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RESEARCH ARTICLE

In vitro Antioxidant and Anticholinesterase Activities of Extracts from the Leaves of *Cassia moschata* Kunth

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ABSTRACT:

Alzheimer's disease (AD) is a neurodegenerative disorder, which is the most common cause of dementia. This disease commonly occurs in elderly people. The increase in life expectancy means that that the number of people suffering from AD is expected to rise each year if there is no effective treatment found. The relation of cholinesterase and oxidative stress to Alzheimer's disease has been reported. In our previous study, we have investigated the potency of the ethanolic extract of *Cassia moschata* leaves as an anticholinesterase. The current study aimed to investigate the antioxidant and anticholinesterase properties of the ethanolic and aqueous extracts of *C. moschata* as well as to determine the total phenolic content (TPC). Two different methods were used to evaluate the antioxidant activity by 2,2-diphenyl-1-picryl hydrazyl (DPPH) and 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assays. The anticholinesterase assay was carried out against acetylcholinesterase (AChE) according to the modified Ellman's method. The TPC was determined by a colorimetric method using Folin-Ciocalteu's phenol reagent, and employing gallic acid as a reference. The ethanolic and aqueous extracts of *C. moschata* demonstrated antioxidant activity in both DPPH and ABTS assays. There were statistically significant differences in the IC_{50} values of the ethanolic and aqueous extracts in both DPPH and ABTS assays. The aqueous extract exhibited a lower IC_{50} value compared to the ethanolic extract. The IC_{50} value for the aqueous extract was 36.46 µg/mL in the DPPH assay, and 10.61 µg/mL in the ABTS method compared to IC_{50} 38.74 µg/mL and 17.17 µg/mL for the ethanolic extract, respectively. Meanwhile, the ethanolic extract showed higher potency as anticholinesterase with the IC_{50} value of 44.43 µg/mL compared to the aqueous extract with an IC_{50} value of 114.60 µg/mL. The TPC measurement revealed that the aqueous extract has a higher amount of phenolic than the ethanolic extract. These data suggest that the aqueous extract from the leaves of *C. moschata* has a higher ability to scavenge free radicals compared to the ethanolic extract, which also contains a higher amount of phenolic compounds. However, the high content of phenolic compounds in the aqueous extract did not correspond to the anticholinesterase activity. The presence of non-phenolic compounds may also contribute to the anticholinesterase activity in the ethanolic extract.

KEYWORDS: Alzheimer's disease, *Cassia moschata*, Medicinal plant, Anticholinesterase, Antioxidant.

INTRODUCTION :

Alzheimer's disease (AD) is a progressive neurodegenerative disorder that has been known as the common cause of dementia[1]. The low level of neurotransmitters especially acetylcholine is one of the common features in this disease which is caused by the loss of cholinergic nerves as well as the presence of senile

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plaques and neurofibrillary tangles[2-6]. Therefore one of the strategies in the treatment of this disease is the use of anticholinesterases such as tacrine, donepezil, rivastigmine, and galantamine. The relation of oxidative stress and neurological diseases, such as Alzheimer's disease has been well documented. Numerous experimental and clinical studies have shown that oxidative stress causes the loss of neurons and the progression of the disease to dementia[7-10]. The presence of a toxic peptide, β-amyloid in the brain of patients with Alzheimer's disease is also caused by oxidative stress[10,11]. Antioxidants are components that can scavenge free radicals so that can prevent and repair the damage caused by oxidative stress[10,12].

Medicinal 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* and lately Chinese medicinal plant *Huperzia serrata*[13-17]. Plant from the genus *Cassia*, the family Caesalpiniaceae consists of more than 500 species worldwide. These flowering plants are distributed in tropical and subtropical regions[18-20]. The genus *Cassia* is not only known as an ornamental plant but also known for its use in folk medicine, such as for skin diseases, gastrointestinal problems, cough, and cardiac disorders[18,20,21]. Metabolites with promising bioactivities have also been reported from several *Cassia*, such as terpenoid, flavonoid, alkaloid, xanthone, and anthraquinone[21-24]. In our continuing study on the search of medicinal plants and marine sources for neurodegenerative diseases, we have found that several *Cassia* species, including the ethanolic extract of *Cassia moschata* have potency as cholinesterase inhibitor[25]. Several *Cassia* species have also been reported as antioxidants, such as the ethanolic and aqueous extracts of *C. angustifolia* seed[26]. The antioxidant activity of four species of *Cassia*, namely *C. auriculata*, *C. siamea*, *C. uniflora*, and *C. italica* have also been reported[27]. Other *Cassia* species that have shown potency as antioxidants including *C. tora*, *C. occidentalis*, *C, javanica*, *C. glauca*, and *C. grandis*[28-32]. To the best of our knowledge, there is no report on the antioxidant potency of the bronze shower plant, *C. Moschata*, as well as its phytochemical contents. Therefore, the current study focus on the evaluation of the antioxidant and anticholinesterase activities of the ethanolic and aqueous extracts of *Cassia moschata* leaves as well as the determination of the phenolic content.

MATERIALS AND METHODS:

Materials:

2,2-Diphenyl-1-picrylhydrazyl (DPPH), 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), gallic acid, quercetin, and potassium persulfate were purchased from Sigma. Folin-Ciocalteu's phenol reagent, aluminum chloride, sodium carbonate, sodium nitrite were purchased from Merck. All chemicals and solvents used were of analytical grade. The leaves of *Cassia moschata* were obtained from Purwodadi Botanic Garden, East Java, Indonesia on March 9th, 2019. The voucher specimen (PWD 04) was deposited at the Institute of Tropical Diseases 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:

The leaves of *Cassia moschata* were air-dried at room temperature for seven days, then powdered in a grinder. Ten grams of the powdered leaves were each extracted with 80 mL ethanol and water by using ultrasonic extraction for 3 x 10 mins, then the filtrate was separated by filtration. The residue was re-extracted with each solvent using the same procedure three times. All collected filtrates were evaporated in a rotary evaporator to obtain the ethanolic (1.40 g) and aqueous (0.77 g) extracts.

DPPH radical scavenging assay:

The assay was carried out in triplicates based on modified protocols[33,34]. The DPPH solution (0.25 mM) was prepared by dissolving DPPH powder in methanol. The extracts were dissolved in methanol at a concentration of 100 µg/mL, this was then further diluted to series of concentrations 2.5 – 60 µg/mL. Gallic acid was used as a standard. The samples (100 µL) were mixed with 0.25 mM DPPH reagent (100 µL) in 96 well plates. DPPH reagent (100 µL) was mixed with methanol (100 µL) to serve as a control, while methanol (200 µL) was used as blank. The reaction mixtures were incubated in the dark at room temperature for 30 mins. The solutions were shaken for 30 s, followed by measurement of absorbance at 517 nm in a microplate reader (Bio-Tek Instrument, USA). The DPPH scavenging effect was calculated by the following formula.

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

where *abs control* is the absorbance of DPPH radical + methanol and *abs sample* is absorbance DPPH radical + extract/standard.

ABTS radical scavenging assay:

The bioassay was performed based on the previous method with some modifications[34]. Briefly, to produce ABTS radical, 5 mL ABTS (7 mM) was mixed with 88 μL potassium persulfate (140 nM), and was kept in the dark at room temperature for 16 h. Samples at the concentration range 2.5 – 60 μg/mL were prepared in

methanol. The samples (100 μL) were then mixed with 100 μL of ABTS in a 96-well microplate and allowed for incubation for 6 mins in the dark at room temperature. The absorbance was measured at 734 nm by using a microplate reader (set to shake for 30 s before reading). Gallic acid was used as standard. Experiments were done in triplicate. The ABTS radical scavenging activity was calculated by using the following equation.

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

abs control is the absorbance of ABTS radical + methanol and *abs sample* is absorbance ABTS radical + extract/standard.

Anticholinesterase Assay:

The assay was carried out according to the modified Ellman's method[35-37]. The extracts were dissolved in methanol at a concentration of 10mg/mL and were then diluted with 50mM Tris buffer to obtain series of concentrations containing not more than 10% of methanol. The final test concentrations in the wells were: 500, 400, 200, 100, 50, 40, 20, 10, 5, 2 and 1 µg/mL. Sample solutions were then 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 enzyme *Ee*AChE (25 µL of 0.22 U/mL) was then added. The solutions were shaken for 30 s in a microplate reader (Bio-Tek Instrument, USA) before measurement. The presence of yellow color due to the formation of 5-thio-2-nitrobenzoate was monitored at 405 nm every 5 s for 2 mins. Every experiment was carried out in triplicates. Galantamine was used as a positive control, and 10% methanol was used as a negative control. The inhibitory activity was calculated by using the equation below:

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

Determination of total phenolic content (TPC):

The TPC in the samples was determined according to the method Herald et al (2012) Zhang et al (2006) with slight modification[33,38]. In brief, twenty-five microlitres of gallic acid standard (25 – 500 µg/mL) or samples (1000 µg/mL) were added to 96-well microplate, followed by water (75 µL) and Folin & Ciocalteu's phenol reagent (25 µL). The mixture was incubated for 6 mins at room temperature. After incubation, 100 µL 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 reading the absorbance at 765 nm in a microplate reader.

Statistical analysis:

The 50% inhibitory concentration (IC_{50}) was determined using GraphPad Prism 6.0 software by plotting log concentrations as axis and % inhibition as ordinate. Results were expressed as mean ± standard deviation (SD) of three parallel measurements. The IC_{50} values from DPPH and ABTS assays were analyzed using independent-sample T-tests. The correlation between the antioxidant capacity and the total phenolic content was determined using Pearson's correlation test using IBM SPSS statistics 21 software. Difference and correlation were regarded as statistically significant when $p < 0.05$.

RESULT:

Antioxidant Activity:

The dose-response inhibition of extracts in DPPH and ABTS assays can be seen in Figures 1 and 2. The data showed that in the DPPH assay the radical quenching abilities of both extracts were increased in a dose-dependent manner (Figure 1). At a concentration of 5 µg/mL, the ethanolic and aqueous extracts scavenged 4.30% and 16.52% of DPPH radical, respectively. These values increased to 66.15% and 66.65%, respectively at a concentration of 50 µg/mL. The same trend was observed in the ABTS assay, the inhibition of ABTS radical for both ethanolic and aqueous extracts increased more than four and two folds, respectively at concentration 50 µg/mL compared to at concentration 5 µg/mL (Figure 2).

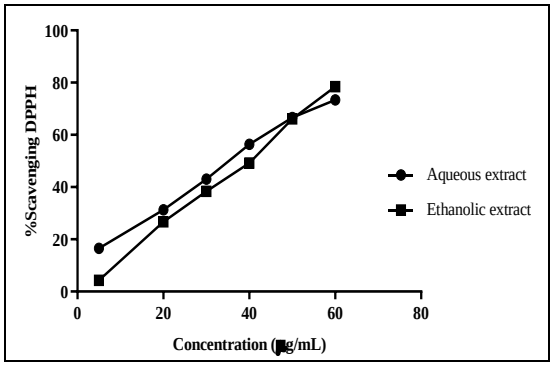

Figure 1. Percentage scavenging of DPPH at different concentrations of *C. moschata* extracts

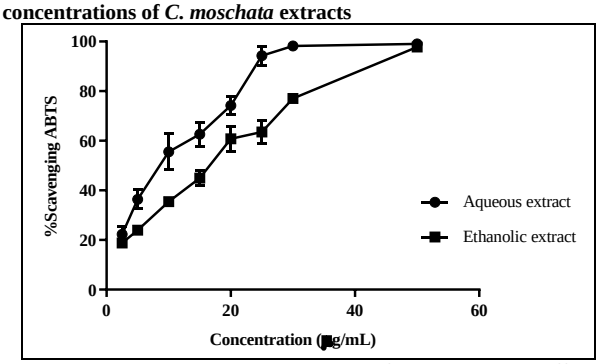

Figure 2. Percentage scavenging of ABTS at different concentrations of *C. moschata* extracts

The IC_{50} values were calculated and presented in table 1 which shows that the aqueous extract gave lower IC_{50} values at 36.46 and 10.61 µg/mL in DPPH and ABTS assays, respectively compared to the aqueous extract.

Table 1. Antioxidant and anticholinesterase of *C. moschata* extracts

Samples	IC_{50} (µg/mL)[a]		
	DPPH	ABTS	AChE
Ethanolic extract	38.74 ± 0.26	17.17 ± 0.41	44.43± 3.58
Aqueous extract	36.46 ± 0.09	10.61 ± 0.50	114.60 ± 2.88
Gallic acid	2.76 ± 0.02	0.97 ± 0.03	n.d
Galantamine	nd	nd	0.63 ± 0.05

[a]Data presented as mean ± SD of three experiments, each done in triplicate. nd: not determine

Anticholinesterase Activity:

The evaluation of the anticholinesterase activity of extracts was carried out against the AChE enzyme. The results as can be seen in Table 1 and Figure 3 showed that the ethanolic extract gave higher potency as anticholinesterase with an IC_{50} value of 44.43 µg/mL compared to the aqueous extract with an IC_{50} value of 114.60 µg/mL.

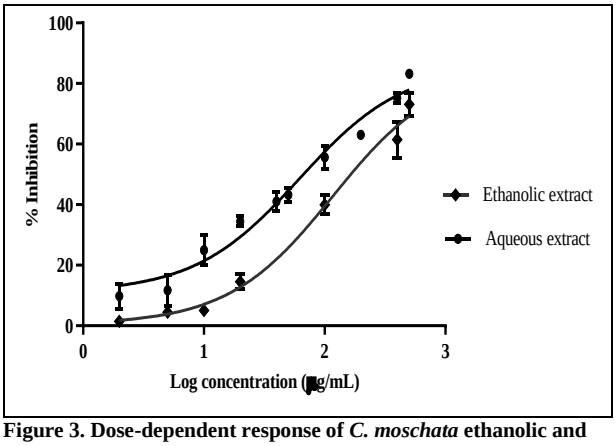

Figure 3. Dose-dependent response of *C. moschata* ethanolic and aqueous extracts against AChE

Total Phenolic Content (TPC):

The total phenolic contents in the ethanolic and aqueous extracts were calculated based on a standard curve of gallic acid ($y = 0.005x+0.029$, $R^2 = 0.999$). The results as can be seen in Table 2 showed that the aqueous extract contains higher phenolic contents (260.0 mg GAE/g extract) compared to that of the ethanolic extract (238.2 mg GAE/g extract**)**.

Table 2. Extract yield and total phenolic content (TPC) of *C. moschata* extracts

Samples	Extract Yield (%)	TPC (mg GAE/g extract)[a]
Ethanolic extract	7.70	238.2 ± 0.06
Aqueous extract	13.97	260.0 ± 0.25

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

DISCUSSION:

Cassia species are higher plants native to Southeast Asia and sub-Saharan Africa. These plants are commonly used as food as well as various traditional medicines. Several *Cassia* species have been reported to possess antioxidant properties in many experimental studies[26-32]. However, there are *Cassia* species, such as *Cassia moschata*, that have limited reports both for biological activities as well as chemical composition.

In this study, the radical-scavenging activities of the ethanolic and aqueous extracts of *C. moschata* leaves were determined using the DPPH and ABTS assays. In the DPPH assay, the antioxidant provides a hydrogen atom that will react with DPPH radical (diphenylpicrylhydrazyl) to form a non-radical diphenylpicrylhydrazin, which has a yellow color. The degree of discoloration indicates the radical-scavenging potential of the sample[39,40]. 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 spectrofotometer[41,42].

The data presented in Figures 1 and 2 suggested that the radical quenching abilities of both extracts are increased in a dose-dependent manner in both ethanolic and aqueous extracts. The potency of samples to scavenge DPPH and ABTS radicals are expressed as IC_{50} value, which is the concentration of sample required to scavenge 50% DPPH or ABTS free radicals. The value was calculated from the inhibition curve. Based on the IC_{50} values (Table 1), it can be seen that the aqueous extract has higher potency as an antioxidant compared to the ethanolic extract. Analysis by using an unpaired t-test suggested that there were statistically significant differences observed between the IC_{50} values for the ethanolic and aqueous extracts in both DPPH and ABTS assays with *p* values of 0.0001 (DPPH) and <0.0001 (ABTS), respectively.

The anticholinesterase activity of the ethanolic extract of *C. moschata* has been reported in our previous study. In this study, the evaluation of anticholinesterase activity was carried out in both ethanolic and aqueous extracts in order to compare the potency. The results as can be seen in Table 1 and Figure 3 showed that the ethanolic extract higher potency as anticholinesterase with an IC_{50} value of 44.43 µg/mL compared to the aqueous extract with an IC_{50} value of 114.60 µg/mL.

The higher contents of phenolic in the aqueous extract compare to the ethanolic extract suggested that the phenolic presents in the leaves of *C. moschata* possibly are polar compounds, such as in the glycoside form. In addition, the results also showed that there is a significant correlation between the IC_{50} values in both DPPH and

ABTS assays and the total phenolic contents in the extracts (Table 3). The higher phenolic content is responsible for the higher antioxidant activity in the aqueous extract. These results are in agreement with the previous studies, which prove that there is a strong correlation between the total phenolic content and the antioxidant activity of some medicinal plants[28]. However, the higher phenolic content in the aqueous extract does not correspond to the anticholinesterase activity. Therefore, this suggests that the presence of other compounds, such as alkaloids that are commonly present in the plant from the genus *Cassia*[21-24], may also be responsible for the anticholinesterase activity in the ethanolic extract.

Table 3. Correlations between the IC_{50} values of antioxidant assays and phenolic content

Assay	Correlation (r) TPC
IC_{50} of DPPH radical scavenging ability	-0,991*
IC_{50} of ABTS radical scavenging ability	-0,994*

*r: correlation coefficient. *indicates significance at p < 0.01*

Phenolics are aromatic secondary plant metabolites that are known for their extensive profitable biological activities[43,44]. These compounds are always considered as the major contributor to antioxidants in plants due to their ability to scavenge free radicals, active oxygen species as well as metal chelators[43,45]. Studies revealed that the antioxidant capacity of phenolic compounds depends on the number and arrangement of the hydroxyl groups in the phenolic compound. Studies on the phenolic content of *Cassia* spp. have been documented[26-28,30,46], however, there is no report for *C. moschata*. Mehta et al (2017) reported the total phenolic contents of various parts of *C. javanica* and *C. siamea*, which stated that *C. javanica* leaves contain higher phenolic compared to other tested extracts[30]. The antioxidant potency, as well as phenolic contents of seven *Cassia* species, have also been reported. *C. glauca* was reported to show the strongest antioxidant capacity compared to the other six *Cassia* species, which was also related to its high content of phenolic compounds[28]. The study on the chemistry of *C. moschata* is very limited, however tracing on the chemistry of the plant from the same genus revealed that anthraquinone, terpenoid, xanthone, flavonoid as well as alkaloid presence in *Cassia* spp.[21-24]. Considering higher anticholinesterase potency in the ethanolic extract, the non-phenolic compounds may contribute to this activity, which needs further study.

CONCLUSION:

The aqueous extract of *Cassia moschata* leaves gave higher free radical scavenging activity compared to the ethanolic extract due to higher phenolic content, however, the ethanolic extract gave better anticholinesterase activity. The presence of other compounds, such as alkaloids in the ethanolic extracts may be responsible for its anticholinesterase activity. Further study is needed to investigate the chemistry of *C. moschata* leaves.

CONFLICT OF INTEREST:

The authors have no conflicts of interest regarding this investigation**.**

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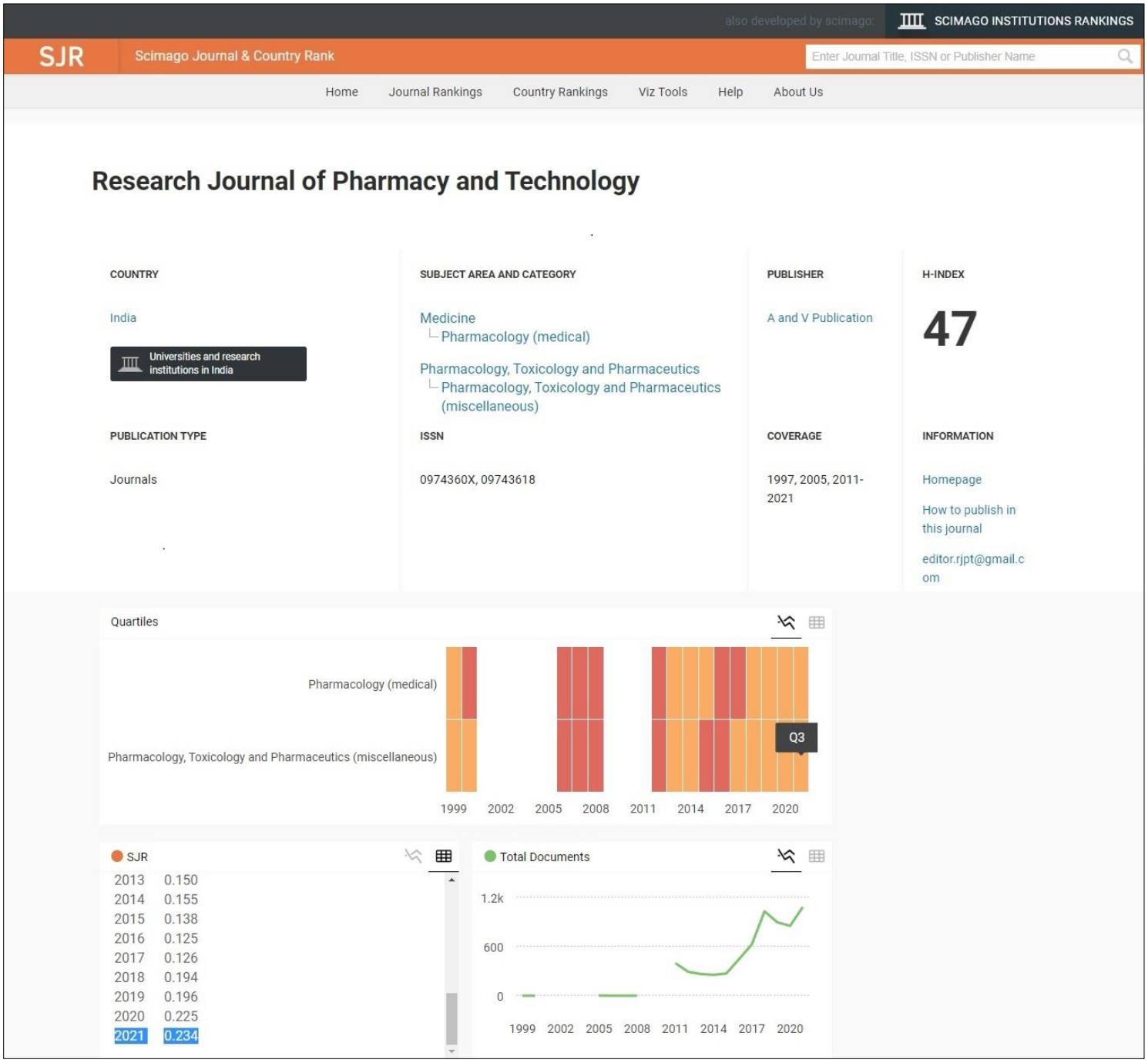
Bukti – Subject Area and Category, Quartile dan SJR



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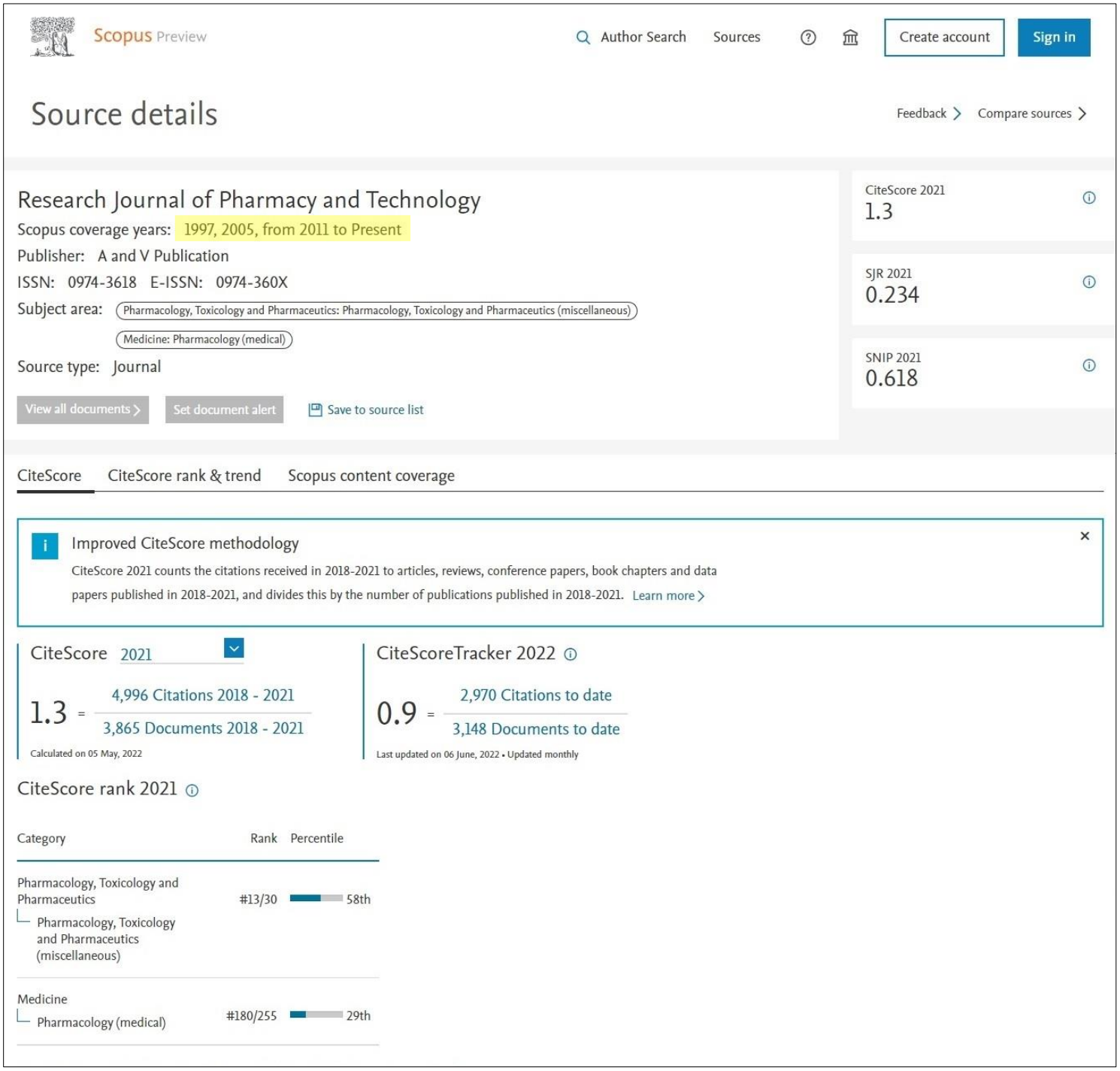

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