

## Hexane neem leaf extract more potent than ethanol extract against *Aspergillus flavus*

Jenny Hidayat\*, Ay Ly Margaret\*, Hanna Yolanda\*\*, and Lies K. Wibisono\*

### ABSTRACT

#### BACKGROUND

*Aspergillus flavus* is one of the causes of aspergillosis, with a high virulence and resistance to standard antifungals, resulting in a high mortality rate. Medicinal plants are increasingly used as they are relatively safer with minimal side effects. Previously we found that the ethanol extract of neem (*Azadirachta indica* A Juss) leaves inhibits *A. flavus* growth in vitro. However, most chemical compounds with antifungal effect are nonpolar. The purpose of this research was to compare the antifungal effect of neem leaves extracted in a nonpolar solvent to that of leaves extracted in a polar solvent.

#### METHODS

An in vitro experimental research was conducted between October 2013 and January 2014. Neem leaves were extracted in ethanol or hexane at various concentrations. A macrodilution test with 48-hour incubation time was done in triplicate on 8 groups of samples. These comprised the neem leaf ethanol extract (NLEE) at 0.5, 1.0, and 2.0 g/dL, neem leaf hexane extract (NLHE) at 0.5, 1.0, and 2.0 g/dL, positive control, and negative control groups. Fungal growth was detected on Sabouroud dextrose agar. Statistical analysis used Chi square and Fisher's exact test.

#### RESULTS

NLHE had a higher, but statistically non-significant, inhibitory effect on *A. flavus* than NLEE (p=0.996). At higher concentrations, the antifungal effect of NLHE is better than that of NLEE.

#### CONCLUSION

There is no significant difference in in-vitro inhibitory effectivity on *A. flavus* of neem leaves between extracts in polar and nonpolar solvents.

**Keywords :** *Azadirachta indica*, neem, *A. flavus*, ethanol, hexane, antifungal

\*Department of Biochemistry, School of Medicine, Atma Jaya Catholic University of Indonesia

\*\*Department of Parasitology, School of Medicine, Atma Jaya Catholic University of Indonesia

#### Correspondence

dr. Jenny Hidayat, M.Biomed.  
Department of Biochemistry,  
School of Medicine, Atma  
Jaya Catholic University of  
Indonesia  
Jl. Pluit Raya no.2,  
North of Jakarta, 14440  
Phone : +62-21 6693168  
ext 203, 204,  
Fax : +62-21 6606123  
Email:  
Jenny.hidayat@atmajaya.ac.id

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## **Ekstrak heksana daun mimba lebih potensial dibandingkan ekstrak etanol terhadap *Aspergillus flavus***

### **ABSTRAK**

#### **LATAR BELAKANG**

*Aspergillus flavus* merupakan salah satu penyebab aspergilosis. Virulensi *A. flavus* yang tinggi dan resistensi terhadap antifungal standar menyebabkan angka mortalitas yang tinggi. Penggunaan tanaman obat semakin meningkat karena relatif lebih aman dan minimal efek samping. Hasil penelitian kami sebelumnya mendapatkan ekstrak etanol daun mimba menghambat pertumbuhan *A. flavus* in vitro. Namun, senyawa kimia yang berefek antifungal lebih banyak bersifat nonpolar. Oleh sebab itu, penelitian ini bertujuan untuk menilai efek ekstrak daun mimba dalam pelarut nonpolar lebih baik dibandingkan pelarut polar terhadap *A. flavus*.

#### **METODE**

Penelitian ini merupakan penelitian eksperimental in vitro yang dilakukan antara bulan Oktober 2013 – Januari 2014. Daun mimba diekstrak dalam pelarut etanol dan heksana pada berbagai konsentrasi. Uji makrodilusi dilakukan pada 8 kelompok percobaan, yang diulang sebanyak 3 kali, dan diinkubasi selama 48 jam. Kelompok terdiri dari ekstrak etanol daun mimba (EEDM 0,5; 1,0; 2,0 g/dL), ekstrak heksana daun mimba (EHDM 0,5; 1,0; 2,0 g/dL), kontrol positif, dan kontrol negatif. Deteksi pertumbuhan jamur dinilai dengan menanam sedikit dari media – media uji tersebut pada agar dekstroza Sabouroud. Uji statistik yang digunakan adalah uji Chi square dan Fisher's exact test.

#### **HASIL**

Ekstrak heksana daun mimba memiliki efek penghambatan terhadap *A. flavus* yang lebih baik dibandingkan EEDM namun tidak bermakna ( $p=0,996$ ). Pada konsentrasi yang lebih tinggi, efek antifungal EHDM lebih baik dibanding EEDM.

#### **KESIMPULAN**

Tidak ada perbedaan efektivitas yang bermakna antara ekstrak daun mimba dalam pelarut polar dan nonpolar terhadap pertumbuhan *A. flavus* in vitro.

**Kata kunci :** *A. indica*, neem, *A. flavus*, etanol, heksana, antifungal

## **INTRODUCTION**

*Aspergillus flavus* is the second most common cause of aspergillosis, after *Aspergillus fumigatus*. Fungal infection caused by *A. flavus* is 100 times more invasive than that by *A. fumigatus*.<sup>(1)</sup>

Infection in aspergillosis occurs through inhalation of fungal spores. The clinical manifestations of aspergillosis are miscellaneous, from non invasive to invasive.<sup>(2)</sup> The incidence of invasive aspergillosis has been increasing.<sup>(3)</sup>

The mortality rate of invasive aspergillosis in treated patients is 50%. Standard antifungals is reported to have various side effects.<sup>(4)</sup> This encourages researchers to find natural sources of antifungal alternative treatment.<sup>(5)</sup> In recent years natural herbal compounds with antifungal properties have received the attention of researchers because they are natural and low risk substances for human health and the environment.<sup>(6)</sup> Medicinal plants are considered to be safer and have fewer side effects than chemical drugs.<sup>(3)</sup> Neem (*Azadirachta indica* A

Juss) is known as one of the medicinal plants that can inhibit *Aspergillus* growth. Neem leaves have *in vitro* antifungal activity towards *A. fumigatus* and *Aspergillus niger*.<sup>(3)</sup>

For more than 2000 years, neem has been known as a medicinal plant with broad aspects of biological activity.<sup>(7)</sup> Neem leaf extract can be used as an immunomodulatory, antiinflammatory, antihyperglycemic, antiulcer, antimalarial, antifungal, antibacterial, antioxidant, antimutagenic, and anticarcinogenic agent.<sup>(8,9)</sup> Neem leaf extract has been reported to be effective for superficial fungal infection.<sup>(9)</sup> However, the information on the effectivity of neem leaf extract against *A. flavus* in systemic fungal infection is still limited.

The result of our previous study was that the minimal inhibitory concentration of neem leaf ethanol extract (NLEE) against *A. flavus* is 0.5 g/dL.<sup>(10)</sup> Ethanol is a polar solvent. The biological compounds that can be extracted by polar and nonpolar solvent are different.<sup>(11)</sup> One of the nonpolar solvents is hexane. Some studies have revealed that neem leaf extracts both in polar and non polar solvents have antifungal activities.<sup>(11-13)</sup> However, the antifungal activities of the extracted compounds may differ, depending on the nature of the compounds.<sup>(13)</sup> Based on these considerations, the purpose of this study was to evaluate the comparative effectivity of neem leaves extracted by hexane and ethanol on *A. flavus*.

## METHODS

### Research design

This study was an experimental research study conducted at the Biochemistry and Parasitology laboratories of the Medical School, Atma Jaya Catholic University of Indonesia, from October 2013 to January 2014.

### Preparation of neem leaf extract

Neem leaves were obtained from the Karyasari botanical garden, Indonesia. Fresh neem leaves were cut into small pieces and then

allowed to dry for at least 2-3 weeks (no direct sunlight), then blended into powder. Each of fifty grams of dried neem leaf powder was extracted with 200 mL of 95% ethanol and 200 mL of hexane by maceration for 72 hours. The extracted solution was evaporated by rotary evaporator with set temperature of 60-65°C for hexane and 70-75°C for 95% ethanol. The ethanol extract that still contained small amounts of alcohol was dried in a dessicator with silica gel. The extracts were stored in the refrigerator at 4°C.

### Fungal preparation

*A. flavus* was cultured on Sabouroud dextrose agar (SDA) (Difco, France) for 7 days at 37°C.<sup>(14,15)</sup> Fungal colonies were suspended in 0.9% sterile NaCl. The turbidity of this suspension was adjusted by spectrophotometry to obtain an optical density of 0.09-0.11 at 530 nm. This adjusted suspension was diluted 1:50 in Sabouroud dextrose broth (SDB).<sup>(14,15)</sup>

### Macrodilution assay

The concentrations of neem leaf extract used in this study were 0.5 g/dL, 1.0 g/dL, and 2.0 g/dL, both for the ethanol and hexane extracts. The total volume of each group was 4 mL, consisting of 1 mL SDB, 1 mL dimethylsulfoxide (DMSO) 1%, 2 mL fungal suspension of 0.09-0.11 OD at 530 nm, 10 µL chloramphenicol 1 mg/mL (to prevent bacterial contamination from neem leaf extract), and neem leaf extract. The positive control was a solution consisting of 1 mL SDB, 1 mL DMSO 1%, 2 mL fungal suspension, and 10 µL chloramphenicol 1 mg/mL. The positive control was used as a standard of fungal growth without neem leaves. The negative control was a solution consisting of 1 mL SDB, 1 mL DMSO 1%, and 10 µL chloramphenicol 1 mg/mL. All of these solutions were incubated for 48 hours at 37°C.

### Antifungal activity of different extracts of neem leaves

Fungal growth was detected by culturing on SDA after serial dilution.<sup>(16)</sup> Serial dilution

was conducted by diluting 0.1 mL solution in 0.9 mL 0.9% sterile NaCl, and then taking 0.1 mL from the first dilution into 0.9 mL 0.9% sterile NaCl. A volume of 20  $\mu$ L from the second dilution was cultured on SDA, with an incubation time of 72 hours at 37<sup>o</sup>C. The area that was covered by *A. flavus* was compared to the area of the petri dish. The results were defined as: no growth, growth of less than half of the petri dish, and growth of more than half of the petri dish area. The tests were done in triplicate.

### Data analysis

Data was analyzed by SPSS 17, with Chi square and Fisher's exact test.

### RESULTS

Fifty grams of dried neem leaf powder produced about 7.85 grams of NLEE in 95% ethanol and 0.55 grams of neem leaf extract in hexane (NLHE). NLEE was dark green in color, and NLHE was yellowish green.

Figures 1 and 2 show the effect of NLEE and NLHE on growth of *A. flavus* at various concentrations. Figure 3 shows the positive and negative controls. Statistically, there were no significant differences between NLHE and NLEE at identical concentrations ( $p=0.996$ ) (Table 1). There were also no significant differences among concentrations in NLEE and NLHE (Table 2).

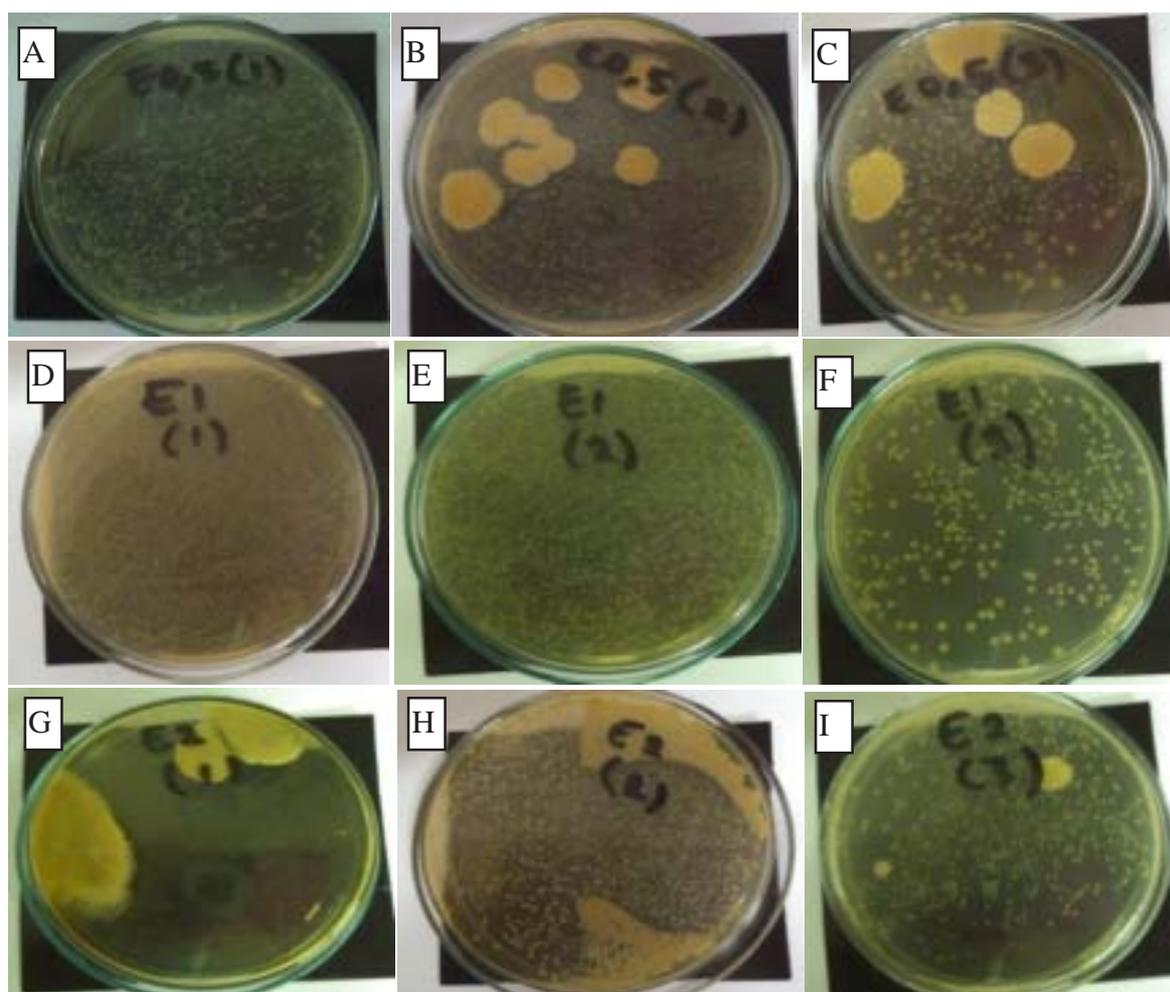


Figure 1. *A. flavus* colonies in various concentrations of NLEE

A = NLEE 0.5 g/dL (1) ; B = NLEE 0.5 g/dL (2) ; C = NLEE 0.5 g/dL (3) ; D = NLEE 1.0 g/dL (1) ;  
E = NLEE 1.0 g/dL (2) ; F = NLEE 1.0 g/dL (3) ; G = NLEE 2.0 g/dL (1) ; H = NLEE 2.0 g/dL (2) ;  
I = NLEE 2.0 g/dL (3)

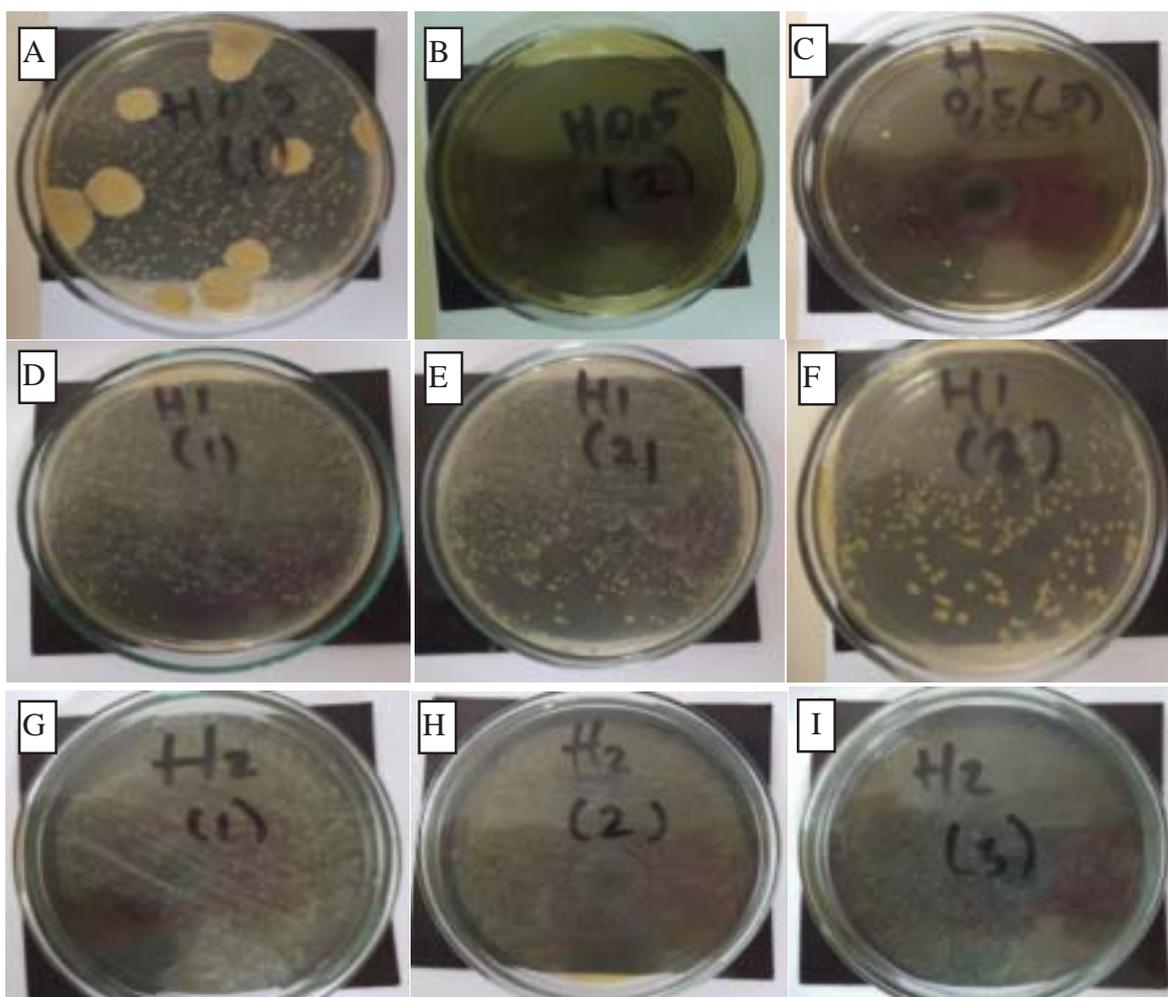


Figure 2. *A.flavus* colonies in various concentrations of NLHE

A = NLHE 0.5 g/dL (1); B = NLHE 0.5 g/dL (2); C = NLHE 0.5 g/dL (3); D = NLHE 1.0 g/dL (1);  
 E = NLHE 1.0 g/dL (2); F = NLHE 1.0 g/dL (3); G = NLHE 2.0 g/dL (1); H = NLHE 2.0 g/dL (2);  
 I = NLHE 2.0 g/dL (3)

**DISCUSSION**

According to some studies, neem leaf extract has antifungal effects.<sup>(11-13)</sup> Neem leaves

contain nimbin, nimbidin, azadirachtin, and tannins that have antifungal activity. In this study, NLEE concentration was inversely proportional to the growth of *A. flavus*, although not

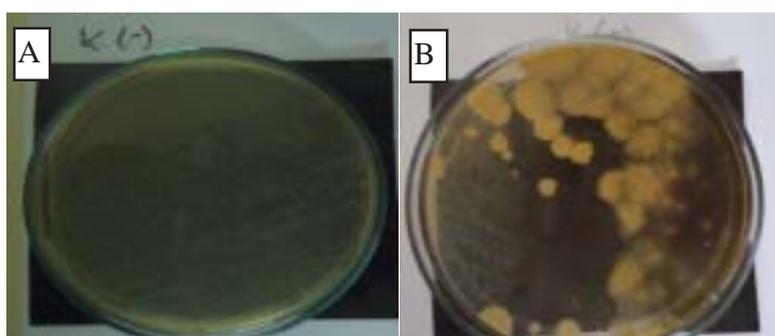


Figure 3. Negative control (A); Positive control (B)

Table 1. Differences on *A.flavus* growth between NLEE and NLHE

Neem leaf	Fungal growth						p
	No growth		< 50%		> = 50%		
	n	%	n	%	n	%	
NLEE 0.5 g/dL	1	33.3	2	66.7	0	0	0.996
NLHE 0.5 g/dL	2	66.7	1	33.3	0	0	
NLEE 1.0 g/dL	2	66.0	1	33.3	0	0	0.996
NLHE 1.0 g/dL	3	100.0	0	0	0	0	
NLEE 2.0 g/dL	1	33.3	2	66.7	0	0	0.518
NLHE 2.0 g/dL	3	100.0	0	0	0	0	

The experiments were done in triplicate.

n = number of replications with results denoted as “no of fungal growth”, “fungal growth on <50% of petri dish area”, or “fungal growth on >=50% of petri dish area”.

NLEE : neem leaf ethanol extract; NLHE : neem leaf hexane extract

statistically significant ( $p=0.996$ ). Fungal colonies in NLEE 0.5 g/dL were more abundant than in NLEE 1.0 g/dL. At higher concentrations, the antifungal effect of NLEE also increased. The density of the fungal growth at NLEE 0.5 g/dL was higher than at NLEE 2 g/dL. This may be due to uneven spread of fungal growth, because the growth was only at the edge of the dish and the number of colonies were less in NLEE 2 g/dL than NLEE 0.5 g/dL.

This study used the antibiotic chloramphenicol at 1 mg/mL to prevent bacterial growth, which is consistent with the research of Akpuaka et al.<sup>(12)</sup> This dose should be sufficient to inhibit the growth of bacteria. Radhika et al.<sup>(8)</sup> used chloramphenicol 0.05 mg/mL, while Jabeen<sup>(9)</sup> used chloramphenicol 0.5 mg/mL. Nevertheless, in all media there was still visible growth of bacterial colonies, although the media were given antibiotics. This may be due to the presence of bacterial resistance to chloramphenicol. At higher concentrations of

NLEE, there was less growth of bacterial colonies, with smaller diameters, than at lower concentration of NLEE. This finding supports the research of Biswas et al.<sup>(17)</sup> which stated that neem leaf extract has antibacterial properties.

The solubility of NLEE and NLHE in the media is improved by using DMSO. Dimethylsulfoxide will bind to the fungal cell membrane and increase its permeability. In this study, we used 1% DMSO, since according to Hazen's research the final concentrations of DMSO used in in vitro antifungal experiments should be 0.5% and 1%.<sup>(18,19)</sup> Randhawa<sup>(19)</sup> stated that DMSO concentrations of 1% and below were safe to use in antifungal experiments. Rauf et al.<sup>(20)</sup> showed that 0.45% DMSO still can be used in antifungal tests. However, Radhika et al.<sup>(8)</sup> used a higher concentration of DMSO (5%) than the recommended ones. It has been reported that DMSO concentrations of 2% and above slow the fungal kinetic growth.<sup>(18)</sup> Therefore, there is the possibility that the fungal growth is

Table 2. Differences on *A. flavus* growth between several concentrations of NLEE and NLHE

Concentrations	Fungal growth				p
	No		Yes		
	n	%	n	%	
NLEE 0.5 g/dL and 1.0 g/dL	3	50	3	50	1.000
NLEE 2.0 g/dL	1	33.3	2	66.7	
NLHE 0.5 g/dL and 1.0 g/dL	5	83.3	1	16.7	1.000
NLHE 2.0 g/dL	3	100	0	0	

NLEE : neem leaf ethanol extract; NLHE : neem leaf hexane extract

influenced by the concentration of DMSO used, apart from the extract itself.

In this study, the number of *A. flavus* colonies in NLHE were inversely proportional to the concentration. Fungal growth was visible in NLHE 0.5 g/dL but not in NLHE 1.0 g/dL and 2.0 g/dL. Bacterial growth was also inversely proportional to the concentration of NLHE. This may be due to the antibacterial effect of the neem leaves.<sup>(12,21)</sup>

In this study, NLHE gave better results in inhibiting *A. flavus* compared with NLEE, although the increase was not statistically significant. At NLHE 0.5 g/dL, fungal growth was less than NLEE. At NLHE 2.0 g/dL, the diameters of bacterial colonies were slightly smaller than those at NLHE 0.5 g/dL and 1.0 g/dL. According to Verma et al.<sup>(21)</sup> neem leaf contains compounds that are antibacterial and antifungal. This allows an increase in the active antibacterial and antifungal compounds with increasing concentrations of neem leaf extract. In the present study, the inhibitory activity on the growth of *A. flavus* appeared to be higher for NLHE compared to NLEE at various concentrations. However, no statistically significant differences were found between NLEE and NLHE inhibitory activity. NLHE contains many bioactive compounds that have antifungal activity.<sup>(12)</sup> According Jeyasakthy et al.<sup>(22)</sup> and Kumar et al.,<sup>(23)</sup> neem leave have tannins, flavonoids, terpenoids, and alkaloids that are antifungal. These compounds can be found in neem leaf extracts using different solvents. Mondali study showed that the antifungal compounds of neem leaf extract in non-polar solvents were better than in polar solvents.<sup>(11)</sup> Akpuaka et al. identified 33 bioactive compounds in the hexane extract of neem leaves which have antifungal activity. Our study found that NLHE activity in inhibiting the growth of *A. flavus* was better than that of NLEE. In contrast, Radhika et al.<sup>(8)</sup> stated that NLEE was more effective in inhibiting the growth of fungi compared to NLHE.

NLHE contains more terpenoids and diterpenoids than NLEE.<sup>(12)</sup> Terpenoid and diterpenoid compounds allegedly have high antifungal activity. Rauf et al.<sup>(20)</sup> stated that the hexane fraction of some medicinal plants from Pakistan has the highest antifungal activity, compared to ethyl acetate and methanol fractions. The results of our study differ from the results of Radhika et al.<sup>(8)</sup> This may be caused by the difference in fungal species used. Radhika et al.<sup>(8)</sup> used a superficial dermatophyte as test fungus, whereas our study used *A. flavus*. The growth of *A. flavus* in NLHE was less than the growth of *A. flavus* in NLEE although not statistically significant. This may because of the inadequate number of trials, or because of contamination in the preparation of DMSO and neem leaf extract.

The clinical implication of this study is to provide information to clinicians and the community about the potential of neem leaves as systemic antifungal agent, especially about the discovery of a new natural and low-risk antifungal drug. Future directions of this study are to investigate the potential of neem leaf extract as alternative treatment for lung cancer with pulmonary aspergillosis.

## CONCLUSION

There is no significant difference in effectivity of neem leaves to inhibit *A. flavus* in vitro, between leaves extracted in polar and nonpolar solvents.

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