

Nepenthes rafflesiana pitcher liquid has antifungal activity against *Candida* spp.

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ABSTRACT

BACKGROUND

To develop new effective antifungals, it is essential to search for antifungal compounds from plants such as *Nepenthes* spp., which have their greatest diversity in Indonesia. Since chitin-induced liquid (CIL) from *Nepenthes khasiana* pitchers has antifungal activity, due to their naphthoquinone content, this study aimed to evaluate antifungal activity of *Nepenthes rafflesiana* pitcher liquids on *Candida* spp.

METHODS

Collected pitcher liquids were of 3 types: non-induced liquid (NIL), prey-induced liquid (PIL), and chitin-induced liquid (CIL). Non-induced liquid (NIL) was collected from fresh naturally opened pitchers, PIL from opened pitchers after 3 hours of induction with *Zophobas morio* larvae, and CIL from closed pitchers after 5 days of chitin solution injection. The antifungal activity of the liquids against *C. albicans*, *C. glabrata*, *C. krusei*, and *C. tropicalis* were detected by disc diffusion and macrodilution methods.

RESULTS

Inhibition zone diameters of NIL, PIL, and CIL against *C. albicans* were 35.00 (35.00 – 39.33) mm, 26.33 (23.00 – 40.00) mm, and 30.00 (28.00 – 32.00) mm, respectively, while for *C. glabrata* the zone diameters were 22.22 ± 3.66 mm, 29.89 ± 2.79 mm, and 28.89 ± 1.17 mm, respectively. No inhibition zones were found for NIL, PIL, and CIL against *C. krusei* and *C. tropicalis*. At concentrations of 80%, almost all samples showed visually apparent inhibition of fungal growth.

CONCLUSION

The pitcher liquid of *N. rafflesiana* has antifungal properties, presumably due to the presence of many potentially active substances, such as naphthoquinones, as has been proven in other studies.

Key words : Antifungal, chitin-induced, disc diffusion, *Nepenthes*, *Candida*

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Aktivitas antijamur cairan kantong *Nepenthes rafflesiana* terhadap *Candida* spp.

ABSTRAK

PENDAHULUAN

Untuk mengembangkan obat antijamur baru yang efektif, adalah sangat penting untuk mencari senyawa antijamur dari sumber alamiah, seperti *Nepenthes* spp., yang paling aneka ragam di Indonesia. Karena cairan kantong *Nepenthes khasiana* yang telah diinduksi kitin memiliki aktivitas antijamur akibat kandungan naftokuinon, penelitian ini bertujuan untuk menilai aktivitas antijamur *Nepenthes rafflesiana* terhadap *Candida* spp.

METODE

Cairan kantong yang dikumpulkan ada 3 jenis yaitu: cairan yang tidak diinduksi (T), cairan yang diinduksi mangsa (M), dan cairan yang diinduksi kitin (K). T diambil dari kantong yang baru terbuka secara alami, M diambil dari kantong terbuka yang telah diinduksi larva *Zophobas morio* selama 3 jam, dan K diambil dari kantong tertutup yang telah disuntikkan larutan kitin selama 5 hari. Aktivitas antijamur ketiga jenis cairan tersebut terhadap *C. albicans*, *C. glabrata*, *C. krusei*, dan *C. tropicalis* dideteksi dengan menggunakan metode difusi diskus dan makrodilusi.

HASIL

Diameter zona inhibisi tampak pada uji T, M, dan K terhadap *C. albicans* [35,00 (35,00 – 39,33) mm, 26,33 (23,00 – 40,00) mm, dan 30,00 (28,00 – 32,00) mm] dan *C. glabrata* (22.22 ± 3.66 mm, 29.89 ± 2.79 mm, dan 28.89 ± 1.17 mm). Pada uji T, M, dan K terhadap *C. krusei* dan *C. tropicalis* tidak tampak adanya zona inhibisi. Pada konsentrasi 80% didapatkan berkurangnya pertumbuhan jamur secara visual pada hampir semua sampel uji.

KESIMPULAN

Cairan kantong *N. rafflesiana* memiliki aktivitas antijamur, karena mengandung beberapa zat potensial, antara lain adalah naftokuinon.

Kata kunci : Antijamur, diinduksi kitin, difusi diskus, *Nepenthes*, *Candida*

INTRODUCTION

The emergence of the human immunodeficiency virus infection, modern patient-management technologies and therapy, such as bone marrow and solid-organ transplants, and the use of more aggressive chemotherapy have resulted in a rapidly expanding number of immunocompromised patients highly susceptible to fungal infections,⁽¹⁾ the most common being candidiasis. Because of increasing use of antifungals and the limited types of antifungals, the development of resistance is an important issue. In addition, adverse effects, drug-drug interactions, and toxicity, are also factors that influence the use

of antifungals. Therefore, exploration to find new and effective compounds for antifungal chemotherapy is of pivotal importance.⁽²⁾

Exploration of natural products that may have antifungal properties has been increasing. Naphthoquinones are a group of secondary metabolites with antifungal properties occurring in a number of plant families, such as the *Nepenthaceae*.⁽⁵⁾ *Nepenthes* are carnivorous plants that have developed insect capturing-traps, evolved by specific modification of the leaf tips, and capable of utilizing digested insects. *Nepenthes khasiana*, commonly found in India, has been found to contain naphthoquinones, especially droserone and 5-O-methyl droserone.^(3,4)

Indonesia, as a tropical country, has many species of *Nepenthes*, such as *Nepenthes rafflesiana*, since the distribution of *Nepenthes* is predominantly in Indonesia, especially Borneo. The liquid inside the pitchers is traditionally used for relieving eye disorders, cough, stomach ache, burn injuries, and skin diseases.⁽⁶⁾

Several studies have been conducted to detect antifungal activity in *Nepenthes*, but none of them have explored antifungal activity in pitcher fluid of *N. rafflesiana*.^(3,7,8) A study by Eilenberg et al.⁽³⁾ on the antifungal effects of chitin-induced liquid (CIL) from *N. khasiana* to *Candida* spp. concluded that only CIL efficiently inhibited fungal pathogens.⁽³⁾ It has been suggested that either chitin itself or its breakdown products induce the synthesis of antifungal agent/s, that are not produced under natural conditions either in closed or opened pitchers. However, subsequently many enzymes, such as endochitinases, were detected inside closed pitchers.⁽⁹⁾ Since chitin is one of the major components of fungal cell walls,⁽¹⁰⁾ these results suggested that closed pitchers may have antifungal effects as a result of compounds other than naphthoquinones, e.g. chitinases.

In this study, we used 3 types of pitcher liquid of *N. rafflesiana*, i.e. non-induced liquid (NIL), prey-induced liquid (PIL), and CIL. The objective of this research was to determine if pitcher liquids of *N. rafflesiana* have antifungal activity on tested *Candida* spp. and have significant differences in activity. *N. rafflesiana* is a lowland pitcher plant and thus easy to cultivate in Jakarta. The pitcher liquid of *N. rafflesiana* has the unique characteristic of being the most viscoelastic. A 95%-dilution in water still retains enough viscoelasticity to capture all insects dropped into the pitcher.⁽¹¹⁾

METHODS

Research design

The design of this research was experimental laboratory. This research was done in Medical School, Atma Jaya Catholic

University of Indonesia, from March 2013 to November 2013. The first six months were used to collect the 3 types of pitcher liquid until we had enough stock. The next months were used to conduct the antifungal activity tests.

Plant materials

N. rafflesiana plants were obtained from Borneo island and looked after in the garden of the Medical School of Atma Jaya Catholic University of Indonesia. We used pitcher liquid from the lower pitchers of *N. rafflesiana* that were more than 7 cm in height, measured from the base to the spur of the pitcher. We used larvae of *Zophobas morio* as prey for induction. The chitin solution was made from dried prawn exoskeletons. For fungal preparation and disc diffusion test, we used Sabouraud dextrose agar (Difco, France), Mueller Hinton agar (Difco, France) with the addition of 2% glucose and 0.5 mg/L methylene blue.⁽¹²⁾ The discs were 5 mm in diameter, cut out of Whatman filter paper No. 42. For the macrodilution method, we used Sabouraud dextrose broth (Difco, France). As test fungi we used four *Candida* species, viz. *Candida albicans*, *Candida glabrata*, *Candida krusei*, and *Candida tropicalis*, obtained from the fungal collection of the Parasitology Department, Medical Faculty, University of Indonesia.

Chitin extraction

The chitin solution was prepared from ground dried prawn exoskeletons, which were washed by boiling with distilled water for 1 hour. The mixture was centrifuged to precipitate the solid phase, which was separated by filtration through filter paper. The solid phase was stirred in an ethanol : ether : HCl mix (38:38:0.8, v:v:v) for 15 minutes, then the mixture was filtered through filter paper. Thereafter, the solid phase was solubilized in 0.2 M NaClO for 1 hour at 75°C. After an additional filtering, the solid phase was solubilized in acetone and 32% HCl (1:150, v:v) at 4°C. After centrifugation, the supernatant (solubilized chitin) was collected and the chitin

was precipitated by adding ice-cold water and incubating at 4°C overnight. The acid was removed by subsequent washes with cold water until the pH of the supernatant reached 2.5. The prepared chitin was allowed to dry on a filter paper, then 80 mg of the chitin was homogenized with 10 mL distilled water, and the pH of the colloidal chitin adjusted to 5 by addition of 1 N NaOH.^(3,4,13)

Collection of pitcher liquid

The NIL, PIL, and CIL were collected as follows. We wrapped target-pitchers with sealed plastic. NIL was collected from fresh naturally opened pitchers (opened less than 24 hours). PIL was collected from opened pitchers after 3 hours of *Z. morio* induction. CIL was collected from closed pitchers after 5 days of 2 ml chitin injection (8 mg/mL chitin solution).^(3,4) All of the liquids were stored in dark bottles at 4°C.⁽¹⁴⁾ Before being used for the disc diffusion tests, the samples were incubated in a water bath at 50°C for an hour. We collected the liquid from 20 plants of *N. rafflesiana*.

Fungal preparation

All *Candida* species were subcultured into Sabouraud dextrose agar at 37°C for 24 hours.^(15,16) The inocula were prepared from 24-hour old cultures of *Candida* spp. The colonies were suspended in sterile saline, then the turbidity of the homogenous suspension was adjusted by spectrophotometry to ~0.5 McFarland standard at 530 nm. This stock suspension was used for the disc diffusion method. For macrodilution, this stock suspension was diluted 1:2000 in Sabouraud dextrose broth.^(12,15,16)

Disc diffusion test

The 24-hour cultures of *Candida* spp. were cultured onto Mueller Hinton agar with the addition of 2% glucose and 0.5mg/L methylene blue, by dropping 10 µL fungal suspension onto the agar and spreading it over the entire surface by sterile swab to obtain a confluent growth.⁽¹²⁾

The sterile discs were soaked in NIL, PIL, or CIL and placed on the seeded surface.⁽¹⁷⁾ A sterile disc soaked in aquabidest and similarly placed on the seeded medium was used as a control. The plates were incubated at 37°C for 24 hours, then examined for inhibition zones around the paper discs, indicating antifungal activity. The inhibition zone diameters (in millimeters) were measured in triplicate at the point at which there was a prominent reduction in growth, repeating the measurements in different diagonals, and calculating their mean value. All disc diffusion tests were performed in triplicate.

Macrodilution

We used pitcher liquid in concentrations of 20%, 50%, and 80%. A volume of 1000 µL fungal suspension was used as control. The mixtures were incubated at 37°C for 24 hours, then their turbidities were compared with that of the control. These tests were also performed in triplicate.

Statistical analysis

Inhibition zone diameters of pitcher liquids were compared using one-way Anova test or Kruskal-Wallis test. For all *Candida* spp. except *C. glabrata*, the test results are shown as median (minimum – maximum) because they were non-normally distributed and were tested by Kruskal-Wallis test. The normally distributed *C. glabrata* data were tested by one-way Anova, with the results shown as mean (\pm standard deviation). Results were considered significant if $p < 0.05$.

RESULTS

The color of NIL and PIL was a cloudy white, with PIL being more cloudy than NIL. On chitin induction, the color of the pitcher liquid changed to orange red (Figure 1). The pH values of NIL, PIL and CIL were 3.1, 3.0, and 2.6, respectively. All samples were slimy.

The tested pitcher liquids exhibited different degrees of antifungal activity against *Candida*



Figure 1. Pitcher liquid of *N. rafflesiana*: non-induced liquid (NIL) (A), prey-induced liquid (PIL) (B), and chitin-induced liquid (CIL) (C)

spp. NIL, PIL, and CIL inhibited the growth of *C. albicans* and *C. glabrata*. The diameters of the inhibition zones found on testing of NIL, PIL, and CIL against *C. albicans* were 35.00 (35.00 – 39.33) mm, 26.33 (23.00 – 40.00) mm, and 30.00 (28.00 – 32.00) mm, respectively. For tests against *C. glabrata*, the inhibition zone diameters of NIL, PIL, and CIL were 22.22 ± 3.66 mm, 29.89 ± 2.79 mm, and 28.89 ± 1.17 mm, respectively. No inhibition zones were found in NIL, PIL, and CIL tests against *C. krusei* and *C. tropicalis*. (Table 1 and Figure 2).

By Kruskal-Wallis test, there were no significant differences in inhibition zones among the 3 types of pitcher liquid against *C. albicans* ($p=0.298$) (Table 1). In contrast, for tests against

C. glabrata, using one-way Anova, significant differences were found between 2 types of pitcher liquid ($p=0.027$) (Table 1). By post-hoc LSD test, there were significant differences in inhibition zones between NIL and PIL ($p=0.014$), and between NIL and CIL ($p=0.025$), but no significant differences between PIL and CIL ($p=0.671$).

The concentrations of NIL and PIL that showed decreased growth of *C. albicans*, *C. glabrata*, *C. krusei*, and *C. tropicalis*, were observed visually to be 80%, 50%, 80%, and 50%, respectively. On the other hand, the CIL concentrations showing decreased growth of *C. albicans*, *C. glabrata*, *C. krusei*, and *C. tropicalis* were observed visually to be 80%, 20%, 80%, and 80%, respectively (Table 2).

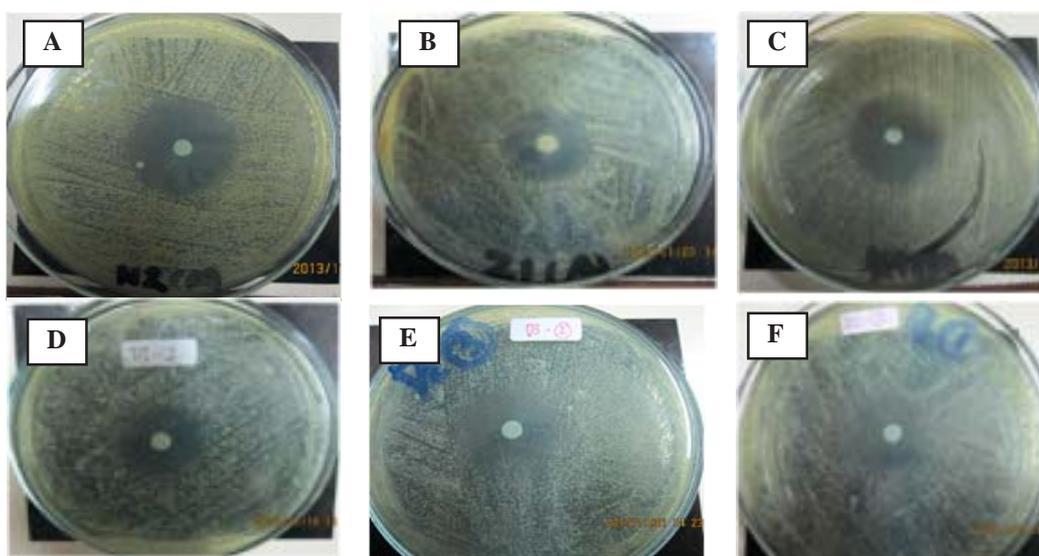


Figure 2. Results of disc diffusion test of non-induced liquid (NIL), prey-induced liquid (PIL), and chitin-induced liquid (CIL) to *C. albicans* and *C. glabrata*. Inhibition zone of NIL to *C. albicans* (A), PIL to *C. albicans* (B), CIL to *C. albicans* (C), NIL to *C. glabrata* (D), PIL to *C. glabrata* (E), CIL to *C. glabrata* (F)

Table 1. Inhibition zone of NIL, PIL, and CIL against *Candida* spp. growth in vitro

Fungi	Pitcher liquid	Diameter of inhibition zone (mm)	p value
<i>C. albicans</i>	NIL	35.00 (35.00 – 39.33) [†]	0.298*
	PIL	26.33 (23.00 – 40.00) [†]	
	CIL	30.00 (28.00 – 32.00) [†]	
<i>C. glabrata</i>	NIL	22.22 ± 3.66 [‡]	0.027**
	PIL	29.89 ± 2.79 [‡]	
	CIL	28.89 ± 1.17 [‡]	
<i>C. krusei</i>	NIL	no zone	
	PIL	no zone	
	CIL	no zone	
<i>C. tropicalis</i>	NIL	no zone	
	PIL	no zone	
	CIL	no zone	
Control		no zone	

NIL : non-induced liquid; PIL : prey-induced liquid; CIL : chitin-induced liquid; [†] Data are shown as median (minimum – maximum) because data distribution was not normal; [‡] Data are shown as mean ± standar deviation because data distribution was normal; * Kruskal-Wallis test; ** One-way Anova. Post-hoc LSD test : NIL vs PIL p=0.014; NIL vs CIL p = 0.025; PIL vs CIL p = 0.671

DISCUSSION

The CIL color change into orange red was similar to that found by Eilenberg et al. in CIL of *Nepenthes khasiana*.⁽³⁾ According to these investigators, the color change was associated with the presence of droserone (3,5-dihydroxy-2-methyl-1,4-naphthoquinone), which has antifungal properties. The pH of NIL, PIL, and CIL was acidic, as has also been determined by several studies, which found that the pH of most pitcher fluid is acidic, rarely neutral.^(18,19) *N. rafflesiana* has the unique characteristic of its

highly viscoelastic fluid, differing in this respect from other *Nepenthes* species.

According to a Clinical Laboratory Standard Institute document, the sensitivity to fluconazole, one of the standard treatments of candidiasis, is defined as an inhibition zone of more than 19 mm.^(12,15) Since the inhibition zones of NIL, PIL, and CIL towards *C. albicans* and *C. glabrata* were more than 19 mm, these results indicate that *C. albicans* and *C. glabrata* were susceptible to *N. rafflesiana* pitcher liquid. In our study, PIL and CIL showed better inhibition than NIL against *C. glabrata*.

Table 2. Macrodilution test NIL, PIL, and CIL against *Candida* spp. (in % sample)

Types of pitcher liquid	Concentration (%)	<i>C. albicans</i>			<i>C. glabrata</i>			<i>C. krusei</i>			<i>C. tropicalis</i>		
		+	±	-	+	±	-	+	±	-	+	±	-
NIL	20	100	0	0	100	0	0	100	0	0	66.67	33.33	0
	50	100	0	0	0	100	0	100	0	0	0	100	0
	80	0	33.33	66.67	0	100	0	0	100	0	0	100	0
PIL	20	100	0	0	100	0	0	100	0	0	33.33	66.67	0
	50	100	0	0	0	100	0	66.67	33.33	0	0	100	0
	80	0	100	0		66.67	33.33	0	100	0	0	100	0
CIL	20	100	0	0	0	100	0	100	0	0	66.67	33.33	0
	50	100	0	0	0	100	0	100	0	0	33.33	66.67	0
	80	0	100	0	0	0	100	0	100	0	0	0	100

NIL : non-induced liquid; PIL : prey-induced liquid; CIL : chitin-induced liquid; + : turbidity equal to control; ± : turbidity less than control; - : no turbidity

Eilenberg et al.⁽³⁾ and Raj et al.⁽⁴⁾ stated that naphthoquinones, which have antifungal properties, are only detected in red-colored CIL. Naphthoquinones are apparently chromatic pigments.⁽⁵⁾ However, in the present study, we found that NIL and PIL, which are not red in color, also have an antifungal effect. It is possible that NIL and PIL of *N. rafflesiana* contain other substances with antifungal properties, which do not give a red color to pitcher liquid.

No inhibition zones were produced in disc diffusion tests of NIL, PIL, and CIL against *C. krusei* and *C. tropicalis*, which agrees with the fact that *C. krusei* and *C. tropicalis* are commonly more resistant to antifungal treatment.⁽²⁰⁾ According to the European Committee on Antimicrobial Susceptibility Testing, the drug dosages for MIC₅₀ and MIC₉₀ of both of fungi, especially *C. krusei*, are higher than those for the other *Candida* species.⁽²¹⁾ The CIL concentration that inhibited *C. albicans* was about 3-fold and 6-fold lower than that required for growth inhibition of *C. krusei* and *C. glabrata*, respectively.⁽³⁾ In this study, we used crude liquids that contain many substances, therefore the concentrations of the active compounds were presumably lower than necessary to obtain inhibition zones against *C. krusei* and *C. tropicalis*. However, this may have also been caused by the intrinsic resistance of the fungi.

In the macrodilution test, we observed the turbidity of the test samples, compared to control. Most of them showed inhibition to tested fungi at 80% pitcher liquid concentration. Decreased growth of *C. glabrata* was shown at 20% CIL, compared with 50% NIL and PIL (Table 2). Pitcher liquids showed growth inhibition against *C. krusei* and *C. tropicalis* at 80%, except for inhibition of NIL and PIL against *C. tropicalis*, which were at 50%. These results support the idea of fungal activity of pitcher liquids towards *C. krusei* and *C. tropicalis*, although no inhibition zones were detected in disc diffusion test.

Chitin injection into closed pitchers triggers the synthesis of antifungals and certain chitinases.⁽³⁾ As yet, the mechanism underlying the pitcher response to chitin in *Nepenthes* is not clear and may include both the induced synthesis of new compounds and/or the release of existing compounds from the pitcher gland cells to the trap liquid. The presence of naphthoquinones in the pitcher of *Nepenthes* is important for chemical defense.⁽³⁾ In addition, chitinases hydrolyze chitin which is a major component of fungal cell walls.^(7,10,22) Chitinase was also identified in the digestion fluid of closed pitchers, not only in chitin-induced pitchers.⁽⁹⁾ Therefore it may be assumed that chitinase is also potentially antifungal.

The difficulties of this study were how to predict the right time to do chitin induction before opening the pitcher and the phenomenon of non-producing pitchers after several times of taking pitcher liquids. Obtaining pitcher liquid at precisely the right time is important to get as much as liquid we can. Taking liquids was only done once, and no repetition was performed because of the contamination risk. Using special treatments to plants may have to be done to promote the production of pitcher liquid.

Exploration about potentially active substances in the pitcher liquid of *Nepenthes* should be encouraged. Naphthoquinones and chitinase are potential future antifungals. Many species of *Nepenthes* in Indonesia have not been explored, although most of *Nepenthes* species originate from Indonesia.

CONCLUSION

The pitcher liquid of *N. rafflesiana* has antifungal properties due to the presence of many potentially active substances, especially against *C. albicans* and *C. glabrata*.

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