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Antibacterial properties of alcohol and water extracts of *Waltheria indica* (L.) plants collected from Binh Thuan province, Vietnam

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Abstract

This study aimed to investigate the antibacterial ability of *Waltheria indica* species collected from two communes of Phan Thiet city, Binh Thuan province. Research methods included making ethanol extracts and decoction and investigating their ability to inhibit bacteria through agar well diffusion method and determining the minimum inhibitory concentration (MIC). The results showed that the ethanol extract was resistant to 5 tested strains of bacteria (*Bacillus cereus*, *Staphylococcus aureus*, *Enterococcus faecium*, *Pseudomonas aeruginosa*, *Escherichia coli*) with diameters of bacterial inhibition zones ranging from 6.76 to 14.50 mm, in which the resistance to Gram-positive strains was better than Gram-negative strains. The decoction inhibited only 3/5 of the tested bacterial strains (*S. aureus*, *E. faecium*, *E. coli*) with MICs from 125 to 750 mg/mL (dry weight of plant per volume of decoction). The antibacterial activities of extracts from the stems, leaves, and roots of the plants were different. Samples obtained from the 2 sites also showed different antibacterial capacities.

Keywords: Bacterial inhibition zone; Binh Thuan; Decoction; Ethanol extract; Minimum Inhibitory Concentration; *Waltheria indica*

1. Introduction

Medicinal plants were a potential source of cure for many diseases for a large number of populations in both developed and developing countries [1]. The survey showed that in Vietnam there were more than 4,000 species of plants and fungi that had been used in traditional medicine, of which many species were listed as rare in the world [2, 3]. Knowledge of medicinal plants in Vietnam was mainly based on the remedies of generations of traditional medicine doctors and ethnic minority communities [3]. Therefore, more modern research works were needed to evaluate the scientific basis for each of the medicinal properties of Vietnamese medicinal plants.

Waltheria indica (L.) of the family Sterculiaceae (synonym *Waltheria americana*, common name "morning sleepy") was a folk medicinal plant widely distributed in tropical regions of the world. There were many studies and reports on botany, phytochemistry, traditional medicinal uses, medicinal properties and toxicity of plants [1, 4]. Particularly in Vietnam, in addition to some basic information on morphology, distribution and medicinal uses in books on traditional

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medicinal herbs [5, 6], this plant was studied for its morpho-anatomical characteristics adapted to the drought conditions of Binh Thuan province [7].

In terms of traditional medicine, the *Waltheria indica* plant had a spicy, slightly sweet taste [6], and astringent property and was used to clear heat, detoxify, and cool the blood [1]. Antidiabetic, asthmatic, anemic, aphrodisiac, and anticancer activities were also listed [1]. Medical applications were based on the composition of caffeic acid, flavonoids, alkaloids, terpenoids, steroids, saponins and tannins that were identified in the whole plant extracts [1]. Antibacterial potential supported the use of this plant in the treatment of urinary tract infections and diarrhea [8, 9].

Following the study of adaptive anatomical morphology [presented in document 7], this research was conducted to investigate the resistance to five strains of *Bacillus cereus*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Escherichia coli*, *Enterococcus faecium* of alcohol and water extracts of *Waltheria indica* in comparison of samples collected at two different ecological sites of Binh Thuan province, Vietnam.

2. Material and methods

This study was carried out at the Biochemistry-Microbiology laboratory of Saigon University in October 2021.

2.1. Sample collection and preparation

Samples of *Waltheria indica* were collected in the wild in Ham Tien (1) and Tien Thanh communes (2), Phan Thiet city (latitude: 10°55'44.00" N; longitude: 108°06'7.49" E), Binh Thuan province (Figure 1). Method of collection and preservation according to the guidance of the Ministry of Health [10]. Samples were delivered to the laboratory the same day. In the laboratory, plant samples were separated into parts (leaves, stems and roots), washed and air-dried. The stem, leaf, and root samples were then individually wrapped in paper bags, weighed, and continued to be dried in an oven at 50 °C until completely dry. Dried samples were stored in zip bags with desiccant packs.

Figure 1 Sampling locations (star shapes) in Phan Thiet city, Binh Thuan province

2.2. Production of ethanol extract

Figure 2 Samples of stem, leaf, and root powder (left) soaked in alcohol (right)

The ethanol extraction method was performed as described by Nguyen Kim Phi Phung [11]. The dried samples were ground to a fine powder. Plant powder was soaked in ethanol solution in a ratio of 1:10. After 48 hours of soaking, the extract was filtered and the residue was removed. The remaining residue was continued to be soaked in 96° ethanol solution with the same volume as above. Extraction process was repeated 3 times to ensure thoroughness. The extract was solvent removed by being placed in a rotary evaporator at 50 °C and 175 mbar pressure (Rotary Evaporator RE301, Yamato Scientific). After solvent was almost completely evaporated (the remaining solution was about 1/60 of the original volume), the extract was poured into a beaker and allowed to evaporate the alcohol naturally until it thickened. This solid was considered to be the crude extract and was stored in a dark vial at 4 °C.

2.3. Production of decoction

The decoction method was performed as described by Ho Huynh Thuy Duong [12]. The dried sample was boiled in distilled water (1:30 w/w) at 70 °C for 90 minutes using an Automatic Herbal Medicine Decoction Thermo Pot. At the end of the first boil, the decoction was collected and then distilled water was added to the pot (2/3 of the volume of the first time). The decoction of the two times of boil was mixed and then incubated in the incubator at 60 °C to naturally thicken (the remaining solution was about 1/30 of the total volume of used water). The decoction was then centrifuged to remove the residue, and then was evaporated spontaneously until a concentration equivalent to 1 g dry matter per 1 mL was obtained.

Figure 3 Collection of decoction from stem samples by using an Automatic Herbal Medicine Decoction Thermo Pot

2.4. Determination of the antibacterial ability of plant extract

Five strains of test bacteria (*Bacillus cereus*, *Staphylococcus aureus*, *Enterococcus faecium*, *Pseudomonas aeruginosa*, *Escherichia coli*) were provided by the Institute of Biotechnology Research and Development, Can Tho University.

2.4.1. Determination of antibacterial activity of ethanol extract by agar well diffusion method

Antimicrobial assay was performed by agar well diffusion method [13]. Five strains of test bacteria were cultured in LB liquid medium within 48 hours. Bacterial suspension of each strain was added to the flask containing warm LB agar (50 °C) to give an adjusted concentration of 5x10^6 CFU/mL and then poured into Petri dishes.

After the agar solidified, wells were made by drilling with a round sterile steel tube (Φ = 6 mm). Each 20 µl of the test compound (alcohol extracts at concentrations of 200 - 1000 mg/ml; negative and positive controls) was injected into each well. The crude extract mentioned above was re-dissolved in 70% v/v ethanol to give a stock solution concentration of 1,000 mg/mL. This stock solution was further diluted in 70% v/v ethanol to give a range of concentrations of 800 mg/mL, 600 mg/mL, 400 mg/mL and 200 mg/mL. The negative control was a 70% v/v ethanol solution corresponding to an extract content of 0 mg/mL. Positive controls were gentamicin (0.5 mg/mL) (Gentamicin Sulfate - Gentamicin 80 mg/2mL - DOPHARMA, Vietnam) and tetracycline (0.5 mg/mL) (Tetracycline 500 mg – Mekophar, Vietnam). Plates were incubated at 37 °C for 48 hours. The diameter of the zone of inhibition was measured as described by Hudzicki [14]

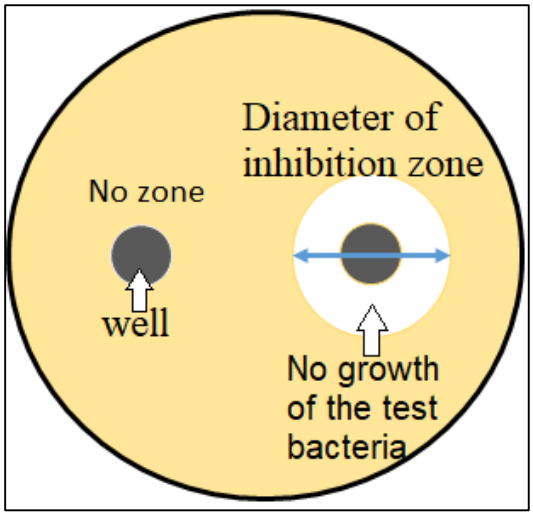


Figure 4 Describe the determination of bacterial inhibition zones in experimental plates [15]

2.4.2. Determination of antibacterial activity of decoction based on Minimum Inhibition Concentration (MIC)

The decoction was filtered through a bacterial filter to obtain a sterile decoction. The antibacterial activity of the decoction was assessed based on the Minimum Inhibition Concentration (MIC) by the Broth Dilution test as described by Huang [15] with some modifications. The decoction (1000 mg/mL) was diluted sequentially in test tubes containing liquid LB medium to produce a range of decoction concentrations ranging from 0 to 100%, with each scale being 12.5% v/v apart (Figure 5). The bacterial suspension was added to the tubes so that the bacterial population reached 5x10^6 CFU/mL. These tubes were incubated at 30° C for 24 hours and determined the lowest concentration of the decoction that did not increase the bacterial population (no significant increase in turbidity), that was, the MIC.

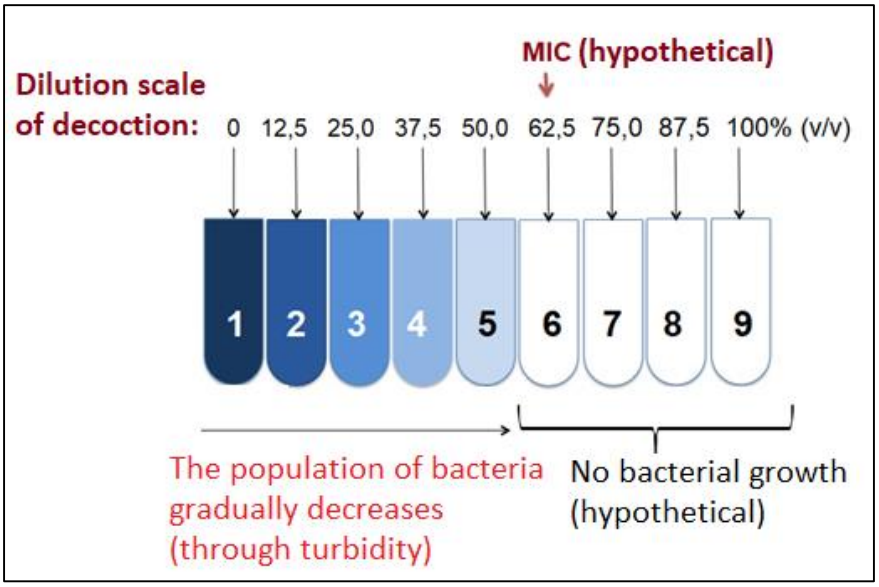


Figure 5 Summary of test setup diagram for MIC

2.5. Processing statistics

Each statistical experiment was repeated 3 times. Statistical methods include one-factor ANOVA and LSD test at α = 0.05 using IBM SPSS Statistics 20.0.

3. Results

3.1. Antibacterial activity of ethanol extract through agar well diffusion method

The average ethanol extraction yield was 52.85%, the average density of the extract was 15.1 g/mL. Observed under an optical microscope at 400x magnification, the leaf powder contained trichomes, mesophyll cells, epidermis and stomata, while the stem meal and root powder mainly contained small fragments of vascular elements (Figure 6).

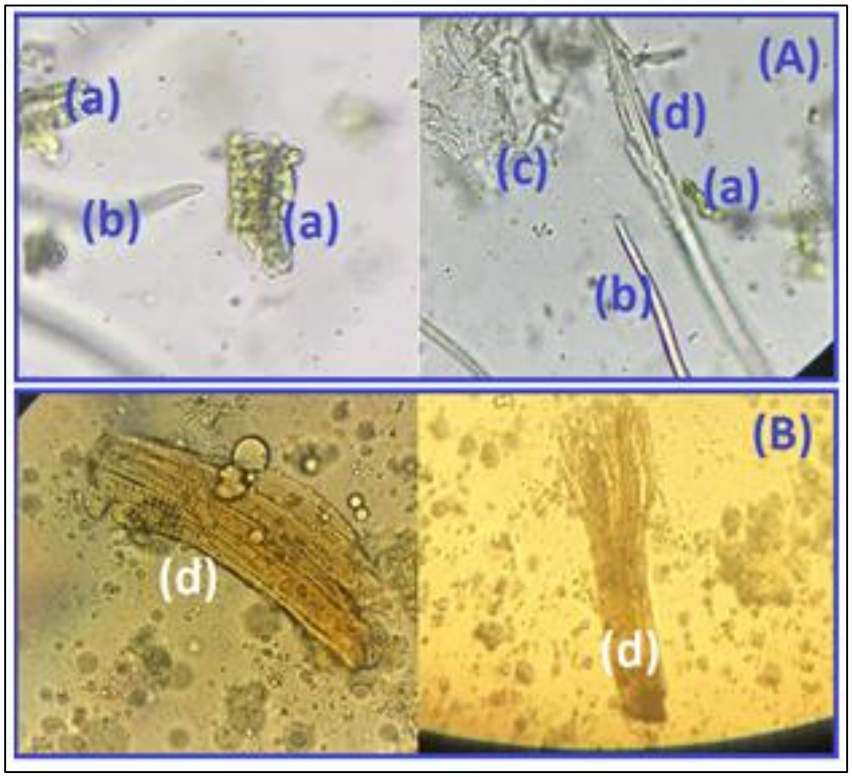


Figure 6 Leave (A) and stem powder (B) observed under a microscope. (a): Mesophyll cells; (b): Trichomes, (c): Epidermis and stomata, (d): Vascular elements

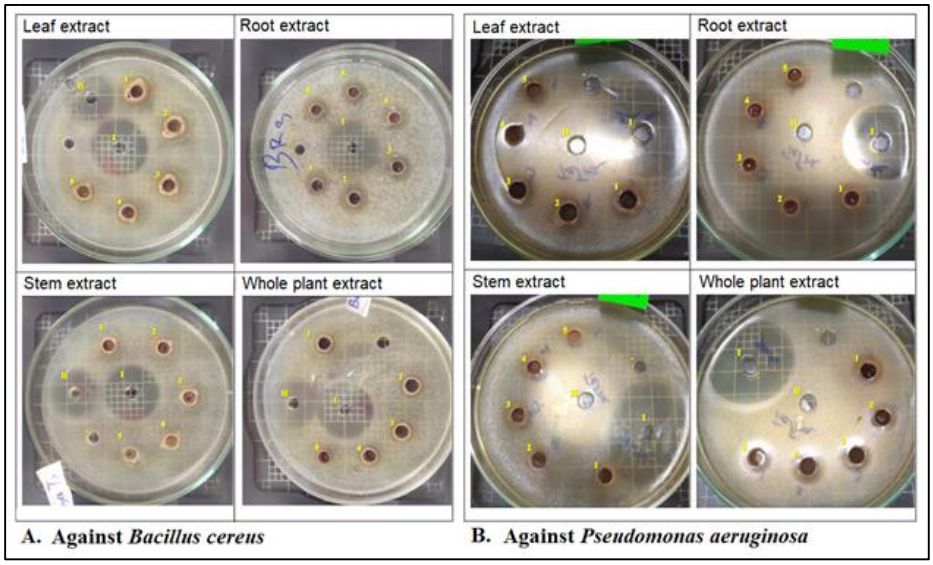
The antibacterial ability of the ethanol extract from *Waltheria indica* was shown in Table 1. Thereby showing that the extract had better inhibitory effect on 3 strains of Gram-positive bacteria than 2 strains of Gram-negative bacteria tested. The leaf extract obtained from Ham Tien commune at a concentration of 400 mg/mL had the ability to inhibit *B. cereus* with an average halo diameter of 12.72 mm; while to achieve that size, the leaf extract obtained in Tien Thanh commune had to be at a concentration of 800 mg/mL (not shown in Table 1) and the highest measured halo diameter was 13.24 mm at the concentration of 1000 mg/mL. Similarly, leaf extract obtained from Ham Tien and Tien Thanh communes also had a good effect on *S. aureus* with the largest diameter of the halo ring measured from 13.26 mm (at a concentration of 600 mg/m) to 14.00 mm (at a concentration of 1000 mg/mL), respectively. For the three strains of Gram-positive bacteria tested, in general, the leaf and whole plant extracts had good effects on *B. cereus*. The leaf and stem extracts had good effects on *S. aureus*, while the stem and root extracts had good effects on *E. faecalis* (Table 1).

For the sampling site, in general, *Waltheria indica* samples collected in Ham Tien showed better resistance to bacteria than those collected from Tien Thanh. In particular, the resistance to Gram negative bacteria *P. aeruginosa* and *E. coli* of the plants collected in Ham Tien, although not high, was also shown while even the antibiotic Tetracycline had no effect (Figure 7B).

Table 1 Antibacterial activity of ethanol extract of *Waltheria indica*

Test samples		Maximum diameter (mm) of bacterial inhibition zone achieved for tested baterial strains				
		B. cereus	***S. aureus***	***E. faecalis***	***P. aeruginosa***	***E. coli***
Leaf extract	(1)	12.72 (E4)	13.26 (E3)	12.50 (E1)	9.26 (E3)	7.76 (E3)
	(2)	13.24 (E1)	14.00 (E1)	12.26 (E2)	9.50 (E3)	-
Stem extract	(1)	12.10 (E4)	14.50 (E3)	13.26 (E1)	9.76 (E3)	7.00 (E3)
	(2)	12.76 (E1)	12.26 (E1)	12.36 (E2)	9.00 (E2)	-
Root extract	(1)	9.76 (E3)	11.76 (E3)	12.76 (E1)	10.50 (E5)	6.50 (E3)
	(2)	10.00 (E3)	11.30 (E1)	13.50 (E1)	11.26 (E2)	-
Plant extract*	(1)	12.50 (E3)	11.50 (E1)	10.66 (E5)	9.50 (E2)	6.76 (E3)
	(2)	10.76 (E4)	9.50 (E2)	10.00 (E5)	10.00 (E1)	-
Ethanol		-	-	-	-	-
Gentamicin (I)		25.88	27.06	25.00	28.50	24.88
Tetracycline (II)		22.66	25.26	23.50	-	-

The data presented in Table 1 were the average results of 3 replicates and had undergone statistical testing. Only the highest results obtained were presented in the Table, with the symbol in parentheses indicating the respective extract concentration. (E5): 200 mg/mL; (E4): 400 mg/mL; (E3): 600 mg/mL; (E2): 800 mg/mL; (E1): 1000 mg/mL; (-): No inhibition zone. (*): The extract was obtained from the whole plant. (1): Samples were collected in Ham Tien; (2): Samples were collected in Tien Thanh. The concentration of antibiotic used was 1,000 mg/mL.



(1) 1,000 mg/mL; (2) 800 mg/mL; (3) 600mg/mL; (4) 400 mg/mL; (5) 200 mg/mL; (-) ethanol; (I) Gentamicin; (II) Tetracycline.

Figure 7 Bacterial inhibition zone of *W. indica* against *B. cereus* (A) and *P. aeruginosa* (B)

3.2. Antibacterial activity of decoction through MIC method

Concentrated decoction when diluted gives a range of concentrations of 125, 250, 375, 500, 625, 750, 875, and 1000 mg/mL (based on dry matter of stem, leaf and root parts, respectively). Through the test results on 5 strains of bacteria, the decoction showed no effect on *B. cereus* and *P. aeruginosa* and weak effect on *E. faecalis* (Table 2). Leaf and root decoction had a good effect on *S. aureus* with the MIC of 125 mg/mL. Root and whole plant decoction had good effects on *E. coli* with MICs of 250 mg/mL and 250 - 500 mg/mL, respectively. Particularly, the decoction of the stem and the whole plant were effective on *E. faecalis* with a fairly high concentration, the MICs were 625 mg/mL and 500 - 750 mg/mL, respectively.

Table 2 MIC results of decoction of *Waltheria indica*

Test samples		**MIC (mg/mL) for tested baterial strains**		
		S. aureus	***E. faecalis***	***E. coli***
Leaf extract	(1)	125	-	-
	(2)	250	-	-
Stem extract	(1)	250	625	-
	(2)	250	625	-
Root extract	(1)	125	750	250
	(2)	125	750	250
Whole plant extract	(1)	-	500	250
	(2)	-	750	500

4. Discussion

Ethanol extract of *Waltheria indica* had the ability to inhibit *S. aureus*, *E. faecalis*, *E. co*li [8, 16, 17], in which the leaf extract was the most active. The aqueous extract was resistant to *S. aureus*, *E. faecalis*, *E. coli* [8, 18, 19]. The aqueous extract from the roots had the best inhibitory effect on *P. aeruginosa* [18]. In particular, the aqueous extract was resistant to *E. coli* with a ring diameter of up to 31 mm [19]. The inhibitory potential of *Waltheria indica* against *B. cereus* had also been reported. For resistance to *S. aureus* as assessed by MIC, the ethanolic and aqueous extracts of *Waltheria indica* roots were recorded at 0.20 and 2.34 mg/mL, respectively [20]. Through the above works, it could be seen that the antibacterial properties of *Waltheria indica* extracts collected from different regions were different. In this study, the extracts obtained from plants grown in Ham Tien were more resistant than those grown in Tien Thanh; although previous research[7] had shown that plants grown in Ham Tien were smaller and sparser because of their response to low-nutrient and acidic soils. Which mechanism involved was still not well understood. The leaves were more active against Gram-negative bacteria such as *E. coli*, *P. aeruginos*a and *S. typhi* than the stem possibly due to the presence of cardiac glycosides. The higher antibacterial activity of *Waltheria indica* leaves compared with other parts was consistent with formulations using this plant extract in skin care.

5. Conclusion

The ethanol extract from *Waltheria indica* showed better resistance against 3 strains of Gram-positive bacteria (*B. cereus*, *S. aureus* and *E. faecalis*) than 2 strains of Gram-negative bacteria (*P. aeruginosa* and *E. coli*) through agar well diffusion method. The decoction from *Waltheria indica* had the ability to inhibit only 3 strains of bacteria (*S. aureus*, *E. faecalis* and *E. coli*) through MIC measurement method, in which the root extract was the best. The inhibitory ability of two types of *Waltheria indica* samples collected from Ham Tien and Tien Thanh sites was different.

Compliance with ethical standards

Acknowledgments

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Disclosure of conflict of interest

All authors declare no conflict of interest in relation to this article.

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