

## Polyphenol content and antibacterial activity of various extracts from apple leaves *Ziziphus mauritiana* in Vietnam

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**Abstract**— Apple leaves (*Ziziphus mauritiana* Lam) are a traditional plant, contain numerous bioactive ingredients. The paper focused on polyphenol content and antibacterial activity of acetone, hexane, and ethyl acetate extracts from apple leaf *Ziziphus mauritiana* Lam selecting in NinhThuan province, Vietnam on four-strain *Pseudomonas aeruginosa*, *Bacillus subtilis*, *Escherichia coli*, and *Staphylococcus aureus*. Antibacterial activity of different fractions was evaluated by using minimum inhibitory concentration. The results showed that apple leaves (*Ziziphus mauritiana* Lam) exhibited high antibacterial activity as the evaluation of antibacterial activity by using MIC method in liquid medium, and MIC of three fractions was in the range of 3.125 to 6.25 mg/ml. Polyphenol content of acetone fraction from apple leaves was  $68.96 \pm 0.53$  mg gallic acid equivalent/g DW. The purity of polyphenols in different fractions was arranged in ascending order, acetone, n-hexane, and ethyl acetate. Apple leaves *Ziziphus mauritiana* was potential in functional food and pharmaceuticals.

**Keywords:** apple leaves, antibacterial activity, MIC, polyphenol, *Ziziphus mauritiana*

### 1. Introduction

Medical plants are usually evaluated high in food, functional food, and pharmaceuticals because they contain numerous bioactive ingredients composed of polyphenol. Polyphenols are secondary metabolisms, possess numerous bioactive activities such as antioxidant activity, antibacterial, anti-virus, and improve resistance. They are valuable for treating diseases, controlling the number of microorganisms, inhibiting the growth of pathogenic bacteria, inhibiting food spoilage bacteria, and acting as a preservative for a safe level for human health.

Apple leaves *Ziziphus mauritiana* Lam are belong to the family *Rhamnaceae*, mainly distributed in Bangladesh, India, Sri Lanka, and Southern East-Asian. *Ziziphus mauritiana* is an abundant resource of carbohydrates, protein, vitamins, and fibers that help laxatives and are beneficial to human health. [1] *Ziziphus mauritiana* is also rich in minerals. Hence, some animals such as cattle, camels, goats use these leaves for their healthy improvement. [2] Outside, apple leaves are rich in vitamin C, triterpene, polysaccharides, and polyphenols. They are valuable factors in some diseases treatment such as liver, asthma, and fever. [3]

Some valuable bioactive ingredients of apple leaf *Ziziphus mauritiana* such as various phytoconstituents are studied and extracted by different solvents such as methanol, n-hexane, chloroform, and ethanol. Methanol fraction contained saponins, anthraquinones, [4] alkaloids, [5] tannins, [6] terpenoids, flavonoids, [7] polyphenols, [8] and phytoconstituents. [9] Studies on the active substances from family *Rhamnaceae* focus on pharmaceuticals, for example, antioxidant, antibacterial, anti-inflammatory, [10] and anti-tumor. [9] Extract from plant *Ziziphus* useful for the prevention of sausage because of their antibacterial. [11] According to Yalewet al., (2016) [12], methanol and chloroform fractions from the family *Rhamnaceae* are an antibacterial ability with a

broad antibacterial spectrum on disease-cause bacterial, for example, *Staphylococcus aureus*, *Streptococcus pyogenes*, *Streptococcus pneumoniae*, and *Salmonella typhi*. The mean values of the minimum inhibitory concentrations of the methanol and chloroform fractions ranged from 8.13 to 32.5 mg/ml and from 8.13 to 16.25 mg/ml, respectively. Therefore, apple leaves are potential in pharmaceutical. However, polyphenol content and antibacterial activity of various fractions such as n-hexane, acetone, and ethyl acetate from *Ziziphus mauritiana* still be less, especially apple leaves in Vietnam.

Therefore, the paper focused on polyphenol content and antibacterial activity of different fractions from apple leaves in Vietnam.

## **2. Materials and methods**

### **2.1. Materials**

Apple leaves (*Ziziphus mauritiana*) were alternate, ovate or elliptical, and size (length x width, 2.5 - 4.0 cm x 1.8 - 3.8 cm, respectively). Apple leaves were selected from trees grown 25 years olds in NinhPhuoc district, NinhThuan province, Vietnam, and the collection time was seven months after harvesting apples. Apple leaves must be fresh, not wilted or crushed. Four strains of microorganisms were used, including two gram-positive bacteria (*Staphylococcus aureus*, *Bacillus subtilis*) and two gram-negative bacteria (*Escherichia coli*, *Pseudomonas aeruginosa*) purchasing from the Institute of Food and Biotechnology, City University of Industry Ho Chi Minh Street.

### **2.2. Sample preparation**

After harvesting, apple leaves drying was at 40 °C until the moisture lower 10%, finally ground to a fine powder and stored in vacuum-sealed PE bags for further studies.

### **2.3. Extract preparation**

Apple leaves powder was vortex-assisted soaked in 80% acetone with the solvent-to-material ratio of 20/1 (v/w) for 120 minutes at 350 rpm and filtered through Whatman No. 1. The filtrate was vacuumed rotary evaporator at a temperature of 40 °C, and a concentrated extract was then fracted by n-hexane and ethyl acetate. Fractions of n-hexane and ethyl acetate were also vacuumed concentration at a temperature of 40 °C. All concentrated extracts were used for further studies.

### **2.4. Microorganism preparation**

#### *Medium preparation*

2.4 g of nutrient broth were accurately weighed and dissolved in 300 mL of distilled-autoclaved water. Then let them cool at room temperature.

#### *Cultivation of increase bacteria*

The original microbiological was kept at -18 to 0°C for 6 hours, incubated then at 0 to 4°C for 6 hours. The microorganisms were then moved into test tubes which contained 15 ml of nutrient broth and autoclaved for 15 minutes at 121°C. Finally, all samples were kept for 24 hours at 37°C for formatting microorganisms in a liquid medium.

#### *Preparation of strains and adjustment of microbial density*

Obtaining bacterial strains were from slanted agar by pouring nutrient broth medium into test tubes

and then using circular culture rods to scrape bacteria from the surface of the agar. Pour the solution from that test tube into the centrifuge tube to get the bacterial solution. Centrifuge at 3,000 rpm for 15 minutes to wash bacteria with sterile distilled water with triplication. The final bacterial culture fluid was vortexed for 1 min, and then the bacterial density was adjusted by photometric at the wavelength of 625 nm and set blank with distilled water. Measure absorbance from 0.08 – 0.1 ~ 0.5 McFarland ( $\sim 10^8$  CFU/ml). The bacterial suspension was adjusted from  $10^8$  CFU/ml to  $10^6$  CFU/ml by aspirating 200  $\mu$ l of the suspension into 19.8 ml saline for obtaining a density of  $10^6$  CFU/ml (4 tubes of 19.8 ml saline + 4 tubes of bacteria centrifuge).

#### *Microorganisms inoculation*

Inoculation on agar is similar to an aqueous medium but was added with 2% agar, and the inoculation medium was incubated at a suitable temperature to achieve the growth of microorganisms. After 24 hours, the grown microorganisms was kept at 4 – 8 °C for further studies. According to the method, the microorganisms has a survival time from 3 to 6 months longer than the liquid inoculation.

#### **2.5. Quantification of polyphenol content**

100  $\mu$ l was separately added into various fractions for vortexing, and then, in turn, poured 0.5 ml Folin reagent and 2.5 ml 10%  $\text{Na}_2\text{CO}_3$  in the mixture and kept for 30 minutes for measuring at the wavelength of 750 nm with the gallic acid standard. [13]

#### **2.6. Antibacterial activity evaluation by MIC method**

10g extracts of each were prepared separately in 8 ml of DMSO. All extracts were separately diluted at concentrations of 100 mg/ml, 50 mg/ml, 25 mg/ml, 12.5 mg/ml, 6.25 mg/ml, 3.125 mg/ml, 1.562 mg/ml, respectively and added into the volume tube of 10 ml which contained  $10^6$  CFU/ml. The mixture was incubated at 37 °C for 24 hours and measured the turbidity. Thereby comparing and evaluating the antibacterial ability of various fractions by MIC method.[14]

#### **2.7. Data analysis**

All experiments were triplication (n=3). Analysis of one factor ANOVA and statistical by software R and MS. Excel 2013. Data were expressed under the mean  $\pm$  standard deviation. Movement of unnormal value was by Duncan method.

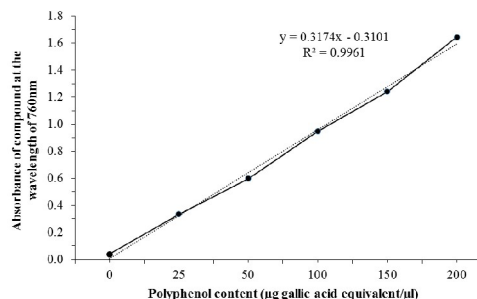
### **3. Results and discussion**

#### **3.1. Polyphenol content**

Polyphenol content of apple leaves was calculated based on the standard curve (Figure 1), corresponding to  $68.96 \pm 0.53$  mg gallic acid equivalent/g DW as extracted by acetone. Fraction of n-hexane and ethyl acetate possessed polyphenol content of  $76.09 \pm 1.31$  and  $87.22 \pm 0.43$  mg gallic acid equivalent/g DW, respectively (Table 1). The thing showed that the ratio of polyphenol content-to-drying weight in the n-hexane fraction was higher than one in the acetone fraction but lower than the ethyl acetate fraction. It meant that polyphenol purification in ethyl acetate fraction was the highest value, following fraction n-hexane and acetone. Polyphenol content ratio in other fractions was a significant difference ( $p < 0.05$ ). These results were suitable for the previous studies on the purification of polyphenol. [15] Polyphenol was focused in the current study because polyphenol was known as antioxidant, antibacterial, and the information on polyphenol of apple leaves is not yet much. [16]

**Table 1.** Polyphenol content of various fractions

Extract	Polyphenol content (mg gallic acid equivalent/g DW)
Acetone	68.96 ± 0.53
n-hexane	76.09 ± 1.31
Ethyl acetate	87.22 ± 0.43



**Figure 1.** Standard curve of polyphenol

**3.2. Antibacteria activity of acetone fraction**

Acetone fraction extracted from apple leaves had antibacterial activity. In terms of turbidity and bacterial number on the four bacterial strains, it exhibited that the minimum inhibitory concentration of the extract against *Escherichia coli* strain (3.125 mg extract/ml,  $7.1 \times 10^3$  CFU/ml), *Bacillus subtilis* strain (3.125 mg extract/ml,  $2.7 \times 10^5$  CFU/ml), *Staphylococcus aureus* strain (6.25 mg extract/ml,  $5.5 \times 10^5$  CFU/ml), and *Pseudomonas aeruginosa* strain (3.125 mg extract/ml,  $5.1 \times 10^3$  CFU/ml) (Table 2). MIC of acetone extract from *Pouzolzia mixta* on the strains of *Bacillus cereus*, *Bacillus pumilus*, *Bacillus subtilis*, *Staphylococcus aureus*, *Enterococcus fecalis*; *Enterobacter cloacae*, *Escherichia coli*, *Pantoea agglomerans*, *Pseudomonas aeruginosa*, *Shigella flexneri*, *Aeromonas hydrophila*, *Proteus mirabilis*, *Klebsiella pneumoniae*, *Salmonella cholerae-suis* and *Serratia marcescens* was more than 12 mg/ml. [17] Hence, the antibacterial activity of all extracts from apple leaves in Vietnam was higher than *Pouzolzia mixta*. Antibacterial activity of various fractions from apple leaves in Bangladesh [16] was lower than one in Vietnam, but in comparison to one in Pakistan, [18] one in Vietnam was lower.

**Table 2.** Antibacteria activity of acetone fraction on four microorganism trains

Extract concentration (mg/ml)	<i>Escherichia coli</i>		<i>Bacillus subtilis</i>		<i>Staphylococcus aureus</i>		<i>Pseudomonas aeruginosa</i>	
	Turbidity	Bacteria number	Turbidity	Bacteria number	Turbidity	Bacteria number	Turbidity	Bacteria number
100	T	$4.3 \times 10^2$	T	$5.2 \times 10^3$	T	$5.6 \times 10^3$	T	$1.2 \times 10^0$
50	T	$6.8 \times 10^2$	T	$5.9 \times 10^3$	T	$7.2 \times 10^3$	T	$4.2 \times 10^1$
25	T	$9.5 \times 10^2$	T	$7.4 \times 10^3$	T	$9.1 \times 10^3$	T	$35 \times 10^2$
12.5	T	$3.4 \times 10^3$	T	$8.3 \times 10^4$	T	$4.3 \times 10^4$	T	$5.4 \times 10^2$
6.25	T	$5.6 \times 10^3$	T	$8.5 \times 10^4$	T	$5.5 \times 10^5$	T	$2.5 \times 10^3$
3.125	T	$7.1 \times 10^3$	T	$2.7 \times 10^5$	N	$4.9 \times 10^5$	T	$5.1 \times 10^3$
1.5625	N	$2.5 \times 10^5$	N	$5.4 \times 10^5$	N	$7.3 \times 10^6$	N	$7.9 \times 10^4$
0	N	$5.1 \times 10^7$	N	$1.4 \times 10^8$	N	$3.5 \times 10^8$	N	$3.5 \times 10^7$
0	N	$7.4 \times 10^8$	N	$3.5 \times 10^8$	N	$7.3 \times 10^8$	N	$7.8 \times 10^8$

\*Note: T: Turbidity; N: Non-turbidity

**3.3. Antibacteria activity of n-hexane fraction**

The hexane fraction had antibacterial activity against four strains of microorganisms. In terms of turbidity and bacterial number on the four bacterial strains, it showed that the minimum inhibitory concentration of the extract against *Escherichia coli* strain (6.25 mg extract/ml,  $1.2 \times 10^3$  CFU/ml), *Bacillus subtilis* strain (3.175 mg extract/ml,  $4.1 \times 10^3$  CFU/ml), *Staphylococcus aureus* strain (6.25

mg extract/ml,  $6.6 \times 10^3$  CFU/ml), and *Pseudomonas aeruginosa* (3.175 mg extract/ml,  $2.1 \times 10^3$  CFU/ml). Luong et al., (2018) [19] noticed MIC of hibiscus leaves (*H. rosa-sinensis*) on three strains *Pseudomonas aeruginosa* (12.5 mg extract/ml), *Proteus mirabilis* (10 mg extract/ml), and *Klebsiella pneumoniae* (15 mg extract/ml), the thing showed that antibacterial activity of hexane extract from hibiscus leaves was lower than one of the apple leaves. n-hexane fraction was interested in the previous study [20-22] because of their antibacterial, antifungal and antioxidant activity.

**Table 3.** Antibacteria activity of n-hexane fraction on four microorganism trains

Extract concentration (mg/ml)	<i>Escherichia coli</i>		<i>Bacillus subtilis</i>		<i>Staphylococcus aureus</i>		<i>Pseudomonas aeruginosa</i>	
	Turbidity	Bacteria l number (CFU/ml)	Turbidity	Bacteria l number (CFU/ml)	Turbidity	Bacterial number (CFU/ml)	Turbidity	Bacteria l number (CFU/ml)
100	T	$3.9 \times 10^1$	T	$1.9 \times 10^2$	T	$4.3 \times 10^2$	T	$1.5 \times 10^0$
50	T	$2.5 \times 10^2$	T	$2.2 \times 10^3$	T	$1.2 \times 10^3$	T	$2.5 \times 10^0$
25	T	$5.6 \times 10^2$	T	$2.2 \times 10^3$	T	$5.7 \times 10^3$	T	$1.6 \times 10^2$
12.5	T	$9.3 \times 10^2$	T	$2.5 \times 10^3$	T	$7.5 \times 10^3$	T	$5.4 \times 10^2$
6.25	T	$1.2 \times 10^3$	T	$3.6 \times 10^3$	T	$6.6 \times 10^3$	T	$1.2 \times 10^3$
3.125	N	$2.1 \times 10^3$	T	$4.1 \times 10^3$	N	$3.1 \times 10^4$	T	$2.1 \times 10^3$
1.5625	N	$5.6 \times 10^4$	N	$1.4 \times 10^5$	N	$5.2 \times 10^5$	N	$5.6 \times 10^4$
0	N	$2.5 \times 10^6$	N	$3.2 \times 10^6$	N	$6.7 \times 10^6$	N	$2.5 \times 10^6$
0	N	$5.8 \times 10^8$	N	$7.5 \times 10^8$	N	$8.5 \times 10^8$	N	$5.8 \times 10^8$

\*Note: T: Turbidity; N: Non-turbidity

### 3.4. Antibacteria activity of ethyl acetate fraction

The ethyl acetate fraction possessed antibacterial activity against four strains of microorganisms. In terms of turbidity and bacterial number on the four bacterial strains, it showed that the minimum inhibitory concentration of the extract against *Escherichia coli* strain (3.125 mg extract/ml,  $4.1 \times 10^3$  CFU/ml), *Bacillus subtilis* strain (3.125 mg extract/ml,  $2.1 \times 10^5$  CFU/ml), *Staphylococcus aureus* strain (6.25 mg extract/ml,  $2.5 \times 10^5$  CFU/ml), and *Pseudomonas aeruginosa* strain (3.125 mg extract/ml,  $3.1 \times 10^3$  CFU/ml) (Table 4). In the study of Luong et al., (2018) on the antibacterial activity of *Hibiscus rosasinensis* L leaves, ethyl acetate fraction exhibited the highest activity against all three strains (*Proteus mirabilis*, *Pseudomonas aeruginosa*, and *Klebsiella pneumoniae*). MIC of ethyl acetate fraction in the study of Luong et al., (2018) [19] corresponded to 7.5 mg extract/ml for two strains of *Proteus mirabilis* and *Pseudomonas aeruginosa*, and 10 mg extract/ml for strain *Klebsiella pneumoniae*. The thing showed that the antibacterial activity of apple leaves was higher than hibiscus leaves. From the above results, all three extracts (acetone, hexane, and ethyl acetate) were antibacterial against four strains of *Escherichia coli*, *Bacillus subtilis*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa* with minimum inhibitory concentrations ranging from 3.175 mg/ml to 6.25 mg/ml. Comparison to the antibacterial activity of *Calotropis gigantea* leaves [23], apple leaves in Vietnam was somewhat higher. Polyphenol content and antibacterial activity of various fractions from apple leaves in Vietnam were presented in the current study but found in previous studies. [24,25]

**Table 4.** Antibacteria activity of ethyl acetate fraction on four microorganism trains

Extract	<i>Escherichia coli</i>	<i>Bacillus subtilis</i>	<i>Staphylococcus</i>	<i>Pseudomonas</i>
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concentration (mg/ml)	<i>aureus</i>				<i>aeruginosa</i>			
	Turbidity	Bacteria number (CFU/mL)	Turbidity	Bacteria number (CFU/mL)	Turbidity	Bacteria number (CFU/mL)	Turbidity	Bacteria number (CFU/mL)
100	T	$2.5 \times 10^2$	T	$3.3 \times 10^3$	T	$4.5 \times 10^3$	T	$1.2 \times 10^0$
50	T	$4.8 \times 10^2$	T	$5.8 \times 10^3$	T	$7.6 \times 10^3$	T	$4.6 \times 10^0$
25	T	$6.6 \times 10^2$	T	$8.6 \times 10^3$	T	$8.3 \times 10^3$	T	$8.5 \times 10^1$
12.5	T	$8.4 \times 10^2$	T	$4.3 \times 10^4$	T	$5.3 \times 10^4$	T	$5.4 \times 10^2$
6.25	T	$1.6 \times 10^3$	T	$7.5 \times 10^4$	T	$2.5 \times 10^5$	T	$1.6 \times 10^3$
3.125	T	$4.1 \times 10^3$	T	$2.1 \times 10^5$	Đ	$4.1 \times 10^5$	T	$3.1 \times 10^3$
1.5625	Đ	$1.4 \times 10^5$	Đ	$4.4 \times 10^5$	Đ	$5.5 \times 10^6$	Đ	$6.7 \times 10^4$
0	Đ	$4.2 \times 10^7$	Đ	$8.9 \times 10^7$	Đ	$2.9 \times 10^8$	Đ	$3.1 \times 10^7$
0	Đ	$5.5 \times 10^8$	Đ	$3.5 \times 10^8$	Đ	$5.5 \times 10^8$	Đ	$6.4 \times 10^8$

\*Note: T: Turbidity; N: Non-turbidity

### 3. Conclusion

Apple leaves (*Ziziphus mauritiana* Lam) contained polyphenol and possessed antibacterial activity on four microorganism strains (*Escherichia coli*, *Bacillus subtilis*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa*). All three fractions (acetone, n-hexane, and ethyl acetate) exhibited polyphenol content and antibacterial activity. The antibacterial activity of ethyl acetate fraction was the highest value on four microorganisms strains, compared to other extracts, lowest in acetone fraction. Minimum inhibitory concentrations of three extracts on four bacterial strains were from 3.125 mg/ml to 6.25 mg/ml. Apple leaves can be used as extracts to supplement functional foods and pharmaceuticals to create safe antibacterial products in the trend of nature, protecting health and the environment.

### 4. Acknowledgements

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### 5. Competing interests

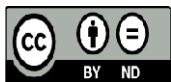
Authors have declared that no competing interests exist.

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