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Optimization of Ultrasonic-Assisted Extraction Methods of *Gymnanthemum Amygdalinum* Del for Antioxidant and Antibacterial Activities

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Abstract

Gymnanthemum amygdalinum Del is one of the medicinal plants that have been shown to have antidiabetic, antimalarial, and anticancer properties. *Gymnanthemum amygdalinum* leaves are expected to be able to limit the increase in free radicals caused by exposure to sunlight because they have flavonoids, which are antioxidant compounds and photoprotective compounds. The purpose of this study was to determine the optimum conditions for *Gymnanthemum amygdalinum* leaf extract using the ultrasonic-assisted extraction method based on the value of antioxidant activity using the ferric reducing antioxidant power (FRAP) method and the possibility that it can be used as an antibacterial against E. coli and B. subtilis using the disc diffusion test method. The ultrasonic assisted extraction method on *Gymnanthemum amygdalinum* leaves was carried out with 4 variations of time and amplitude conditions, among others: condition A (30 minutes, 60%); condition B (30 minutes, 65%); condition C (45 minutes, 60%); and condition D (45 minutes, 65%). The IC_{50} value for the ability of *Gymnanthemum amygdalinum* leaf extract as an antioxidant is 94.83 ± 0.04 mg/L (A); 107.44 ± 0.03 mg/L (B); 145.06 ± 0.39 mg/L (C); and 153.46 ± 0.04 mg/L (D). Ultrasonic leaf extraction using 30 minutes and 60% amplitude yielded the best results for *Gymnanthemum amygdalinum* (condition A). Clinical and Laboratory Standards Institute (CLSI) criteria indicate that E. coli and B. subtilis do not have particularly potent antibacterial properties. *Gymnanthemum amygdalinum* leaves have been shown to have antibacterial activity against E. coli and B. subtilis, with average zones of inhibition of 1 mm (A), 2 mm (B), and 1 mm (C), and 1 mm (D).

***Keywords*:** *Gymnanthemum amygdalinum*; ultrasonic-assisted extraction; antioxidant; FRAP; antibacterial

1. Introduction

Medicinal plants are plants that are known to have good properties for helping to maintain health or treat a disease. Medicinal plants are very closely related to traditional medicine, because most of the utilisation of medicinal plants has not been based on laboratory clinical trials but rather on experience of use [1]. The human body produces antioxidants, but the amount is often not balanced with the amount of free radicals in the body, so antioxidants are needed that come from outside the body. The need for antioxidants can be met by natural antioxidants derived from plants [2]. The balance between oxidants and antioxidants in cells is controlled by the body's antioxidant defence system. If this system does not function properly, it will cause an increase in the production of free radicals, which can cause oxidative stress. Oxidative stress results in tissue damage [3]. Diseases like diabetes, Alzheimer's, Parkinson's, atherosclerosis, cancer, liver disease, inflammation, arthritis, neurodegenerative disorders, and others can be caused by this condition [4-5].

Gymnanthemum amygdalinum Del (GAD) is part of the Gymnanthemum genus and has outstanding benefits for the human diet. There are roughly a thousand different species of Asteraceae plants, of which GAD is one [6]. For centuries, this plant has served as an essential medicine, particularly in sub-Saharan Africa, where it has been used to treat

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a wide range of conditions, including malaria, stomach disorders, hiccups, diabetes, inflammation, fertility issues, and bacterial infections [7]. This plant contains fat, protein, fiber, minerals, amino acids, carbohydrates, and vitamins [8]. A study found that plant extract yield and bioactivity can be affected by geographic location [9]. The main chemical components of GAD contain steroids, polyphenols, alkaloids, anthraquinones, lignans, flavonoids, and coumarins [10]. Biological activities of extracts and isolation of compounds from GAD have been reported, such as antibacterial, antifungal, antiparasitic, anticancer, anti-obesity, antioxidant, antihypertensive, antidiabetic, anti-inflammatory, anthelmintic, cardiac protective activity, and liver protective activity [11-23]. However, several previous studies related to GAD leaves still used conventional methods to obtain the desired extract, such as maceration [24-26] and soxhlet methods [27].

The main challenges of conventional extraction are the long extraction times, the high and expensive requirements for solvent purity, the evaporation of large amounts of solvent, the low selectivity of extraction, and the thermal decomposition of thermolabile compounds. To overcome these limitations, prospective extraction techniques have been extensively introduced. Ultrasonic extraction is one example of the so-called unconventional extraction techniques [28]. Ultrasound-Assisted Extraction (UAE) is a solid-liquid extraction method that uses high-frequency sound waves to break up molecules. The ultrasonic method is an effective and efficient non-thermal extraction method [29]. This technique is known as sonochemistry, which is the utilisation of the effects of ultrasonic waves to affect changes that occur in chemical processes [30].

Ultrasonic extraction could have a powerful mechanical effect, hence boosting mass transfer during the extraction process [31]. Ultrasonic extraction has been applied to food and related industries, including herbal, protein, and oil, as well as bioactives from plant and animal materials (e.g., polyphenols, anthocyanins, polysaccharides, aromatic compounds, and functional compounds), with increased yields of extracted components, increased reaction rates, reduced extraction time, reduced temperature, reduced use of solvents, and higher processing as compared to other methods [32-34]. The use of the ultrasonic extraction method to obtain GAD leaf extract and test its antioxidant and antibacterial activity has never been reported before. In this study, ultrasonic extraction used ethanol as a solvent, and the extraction process was carried out using several variations of conditions by optimising time and amplitude percent.

2. Experimental

2.1. Instruments

A UV-VIS spectrophotometer (Shimadzu UV-VIS 1700), an ultrasonic vibration probe (Sonics VCX 750), an analytical balance (Shimadzu), a blender (Philips), a sieve (40 mesh), an oven (Memmert), a pH metre (Eutech 510 Instrument), a micropipette (Eppendorf), and a set of Pyrex glassware were used.

2.2. Materials

The test material used in this study was GAD leaves collected from the Cimenyan area, Bandung Regency, West Java. The chemicals used were distilled water, ethanol pa (Merck), gallic acid (Sigma-Aldrich), o-phenanthroline (Sigma-Aldrich), 0.001 M citric acid solution, and $FeCl_3.6H_2O$ (Merck).

2.3. Methods

2.3.1. Sample preparation

The part of the plant used for the research was the leaves of GAD. The collected leaves were then sorted to separate other parts from plants, insects, and other impurities, then washed. We measured the dry weight of GAD leaves after they had been air-dried in the open air away from direct sunlight for three weeks. We weighed each the sample, blended it until it was powdery, and sieved it through a 40-mesh screen. Every powdered sample was kept in its own airtight, labelled, and dark container until extraction could be performed.

2.3.2. Sample extraction by ultrasonic method

Weighted GAD leaf sample powder was placed in a beaker glass. A total of 100 mL of a 70% ethanol solvent was poured into each beaker. Afterwards, the samples were sonicated using an ultrasonic probe at room temperature with a range of sonication times and amplitudes:

- 30 minute extraction time with an amplitude of 60%
- 30 minute extraction time with an amplitude of 65%
- 45 minute extraction time with an amplitude of 60%
- 45 minute extraction time with an amplitude of 65%

2.3.3. Antioxidant activity with FRAP method [35]

By dissolving 5 mg of the extract in ethanol in a 5 mL measuring flask, we were able to create a sample solution with a concentration of 1,000 mg/L. After being pipetted into a 5 mL measuring flask with 0.001 M citric acid, 0.002 M $FeCl_3$, and 0.2% o-phenanthroline in 0.4 mL, it was then measured and homogenised (sample concentrations of 20, 40, 80, 120, and 180 mg/L). After incubating the solution for

35 minutes at 37 °C, the spectrophotometer reading was taken at 550 nm to determine the solution's absorption. Three separate instances of the work were performed. The same work was carried out on gallic acid as a comparator with various concentrations of gallic acid: 1, 1.5, 2, 2.5, and 3 mg/L.

Here's an equation that can be used to determine how much of an impact reduction you'll see:

$$\% \text{ Power Reduction } = \frac{A_{Sample} - A_{Blank}}{A_{Sample}} \times 100\%$$

Description:

A_{blank} = Blank Absorbance
A_{sample} = Sample Absorbance

The resulting concentration (in ppm or mg/L) was used as the abscissa in a linear equation (Y = bX + a), while the ordinate (Y) represents the result (% reduction). For a 50% concentration of the reducing agent, the calculation yielded the IC_{50} value.

2.3.4. Antibacterial test by diffusion method [36]

A total of 1 mL of GAD leaf extract was pipetted into a petri dish. The disc paper was put into a petri dish containing African leaf extract until it was submerged, then left for 1-2 minutes. Tweezers were used to remove paper discs one at a time. Paper discs were placed on each medium, which had been labelled or marked with the position of the test bacteria (in the middle). After that, the samples were incubated upside down for 18–24 hours at 37 °C. Observations were made on the presence or absence of an inhibition zone. If there was an inhibition zone or clear zone, then the overall diameter, disc diameter, and inhibition zone diameter were measured using a ruler. If there was no inhibition zone or clear zone, then (-) was entered in the measurement column. Measurements and observations were calculated in mm.

3. Result and Discussion

3.1. Extraction Result Using UAE

Fresh GAD leaves were imported from the Cimenyan area, Bandung Regency, West Java. The leaf sample of GAD belongs to the Asteraceae tribe. The sample was authenticated at the Bogoriense Herbarium, National Research and Innovation Agency Biology Research Center, Indonesia. Using the UAE technique, leaves of GAD were extracted with 70% ethanol. Adjustments to the ultrasonic probe's extraction time and amplitude were made at this stage. The time variations used were in a 30 and 45 minute range while the amplitude variations were 60% and 65%. Table 1 displays the outcomes of the extracting procedures for all of the different GAD leaf variants.

Table 1
Crude extract yield using the UAE method

Sample	Variation	Variation of time (minutes) and amplitude (%)	Sample Weight (g)	Crude Extract Weight (g)	Yield (%)
GAD Leaves	A	30-60	14,7810	4,3269	**29,27**
	B	30-65	14,9420	3,2633	21,84
	C	45-60	14,8002	2,4541	16,58
	D	45-65	14,1385	3,5222	24,91

Table 1 shows that the variation of the time parameter with amplitude on GAD leaves causes an increase in the extraction yield. In GAD leaves, the optimum yield of sample A was 29.27% in 30 minutes at an amplitude of 60%. Increasing the extraction time and amplitude lets more energy into the cell, which makes the cell wall easier to break down and lets more substance be taken out [37]. However according to Jerman et al. [38], bioactive compounds exposed to ultrasonic waves for more than 35 minutes exhibited structural degradation and yield loss. In addition, higher amplitudes and longer sonication times encourage the production of free radicals. Metabolic chemicals neutralize these radicals, lowering the overall extraction yield. That's why it is necessary to find the optimum conditions for each extraction using the UAE method so that the time and amplitude parameters can be adjusted according to the type of sample.

When compared to the results of extracting GAD leaves using the UAE method, which we carried out with other extraction methods that have been previously reported [39], namely conventional methods (maceration and soxhlet) and other non-conventional methods (microwave-assisted extraction), the results are as follows:

Table 2
Comparison crude extract yield using the UAE, MAE, maceration and soxhlet method

Sample	Extraction Method	Duration Extraction (Min)	Volume of Solvent (mL)	Yield (%)
Ethanol Extract GAD Leaves	Maceration	2880	250	15.60
	Soxhlet	265	150	18.50
	MAE	45	100	20.55
	UAE	30	100	29.27

In Table 2, it can be seen that the extraction results using conventional methods such as maceration and soxhlet that have been reported [39] require a larger volume of solvent and the extraction time is much longer than non-conventional methods. Another non-conventional extraction method other

than the UAE that has been widely reported is the microwave-assisted extraction (MAE) method. The yield results obtained by the MAE and UAE methods were higher than the conventional methods, namely 20.55 and 29.27%, respectively. From these data, it can be concluded that non-conventional methods, especially the UAE method, are more efficient for extracting GAD leaves than conventional methods.

The UAE technique utilises ultrasonic waves resulting in microcavitation around the material being extracted, which heat it and release its compounds [40]. There is a double effect produced, namely the disruption of the cell wall so as to free the compounds contained in the cells, local heating of the fluid, and increased diffusion of the extract [41]. As the fluid's kinetic energy spreads, cavitation bubbles form on the walls or surfaces, increasing the mass transfer between the solid and liquid phases. Mechanically, this serves to facilitate the release of cellular components, boost mass transfer, and deepen fluid penetration into the cell membrane [29]. Ultrasonic cavitation generates fracture forces that will break down the cell walls mechanically and increase material transfer [42].

The ability of ultrasonics to cause a cavitation effect in the extraction process is influenced by several factors. The first is frequency; increasing the frequency will reduce the minimum pressure so that more energy is required to form cavitation in the system. The frequency commonly used is between 20 and 40 kHz. The second is the viscosity of the solvent; the thicker the solvent, the more difficult it is to form cavitation, so that the process efficiency decreases. Furthermore, the lower the surface tension and vapour pressure of the solvent, the more difficult it is for cavitation to occur. High vapour pressure makes it easier for cavitation to form, so volatile solvents are often used.

External pressure is another factor that influences the activation effect; an increase in external pressure causes the intensity to rise and the cavitation phenomenon to break. The amplitude intensity is also very influential; increasing the sonication intensity will increase the sonochemical process, but this is limited by the ultrasonic energy entering the system. The cavitation effect is also affected by temperature; at high temperatures, the vapour pressure in the medium rises, allowing cation bubbles to form easily, but if the temperature is too high, accompanied by a decrease in viscosity and surface tension, only a few bubbles break. At temperatures close to the boiling point, cavitation bubbles appear simultaneously in large numbers; this will hinder sound transmission and reduce the effectiveness of energy entering the liquid medium, so that the sonochemical process is less efficient [43].

3.2. Antioxidant Activity with FRAP Method

This method is based on the antioxidant activity of electron donors at low pH to reduce the Fe^{3+}-TPTZ complex (colorless complex) to Fe^{2+}-tripyridyltriazine (blue complex) [44]. FRAP measures antioxidant activity as a percentage of reducing power, which is then plotted against a standard concentration series to obtain a linear regression equation (Table 3).

The regression equations for gallic acid and ethanol extract of GAD leaves with treatments A, B, C, and D, respectively, are as follows: y = 27.898x - 22.738; y = 0.2973x + 21.806; y = 0.4148x + 5.4359; y = 0.3345x + 1.4761; and y = 0.3137x + 1.8602 (Figure 1). Using this formula, treatments A, B, C, and D all have different IC_{50} values for gallic acid, which we can calculate to be 2.61 ± 0.01 mg/L, 94.83 ± 0.04 mg/L, 107.44 ± 0.03 mg/L, 145.06 ± 0.39 mg/L, and 153.46 ± 0.04 mg/L.

Basically, the presence of a chemical with reducing ability donates a hydrogen atom, which results in the breaking of the free radical chain. The presence of antioxidants in the sample acts as a reducing agent in the redox-related colorimetric reaction in the FRAP test [45]. The total antioxidant status of a sample is commonly evaluated using the FRAP test. The antioxidant activity based of power reduction extract of GAD leaves as shown in IC_{50} by the FRAP method (table 3) was in the range of 94.83 ± 0.04 to 153.46 ± 0.04 mg/L. According to the extraction conditions in the UAE, this indicates strong to weak activity. Optimal results for testing antioxidant activity with the FRAP method were obtained from samples of ethanol extract variation A with extraction conditions of UAE 30 minutes and 60% amplitude.

The results of the antioxidant reducing power test that we obtained from the GAD extract were fairly good. Previous research [46-47] on the reducing power test of GAD extract obtained from the extraction process using conventional methods showed that the antioxidant activity was in the weak category, namely 208.652 µg/mL, and 717.50 µg/mL. This is likely to occur because the UAE extraction method can carry more bioactive compounds.

Table 3

The antioxidant activity of the ethanol extract of GAD leaves using the FRAP method is based on reducing power

Sample	Concentration (mg/L)	Reduction Power (%)	Regression Equation	IC_{50} (mg/L)
Gallic Acid	1	2.60 ± 0.13	y = 27.898x - 22.738	2.61± 0.01
	1.5	19.61 ± 0.23		
	2	36.44 ± 0.05		
	2.5	48.98 ± 0.01		
	3	57.66 ± 0.03		
A	20	21.33 ± 0.05	y = 0.2973x + 21.806	**94.83 ± 0.04**
	40	34.94 ± 0.08		
	80	51.63 ± 0.02		
	120	61.64 ± 0.01		
	180	70.31 ± 0.01		
B	20	15.35 ± 0.10	y = 0.4148x + 5.4359	107.44 ± 0.03
	40	18.92 ± 0.09		
	80	30.66 ± 0.10		
	120	71.40 ± 0.02		
	180	73.34 ± 0.01		
C	20	7.52 ± 0.20	y = 0.3345x + 1.4761	145.06 ± 0.39
	40	11.87 ± 0.18		
	80	32.59 ± 0.03		
	120	43.04 ± 0.50		
	180	59.53 ± 0.13		
D	20	5.55 ± 0,06	y = 0.3137x + 1.8602	153.46 ± 0.04
	40	13.76 ± 0,01		
	80	30.58 ± 0,01		
	120	41.86 ± 0,01		
	180	55.57 ± 0,01		

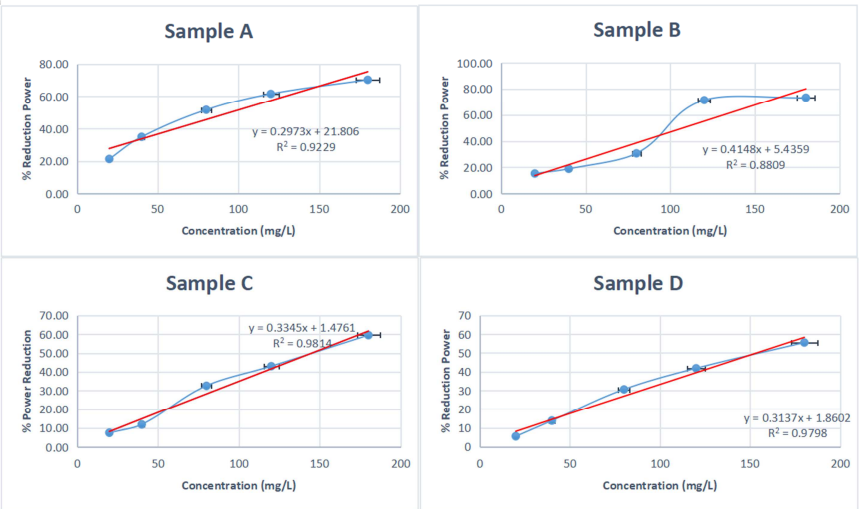

Figure 1: FRAP Test Results of GAD Leaf Extract with Various Conditions

3.3. Antibacterial Test by Diffusion Method

The level of activity of an antimicrobial compound can be determined by several methods, including the agar diffusion method. For an antibacterial compound to interact with bacterial cells, there must be a hydrophilic-lipophilic balance (HLB: hydrophilic-lipophilic balance). This is why compounds with the most favourable polarity tend to be the most effective against microbes [48–49]. Therefore, the polarity of the antibacterial compound is an important physical property. The antimicrobial compounds must dissolve in the water phase, where microbial life can exist, so a hydrophilic property is essential. However, compounds that act on cell membranes that are hydrophobic in nature require lipophilic properties as well. Factors that influence the presence of an inhibition zone are the diffusion ability of the inoculated antimicrobial substance, the growth rate of the tested microbe, and the level of sensitivity of the microbe to the antimicrobial agent concerned.

Figures 2 and 3 show the results of the antibacterial test and the results of the measurement of the inhibition zone of the influence of the ethanol extract of GAD leaves on E. coli and B. subtilis bacteria, respectively. The inhibition zones formed from each PDA media had varying sizes but could still be counted in units of mm.

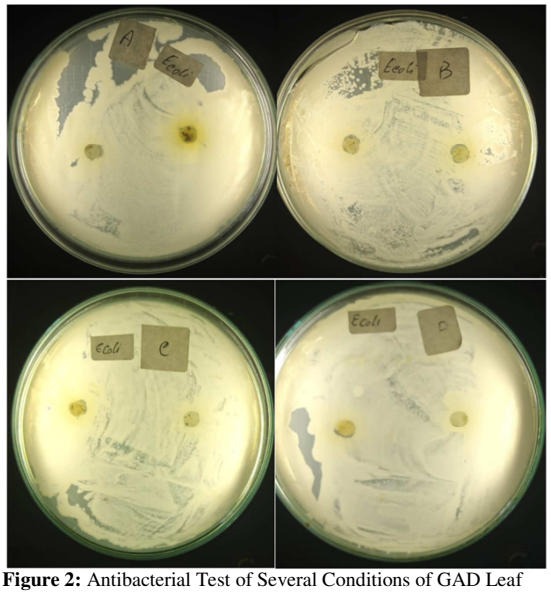

Figure 2: Antibacterial Test of Several Conditions of GAD Leaf Extract against E.coli Bacteria

The ethanol extract of GAD leaves showed weak inhibition against gram-negative E. coli and gram-positive B. subtilis, as measured by the diameter of the inhibition zone (table 4). These standards are established by the Clinical and Laboratory Standards Institute (CLSI) and include the following provisions for the potency of antibacterial power: Very strong inhibition occurs at an area of 20 mm or more, strong inhibition occurs between 10 and 20 mm, medium inhibition occurs between 5 and 10 mm, and weak inhibition occurs at an area of 5 mm or less [50].

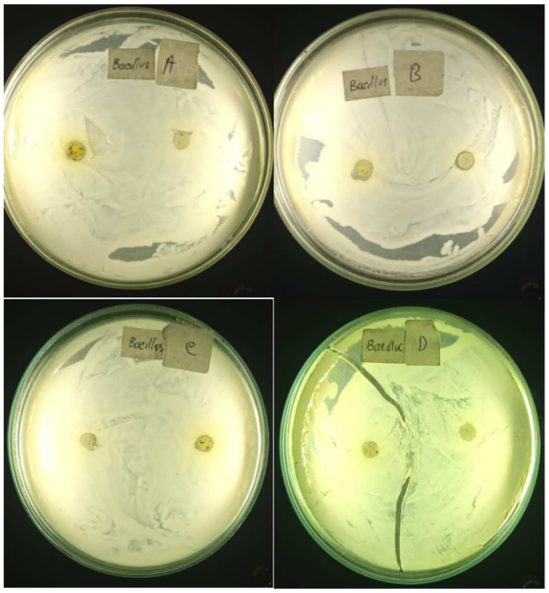

Figure 3: Antibacterial Test of Several Conditions of GAD Leaf Extract against B. subtilis Bacteria

Although in this study the results showed strong antioxidant activity, the antibacterial activity was very weak. This is possible because the active compounds that act as antioxidants and antimicrobials in GAD leaf extract are different, and the types of bacteria used as samples are also very influential. In the previously reported GAD leaf antibacterial study, it can be seen that GAD leaf extract has antibacterial activity on S. aureus and P. aeruginosa bacteria, which are categorized as strong according to CLSI, and the diameter of the inhibition zone is between 20 mm and 11.5 mm [25-26]. From these data it can be stated that GAD leaf extract has more antibacterial potential against S. aureus and P. aeruginosa types of bacteria.

Table 4
The diameter of the inhibition zone produced by GAD leaf extract under different conditions was measured

Sample	Variation	Resistance Zone *E. colli* (mm)	Resistance Zone *B. subtilis* (mm)	CLSI Standard
Standard		21	24	Very Strong
GAD leaves	A	1	1	Weak
	B	2	2	Weak
	C	1	2	Weak
	D	1	1	Weak

4. Conclusion

The ultrasonic assisted extraction method on *Gymnanthemum amygdalinum* leaves was carried out with 4 variations of time and amplitude conditions, among others: condition A (30 minutes, 60%); condition B (30 minutes, 65%); condition C (45 minutes, 60%); and condition D (45 minutes, 65%). The IC_{50} value for the ability of *Gymnanthemum amygdalinum* leaf extract as an antioxidant is 94.83 ± 0.04 mg/L (A); 107.44 ± 0.03 mg/L (B); 145.06 ± 0.39 mg/L (C); and 153.46 ± 0.04 mg/L (D). *Gymnanthemum amygdalinum* leaves gave optimum results using the ultrasonic method at an extraction time of 30 minutes and an amplitude of 60% (condition A). When compared to other bacteria, such as S. aureus and P. aeruginosa, *Gymnanthemum amygdalinum* extract do not have high antibacterial activity against E. coli and B. subtilis bacteria, at least not when measured against CLSI guidelines. 1 mm (A), 2 mm (B), 1 mm (C), and 1 mm (D) is the average zone of inhibition of antibacterial activity of *Gymnanthemum amygdalinum* leaves against Escherichia coli and Bacillus subtilis, respectively.

5. Conflict of Interest

There are no conflicts to declare.

6. Acknowledgment

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