Phytochemical Analysis and Screening of Antibacterial Activity of White Pacing Flower (*Costus Speciosus* (J. Koening) Sm.)

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Abstract. This study was conducted with the aim of identifying secondary metabolite compounds and antibacterial activity of white pacing flower extract (*Costus speciosus* (J. Koenig) Sm.). Phytochemical screening tests are performed qualitatively and antibacterial activity tests are carried out by agar well diffusion methods with modification against *Staphylococcus aureus* ATCC 25923*, Propionibacterium acnes* KCCM 41747, *Streptococcus mutans* ATCC 25175*, Streptococcus sobrinus* KCCM 11898 and *Salmonella typhi* ATCC 422. The results of phytochemical screening showed that crude ethanol extract containing flavonoids, phenolics, and saponins, in the methanol:water fraction containing flavonoids, phenolics, and tannins while the n-hexane and ethyl acetate fractions containing steroid compounds. Antibacterial screening showed the highest antibacterial activity in the ethyl acetate fraction against *P. acnes* with an inhibition zone diameter of 11.3 mm and a minimum inhibitory concentration value of 0.25%.

Keywords: Costus speciosus · Antibacterial · Minimum Inhibitory Concentration

1 Introduction

One of the most widely developed bioassays is antibacterial activity using wild plants as the main ingredient. White pacing plant (Costus speciosus (J. Koenig) Sm.) is one of the plants that contains many secondary metabolites which are used as folk medicines. White pacing plant is a wild plant that grows in bushes. A single, erect pacing stem can grow 1 to 3 m in height. The crown at the end of the tubular stem has large spikelet containing flowers [1]. This plant is widely used as traditional medicine such as being processed into rhizome juice with sugar which is consumed for leprosy patients and relieves pain [2]. Several previous studies revealed the content of secondary metabolites contained in white pacing plants. These secondary metabolites can be used as antibacterial agents to treat various diseases caused by bacteria.

Bacteria are living organisms without a cell nucleus that can live in various places. Bacteria can cause disease by producing toxins that can harm humans. Several bacteria

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are harmful to humans [3] such as Staphylococcus aureus which causes skin infections [4], Propionibacterium acnes which causes acne [5], Streptococcus mutans [6] and Streptococcus sobrinus which causes dental caries [7], and Salmonella typhi which causes typhoid fever [8]. The losses caused by these bacteria can be prevented by utilizing antibacterial agents from active compounds in plants. Antibacterial constituents are toxic selectively able to fight pathogenic bacteria [9]. Antibacterial compounds will suppressed the bacterial growth by damaging the bacterial wall and cause the bacteria to eventually die [10].

The well diffusion method is one of the methods used to determine antibacterial activity based on diffusion of antibacterial into the solid media that have been inoculated with the tested microbe. A clear zone will be formed around the well which indicates the presence of antibacterial activity of the sample used [11]. The lowest concentration of the sample that is still able to inhibit bacterial growth is called the MIC (Minimum Inhibitory Concentration) value [12].

Previous research by Dilaga (2014) [2] showed that white pacing plants contain alkaloids, flavonoids, polyphenols, quinones, tannins, saponins and monoterpenes/sesquiterpenes. Research by Rahmiyani & Zustika (2016) [13] also reported the content of secondary metabolites in the extract of n-hexane, ethyl acetate and methanol from pacing leaves, namely alkaloids, flavonoids, polyphenols, steroids, mono and sesquiterpenoids and quinones. The results of the study [14] demonstrated that the three isolates of endophytic fungi from white pacing leaves had potential as antibacterial agents.

This study was aimed to determine the secondary metabolite compounds contained in white pacing plant flowers and to determine its antibacterial activity and the MIC value. It is hoped that this research can explain the natural benefits of white pacing plant flowers and can be used as a basis for further research related to the use of white pacing plants in the health sector.

2 Methods

2.1 Research Materials and Methods

This study utilized the white pacing plant (Costus speciosus (J. Koenig) Sm.) and used the agar diffusion method with modifications to conducted its antibacterial activity.

2.2 Research Procedures

Sample Preparation

The white pacing plant flower parts are cleaned, then dried and mashed until almost smooth. The mashed sample was then macerated using 96% ethanol. The results of the macerate were filtered and concentrated using a rotary evaporator. The crude ethanol extract was then dissolved in methanol:water, then fractionated in stages using n-hexane

and ethyl acetate as solvents. The results of the fractionation are then concentrated using a rotary evaporator.

Phytochemical Screening

1. Flavonoid Test
   Each sample was dissolved in a suitable solvent and then heated. Then Mg tape was added and 1 drop of HCl(p) solution was added. A positive result is indicated by a change in the color of the solution from red to orange [15].
2. Phenolic Test
   Each sample was dissolved with the appropriate solvent and added FeCl3 solution. A positive result is indicated by a change in the color of the solution to greenish black [16].
3. Saponin Test
   Each sample was dissolved with the appropriate solvent and hot distilled water was added, then shaken and added 1 drop of 2 N HCl solution. Positive results were indicated by the formation of stable foam for ±30 s [15].
4. Tannin Test
   Each sample was dissolved with the appropriate solvent and added 3 drops of 1% FeCl3 solution. A positive result is indicated by a change in the color of the solution to bluish black or green [15].
5. Alkaloid Test
   Each sample was dissolved with the appropriate solvent and added 3 drops of 2 M HCl solution, then added 3 drops of Dragendorff's reagent. A positive result is indicated by the formation of an orange precipitate [2].
6. Steroid Test
   Each sample was dissolved with the appropriate solvent then added chloroform ammonia and 2 N H2SO4 solution then shaken and allowed to stand until 2 phases were formed. The lower phase in the form of chloroform was added with Liebermann-Burchard reagent. A positive result is indicated by a change in the color of the solution to green-blue [17].

Antibacterial Activity Test

1. Equipment Sterilization
   The tools used were washed and covered with aluminum foil, then sterilized in an autoclave at 121 °C for 15 min and a pressure of 1 atm [18].
2. Making Basic Agar Media
   Nutrient Agar (NA) was used as much as 2.8 g dissolved in 100 mL of distilled water and homogenized by heating. The homogenized NA was then autoclaved at 121 °C for 15 minutes and a pressure of 1 atm. The sterile NA was cooled to the required temperature and the unused NA was stored in the refrigerator [19].
3. Liquid Media Making
   Nutrient Broth (NB) was used as much as 0.9 g dissolved in 100 mL of distilled water and homogenized by heating. The homogenized NB was then autoclaved at 121 °C for 15 minutes and a pressure of 1 atm [20].

4. Rejuvenation of Pure Bacterial Culture
   Pure cultures of the test bacteria were taken with sterile cotton buds and swabbed on the surface of the solid agar medium, then incubated for 24 hours at 37 °C in an incubator [18].
5. Preparation of Bacterial Inoculum
   With 10 mL of NB was poured into a test tube up to one third of the tube and allowed to stand at room temperature. A total of one needle of test bacteria from agar medium was inoculated into a test tube, then shaken in a water bath and incubated for 24 hours at 37 °C in an incubator [21].
6. Screening of Antibacterial Activity
   The NA was poured into a 10 mL petri dish and allowed to solidify. The solid media was made 5 wells spaced using a steel backer. The test bacteria were taken using a sterile cotton bud and swab on the surface of solid media. With 3% of sample, positive control (ampicillin) 0.5% and negative control (methanol) was poured into the well as much as 30 uL. The media was incubated for 18–24 h at 37 °C in an incubator. The clear zone formed from the edge of the well to the boundary of the clear zone circle was measured using a ruler. The procedure was carried out in triples for each test bacterium [19].
7. Determining the Minimum Inhibitory Concentration (MIC) Value
   The MIC value is determined based on the smallest concentration of the sample that still has antibacterial activity. The procedure for determining the MIC value uses the same procedure as screening for antibacterial activity and is carried out with various concentrations of sample, namely 2; 1.5; 1; 0.5; 0.25 and 0.625%. The positive control (ampicillin) was also carried out with various concentration of 0.25; 0.125; 0.0625; 0.0312; 0.0156 and 0.0078% and triple negative control.

3 Results and Discussion

3.1 Extraction and Fractionation of White Pacing Flower Samples

Samples of dried white pacing flowers (Costus speciosus (J. Koenig) Sm.) with 1075 g were extracted by using ethanol to obtained 52 g of ethanol extract. According to Alhaddad et al., (2019), the maceration process can separate the active compounds from more complex compound in the sample [2] without damaging the compound due to the heating process. In addition, organic solvent was used can penetrate the cell wall of the sample and then attract the active substance in the cell and dissolve with the solvent [3]. After that, partition of ethanol extracts were conducted by fractionation and obtained n-hexane, ethil acetate and methanol:water fractions. The results of the fraction yield can be seen in Table 1.

3.2 Phytochemical Screening

In the phytochemical screening test, the white pacing flower sample showed several compounds contained in the extract and sample fractions. Some compounds were not detected in the crude ethanol extract but were detected in the methanol:water, n-hexane

Table 1. Yield of White Pacing Flower Fraction

Fraction	Final Mass (g)	Yield (%)
n-Hexane	4	11.33
Ethyl acetate	10	33.33
Methanol;water	2	6.67

and ethyl acetate fractions. The results of phytochemical screening of white pacing flower extracts and fractions are shown in Table 2.

Based on Table 2, the phytochemical results revealed that flavonoids were detected in the ethanol extract and methanol:water fraction. Samples containing flavonoid compounds will show a change in the color of the solution after adding HCl and Mg bands to form flavilium salts [15] (Fig. 1).

Phenolic compounds were also detected in the ethanol extract and methanol:water fraction. Samples that have been added with FeCl3 will change the color of the solution to black, which indicates a positive sample containing phenolics. The reaction of FeCl3 with phenolic compounds occurs as a result of the aromatic -OH group on the phenolic reacting with FeCl3 to form a complex compound which is thought to be iron (III) hexaphenolate [23] (Fig. 2).

Saponin compounds were only detected in the ethanol extract. The formation of stable foam for ±30 s in the sample solution after the addition of HCl occurs because the hydrophilic (OH) group on the saponins causes saponins to dissolve in water and then reacts with water molecules and forms hydrogen bonds (Fig. 3).

Tannins were also only detected in the methanol:water fraction. The thing that indicates the presence of tannins is a change in the color of the sample solution to blackish color due to the reaction of the hydroxy group on the tannins with 1% $FeCl_3$ solution. The color of the green-black solution indicates the group of condensed tannin compounds [26] (Fig. 4).

Table 2. Phytochemical Screening of White Pacing Flower Extract and Fraction

Secondary metabolites	Ethanol extract	Methanol;water fraction	n-Hexane fraction	Ethyl acetate fraction
Flavonoids	+	+	–	–
Phenolic	+	+	–	–
Saponins	+	–	–	–
Tannins	–	+	–	–
Alkaloids	–	–	–	–
Steroids	–	–	+	+

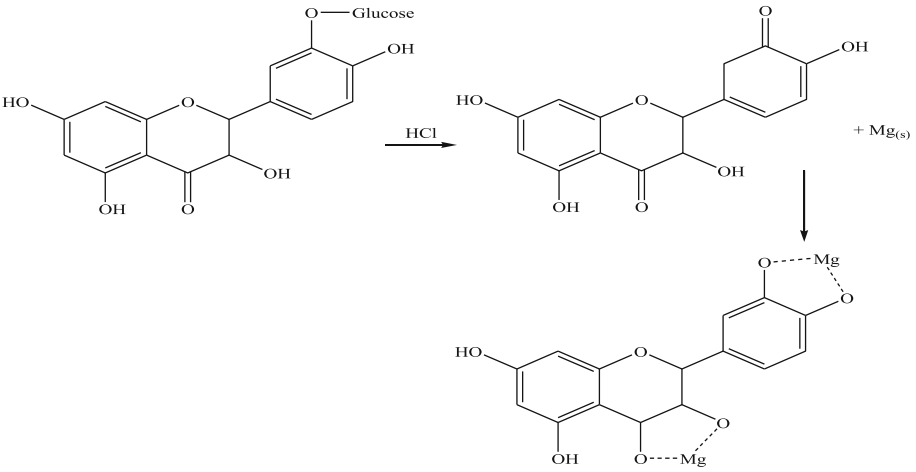

Fig. 1. Flavonoid reaction with HCl and Magnesium [22].

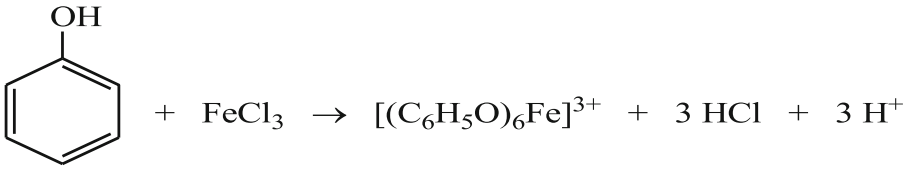

Fig. 2. Reaction of Phenol with $FeCl_3$ [24].

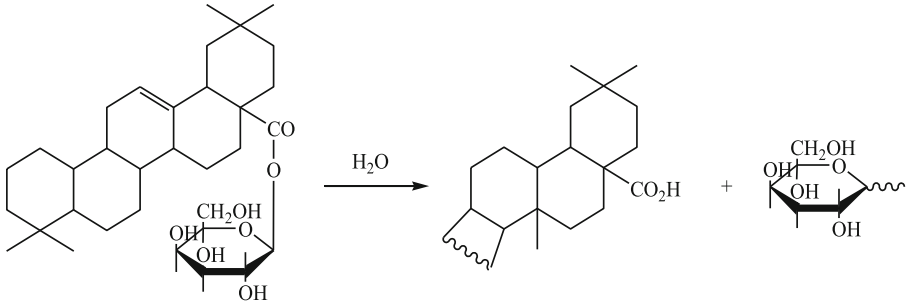

Fig. 3. Hydrolysis of Saponins in Water [25]

Alkaloid compounds were not detected in the four samples tested. According to Yeti (2021), the presence of alkaloids will be shown from reddish to orange precipitate formed after adding hydrochloric acid and Dragendorff's reagent [28] (Fig. 5).

Moreover, steroids were detected in the n-hexane and ethyl acetate fractions. After the addition of the sulfuric acid to the sample solution, 2 phases will form and at the boundary of the two phases a brown ring is formed. The lower phase in the form of chloroform after adding the Liebermann-Burchard reagent will change color to dark

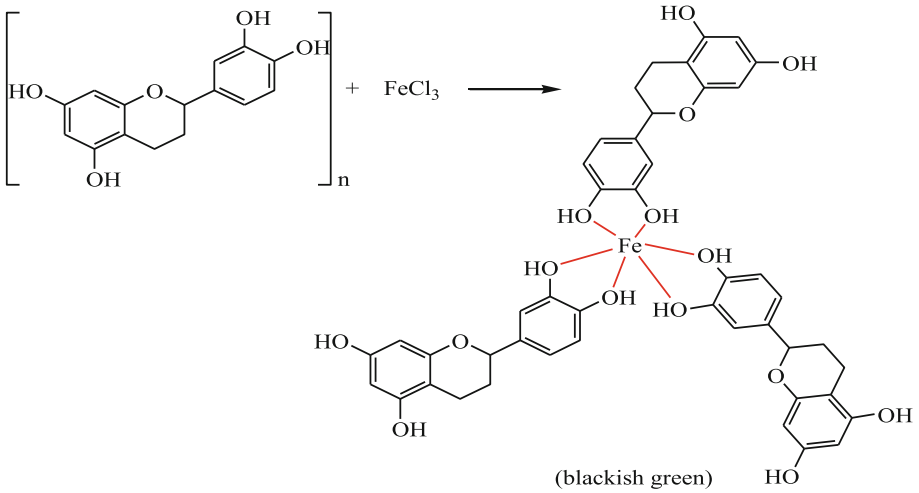

Fig. 4. Reaction of tannins and $FeCl_3$ [27]

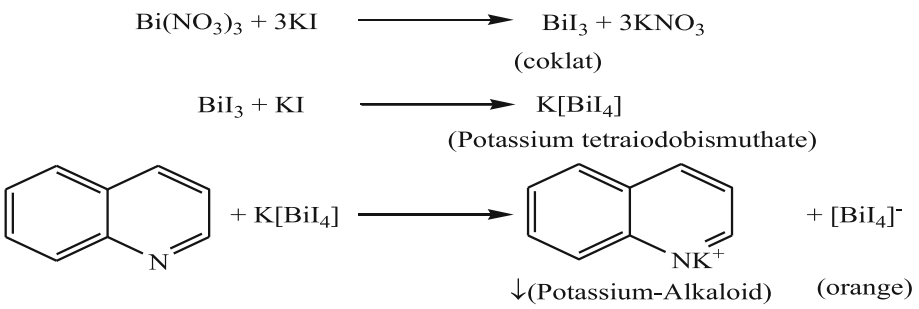

Fig. 5. Alkaloid Reaction and Dragendorff's Reagent [27]

green due to the oxidation reaction of steroid compounds resulting from the formation of conjugated double bonds [27] (Fig. 6).

3.3 Antibacterial Activity Screening

In screening for antibacterial activity, the diffusion method of agar pits was used with modifications where this method has the advantage that it is easier to measure the diameter of the inhibition zone formed due to the overall spread of antibacterial activity from the surface to the bottom of the agar medium. The results of the antibacterial activity screening are shown in Table 3.

The highest antibacterial activity was shown by the ethyl acetate fraction against P. acnes with an inhibition zone diameter of 11.3 ± 1.2 mm. Based on the classification for antibacterial activity criteria by Kingkaew (2018) [30] where the inhibition zone diameter of 11.3 mm is included in the strong category. Steroids contained in the ethyl acetate fraction are suspected as antibacterial agents that can inhibit the growth of P.

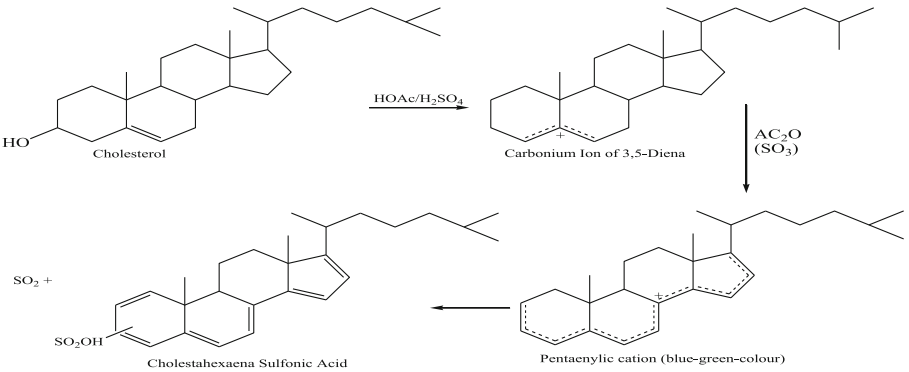

Fig. 6. Steroid reaction and Liebermann-Burchard reagent [29]

Table 3. Results of Antibacterial Activity Screening

Sample 30%	Diameter of Inhibition zone (mm ± SD)				
	S. aureus	P. acnes	S. mutans	S. sobrinus	S. typhi
Ethanol crude extract	6.0 ± 0.0	6.0 ± 0.0	6.0 ± 0.0	6.0 ± 0.0	6.0 ± 0.0
Methanol:water fraction	6.0 ± 0.0	6.0 ± 0.0	6.0 ± 0.0	6.0 ± 0.0	6.0 ± 0.0
n-Hexane fraction	6.0 ± 0.0	6.0 ± 0.0	6.0 ± 0.0	6.0 ± 0.0	6.0 ± 0.0
Ethyl acetate fraction	6.0 ± 0.0	11.3 ± 1.2	6.0 ± 0.0	6.0 ± 0.0	6.0 ± 0.0
Ampicilin 0,5%	26.7 ± 1.5	20.7 ± 0.6	22.3 ± 2.5	24.0 ± 1.0	18.0 ± 2.7
Methanol	6.0 ± 0.0	6.0 ± 0.0	6.00 ± 0.0	6.00 ± 0.0	6.00 ± 0.0

Diameter of the well is 6 mm

acnes. Khoiriyah (2014) [4] revealed that one of the secondary metabolites that have potential as an antibacterial agent is from the class or derivative of terpenoids such as steroids. The results of Madduluri (2013) [31] mentioned the mechanism of action of steroids, namely by combining with lipid membranes, liposomes leak and reduce the integrity of the cell membrane. Therefore, the cell will be fragile and then lysed due to the changed cell membrane morphology.

Antibacterial activity was also shown in the positive control ampicillin 0.5%. The growth inhibition of the test bacteria by ampicillin was caused by ampicillin which prevented the tRNA from binding to the ribosome. This causes the protein synthesis process to be inhibited and the constituent components of the bacterial cell to change so that the cell will lyse [32].

Other samples did not perform antibacterial activity, perhaps because the samples tested were still using relatively low concentrations. As a result, the growth of the test bacteria has not been able to be inhibited by the sample at a concentration of 3%. This is also stated by Rastina (2015) [33] that samples using high concentrations will form a large inhibition zone. Another thing that is suspected as the cause of the absence of antibacterial activity is that the secondary metabolite compounds in the sample are not

Table 4. Results of Antibacterial Activity Screening

Concentration (%)	Diameter of inhibition zone (mm ± SD)
2	10.7 ± 1.2
1.5	10.3 ± 1.5
1	10.0 ± 2.7
0.5	8.7 ± 1.5
0.25	6.7 ± 0.6
0.125	6.0 ± 0.0
Methanol	6.0 ± 0.0

Diameter of the well is 6 mm

compounds that have antibacterial activity. Rastina (2015) [33] also stated that one of the factors for the formation of the inhibition zone was the type of antibacterial compound produced.

3.4 Determination of MIC Value (Minimum Inhibitory Concentration)

The MIC value was used to determine the lowest concentration of the sample that was still able to inhibit the growth of the test bacteria. The MIC value can be used to measure the level of bacterial resistance to antibiotics [34]. The MIC value of the ethyl acetate fraction was at a concentration of 0.25% and the diameter of the inhibition zone was 6.7 ± 0.6 mm. This indicates that the sample of the ethyl acetate fraction of white pacing flowers was still able to inhibit the growth of P. acnes up to a concentration of 0.25%. The results of determining the MIC values in the ethyl acetate fraction samples against P. acnes are shown in Table 4.

4 Conclusion

White pacing plant contains several secondary metabolites such as flavonoids, phenolics, saponins, tannins and steroids. Antibacterial activity was shown in the ethyl acetate fraction against *P. acnes* with an inhibition zone diameter of 11.3 mm. The MIC value of the ethyl acetate fraction against *P. acnes* was 0.25% with an inhibition zone diameter of 6.7 mm.

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