doi: 10.11594/jtls.08.02.08
THE JOURNAL OF TROPICAL LIFE SCIENCE
OPEN ACCESS Freely available online
VOL. 8, NO. 2, pp. 151 – 157, April 2018
Submitted March 2018; Revised March 2018; Accepted April 2018

Antioxidant Activity and Toxicity of *Puspa* (*Schima wallichii*) Leaves Extract from Indonesia

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ABSTRACT

Antioxidant and toxicity of *Puspa* (*Schima wallichii*) were studied. The plant extracts were prepared for biological screening. Antioxidant activity of extracts was analysed by 1,1-diphenyl-2-picrylhydrazyl (DPPH) as stable free radical, while toxicity of extracts was analysed by Brine Shrimp Lethality Test (BSLT) method. The result showed that ethyl acetate, methanol and butanol extracts were very active as antioxidant, as indicated by IC_{50} value of 11.44, 17.97 and 12.55 µg/mL for DPPH inhibition, respectively. Whilst n-hexane extract was not active as antioxidant indicated by IC_{50} value of 257.91 µg/mL, but aquadest extract was less active as antioxidant indicated by IC_{50} value of 171.94 µg/mL. The toxicity test result showed that all extracts were active against *Artemia salina* Leach with LC_{50} value of 107.35 – 902 µg/mL. Furthermore, isolation of individual bioactive compound of ethyl acetate extract that most active as antioxidant was carried out using column chromatography followed by preparative Thin Layer Chromatography (TLC). The active isolate was then identified using TLC, Fourier Transform Infra-Red (FTIR) and Liquid Chromatography Mass Spectroscopy (LCMS). The identification result showed that the major compound contained a putative compound known as theanine with MS 174.26 g/mol. The isolate was very active as antioxidant with IC_{50} value of 48.04 µg/mL, although it was less active than the extract. The isolate was also toxic with LC_{50} value of 27.71 µg/mL so that *puspa* leaf potentially to be developed as a natural antioxidant and anticancer agent for preventing of human diseases.

Keywords: *Puspa, maceration-partition, DPPH, BSLT, ethyl acetate extract, theanine*

INTRODUCTION

Toxicity and antioxidant analysis is a biological screening of medicinal plants extract to discovery natural compounds that potentially as a natural antioxidant and anticancer.

An antioxidant is a compound that able to inhibit oxidation process caused by free radicals [1, 2]. Free radical is a molecule that has unbonding electrons which produced from an oxidizing process such as smoking, cooking, and pollution. Besides that, free radical can also produce by living things. The free radical is one of the products from energy production process. Energy produced from body metabolism by oxidizing food substances such as carbohydrates, lipids, and proteins [3]. Free radical is very harmful because they can react with an important component of cells, for example, DNA and cell membrane so that causing disfunction or cell death even diseases in human such as cancer. The preventive way is using an antioxidant.

An antioxidant will produce a stable compound by reacting with free radicals. Antioxidant, generally classed as a natural antioxidant and synthetic antioxidant. Synthetic antioxidants have a tendency for side effects when used excessively, but natural antioxidant will never have any effects, therefore it is safer to use. Natural antioxidants can be obtained from various types of plants. Indonesia is a country rich in medicinal plants. One of the interesting plants under study as a potential antioxidant is *Puspa* (*Schima wallichii*) Theaceae [4].

Puspa also was known as cheloni is one of the plants that used to produce carpentry wood which belongs to the genus of *Schima* and tea family (Theaceae). Puspa is one of the Indonesian original plants that grow in most parts of Indonesia, but its utilization is currently limited

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How to cite:
Widiyarti G, Supiani, Tiara Y (2018) Antioxidant Activity and Toxicity of *Puspa* (*Schima wallichii*) Leaves Extract from Indonesia. J. Trop. Life. Science 8 (2): 151 – 157.

to the stem, to make a wood, while the leaves for primate feed [5, 6]. The leaves of *puspa* have been known anti-breast cancer activity due to kaempferol-3-O-rhamnoside bioactive compound through the mechanism of inducing apoptosis of MCF-7 breast cancer cells [7, 8]. Previous phytochemical studies on *Puspa* leaves have revealed the presence of various compounds including flavonoids, tannins, saponins, terpenes, quinone, and anthraquinone. The rendement of methanol, n-hexane, ethyl acetate, butanol and aquadest of *puspa* extracts were 23.22, 1.05, 11.81, 11.03, and 21.84%, respectively [9].

In the present study, we analyzed of antioxidant and toxicity of *puspa* leaves extracts. Furthermore, we have isolated some bioactive compounds contained in the extract that most active as an antioxidant. Isolation of bioactive compound was carried out using column chromatography and then preparative thin layer chromatography (TLC). The isolate was then identified using TLC and spectroscopy Fourier Transform Infra-Red (FTIR) and Liquid Chromatography Mass Spectroscopy (LCMS).

Antioxidant activity analysis

Antioxidant activity of the plant extracts was done on the basis of the radical scavenging effect of the stable DPPH (2 2-diphenyl-2-picrilhydrazyl) radical formula [10]. DPPH as sources of free radical, which captured hydrogen from a plant extract or isolate containing an antioxidant. The DPPH solution color turned from purple to yellow, indicating the conversion of 1,1-diphenyl-2-picrylhydrazyl into 1,1-diphenyl-2-pikrilhidrazin [10, 11, 12, 13].

Whilst the toxicity of plant extract was performed using BSLT (Meyer's) method. BSLT that conducted using nauplii of *Artemia salina* Leach was generally considered a preliminary assay of cytotoxicity for pharmacological activities screening and discovery of natural compounds [14, 15].

MATERIALS AND METHOD

Materials

Puspa leaves were collected from PUSPIPTEK provincial garden. A total of 141.29 g of the *puspa* leaves extracts obtained by percolation-partitions process. Extraction was first done using methanol, and then the extract was partitioned using n-hexane, ethyl acetate, and butanol, respectively. Chemicals used in this research were distilled water and technical grade of methanol, butanol, ethyl acetate, and n-hexane. Some analytical grade chemicals were used such as H_2SO_4 for TLC analysis, methanol and acetate acid for mass spectra (MS) analysis, DMSO for BSLT analysis and DPPH for radical scavenging activity analysis.

Equipment

Equipment used in this experiment were extraction unit, evaporation unit, and one set of bioactive compound identification unit. TLC was carried out using precoated silica gel plates (Merck Kieselgel 60F 254, 0.25 mm). The functional groups of the isolate were determined using FTIR, spectrophotometer IR Schimadzu prestige 21 using KBr pellet. Mass spectra (MS) or molecular weight was obtained with LCMS Mariner Biospectrometry spectrometer using Electrospray Ionization (ESI) System and positive ion mode. DPPH radical scavenging activity analysis was evaluated by using spectrophotometer Spechtronic Hitachi U2000 at 517 nm.

Methods

Isolation and purification of bioactive compounds from extract were carried out using chromatography column techniques on silica gel 60 GF_{254} with a gradient solvent system of n-hexane : ethyl acetate as the mobile phase to obtain a number of fractions and continued by preparative TLC.

Identification of individual chemical isolates

The isolate obtained was then analyzed by FTIR and LCMS spectroscopy. FTIR spectroscopy was used to determine the functional groups of the isolate compound, while LCMS was used to determine the mass spectra (MS) of the isolate.

The antioxidant activity analysis

Activity of antioxidant was evaluated by using DPPH method. Sample solutions including n-hexane, ethyl acetate, methanol, butanol, aquadest *puspa* leave dried extracts and isolate were prepared in methanol. An approximately 0.01 g of plant extracts were dissolved in 1 mL of methanol, while a solution 0.004% of DPPH was also prepared in the same solution. Different concentrations of the sample were prepared by mixing the DPPH stock solution and the respective amount of the sample to obtain a final concentration of 10, 50, 100, and 200 μg/mL, in order to find the IC50 value of the tested sample solutions of 10 – 200 μg/mL. Whilst quercetin as a standard antioxidant and the positive control was dissolved in methanol with a final concentration of 10 – 25 μg/mL. The sample solution was then reacted with

1 mM DPPH in methanol with total volume of 10 – 25 mL in a water bath at 37°C. The blank was 1 mM DPPH in 2.5 mM of methanol. Incubation was done at 37°C in dark room for 30 minutes. After that, absorbance measurements performed using spectrophotometer UV-VIS at a wavelength of 517 nm was recorded and the percent inhibition was calculated as follows:

$$\% \, inhibition = \left[\frac{(C - S)}{C} \right] \times 100\%$$

where C is absorbance of the blank and S is the sample absorbance. The IC_{50} value was calculated as the concentration that caused 50% inhibition of DPPH [11, 16].

Toxicity analysis

Toxicity analysis was carried out using BSLT (Meyer's) method. The method was conducted using nauplii of *A. salina* Leach that was generally considered as a bench top assay aiming at the discovery of cytotoxic compounds. About 50 – 100 mg brine shrimp's eggs were placed in seawater in the vessel and allowed the shrimps to mature as shrimp larvae for 48 hours. An amount of 10, 100, 500, and 1000 µg/mL of samples concentration were prepared. About 100 mL of seawater containing 10 – 11 shrimp larvae of *A. salina* L. were transferred into the test container. About 100 µL of each sample concentration of 10, 100, 500, and 1000 µg/mL was added to the test container. Each concentration was carried out three replicates. Solutions were incubated for 24 hours at room temperature under illumination. The number of dead and life of shrimp larvae in every hole was counted, and LC_{50} value was determined after 24 hours exposure. LC_{50} value is the concentration of a substance that caused 50% of the death of shrimp larvae [14, 17].

RESULTS AND DISCUSSION

Antioxidant activity was performed using DPPH radical, due to the easy, rapid, and economical method of establishing antioxidant activity. 1,1-diphenyl-2-picrylhydrazyl (DPPH) has a distinctive purple color. After reacting with the antioxidant compound, DPPH will be reduced to 1,1-diphenyl-2-pikrilhidrazin that was indicated by changing the DPPH solution color turn a yellow color. The color change from purple to yellow was proportional to the number of captured electrons or radicals and can be measured by visible light spectrophotometry at wavelength 515 – 517 nm [18, 19, 20, 21, 22]. The antioxidant activity test in this study, we used quercetin as standard antioxidant/material which has been

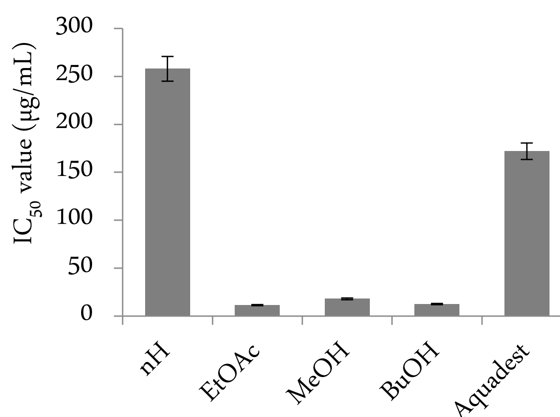

Figure 1. IC_{50} value of *puspa* leaves extracts as an antioxidant (µg/mL) (Note: nH: n-hexane, EtOAc: ethyl acetate, MeOH: methanol, BuOH: butanol)

known as an antioxidant with IC_{50} less than 10 µg/mL, so we know if the tested solutions had the same, less, or higher activity compared to the standard material [8].

The antioxidant activity test result showed that ethyl acetate, methanol, and butanol extracts of *puspa* leaves were active as antioxidant indicated by changing of the DPPH solution color, from purple to yellow color. The results of the antioxidant activity test of extracts were presented in Figure 1.

The IC_{50} value was a number of the concentration of sample that can inhibit 50% of the oxidation process. Based on the IC_{50} value, it showed that ethyl acetate, methanol, and butanol extracts have an IC_{50} value less than 50 µg/mL, thus the extracts categorized as very active as an antioxidant, but less active than quercetin as a standard antioxidant with IC_{50} value 9.35 µg/mL. The sample was very active/strong as antioxidant if its IC_{50} value 50 – 100 µg/mL. The extract was categorized as active/moderate antioxidant if its IC_{50} value of 100 – 150 µg/mL and as weak antioxidant if its IC_{50} value of 151 – 200 µg/mL [8, 16]. Based on the IC_{50} value, the aquadest extract with IC_{50} value 171.94 µg/mL were categorized as a weak antioxidant, while the n-hexane extract was not active as antioxidant indicated its IC_{50} value of 257.91 µg/mL. The ethyl acetate extract that most active as an antioxidant than the other extracts was further isolated.

The preliminary assay of cytotoxicity of *puspa* leaves extracts was toxicity test using Brine Shrimps Lethality Test (BSLT) method. BSLT is an easy, cheap, and reliable to be used as a preliminary assay of an anticancer or cytotoxic activity of natural products screening. The extract is active or toxic if the value of LC50

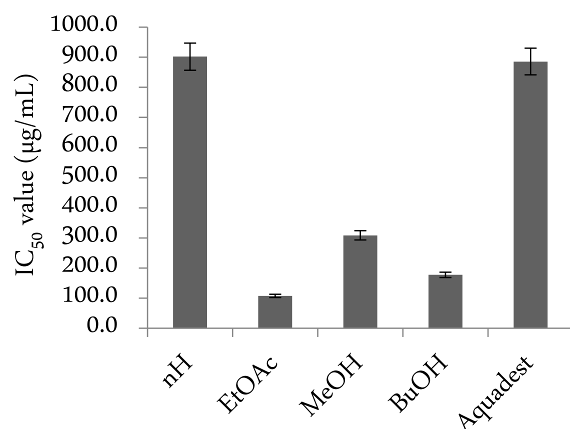

Figure 2. LC_{50} value of *puspa* leaves extracts as an antioxidant (μg/mL) (Note: nH: n-hexane, EtOAc: ethyl acetate, MeOH: methanol, BuOH: butanol)

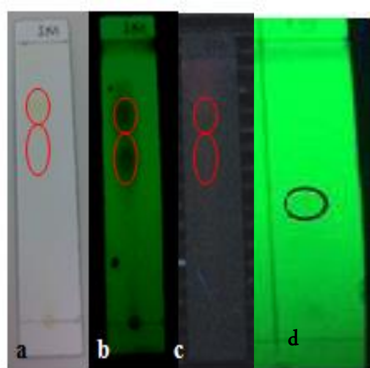

Figure 3. TLC analysis of the TLC preparative result (a) no irradiation, (b) radiation using UV at 254 nm, and (c) radiation using UV at 365 nm, and (d) control (the starting compound was the combined fraction of B8)

less than 1000 μg/mL (≤ 1000 μg/mL), and for a compound is active if of LC50 less than 30 μg/mL (≤ 30 μg/mL) [14, 15]. In this BSLT test no shrimp mortality was found in DMSO as a negative control. The result of BSLT of *puspa* leaves extracts showed at Figure 2. It showed that *n* shrimp model, no death of the nauplii was recorded in negative control (DMSO) group. It also showed that all of the extracts were active against *A. salina* L. with a LC_{50} value of 107.4 – 902 μg/mL which was considered moderately toxic. Therefore, *puspa* leaves extracts were potential sources of the cytotoxic agent.

Based on the antioxidant activity and toxicity test result, so that the ethyl acetate extract that most active as antioxidant and potential as a cytotoxic agent than the other extracts were then isolated. Isolation of the compound was carried out by silica gel column chromatography eluting with n-hexane, a gradient of ethyl acetate to 100%. The separation by column chromatography yields 135 fractions (F1 – F135) than combined into 10 combined fractions of B8 – B17. The result of isolation by column chromatography has not produced a pure compound yet so that further purification was carried out. Based on the TLC analysis, the suspected compound as the target compound obtained at the combined fraction of B8 – B17 was then separated by preparative TLC [22]. The preparative TLC resulted in 20.2 mg of the active isolate. The active isolate was analyzed using TLC again to ensure the purity of the compound. The TLC analysis results of the active isolate product were presented in Figure 3.

The isolate compound was analyzed by FTIR spectrophotometer to determine the presence of a particular functional group of the isolate compound. The compound exhibited absorption bands at 3151.69 cm^{-1} – 3473.80 cm^{-1} indicating the presence of hydroxyl (OH) groups. At 2926.01 cm^{-1} and 2860.43 cm^{-1} indicating the presence of C-H stretch. Furthermore at 1726.29 cm^{-1} indicating the presence of carbonyl (C=O) group and at 1606.70 cm^{-1} indicating the absorption of the C=N bending group [24]. At 1452.40 cm^{-1} indicating the presence of methyl (CH_3) group and at 1236.37 cm^{-1} and 1178.51 cm^{-1} was the absorption of the C-O group. Based on all of the functional groups contained in the isolate, it can be predicted that the structure of the isolate belongs to the flavonoid. The presence of C=N bending group on the spectrum also indicates the possibility of the presence of amino groups or alkaloids of the isolate.

The mass spectra (MS) or molecular weight (MW) of the isolate was analyzed by LC-MS. The chromatogram of isolate illustrated 1 peak that is not sharp but widened, which the retention time of 1.68 minute and MS/MW of 174.26 g/mol was recognized as theanine, as the molecular weight of theanine according to literature was 174.20. Theanine was an amino acid that commonly found in the *puspa* leaves. Theanine has been known as a functional drink additive for disease prevention and health promotion due to its antioxidant, antimutagenic and anticarcinogenic effects [25, 26, 27]. Another MS most likely were impurity. The LC-MS chromatogram of isolate presented as Figure 4.

Antioxidant activity of isolate

The active isolate contained a major compound known as theanine was then tested as an antioxidant using DPPH method and toxicity against *A. salina* L. using BSLT method. The antioxidant activity of the active iso-

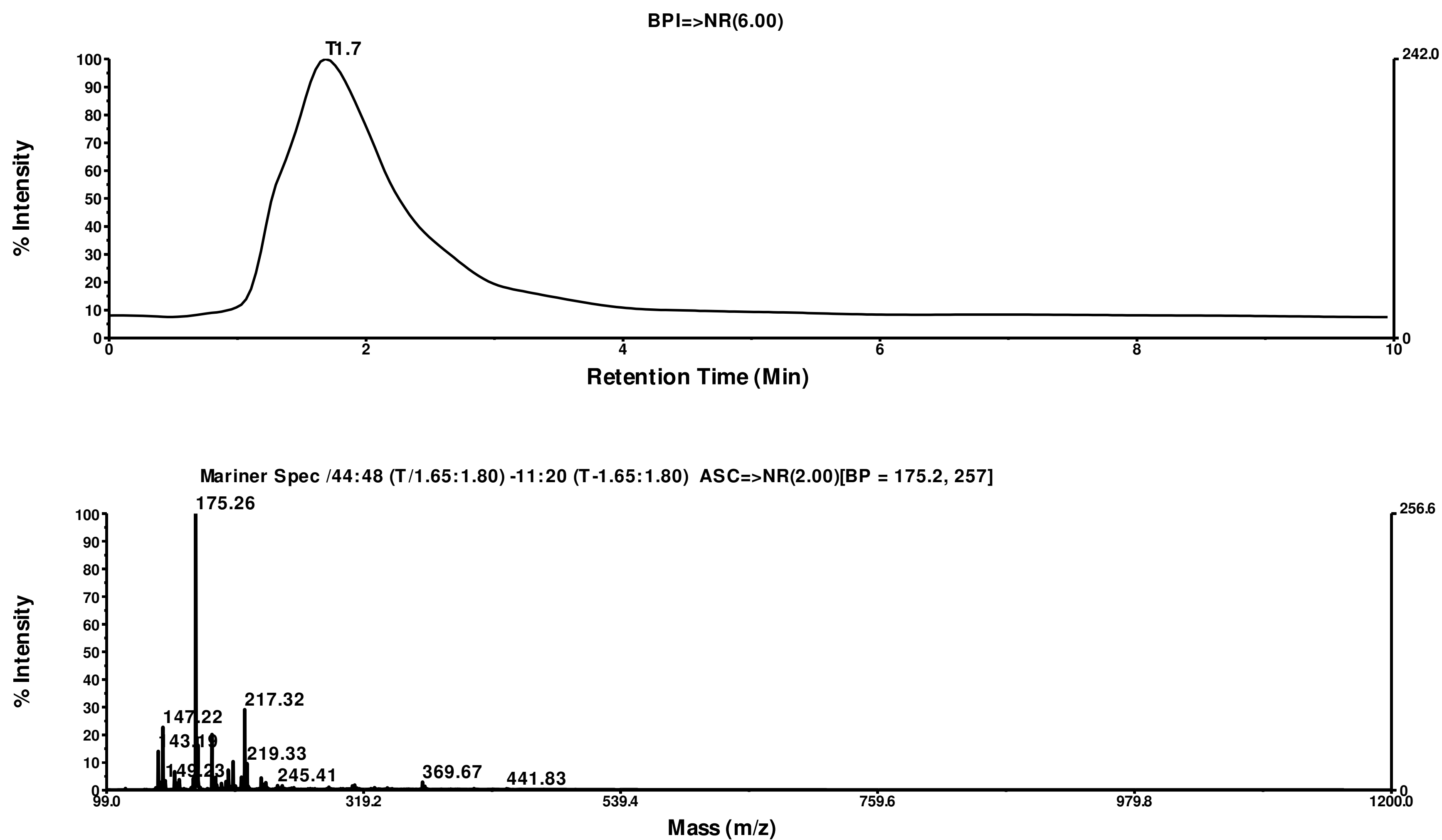

Figure 4. LCMS chromatogram of isolate

Table 1. IC_{50} value of antioxidant and LC_{50} value of isolate

Sample Name	IC_{50} (μg/mL)	LC_{50} (μg/mL)
Ethyl acetate extract of *puspa* leaves	17.07	107.35
An active isolate of *puspa* leaves ethyl acetate extract	48.04	27.71
Quercetin	9.35	-

late compared to *puspa* leaves extract and quercetin as standard antioxidant. The active isolate can change the color of DPPH solution, purple to yellow, which means that the active isolate can inhibit the DPPH radical. The antioxidant and BSLT test result of the active isolate showed in Table 1.

The IC50 value of isolate higher than the IC50 values of extract and quercetin. It means, the antioxidant activity of isolate containing theanine compound weaker than antioxidant activity of the extract. The smaller of the IC50 value means the stronger of the antioxidant activity [11, 13]. It may be due to the purity of compounds, where the isolate was still a mixture compound and the minor compound was maybe antagonistic to the antioxidant activity of major compound so that decrease the antioxidant activity of the active isolate. Nevertheless, the IC50 value of the active isolate was still below 50 μg/mL (≤ 50 μg/mL) so that the isolate was still categorized as very strong/active antioxidant [11].

The BSLT test result of the active isolate showed that the active isolate was active against *A. salina* L. with LC_{50} value of 27.71 μg/mL, so that the active isolate was a potential source of cytotoxic agents based on the value of LC_{50} whereas for a compound was active if its LC_{50} value of ≤ 30 μg/mL [14, 15].

CONCLUSION

Based on IR and MS spectrums, the major compound contained in the active isolate of ethyl acetate extract of *puspa* leaves might be theanine compound. Based on the IC_{50} values, both of extract and the active isolate of ethyl acetate extract of *puspa* leaves were categorized as a very strong antioxidant. Based on the LC_{50} value, the isolate has potency as a cytotoxic agent also. Therefore, *puspa* leaf potentially to be developed as a natural antioxidant and anticancer agent for preventing human diseases. In future identification or molecular structure elucidation of the active isolate by using ^{1}H and ^{13}C-NMR should be conducted.

ACKNOWLEDGMENT

The authors were grateful to Indonesian Ministry for Research Technology and Higher Education (Ristekdikti) for funding this research. We also thanks Mrs. Puspa Dewi Lotulung and Mrs. Yeni Apriliani for her help in LC-MS and IR analysis, Prof. Dr. Muhammad Hanafi for suggesting this research.

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