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RESEARCH ARTICLE

Total phenolic content and *in vitro* evaluation of antioxidant activity of ethanol extract of *Ganoderma amboinense*

Nina Salamah, Mustofa Ahda, Sidiq Bimantara, Rizka Hanar

Department of Analitical Chemistry, Faculty of Pharmacy, University of Ahmad Dahlan, Semaki, Umbulharjo, Yogyakarta, Indonesia

Correspondence to: Nina Salamah, E-mail: ninasalamah1996@gmail.com

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ABSTRACT

Background: *Ganoderma amboinense* is rich in fibers, minerals, and vitamins. This antler-shaped mushroom contains various antioxidant compounds called polyphenols and flavonoids. Antioxidants can counteract or deactivate free radicals in plants and animals by turning them into less reactive compounds. The methods of compound extraction are numerous and with different effects on the concentration of active compounds. Hence, selecting a correct extraction method becomes necessary. **Aims and Objectives:** The aim of this stuidy is to compare two extraction methods, namely, extraction by maceration and Soxhlet extraction, based on the total phenolic contents and the antioxidant activities of the ethanol extracts (96%) of *G. amboinense.* **Materials and Methods:** The ethanol extract was obtained from maceration and Soxhlet extraction. The total phenolic content of 96% ethanol extract of *G. amboinense* was determined using ultraviolet-visible spectrophotometry with Folin–Ciocalteu reagent, while the antioxidant activity was assessed using the 2,2-diphenyl-1-picrylhydrazyl method. **Results:** Thin-layer chromatography produces ethanol extracts, from either maceration or Soxhlet extraction, that contain phenolic compounds. The total phenolic contents of the ethanol extracts from maceration and Soxhlet extraction are 26.72 ± 1.13 mg gallic acid equivalents (GAE)/g of extract and 23.27 ± 1.07 mg GAE/g of extract, respectively. The effective scavenging 50% values of gallic acid and ethanol extract are, respectively, 2.476 µg/ml and 197.6 µg/ml. **Conclusion:** Maceration produces ethanol extract of *G. amboinense* with higher total phenolic content than Soxhlet extraction. The research findings indicate that the ethanol extract has *in vitro* antioxidant activity.

KEY WORDS: Phenolic; Antioxidant; *Ganoderma*; Mushroom

INTRODUCTION

Extraction is a process of removing or separating active compounds in stems, leaves, and fruits. It is very important in determining the amount of extractable active substance. The efficiency of conventional and non-conventional extraction depends on critical parameters such as plant matrix properties, chemical compounds, bioactive, and scientific expertise.[1] There are various types of extraction methods, namely, maceration, infusion, digestion, decoction, percolation, and hot continuous extraction (Soxhlet).[2,3] Each method has different strengths and weaknesses. The latest development of extraction method in medicinal plants uses liquid nitrogen as solvent.[4] Odey *et al*.[5] found that the effectiveness of medicinal plants highly depends on extraction method and extract preparation.

This research analyzes two extraction methods, namely, maceration and Soxhlet extraction, for removing active compounds in *Ganoderma* mushrooms. These two methods are selected because they are simple and widely used. Meanwhile, *Ganoderma* mushrooms are sampled in this

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research because they have various pharmaceutically active compounds that exhibit antitumor, anticancer, antihypertensive, hypocholesterolemic, antihistaminic, antiplatelet, and antiretroviral/anti-HIV effects as well as immunomodulatory proteins.[6] According to Boh *et al*.,[7] the active compounds are ganoderic acid, lucideric acid, ganodermic acid, ganoderenic acid, ganolucidic acid, polysaccharide, protein, amino acid, nucleotide, alkaloid, steroid, lactone, fatty acid, and enzyme. In addition, Rajasekaran and Kalaimagal[8] state that the ethanol extract of *Ganoderma* has many phenolic compounds, flavone, and ascorbic acid that have protective effects against free radicals.

Considering the enormous potentials of *Ganoderma*, studies that aim to identify the effective and accurate extraction process for acquiring the active compounds of *Ganoderma amboinense* become necessary. This research compares a conventional extraction method, i.e., by maceration, with Soxhlet extraction and analyzes non-specific parameters, total phenolic contents, and antioxidant activities to determine the effectiveness of each method. Furthermore, ethanol extract with the highest total phenolic content is analyzed using diphenylpicrylhydrazyl 2,2-diphenyl-1-picrylhydrazyl (DPPH) method to identify its antioxidant activities.

MATERIALS AND METHODS

Loss on Drying (LOD) Test of the Ethanol Extract of *G. amboinense*

1 g sample of *G. amboinense* extract was tested for LOD using halogen moisture analyzer. The amount of loss from the sample was recorded.

Qualitative Test for Active Compounds using Thin-layer Chromatography (TLC)

The extract solutions from maceration and Soxhlet extraction as well as a standard solution of gallic acid with a concentration of 2 mg/mL were sampled as many as 10 µL and spotted to silica gel GF_{254} (stationary phase) and with a solvent mixture (mobile phase) of toluene, ethyl acetate, and formic acid (ratio 6:4:0.8). After drying, the TLC plate was sprayed with two reagents, namely, form purple coloration in ferric chloride test ($FeCl_3$) and vanillin-sulfuric acid, and observed under visible lights.

Determination of Total Phenolic Content[9]

The total phenolic content of *G. amboinense* was determined using the method proposed by Murtijaya and Lim (2007) with modification. The gallic acid solution was made in various concentrations, i.e. 20-50 µg/mL. Afterward, 300 µl samples of each gallic acid solution were added by 1.5 ml of Folin–Ciocalteu reagent, and then, shaken. The mixture was allowed to stand for 3 min, followed by the addition of 1.2 ml of 7.5% Na_2CO_3. After 30 min, the absorbance was measured at 598.5 nm. Then, 25.0 mg of the ethanol extract of *G. amboinense* was dissolved up to 10.0 ml with 96% ethanol. This solution was dispensed as many as 300 µl into the tube, added by 1.5 ml of Folin–Ciocalteu reagent, and shaken. It was allowed to stand for 3 min, followed by the addition of 1.2 ml of 7.5% Na_2CO_3. This mixture was allowed to stand within the range of operating time (OT) for 30 min at room temperature. The absorbance of the extract solution was measured using ultraviolet visible (UV-Vis) spectrophotometer at a maximum wavelength of 598.5 nm.

Determination of DPPH Radical Scavenging Activity

Determination of OT

Each sample was dispensed as many as 1 ml, added with 1 ml of 0.15 mM DPPH, and shaken. Then, the absorbance of this solution was observed until it was stable at minute *t* at a wavelength of 517 nm.[10]

Determination of the wavelength of maximum absorbance

The wavelength of maximum absorbance of DPPH solution was determined using the following technique: 1.0 ml of 0.15 mM DPPH solution was added with 1.0 ml of ethanol p.a. and shaken homogeneously. The absorbance was measured at 400-600 nm.[10]

Measurement of the absorbance of free radical scavenging using DPPH method

Each concentration of ethanol extract was sampled by 1.0 ml. The negative control (DPPH + ethanol p.a.) and the positive control (DPPH + gallic acid) were made in various concentrations. The sample, negative control, and positive control were added by 1.0 ml of 0.15 DPPH solution and then shaken vigorously. The solution was stored in the darkness for as long as the OT. The absorbance of the solution was measured at the maximum wavelength of DPPH with UV-Vis spectrophotometry. Blank was concomitantly prepared, containing ethanol p.a. solution.[10]

Data analysis

The data obtained from the aforementioned measurements were effective scavenging and the concentration of tested compounds. These data were then analyzed using linear regression to identify the concentration at which DPPH radicals were scavenged by 50% effective scavenging 50% (ES_{50}).

RESULTS

In this study, identification of *G. amboinense* includes some parameter such as qualitative test, total phenolic content, and

Table 1: The results of qualitative test on phenolic content in ***G. amboinense***

Test	Qualitative tests of phenolic contents						
	FeC_{l3}	Folin–Ciocalteu	TLC				Remarks
			Rf	UV 254 nm	UV 366 nm	FeC_{l3} reagent	
Maceration	Purple	Blue	0.25, 0.42	Black	Green	Violet	Positive
Soxhlet extraction	Purple	Blue	0.25, 0.42	Black	Green	Violet	Positive

G. amboinense: Ganoderma amboinense, UV: Ultraviolet, TLC: Thin-layer chromatography

antioxidant activity of DPPH. The qualitative test appears the specific color and some count of spot on KLT plate [Table 1 and Figure 1] . The result of this research showed that the *G. amboinense* has phenolic compound which it showed from coloring test and Korean language test (KLT) test on the spot with Rf 0.25 and 0.42.

With mobile phase (toluene:ethyl acetate:formic acid = 6:4:0.8) and (A) $FeCl_3$ reagent, (B) Vanillin-sulfuric acid reagent. Spotting: (1) and (5) extracts from maceration, (2) and (4) extracts from Soxhlet with 6 circulations, (3) a standard Solution of gallic acid, and (6) powdered crude drugs.

Other parameters for testing the *G. amboinense* quality are determinant the total phenol content, chemical yield, LOD, and antioxidant activity of DPPH. The result of this research was showed on Tables 2 and 3.

DISCUSSION

The extraction of active compounds in plants is influenced by several factors. The amount of the extracted compound has a significant influence on extraction method, solvent, and time possess.[11] Therefore, this research describes the influence of different extraction methods, namely, maceration and Soxhlet extraction, on the active compounds of *G. amboinense*. Table 1 shows that the properties of the *G. amboinense* extracts produced in maceration and Soxhlet extraction have similarity. The active compounds that are extracted with ethanol in both methods consist of a phenyl group, which is not found in the aqueous and ethanolic extracts of *Ganoderma lucidum*. The active compounds of *G. lucidum* that are extracted using water and ethanol are not phenolic but petroleum-based.[12] Phenolic content in the active compounds is identified using two qualitative tests in which the extracted active compounds $FeCl_3$ reagent forms a complex of compounds with phenyl group. Furthermore, the phenyl group of the active compounds also reacts to Folin–Ciocalteu reagent (oxidation-reduction reaction) and forms blue coloration, i.e. color indicator for phenyl group. However, there is a slight difference between the results of maceration and Soxhlet extraction in the removal of active compounds. Both of these methods produce two purple spots on the TLC plates, indicating the presence of two active compounds with phenyl group [Table 1] . Each spot on the

Table 2: The test results of parameters in ***G. amboinense***

Parameters	Extraction methods	
	Maceration	Soxhlet extraction
Chemical yield (%)	10.88	7.86
LOD (%)	4.72±0.22*	5.72±0.55*
Total phenolic content (mg GAE/g of extract)	26.72±1.13**	23.27±1.07**

Notes: *3-time replications; **6-time replications.
G. amboinense: Ganoderma amboinense, GAE: Gallic acid equivalents, LOD: Loss on drying

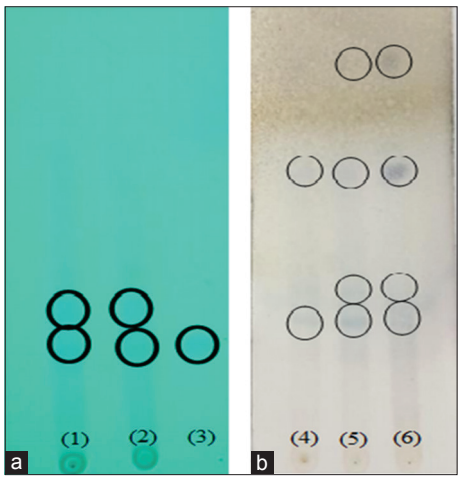

Figure 1: (a and b) The extraction of active compounds of *Ganoderma amboinense* using thin-layer chromatography

TLC plates shows dark color under 254-nm UV light and green under 366-nm UV light.

Spots on the TLC plate show that one of the active compounds has similar characteristics to gallic acid because its Rf value is 0.25, which is close to the Rf value of gallic acid [Figure 1a]. Figure 1b depicts the different numbers of spots when the TLC plate is sprayed with vanillin-sulfuric acid. Maceration and powdered crude drug have similar number of spots. Meanwhile, the Soxhlet technique only extracts two active compounds. However, this number of active compounds does not necessarily mean that Soxhlet extraction with six or more circulations is less effective than 24-h maceration in extracting the active compounds of *G. amboinense* because the active compounds may not react to the vanillin-sulfuric acid reagent. Alcohol compounds, phenols, steroids, and essential oils exhibit positive reactions to vanillin-sulfuric

Table 3: Free radical scavenging and ES_{50} (%) of ethanol extract and standard solution of gallic acid

Sample concentrations (µg/ml)	% inhibitions (µg/ml)	ES_{50} (µg/ml) EtOH extract	% inhibitions (µg/ml)	ES_{50} (µg/ml) Gallic acid
0.39			5.6±2.9	
0.78			21.8±1.3	
1.56			46.8±2.5	2.476
3.12			83.3±2.8	
6.25			84.2±2.9	
12.5	0.9±2.6			
25	13.3±4.2			
50	21.9±2.7	197.6		
100	33.1±2.0			
200	50.5±1.0			
400	65.7±1.8			

ES_{50}: Effective scavenging 50%

acid.[13] Aside from producing different spots on the TLC plates, maceration and Soxhlet extraction produce different parameters such as chemical yield, LOD, and total phenolic content. The chemical yield of maceration (10.88%) is larger than the yield of Soxhlet extraction (7.86%). As for the LOD of the ethanol extract of *G. amboinense*, maceration and Soxhlet extraction produce slightly different percentages, i.e., 4.75% and 5.72%, respectively. The total phenolic contents produced in the two extraction methods are also different. The total phenolic content from maceration is 26.72 mg gallic acid equivalents/g of extract, which is higher than Soxhlet extraction [Table 2] .

Soxhlet extractor produces extracts with low phenolic content due to the presence of heat and oxygen in the process. Total phenolic compounds are oxidized in alkaline solution. Moreover, the polyphenol oxidase activity forms ortho-semiquinone radicals that are reactive and may further react with amino compounds creating brown-colored products with high molecular weight.[14] Heating vegetables at 60°C reduce the phenolic contents of several compounds significantly. Some phenolic compounds are easily oxidized particularly in alkaline environment and due to polyphenol oxidase activity.[15] Compared to 24-h maceration, the chemical yield of Soxhlet extraction is lower. In prolonged Soxhlet extraction, the total phenolic contents decline significantly. When the total phenolic contents of the extract decrease, the effectiveness of *G. amboinense* as an antioxidant also decreases.[16] Therefore, a high total phenolic content commonly associates with high antioxidant activity. Maceration produces extracts with higher total phenolic content than Soxhlet extraction. The antioxidant activities were analyzed using the DPPH method. The comparison between the antioxidant activities of the ethanol extract of *G. amboinense* and gallic acid is indicated by ES_{50}. ES_{50} represents the concentration of tested compound required for reaching an effectiveness of 50% free radical scavenging. This value is determined using linear regression analysis between the concentration of the tested compound and the percentage of free radical scavenging. Table 3 shows that gallic acid has more free radical-scavenging potential (ES_{50} 2.476 µg/ml) than the ethanol extract of *G. amboinense* (ES_{50} 197.6 µg/ml). Gallic acid establishes higher scavenging activities than the ethanol extract, as indicated by the small ES_{50} value of gallic acid. The ES_{50} value is inversely proportional to the percentage of free radical scavenging. The higher the ES_{50} value, the higher the concentration required in producing 50% free radical-scavenging activities, and hence, the smaller the scavenging potential. *G. lucidum* is the same family with *G. Amboinense* and it is reported that the antioxidant activity of ethanolic extract *G. lucidum* with ES50 13, 16 µg/ml.[17] Based on it, if we compare the potensial antioxidant of *G. lucidum* higher than *G. amboinense*.

The antioxidant activities of gallic acid and the ethanol extract are analyzed statistically using normality test, Levene's test, Kruskal–Wallis test, and Mann–Whitney U-test. The significance value (α) from the normality test is <0.05 (i.e., 0.231), meaning that the data are normally distributed. The test for homogeneity of variance with Levene's test shows a significance value between 0.005 and 0.05, which represents data with non-homogeneous variance. In addition, the significance value produced in the non-parametric test, i.e. the Kruskal–Wallis test, with a confidence level of 95% is 0.0009. Since it is <0.05, the test confirms that the difference between the various treatment groups is significant. The last test is the Mann–Whitney U-test that compares the treatment results between the treatment groups. The p-value of Mann–Whitney U-test is 0.008, which is <0.05, indicating that the antioxidant activities between gallic acid and the ethanol extract are significantly different.

CONCLUSION

Extraction by maceration produces a higher total phenolic content than Soxhlet extraction. The total phenolic content

shows antioxidant potentials of the ethanol extract of *G. amboinense*. The antioxidant activity of the ethanol extract is smaller than gallic acid, which is a type of polyphenol.

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