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Antifungal activity of neem leaf ethanol extract on *Aspergillus flavus*

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ABSTRACT

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BACKGROUND
Aspergillosis is an infectious disease due to inhalation of *Aspergillus* spores. Drug resistance to aspergillosis has been reported since 14 years ago. Therefore, alternative treatment using herbals began to attract the attention of the medical profession and the community, because herbals are relatively safe and have minimal side effects. Neem (*Azadirachta indica)* is an herb with broad spectrum biological activity, but studies on the antifungal activity of neem leaves are lacking. The objective of this study was to evaluate the activity of neem leaves on *Aspergillus flavus (A. flavus)*.

METHODS
This was a laboratory experimental study using *A. flavus* samples obtained from bronchial washing specimens of inpatients. Neem leaf extract in ethanol (NLE) was prepared in various concentrations. *Aspergillus. flavus* antifungal activity of NLE were assessed by disc diffusion (using NLE at 1.0; 0.75; 0.50; 0.25 g/dL) and macrodilution (using NLE at 1.0; 0.75; 0.5; 0.25; 0.125 g/dL). Ketoconazole 0.1 g/dL was used as antifungal positive control. Disc diffusion and macrodilution were conducted using Sabouraud dextrose agar and broth, to measure inhibition zone diameter and minimal inhibitory concentration (MIC), respectively. Statistical analysis was performed using Mann-Whitney test from SPSS 15.

RESULTS
There were significant differences in inhibition zone diameter ($p = 0.034$) between *A. flavus* samples exposed to NLE (1 g/dL) and the control group exposed to ketoconazole (0.1 g/dL). The MIC of NLE against *A. flavus* was 0.5 g/dL.

CONCLUSIONS
Neem leaf ethanol extract has antifungal activity against *A.flavus* in vitro.

Key words : *A. flavus*, neem, *Azadirachta indica*, antifungal

Aktivitas antijamur ekstrak daun mimba dalam etanol terhadap Aspergillus flavus

ABSTRAK

LATAR BELAKANG

Aspergilosis adalah penyakit infeksius yang disebabkan oleh inhalasi spora Aspergilus. Resistensi obat terhadap aspergilosis telah dilaporkan sejak 14 tahun yang lalu. Oleh sebab itu, alternative pengobatan menggunakan tanaman obat mulai menjadi perhatian di kalangan medis dan masyarakat awam karena sifatnya relatif aman dan efek samping yang minimal. Mimba (Azadirachta indica) merupakan tanaman obat yang memiliki aktivitas biologis yang sangat banyak, namun demikian informasi mengenai aktivitas antijamur dari daun mimba masih jarang. Tujuan penelitian ini adalah untuk mengevaluasi aktivitas daun mimba terhadap pertumbuhan A.flavus.

METODE

Desain penelitian adalah eksperimental in vitro. A. flavus berasal dari bilasan bronkus pasien. Ekstrak etanol daun mimba (EEDM) dibuat dalam berbagai konsentrasi. Metode yang digunakan adalah tes disc diffusion EEDM (1,0; 0,75; 0,5; 0,25 g/dL) dan makrodilusi EEDM (1,0; 0,75; 0,5; 0,25; 0,125 g/dL). Antifungi yang digunakan sebagai kontrol positif adalah ketoconazole 0,1 g/dL. Medium yang digunakan untuk tes disc diffusion dan makrodilusi adalah Sabouroud dextrose agar (SDA) dan Sabouraud dextrose broth (SDB). Tes tersebut untuk mendapatkan diameter zona inhibisi dan konsentrasi inhibisi minimal. Analisa statistik menggunakan Mann-Whitney dari program SPSS 15.

HASIL

Terdapat perbedaan zona inhibisi yang bermakna (p=0,034) antara A. flavus yang terpajan kelompok EEDM (1 g/dL) dan kelompok ketoconazole (0,1 g/dL). Konsentrasi inhibisi minimal EEDM terhadap pertumbuhan A. flavus adalah 0,5 g/dL.

KESIMPULAN

Ekstrak etanol daun mimba memiliki aktivitas antijamur terhadap pertumbuhan A. flavus pada percobaan in vitro.

Kata kunci : *A. flavus, mimba, Azadirachta indica, antijamur*

INTRODUCTION

Aspergillosis is a fungal disease caused by inhalation of *Aspergillus* spores.[1] Aspergillosis morbidity and mortality have been increasing in recent years. Aspergillosis varies in clinical manifestations according to its non-invasive or invasive nature.[1-4] Amphotericin B and itraconazole are the drugs of choice that are currently widely available for treatment of aspergillosis.[2-4] However, these drugs can cause side effects, such as dry mouth, nausea, vomiting, extreme drowsiness, muscle pain or weakness, more or less than normal urinary output, fever, and bleeding.[2-4] The mortality rate of invasive aspergillosis is 50% in patients who have received antifungal therapy. Cases of drug resistance have been reported since 14 years ago.[3-4]

Aspergillus flavus is the second leading cause of invasive aspergillosis after *Aspergillus fumigatus*. Experiments in mice showed that invasive infection caused by *A. flavus* was 100 times more virulent than infection by *A.*

fumigatus.[5] Recently, drug resistance has attracted the attention of the medical profession and the public. These have turned to the use of medicinal plants as alternative therapy for treating infectious diseases,[1] and the use of medicinal plants has increased due to their minimal side effects.[6] For more than 2000 years, neem (*Azadirachta indica* A. Juss., Meliaceae) has been known as an herb with a broad spectrum of biological activity.[6] Neem leaves have been used for their antimicrobial, antifungal, antiviral, or anthelminthic activities, and also to lower blood glucose levels, for healing of skin diseases, for their gastroprotective effect, and to lower total serum cholesterol.[7-8] In this study, we examined the antifungal activity of an ethanolic extract of neem leaves (which were obtained from Karyasari Botanical Garden, Indonesia) on *A. flavus* samples from humans. This is in contrast to most of the other studies that used *A. flavus* from plants.[9-11]

METHODS

Research design

This was a laboratory experimental study carried out at the Laboratory of Chemistry, Faculty of Medicine, University of Indonesia, and the Laboratories of Biochemistry and Parasitology, School of Medicine, Atma Jaya Catholic University of Indonesia. The study began in September and was completed in December 2012.

Preparation of neem extract

Fresh neem leaves were cut into small pieces and then allowed to dry for at least 2-3 weeks (no direct sunlight), then blenderized into powder. One hundred grams of dried neem leaf powder was extracted with 500 mL of 90-95% alcohol for 8 hours using a Soxhlet extractor. The extracted solution was evaporated by distillation (by heating in a water bath at a temperature of 70-75^{0}C). The extract, still containing traces of alcohol, was dried in a dessicator with silica gel. One hundred grams of dried neem leaf powder produced about 10 grams of neem leaf extract (Figure 1). The pure neem leaf extract was diluted with S*abouraud dextrose broth* (SDB) for assessment of the minimal inhibitory concentration (MIC) and with twice-distilled water for disc diffusion, in both cases to concentrations of 1.0 g/dL, 0.75 g/dL, 0.5 g/dL, 0.25 g/dL, and 0.125 g/dL.

Figure 1. Neem leaf ethanol extract (NLE)

In vitro tests

A.flavus samples were collected at the Parasitology Department, Faculty of Medicine, University of Indonesia, from bronchial washing specimens and then cultured. *A.flavus* samples were incubated at 37^{0}C for 48 hours before the tests. The 5-mm diameter discs were made from Whatman paper no 42 and impregnated with NLE. A fungal suspension equivalent to a McFarland no. 0.5 standard was prepared in 2 mL NaCl 0.9%.[12] The diameters of the inhibition zones were measured to the nearest millimeter. MICs were determined visually. All test groups were incubated for 24 hours. All experiments were performed in triplicate. In the disc diffusion method to detect antifungal activity, 6 groups were used: a negative control group consisting of *A. flavus* culture (AFC) in Sabouraud dextrose agar (SDA) only, group A (AFC and neem leaf ethanol extract (NLE) discs at 1.0 g/dL), group B (AFC and NLE 0.75 g/dL), group C (AFC and NLE 0.50 g/dL), group D (AFC and NLE 0.25 g/dL), and a positive control group (AFC and ketoconazole 0.1 g/dL discs).

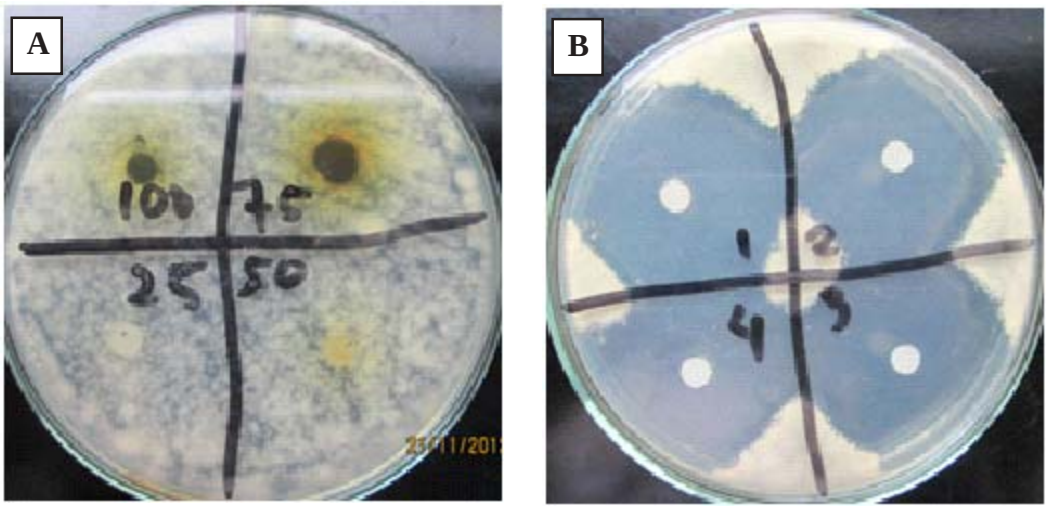

Figure 2. Disc diffusion test results of NLE (A) at concentrations of 1.0 g/dL; 0.75 g/dL; 0.50 /dL; 0.25 g/dL and ketoconazole (B) at concentrations of 0.1 g/dL (1); 0.05 g/dL (2); 0.025 g/dL (3); 0.0125 g/dL (4)

There were 7 groups in the macrodilution method: a negative control group (AFC in SDB), group A (AFC and NLE 1.0 g/dL), group B (AFC and NLE 0.75 g/dL), group C (AFC and NLE 0.50 g/dL), group D (AFC and NLE 0.25 g/dL), group E (AFC and NLE 0.125 g/dL), and a positive control group (AFC and ketoconazole 0.1 g/dL).

Data analysis

Statistical analysis was performed using the Mann-Whitney test in SPSS 15, for analyzing disc diffusion results between the groups exposed to NLE 1.0 g/dL and ketoconazole 0.1 g/dL, respectively.

RESULTS

Disc diffusion test

Disc diffusion test results of NLE against *A. flavus* showed no inhibition zone (Figure 2A), compared to the control group which showed inhibition zones 35-40 mm in diameter (Figure 2B). Statistical analysis by Mann-Whitney test showed significant differences in inhibition zone diameters (p=0.034) between groups of *A. flavus* exposed to 1.0 g/dL NLE and those exposed to 0.1 g/dL ketoconazole (Table 1).

Macrodilution test

At the concentrations of 1.0 g/dL, 0.75 g/dL, and 0.50 g/dL NLE (tubes number 1-3 in Figure 3), the test showed no turbidity visually. At the concentrations of 0.25 and 0.125 g/dL NLE, the test revealed turbidity at the surface of the medium. All of the ketoconazole concentrations revealed no turbidity. Therefore the minimum concentration of NLE needed to inhibit *A. flavus* growth was 0.5 g/dL.

DISCUSSION

In this study, the final neem leaf ethanol extract (NLE) was a dark green sticky solution. The extraction process used ethanol as a polar solvent, which has the ability to extract most of the compounds contained in neem leaves, such as triterpenoids (nimbin, nimbidin, azadirachtin).[9,13] Mondali et al.[9] reported that neem extracted with ethanol had a greater the inhibitory effect on *A. flavus* than neem extracted with water. In the disc diffusion test for NLE against *A. flavus*, no inhibition zones were found, which were however present in the test for ketoconazole (Figure 2B). An inhibition zone of more than 10 mm in diameter indicates that the test substance effectively

Table 1. Median *A. flavus* inhibition zone (mm) difference between 1.0 g/dL NLE and 0.1 g/dL ketoconazole

	n	Median	p
Inhibition zone of NLE groups	3	0	0.034*
Inhibition zone of ketoconazole groups	3	40	

*Mann-Whitney test

inhibits fungal growth.[14] The present study differs from a previous study conducted by Rai et al. who concluded that neem extract expressed an inhibition zone of 0.4 cm in diameter.[15] However, these authors did not specify which solvent they used in the preparation of the neem extract. Among the many factors that could influence the results of the disc diffusion test are the ability of the test substance to diffuse into the medium, interaction between test substance and test microbes, the amount of inoculated microbes, the growth rate of the microbes, and the sensitivity of the microbes to the test substance.[12,16-19] In our experimental study, the reason why no inhibition zones were produced by NLE in disc diffusion method was probably the low ability of NLE to diffuse. The differences in results between our study and that of Rai et al. were presumably caused by differences in the human specimens used and the origin of the neem plants. Rai et al. used *A.flavus* obtained from otomycotic patients.[15]

Observations in the macrodilution test were made after incubating the tubes for 48 hours. No turbidity was seen in the 1.0 g/dL, 0.75 g/dL, and 0.50 g/dL tubes (tubes 1-3 in Figure 3), signifying absence of visible fungal growth at these concentrations. These results are consistent with the findings of Mahmoud et al., where 5% neem extract in ethanol yielded inhibition of about 44%.[20] The higher concentrations of neem extract in ethanol inhibited *A.flavus* growth to a larger extent.[20] The limitations of this study were the difficulties in the preparation of the various concentrations of neem extract, because of the extremely sticky nature of NLE, thus hindering the achievement of consistency of fungal suspension turbidity for each triplicate. The clinical implication of this study is to increase the use of natural resources for healing diseases. We hope that neem leaf ethanol extract can be used as an alternative treatment for aspergillosis. Further studies are needed to determine the antifungal activity of neem extract in other solvents, achieve

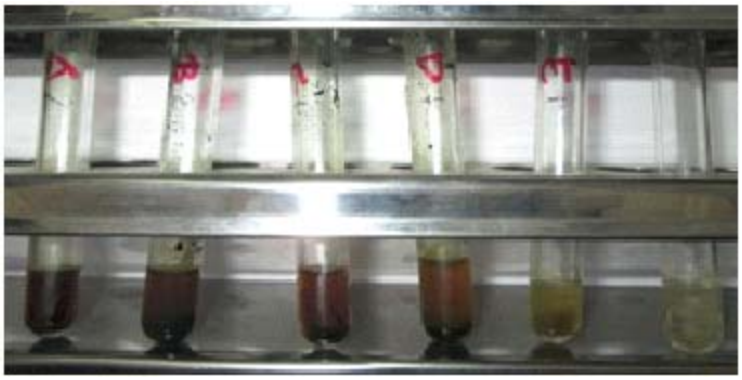

Figure 3. Macrodilution test results (from left to right) of NLE at 1.0 g/dL; 0.75 g/dL; 0.50 g/dL; 0.25 g/dL; 0.125 g/dL; negative control

consistency of fungal suspension turbidity by spectrophotometry, and use more diverse and more numerous fungal samples.

CONCLUSION

Neem leaf ethanol extract has antifungal activity against *A. flavus*. The minimum concentration of NLE to inhibit *A. flavus* growth was 0.50 g/dL.

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