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Phytochemical Analysis and Antioxidant Activities Using DPPH (1,1-Diphenyl-2-Picrylhydrazine) Assay of *Averrhoa bilimbi* L. growing in Indonesia

Análisis fitoquímico y actividades antioxidantes utilizando el ensayo DPPH (1,1-difenil-2-picrilhidrazina) de *Averrhoa bilimbi L.* que crece en Indonesia

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SUMMARY

Introduction: *To investigate Averrhoa bilimbi L. growing in Indonesia by phytochemical analysis and antioxidant activities using the 2,2-diphenyl-1-picrylhydrazine (DPPH) test.*
Methods: *extraction was carried out by sequential maceration with n-hexane, chloroform, and methanol as solvent. Meanwhile, phytochemical analysis was carried out by various chemical tests that have been investigated in different in vitro methods. Furthermore, its antioxidant activity was assessed by the 2,2-diphenyl-1-picrylhydrazine (DPPH) test. Moreover, the results were evaluated to analyze the new compounds.*
Results: *Chemical analysis of steroids and terpenoids revealed the presence of n-hexane extract, while phenols and terpenoids were found in the chloroform extract. Methanol extract contained flavonoids, steroids, saponins, phenols, and terpenoids. Among the extracts tested, the methanol extract showed the highest activity at the strong DPPH radical scavenger with an IC50 value of 11.2 g/mL.*
Conclusion: *This study provides a scientific essential for using Averrhoa bilimbi L. growing in Indonesia as an antioxidant from natural resources.*

Keywords: *Antioxidant, Averrhoa bilimbi L., DPPH (1,1-Diphenyl-2-Picrylhydrazine) assay phytochemical analysis*

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RESUMEN

Introducción: *Se investiga el cultivo de Averrhoa bilimbi L. en Indonesia mediante análisis fitoquímicos y actividades antioxidantes utilizando la prueba de 2,2-difenil-1-picrilhidrazina (DPPH).*
Métodos: *la extracción se realizó por maceración secuencial con n-hexano, cloroformo y metanol como solvente. Por su parte, el análisis fitoquímico se llevó a cabo mediante diversas pruebas químicas que han sido investigadas en diferentes métodos in vitro. Además, su actividad antioxidante se evaluó mediante la prueba de 2,2-difenil-1-picrilhidrazina*

(DPPH). Además, se evaluaron los resultados para analizar los nuevos compuestos.
Resultados: *El análisis químico de esteroides y terpenoides reveló en presencia de extracto de n-hexano, mientras que los fenoles y terpenoides se encontraron en el extracto de cloroformo. El extracto de metanol contenía flavonoides, esteroides, saponinas, fenoles y terpenoides. Entre los extractos probados, el extracto de metanol mostró la actividad más alta en el eliminador de radicales DPPH fuerte con un valor IC50 de 11,2 g/mL.*
Conclusión: *Este estudio proporciona un fundamento científico para el uso de Averrhoa bilimbi L. que crece en Indonesia como antioxidante de los recursos naturales.*

Palabras clave: *Antioxidante, Averrhoa bilimbi L., análisis fitoquímico del ensayo DPPH (1,1-difenil-2-picrilhidracina)*

INTRODUCTION

The reactive oxygen species (ROS) includes superoxide (O-), hydroxyl (HO), and peroxide radicals (ROO), defined as chemically reactive molecules (1-3). The consequence of oxygen metabolism is the production of ROS. Excessive stress leads to severe damage to the cells and tissues and stimulates oxidative stress, apoptosis, and necrosis. The damage is associated with heart disease and carcinogenesis (4,5). The antioxidants neutralize the excessive free radicals, maintain the biomolecules from the ROS attack, protect cells from the toxic effects, and prevent severe impacts – such as arthritis, stroke, and chronic bronchitis (6-8).

Researchers have examined plants as a natural and safe source of antioxidants. The natural antioxidants contain phenolic compounds (i.e., flavonoids, phenolic acids, and tannins) (9,10), nitrogen-containing compounds (i.e., alkaloids, amino acids, peptides, and amines), carotenoids, tocopherols, or ascorbic acid and their derivatives (11,12). The previous studies reported antibacterial activities in *Averrhoa bilimbi L.* consisting of active compounds in both stems, fruits, flowers, and leaves (13-17). The compounds in its flowers played a significant role in inhibiting growth and killing bacteria. The active compounds included saponins, polyphenols, and flavonoids (18-20) but few or no OCm, phagocytized and killed Escherichia coli in association with the production of reactive oxygen species (ROS.

Many investigations focused on the antibacterial activities of *Averrhoa bilimbi L.* However, no study has investigated the details of phytochemical analysis and antioxidant activity of *Averrhoa bilimbi L* (21-23). In addition, there is no pharmacological study on the effect of the chemical composition of those plants based on the geographical distribution (24,25). Thus, this paper describes the phytochemical analysis and investigates antioxidant activity in the *Averrhoa bilimbi L.* – especially from the flower extract. The Averrhoa bilimbi L. used in this study is growing in Madura Island, Indonesia.

METHODS

Preparation, extraction, and screening of phytochemical

Averrhoa bilimbi L. was obtained from Madura Island, Indonesia. In brief, the flowers of *Averrhoa bilimbi* L. were extracted as a powder, and the extracts were saturated with chemicals consisting of n-hexane (2 L), chloroform (2 L), and methanol (2 L) for three days. The extracts were filtered using Whatman paper number One and concentrated using a rotatory evaporator to obtain the chemicals. Then the phytochemical test was carried out on the extract. Phytochemical analysis determines the presence of alkaloids, flavonoids, saponins, triterpenoids, and steroids. Hence, all reagents are analytical.

Determination of total Wagner, Ferric, Salkowski, and Foam test

Alkaloid test (Wagner test)

Six drops of Wagner's reagent were added to 2 mL of the crude extract. Wagner's reagent is prepared by mixing iodine in a solution of potassium iodide. The formation of a brown or reddish precipitate indicates the presence of alkaloids.

Flavonoid test (Ferric chloride test)

The crude extract (2 mL) was given a few drops of ferric chloride solution. The intense

green color of the solution indicates the presence of flavonoids.

Phenolic test (Ferric chloride test)

A few drops of ferric chloride solution were added to the crude extract (2 mL). The appearance of a bluish-black color indicates phenolic compounds (Khanam, Wen, and Bhat, 2015).

Terpenoid test (Salkowski test)

The crude extract (1 mg) was mixed with chloroform (2 mL) and concentrated sulfuric acid (1 mL). The formation of reddish-brown color at the interface indicates the presence of terpenoids.

Test for steroids (Salkowski test)

About 100 mg of dry crude extract was dissolved in 2 mL of chloroform. About 2-3 drops of sulfuric acid were carefully added to the extract to form a bottom layer. A reddish-brown color at the interface indicates the presence of a steroid ring.

Saponin test (Foam test)

Distilled water (2 mL) was added to 2 mL of the extract. The mixture was shaken vigorously for 15 minutes. The resistance of the foam produced after 15 minutes indicates the presence of saponins.

***In vitro* methods of Antioxidant activity**

2,2-Diphenyl-1-picrylhydrazyl (DPPH) assay is widely used to investigate the scavenging activity of several natural compounds (Yang *et al*., 2020). The extract solution was mixed with 100 L of freshly prepared DPPH methanol solution (250 mM) on a 96-well microplate, then replicated three times. After incubation in the dark for 30 min, the remaining DPPH radicals were evaluated for absorbance at 515 nm, using a Multiscan Go Thermo Scientific microplate reader. Dimethylsulfoxide (DMSO) was used as a negative control and Trolox as a positive control.

Statistical analysis

SPSS statistics calculated the statistical analysis. The half-maximal inhibitory concentration (IC50) was calculated, then analyzed with linear regression. The pharmacological outcome was presented in four experiments. In addition, A p-value less than 0.05 is statistically significant. (Krousel-Wood, Chambers, and Muntner, 2006).

RESULTS AND DISCUSSION

The flower extract of *Averrhoa bilimbi* L. growing in Indonesia

Extract fractionation was carried out by removing the solvent under reduced pressure. It resulted in n-hexane, chloroform, and methanol extracts. A higher extraction yield was observed in methanol extract (11.14 %), followed by chloroform extract (2.74 %) and n-hexane (1,74 %). It indicates that the extraction increases as the polarity of the solvent are used in the extraction process (26,27). Therefore, the higher percentage result in methanol extract might be related to the high polarity of the methanol solvent. It can be extracted as an abundant type of compound (28,29). In conclusion, the highest total of extraction of *Averrhoa bilimbi L.* growing in Indonesia was methanol.

Preliminary phytochemical examination

Table 1 describes the results of the phytochemical analysis in n-hexane, chloroform, and methanol extracts. The n-hexane extract contains steroids and terpenoids, while the chloroform extract has substance phenols and terpenoids. In addition, flavonoids, steroids, saponins, phenols, and terpenoids are detected in the methanol extract. Furthermore, the result reveals terpenoids in all extracts, characterized by a reddish-brown color. However, those three extracts do not represent a brown or reddish precipitate when treated with Wagner's reagent, indicating the absence of alkaloids. In this study, the phytochemical analysis strongly shows flavonoids and phenols. It is in line with the reports in the previous studies (30,31).

Previous reports described those phytochemicals also detected in *P. guajava* leaves that

Table 1

Phytochemical analysis of *Averrhoa bilimbi L.* growing in Indonesia

Phytochemicals	Extract		
	n – Hexane	Chloroform	Methanol
Alkaloids	-	-	-
Flavonoids	-	-	+
Steroids	+	-	+
Saponins	-	-	+
Phenols	-	+	+
Terpenoids	+	+	+

had been reported to have medicinal benefits. Studies revealed that phenolics and flavonoids exhibited many biological activities, such as antioxidant (1,2), antimicrobial, anticancer, anti-inflammatory, and wound healing (4-6). Meanwhile, steroids had antibacterial, insecticidal, and cardiotonic properties (7,9). On the other hand, saponins could medicate diabetes (10,11). Similarly, terpenoids were used to treat cancer, malaria, inflammation, and various infectious diseases (12-14). We conclude that the *Averrhoa bilimbi L.* growing in Indonesia has benefits in the medical field.

In Vitro Antioxidant activity judgment

The result showed that the DPPH radical scavenging assay (1,1-diphenyl-2-picrylhydrazyl radical) is a stable free radical characterized by the dark purple color. The reaction of DPPH and hydrogen atoms results in DPPHH represented in yellow (18,19,21). In addition, Trolox is an analog of vitamin E and is used as a reference compound (22,24,25). The dose depends on each n-Hexane, chloroform, and methanol extract evaluated by in vitro assay (Figure 1). Both Trolox and extract of *Averrhoa bilimbi. L* growing in Indonesia showed a nearly linear response with a correlation coefficient value (r) > 0.95 (Table 2).

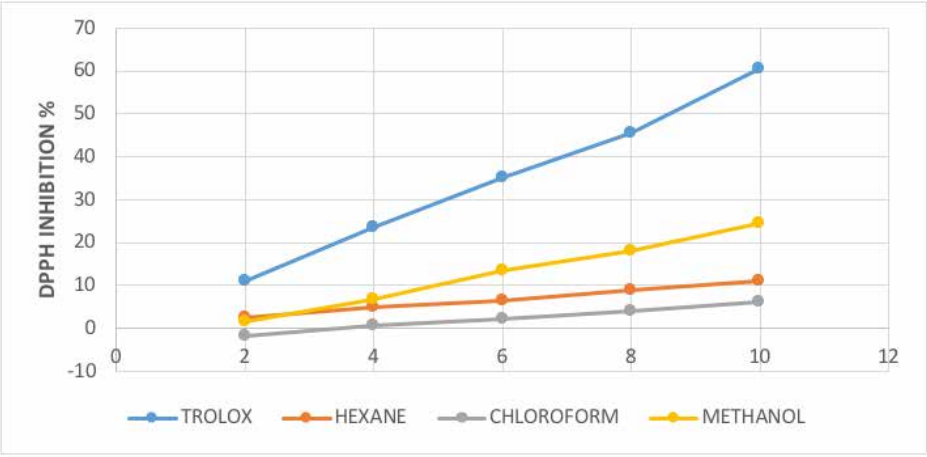

Figure 1. Dose-dependent antioxidant activity in flower extract of *Averrhoa bilimbi. L* growing in Indonesia (n=3).

Table 2

The IC50 in DPPH radical scavenging assay

Sample	Slope	Intercept	r2	Range Concentration	IC50 (μg/mL)	p
n-Hexane	1.0546	0.3451	0.9933	1-10 (μg/mL)	47.7	
Chloroform	0.9914	3.6111	0.9914	1-10 (μg/mL)	46.7	0.01
Methanol	4.2077	2.8409	0.9974	1-10 (μg/mL)	11.2	
Trolox	3.0619	0.4681	0.9983	1-10 (μg/mL)	8.1	

Table 2 describes that the IC50 value in the n-Hexane extract is 47.7 g/mL, the chlorophyll extract is 46.7 g/mL, and the methanol extract is 11.2 g/mL, while Trolox is 8.1 g/mL. The linear regression test obtains p=0.01 (p<0.05), indicating statistical significance. Thus, the antioxidant activity of the methanol extract of the flower of *Averrhoa bilimbi. L* growing in Indonesia was lower than Trolox. It is in line with previous studies (26-28). The authors consider that the extracts are a mixture of several compounds. Therefore, our study results reveal the antioxidant activity in *Averrhoa bilimbi. L* seems promising to control oxidative stress.

CONCLUSION

This study concludes the phytochemical analysis of *Averrhoa bilimbi. L* growing in Indonesia consists of various beneficial phytochemical compounds, such as flavonoids, steroids, saponins, phenols, and terpenoids. In addition, the methanol extract produces the highest extraction and shows an excellent antioxidant activity in the DPPH radical scavenging assay. These significant findings are beneficial as preliminary steps to determine the potential of natural antioxidants that can be applied in the pharmaceutical and therapeutic industries. Further study should describe the isolation and characterization of *Averrhoa bilimbi. L* to identify antioxidant activity.

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