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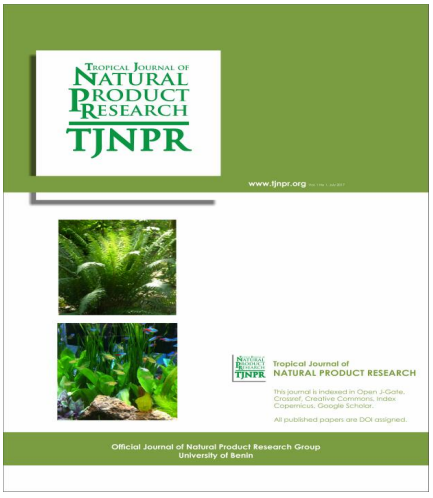

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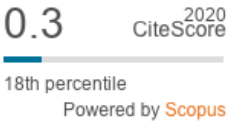

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Original Research Article

Evaluation of Antioxidant and Cholinesterase Inhibitory Activities of Various Extracts of *Cassia spectabilis*

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ABSTRACT

Alzheimer's disease (AD) is a progressive neurodegenerative disorder that mainly occurs in elderly people. The increasing number of people suffering from AD causes health, social and economic problems. Therapeutic strategies implemented to slow down the progress of AD are by using cholinesterase inhibitors and antioxidants. The current study aimed to investigate the antioxidant and cholinesterase inhibitory properties of the leaves and stems of *Cassia spectabilis* as well as to determine the total phenolic contents in the samples. The leaves and stems of *C. spectabilis* were extracted with 96% ethanol by the maceration method. The cholinesterase inhibitory assay was performed by using the modified Ellman's method against two cholinesterase enzymes, namely acetylcholinesterase (AChE) and butyrylcholinesterase (BChE). The antioxidant properties of the samples were evaluated using 2,2-diphenyl-1-picryl hydrazyl (DPPH) and 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) methods. The total phenolic contents (TPC) were determined by a colorimetric assay using gallic acid as a reference. The results showed that the leaves and stems of *C. spectabilis* exerted significant inhibitory effects against both AChE and BChE enzymes with IC_{50} values of 24.3 and 9.8 µg/mL for the leaves and 58.6 and 47.8 µg/mL for the stems, respectively. The extracts showed moderate antioxidant activity in both DPPH and ABTS assays with IC_{50} ranging from 117 – 313 µg/mL. The presence of phenolic content in the samples may contribute to the antioxidant potency of the samples. The presence of non-phenolic compounds, such as alkaloids may be responsible for the cholinesterase inhibitory properties of the extracts.

Keywords: Alzheimer's disease, *Cassia spectabilis*, Cholinesterase inhibitor, Antioxidant.

Introduction

For many years plants have shown a great contribution to the treatment of many diseases. Herbal medicines have been reported to show significant effects in the treatment of Alzheimer's disease, such as the well-known *Ginkgo biloba*, *Bacopa monnieri*, and lately Chinese medicinal plant *Huperzia serrata*.[1,2] Alzheimer's disease (AD) is a neurodegenerative disorder that commonly affects elderly people. AD produces progressive and irreversible memory and cognitive decline and is associated with progressive behavioral disturbances and restrictions in activities of daily living. One of the common features of this disease is the low level of acetylcholine in the brain. Acetylcholine (ACh) is a neurotransmitter produced in the nerve ending of the presynaptic nerve from choline and acetyl coenzyme A. ACh is hydrolyzed to choline and ethanoic acid by AChE in the postsynaptic nerve.[3] Another enzyme that also plays an important in the pathogenesis of AD is BChE which is co-regulated with AChE in the metabolism of ACh.[4] Growing evidence showed the relation between oxidative stress and AD.

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The overproduction of reactive oxygen species (ROS) or lack of antioxidants leads to oxidative stress which eventually damages the brain cells, and leads to the progression of AD to dementia.[5,6] The presence of a toxic peptide, β-amyloid in the brain of patients with Alzheimer's disease is also contributed by oxidative stress.[5] Therefore, strategies for the treatment of AD have involved the use of cholinesterase inhibitors such as donepezil, rivastigmine, and galantamine, as well as the use of antioxidants.[7,8]

Cassia spectabilis (sin. *Senna spectabilis*) (DC.) H.S.Irwin & Barneby family Fabaceae is a flowering plant that can be found in tropical and sub-tropical regions.[9] The flowers are bright yellow therefore it is usually used as ornamental plants in some regions. *C. spectabilis* also known for its medicinal purposes in the folk medicines of several countries such as Indonesia, Brazil, and Thailand. The leaves are commonly used as laxatives and purgatives. Other traditional uses are for the treatment of skin diseases, edema, as well as poisoning, and protozoic infection of the gut.[10] A phytochemical study of the plant revealed the presence of alkaloids, terpenoids, flavonoids, anthraquinones, and steroids from various parts of *C. spectabilis* including the leaves, flowers, fruits, seeds, stems, and roots.[10-12] Pharmacological studies of the plant have shown promising antimicrobial[13-16] anticonvulsant,[17,18] antinociceptive, and anti-inflammatory activities.[19] In our previous study, we screened the potency of the leaves of several Cassia species as cholinesterase inhibitors. It was discovered that *C. spectabilis* showed the best potency among other Cassia species tested.[20] Leaves and stems are the part of this plant that show in abundance the presence of various metabolites. Therefore, in the current study, the potency of the leaves and the stems of *C. spectabilis* as cholinesterase inhibitors was

compared as well as to determine the antioxidant and quantify the phenolic contents in the extracts.

Materials and Methods

Materials

The reagents used for cholinesterase assays were acetylcholinesterase from electric eel (AChE type VI-S), acetylthiocholine iodide (ATCI), horse-serum butyrylcholinesterase (BChE), butyrylthiocholine iodide (BTCI), 5,5′-dithiobis[2-nitrobenzoic acid] (DTNB), bovine serum albumin (BSA), tris buffer, and galantamine. The chemicals used for antioxidant assays were 2,2-Diphenyl-1-picrylhydrazyl (DPPH), 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), and potassium persulfate. Folin-Ciocalteu's phenol reagent, sodium carbonate, and gallic acid were used for the determination of total phenolics. All reagents were purchased from Sigma-Aldrich.

Sample collection

The leaves of *Cassia spectabilis* were collected from Purwodadi Botanic Garden, East Java, Indonesia on March 9th, 2019. The voucher specimen (PWD 02) was stored at the Faculty of Pharmacy, Universitas Airlangga. The plant was identified by Purwodadi Botanic Garden, Indonesian Institute of Sciences with identification letter number: 0371/IPH.06/HM/III/2019.

Preparation of extracts

Freshly collected leaves and stems of *Cassia spectabilis* were air-dried at room temperature for approximately seven days and then pulverized. Four hundred grams of the powdered leaves and stems were each extracted with 2 L of 96% ethanol. The samples were soaked in the solvent for 24 hours, followed by vacuum filtration. Then residues were each re-extracted with 1 L of ethanol using the same procedure. This process was repeated twice. All collected filtrates were then concentrated in a rotary evaporator at 40ºC to yield crude ethanolic extracts of the leaves (27.5 g) and stems (9.6 g).

Anticholinesterase Assay

The assay was carried out according to the modified Ellman's method.[21-23] The extracts were dissolved in methanol at a concentration of 10 mg/mL and were then diluted with water to obtain serial concentrations of samples containing not more than 10% of methanol. The final test concentrations in the wells were: 300, 200, 100, 30, 20, 10, 3, 2, 1, 0.3 and 0.1 µg/mL. Twenty-five microliters of sample solutions were added to a 96-well microplate, followed by the addition of 25 µL substrates 1.5 mM ATCI or 1.5 mM BTCI, 125 µL of 3 mM DTNB, and 50 µL Tris buffer, and finally 25 µL of 0.22 U/mL AChE or BChE. The solutions were placed in a microplate reader (Thermo Scientific Multiskan FC) and shaken for 30 s before measurement. The absorbances were measured at 405 nm every 5 s for 2 mins. Experiments were carried out in triplicates. Galantamine was used as a positive control, and 10% methanol was used as a negative control. The percentage of inhibition was then calculated as follows:

$$\%Inhibition = \frac{(Mean\ velocity\ of\ control - Mean\ velocity\ of\ sample)}{Mean\ velocity\ of\ control}\ x\ 100$$

DPPH radical scavenging assay

The DPPH assay was performed according to the modified method of Herald *et al*. (2012) and Lee *et al*.[24,25] The extracts were dissolved with methanol to make a series of concentrations of 5 – 500 µg/mL. Gallic acid was employed as a standard. The samples (100 µL) were added to 96 microwell plates and mixed with 0.25 mM DPPH reagent (100 µL). The DPPH reagent (100 µL) was mixed with methanol (100 µL) as a control, while methanol (200 µL) was used as a blank. The mixtures were then incubated in the dark at room temperature for 30 mins. The solutions were shaken for 30 s in a microplate reader (Thermo Scientific Multiskan FC). The absorbances were then recorded at 517 nm. The DPPH scavenging effect was calculated using the following formula.

$$DPPH\ Radical\ Scavenging\ activity\ (\%) = \frac{(abs\ control - abs\ sample)}{abs\ control}\ x\ 100$$

ABTS radical scavenging assay

The ABTS assay was carried out based on Lee *et al*. with some modifications.[25] ABTS solution (5 mL, 7 mM) was mixed with potassium persulfate (88 μL, 140 nM), and the mixture was kept in the dark at room temperature for 16 h to produce ABTS radical. Solution of samples at the concentration range 2.5 – 250 μg/mL was prepared in methanol. The samples (100 μL) were then mixed with 100 μL of ABTS in a 96-well microplate followed by incubation for 6 mins in the dark at room temperature. The absorbance was measured at 734 nm in a microplate reader and the plates were shaken for 30 s before reading. Gallic acid was used as standard. Experiments were done in triplicate. The ABTS radical scavenging activity was calculated using the equation as follows.

$$ABTS\ Radical\ Scavenging\ activity\ (\%) = \frac{(abs\ control - abs\ sample)}{abs\ control}\ x\ 100$$

Determination of total phenolic content (TPC)

The TPC of the extracts was determined according to the method by Herald *et al*. with slight modification.[24] Briefly, twenty-five microlitres of serial concentrations of gallic acid (25 – 500 µg/mL) or samples (1000 µg/mL) were added to a 96-well microplate, followed by the addition of water (75 µL) and Folin-Ciocalteu's phenol reagent (25 µL). The solutions were incubated for 6 mins at room temperature. Then 100 µL of Na_2CO_3 solution (75 g/L) was added to each well, followed by incubation for 90 mins in the dark at room temperature. The mixtures were shaken for 30 s before measurement of the absorbance at 765 nm in a microplate reader (Thermo Scientific Multiskan FC). The TPC of samples was expressed as milligrams of gallic acid equivalents (GAE) per gram extract.

Statistical analysis

The 50% inhibitory concentration (IC_{50}) values were determined using GraphPad Prism 8.0 software by plotting log concentrations as axis and % inhibition as ordinate for the cholinesterase inhibitory assays, and concentration of extracts as axis and % scavenging DPPH or ABTS for the antioxidant assays. Results were expressed as mean ± standard error of the mean (SEM) of three experiments.

Results and Discussion

Cholinesterase Inhibitory Activities

Despite the multifactorial nature of Alzheimer's disease, the most current therapeutic approach used is based on the cholinergic hypothesis. The use of cholinesterase inhibitors becomes the main therapeutic agent for this disease. Cholinergic neurotransmission is terminated by two cholinesterase enzymes, namely acetylcholinesterase (AChE) and butyrylcholinesterase (BChE), that play an important role in the hydrolysis of acetylcholine (ACh).[26]

Several plants from the genus Cassia have been reported as cholinesterase inhibitors such as *C. obtusifolia* inhibit AChE, BChE, and BACE1[27,28] and *Cassia alata* leave which showed inhibition against AChE.[29] In the present study, the *in vitro* cholinesterase inhibitory activity of the ethanolic extracts of the leaves and stems of *C. spectabilis* was evaluated based on the modified Ellman's method. The results show that all samples tested were able to inhibit both AChE and BChE enzymes in a concentration-dependent manner (Figures 1 and 2). As can be seen in Table 1 and Figures 1 and 2 the ethanolic extracts of the leaves and the stems demonstrated stronger inhibition against BChE compared to AChE. Inhibition of both AChE and BChE enzymes will prevent rapid hydrolysis of acetylcholine so that the amount of ACh will increase in the brain. BChE is also associated with neuritic plaques and the accumulation of fibrillar Aβ plaques. Therefore, inhibition against BChE will serve two roles, increasing the amount of acetylcholine as well as inhibiting fibrillar Aβ deposition.[4]

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Table 1: Cholinesterase inhibitory activity of *C. spectabilis* extracts

Samples	IC_{50} (µg/mL)[a]	
	AChE	BChE
Leaves extract	24.3 ± 0.4	9.8 ± 0.8
Stems extract	58.6 ± 0.4	47.8 ± 1.2
Galantamine	0.4 ± 0.1	2.2 ± 0.3

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

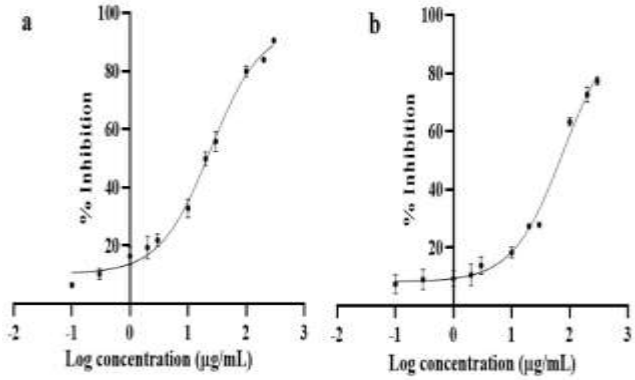

Figure 1: Concentration-dependent response of *C. spectabilis* leaves (a) and stems (b) extracts against AChE

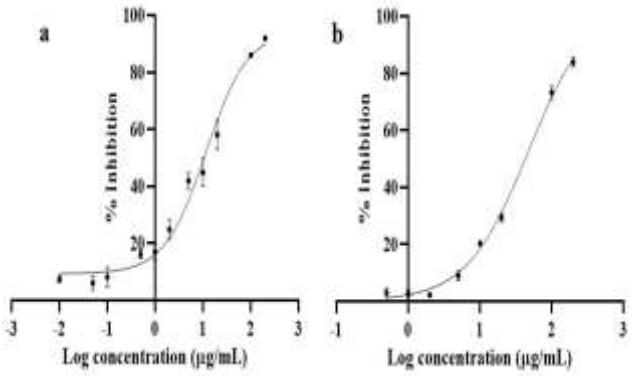

Figure 2: Concentration-dependent response of *C. spectabilis* leaves (a) and stems (b) extracts against BChE, each value is expressed as means ± SEM (n = 3)

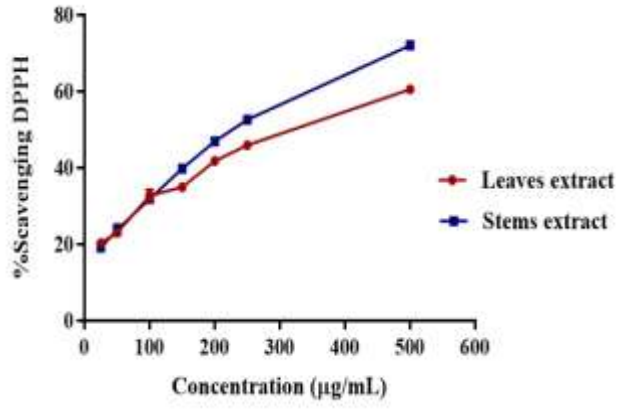

Figure 3: Scavenging effect of *C. spectabilis* extracts on DPPH free radicals, each value is expressed as mean ± SEM (n = 3)

The leaves extract gave IC_{50} values of 24.3 and 9.8 µg/mL against AChE and BChE, respectively compared to the stem extracts with IC_{50} values of 58.6 and 47.8 µg/mL. These results suggested that the leaves extract of *C. spectabilis* has better potency compared to the extract of the stems. Selegato *et al*. (2017) have summarized secondary metabolites reported from various parts of *C. spectabilis*.[11] It was found that the leaves contain more alkaloids compared to other parts of this plant, and there are no alkaloids reported from the stem. Numerous classes of compounds have been reported as cholinesterase inhibitors, however, the majority of these are alkaloids.[2,30] Alkaloids have shown promising anticholinesterase activities, even the current drug uses for AD therapy are alkaloids, galantamine, rivastigmine, and donepezil. The higher cholinesterase inhibitory activities of the leaves of *C. spectabilis* compared to the stems are possibly due to the presence of alkaloids in the extracts. Non-alkaloidal compounds present in the stem may also contribute to the anticholinesterase activity of the extract. This finding is in accordance with our previous study that based on the LC-MS/MS analysis the leaves of *C. spectabilis* contain alkaloid cassine, spectaline, and 3-*O*-acetylspectaline.[20] Further study is needed to investigate the chemical composition of the stems of *C. spectabilis* since there is a limited report.

Antioxidant Activity

In this study, the radical-scavenging activities of the ethanolic and aqueous extracts of *C. spectabilis* leaves were evaluated using the DPPH and ABTS assays. In the DPPH assay, the samples with antioxidant compounds provide a hydrogen atom, which can react with the stable radical DPPH to form a yellow-colored non-radical diphenylpicrylhydrazine. The degree of discoloration indicates the radical-scavenging potential of the sample.[31] The principle of ABTS assay is similar to that of DPPH assay, in which the antioxidant acts as a hydrogen donor to form a non-radical ABTS. The reduction of a dark-bluish color of ABTS radical can be monitored by spectrophotometer.[32] The results of the DPPH and ABTS scavenging activity of the extracts are shown in Table 2, Figures 3 and 4. The ethanolic extracts of *C. spectabilis* exhibited concentration-dependent antiradical activities in both DPPH and ABTS assays. The extracts showed lower antioxidant potency compared to the standard antioxidant gallic acid with inhibitory concentration 50% (IC_{50}) values ranging from 233.2-313.8 µg/mL and 117.2-214.0 µg/mL in the DPPH and ABTS assays, respectively.

Total Phenolic Content (TPC)

The total phenolic contents in the leaves and stems extracts were evaluated using a Folin-Ciocalteu reagent with gallic acid as a standard. The calculation was based on the standard curve of equation ($y = 0.0059x-0.1105$, $R^2 = 0.998$). The results as can be seen in Table 3 showed that the leaves and stems of *C. spectabilis* contain a slightly low amount of phenolics, which may contribute to the moderate antioxidant activities of the extracts.

Phenolic compounds have been reported to play a significant role in the antioxidant activities of plants. The antioxidant potential of the phenolic compound is predominantly due to its redox capability so that it can absorb and neutralize free radicals, decompose peroxide, and quench singlet or triplet oxygen.[33] Studies revealed that the antioxidant capacity of the phenolic compounds depends on the number and arrangement of the hydroxyl groups in this compound. The relation between phenolic contents and the antioxidant activity of several Cassia species has been documented.[34-38] The antioxidant potency, as well as phenolic contents of seven *Cassia* species, have been reported. *C. glauca* was reported to show the strongest antioxidant capacity compared to the other six *Cassia* species, which was related to its high content of phenolic compounds.[34] Several phenolic compounds such as anthraquinones and flavonoids have been reported from *C. spectabilis*.[11] The results from our study are in accordance with that reported in the previous study. Jothy *et al.* investigated the antioxidant potency of the leaves of *C. spectabilis* which showed moderate antioxidant activity with an IC_{50} value of 30.178 ± 0.129 mg/mL in the DPPH assay. The antioxidant activity of the stems of *C. spectabilis* has not been reported.[33]

Table 2: Antioxidant activity of *C. spectabilis* extracts

Samples	IC_{50} (µg/mL)[a]	
	DPPH	ABTS
Leaves extract	313.8 ± 5.9	214.0 ± 1.0
Stems extract	233.2 ± 2.2	117.2 ± 0.4
Gallic acid	2.76 ± 0.02	0.97 ± 0.03

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

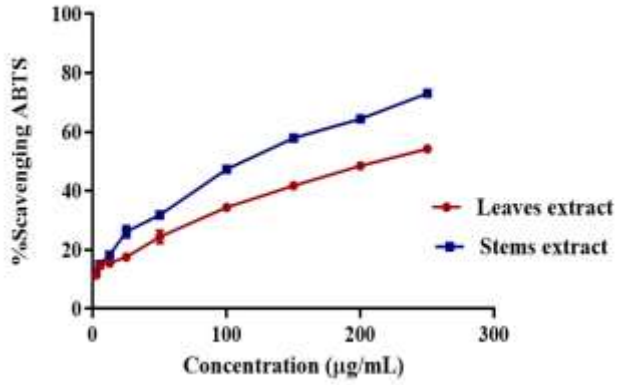

Figure 4: Scavenging effect of *C. spectabilis* extracts on DPPH free radicals, each value is expressed as mean ± SEM (*n* = 3)

Table 3: Extract yield and total phenolic content (TPC) of *C. spectabilis* extracts

Samples	Extract Yield (%)	TPC (mg GAE/g extract)[a]
Leaves extract	6.9	29.9 ± 0.4
Stems extract	2.4	25.2 ± 0.2

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

Conclusion

The leaves and stems extracts of *Cassia spectabilis* exhibited significant cholinesterase inhibitory activity against AChE and BChE. Both extracts demonstrated moderate antioxidant activity. The presence of non-phenolic compounds, such as alkaloids in the leaves may be responsible for the higher potency of the extract as a cholinesterase inhibitor.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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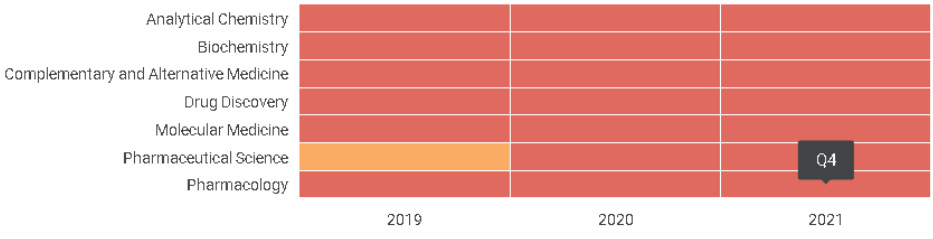

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