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Antibacterial activity of ethanol extract and decoction from *Avicennia alba* Blume growing in the Can Gio Mangrove Biosphere Reserve, Vietnam

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Abstract

This study was conducted to find out the antibacterial ability of a folk medicinal plant in Can Gio Mangrove Biosphere Reserve, *Avicennia alba* Blume. Leaf powder of *A. alba* derived from 3 different locations in the Biosphere Reserve was used to make ethanol extracts and decoctions. Methods to evaluate antibacterial ability included agar well diffusion, and Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC). The results showed that the ethanol extract from Dan Xay site was resistant to 6 tested bacterial strains including *Bacillus cereus*, *Bacillus subtilis*, *Staphylococcus aureus*, *Enterococcus faecium*, *Pseudomonas aeruginosa*, *Escherichia coli*. Most of the decoction samples had bacteriostatic and bactericidal properties with MBC/MIC rates ranging from 1.43 to 10.0.

Keywords: *Avicennia alba* Blume; Can Gio Mangrove Biosphere Reserve; Decoction; Ethanol extract; Minimum Inhibitory Concentration; Minimum Bactericidal Concentration

1. Introduction

Can Gio Mangrove Biosphere Reserve (Can Gio MBR) was the first World Biosphere Reserve of Vietnam recognized by UNESCO on January 21, 2000. Among the 37 main mangrove species of Can Gio MBR, *Avicennia alba* (Blume, 1826) (family Acanthaceae) was an important species that played a pioneering role and stabilized new mudflats [1, 2]. *A. alba* was widely distributed in estuaries and coastal regions from Western India to Indochina, the Malay Archipelago, the Philippines, New Guinea and even Northern Australia [3, 4]. In Vietnam, the tree was commonly found in mangrove forests in the South. The tree was a shrub to woody, up to 20 m tall, much branched. Bark was smooth or slightly rough, gray-brown, with irregular pale gray spots. The pencil-like aerial roots known as the pneumatophores grew up to 30 cm tall. Leaves were simple and opposite. The leaf blade was lanceolate, 2.5 - 3 cm wide and 7 - 8 cm long, green and glossy on the upperside and silvery white on the underside due to the covering of tiny hairs (origin of the Latin "*alba*" and of the word "trắng" in Vietnamese name "Mắm trắng"). The inflorescence had about 10 - 30 orange-yellow flowers with a diameter of each flower only about 0.5 cm. The flower had small bract, 5 sepals that did not fall, corolla with 4 equal lobes, 4 stamens, and an ovary with 4 cells and 4 ovules. Cone-shaped fruit was about 3.5 - 4 cm long with a distinctive pointed tip that curved to the side. The fruit has a rind with velvety hairs and a seed. Seedlings germinated through the fruit to take root as soon as there was a period of not being waterlogged and developed strong roots to resist sediment erosion and successfully cling to the soil [2, 3, 5].

In addition to the general use of a main species of mangrove tree, *Avicennia alba* was also a medicinal plant in the folk medicine documents of many countries such as India, Myanmar, Indonesia. Many uses in folk medicine in countries such

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as aphrodisiac, asthma, rheumatism, infertility, polio, snakebite, and cancer also needed to be scientifically verified [6]. New naphthoquinones, a flavone and a triterpene were isolated from *A. alba* [7-9]. Studies on the pharmacological activities of *A. Alba* extract including euphoria, analgesic, antipyretic, against ethanol-induced gastric mucosal injury, antidiarrheal and antibacterial activities had also been investigated [4]. The antidiabetic, anti-inflammatory, analgesic and antidiarrheal activities of the methanolic extract of *A. alba* leaves were tested in an albino mouse model [6]. In Vietnam, the plant was said to be used to treat scabies, leprosy and dysentery associated with bacteria [10]. Silver nanoparticles of the leaves of *A. alba* were synthesized and showed a significantly better inhibitory region against the tested bacteria (*Arthrobacter protophormiae* and *Proteus mirabilis*) than the positive control, streptomycin [11]. A triterpene, named as *Albain 1*, extracted from the wood of *A. alba* had also shown remarkable antibacterial activity against *Bacillus cereus*, *B. polymyxa*, and *B. pumilus* [9].

On the basis of the assumption that the pharmacological properties of a plant species could vary depending on the locality (geographical variety, chemical variety) [12], this study was conducted to investigate the antibacterial activity of the extracts from *A. alba* leaves collected in different buffer zones of Can Gio MBR with different salinities of the intertidal zone. The results obtained would contribute to enriching the database of medicinal plants in Vietnam as well as the mangrove biosphere.

2. Material and methods

2.1. Samples collection and preparation

Samples of *A. alba* leaves were collected in the wild and preserved according to the instructions of the Ministry of Health [13]. There were 3 sampling points, respectively, An Nghia, Dan Xay and Long Hoa belonging to buffer zones 5a, 10c and 17 of Can Gio Mangrove Biosphere Reserve (coordinates were 10°22'14" – 10°37'39" North latitude and 106°46'12" – 107°00'59" East longitude), Can Gio District, Ho Chi Minh City (Figure 1). In the laboratory, leaf samples were cleaned and air-dried, then individually wrapped in paper bags and dried in an oven at 50 °C. Dried samples were stored in zip lock plastic bags with desiccant. [14].



Figure 1 Map of Can Gio MBR [1] and three sampling sites (star shapes)

2.2. Production of ethanol extract and decoction of *A. alba*

2.2.1. Production of ethanol extract

The dried leaf samples after grinding to a fine powder (300 g) were immersed in a 96° ethanol solution (1 L) for 48 hours. After soaking, the extract was collected and the residue was further immersed in the ethanol solution according to the above procedure, repeated 3 times to obtain a more thorough extract. The extract was solvent removed by placing it in a Yamato Scientific RE801 rotary evaporator, at 90 rounds per minute, 130 Pa pressure and 50 °C until about 1/10

of the original volume remained. This extract was centrifuged to remove residue and allowed to evaporate naturally until 50 mL remained. The concentrated crude extract was stored in a dark vial at 4 °C for future study [15]. Thus, this extract had a concentration equivalent to 6 g of leaf powder per mL of solution (6000 mg/mL).

2.2.2. Production of decoction

The leaf powder (100 g) was boiled in distilled water (1 L) at 70 °C for 90 minutes using an Automatic Herbal Medicine Decoction Thermo Pot [14]. The decoction was collected and then incubated in an incubator at 50 °C to evaporate the water until the solution was about 200 mL. The decoction was further centrifuged to remove residue and left to evaporate naturally until 100 mL remained [16]. Thus, this decoction (known as the stock of extract) had a concentration equivalent to 1000 mg of leaf powder per mL of solution (1000 mg/mL).

2.3. Determination of antibacterial activity of ethanol extract of *A. alba*

Antimicrobial assay was performed by agar well diffusion method. Six experimental strains of bacteria (*Bacillus cereus*, *Bacillus subtilis*, *Staphylococcus aureus*, *Enterococcus faecium*, *Pseudomonas aeruginosa*, *Escherichia coli*) were cultured in LB liquid medium for 48 hours. The bacterial suspension of each strain was added to a flask containing a warm LB agar (about 50 °C) such that the adjusted bacterial density was 5×10^6 CFU/mL (according to McFarland standards). Bacterial agar was poured into Petri dishes and holes (wells) ($\Phi = 5.5$ mm) were drilled after agar solidification [17].

The stock of extract of *A. alba* mentioned in section 2.2.1 (1 g) was dissolved in 70° alcohol to form a concentration range of 20, 40, 60, 80 and 100 mg/mL. Each 20 μ L of test solution (alcohol extract at test concentrations; negative and positive controls) was injected into each well. The negative control was a 70° alcohol 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). The Petri dishes were incubated at 5 °C for 3 hours so that the well contents diffused into the agar, then incubated at 28 °C for 48 hours to observe bacterial growth and measure the diameter of the inhibition zone [14, 17].

2.4. Determination of antibacterial activity of decoction of *A. alba*

The antibacterial activity of the decoction was evaluated based on the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC).

Resazurin reagent solution 0.1% was prepared in sterile DPBS solution [18]. The decoction was diluted with liquid LB in sterile eppendorf tubes to give a sequential concentration range from 0 mg/mL to 1000 mg/mL, spaced at 100 mg/mL intervals. The suspension of each test strain (six strains: *Bacillus cereus*, *Bacillus subtilis*, *Staphylococcus aureus*, *Enterococcus faecalis*, *Pseudomonas aeruginosa*, *Escherichia coli*) was added to each eppendorf containing the decoction mentioned above to obtain an adjusted bacterial density of 5×10^6 CFU/mL (according to McFarland standards).

The 96-well templates were used in the MIC experimental setup by injecting each well with 100 μ L of a mixture of decoction and bacterial suspension. Templates were incubated at 38 °C for 24 hours and then added 20 μ L of 0.1% resazurin reagent solution to each well. After 30 minutes, the color change of the reagent was observed for MIC determination [19]. The Minimum Bactericidal Concentration (MBC) was determined by the spread method, where every 10 μ L of solution in each well without discoloration was spread on a nutrient agar plate (3 g extract yeast, 5 g peptone, and 15 g agar per liter) and incubated at 38 °C for 24 hours. The MBC value was the lowest concentration in the concentration range of the decoction that could kill all bacteria, i.e. no bacteria grew on the agar plate [19].

2.5. Processing statistics

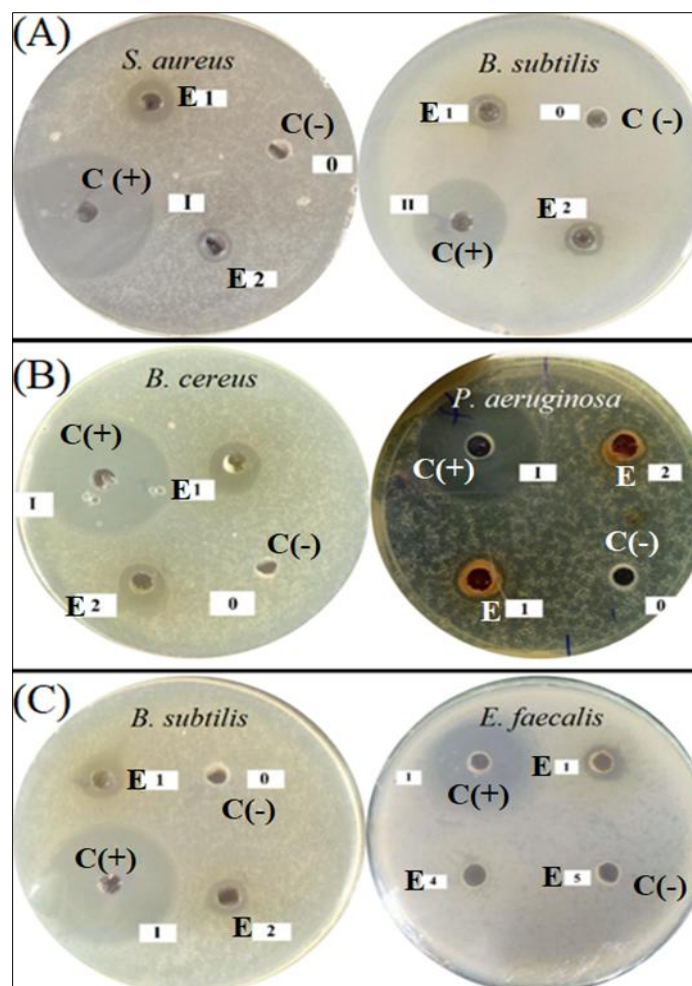
The experiment was arranged in completely randomized design, repeated 3 times. All quantitative data were analyzed for One-way ANOVA (Analysis of Variance) and Least Significant Difference (LSD) with $\alpha=0.05$ by using IBM SPSS Statistics 20.0.

3. Results and discussion

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

For the ethanol extraction from *A. alba* leaf powder, the average extraction yield was 23.81% and the density of the extract was 1.43 g/mL. Under the optical microscope at 400x magnification, the obtained extract had almost no solid components. The antibacterial ability of the ethanol extract from *A. alba* presented in Table 1 showed that the leaf

extract obtained from plants growing in An Nghia and Long Hoa had an inhibitory effect on 3 strains of Gram-positive bacteria including *Bacillus subtilis*, *Staphylococcus aureus*, and *Enterococcus faecalis*, but the extracts obtained from plants growing in Long Hoa also inhibited Gram-negative bacteria *Pseudomonas aeruginosa*. Meanwhile, the leaf extracts from plants collected in Dan Xay was effective on all 6 strains of tested including *Escherichia coli*.



(E1): 100 mg/mL; (E2): 80 mg/mL; (E3): 60 mg/mL; (E4): 40 mg/mL; (E5): 20 mg/mL. (0): negative control (C-) ethanol 70°; (C+) positive control: (I)-Gentamicin and (II)-Tetracycline. Samples were collected in An Nghia (A), Dan Xay (B), and Long Hoa (C).

Figure 2 Some results on the halo zones of ethanol extracts of *A. alba*

Table 1 Antibacterial activity of leaf extract of *A. alba*

Test samples		Maximum diameter (mm) of bacterial inhibition zone achieved for tested bacteria strains					
		<i>B. cereus</i>	<i>B. subtilis</i>	<i>S. aureus</i>	<i>E. faecalis</i>	<i>P. aeruginosa</i>	<i>E. coli</i>
Leaf extract from	An Nghia	-	18.7 (E1)	21.0 (E1)	3.7 (E1)	-	-
	Dan Xay	18.7 (E1)	25.3 (E1)	23.0 (E2)	15.3 (E1)	4.7 (E5)	6.3 (E2)
	Long Hoa	-	18.7 (E2)	19.7 (E1)	9.3 (E1)	8.7 (E1)	-
Ethanol 70°		-	-	-	-	-	-
Gentamicin		48.5	59.8	61.3	36.8	35.8	34.1
Tetracycline		38.0	54.6	52.0	25.1	41.1	40.9

Only the highest results obtained were presented in the Table, with the symbol in parentheses indicating the respective extract concentration. (E5): 20 mg/mL; (E2): 80 mg/mL; (E1): 100 mg/mL; (-): No inhibition zone.

Most of the halo rings with the largest diameter were obtained when tested with extracts at a concentration of 100 mg/mL. However, the ability to inhibit *B. subtilis* of *A. alba* leaf extract collected from Long Hoa had the best inhibitory effect on bacteria even at the concentration of 80 mg/mL (E2). The obtained diameter of halo ring was 18.7 mm with a non-statistically significant difference when compared with the results obtained at a concentration of 100 mg/mL (E1) of 20.7 mm (not shown in Table 1). The leaf extract obtained from *A. alba* plants growing in Dan Xay had better effect on four strains of Gram-positive bacteria than on the two strains of Gram-negative, with the largest halo diameter measured from 4.7 mm to 25.3 mm. In particular, the difference in values obtained at extract concentrations tested on *Pseudomonas aeruginosa* strains between 20 and 100 mg/mL (E1 to E5) was not statistically significant. In Table 1, 4.7 mm was the value obtained at the test concentration of 20 mg/mL (E5). Similarly, for *E. coli*, the halo ring diameter obtained at a test concentration of 80 mg/mL (E2) of 6.3 mm was not significantly different from the 7.0 mm result of a test concentration of 100 mg/mL (E1) (not shown in Table 1).

The antibacterial activity of the ethanol extract from the leaves of *A. alba* collected in India [20] was only effective against *B. subtilis* (diameter of halo zone ranged from 6 to 9.9 mm) but not against *E. coli*. The wood extract showed better results with halo zones diameters ranged from 10 to 14.9 mm, also on the test bacteria *B. subtilis* [20]. Extractions with different solvents also showed different effects on the tested bacterial strains [20, 21]. Nagababu and Umamaheswara [21] found that the resistance of *A. alba* leaf extract to different solvents (excluding ethanol) had a better effect on Gram-negative than Gram-positive bacteria, especially against *P. aeruginosa* but this contradicts the results reported by some other authors [20, 22, 23]. Together with the results obtained in the present study, the above summary demonstrated the complexity of the antimicrobial mechanism of extracts from *A. alba* associated with phytochemical variants, extraction solvents and collected plant organs [12, 21].

3.2. Antibacterial activity of decoction through MIC and MBC

The antibacterial activity of decoction of *A. alba* collected from 3 different sites was presented in Table 2. Thereby, the MIC of the decoction was effective on tested bacterial strains ranging from 200 - 1000 mg/mL. The MICs for tested bacterial strains of the decoction samples from *A. alba* of 3 sampling sites including An Nghia, Dan Xay, and Long Hoa were 300 - 900, 100 - 400, and 600 - 1000 mg/mL, respectively. The results showed that *A. alba* decoction collected in Dan Xay had better antibacterial effect than An Nghia and Long Hoa, similar to the halo zones results presented in Section 3.1. An interesting thing was that the salinity of the waters of these 3 sites increases gradually in the direction of the sea (10, 15, and 20‰), in which Dan Xay had higher salinity than An Nghia and lower than in Long Hoa. However, the influence of ecological conditions including salinity on the biological properties of the plant, which in turn affected the medicinal properties, also needed to be further investigated.

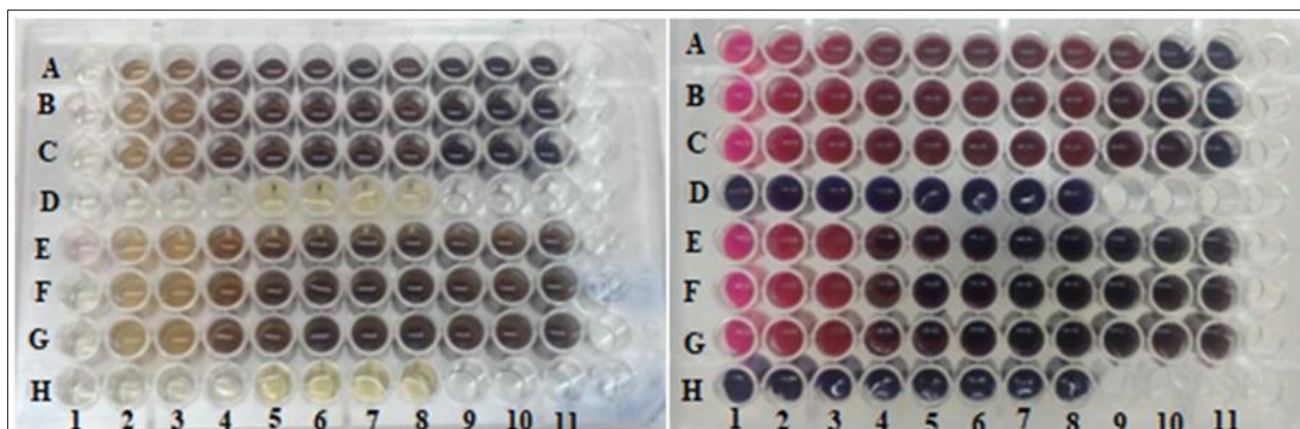
Table 2 Minimal inhibitory concentration (MIC) and minimal bacterial concentration (MBC) of decoction of *A. alba*

Bacterial strains	Samples								
	An Nghia			Dan Xay			Long Hoa		
	MIC	MBC	MBC/MIC	MIC	MBC	MBC/MIC	MIC	MBC	MBC/MIC
<i>B. cereus</i>	300	800	2.67	400	1000	2.50	600	900	1.50
<i>B. subtilis</i>	300	700	2.33	400	1000	2.50	600	1000	1.67
<i>S. aureus</i>	800	>	nd	200	700	3.50	1000	>	nd
<i>E. faecalis</i>	900	>	nd	400	700	1.75	1000	>	nd
<i>E. coli</i>	800	>	nd	200	800	4.00	700	1000	1.43
<i>P. aeruginosa</i>	300	900	3.00	100	1000	10.0	900	>	nd

All results for MIC and MBC were expressed in mg/mL. (>): values greater than 1000mg/mL. (nd): not determined

In the present study, the amount of leaf powder to extract 1 mL of decoction was 1000 mg. Although the maximum MIC was 1000 mg, for MBC, many samples showed that this maximum concentration did not kill bacteria (samples with symbol >). Therefore, in another trial, it was necessary to increase the dose of leaf powder to above 1000 mg per 1 mL of decoction. Except for Dan Xay sample for *P. aeruginosa* with MBC/MIC value of 10, the remaining data ranged from 1.43 to 4.00, many of which were close to 1 (Long Hoa samples). This showed the ability of the decoction from *A. alba* to be bacteriostatic or bactericidal.

The following Figure 3 depicted the MIC experimental setup and the results of the decoction of *A. alba* plant samples collected from An Nghia. In which the meanings of letters and numbers were as follows. “A” to “H” were *E. faecalis*, *S. aureus*, *E. coli*, gentamicin, *P. aeruginosa*, *B. subtilis*, *B. cereus*, and tetracycline, respectively. From “0” to “11” in rows A, B, C, E, F, and G corresponded to increasing values of decoction concentration from 0 to 1000 mg/mL with 100 mg/mL intervals. The remaining wells were according to the caption in the figure.



(D1 and H1): gentamicin; (D2 to D4): gentamicin and bacterial strains were *E. faecalis*, *S. aureus*, *E. coli*, respectively; (H2 to H4): gentamicin and bacterial strains were *P. aeruginosa*, *B. subtilis*, *B. cereus*, respectively. (D5 and H5): tetracycline; (D6 to D8): tetracycline and bacterial strains were *E. faecalis*, *S. aureus*, *E. coli*, respectively; (H6 to H8): tetracycline and bacterial strains were *P. aeruginosa*, *B. subtilis*, *B. cereus*, respectively.

Figure 3 MIC test of decoction from *A. alba* growing in An Nghia (left) and results (right)

For the antibacterial test according to the MIC and MBC evaluation methods, there were not many published yet. According to the results of Gupta and Roy [20], the MIC and MBC tested on *B. subtilis* of chloroform leaf extracts of *A. alba* were 3.91 and 7.81 mg/mL, respectively, and of the methanol extracts from the stems were 7.81 and 15.63 mg/mL, respectively. Especially, ethanol extract from the roots had MBC/MIC equal to 1.0 at a dose of 1.95 mg/L. It was worth noting that these results were recorded on total extracts. In another study [9], a phytochemical isolated from the total wood extract of *A. alba* called *Albain 1* was effective against *Bacillus* including *B. cereus*, *B. polymyxa* and *B. pumilus* with a MIC of 0.125 mg/mL. This showed that it was necessary to conduct intensive research on the chemical composition in different types of extracts on the origin of the plant parts collected and the extraction solvent in order to more effectively exploit the folk medicinal herbs.

4. Conclusion

In this study, all samples of ethanol extracts showed bacterial inhibition against 3 strains *B. subtilis*, *S. aureus*, *E. faecium*, through agar well diffusion method. Particularly, the *A. alba* leaf extract derived from Dan Xay showed halo zones expression on all 6 tested bacterial strains. For the MIC and MBC methods, except for the decoction derived from Dan Xay and tested on *P. aeruginosa*, the samples showed the MBC/MIC rates in the usual range, from 1.43 to 4.0. Some samples tested on *E. coli*, *E. faecalis*, *B. cereus*, and *B. subtilis* had these rates close to 1.0, indicating the high bactericidal ability of the decoction from the leaves of *A. alba*.

Compliance with ethical standards

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Disclosure of conflict of interest

There is no conflict of interest.

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