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Phytochemical and antioxidant properties of *Syzygium zollingerianum* leaves extract

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Abstract. *Sujana PKW, Wijayanti N. 2022. Phytochemical and antioxidant properties of* Syzygium zollingerianum *leaves extract. Biodiversitas 23: 916-921.* The genus *Syzygium* is known as a medicinal plant that has broad bioactive properties due to the abundance of bioactive compounds. *Syzygium zollingerianum* (Miq.) Amshoff could be considered as one of the important species to study because recent reports indicate that this species only grows in Indonesia and studies on its health benefits are still very limited. This study aimed to determine the phytochemical content of the ethanolic extract of *S. zollingeranum* leaves and to evaluate its antioxidant activity and cytotoxic effects on Vero cell lines. Phytochemical content was analyzed using spectrophotometry, DPPH assay was used to determine antioxidant activity and MTT assay was carried out to determine its cytotoxicity. The results of the spectrophotometric test showed the presence of phenolic, flavonoids, alkaloids, and tannins in the ethanolic extract of *S. zollingeranum* leaves. The ability to inhibit free radicals was very strong with antioxidant activity index (AAI) of 68.75 ± 31.386 and IC_{50} of 0.57 ± 0.211 µg/mL. The results of cytotoxicity examination showed that *S. zollingerianum* leaves extract has moderate cytotoxicity against Vero cells (101.42 ± 6.823 µg/mL). This study showed that *S. zollingerianum* leaves extract has great potential as a source of natural exogenous antioxidants and conservation for the sustainability of this plant needs to be considered.

Keywords: Antioxidant, cytotoxicity, DPPH, *Syzygium zollingerianum*, Vero

INTRODUCTION

The genus of *Syzygium* is known as the general basis of complementary medicines because of its abundant bioactive compounds (Chua et al. 2019). This genus also has many benefits for human health such as antioxidant, anticancer, antidiabetic, anti-inflammatory, antifungal, antibacterial (Annadurai et al. 2012), antiviral, anti-HIV, antidiarrheal (Abera et al. 2018), and reduce the blood triglyceride (Nugroho et al. 2012). Various metabolites have been found that act as important exogenous antioxidants, including phenolic compounds (e.g. phenolic acids, coumarins, flavonoids, lignans, stilbenes, tannins), terpenoids (e.g. carotenoids), and vitamins (e.g. vitamin C, vitamin E) (Baiano and Del Nobile 2016). The genus *Syzygium* is reported to contain phenolic compounds with antioxidant activity such as chalcones, flavonoids, lignans, alkyl phloroglucinols, hydrolyzable tannins, and chromone derivatives (Memon et al. 2015). In addition, many terpenoid compounds that have antioxidant properties such as oleanolic acids and betulinic acids are also found in the genus *Syzygium* (Chua et al. 2019). Previous studies showed that several species of the genus *Syzygium* have been reported as potent antioxidants and anticancer (Twilley et al. 2017; Mahomoodally et al. 2020). The ethanolic extract of *Syzygium aromaticum* flower buds has high antioxidant activity, which is almost the same as ascorbic acid activity (Singh et al. 2018).

Syzygium zollingerianum was first discovered by Miquel in 1855 on a riverside of Sumbawa Island, Indonesia. Based on the recent report, *S. zollingerianum* (Miq.) Amshoff has only been discovered in Indonesia and distributed in Sumatra and the Lesser Sunda Islands such as Java, Bali, and Sumbawa. This plant is found at the altitudes between 400-475 MASL on the island of Sumatra, Java (e.g. Mount Slamet) at the altitudes of 700-1000 MASL, and on Bukit Tapak Bali at the altitudes higher than 1000 MASL (Widodo et al. 2011; Dharma et al. 2017). In Sumatra, it is found in Sibolangit, Lampung, Mount Reti Berenong, and Simeulue Island (Widodo et al. 2011), and it is also found in the western part of Central Java (Backer and Brink 1963), and Kalimantan (Royyani and Efendy 2015). However, studies on phytochemical profiles and potential bioactivity of *S. zollingerianum* have not been carried out in any previous research. Therefore, this study was carried out to determine the phytochemical content of ethanolic extract of *S. zollingerianum* leaves, antioxidant properties, and its cytotoxic effects on Vero cell lines.

MATERIALS AND METHODS

Preparation of sample and extraction

Mature leaves of *S. zollingerianum* were collected from the top of hills in Sepang Village, Busungbiu District, Buleleng Regency, Bali, Indonesia. The leaves were

cleaned with water and air-dried at room temperature. The dried leaves were ground with a blender to obtain leaf powder. The leaf powder was macerated with 70% ethanol (1:10 w/v) and stirred with an orbital shaker for 3 days. The solution was filtered using Whatman filter paper No.1 and the filtrate was evaporated using a rotary evaporator (50-65°C) for about 4 hours. The dark brown concentrated extract was dried using an incubator at a temperature of 50°C and stored in a refrigerator.

Determination of total phenolics content (TPC)

50 mg of sample extract was mixed with 0.5 mL of Folin-Ciocalteu reagent and 7.5 mL of distilled water. The mixture was allowed to stand at room temperature for 10 minutes. Afterward, 1.5 mL of 20% Na_2CO_3 was added. Distilled water was added until it reached 10 mL of volume. The absorbance was measured at a wavelength of 760 nm (Kumar et al. 2017). A standard curve was obtained from various concentrations of gallic acid (6.25, 12.5, 25, 50 µg/mL).

Determination of total flavonoids content (TFC)

50 mg of sample extract was mixed with 0.3 mL of 5% $NaNO_2$, left for 5 minutes and as much as 0.6 mL of 10% $AlCl_3$ was added and left for 5 minutes. Two mL of 1 M NaOH was added, followed by the addition of distilled water until it reached 10 mL of volume (Lim et al. 2019). The absorbance was measured at a wavelength of 510 nm. A standard curve was obtained from various concentrations of quercetin (0.5, 1, 2, 5, 10, 25, 50, 75, 100 µg/mL).

Determination of total alkaloids content (TAC)

100 mg of extract was added with 5 mL of 2N HCl. The solution was washed with 10 mL of chloroform 3 times in a funnel separator and then the chloroform phase was discarded. The solution was neutralized by adding 0.1 N NaOH. After that, 5 mL of bromocresol green solution and 5 mL of phosphate buffer were added. The solution was extracted with 5 mL of chloroform 2 times and stirred using a magnetic stirrer at 500 rpm for 15 minutes. The chloroform phase was collected and evaporated with nitrogen gas. The extract was added with chloroform until it reached 10 mL of volume and diluted 5 times. The absorbance was measured at a wavelength of 470 nm (Tabasum et al. 2016). A standard curve was obtained from various concentrations of quinine (3.125, 6.25, 12.5, 25, 50, 100, 200, 400 µg/mL).

Determination of total tannins content (TTC)

50 mg of sample extract was extracted with 10 mL of diethyl ether for 20 hours by maceration. The extract was filtered and diethyl ether was evaporated. Distilled water was added until it reached 10 mL of volume. One mL of sample solution was added with 0.1 mL of Folin Ciocalteu reagent and vortexed for 5 minutes. After that, 2 mL of 20% Na_2CO_3 was added and vortexed for 5 minutes, followed by the addition of distilled water until the volume reached 10 mL. The solution was diluted 20 times and incubated for 30 minutes at room temperature. The absorbance was measured at a wavelength of 760 nm (Wahyuni et al. 2020). A standard curve was obtained from various concentrations of tannic acid (0.125, 0.25, 0.5, 1, 2, 4, 8, 16 µg/mL).

Antioxidant activity assay

The antioxidant activity assay was carried out by the DPPH method based on Zhou et al (2020) with slight modifications. The extract was made with various concentrations (1, 3, 6, 9, 12, 15 µg/mL). Vitamin C (ascorbic acid) was used as a standard at the concentrations of 0.5, 1, 2, 4, 6, 8 µg/mL. One mL of each concentration of extract and the ascorbic acid solution was mixed with 1 mL of 0.1 mM DPPH solution in methanol (1:1 v/v). Methanol was used as a blank and 0.1 mM DPPH solution was used as a control. The absorbance was measured at a wavelength of 517 nm after incubating at room temperature in dark conditions for 30 minutes. Analysis was carried out in triplicate for each concentration of extract and standard. The radical scavenging activity was calculated using formula [1]. The 50% inhibitory concentration (IC_{50}) of extract that can scavenge 50% of DPPH radicals was calculated by linear regression. The antioxidant activity of the extract was expressed as the antioxidant activity index (AAI) which was calculated by the formula [2].

$$\text{Scavenging activity (\%)} = [(A_C - A_S) / A_C] \times 100\% \quad [1]$$

Where: Ac was the absorbance of DPPH control, As was the absorbance of DPPH mixed with extract or ascorbic acid solution

$$\text{AAI} = \text{final concentration of DPPH in reaction (µg/mL)} / IC_{50} \text{ (µg/mL)} \quad [2]$$

MTT assay for cytotoxicity assessment

Vero cell lines were cultured with Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS) and 1% antimycotic antibiotic solution (10000 units/mL penicillin, 10 mg/mL streptomycin, and 25 µg/mL amphotericin B). Vero cell lines were seeded in 96 well plates and incubated at 37°C, 95% humidity, and 5% of CO_2. Treatment with *S. zollingerianum* extract and doxorubicin was performed when the cells had reached 80-90% confluence. Concentrated extracts (5000 µg/mL) were serially diluted in methanol. MTT solution (0.5 mg/mL) was added after the cells were exposed to the test solution for 24 hours. The cells were incubated for 4 hours. After incubation, 10% SDS in 0.01 N HCl was added and incubated overnight in dark conditions. The absorbance was measured at a wavelength of 595 nm. The percentage of viable cells was calculated using formula [3]. The cytotoxicity of the extract was expressed as IC_{50} as the concentration of the extract that caused inhibition of metabolic activity in 50% of the cell.

$$\text{Viable Cells (\%)} = [(A_{TC} - A_M) / (A_{UC} - A_M)] \times 100\% \quad [3]$$

Where: A_{TC} is the absorbance of the medium containing treated cells, A_{UC} is the absorbance of the medium containing untreated cells, A_M is the absorbance of the medium without cells.

Data analysis

Quantitative data were statistically analyzed using SPSS version 17 and Microsoft Office Excel Professional Plus 2016. IC_{50} values were calculated by linear regression using Microsoft Excel. The differences of IC_{50} values between extracts and positive controls were statistically analyzed using unpaired t-test analysis at significance levels of $p<0.05$. The differences in phytochemical contents were analyzed using one-way ANOVA followed by the Duncan test at a significance level of $p<0.05$. Data were presented as mean ± standard deviation (SD) based on 3 replications.

RESULTS AND DISCUSSION

Phytochemical content of *S. zollingerianum* leaves extract

The quantitative analysis of phytochemical content focused on total phenolic, flavonoid, alkaloid, and tannin content using spectrophotometric methods. Statistical analysis showed that the chemical compounds were significantly different ($p<0.05$) (Table 1). The total tannin content of ethanolic extract of *S. zollingerianum* leaves was significantly higher ($p<0.05$) compared to total phenolic, flavonoid, and total alkaloids content.

Antioxidant activities

The results of the DPPH free radical scavenging method showed that *S. zollingerianum* leaves extract possesses antioxidant activity. The IC_{50} value of ethanolic extract of *S. zollingerianum* was not significantly different ($p>0.05$) from ascorbic acid as a standard antioxidant. Antioxidant Activity Index (AAI) value of extract and standard were > 2 (Table 2) indicates strong antioxidant activity.

Cytotoxic activities

The cytotoxic activity of *S. zollingerianum* leaves extract was evaluated on Vero cell lines. Figure 1 shows the viability of Vero cells based on the log dose series. The IC_{50} values for cytotoxic activity of *S. zollingerianum* leaves extract were 101.42 ± 6.823 µg/mL. This value was significantly higher than doxorubicin (23.79 ± 3.659 µg/mL). This indicated that *S. zollingerianum* leaves extract was significantly less toxic ($p<0.05$) compared to doxorubicin on the Vero cell line (Figure 2).

Discussion

The bioactive potential of plant extracts is closely related to their phytochemical composition. Quantitative analysis of bioactive compounds from ethanolic extract of *S. zollingerianum* leaves showed a significant difference in content ($p < 0.05$) of the analyzed chemical compounds (Table 1). Several factors affected the distribution and accumulation of bioactive compounds in plants, i.e., environmental factors (e.g. air temperature, intensity and quality of light, rainfall, humidity, soil type, and composition), the stage of plant development (Li et al. 2020), the presence of microbes, the threat of predators, etc. (Mohiuddin 2019). However, the composition of bioactive compounds is also closely related to a taxon (i.e. genes, enzymes) (Li et al. 2020). Chemical compounds in the plant may be more common or unique in certain genera and species and can be similar within genus and family (Liu et al. 2017). On the other hand, the composition of compounds obtained from plant extracts is also determined by the part of the plant being used as extract (Li et al. 2020), the type of solvent, and the extraction method (Hosyar et al. 2016).

Our study showed that the total tannin content (23.71 ± 0.076% w/w) was significantly higher ($p<0.05$) than the other analyzed compounds (Table 1). Based on research by Rezende et al. (2015), high levels of tannins in *S. jambos* have a negative correlation with temperature. A high level of phenolics, including tannins in the leaves *of S. jambos*, may be associated with the increase of phenylalanine ammonia-lyase (PAL) activity at low temperatures (Rezende et al. 2015). Similar to this study, the leaves of *S. zollingerianum* were collected from a tree that grew on hilltops (more than 700 MASL) with an air temperature range of 17-26°C at the time of collecting samples, therefore phenolic and tannin content were also increased along with increasing CO_2 levels and greater light intensity (Kraus et al. 2003). However, investigations into other abiotic and biotic factors are still needed.

Phenolic compounds have been known to have excellent antioxidant abilities through the number and position of phenolic hydroxyls, methoxy, and carboxylic acid groups (Chen et al. 2020). Phenolic compounds that have been found in the genus of *Syzygium* include gallic acid and this compound is widely used as a positive control for antioxidant activity tests with an IC_{50} value of 25.0 ± 0.1 µM by DPPH method (Simirgiotis et al. 2008). The total phenolic content in the leaves extract of *S. zollingerianum* (5.97 ± 0.284% w/w) (Table 1) was similar to *S. samarangense* (5.99± 0.061% w/w), but higher than leaves extract of *S. aqueum* (3.91 ± 0.055% w/w) and lower than *S. cumini* (8.52 ± 0.055 % w/w), *S. jambos* (7.59± 0.040% w/w), and *S. malaccense* (6.11± 0.083% w/w) (Sheela and Cheenickal 2017). Several studies have shown a significant positive correlation between the total phenolic contents and the antioxidant activity in several plant tissues in the genus *Syzygium* (Sultana et al. 2014; Sathyanarayanan et al. 2018). So, the antioxidant activity of the leaves extracts of *S. zollingerianum* seems to have a positive correlation with the total phenolic content.

Table 1. Phytochemical content of ethanolic extract of *S. zollingerianum* leaves

Compound	Content (% w/w)
Phenolics	5.97 ± 0.284^{a}
Flavonoids	8.00 ± 0.515^{b}
Alkaloids	1.44 ± 0.014^{c}
Tannins	23.71 ± 0.076^{d}

Note: All data expressed as mean ± SD; Differ significantly by one-way ANOVA and followed by Duncan test to compare the mean difference between each group ($p < 0.05$). Mean ± SD with different letters showed significantly different values.

Table 2. The IC_{50} value and AAI of ethanolic extract of *S. zollingerianum* leaves for antioxidant activity

Sample	IC_{50} (µg/mL)	AAI
S. zollingerianum	0.57 ± 0.211[a]	68.75 ± 31.386[a]
Ascorbic acid	0.70 ± 0.423[a]	56.06 ± 36.580[a]

Note: All data expressed as mean ± SD; Not significantly different by unpaired t-test analysis at p-value < 0.05. Mean ± SD with the same letter showed no significantly different value

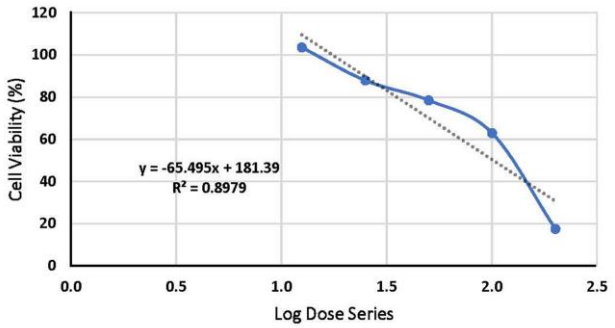

Figure 1. The effect of *S. zollingerianum* leaves extract on cell viability of Vero cell line. The data represented 3 independent experiments

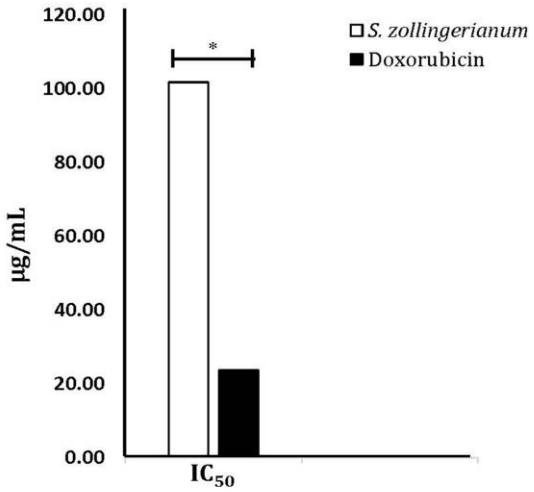

Figure 2. The IC_{50} value for the cytotoxic effect of *S. zollingerianum* leaves extract compared to currently used chemotherapy agent Doxorubicin on Vero cell line. All data expressed as mean ± SD based on 3 replicates (n=3); significantly different by unpaired t-test analysis at p-value <0.05*

The leaves extract of *S. zollingerianum* has the highest total flavonoid content (8.00 ± 0.515% w/w) (Table 1) than other *Syzygium* species that were previously reported by Sheela and Cheenickal (2017), including the leaves extract of *S. aqueum* (0.423± 0.021% w/ w), *S. cumini* (0.468± 0.005% w/w), *S. jambos* (0.496± 0.014% w/w), *S. malaccense* (1.044± 0.007% w/w), and *S. samarangense* (1.117± 0.006 w/w). Several studies have confirmed that several compounds belonging to the flavonoid group are found in the *Syzygium* genus including quercetin (Batista et al. 2017) and myricetin, (Nguyen et al. 2016). Quercetin is a compound with very strong antioxidant activities (IC_{50} 0.87 µg/mL) on DPPH assay (Meda et al. 2005) and it was used as a standard to determine the flavonoid content in this study. According to Eshwarappa et al. (2014), the antioxidant activity of *S. cumini* leaf gall extracts has a strong correlation to both the total phenolic and flavonoid contents In this study, the total flavonoid content may also have a positive correlation with the antioxidant activity of the *S. zollingerianum* extract.

The total alkaloid content in the extract of *S. zollingerianum* leaves (1.44 ± 0.014% w/w) (Table 1) was lower than that of *S. cumini* leaves extract (3.326 ± 0.235% w/w) (Zahra et al. 2019). The antioxidant activity of alkaloids may be determined by the number of aromatic hydroxyl groups. At the cellular level, the antioxidant effects of alkaloids can influence oxidative stress pathways through several possible mechanisms described as follows: inhibition of synthesis, activation, or translocation of NADPH-oxidase subunits; activation of the nuclear factor Nrf2; activation of transcription factors FOXOs and PPARs; epigenetic effects (influence on histone acetylation/methylation, DNA methylation, or expression of microRNA); and directly inhibits the myeloperoxidase (Macáková et al. 2019). The alkaloids compounds such as sanguinarine that have been isolated from *S. aromaticum* (Batiha et al. 2020) have potential as antioxidants, anti-inflammatory, proapoptotic, and growth inhibitory agents on various cancer cells, and have antiangiogenic and anti-invasive properties (Fu et al. 2018).

The total tannin content from *S. zollingerianum* leaves extract (23.71 ± 0.076% w/w) (Table 1) was three times higher than in the leaves extract of *S. cumini* (7.968 ± 0.164% w/w) (Silva et al. 2021). Medini et al. (2018) reported that biological factors, environmental conditions, extraction methods, and solvent will affect different metabolite content and amount. The tannin content of *S. guineense* showed very strong antioxidant activity with an IC_{50} 4.5 ± 0.3 µM (Nguyen et al. 2016). Therefore, we suspect that the presence of tannins has a major influence on the results of the antioxidant activity of the leaves extract of *S. zollingerianum* as shown in Table 2 (AAI 68.75 ± 31.386, IC_{50} 0.57 ± 0.211 µg/mL).

The antioxidant activity of *S. zollingerianum* leaves extract was classified as very strong (AAI > 2.0) according to Scherer and Godoy (2016). The AAI value of *S. zollingerianum* leaves extract was not significantly different (p>0.05) with ascorbic acid (56.06 ± 36.580). It explains that the leaves extract of this plant has a very strong antioxidant activity and even higher than ascorbic acid. This activity is closely related to the compounds contained in the leaves of this plant. Although the abundance of tannins may have a major influence on the antioxidant activity of *S. zollingerianum* leaves extract, however, the accumulative or synergistic effects of the other compounds should be considered.

We also found that the leaves extract of *S. zollingerianum* had moderate cytotoxic activity (IC_{50} 101.42 ± 6.823 µg/mL) compared to doxorubicin on the Vero cell. Based on U.S. National Cancer Institute (NCI) and Geran protocol, the cytotoxic activity of the compound was classified as follows: very high cytotoxic activity if the IC_{50} value is <20 µg/ml, moderate cytotoxic if IC_{50} is ranged between 21 and 200 µg/ml, weak cytotoxic if IC_{50} is ranged between 201 and 500 µg/ml, and not toxic if IC_{50} >501 µg/ml (Sajjadi et al. 2015). However, it should be considered that compounds in plants might have synergistic, antagonistic, or accumulative effects (Martin et al. 2021) when they act as bioactive agents.

This study showed that the leaves extract of *S. zollingerianum* has the potential to be a promising source of natural exogenous antioxidants agents due to its strong antioxidant activity and moderate cytotoxic effect on Vero cell lines. Therefore, further studies are needed to isolate the potential chemical compounds and determine the antioxidant potential from pure compounds or fractions from leaves extract of *S. zollingerianum.*

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