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Phytochemical and Antifungal Activity Combination of *Costus speciosus* Rhizome and *Bryophyllum pinnatum* In Vitro

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Abstract. The herbs use plants from Dayak ethnicity often used to solve infertility for women. The plants are *Costus specious* and *Bryophllum pinnatum*. This study aimed to determine the content of compounds present in the combination of *Costus specious* and *Bryophllum pinnatum* and the antifungal activity of *Candida albicans*. This research is qualitative descriptive. Costus specious and Bryophllum pinnatum extracted using a single maceration method with a water solvent. Phytochemical tests used the Meyer Test method, Dragendorf Test for alkaloids, Wilstater Test for flavonoids, Lieberman Burchard Test for triterpenoids, Forth Test for saponins, and $FeCl_3$ Test for tannins. Antifungal activity test of *Candida albicans* used in vitro test the 100% diffusion method and microdilution with a concentration of 50%, 25%, 12.5%, 6.25%, 3.13%, 1.56%, 0.78%, and 0.39%. The results of phytochemical tests showed that the combination of aqueous extracts of *Costus specious* and *Bryophllum pinnatum* is flavonoids and triterpenoids. The antifungal test showed that the combination extract inhibited the growth of *Candida albicans* with an inhibitory zone of 8.91 (moderate), 10.42 (moderate), and 8.23 (moderate). The MIC value at a concentration of 1.56% and the KBM value at a concentration of 3.13%.

INTRODUCTION

Infertility condition is the inability of a woman, over 35 years of age, to get pregnant after trying to get pregnant for at least six months or one year, without using birth control while having normal sexual relations [1]. In Indonesia, the incidence of infertile women is 15% at 30-34 years old, increasing by 30% at 35-39 years old and 55% at 40-44 years old. Possible causes of infertility are diseases affecting ovarian function, problems with eating habits, excessive weight gain, or exposure to radiation [1]. Free radicals can cause infertility, one of which is caused by leucorrhoea.

Leucorrhoea can be caused by bacteria, *Staphylococcus saprophyticus*, *Trichomonas vaginalis* parasites, and *Neisseria gonorrhoeae* virus, but abnormal leucorrhoea often caused by fungal infections, *Candida albicans.* Leucorrhoea is caused by *C. albicans*, namely vaginalis candidiasis. Vaginalis candidiasis is an infectious disease often experienced by most women that cause infertility. Vulvovaginitis candidiasis (CVV) is an infection of the vaginal and vulvar mucosa (unrelated epithelium) caused by Candida species. The most common cause of vaginitis (80-90%) is *Candida albicans* [2]. In recent years, candidiasis has increased due to factors that can affect *C. albicans* to become pathogens. This can be due to the increasing population of people with disorders of the immune system (HIV-AIDS), invasive medical procedures, organ transplants, and the use of antibiotics [3].

Treatment of infertility with existing diagnostic methods is usually treated by surgery, drug therapy, in vitro fertilization (IVF), or artificial reproductive technology (Assisted Reproduction Technology) [4]. Lately, there is a tendency for people to return to nature (back to nature) to use traditional medicines and herbal medicines for health

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[5]. This fact is also stated by the World Health Organization (WHO) reporting that 80% of people in the world currently depend on medicinal plants for their health [6]. There are more than 7,000 species of plants that are known and can be used for medicine. However, only about 250 types of plants are used as raw materials for medicine by the traditional and modern medicinal industries [7].

Plants can be used as medicine and ingredients for herbal medicine. One of the herbal products utilizing plants is the herbal Dayak herb often used as a solution to female infertility. The plants used in the Dayak tribe are *Bryophyllum pinnatum* and *Costus specious* plants. The Dayak tribe uses these plants for the treatment of women's infertility by boiling, soaking, and making strands or pills [8]. *B. pinnatum* contains several compounds from phytochemical test results, namely flavonoids, steroids, and phenols. The content of flavonoids is used for anti-inflammatory, antioxidant, antiseptic, and antifungal properties [9]. Alkaloids, flavonoids, saponins, and tannins are compounds found in *C. specious* extracts [10]. The rhizome of the *C. specious* has anti-fertility, anti-inflammatory, and anthelminthic activities. Rhizome essential oil shows microbial activity. Steroid saponins and sapogenins from the rhizome of the *C. specious* exhibit antifungal activity [11].

Plant extraction used water solvents because water is polar, generally, polar solvents will dissolve sugar, amino acids, proteins, polyglycosides, tannins, alkaloid salts, flavonoids, saponins, and polyphenols [12]. This researchis expected to provide information about the potential of natural ingredients combining the ingredients of *B. pinnatum* and *C. specious* scientifically as an anti-fungal, and to the knowledge of researchers, there has been no research combining the two plants for antifungal testing.

EXPERIMENTAL DETAILS

Material Extraction

Three combinations were prepared: A total of 10 g of *B. pinnatum* rhizome powder and 10 g of *C. specious* rhizome powder for combination I (C1), 5 g of *B. pinnatum* rhizome powder and 15 g of *C. specious* rhizome powder for combination II (C2), and 15 g of *B. pinnatum* rhizome powder and 5 g of *C. specious* rhizome powder for combination III (C3). Then, those three combinations were mixed and put into 500 ml Erlenmeyer and added with 160 mL of water solvent, then soaked for 24 hours. Then, in the shaker for three hours, the mixture was filtered with a Buchner filter and the pulp obtained was reasserted with the same solvent. This stage was carried out three times until the filtrate was clear. The macerated filtrate was concentrated by rotary evaporation at 50 °C until obtaining a concentrated extract. A combined water concentrated extract was used for the anti-fungal test.

Phytochemical Test

Phytochemical test used alkaloids, flavonoids, triterpenoids, steroids, tannins, and saponins. In the alkaloid test, the concentrated extract of each sample was added with 0.5 mL of HCl 2% and Dragendrof and Meyer reagents each (three drops each), the white-yellowish precipitate indicated the presence of alkaloids. Flavonoid test was carried out by dissolving the sample in 1-2 mL of hot methanol (50% v/v) after Mg metal and 0.5 mL concentrated HCl was added. The red or orange solution formed indicated the presence of flavonoids. In the Triterpenoid and Steroid test, the concentrated extract of each sample was put in a test tube dissolved in 0.5 mL chloroform, and then added with 0.5 mL of anhydrous acetic acid. The mixture was then added with 1-2 mL of concentrated H_2SO_4 through the tube wall. Positive results for steroids were indicated by the presence of brownish or violet rings while steroids were demonstrated by the formation of a bluish-green color. The saponin test was carried out by making a mixture of concentrated sample extract with water (1 : 1) chili shaking for one minute, if a stable foam was formed after adding two drops of 1 N HCl, then the extract was positive. The Tannin test carried out was that each sample was put in a test tube, and added 2-3 drops of 1% $FeCl_3$ solution. If the solution produced a green-black color, it indicated the presence of catechol tannin compounds and the blue-black color showed the presence of a tannin compound.

Antifungal Activity Test

The antifungal activity test was carried out by the agar diffusion method using disc paper (6 mm diameter). Fifteen petri-dishes were provided (nine for Candida albicans, and six for control). Pouring the diffusion method media into the petri dish was performed by pour plate. 100 µL of the Candida albicans suspension was poured into a petri dish then put into 20 ml of SDA (Himedia) media. A sterile disc paper soaked with water extract of *B. pinnatum* and *C.*

specious plants were prepared according to the composition for 60 mins. Positive control by immersing disc paper in nystatin and negative control using DMSO solvent. This negative control was used to ensure that the resulting inhibition zone did not come from solvents. Then, the paper disc was placed on the surface of the SDA media that had been inoculated with the fungus. Incubation at 37 °C for 24 hours. After 24 hours, a clear zone was observed around the disc paper. The diameter of the clear zone formed was measured using a caliper. The presence of a clear area around the disc paper indicated antifungal activity [13]. The inhibiting zone was measured by Formula 1 [13]:

$$\text{Inhibition zone} = \text{clear zone diameter} - \text{Paper disc diameter} \quad (1)$$

Minimum Inhibitory Concentration (MIC) and Minimum Killing Concentration (MKC) Test

The MIC method used ten wells on the microplate for each fungus with three replications. The first well was called material control. The 2nd to 9th wells were a test treatment, while the last wells were called microbial controls. The concentration series used were 50%, 25%, 12.5%, 6.25%, 3.13%, 1.56%, 0.78%, and 0.39%. The research conducted using concentration carried out on *C. albican* antifungals [14]. The 100% herbal medicine was put into 200 µL of the first well (as control material) and into 100 µL of the second well. Then, the 3rd to 10th wells were filled with 100 µL of sterile water. After that, the well 100 µL was taken and placed in the 4th well. This process was carried out until the 9th well. In the 9th well, 100 µL of a solution was discarded. Then, from the 2nd to the 10th well, for the test of antifungi 100 µL *C. albican* was added.

RESULTS AND DISCUSSION

Phytochemical Test Combination of *C. specious* and *B. pinnatum* Extracts

Based on the results of research, phytochemical tests that have been carried out show several chemical compounds contained in the combination of *C. specious* and *B. pinnatum* water extract (Table 1). The combination of those plant extracts contains flavonoids and triterpenoids. The identification of flavonoids using the wilstater test showed an orange color meaning that there were positive flavonoids. The magnesium and hydrochloric acid in the wilstater reacted to form bubbles which were H_2 gas, while the concentrated Mg and HCl metals in this test functioned to reduce the benzopyrone core contained in the flavonoid structure so that a color change was formed to orange. If a plant extract contains flavonoid compounds, flavilium salts will be formed when the orange Mg and HCl are added. The red or orange solution formed indicated the presence of flavonoids.

TABLE 1. Phytochemical test results of the combination of *C. specious* and *B. pinnatum* water extract

Sample combination	Phytochemical Test Results					
	Alkaloids		Flavonoids	Triterpenoids	Saponins	Tannins
	Meyer	Dragendrof	Wilstater	Lieberman Burchard	Forth	$FeCl_3$
I	-	-	+	+	-	-
II	-	-	+	+	-	-
III	-	-	+	+	-	-

The combination of *C. specious* and *B. pinnatum* extracts gave positive results for the terpenoid test, indicated with the formation of a violet ring at the solution boundary when added with H_2SO_4 and green color when the solution was dropped on the drop plate. The color change occurred due to oxidation in the terpenoid group of compounds through the formation of conjugated double bonds. The principle of reaction in the reaction mechanism of the terpenoid test is the condensation or release of H_2O and the incorporation of the carbocation. This reaction begins with the acetylation process of the hydroxyl group using acetic acid anhydride. The acetyl group which is the good leaving group will break free, forming a double bond. Then, there is the release of the hydrogen group and its electrons causing the double bond to move. These compounds undergo resonances acting as electrophiles or carbocation. The carbocation attack causes electrophilic addition, followed by the release of hydrogen. Then, the hydrogen group and its electrons are released, as a result of which the compound undergoes a conjugation extension which shows the appearance of a violet ring. If the result is a brownish or violet ring on the border of the two solvents, it shows

triterpenoids, whereas if it forms a bluish-green color, it indicates the presence of steroids. The compounds contained in the ethanol extract of *B. pinnatum* are flavonoids, triterpenoids, phenols, and tannins while in this study, the chemical compounds found were flavonoids and terpenoids. The results of ethanol extraction produced more compounds because ethanol is a polar solution, has a hydroxyl group that can bind chemical compounds easily. Water is semi-polar and becomes a universal solution because it is easily available and used by the public at large.

The biological activity of flavonoid compounds can damage the cell walls of *C. albicans* which consist of lipids and amino acids. This cell wall arrangement will react with alcohol groups in flavonoid compounds so that the cell walls will be damaged and these compounds can enter the nucleus of fungal cells [15]. Furthermore, through the fungal cell nucleus, this compound will contact with the DNA in the nucleus of *C. albicans* fungal cells. The polarity difference between the lipids that make up DNA and the alcohol groups in flavonoid compounds will cause a reaction so that it will damage the lipid structure of the DNA of the *C. albicans* fungus and cause the fungal cell nucleus to also lysis. Meanwhile, triterpenoids work as antimicrobials by reacting with porin (transmembrane protein), forming strong polymer bonds resulting in pore damage, porin damage resulting in reduced permeability, so that microbes lack nutrients and stunted growth [16].

Candida albicans Antifungal Test Results

The combination of *C. specious* and *B. pinnatum* can inhibit the growth of the fungus (Table 2) although it is smaller than the positive control used, namely nystatin. This is because nystatin is a single chemical compound as an antifungal, while the combination of *C. specious* and *B. pinnatum* is a macerate product that still contains many other compounds that have nothing to do with antifungal. The more effective the combination of *C. specious* and *B. pinnatum* in killing *C. albicans* colonies compared to nystatin in the inhibitory or killing power test, the chances of the extract being an antifungal source became greater as well.

TABLE 2. Antifungal test results combination of *C. specious* and *B. pinnatum* extracts against *C. albicans*

Sample	Inhibition zone diameter (mm) ± SD	Category
C 1 (*C. specious* 50 : 50 *B. pinnatum*)	9.10 ± 1.56	Moderate
C 2 (*C. specious* 75 : 25 *B. pinnatum*)	10.53 ± 0.76	Moderate
C 3 (*C. specious* 25 : 75 *B. pinnatum*)	8.67 ± 1.27	Moderate
Nystatin (Control +)	18.87 ± 0.75	Strong
DMSO (Control -)	0	-

The combination of *C. specious* and *B. pinnatum* aqueous extracts C1, C2, and C3 can produce medium category inhibition zone diameters (Figure 1). Although it has not shown optimal results, C2 can produce an average inhibition zone of 10.53 mm, while C1 and C3 only can produce an inhibition zone with an average of 9.10 and 8.67 mm. The ability of C2 having the stronger category than others is probably due to the strength of the chemical compounds contained in *C. specious* because in the C2 combination of *C. specious* composition is greater.

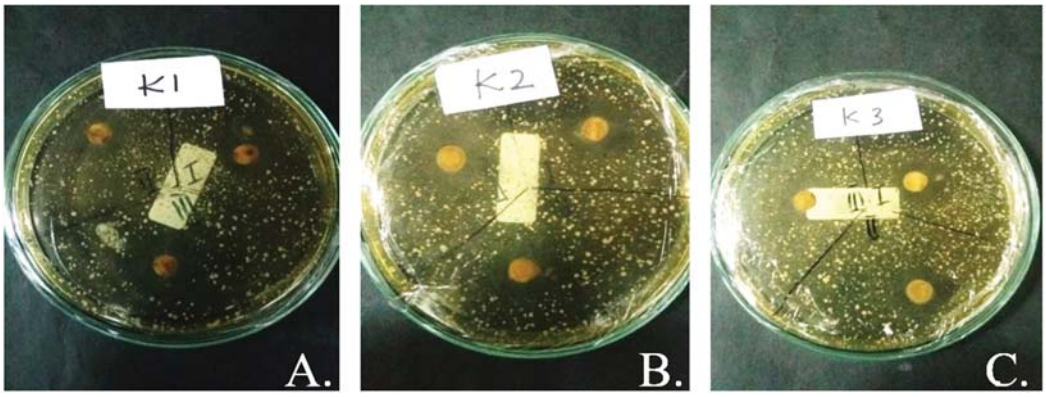

FIGURE 1. Result of A. C1 test treatment, B. C2 test treatment, and C. C3 test treatment against *C.albicans*

The concentration of the combined water extract of *C. specious* and *B. pinnatum* against *C. albicans* was still very far compared to the comparative concentration of nystatin. This is because the combination of the plant extract used was still a natural extract and not a pure compound, whereas nystatin is a relatively pure antimicrobial active substance. The antibiotic control used was nystatin with a concentration of 100% indicating an inhibition zone of 18.87 mm affecting the *C. albicans*; its inhibitory activity was in a strong category. Nystatin has antifungal activity by inhibiting sterols (especially ergosterol) in the fungal cell membrane. Nystatin is not active against bacteria because bacteria do not have sterols in their cell membranes. This is under the statement that the antifungal mechanism is grouped into four, namely interference with cell membranes, inhibition of ergosterol biosynthesis in fungal cells, inhibition of nucleic acid synthesis, and potential for fungi and inhibition of fungal mitosis [17].

Fungistatic substances inhibit the work of certain enzymes that disrupt the metabolism of fungal cells so that the process of elongation of the hyphae (mycelium) of the fungi is inhibited. If the growth of fungal cells characterized by hyphal elongation (mycelia) is inhibited, then hyphal fragmentation becomes disrupted so that it can be said that the fungal cells cannot reproduce. Hyphae that cannot undergo fragmentation is caused by the destruction of the hyphae tissue of the cell resulting in the fungal cells being sensitive and susceptible to environmental changes at the same time so that the fungal cells die easily. Adding that, compounds that are fungistatic such as phenolic compounds can denature proteins, namely damage to the tertiary structure of the protein so that the protein loses its original properties. Denaturation of the *C. albicans* wall protein will cause brittleness in the cell wall so that it is easily penetrated by other active substances that are fungistatic. If the denatured protein is an enzyme protein, the enzyme cannot work which causes metabolism and nutrient absorption to be disrupted [18].

Results of Determination of Minimum Inhibitory Concentrations (MIC) and Minimum Killing Concentrations (MKC)

The three treatment combinations gave almost the same results in the Minimum Inhibition Concentration (MIC) test. At a concentration of 0.39%, the microplate still looked cloudy, which indicated that there were still microorganisms while at a concentration of 0.78%, it showed a bit cloudy. At a concentration of 1.56%, the solution was clear, so it was stated that there was no growth of microorganisms at that concentration (Table 3). Minimum Inhibitory Concentration is the minimum concentration that can inhibit the growth of target microorganisms or prevent multiplication without killing the microorganisms [19]. MIC value lies in the last concentration where microorganism growth is still present before the concentration where there is no microorganism growth. The MIC value in the combination of *C. specious* and *B. pinnatum* extract on the growth of *C. albicans* is a concentration of 0.78%.

TABLE 3. Results of Minimum Inhibitory Concentration (MIC) combination of *C. specious* and *B. pinnatum* extract against *C. albicans*

Concentration	C1	C2	C3
Control microbes	+	+	+
0.39%	++	++	++
0.78%	+++	+++	+++
1.56%	++++	++++	++++
3.13%	++++	++++	++++
6.25%	++++	++++	++++
12.50%	++++	++++	++++
25%	++++	++++	++++
50%	++++	++++	++++
Control samples	++++	++++	++++

Notes: Very turbid: + ; Turbid: ++; Almost turbid: +++, Clear: ++++

Based on the results in Table 4, the concentration that has a minimum kill value is a concentration of 3.13%. Active extracts of *C. specious* and *B. pinnatum* could kill the *C. albicans* at a concentration of 3.13%, so it can be said that the combination of extracts was proven to be sensitive as an antifungal against *C. albicans*. The absence of a difference between the three combinations was probably due to the random combination so we did not know whether the chemical compounds contained in the three combinations were in synergy or opposite.

TABLE 4. Results of MKC combination of *C. specious* and *B. pinnatum* extract against *C. albicans*

Concentration	C1	C2	C3
Control +	1.43×10^{11}	1.74×10^{11}	1.55×10^{11}
0.390 %	1.11×10^{8}	1.43×10^{8}	1.40×10^{8}
0.780 %	1.50×10^{8}	1.26×10^{8}	1.31×10^{8}
1.56 %	5.6×10^{7}	6.3×10^{7}	6.0×10^{7}
3.13 %	0	0	0
6.25 %	0	0	0
12.50 %	0	0	0
25.00 %	0	0	0
50.00 %	0	0	0
Control -	0	0	0

SUMMARY

Water extracts of *C. specious* and *B. pinnatum* contained flavonoids and triterpenoids. The results of the measurement of disc showed that combination 2 (C2) was stronger in inhibiting the growth of *C. albicans* with an inhibition zone diameter of 10.53 mm in the moderate category. Followed by Combination 1 (C1) producing an inhibition zone of 9.10 mm in the medium category and combination 3 (C3) producing an inhibition zone of 8.67 mm in the moderate category. The combination of *Costus specious* water extract and *Bryophyllum pinnatum* had antifungal activity. The three combinations (1, 2, and 3) had the same Minimum Inhibitory Concentration (MIC), namely at a concentration of 0.78%. Meanwhile, the value of Minimal Killing Concentration (MKC) of the combination of *C. specious* and *B. pinnatum* water extract lied at a concentration of 3.13%. The best antifungal potential of the three combinations lied in combination 2 (C2).

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