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Antimicrobial activity and chemical investigation of *Pongamia pinnata* L. Leaf

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Abstract
Pongamia pinnata (L.) Pierre grows in many places in Danang city, Vietnam. Local people normally boil fresh leaves of this species with hot water to obtain a solution used for showering to treat skin diseases effectively. Our study aimed to carry out the preliminary phytochemical screening of methanol extract and evaluate the antimicrobial activity of various fractions from *Pongamia pinnata* L. leaf, as well as to investigate the chemical compostion of the most effective fraction. Methanol extract and various fractions including *n*-hexan extract, chloroform extract, and ethyl acetate extract from leaves *of Pongamia pinnata* L. were prepared. The preliminary phytochemical screening of the extracts reveals the presence of alkaloids, flavonoids, organic acids, saponins and reducing sugar. Antibacterial activity of these extracts been studied by disc diffusion method against two bacterial strains, including *Staphylococcus aureus* ATCC 25923 and Methicillin-Resistant *Staphylococcus aureus* (MRSA). Among various extracts, the ethyl acetate extract exhibited the best antibacterial activity of. It can effectively inhibit the two strains of pathogenic bacteria, including *S. aureus* ATCC 25923 and MRSA with the sterile ring diameters of the test solution were 16.33 mm and 16.5mm, respectively. GC-MS analysis of ethyl acetate fraction led to the identification of nine compounds.

Keywords: *Pongamia pinnata*, chemical constituents, ethyl acetate extract, antibacterial activity, GC/MS

1. Introduction
Pongamia pinnata (L.) Pierre (Leguminosae) (vietnamese name: Đính, Đậu dầu, Dây lim) is a species that grows scatteredly in some places in Danang city, Vietnam, but mostly along the riverside of Cam Le river. For a long time, local people in this area have cooked leaves of *P. pinnata* to bath and treat skin diseases in children with infections very effectively. In India and some countries in Southeast Asia, *P. pinnata* had also been used as a medicine to treat respiratory diseases, colds, diarrhea, flatulence, gonorrhea, leprosy, kill worms, laxatives, help prevent worms. digestive, anti-inflammatory [6].
So far, there have been very few domestic studies on *P. pinnata*, especially on the antibacterial ability of this plant. In this stuty, we investigated the chemical components and antibacterial activities of the extracts from leaves of *P. pinnata* L.

2. Material and method
2.1. Plant material
Leaves of *Pongamia pinnata* (L.) Pierre were collected from mature trees in Cam Le river basin, Danang city (15°59'54.8"N 108°11'52.1"E) from April to August in 2020. The collected fresh leaves were washed and dried at a temperature of about 45-50 ^{o}C, then stored in P.E bags, vacuumed to conduct research on chemical composition and antibacterial activity.

2.2. Extraction
1800 grams (g) of powdered material (humidity of 9.54%) were extracted with methanol (6 Litres x 4 time). The total extract was concentrated *in vacuo* to obtain 220,05 g of crude extract. Then the extract was suspended in water (2 Litres) and successfully partionized with *n*-hexane (2 Litres x 4 times), chloroform (2 Litres x 4 times), ethyl acetate (2 Litres x 4 times). The organic solvent partionations were evaporated *in vacuo* to obtain respective residues.

2.3 Preliminary phytochemical screening
In the study, we carried out the preliminary phytochemical screening of the methanol extract and fractionations from *P. pinnata* leaves using simple qualitative method. The presence or absence of thirteen groups of metabolites were tested.

2.4. Gas Chromatography (GC)

The ethyl acetate residue was dissolved in DMSO 20%. The samples were analyzed using a GC-MS system (Agilent Gas chromatograph model 7890A equipped with MSD 5975C). Helium (1.2 mL/min, 11.05 psi) was used as a carrier gas. Injector and detector temperatures were 250°C and 230 °C, respectively. Column HP-5MS (30 m x 0.25 mm x 0.25 µm) (Agilent Technologies) was used. The column temperature was programmed from 70°C, hold for 1 minutes, after that gradually increase 15°C/min to 200°C and hold for 5 minutes. After that the column temperature continously increase 7°C/min to 310°C and hold for 10 minutes. Ionization energy was 70 ev with a scan range from 40 to 500 amu.

Individual compounds in the ethyl acetate fraction were identified by comparision of their mass spectra with those in GC/MS libraries (NIST 08, Wiley 09) and/or with those reported in the literatures.

2.6. Biological assay

Preliminary biological testing and previous studies showed that the ethyl acetate fraction possessed the best antibacterial activity. The high mass of ethyl acetate obtained was 7.32g high (moisture was 11.23%). Weigh accurately 5.0 g high fraction ethyl acetate. Add 10ml of diluent, shake well until completely dissolved in diluent to obtain test solution C5 with a high sample/dilution ratio (w/v) equivalent to 1/2. Dilute C5 with diluent to get solutions of C4 (1/4), C3 (1/8), C2 (1/16), C1 (1/32). After the survey process, choose solution C5 as the solution for the official test.

Staphylococci strains were provided by the Laboratory Department, Danang University of Medical Technology and Pharmacy, including: *Staphylacocus aureus* ATCC 25923 and Methicillin-Resistant *Staphylococcus aureus* – MRSA. Culture and proliferation of bacterial strains on sterile Mueller- Hinton agar, incubated at 37ºC for 18-24 hours. After incubation, add 5ml of sterile physiological saline to each tube, shake well to reap the biomass to obtain the stock suspension. Dilute the resulting inoculum with physiological saline and compare with the McFaland standard turbidity No. 2 to obtain a standard microbial suspension with a concentration of about 6 × 108 CFU/ml.

Disc diffusion method (perforated agar method) was used to investigate the antibacterial effect of herbal extracts. Based on the diffusion of the reagent into the medium, if the reagent has the effect of inhibiting the growth of bacteria, then around the agar hole with the reagent, bacteria will not grow.

The procedures of the test were carried out in following steps: The agar hole was made by using a sterile metal cylinder, 6 mm diameter chiseled onto the agar surface. Use a micro pipette to aspirate 100 µl of test hole. Concentration of strains added to the medium: 1.0% active inoculum suspension. After inserting the reagent into the hole in the plate covered with bacteria, incubate at 37 ºC. Read results after 18-24 hours for bacteria. The experiments were arranged in randomized design and repeated 3 times. The diameter of the sterile rings (D – d) were determined by the diameter of the outer ring minus the diameter of the hole (6mm). The antibacterial activity of medicinal herbs is shown by the diameter of the ring that inhibits the growth of bacteria. The larger the diameter, the stronger the reagent and vice versa. Assess the degree of bacterial inhibition according to the diameter of the sterile ring using the rating scale of Manuanza [4].

Gentamicin sulfate was selected as positive control. This is a broad-spectrum antibiotic of the Aminoglycoside group, sensitive to many gram (-) and gram (+) bacteria, especially Methicillin-resistant *Staphylacocus aureus*, and blue pus *Bacilli* [5].

3. Results and discussions

3.1. Preliminary phytochemical screening

Among thirteen metabolites, the phytochemical screening showed the presence of five metabolites including alkaloids, flavonoids, saponins, organic acids and reducing sugars in methanol extract of *P. pinnata* leaves. Reducing sugars were present in all fractions. Organic acids were absent in *n*-hexan extract. Flavonoids and saponins were present in ethyl acetate extract and water residues. Alkaloids were detected in water residue.

Table 1: The results of preliminary phytochemical screening of *P. pinnata*

No.	Metabolites	Methanol extract	*n*-Hexan extract	Chloroform extract	Ethyl acetate extract	Water residue
1	Alkaloids	+	-	-	-	+
2	Flavonoids	+	-	-	+	-
3	Saponins	+	-	-	+	+
4	Organic acids	+	-	+	+	+
5	Reducing sugars	+	+	+	+	+

3.2. Antimicrobial activity of *P. pinnata*: The antibiogram of ethyl acetate extract from *P. pinnata* against *S. aureus* and MRSA was shown in figure 1. The measured diameters of sterile ring were exhibited in table 2.

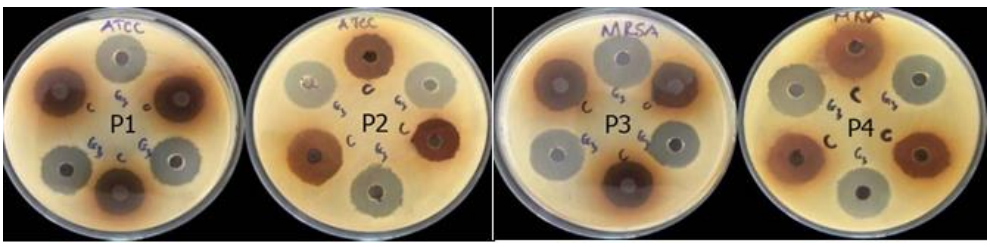

Fig 1: The antibiogram of ethyl acetat extract from *P. pinnata* against *S. aureus* and MRSA

Table 2: Evaluation of antimicrobial activity of ethyl acetat extract from *P. pinnata* against *S. aureus* and MRSA

***Staphylococci* strain**	**Plate**	**Diameter of sterile ring (mm)**	$\overline{X}$ **± SD (mm)**
S. aureus ATCC 25923	Plate 1	17	16.3 ± 1.1
		17	
		15	
	Plate 2	16	16.3 ± 0.5
		16	
		17	
Methicillin-Resistant Staphylococcus aureus – MRSA	Plate 3	18	17.0 ± 1.0
		16	
		17	
	Plate 4	16	16.0 ± 1.0
		17	
		15	

The results of the evaluation of antibacterial activity of the leaf extract of *P. pinnata* against two *Staphylococci* studied strains showed that ethyl acetate fraction at the concentration C5 (1/2 w/v) strongly inhibited the two strains. Solution C5 showed antibacterial activity equivalent to Gentamicin sulfate standard solution with concentration of 0.106 µg/ml with *S. aureus* ATCC 25923 strain and 0.089 µg/ml with Methicillin-Resistant *Staphylococcus aureus* (MRSA) strain.

The results of our study are quite similar to the previous studies. The *n*-hexan extract almost exhibited no activity against *Staphylococci* strains while the ethyl acetate extract showed quite strong anti-Staphylococcal activity. Specifically, the study of Bajpai KV *et al*. showed that the ethyl acetate extract exhibited strong antibacterial activity against *S. aureus* ATCC 6538 strain, with antibacterial diameter from 13 to 18 mm [9].

3. Gas chromatography analysis

Totally, nine compounds were identified in the GC chromatogram of ethyl acetat of *P. pinnata* (Table 3). Some fatty acids such as *n*-hexadecanoic acid (8.93%), octadecanoic acid (3.14%) and their derivates could be detected. Besides, Eugenol (1.24%) could be identified. This compound showed strong inhibitory effects against 15 spoilage bacteria [10].

Table 3: The results of GC chromatogram of ethyl acetat of *P. pinnata*

No.	**RT (min)**	**Compound**	**% Area**
1	6.550	Eugenol	1.24
2	10.246	Neophytadiene	3.25
3	10.458	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	0.85
4	11.040	Hexadecanoic acid, methyl ester	2.17
5	11.448	n-Hexadecanoic acid	8.93
6	11.803	Hexadecanoic acid, ethyl ester	1.85
7	13.831	Methyl stearate	0.82
8	14.567	Octadecanoic acid	3.14
9	15.208	Octadecanoic acid, ethyl ester	1.07

4. Conclusions

Phytochemical study of methanol extract of *Pongamia pinnata* L. leaves collected in Da Nang led to the presence of alkaloids, flavonoids, organic acids, saponins and reducing sugars. Besides, *n*-Hexane fraction contained only reducing sugars; chloroform fraction showed the presence of reducing sugars and organic acids while saponins, flavonoids, reducing sugars and organic acids were present in ethyl acetate extract and alkaloids, saponins and organic acids and reducing sugars could be detected in water residues.

The ethyl acetate extract of *Pongamia pinnata* L. leaves has strong antibacterial activity against *S. aureus* strain ATCC 25923 and Methicillin-Resistant *Staphylococcus aureus* – MRSA strain, with the corresponding sterile ring diameter of the test solution, respectively. 16.33mm and 16.5mm. GC analysis of ethyl acetate fraction of *P. pinnata* led to the identification of nine compounds.

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