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Antifungal Effect of Malaysian Neem Leaf Extract on Selected Fungal Species Causing Otomycosis in *In-Vitro* Culture Medium

Puvan Arul Arumugam,[1] Irfan Mohamad*,[1] Rosdan Salim,[1] Zeehaida Mohamed[2]
[1]Department of ORL-HNS, School of Medical Sciences, [2]Department of Microbiology, Universiti Sains Malaysia Health Campus, 16150 Kota Bharu, Kelantan, Malaysia

ABSTRACT

Azadirachta indica (neem) has been used for a long time in agricultural and alternative medicine. Neem had been proved effective against certain fungi that could infect human body. This pilot study aims to demonstrate the antifungal effect of Malaysian neem leaf extracts on the pathogenic fungi in otomycosis, *Aspergillus niger* and *Candida albicans*. This is a laboratory-controlled prospective study conducted at Universiti Sains Malaysia. The powder form of Malaysian neem leaf was prepared. Ethanol and aqueous extracts of the neem leaf was diluted with sterile water to establish five different concentrations of 50 g/ml, 25 g/ml, 12.5 g/ml, 6.25 g/ml and 3.125g/ml. The extract was tested on Sabouraud Dextrose Agar suspended with *Candida albicans* and *Aspergillus niger* respectively. Well diffusion method was used and zone of inhibition was measured. Growth of the fungi was inhibited in both alcohol and aqueous extract concentrations. The minimum inhibitory concentration (MIC) of Malaysian neem aqueous extract against *Candida albicans* was 11.91 g/ml, neem ethanol extract against *Candida albicans* was 5.16 g/ml, neem aqueous extract against *Aspergillus niger* was 7.73 g/ml and neem ethanol extract against *Aspergillus niger* was 9.25 g/ml. Statistical analysis showed that the antifungal activity of *Candida albicans* is better in alcohol neem than aqueous extract ($p<0.001$) but aqueous neem extract is better than alcohol extract ($p<0.001$) for *Aspergillus niger*.Malaysian neem has significant antifungal effect towards *Aspergillus niger*, best in aqueous extract and towards *Candida albicans,* was best in alcohol extract.

Keywords: Malaysian neem, *Azadirachta indica,* Otomycosis, *Aspergillus niger, Candida albicans*

INTRODUCTION

Herbal medicines have been known to man for centuries. Therapeutic efficacy of many indigenous plants for several disorders has been described by practitioners of traditional medicine. *Azadirachta indica* (neem) is a tree which has been used for a long time in agriculture and medicine.[1] Neem is an attractive evergreen tree native to the Indian subcontinent. It is also cultivated throughout Southeast Asia, Australia, East and sub-Saharan Africa, Fiji, Mauritius, and many countries in Latin America. Neem is called 'arista' in Sanskrit a word that means 'perfect, complete and perishable'.[2] The tree is regarded as village pharmacy in India. The importance of the neem tree has been recognised by US National Academy of Sciences, which published a report in 1992 entitled 'Neem-a tree for solving global problems'.[3] All parts of neem tree including the leaf, bark, flower, fruits, seed pulp and root has medical properties.[3] Neem leaf and its constituents have been demonstrated to exhibit immunomodulatory, anti-inflammatory, antihyperglycaemic, antiulcer, antimalarial, antifungal, antibacterial, antioxidant, antimutagenic and anticarcinogenic properties.[4]

In Malaysia, neem leaves are commonly used as an alternative treatment of chicken pox, a highly contagious disease caused by primary infection with varicella zoster virus. There were very limited studies on antifungal effect of Malaysian neem leaf especially to *Candida albicans* and *Aspergillus niger.* Malaysian neem and Indian neem have almost similar characteristics but whether Malaysian neem has similar medicinal properties as the Indian neem was not explored till today. This is a pilot study to determine the antifungal effect of Malaysian *Azadirachta indica* (neem) leaf extracts on the two commonest pathogenic fungi in otomycosis, *Aspergillus niger* and *Candida albicans.*

METHODOLOGY

Study design

This is a laboratory-controlled prospective study. It was fully conducted under a well-controlled environment in the medical microbiology and pharmacology laboratories in the School of Medical Sciences, Universiti Sains Malaysia.

*Corresponding author: Dr Irfan Mohamad
irfankb@usm.my

Sample preparation method

One kilogram of Malaysian neem leaves was collected from single 10-12 years old tree located near cafeteria of Hospital USM (USM Herbarium voucher no: 11337). The leaf that was collected was healthy without any fungal or bacterial infection. The leaves were washed with distilled water and dried in oven at 45°C for two days. Dried neem leaves should maintain the original green colour. Then, the dried leaves were grinded using leaf grinder machine to smaller course powder form and stored in tightly sealed glass container.

Sample extraction method

The method of choice for extraction was the Soxhlet extraction by using Soxhlet apparatus. This method uses a solvent for extraction and at completion the solvent will be removed fully. Two types of solvent, the aqueous and ethanol 70%, was used.[5,6] The dried powder form of the tested neem leaves was inserted into the Soxhlet thimble and closed with white thin gauze. The thimble was inserted into the Soxhlet main chamber and closed. The solvent chamber was filled with 1 litre of ethanol 70%, and attached to Soxhlet apparatus. Solvent chamber should not be overfilled and the volume of solvent in the vessel should be 3 to 4 times the volume of the Soxhlet chamber. The solvent chamber was heated with suitable temperature and solvent vapour travels up a distillation arm, and fills into the main Soxhlet chamber. The condenser ensures that any solvent vapour cools, and drips back down into the chamber housing the neem powder. Some of the desired compound was then dissolved in the warm solvent. When the Soxhlet chamber was almost full, the chamber was automatically emptied by a siphon side arm, with the solvent running back down to the distillation flask. This extraction allowed for 4-6 cycles. By 6th cycle the solvent chamber was dark in colour and Soxhlet chamber was clearer. The solvent with desired compound was extracted and in solvent chamber. The non-soluble portion of the extracted solid remains in the thimble, and was discarded. The extracted neem leaves in alcohol solvent was then evaporated with rotary evaporator at 30°C. The volume of extract was concentrated till 50 ml and inserted into multiple sterile container. It was freeze dried to avoid further heat damage on a freeze dryer machine. After almost 2-3 days the extracts were in powder form and kept in freezer to maintain its compound. As for water solvent, same process done whereby the ethanol was replaced with distilled water in solvent chamber.

A total of 278 grams of ethanol neem extract and 292 grams of aqueous neem extract after intensive freeze drying. This powder form extracts were used to establish different concentration as required in this research. The concentration required were 50 g/ml, 25 g/ml, 12.5 g/ml, 6.25 g/ml and 3.125 g/ml. It can be achieved by serial dilution using initial 50 g/ml concentration. Initial 100 g mixed with two ml of distilled water to produce 50 g/ml concentration. Then one ml from the 50 g/ml concentration taken and distilled water added till two ml level to make 25 g/ml concentration. Similarly, one ml taken from 25 g/ml concentration to make up 12.5 g/ml. The dilution continued till 3.125 g/ml concentration. The left over extracts was discarded and a new preparation done for new replicates using the same powder extract from one single neem tree.

Preparation of *in vitro* culture medium

The fungal isolates were taken from archives of microbiology laboratory in School of Medical Sciences, Universiti Sains Malaysia, which already been identified earlier from otomycosis patients of otorhinolaryngology clinic, Hospital USM. Both *Candida albicans* and *Aspergillus niger* were used as tested fungi. *Candida albicans* from SDA plates were suspended in sterile distilled water and adjust to 10^6 cells with colony forming units (CFU)/ml^9 (0.5 McFarland standard). Nephelometer was used to adjust the turbidity of fungal suspensions so that the number of fungal was within a given standard McFarland range. Similar process done for *Aspergillus niger*.

The suspended sterile fungal organism labelled and used for the next step to lawn and prepare for testing in SDA plates. Within 15 minutes of diluting the organism, sterile swab was dipped into the properly adjusted inoculums of tested fungal organism. The sterile swab was lifted up slightly out of the suspension and firmly rotate the swab several times against the upper inside wall of the tube to express excess fluid. The entire surface of plate was lawn three times with the swab after drawing linear line, turning the plate 60 degrees between lawns. The swab was turned to obtain an even inoculation. Then the lid was closed and let sit for 3-5 minutes creating a well in the agar. Later, by using glass pipette, four wells were created in four quadrants.

Initiation of *in vitro* test and data collection

The SDA plate was kept lid side up in a 30°C incubator. Before inserting in an incubator, micropipette was used to drop 100 microlitre of extracts into wells. The upper quadrant well, the aqueous neem extract inserted with its control aqueous solution at its opposite site. The lower quadrant well, the alcohol neem extracts was inserted with its control

at opposite site. This was done using different concentration which was diluted prior to this. Five different replicates done. The plates were examined every day to make sure no spillage or growth of other organism. The measurement was done on third day whereby this was the perfect time to visualize the margin of inhibition. After the third day, there was overgrowth especially *Aspergillus niger* and may jeopardize the safety of the staff. Measurement done in millimetres of zones showing complete inhibition by gross visual inspection. Measurement done by holding the vernier caliper over the back of the inverted plate over a black non-reflective surface and illuminate from above.

Determination of minimum inhibitory concentration (MIC)

Minimum inhibitory concentration can be determined in many ways including serial dilution method, agar diffusion method and E-test. In this research we had used the agar diffusion method to determine MIC of each tested Malaysian neem extracts. After measurement of all the result, a scattered plot graph X^2 Versus Log Concentration was plotted. A linear line, which represents the mean value, was drawn and the area where X^2 equal to zero was taken for the MIC level of the extract. Antilog of the selected value was mathematically identified as MIC. The X value represent zone of inhibition diameter subtracts the well diameter (6 mm) and divided by two.

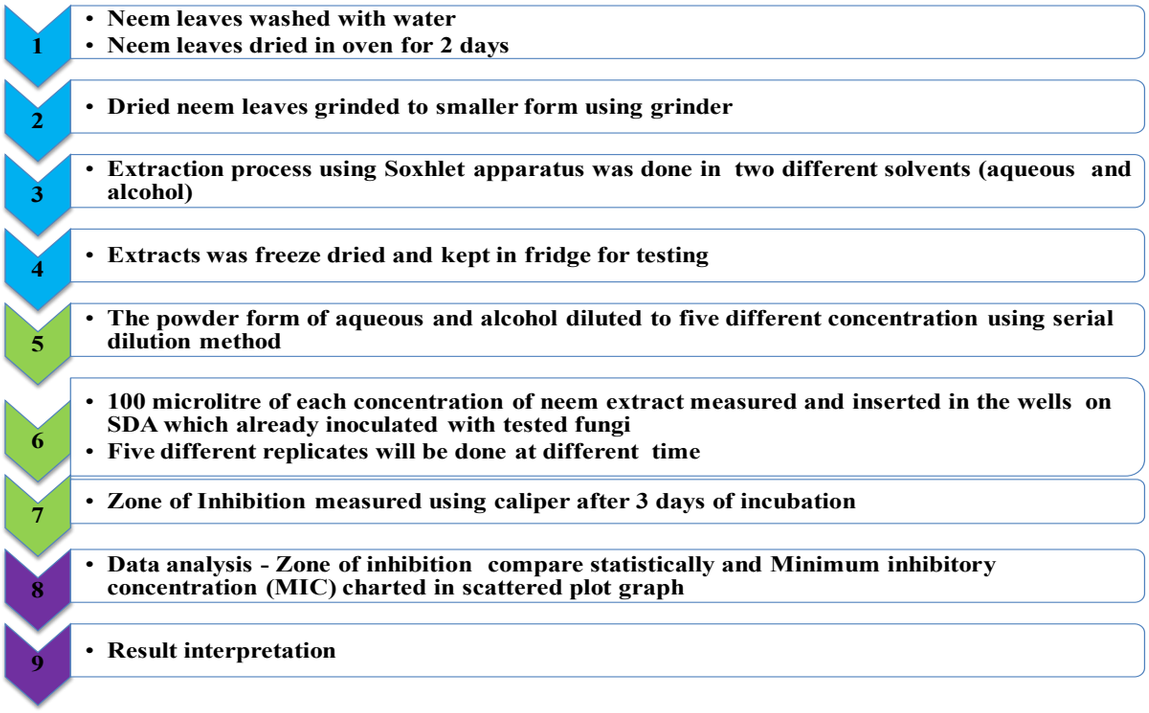

Figure 1: Flow chart of the study

RESULTS

There were presence of zone of inhibition for both aqueous and alcohol neem extracts more than 6 mm after three days of incubation on *Aspergillus niger.* Similarly, *Candida albicans* growth was inhibited more than 6 mm in aqueous and alcohol neem extracts. However, not all concentration of extracts effectively inhibits growth in both pathogens. The highest concentration at 50 g/ml of all extracts gave the highest inhibition to all tested pathogen. The entire sample revealed negative control (wells contain either only alcohol or only aqueous) showed no zone of inhibition against *Aspergillus niger* and *Candida albicans*. Figure 2 shows example of positive zone of inhibition and negative control (magnified view in *Aspergillus niger*- Figure 2.1 & normal view in *Candida albicans*- Figure 2.2). Zone of inhibition, which is the clear zone where growth of fungal does not occurred, was measured for each tested neem extracts. This is the base of calculation for MIC.

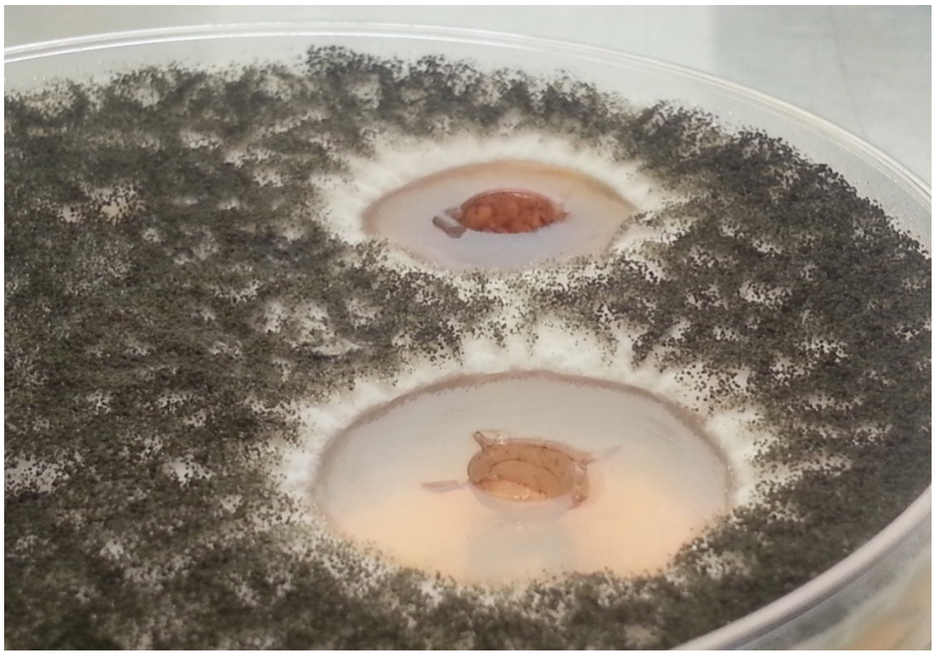

Figure 2.1: Magnified view of zone of inhibition against *Aspergillus niger*

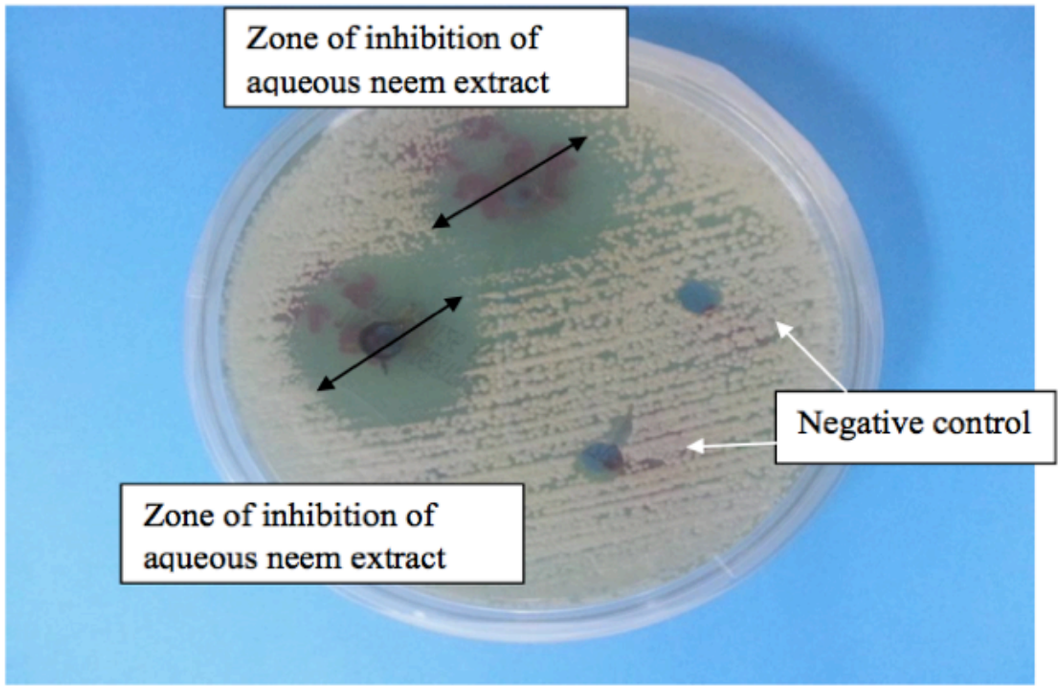

Figure 2.2: Positive zone of inhibition for *Candida albicans* with negative control in neem aqueous and alcohol extract of 25 g/ml concentration.

Inhibitory efficacy of Malaysian neem on *Aspergillus niger*

Aspergillus niger showed sensitivity to both aqueous and alcohol neem extracts (Table 1). Growth of *Aspergillus niger* was inhibited in neem alcohol and aqueous extract concentrations of 50 g/ml, 25 g/ml and 12.5 g/ml. However the growth was resistance to concentration of 6.25 g/ml and 3.125 g/ml. Mean zone of inhibition increases in proportion to extract concentration. There was an increase in diameter zone of inhibition in proportion to concentration.

Table 1: Measurement of zone of inhibition of alcohol and aqueous neem extracts on *Aspergillus niger* growth at five different concentrations.

Extracts	**Concentration (g/ml)**	**Zone of Inhibition (mm)**					**Mean (mm)**
Aqueous	50.000	32	33	33	31	32	**32**
	25.000	25	26	25	25	24	**25**
	12.500	21	20	20	20	19	**20**
	6.250	0	0	0	0	0	**0**
	3.125	0	0	0	0	0	**0**
Alcohol	50.000	26	27	27	27	27	**27**
	25.000	22	22	23	21	21	**22**
	12.500	14	15	15	15	16	**15**
	6.250	0	0	0	0	0	**0**
	3.125	0	0	0	0	0	**0**

Statistically using one way analysis of variance (ANOVA), denotes significant mean differences between groups of concentration for aqueous neem extracts. From the Table 2, we can interpret that mean zone of inhibition of concentration 50 g/ml was 32.2 mm, 25 g/ml was 25 mm and 12.5 g/ml was 20 mm with a significant difference between the three groups ($p < 0.001$). In order to find out which pair of group shows a significant difference, Post-hoc comparison done using Scheffe test. Table 3 showing Post-hoc comparison with significance difference 50 g/ml & 25 g/ml ($p< 0.001$), 50 g/ml & 12.5 g/ml ($p <0.001$) and 25 g/ml & 12.5 g/ml ($p<0.001$). Statistically the results conclude that higher mean of inhibition was observed in 50 g/ml & 12.5g/ml group followed by 50 g/ml & 25 g/ml group and 25 g/ml & 12.5 g/ml respectively. The higher neem aqueous concentrations gave high effect to mean of inhibition of growth of *Aspergillus niger*.

Table 2: Comparison of mean zone of inhibition against *Aspergillus niger* among three concentration groups in aqueous neem extracts.

Concentration of extracts	Mean (SD)	F-statistic[a] (df)	p-value
50.0 g/ml	32.20 (0.84)		
25.0 g/ml	25.00 (0.71)	331.88 (2)	<0.001
12.5 g/ml	20.00 (0.71)		

[a]One-Way ANOVA test was applied

Table 3: Post-hoc comparison of mean difference of zone of inhibition against Aspergillus niger among three concentration groups in aqueous neem extracts.

Concentration of extracts	Mean difference (95% Confidence Interval)	p-value[a]
50.0 g/ml vs 25.0 g/ml	7.20 (5.87, 8.53)	<0.001
50.0 g/ml vs 12.5 g/ml	12.20 (10.87, 13.53)	<0.001
25.0 g/ml vs 12.5 g/ml	5.00 (3.67, 6.33)	<0.001

[a]Scheffe test was applied

The results of mean differences between groups of concentration for alcohol neem extracts also showed a significant difference. From the Table 4, we can interpret that mean zone of inhibition of concentration 50 g/ml was 26.80 mm, 25 g/ml was 21.80 mm and 12.5 g/ml was 15 mm with significant difference between the three groups ($p< 0.001$).

Table 5 showing Post-hoc comparison with significance difference 50 g/ml & 25 g/ml ($p< 0.001$), 50 g/ml & 12.5 g/ml ($p<0.001$) and 25 g/ml & 12.5 g/ml ($p<0.001$). Higher mean of inhibition was observed in 50 g/ml & 12.5 g/ml group followed by 25 g/ml & 12.5 g/ml group and 50 g/ml & 25 g/ml respectively. Statistically using one way analysis of variance (ANOVA), p-value < 0.001 denotes significant mean differences between group of concentration for both alcohol and aqueous neem extracts.

Table 4: Comparison of mean zone of inhibition against *Aspergillus niger* among three concentration groups in alcohol neem extracts.

Concentration of extracts	Mean (SD)	F-statistic[a] (df)	p-value
50.0 g/ml	26.80 (0.45)		
25.0 g/ml	21.80 (0.84)	375.86 (2)	<0.001
12.5 g/ml	15.00 (0.71)		

[a]One-Way ANOVA test was applied

Table 5: Post-hoc comparison of mean difference of zone of inhibition against *Aspergillus niger* among three concentration groups in ethanol neem extracts.

Concentration of extracts	Mean difference (95% Confidence Interval)	p-value[a]
50.0 g/ml vs 25.0 g/ml	5.00 (3.80, 6.20)	<0.001
50.0 g/ml vs 12.5 g/ml	11.80 (10.60, 13.00)	<0.001
25.0 g/ml vs 12.5 g/ml	6.80 (5.60, 8.00)	<0.001

[a]Scheffe test was applied

Inhibitory efficacy of Malaysian neem on *Candida albicans*

Candida albicans showed sensitivity to both aqueous and alcohol neem extracts as presented in Table 6. Growth of *Candida albicans* inhibited in neem alcohol extract concentrations of 50 g/ml, 25 g/ml, 12.5 g/ml, and 6.25 g/ml and demonstrated resistance only to concentration of 3.125 g/ml. However, growth of *Candida albicans* inhibited in neem aqueous extract concentrations of 50 g/ml, 25 g/ml and 12.5 g/ml. The growth was resistance to concentration 6.25 g/ml and 3.125 g/ml almost similar to *Aspergillus niger.* Mean zone of inhibition increases in proportion to extract concentration. There was increase diameter zone of inhibition in proportion to concentration.

Table 6: Measurement of zone of inhibition of alcohol and aqueous neem extracts on *Candida albicans* growth at 5 different concentrations.

Extracts	**Concentration (g/ml)**	**Zone of Inhibition (mm)**					**Mean (mm)**
Aqueous	50.000	30	31	30	29	30	**30**
	25.000	19	20	21	20	20	**20**
	12.500	14	15	13	13	14	**14**
	6.250	0	0	0	0	0	**0**
	3.125	0	0	6	0	0	**0**
Alcohol	50.000	33	34	33	33	32	**33**
	25.000	28	29	28	28	27	**28**
	12.500	22	23	21	22	21	**22**
	6.250	15	14	15	16	15	**15**
	3.125	0	0	0	0	0	**0**

Statistically one-way ANOVA showed significant mean differences between groups of concentration for aqueous neem extracts. From the Table 7, we can interpret that mean zone of inhibition of concentration 50 g/ml was 29.80 mm, 25 g/ml was 20 mm and 12.5 g/ml was 13.80 mm with significant difference between the three groups ($p<0.001$). Post-hoc comparison was done using Scheffe test. Table 8 showing Post-hoc comparison with significant difference 50 g/ml & 25 g/ml ($p< 0.001$), 50 g/ml & 12.5 g/ml ($p<0.001$) and 25 g/ml & 12.5 g/ml ($p<0.001$). Statistically results conclude that higher mean of inhibition was observed in 50 g/ml & 12.5 g/ml group followed by group 50 g/ml & 25 g/ml and 25 g/ml & 12.5 g/ml respectively. The higher neem aqueous concentrations gave high effect to mean of inhibition of growth of *Aspergillus niger*.

Table 7: Comparison of mean zone of inhibition against *Candida albicans* among three concentration groups in aqueous neem extracts.

Concentration of extracts	Mean (SD)	F-statistic[a] (df)	p-value
50.0 g/ml	29.80 (0.84)		
25.0 g/ml	20.00 (0.71)	513.79 (2)	<0.001
12.5 g/ml	13.80 (0.84)		

[a]One-Way ANOVA test was applied

Table 8: Post-hoc comparison of mean difference of zone of inhibition against *Candida albicans* among three concentration groups in aqueous neem extracts.

Concentration of extracts	Mean difference (95% Confidence Interval)	p-value[a]
50.0 g/ml vs 25.0 g/ml	9.80 (8.40, 11.20)	<0.001
50.0 g/ml vs 12.5 g/ml	16.00 (14.60, 17.40)	<0.001
25.0 g/ml vs 12.5 g/ml	6.20 (4.80, 7.60)	<0.001

[a]Scheffe test was applied

The result mean differences between groups of concentration for alcohol neem extracts showed significant difference too. From the Table 9, we can interpret that mean zone of inhibition of concentration 50 g/ml was 33.00 mm, 25 g/ml was 28.00 mm and 12.5 g/ml was 21.80 mm with significant difference between the three groups ($p< 0.001$). Table 10 showing post-hoc comparison with significant difference 50 g/ml & 25 g/ml ($p< 0.001$), 50 g/ml & 12.5 g/ml ($p<0.001$) and 25 g/ml & 12.5 g/ml ($p<0.001$). Higher mean of inhibition was observed in 50 g/ml & 12.5 g/ml group followed by 25 g/ml & 12.5 g/ml group 50 g/ml & 25 g/ml and respectively. Statistically using one way analysis of variance (ANOVA), p-value < 0.001 denotes significant mean differences between group of concentration for both alcohol and aqueous neem extracts.

Table 9: Comparison of mean zone of inhibition against *Candida albicans* among three concentration groups in alcohol neem extracts.

Concentration of extracts	Mean (SD)	F-statistic[a] (df)	p-value
50.0 g/ml	33.00 (0.71)		
25.0 g/ml	28.00 (0.71)	277.77 (2)	<0.001
12.5 g/ml	21.80 (0.84)		

[a]One-Way ANOVA test was applied

Table 10: Post-hoc comparison of mean difference of zone of inhibition against *Candida albicans* among three concentration groups in ethanol neem extracts.

Concentration of extracts	Mean difference (95% Confidence Interval)	p-value[a]
50.0 g/ml vs 25.0 g/ml	5.00 (3.67, 6.33)	<0.001
50.0 g/ml vs 12.5 g/ml	11.20 (9.87, 12.53)	<0.001
25.0 g/ml vs 12.5 g/ml	6.20 (4.87, 7.53)	<0.001

[a]Scheffe test was applied

Comparison on antifungal activity of Malaysian neem aqueous and alcohol extract against *Aspergillus niger*

Both alcohol and aqueous neem extracts showed significant antifungal activities towards *Aspergillus niger* with mean zone of inhibition as 32 mm in aqueous extract. Anti fungal activity in aqueous neem extract is better than alcohol neem extract. In each concentration, 50 g/ml, 25 g/ml and 12.5 g/ml zone of inhibition aqueous neem extracts was bigger as compared to alcohol neem extracts (Table 1).

Independent t-test was done to compare the mean between two groups. Table 11 showed that in all three concentrations aqueous extracts has bigger mean of inhibition as compared to alcohol extract with significance of p<0.001 against *Aspergillus niger.* Ninety five percent confidence interval of mean difference of inhibition does not include zero. Therefore statistically antifungal activity of *Aspergillus niger* is better in aqueous neem extract than alcohol extract.

Table 11: Comparison of mean of zone inhibition between aqueous and ethanol extracts against growth of *Aspergillus niger* at three different concentrations.

Concentration	Group	Mean (SD)	Mean difference (95% Confidence Interval)	t-statistic(df)	p-value[a]
50.0 g/ml	Aqueous	32.20(0.84)	(4.42, 6.38)	12.73(8)	<0.001
	Alcohol	26.80(0.45)			
25.0 g/ml	Aqueous	25.00(0.71)	(2.07, 4.33)	6.53(8)	<0.001
	Alcohol	21.80(0.84)			
12.5 g/ml	Aqueous	20.00(0.71)	(3.97, 6.03)	11.18(8)	<0.001
	Alcohol	15.00(0.71)			

[a] Independent t-test was applied

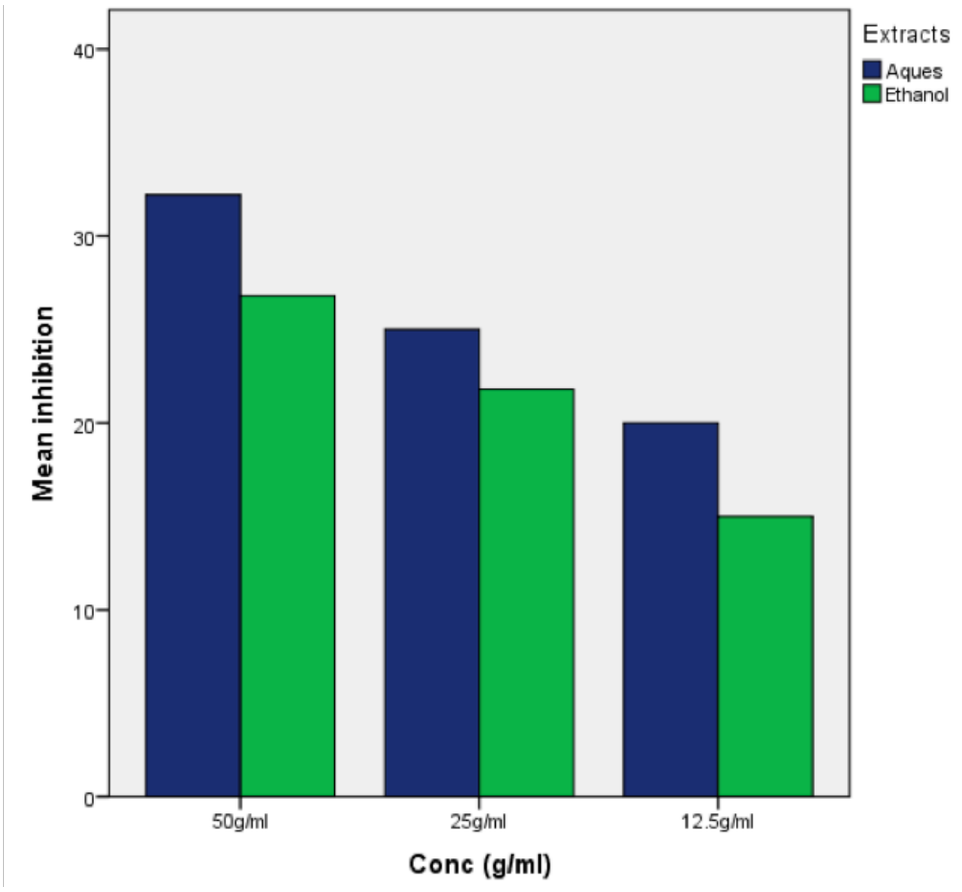

Figure 3: Zone inhibition between aqueous and alcohol extracts against growth of *Aspergillus niger* at different concentrations.

Comparison on antifungal activity of Malaysian neem aqueous and alcohol extract against *Candida albicans*

Both alcohol and aqueous neem extracts showed significant antifungal activities towards *Candida albicans* with widest zone of inhibition as 31 mm in alcohol extract. Anti fungal activity in alcohol neem extract is better than aqueous neem extract. In each concentration, 50 g/ml, 25 g/ml and 12.5 g/ml, zone of inhibition alcohol neem extracts was more as compared to aqueous neem extracts (Table 6). Furthermore, in alcohol neem extract concentration 6.25 g/ml presence of zone of inhibition, mean zone of inhibition was 15 mm shows antifungal activity in more diluted concentration.

Table 12: Comparison of mean of zone inhibition between aqueous and ethanol extracts against growth of *Candida albicans* at three different concentrations.

Concentration	Group	Mean (SD)	Mean difference (95% Confidence Interval)	t-statistic(df)	p-value[a]
50.0 g/ml	Aqueous	29.80(0.84)	(-4.33, -2.08)	-6.53(8)	<0.001
	Alcohol	33.00(0.71)			
25.0 g/ml	Aqueous	20.00(0.71)	(-9.03, -6.97)	-17.89(8)	<0.001
	Alcohol	28.00(0.71)			
12.5 g/ml	Aqueous	13.80(0.84)	(-9.22, -6.78)	-15.12(8)	<0.001
	Alcohol	21.80(0.84)			

[a] Independent t-test was applied

Independent t-test was applied to compare mean between two groups. Table 12 showed us that in all three concentrations alcohol extracts has bigger mean of inhibition as compared to aqueous extract with significance of $p<0.001$ against *Candida albicans*. Ninety five percent confidence interval of mean difference of inhibition does not include zero. Therefore statistically antifungal activity of *Candida albicans* is better in alcohol neem extract than aqueous extract.

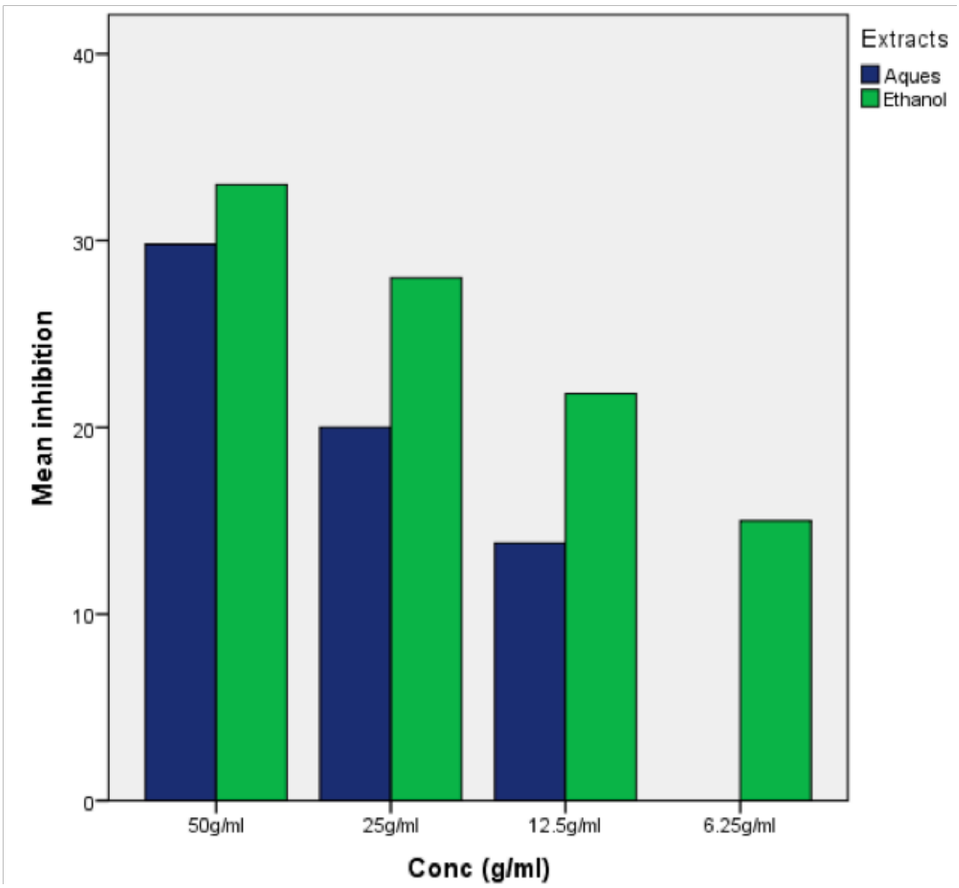

Figure 3: Mean of zone inhibition between aqueous and alcohol extracts against growth of *Candida albicans* at different concentrations.

Comparison on antifungal activity of Malaysian neem between *Candida albicans* and *Aspergillus niger*

Using independent t-test, comparison on antifungal effect between the mentioned two fungal pathogenic otomycosis can be established. *Aspergillus niger* was inhibited better as compared to *Candida albicans* in aqueous neem extracts as shown in Table 13 with significant $p<0.001$ for all three concentrations. However *Candida albicans* was inhibited better as compared to *Aspergillus niger* in alcohol neem extract. Comparison with significant difference are shown in Table 14. Both ninety five percent confidence interval of mean difference of inhibition does not include zero.

Table 13: Comparison of mean of zone inhibition against *Candida albicans* and *Aspergillus niger* on aqueous neem extract at three different concentrations.

Concentration	Group	Mean (SD)	Mean difference (95% Confidence Interval)	t-statistic(df)	p-value[a]
50.0 g/ml	Candida albicans	29.80(0.84)	(-3.62, -1.180)	-4.54(8)	<0.001
	Aspergillus niger	32.20(0.84)			
25.0 g/ml	Candida albicans	20.00(0.71)	(-6.03, -3.97)	-11.18(8)	<0.001
	Aspergillus niger	25.00(0.71)			
12.5 g/ml	Candida albicans	13.80(0.84)	(-7.33, -5.07)	-12.66(8)	<0.001
	Aspergillus niger	20.00(0.71)			

[a] Independent t-test was applied

Table 14: Comparison of mean of zone inhibition against *Candida albicans* and *Aspergillus niger* on alcohol neem extract at three different concentrations.

Concentration	Group	Mean (SD)	Mean difference (95% Confidence Interval)	t-statistic(df)	p-value[a]
50.0 g/ml	Candida albicans	33.00(0.71)	(5.34, 7.06)	16.57(8)	<0.001
	Aspergillus niger	26.80(0.45)			
25.0 g/ml	Candida albicans	28.00(0.71)	(5.07, 7.33)	12.66(8)	<0.001
	Aspergillus niger	21.80(0.84)			
12.5 g/ml	Candida albicans	21.80(0.84)	(5.67, 7.93)	13.88(8)	<0.001
	Aspergillus niger	15.00(0.71)			

[a] Independent t-test was applied

Minimum inhibitory concentration of neem extracts against *Aspergillus niger* and *Candida albicans*

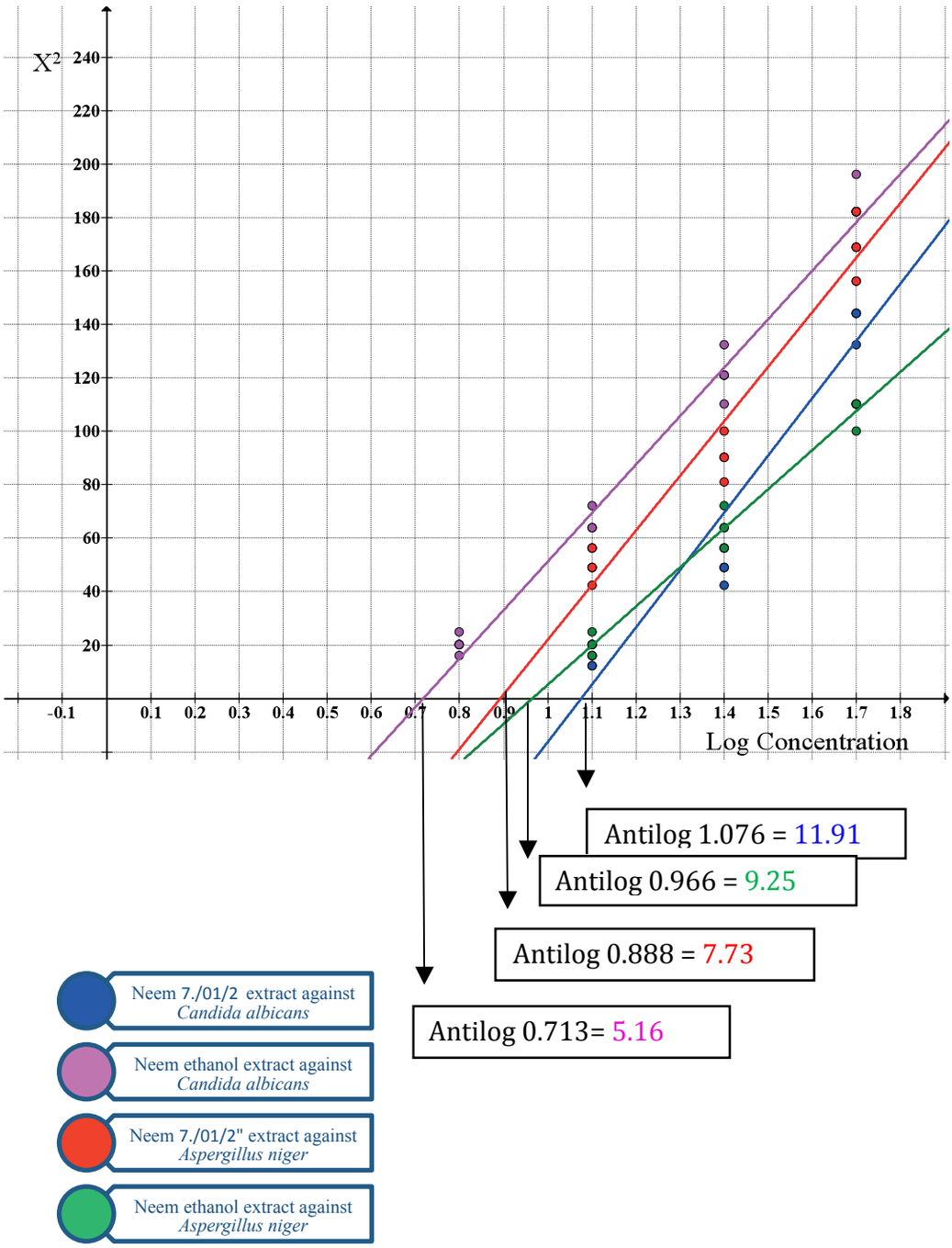

Figure 4: Scattered plot graph with linear line showing zone of inhibition of different concentrations of aqueous and ethanol Malaysian neem extracts against *Candida albicans* and *Aspergillus niger.*

Minimum inhibitory concentration (MIC) as defined earlier was established using agar diffusion method with graph X^2 versus Log concentration. The linear line in the graph, which crosses value zero, was taken and antilog calculated for MIC of each tested organism and types of extract used. With reference to the scattered plotted graph (Figure 4), the MIC of neem aqueous extract against *Candida albicans* was 11.91 g/ml, neem ethanol extract against *Candida albicans* was 5.16 g/ml, neem aqueous extract against Aspergillus niger was 7.73 g/ml and neem ethanol extract against *Aspergillus niger* was 9.25 g/ml.

DISCUSSION

In this study, both alcohol and aqueous extract of Malaysian neem leaf showed significant antifungal activities against *Aspergillus niger* and *Candida albicans*. The efficacy of antifungal towards the pathogenic otomycosis was noted solvent dependant. Antifungal activity of *Candida albicans* in alcohol extracts is more remarkable than in aqueous extract similar to most of the literature reviews. The MIC of Malaysian neem aqueous extract against *Candida albicans* was 11.91 g/ml and MIC neem alcohol extract was 5.16 g/ml. Therefore, there was more inhibition in alcohol extract as compared to aqueous extract. This also being supported with comparative zone of inhibition was bigger in alcohol extracts. In a study by Nayak Aarathi *et al.* (2011) almost similar study to evaluate anti microbial and anti fungal effect on Indian neem leaf aqueous and alcoholic extract as potential agent in inhibition of *Streptococcus mutans, Enterococcus faecalis* and *Candida albicans*. They used serial broth dilution method and determined MIC of the different extracts. MIC of Indian neem aqueous extract against *Candida albicans* documated as 7.5% and MIC of alcohol extract was 3.75%. However, this difference was not significant statistically. They used serial broth dilution method to determine MIC because of failure of neem diffuse effectively through agar medium.[5]

Malaysian neem provides almost close MIC to Indian neem. Similarly, alcohol extract had lower MIC as compared to aqueous extract against *Candida albicans*. In our study, the zone of inhibition of aqueous and alcohol extract against *Candida albicans* was statistically significant ($p<0.001$) and MIC was identified using mathematical logarithm concentration. In a separate study by Mahmoud *et al.* (2011) who conducted a study using 4 different concentrations (5%, 10%, 15%, 20%) of extracts to evaluate anti fungal effects on growth of human pathogens (*Aspergillus flavus, Aspergillus fumigatus, Aspergillus niger, Aspergillus terreus, Candida albicans* and *Microsporum gypseum*) in-vitro. *Candida albicans* growth was inhibited 4.0% on 5.0% aqueous neem leaf extract and higher concentration give higher percentage of inhibition. *Candida albicans* growth was inhibited at 20.30% by alcohol neem extract at 5% concentration. He demonstrated alcohol extracts inhibit more as compared to aqueous extracts. No MIC was stated in their study.[6]

In our study same findings were noted and statistically was significant with $p<0.001$. Another study by Khan *et al.* (1987) reported some leaf extract including those from neem leaf had characteristics effect on dermatophytes especially for low polar extracts over high polar ones. Alcohol extracts have low polarity as compared to aqueous. The author proposed the reason for this was flavonoid quercetin contained in the extracts.[7]

The greater effectiveness of alcoholic as compared with aqueous extracts of neem leaf may be due to differences in constituent extraction.[8] In addition to that, a study conducted on fungicidal activity of some weed extracts against different plant pathogenic fungi by Qasem and Aau-Blan in 1996 suggested that difference in antifungal activities attributed to presence of active principles that are extracted by different solvents, which may be influenced by several factors. The factors are age of plant, method of extraction and type of extracting solvent.[9]

Another study by Sheila *et al* in 2006 on the effect of aqueous extract from Neem on hydrophobicity, biofilm formation and adhesion in composite resin by *Candida albicans* was undertaken to evaluate the effects of the aqueous extract in adhesion, hydrophobicity and biofilm formation by *Candida albicans*. The lack of fungicidal effect of neem extract in assays may be due to low concentration of bioactive compound, which may be increased when alcoholic extracts involved. The adhesion process of *Candida* is a complex issue and involves biological and non-biological factors. Cell surface hydrophobicity, which contributes to interaction between the cells and the surfaces, seems to be important factor in adhesion of *Candida albicans*. Neem leaves have a potential anti-adhesive effect on the samples studied in vitro and anti-adhesive mechanism of action by *Azadirachta indica* was proposed.[10] A study on neem oil of the flowers proposed antifungal activity against *Candida albicans* because of sulphur compound contents.[11]

As for *Aspergillus niger,* we noted there was significant antifungal activity towards *Aspergillus niger* but it was more remarkable in aqueous extract as compared to alcoholic extract, a total opposite to *Candida albicans*. The MIC of Malaysian neem aqueous extract against *Aspergillus niger* was 7.73 g/ml and neem alcoholic extract against *Aspergillus niger* was 9.25 g/ml. In a study on antifungal activity of different neem leaf extract by Mahmoud *et al.* (2011) revealed marked growth inhibition in growth of test fungi at neem extract concentration of 15% characteristically inhibits *Aspergillus niger* up to 88.5%. The inhibition of aqueous extracts showed higher inhibition of all the tested concentrations (5%, 10%, 15%, 20%) as compared to ethanol neem extracts against *Aspergillus niger*.[6] This supports the findings of our study with projected similar results.

Possible explanation regarding inhibition more in aqueous extract rather than alcoholic is depending on the bioactive constituent in the extract. Extraction using water as solvent will preserve certain substance which will strongly inhibit *Aspergillus niger* growth. In study by Mahmoud *et al.* (2011) using HPLC (High Performance Liquid Chromatographic) also mentioned that pure nimonol as single isolated compound from neem leaf has no inhibitory effect on the growth of all tested fungi.[6] This reflects a possible potent synergism for different constituents present in this organic extract which together responsible for characteristic antifungal activity.[12] The author attributed the lowering in antifungal activity to an important fact that triterpenoids when purified, lost its effect whereas in combination provides synergism and gives excellent antifungal activity.

Aspergillus niger unlike *Candida albicans* do not exhibit biofilm formation or adhesion composite resin. Some of the selected *Aspergillus* spp. are known to produce secondary metabolites and are capable of eliciting toxicity in animals and human. Among those toxic substances are the well-known mycotoxins namely 3-nitropropionic acid and ochratoxin A.[13,14] The pathogenicity of *Aspergillus* spp. closely related to these mycotoxins. In a study by Abyaneh *et al.* (2003) on mode of action of neem leaf and seed extraction morphology and aflatoxin production ability of *Aspergillus parasiticus* revealed two important findings. First, the neem extracts influence early steps in aflatoxin biosynthesis pathways due to deformation of the fungal mycelia and vesicles. Secondly, mycelia cell wall was the main target of neem components causing cell wall destruction.[15] Similarly, Zeringue *et al.* (1994) found that aflatoxin was inhibited by neem leaf volatiles on submerged cultures of aflatoxigenic *Aspergillus parasiticus.*[16] Although *Aspergillus niger* does not have the ability to produce aflatoxin, this fungal produces almost similar mycotoxin such as 3-nitropropionic acid and ochratoxin A.[14] Antifungal activity of *Aspergillus niger* inhibited by Malaysian neem extract probably due to inhibition of mycotoxins synthesis similar to *Aspergillus paraticus.* Certain bioactive compound found from neem leaf extracts that has high polarity inhibits mycotoxin production. These compounds also directly damage the mycial cell wall of *Aspergillus niger.* High polarity compounds found more in aqueous extracts.

In addition, study by Mondali *et al.* (2009) on antifungal activities and chemical characterization of neem leaf extracts on growth of some selected fungal species in vitro culture medium had demonstrated an opposite results towards *Aspergillus* spp.[17] The alcohol extracts more effectively inhibits growth of fungus than aqueous extracts. This study conducted using crude neem leaf extracts with either water or ethanol for dilution. Ethanol itself will produce some inhibitory effect to *Aspergillus niger* as noted the control was 38 mm after three days of incubation. The author also mentioned the use of Potato Dextrose Agar (PDA) for inoculation, which was different to our study, which can be another cause of differences in the results. Furthermore, in our study controls for all tested fungi were no growths.

The compounds in neem have been divided into two major classes: isoprenoids and others. The isoprenoids include diterpenoids and triterpenoids containing protomeliacins, limonoids, azadirone and its derivatives, gedunin and its derivatives, vilasinin type of compounds and Csecomeliacins such as nimbin, salanin and azadirachtin. The nonisoprenoids include proteins (amino acids) and carbohydrates (polysaccharides), sulphurous compounds, polyphenolics such as flavonoids and their glycosides, dihydrochalcone, coumarin and tannins, aliphatic compounds, etc.[3] Singh et al stated that fungicidal and bactericidal properties of extracts from neem leave either in vitro or in vivo trials to presence of several ingredients such as desactylimbin, quercetin and sitosterol.[18] Other studies also mentioned antifungal activities in neem leaf due to presence of active ingredients like triterpenes or limonoids compounds.[19] In our study, we believe that synergistic effect of different constituents either isoprenoids or non isoprenoids responsible of antifungal effect towards both *Candida albicans* and *Aspergillus niger.*

From the data that we had gained, Malaysian neem had same antifungal effect toward *Candida albicans* and *Aspergillus niger* compared to Indian neem. However we can't conclude the superiority of the antifungal effect due to differences in methodology such as method of extraction, type of solvent and method to determine MIC. In Malaysia, the weather is very uniform all year round, except there is a distinct rainy and dry season as influence by the North East monsoon and South West monsoon. The vegetation throughout the country is very uniform, called tropical rain forest. The weather and soil constituents factor play an important role in the nutritional gain in each neem tree grown in Malaysia. In the south, India has a tropical climate similar to ours but to north, the climate is changing from tropical to sub tropical and to temperate in the very north part of India. This was the factor that causes different antifungal efficacy in huge continent of India. The neem trees in these areas are subjected to the climatic condition of the respective places.

CONCLUSION

The higher neem aqueous concentrations gave high effect to mean of inhibition of growth of *Aspergillus niger.* All three concentrations aqueous extracts have bigger mean of inhibition as compare to alcohol extract with significance $p<0.001$ against *Aspergillus niger.*

The higher neem aqueous concentrations gave high effect to mean of inhibition of growth of *Candida albicans.* Statistically antifungal activity of *Candida albicans* is better in alcohol neem extract than aqueous extract ($p<0.001$). *Aspergillus niger* was inhibited in aqueous neem extracts better than *Candida albicans* with significant $p<0.001$ for all three concentrations. However *Candida albicans* was inhibited better as compared to *Aspergillus niger* in alcohol neem extract.

The greater effectiveness of alcoholic as compared with aqueous extracts of neem leaf may be due to differences in constituent extraction. Differences in antifungal activities attributed to presence of active principles that are extracted by different solvents, which may be influenced age of plant, method of extraction and type of extracting solvent. Possible explanation regarding inhibition more in aqueous extract rather than alcoholic are depending on the bioactive constituent in the extract and a possible potent synergism for different constituents present in this organic extract which together responsible for characteristic antifungal activity which needs to be explored further.

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