solution. The precipitates were formed if tannins were observed.

3.2.3 Biological activity test

DPPH radical scavenging assay

DPPH radical scavenging assay was performed on flat-bottomed 96-well microplates. The assay was done in triplicate. Reference standard was 0.1 mg/mL quercetin. Each extract solution at the concentration of 0.1 mg/mL was evaluated. Firstly, 100 µL of 0.3 mg/mL DPPH ethanolic solution was mixed with 100 µL of each extract. The reaction was then incubated in the dark place at room temperature for 30 minutes. The absorbance at 517 nm was measured. The percentage of radical scavenging activity was calculated based on the following equation: %Radical scavenging = {1-[(A-B)/(C-D)]} × 100, where A is the absorbance of the extract with DPPH, B is the absorbance of the extract without DPPH, C is the absorbance of solvent with DPPH, and D is the absorbance of solvent without DPPH.

Cholinesterase inhibition assays

Acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) inhibitory activities were determined in flat-bottomed 96-well microplates using the modified Ellman's method (Miao, He and Zhu, 2010; Yanaso *et al.*, 2018). The assay was performed in triplicate. Reference standard was 10 µg/mL donepezil HCl. The extracts were dissolved in 20% v/v polysorbate 20 at the final concentration in the reaction of 0.5 mg/mL and 0.2 mg/mL were determined. AChE and BuChE originated from electric eel and equine serum, respectively were applied. According to AChE inhibition assay, the reaction mixture consisted of 25 µL of each tested solution, 25 µL of 15 mM acetylthiocholine iodide (ATCI), 50 µL of 0.1% w/v bovine serum albumin in 50 mM Tris(hydroxymethyl)aminomethane pH 8, 125 µL of 3 mM 5,5'-dithio-bis-[2-nitrobenzoic acid] (DTNB) and 25 µL of 0.22 units/mL AChE. The final volume of this test was 250 µL. After incubation at 25°C for 20 minutes, the absorbance of the reaction mixture was measured at 405 nm using microplate reader UV scan. The tested sample solution was replaced with sample-dissolving solvent as control. The control and tested samples were examined in triplicate. Percentage of AChE inhibition was calculated by the following equation: %AChE inhibition = {1-[(A-B)/(C-D)]} × 100, where A is the absorbance of the extract with AChE, B is the absorbance of the extract without AChE, C is the absorbance of solvent with AChE, and D is the absorbance of solvent without AChE. Only the extract presented the greatest AChE inhibitory activity was evaluated for concentration providing 50% of inhibition (IC_{50}) by using Microsoft Excel 2016.

Similar to the above protocol, BuChE evaluation employed 25 µL of 0.50 units/mL BuChE to start the enzymatic reaction and 25 µL of 1.5 mM butyrylthiocholine iodide instead of ATCI in each well. BuChE inhibition was calculated by the following equation: %BuChE inhibition = {1-[(A-B)/(C-D)]} × 100, where A is the absorbance of the extract with BuChE, B is the absorbance of the extract without BuChE, C is the absorbance of solvent with BuChE, and D is the absorbance of solvent without BuChE.

3.2.4 Statistical analysis

All biological activity experiments were done in triplicate. Therefore, the results were expressed as mean along with standard deviation (SD) which were calculated by Microsoft Excel 2016. Additionally, one-way analysis of variance (ANOVA) followed by Fisher's Least Significant Difference (LSD) post hoc test was performed using SPSS Statistics 17.0. The results were statistically significance when $p < 0.05$.

4. Results and discussion

4.1 Plant extraction

Rhizomes and pseudostems of *A. galanga* cultivated in Samut Prakan, Thailand were extracted successively with four different organic solvents, including hexane, dichloromethane, ethyl acetate, and ethanol. The percentage extraction yield of eight crude extracts was ranged from 0.73 – 4.10 %w/w, as displayed in Table 1. For rhizome extractions, the hexane fraction provided the highest extraction yield followed by ethanol, ethyl acetate, and dichloromethane, respectively. Meanwhile, the pseudostem crude extract obtained from hexane fraction revealed

the maximum extraction yield followed by dichloromethane, ethanol, and ethyl acetate fractions, respectively. When comparing the extraction yield of rhizome and pseudostem of *A. galanga* with the same extraction solvent, the results indicated that the rhizome extracts offered a greater yield.

Table 1 Percentage yield of crude extracts of *A. galanga* rhizome and pseudostem

***A. galanga* extracts**		**Extraction yield (%w/w)**
Rhizome	Ethanol	4.10
	Ethyl acetate	1.03
	Dichloromethane	0.99
	Hexane	6.24
Pseudostem	Ethanol	0.83
	Ethyl acetate	0.73
	Dichloromethane	1.01
	Hexane	1.79

4.2 Phytochemical screening test

All of the extracts were analyzed for carbohydrates, proteins, alkaloids, polyphenols, flavonoids, anthocyanins, and tannins. The results, as depicted in Table 2, showed that *A. galanga* ethanolic rhizome and pseudostem extracts included carbohydrates and proteins. Furthermore, polyphenols were found in pseudostem when extracting by ethanol, ethyl acetate, and dichloromethane. Polyphenols play an important role in protecting the body from external stimuli and in the removal of reactive oxygen species, which are the cause of many illnesses (Rana *et al.*, 2022). As a result, pseudostem extracts containing polyphenols have a significant potential for future biological activity investigation.

Table 2 Phytochemical screening test of *A. galanga* rhizome and pseudostem extract

Test		***A. galanga* extracts**							
		Rhizome				**Pseudostem**			
		A	**E**	**D**	**H**	**A**	**E**	**D**	**H**
Carbohydrates	Molisch's test	+	-	-	-	+	-	-	-
	Fehling's test	-	-	-	-	-	-	-	-
Proteins	Ninhydrin	+	-	-	-	+	-	-	-
Alkaloids	Dragendorff's	-	-	-	-	-	-	-	-
	Hager's	-	-	-	-	-	-	-	-
	Mayer's	-	-	-	-	-	-	-	-
	Wager's	-	-	-	-	-	-	-	-
	Tannic acid	-	-	-	-	-	-	-	-
Polyphenols	Ferric chloride test	-	-	-	-	+	+	+	-
Flavonoids	Shinoda's test	-	-	-	-	-	-	-	-
	Pew's test	-	-	-	-	-	-	-	-
Anthocyanins	Acid-base test	-	-	-	-	-	-	-	-
Tannins	Gelatin test	-	-	-	-	-	-	-	-

Note: + = presence, - = absence, A = ethanol, E = ethyl acetate, D = dichloromethane, H = hexane.

4.3 Biological activity test

Biological activities, i.e., DPPH radical scavenging, AChE and BuChE inhibition, were investigated in this study. For DPPH radical scavenging experiment, 0.1 mg/mL of the extracts and quercetin (positive control) were examined. The radical scavenging

activities of *A. galanga* extracts and quercetin are depicted in Table 3. The percentage of DPPH radical scavenging of rhizome and pseudostem galangal extracts varied from 16.79% to 85.94%. The dichloromethane extract of *A. galanga* pseudostem had the greatest radical scavenging activity (95.48%) followed by the rhizome extracted in ethyl acetate (89.85%). These extracts exhibited DPPH radical scavenging activity different from that of quercetin (94.66%), with no statistical significance. Moreover, the ethanolic rhizome extract also demonstrated high radical scavenging activity (85.94%). Likewise, some previous studies reported the DPPH scavenging activities of galangal rhizome extracted using different solvents. The aqueous, 60% ethanolic, and 60% methanolic extracts at the concentration of 18 mg/mL exhibited 81.75%, 86.61%, and 92.4% DPPH scavenging, respectively (Devi *et al.*, 2018). Moreover, ethanolic extract of galangal rhizome from Malaysia exhibited 78.47% DPPH scavenging when evaluated at the concentration of 2.0 mg/mL (Manoharan *et al.*, 2012). Ethyl acetate-extracted galangal rhizome was also evaluated which exhibited DPPH scavenging activity with IC_{50} at 475 µg/mL (Sani *et al.*, 2019). While previous studies primarily focused on assessing DPPH scavenging activity in *A. galanga* rhizome extracts, our research expands this scope to include pseudostem extracts. Interestingly, the dichloromethane pseudostem extract potentially exhibited capabilities against free radicals. Therefore, it could be a promising candidate in the treatment of a variety of chronic and degenerative disorders such as cancer, aging, rheumatoid arthritis, cardiovascular and neurological diseases (Pham-Huy, He and Pham-Huy, 2008).

Table 3 Radical scavenging activities of *A. galanga* rhizome and pseudostem extracts

Tested sample		**% radical scavenging (mean ± SD)**
Rhizome extract	Ethanol	85.94 ± 3.076 [a]
	Ethyl acetate	89.85 ± 0.323 [a,b]
	Dichloromethane	48.94 ± 1.417 [c]
	Hexane	16.79 ± 2.104 [d]
Pseudostem extract	Ethanol	72.94 ± 2.935 [e]
	Ethyl acetate	47.17 ± 6.195 [c]
	Dichloromethane	95.48 ± 1.729 [f]
	Hexane	69.23 ± 6.786 [e]
Quercetin		94.66 ± 1.346 [b,f]

Note: Tested concentration = 0.1 mg/mL. SD = standard deviation from triplicate analysis. Different letters (a–f) indicate significant differences ($p < 0.05$) among means.

For AChE and BuChE inhibition of the galangal extracts, 0.5 mg/mL of all rhizome extracts and ethanolic extract of pseudostem were evaluated. Due to color disturbance and solubility concerns, pseudostems extracted with ethyl acetate, dichloromethane, and hexane were determined at the concentration of 0.2 mg/mL. The inhibitory activities of all extracts are detailed in Table 4. The results indicated significant variations in anticholinesterase activities based on the galangal part and the type of extraction solvent ($p < 0.05$).

The AChE inhibition of galangal extracts ranged from 2.91% to 88.57%. Unfortunately, the ethanolic rhizome and pseudostem extracts showed no detectable and negligible activity against AChE, respectively. The results were corresponding to previous studies which reported that 100 µg/mL of methanolic rhizome extract showed only 16.98% inhibitory activity against AChE (Adewusi, Moodley and Steenkamp, 2010). Similarly, the ethanolic rhizome extract was also reported as a weak AChE inhibitor (Khattak *et al.*, 2005). However, not only the rhizome but also the pseudostem which was typically considered as agricultural waste were examined for AChE inhibition in this study. Remarkably, under identical extraction solvent conditions,

the pseudostem extracts expressed greater AChE inhibition than the rhizome extracts. The dichloromethane extract from the pseudostem displayed the highest AChE inhibitory activity with an IC_{50} value of 0.158 mg/mL (Figure 1).

For BuChE inhibitory test, the results of the galangal extracts ranged from 4.92% to 57.84%. Remarkably, the ethanolic and hexane extracts from the rhizome exhibited higher BuChE inhibitory activity than those from the pseudostem when using the same extraction solvent. However, no significant difference was observed between the ethyl acetate rhizome and ethyl acetate pseudostem extracts, as between the dichloromethane rhizome and dichloromethane extracts. According to prior research, the significant inhibition activities (≥ 50%) were found in ethanolic extracts derived from several plants, including rhizome of *A. galanga* (Khattak *et al.*, 2005). However, the hexane rhizome extract displayed the highest BuChE inhibitory activity in this study (57.84%) followed by dichloromethane pseudostem extract (39.09%) and dichloromethane rhizome extract (36.39%).

While AChE inhibitors are the primary choice for treating Alzheimer's disease, they have some defects, such as dose limitations and unsatisfactory long-term treatment effects. To enhance therapeutic outcomes for Alzheimer's, dual-target cholinesterase inhibitors, addressing both AChE and BuChE, have emerged as a recent research focus in drug development due to their lower side effects compared to specific AChE inhibitors (Zhou and Huang, 2022). As a result, the dichloromethane pseudostem extract, which strongly inhibited AChE and partially inhibited BuChE had a potential to develop for Alzheimer's disease treatment.

Table 4 AChE and BuChE inhibition of *A. galanga* rhizome and pseudostem extracts

Tested sample		**% inhibition (mean ± SD)**	
		AChE	**BuChE**
Rhizome extract	Ethanol*	N.D. [a]	24.12 ± 0.929 [i]
	Ethyl acetate*	12.18 ± 1.949 [b]	16.24 ± 0.103 [j]
	Dichloromethane*	10.07 ± 0.429 [c]	36.39 ± 0.103 [k]
	Hexane*	2.91 ± 0.713 [d]	57.84 ± 3.620 [m]
Pseudostem extract	Ethanol*	2.36 ± 0.202 [d]	N.D. [n]
	Ethyl acetate**	77.19 ± 2.447 [e]	14.02 ± 1.548 [j]
	Dichloromethane**	88.57 ± 0.733 [f]	39.09 ± 4.340 [k]
	Hexane**	66.23 ± 0.000 [g]	4.92 ± 0.207 [p]
Donepezil (10 µg/mL)		95.05 ± 0.940 [h]	65.87 ± 3.89 [q]

Note: N.D. = not detected. SD = standard deviation from triplicate analysis. * = 0.5 mg/mL, ** = 0.2 mg/mL. Different letters (a–q) indicate significant differences ($p < 0.05$) among means.

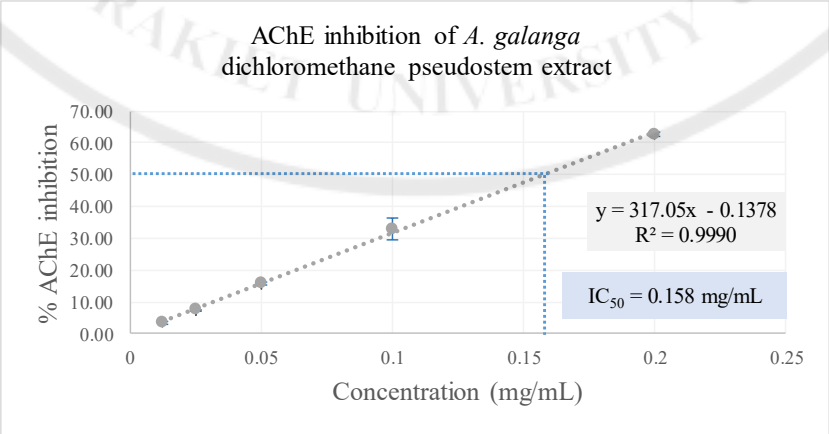

Figure 1 IC_{50} value of *A. galanga* dichloromethane pseudostem extract against AChE.

5. Conclusion

This study revealed intriguing data from galangal extracts, highlighting the presence of polyphenols in the pseudostem extracts. Notably, the dichloromethane pseudostem extract exhibited the highest anti-free radical activity in the DPPH assay. Additionally, it demonstrated the most potent inhibition against AChE, along with partial inhibition against BuChE, suggesting potential for Alzheimer's disease treatment. Accordingly, the extraction development of *A. galanga*, and the identification of bioactive components should undergo further investigation.

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