Strong potential of white cambodia (*Plumeria acuminata*) phyllosphere bacteria which inhibit *Candida albicans* growth

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Abstract. *Candida albicans* is the most common fungal species that infect humans. Over time pathogenic fungi can evolve to become more resistant to current antimicrobials. Therefore, the search for new antimicrobials needs to be carried out continuously. White cambodia leaves (*Plumeria acuminata*) contain several secondary metabolite compounds with antimicrobial properties. The leaf surface is a habitat for microbes such as phyllosphere bacteria, which are known to have the ability to produce secondary metabolite compounds that are the same as the host where they live. This study aims to determine the antifungal potential produced by *P. acuminata* phyllosphere bacterial isolates in inhibiting the growth of *C. albicans*. This research uses experimental laboratory methods. The initial stage was the isolation of *P. acuminata* phyllosphere bacteria using the serial dilution method. After that, observation of colony characteristics based on shape, edge, elevation, texture, and pigment, as well as Gram staining of *P. acuminata* phyllosphere bacterial isolates. The final stage was an antagonistic test of phyllosphere bacterial isolates against *C. albicans* using the two-layer agar method. Isolation of *P. acuminata* phyllosphere bacteria produced 151 isolates, and 66 isolates were selected as samples. Based on the observation of isolate characteristics and morphology of bacteria from 66 isolates, the isolate characteristics and morphology of bacteria were diverse. Based on the Gram staining test, it is known that Gram-positive bacteria in the form of cocci are dominant. Fourteen isolates of phyllosphere bacteria could inhibit the growth of *C.albicans* based on the results of the antagonist test. White Cambodia (*P. acuminata*) phyllosphere bacterial isolates can produce antifungal compounds inhibiting *C. albicans* growth.

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1 Introduction

The fungus infects about a quarter of the world's population (20-25%), and since 1980 continued to increase [1]. The most common fungal species infecting humans is *Candida albicans* [2]. Candida albicans is one of the microbiomes in humans found in the oral cavity, mucous membranes, skin, and other body parts [3]. However, when a person's immune system is weakened, this microbiome can become a pathogen that can potentially infect and cause candidiasis [2].

Candidiasis can occur in all age groups [4], especially in vulnerable groups with low immunity, such as babies, toddlers, children, elders, and immunodeficiency people [5]. *Candida* infections can occur in the oral cavity, vagina, penis, or other parts of the body. Candidiasis that lasts long and is not appropriately treated can develop into pre-malignant lesions (candidiasis leukoplakia) that can become squamous cell carcinoma. The manifestations are massive at this stage because they can attack vital organs due to systemic infection via lymph flow [6].

Fungal infections often recur and require a relatively long period of therapy [7]. Currently, antifungal therapy using synthetic drugs is the primary choice for treating fungal infections [8]. Synthetic antifungal drugs have good potential in treating various fungal infections. The great benefits of synthetic antifungal drugs cannot be separated from the various side effects that may be obtained, apart from their use, which is prolonged and irrational and can lead to resistance [9]. Due to the side effects of synthetic drugs and the potential for resistance, it is necessary to conduct research on natural antifungal sources that can be alternative therapies. They are expected to provide the maximum possible benefits but with minimal risks or losses.

White cambodia leaves (*Plumeria acuminata*) contain secondary metabolite compounds in flavonoids, tannins, saponins, and alkaloids [10], [11]. Based on research conducted regarding the extraction of *P. acuminata* on the growth of *Candida albicans* in vitro, positive results were obtained, with the diameter of the inhibition zone against *C. albicans* being in the strong to powerful category [11]. Apart from extraction, antimicrobial compounds can be produced through secondary metabolism resulting from a combination of several primary metabolites from two different organisms that interact with each other, such as the interaction between plants and microorganisms that live on the structure of the plant [12].

The largest habitat for microbes on Earth is in the phyllosphere [13], [14]. The phyllosphere is the environment on the surface of plant leaves, which is a habitat for various microorganisms [13]. The phyllosphere is an extreme and fluctuating environment due to ultraviolet radiation, changing temperatures, and exposure to antimicrobials from the external environment. It causes the diversity of microorganisms in the phyllosphere to be relatively lower than in the endosphere (plant tissue environment) and rhizosphere (plant root environment). As a result of these factors, only competitive and highly resistant microbes inhabit the phyllosphere [14].

The dominant microorganisms in the phyllosphere are bacteria [15]. Both phyllosphere bacteria, endophytic bacteria (bacteria that live in the endosphere of plants), and rhizosphere bacteria are generally commensals or symbionts for the host plant. These microorganisms are essential in protecting plants against attacks by pathogenic microorganisms through competition mechanisms. Besides, these microorganisms can produce growth-promoting substances such as indole acetic acid, gibberellins and cytokinins, essential in supporting plant growth [16]. Phyllosphere bacteria obtain various chemical components from the environment and host plants, which causes them to produce certain secondary metabolite compounds and even the same secondary metabolite compounds as the host where they live [17]. Therefore, secondary metabolite compounds in flavonoids, tannins, saponins, and alkaloids in *P. acuminata* can inhibit the growth of *C. albicans* in a potent category. It can also be contained in phyllosphere bacteria that live on the leaves of these plants. The use of

isolates of phyllosphere bacteria that produce antimicrobial bioactive compounds will be more environmentally friendly than using the leaves because it does not require exploiting nature or requiring large areas of cultivation land. Therefore, researchers are interested in researching the antifungal potential of *P. acuminata* phyllosphere bacteria on the growth of *C. albicans*. This study aims to isolate *P. acuminata* phyllosphere bacteria and test its antifungal activity against *C. albicans*.

2 Methods

The research used a quantitative data collection method to determine the antifungal activity of *P. acuminata* phyllosphere bacteria against *C. albicans*. The research was conducted at the Microbiology Laboratory, Faculty of Medicine and Health Sciences, Bengkulu University. The research population was phyllosphere bacteria on the leaves of *P. acuminata*, which grow in the West Lingkar area, Gading Cempaka District, Bengkulu City. The sample isolates in this study were from the leaves of three *P. acuminata* plants, which had no structural damage, with one leaf per plant each to obtain the quantity of bacterial variation.

2.1 Isolation of White Cambodian Phyllosphere Bacteria

The method used to transfer phyllosphere bacteria to the media is the flush method. The leaves used as samples were cleaned from dust and dirt with running water. The clean leaves are then put into a measuring cup and then doused with 10 ml of 85% NaCl, evenly wetting the entire surface of the leaves. The suspension from watering is then carried out in gradual dilution up to $1x10^{-4}$. Then, 0.1 ml of the suspension from each dilution was taken and spread on King's B media, which had been prepared and then incubated for 24 hours at room temperature [13].

2.2 Colony Characterization and Morphology of Phyllosphere Bacteria

Phyllosphere bacteria grown on King's B media were observed macroscopically and microscopically. Macroscopic observations involve observing elevation, edges, shape, texture, and pigment, while microscopic observations involve observing the shape of bacterial cells and the Gram type of bacteria through Gram staining. The growing phyllosphere bacteria are taken using a tube and placed on a glass slide. The preparations were dripped with crystal violet for 1 minute and washed. The preparations were dropped with iodine for 10-60 seconds and washed. The preparation was dripped with alcohol as decolourization and washed with water. The preparations were dripped with safranin for 40-60 seconds, washed with water, and dried. The preparations were observed under a microscope with 1000x magnification.

2.3 Antagonist Test

The antagonist test uses a two-layer agar method. The media used in this procedure are Potato Dextrose Broth (PDB) and Potato Dextrose Agar (PDA). The first layer of the cup is filled with PDA. After the PDA in the first layer has solidified, a mixture of semi-solid NA and PDB, which contains *C. albicans,* is poured. After hardening, the phyllosphere bacteria isolate spotted on top of the layer. Incubate for 24 hours and observe the inhibition zone that forms. The inhibition zone formed was assessed based on the categories of Rizqoh et al. (2024) [18], [19].

3 Results

3.1 Isolation of Phyllosphere Bacteria from *P. acuminata* Leaves

The process of isolating phyllosphere bacteria from 3 three *P. acuminata* leaves from 3 different plants resulted in 151 colonies. The number of colonies obtained in this study can be seen in Table 1.

Table 1 Number of White Cambodian Phyllosphere Bacterial Colonies

No. Plant	Dilution Rate	Number of Colonies
K 1 F	10 -1 (Plate 1)	27
	10 -1 (Plate 2)	34
K 1 F	10 -2 (Plate 1)	2
	10 -2 (Plate 2)	3
K 1 F	10 -3 (Plate 1)	28
	10 -3 (Plate 2)	2
K 1 F	10 -4 (Plate 1)	3
	10 -4 (Plate 2)	2
K 2 F	10 -1 (Plate 1)	4
	10 -1 (Plate 2)	3
K 2 F	10 -2 (Plate 1)	1
	10 -2 (Plate 2)	3
K 2 F	10 -3 (Plate 1)	23
	10 -3 (Plate 2)	0
K 2 F	10 -4 (Plate 1)	0
	10 -4 (Plate 2)	0
K 3 F	10 -1 (Plate 1)	3
	10 -1 (Plate 2)	4
K 3 F	10 -2 (Plate 1)	3
	10 -2 (Plate 2)	3
K 3 F	10 -3 (Plate 1)	1
	10 -3 (Plate 2)	4
K 3 F	10 -4 (Plate 1)	0
	10 -4 (Plate 2)	0
Total		151

3.2 Characterization and Morphology of *P. acuminata* Phyllosphere Bacteria

Phyllosphere bacterial isolates that had grown after a 24-hour incubation process were then characterized according to Leboffe and Piece (2012) by observing the isolate's shape, edge shape, elevation, texture, and pigment. Based on morphological characterization, 66 isolates were obtained. The dominant characteristics of the phyllosphere bacterial isolates obtained were regular round shape (67%), regular smooth edges (72%), convex elevation (71%), moist texture (77%), and tended to form shiny yellow (27%) (Figure 1).

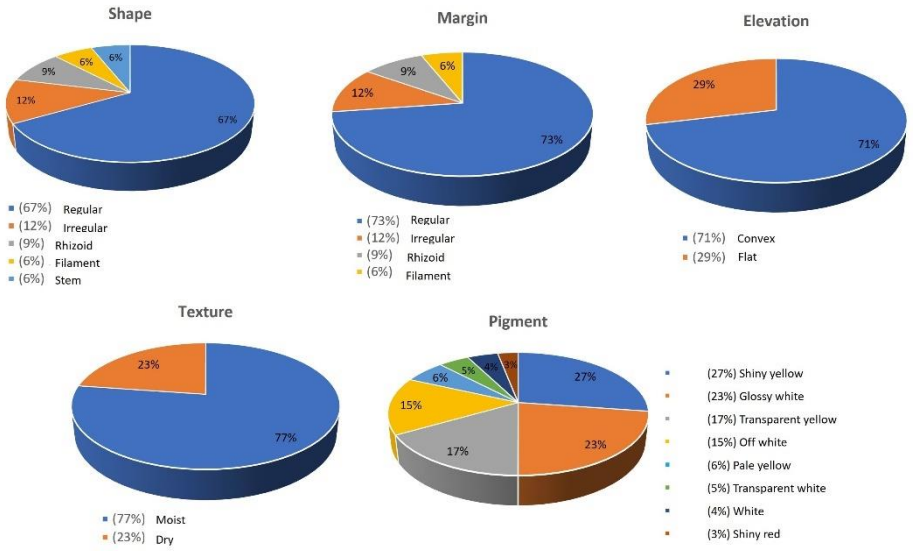

Fig. 1. Variation Characteristics *P acuminata* Phyllosphere Bacterial Isolate. (A) Shape Variation of Bacterial Isolate, (B) Margin Variation of Bacterial Isolate, (C) Elevation Variation of Bacterial Isolates, (D) Texture Variations of Bacterial Isolates, (E) Pigment Variation of Bacterial Isolates.

Gram staining is carried out to determine the shape and type of Gram bacteria. Gram staining was carried out on 66 bacterial isolates that had previously been purified and rejuvenated. The Gram staining results of the 66 isolates can be seen in Table 2. The most dominant types of Gram bacteria were cocci with the Gram-positive type, namely 48 (72.72%) isolates, cocci with the Gram-negative type, 9 (13.63%) isolates, coccobacilli with the Gram-positive type, 5 (7.57%) isolates and Gram-positive bacilli were 4 (6.06%) isolates.

Table 2. Morphology of White Cambodi three *P. acuminata* Phyllosphere Bacteria from Gram Staining Results

Form	Isolate Code	Gram Type	Number of Isolates
Cocci	KF2, KF5, KF6, KF7, KF8, KF9, KF10, KF11, KF12, KF13, KF14, KF15, KF16, KF17, KF22, KF25, KF26, KF27, KF28, KF31, KF32, KF34, KF35, KF37, KF38, KF39, KF40, KF42, KF43, KF45, KF46, KF47, KF48, KF49, KF50, KF51, KF52, KF53, KF54, KF55, KF56, KF57, KF58, KF59, KF60, KF61, KF65, KF66	Positive	48
	KF1, KF3, KF4, KF21, KF23, KF24, KF29, KF62, KF64	Negative	9
Basil	KF19, KF30, KF36, KF63	Positive	4
Cocobacilli	KF18, KF20, KF33, KF41, KF44	Positive	5

3.3 Antagonist Test of *P. acuminata* Phyllosphere Bacteria Against the Growth of *C. albicans*

From the results of the antagonist test of 66 isolates of three *P. acuminata* phyllosphere bacteria two times and the average calculation carried out, 11 isolates inhibited the growth of *C. albicans*. Positive results are indicated by an inhibition zone around the test phyllosphere bacteria spotted on semi-solid media.

Table 3 shows that the zone of inhibition resulting from the inhibitory test of isolates of three *P. acuminata* phyllosphere bacteria against *C. albicans* is in the weak to powerful category. The most significant inhibition zone diameter was formed by isolates KF33, KF9, and KF34, with each inhibition zone formed 33.7 ± 11.03 mm, 28.95 ± 6.86 mm, and 22.45 ± 4.59 mm (powerful category). An overview of the zone of inhibition in the inhibitory test of isolates of three *P. acuminata* phyllosphere bacteria on the growth of *C. albicans* can be seen in Figure 2.

Table 3 Results of the Antagonist Test of Phyllosphere Bacterial Isolates

No.	Isolate Code	Isolate Diameter (mm)	Isolate Diameter + Inhibition Zone (mm)	Inhibition Zone Diameter (mm)	Category
1.	KF9	6.85 ± 0.47	35.8 ± 6.37	28.95 ± 6.86	Powerful
2.	KF28	6.45 ± 0.35	16.15 ± 1.06	9.70 ± 0.71	Moderate
3.	KF33	8.80 ± 2.21	42.50 ± 8.92	33.70 ± 11.03	Powerful
4.	KF34	8.60 ± 0.28	31.05 ± 4.26	22.45 ± 4.59	Powerful
5.	KF35	7.95 ± 0.49	20.20 ± 7.36	12.25 ± 7.89	Strong
6.	KF36	7.75 ± 0.78	11.70 ± 6.36	3.95 ± 5.58	Weak
7.	KF58	8.20 ± 1.72	27.35 ± 8.30	19.15 ± 9.99	Strong
8.	KF59	8.20 ± 1.41	26.4 ± 1.70	18.2 ± 3.11	Strong
9.	KF60	10.15 ± 1.77	26.55 ± 8.50	16.40 ± 7.78	Strong
10.	KF62	8.80 ± 1.73	18.65 ± 4.04	9.85 ± 5.88	Moderate
11.	KF63	8.50 ± 1.74	21.80 ± 0.57	13.30 ± 2.41	Strong

Fig. 2. Results of the Inhibition Test for three *P. acuminata* Phyllosphere Bacteria Bacterial Isolates against *C. albicans*.

4 Discussion

Many factors influence the growth of bacteria in a place. The complexity of factors in the phyllosphere environment greatly influences the number of bacteria that inhabit that environment. King'B media was chosen as the isolation medium because it contains a complex composition and is non-selective, so a growth environment is provided that is quite similar to the native habitat of phyllosphere bacteria [13], [16]. Even though King's B has good content to support the growth of phyllosphere bacteria, this content cannot fully match

the complexity of factors in the phyllosphere environment, so not all bacteria in leaf samples can grow on this medium [16].

Bacteria that were successful in growing were then characterized in terms of isolate and bacterial morphology. Characterization of isolates starts from shape, margin, elevation, texture, and pigment. On the same plate, it finds colonies of bacteria with similar characteristics. Therefore, 2 to 3 colonies were selected from a collection of bacterial colonies with similar characteristics on a plate as representative samples for the next step. A total of 66 bacterial isolates were used as test samples, each of which had been given a code name and divided into 24 groups (each group had the same isolate characteristics).

Several factors influence variations in the characteristics of bacterial isolates, including the shape of the bacteria, the direction of bacterial cell division, which influences the arrangement of the isolates (in pairs, clusters, chains, or like filaments) and the physiological characteristics of the bacteria themselves such as motility, pigments, enzymes and bacterial capsules which influence the appearance of the shine, color and texture of the bacterial isolate [1]. The characteristics of the dominant bacterial isolates in this study were a regular round shape, regular smooth edges, convex elevation, and moist texture with a shiny yellow pigment color of 13 isolates.

The bacterial isolates obtained were then subjected to Gram staining to identify the morphology of the bacteria and their Gram type. Based on the shape of the bacteria and the Gram type, it is known that the most dominant bacterial morphology obtained in this study was cocci with the Gram-positive type, a total of 48 isolates.

Various factors influence the diversity of types of bacteria that inhabit a plant's phyllosphere environment. Factors that influence include the environmental conditions where the plant grows and the availability of nutrients obtained by phyllosphere bacteria from their habitat [20]. Several similar studies related to the identification of morphology and Gram types of phyllosphere bacteria in other plants also found that the morphology of the bacteria that grew was dominated by cocci-shaped bacteria of the Gram-positive type [13], [21].

White Cambodia (*P. acuminata*) leaves have the potential to be an antifungal agent because they contain flavonoids, tannins, saponins, and alkaloids. Based on research that has been carried out regarding tests of the inhibitory power of *P. acuminata* leaf extract on the growth of *C. albicans* in vitro, positive results were obtained, with the diameter of the inhibition zone against Candida albicans being in the strong to powerful category [10], [11].

The antifungal activity of phyllosphere bacteria is closely related to the environment in which the bacteria live. Phyllosphere bacteria obtain various chemical components, including environmental and host plants' nutrients. It causes it to produce certain secondary metabolite compounds and even the same secondary metabolite compounds as the host where he lives [17], [22]. Therefore, secondary metabolite compounds in flavonoids, tannins, saponins, and alkaloids in *P. acuminata* leaves, which are antagonistic to the growth of *C. albicans,* can also be contained in the plant's phyllosphere bacteria.

Compounds contained in phyllosphere bacteria will attack *Candida albicans* using various mechanisms. Flavonoids will denature fungal cell proteins, increasing the permeability of fungal cell walls. Alkaloids are alkaline, which can damage fungal cell walls. Saponins cause a decrease in the surface tension of fungal cells, resulting in increased permeability of fungal cell membranes. Tannins form stable bonds with fungal cell proteins, resulting in coagulation of fungal protoplasm. These compounds ultimately cause the death of the *C. albicans* fungus by various mechanisms [17].

Apart from the antimicrobial compounds produced by phyllosphere bacteria, the formation of a clear zone around the viewing area is due to the competition mechanism between phyllosphere bacteria and *C. albicans* in obtaining nutrients for their survival. It is

why *C. albicans* cannot grow around the viewing area due to limited nutrition, forming a clear zone on the media [23], [24].

In this study, 11 isolates were obtained which were able to form a clear zone namely isolates with codes KF9, KF28, KF33, KF34, KF35, KF36, KF58, KF59, KF60, KF62, KF63, where isolates with codes KF33, KF9, KF34 are in the powerful category with each inhibition zone formed, 33.7 ± 11.03 mm, 28.95 ± 6.86 mm and 22.45 ± 4.59 mm, while the minor inhibition zone diameter was formed by isolate KF36 with an inhibition zone formed of 3.95 ± 5.58 mm (weak category).

Rizqoh et al. (2016) previously researched phyllosphere bacteria in one type of fresh vegetable, namely reundeu leaves (*Staurogyne longata*), which produce antimicrobials [12]. The report also states that reundeu phyllosphere bacteria actively inhibit gram-positive bacteria such as *Escherichia coli* and gram-negative bacteria such as *Bacillus subtilis* and *Staphylococcus aureus* as well as pathogenic bacteria such as Enteropathogenic *E. coli* (EPEC), pathogenic *S. aureus*, *C. albicans*, and *C. tropicalis* [12].

Research related to testing the inhibitory power of other plant phyllosphere bacteria on the growth of *C. albicans* has never been carried out before. Several similar studies have been conducted, including testing the inhibitory power of endophytic bacteria on the growth of *C. albicans*. Based on research conducted by Nindhia et al. (2017) regarding the growth inhibition test for *C. albicans* using supernatant from isolates of the endophytic bacteria of the medicinal plant *Ageratum conyzoides*, positive results were obtained with the diameter of the most robust inhibition zone formed being 11mm [25]. Other research related to the same thing regarding testing the inhibitory power of endophytic bacteria of Javanese wood plants (*Lannea coromandelica*) on the growth of *C. albicans* also obtained positive results with the diameter of the largest inhibitory zone formed, namely 20.75mm [11], [26].

As biocontrol agents, phyllosphere bacteria will create bioactive chemicals that inhibit the activity of pathogenic germs [27]. Okafor (2017) explains that the bioactive compounds produced by living organisms are secondary metabolites [28].Thus, competition for nutrients or competition for growing areas and secondary metabolites from phyllosphere bacteria might induce the mechanism of limiting fungal growth by phyllosphere bacteria. Several studies have found that bioactive substances produced by phyllosphere bacteria in various plants have antibacterial effect against pathogenic germs. According to published research, phyllosphere bacteria aid in plant defense mechanisms, biological nitrogen fixation, plant mineral absorption, disease severity reduction, and plant growth [29].

5 Conclusion

The total isolates of phyllosphere bacteria obtained from the isolation of *P. acuminata* leaves were 151 colonies, with 66 isolates used as test samples. The most dominant characteristics of all the phyllosphere bacterial isolates obtained were a regular round shape, smooth edges, convex elevation, and moist texture with a shiny yellow pigment color of 13 isolates. The majority of bacterial morphology obtained was in the form of cocci with a Gram-positive type of 48 isolates.

Of the 66 isolates of phyllosphere bacteria tested, 11 isolates had the potential to inhibit the growth of *C. albicans*. Isolates with codes KF33, KF9, and KF34 are in the powerful category with each inhibition zone formed, namely 33.7 ± 11.03 mm, 28.95 ± 6.86 mm, and 22.45 ± 4.59 mm, while the zone diameter The smallest inhibition was formed by isolate KF36 with an inhibition zone formed of 3.95 ± 5.58 mm (weak category).

Further research must be conducted to identify secondary metabolites produced by white frangipani phyllosphere bacteria isolates, which potentially inhibit *C. albicans'* growth. In addition, research needs to be carried out regarding the identification of biochemical and

biomolecular characteristics of white frangipani phyllosphere bacteria isolates, which have the potential to inhibit the growth of *C. albicans*.

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