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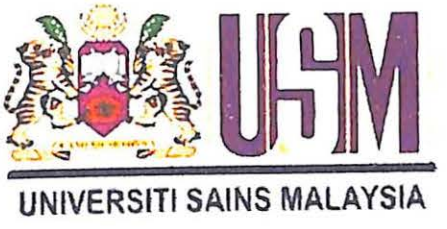

Antifungal properties in some Malaysian herbal plants (*Cassia obtusifolia*)

Dissertation submitted in partial fulfillment for the Degree of Bachelor of Science (Health) in Biomedicine

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2004

CERTIFICATE

This to certify that the dissertation entitled

"Anti-fungal properties of some Malaysian herbal plant (*Cassia obtusifolia*)"

is the bonafide record of research work done by

Mr./ Mrs./ Ms. Norhalini binti Aziz

During the period of June to March 2004

under my/our supervision.

.....

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Date:

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ABSTRACT

Cassia obtusifolia is a plant that belongs to bean family (Leguminosae). Old folks used to use this plant as a treatment for skin diseases such as ringworm. The common part that is used by the folks is the leaf. Thus, I only use *C.obtusifolia* leaves for my research study. Water extract and ethanol extract of *C.obtusifolia* leaves is performed in this study.

The *C.obtusifolia* leaves were collected at Kampung Sungai Keladi, 2 kilometer from Kota Bharu. The leaves were then washed with tap water before it is rinsed with distilled water. The cleaned leaves were dried in an oven to remove the water contained in the leaves so that the pure component of the plant can be collected. The dried leaves were grinded and ready for extraction process by using sohlex apparatus. Distilled water is used as solvent to form water extract. The extraction process takes approximately 7 days. The residue of the leaves was dried in an oven with temperature of 42°C. After 3 days, when the residue dried, it was mixed with ethanol 95% to form ethanolic extract. Then it was filtered to separate the residue and the extract solution. This is done by using 2 layers of gauze which is placed on top of filter paper in a funnel. These two extracts were concentrated by means of evaporation. This process is done by using evaporator apparatus.

The concentrated water extract was put in universal bottles approximately 1/3 full and it was kept in a freezer before it was lyophilysed. As for ethanol extract, it was put in petri dishes and placed in an oven in order to remove the excess ethanol. Since 95% ethanol was used as solvent, the ethanol extract is naturally oily. The process takes several weeks (approximately 3 weeks).

The water extract will be in powder form while ethanol extract will be in oily liquid form. Three concentration of both extract was prepared (1.00 mg/ml, 0.10 mg/ml and 0.01 mg/ml). Since the folks used to use this plant together with the lime ("kapur"), it is believed that the lime helps in enhancing the effect of the plant. Based on this practice, I tried to add the lime to the water extract and ethanol extract to see their reaction. This mixed-extract was also prepared in three concentrations as water and ethanol extract.

The extracts were tested in vitro to fungi. Three types of pathogenic fungi were used for this research namely *Cryptococcus neoformans*, *Candida albican* and *Candida tropicalis*. The test is done by means of agar disc diffusion method. Sabauraud Dextrose Agar (SDA) was used as medium for fungus.

Fungal inoculum suspension was prepared from 48 hours old cultures and sterile distilled water. The suspension was adjusted with saline to approximate a density of 1.0 McFarland turbidity standard. The sterile swab was moisten with adjusted inoculum suspension and the surface of the plates was streaked with the swab in two different directions (at 90 degree angles) to cover the entire surface.

The tests were done by means of disc diffusion method with three incubation periods (3, 5 and 7 days) and three incubation temperature (28°C, 30°C and 37°C). The result was red by measuring the zone of inhibition (clear zone) around the test disc. Amphoterisin B was used as standard antifungal.

TLC was performed at the end of this research.

INTRODUCTION

The word Cassia in ancient Greek means "aromatic". Cassia trees are also known as "gelenggang" among the folks in Malaysia. They are known for their ability to treat ringworm and have been used as a laxative. There are two types of "gelenggang" commonly used in Malaysia namely:

1. "Gelenggang Besar" (*Cassia alata*)
2. "Gelanggang Kecil" (*Cassia obtusifolia*)

As for *Cassia alata*, it is generally used against parasite and act as a laxative. It is also well known as traditional remedy for ringworm and also used to treat bacterial infections caused by *E.coli*, fungal infection caused by *Candida albican* and dermatophytes. Previously, a lot of studies have been done on *Cassia alata* that showed the presence of Antraquinones (responsible for the laxative activity) and Chrypsophanic acid (which is claimed to have antifungal properties and useful for skin complaints such as ringworm).

Another species of Cassia plant is *Cassia obtusifolia* which was chosen the present study subject. *C.obtusifolia* is also called sicklepod (English) and Jue Ming Zi (Chinese). *Cassia obtusifolia* is belongs to bean family (Leguminosae).

The taxonomy of this plant is presented as follow:

Kingdom	Plantae – Plants
Subkingdom	Tracheobionta – Vascular plants
Superdivision	Spermatophyta – Seed plants
Division	Magnoliophyta – Flowering plants
Class	Magnoliopsida – Dicotyledons
Subclass	Rosidae –
Order	Fabales –
Family	Fabaceae – Pea family
Genus	*Senna* P. Mill. – senna
Species	*Senna obtusifolia* (L.) Irwin & Barneby – Java-bean

Morphology

Sicklepod plant is an annual with erect, nearly hairless stems. The leaves are compound with 3-10 leaflets and a conspicuous gland about 2 mm long between, or just above, the lowest pair of leaflets. The terminal pair of leaflets is frequently larger than the lower pairs of leaflets. The leaflet blades are broader toward the tip and smooth above and below. The leaflet margins have short, appressed hairs. The leaflet base is asymmetrical and the leaflet tip is usually rounded with a tiny sharp point. The leaflets are also photosensitive. (The leaflets fold upward by flexible petioles at night or on cloudy days.) The stipules are hairy, linear and about 1-2 cm long. The flowers are axillary and usually solitary with yellow petals 8-17 mm long. The petals are unequal in shape and size. The sepals are unequal, 5-10 mm long and 2-5 mm wide. The fruit is a slender pod up to 18 cm long, 5 mm wide, 4-angled in cross section and usually curved downward. The pods are green and turn brown as the

seeds mature. The seeds are 4-6 mm long, angular, light to dark brown in color, with 2 scars on the opposite surfaces oriented along the longest axis of the seed.

Figure 1: a picture of *C.obtusifolia* plant

Therapeutic Actions

"Jue Ming Zi" or the seed of *Cassia obtusifolia* is claimed by the Chinese to clear the vision, and expels wind-heat for itchy, red, and painful eyes and sensitivity to light due to externally-controlled wind-heat, lowers blood cholesterol, lowers blood pressure, and prevents the formation of atherosclerotic plaque in the artierial wall. It is also claimed to have laxative and anti-bacterial effects. Major chemical constituents of Cassia are: anthraquinones, M.R Khan et al., (2001), betulinic acid, Guo H et al., (1998), chrysophanol , obtusin,Takido M et al., (1990), and many others.

In China, it is traditionally used to remove 'heat' from liver as well as to improve visual acuity. Some of them use it to treat hypercholesterolemia and hypertension.

Since both plant are in same family, It was planed that investigation of *C obtusifolia* in relation to its antifungal activities be performed as a comparison to that of *Cassia alata.*

REVIEW OF LITERATURE

Recently, lot of studies has been done on *C.obtusifolia* plant. Most studies had proved the presence of antraquinones that is responsible for their laxatives activity. According to Takido M. et al, (1990), three anthraquinone glycosides, gluco-obtusifolin, gluco-chryso-obtusin, and gluco-aurantioobtusin, was found to be platelet anti-aggregatory constituents of the seeds of *Cassia obtusifolia.* Various other anthraquinone analogues were also tested.

Zhang Z. et al, (2003) investigate that Toralactone 9-O-beta-d-glucopyranosyl-(1-->6)-beta-d-glucopyranosyl-(1-->3)-beta-d-glucopyranosyl-(1-->6)-beta-d-glucopyranoside (1, cassiaside C(2)), isolated from *Cassia obtusifolia L.* showing strong antiallergic activity, it was concisely synthesized employing glycosyl trifluoroacetimidates as glycosylation agents.

The idea of adding lime to the *C.obtusifolia* extract was based on the tradisional used of the plant by the local folks. Furthermore, Burkill, (1935) said that to treat ringworm is to rub the place with *Cassia alata* leaves or to pound and mix it with lime or other substances before apply it to affected area.

Several parts of *C.obtusifolia* plant has been used in recent study, for example, research done by Guo H. et at, (1998) which used hairy root cultures of *C.obtusifolia* to investigate the anthraquinone production while Lewis DC and Shibamoto T., (1989) used aqueous suspensions and organic extracts of *C.obtusifolia* seeds in their research. The seeds were shown to elevate plasma creatine kinase levels of Sprague-Dawley rats.

A research done by Chopra et al., 1956 stated that the antifungal activity of *Cassia alata* extract may be due to the presence of chrysophanol, a metaboloic product of the powerful antifungal agent.

According to Nantachit, (1988), 35% of ethanolic extract from *C.alata* leaves showed antifungal activity while Crockett et al., (1992) investigated antibacterial and antifungal activities of water extracts of *C.alata* in their search for therapeutic agents.

Lacuna found in literature

- So far, no study of *Cassia obtusifolia* leaves has been done for the antifungal activity.

OBJECTIVES OF THE STUDY

- To investigate whether *Cassia obtusifolia* has antifungal activity as compare to *Cassia alata.*
- To compare the relative effectiveness of *Cassia obtusifolia* and *Cassia alata* on the growth of 3 types of pathogenic fungi.
- To test whether the mixture of *Cassia obtusifolia* extracts and lime has synergistic effect on growth of fungus.

MATERIALS AND METHODS

Materials

Plant materials

Cassia obtusifolia plant was collected at Kampung Sungai Keladi, 2 kilometers from Kota Bharu. The plant was collected in the morning between 10 am to 11 am. Since I was just used the leaves for my present study, the plant leaves was immediately isolated after it was collected. The leaves were put in a clean plastic bag before it was taken to pharmacology laboratory for the cleaning process. 1633 grams of *C.obtusifolia* leaves were collected.

Test organism

Three types of fungi of the yeast type namely *Candida albican, Candida tropicalis* and *Cryptococcus neoformans* were used in this study. These fungi are subcultured from the collection of the Department of Microbiology and Parasitology, PPSP, USM.

Reagent and chemicals

a) Antifungal agent (Amphotericin B)

Amphotericin B (E.R.Squibb & Sons.Inc, USA) was used as standard antifungal agent. It was obtained from the Pharmacy Department of HUSM. Amphotericin B is the most common drug used against the fungal infection. It alters cell membrane permeability, resulting in a leakage of cellular constituents and ultimate lysis and death of the fungal cells.

b) Organic solvent

Ethanol 95% was used as reagent for organic extraction. It was prepared by mixing the ethanol with distilled water. 950 ml of absolute ethanol was mixed with 50 ml of distilled water.

c) Dimethil Sulphoxide (DMSO)

DMSO was also used as solvent for ethanol extract.

Medium

Sabaraud Dextrose Agar (SDA) was media of choice since fungi were used in the present study. SDA was prepared using SDA powder (Oxoid LTD,Basingstoke, Hampshire, England) that is commercially available. Typical formula of SDA is mycological peptone (10.0 g), glucose (40.0G) and agar (15.0g).

Laboratory equipment

There was several laboratory equipment used in the present study. This include blender (model Khind BL 310N), digital balance (AND, GR-200), oven (Memmert, Trawlab, Malaysia), sohlex apparatus, evaporator apparatus (ROTAVAP, Heidolph VV2011), filter paper (Whatman®, Cat No.1001 125), aluminium foil (MCMC XXVII R.M.C), freeze dry apparatus (FTS System INC, Flexi-dry™), freezer (model Panasonic), shaker (Bigger Bill, Thermolyne), autoclave (TOMY SS-325), incubator (Binder GmbH, Bergstr 14D-78532), McFarland reader (Crystal Spec™, RECTON DICKINSON, Serial No. 00002070), purifier

Class II Biosafety Cabinet (Lab. Conco, Kansas City, Missouri), vortex mixer (JK MSI minishaker) and UV light (Betracher, Camag, NS: 29230)

Methodology

Basically, work was carried out at two laboratories. Firstly the Pharmacology laboratory is where the extract was prepared and secondly the Microbiology laboratory was where the extracts were tested on fungus.

The processes involved in the Pharmacology laboratory were:

Drying the leaves

The collected leaves is weigh on a balance to get its wet weight (1633 g) before it is washed using tap water to cleanse the leaves from any insects or other unwanted particles. Then it is rinsed with distilled water and the leaves were put on a tray and placed in an oven at temperature of 42°C to. Oven (Memmert, Trawlab Malaysia) was used for the drying process instead of air drying because it can provide a constant temperature. It can also avoid contamination caused by dust or any unwanted particles. The drying process takes about 4 days. After 4 days, leaves were weighed once again in order to get its dry weight. Then it was ground by using blender to form small pieces so it can readily be use for the extraction.

Weight measurement:

Wet weight = 1774 gram

Dry weight = 293 gram

Extraction (Sohlex extraction)

The extract is prepared by using sohlex apparatus where the ground leaves are put in a permeable cellulose extraction timble in a sohlex chamber. 8 layers of gause are placed on top of the timble so the leaves won't come out from the timble. Distilled water (3500 ml) is used as solvent. The extraction takes 8 days to complete.

Solvent is heated to boiling in a round bottom flask. As a result, the vapor rose through the outer chamber and into the condenser. The vapor condensed into liquid and fall back into the bottom of the sohlex chamber. As the distilled water rose in the chamber, it seeped through the permeable cellulose extraction timble that hold the C.obtusifolia leaves. The water extracted the compound of interest and leaves a solid mass behind. As the water level rose, the solution is forced through the small inner tube and the chamber is flushed due to a siphoning effect. The flushed water returned to the flask, taking the extracted compounds with it. The water is redistilled from the solution in the flak and condensed in the chamber, repeating the extraction with fresh water. The process is repeated as many times as necessary. Each time the process is repeated, the more concentrated the solution in the flask becomes because more is being extracted from the solid mass. This process is stopped when the water in the extraction timble became as clear as before.

Ethanol extract preparation

The residue from the water extracted sample was used to get the ethanol extract. Firstly, the residue was dried using an oven. It takes 3 days to dry the residue. The dried residue was filled in conical flask about ¼ full. Then 95% ethanol was added until it submerges the residue. The residue was mixed well using shaker (Bigger Bill, Thermolyne)

for 15 minutes. This step was repeated 3 times. The solution formed (ethanol extract) was filtered using two layers of gauze which were placed on top of filter paper in a funnel.

Evaporate the extract

Evaporation of the extract was done right after the extraction process. The main reason of this process is to concentrate the extraction by evaporate the excess water (for water extract) and ethanol (for ethanol extract). This process was done by using ROTAVAPOR (VV2000), Heidolph VV2011.

Freeze dry the water extract

Frozen water extract was obtained (using a freezer) before it was lyophylised by freeze dry apparatus. This process takes approximately 2 days.

Dry the ethanol extract by using oven

Concentrated ethanol extract was put in glass petri dish before it was placed in an oven with temperature of about 52°C.The final form of the extract will be an oily liquid.

Extract dilution

As mentioned before, 3 concentration of each extract were tested for antifungal properties. Serial dilution was performed to each extract in order to get concentration that is required.

For the water extract, distilled water was used as solvent. Firstly, 10 mg of water extract (powder form) were weight using the balance (AND, GR-200) before it was added

with 10 ml of distilled water in universal bottle. These will form a concentration of solution to 1mg/ml. Then, from this solution, 1 ml was taken and was put to another universal bottle contained 9 ml of distilled water. The concentration formed was 0.1 mg/ml. Lastly, 1 ml of 0.1mg/ml solution were taken and added with 9 ml distilled water in another universal bottle. The final concentration was 0.01 mg/ml solution.

The same procedure was done to ethanol extract except for the solvent where DMSO (Dimethil sulphoxide) has been used.

Another serial dilution was prepared for both extracts. These solutions were made to add with lime solution. Before that, lime solution need to be prepared first. It was done by adding lime (commercially available) to 100 ml of distilled water. Since no measurement of lime used traditionally by the folk, approximately 1 tea spoon of lime (product of KVS Industries) were used. Lime is partially soluble in water. So, after mixed it with water, the solution was filtered to left the sediment behind. The filtered solution was used in mixing it with extract solutions. 5 ml of lime solution was added to each extract solution and it was well mixed using vortex mixer.

TLC (Thin Layer Chromatography)

TLC is a technique used to indicate the extent of progress of a chemical reaction in which pure substances are separated into the individual substances by using a mobile phase to push the mixture along a stationary phase. Because the individual substances have different molecular structures, they interact differently with both the stationary and mobile phases, and consequently are "pushed" at different rates by the mobile phase separate the component present in the extract. Alumina sheets were used in the present study.

Since there were 4 types of extracts (water extract, water extract with lime, ethanol extract, and ethanol extract with lime) and a positive control (Amphotericin B) used in the present study, the sheets were cut in 7 cm X 10 cm. By using a pencil, a straight line was drawn parallel to the short dimension of the plate approximately 1 cm from the end of the plate. Then the line was marked alternately, 1 cm from another as the guide for placing the extracts spot. By using the narrow end of hematocrit capillary 75 mm/ 75µl (Hirschmann Laborgerate), the solution of extracts was loaded before it was spotted on the marked line. The solvent was allowed to evaporate before the next deposit was applied at the same spot. Again the solvent was allowed to evaporate completely.

Since no mobile phase has been used for antifungal properties, the possible mobile phases were tested according to polarity of the extract. 2 mobile phases were used. First is chloroform plus ethanol with 50:50 ratios and second is water plus methanol with 50:50 ratios. The mobile phase was poured into a chamber. The mobile phase must not exceed the line drawn at TLC (1 cm). The TLC plates were stand in the chamber until solvent has advanced to the top (approximately 1 cm) of the TLC plate. The plate was then placed in a clean dry surface to allow the solvent completely evaporate.

Color reagent was performed and sprayed on TLC surface to monitor the presence of individual component. 3 color reagent were used namely Iodoplatinate, Dragendorff and Vanillin.

The processes involved in microbiology laboratory are:

Sabauroud Dextrose Agar (SDA) plate preparation

For this research, I need to prepare about 100 agar plates. SDA is prepared by using 9 cm diameter plastic Petri dish, distilled water, and most important is Sabauraud Dextrose Agar powder (Oxoid LTD, Basingstoke, Hampshire England). 65 grams of SDA powder was suspended in 1 liter of distilled water. The suspension is bringing to the boil to dissolve completely. Then it was poured into Scott Duran bottles to sterilize it by autoclaving at 121°C for 15 minutes using Autoclave TOMY SS-325. The sterile SDA was left to cool before it was poured into Petri dish. 10 ml is needed for every dish. The plate was labeled and left at room temperature for about 4 hours in order for it to solidify.

Since the plate will be used for the antifungal test, the preparation of the plate should be aseptic. The agar plate was prepared in media preparation room. The solidified agar plates were incubated at 30°C overnight using incubator (Binder GmbH Bergstr. 14D-78532) to screen if there were any contamination occurs during the preparation of the plates.

Preparation of fungal suspension

The yeast inoculum was prepared from 48 hours old cultures on SDA. Approximately 2 ml of sterile distilled water were pipetted into three sterile tubes. Using sterile wire loop, inoculate the single colonies of fungal from SDA and mixed it well with sterile water using vortex mixer (JK MSI minishaker). The suspension was adjusted due to 1.0 McFarland turbidity standard by using McFarland reader. Since the fungal can be infected by inhalation, the preparation of fungal suspension was done in lamina flow ().

Antifungal susceptibility test

The test was done by means of disc diffusion method. The blank disc (6 mm in diameter) was prepared by using 5 layers of filter paper. The disc was placed in bijou bottle before it was sterilized by autoclaving it at 121°C for 15 minutes using Autoclave TOMY SS-325. Sterile disc was compiled in a petri dish. The 100 µl pipette was adjusted to 25 µl and was loaded with extracts solutions. The solutions were pipette slowly to 4 different discs for 3 different concentrations of the extract and a control (Amphotericin B). The disc containing antifungal agent was applied onto the surface of the agar plate using a pair of flamed sterilized forceps. Before that, the plate were divided into 4 section by drew 2 lines perpendicular to each other at the bottom of the plate so that 3 concentrations of extract and a control can be placed at the same plate. The disc was pressed lightly to insure complete contact with agar. Then the plates were placed in incubators with temperature of 28°C, 30°C and 37°C.

Reading the result

The results were taken by measuring zone of inhibition around the disc containing test solution of extract. The results were taken at different intervals (2 days, 5 days and 7 days).

RESULT

Antifungal susceptibility test

The test results were obtained by measuring diameter of clear zone around the disc.

Day 2

Below are the results obtained on day 2.

Water extract

Candida albican (table 1a)

T° / [Extract]	28°C	30°C	37°C
1.00 mg/ml	-	-	-
0.10 mg/ml	-	-	-
0.01 mg/ml	-	-	-
Control	1.3 cm	1.2 cm	1.9 cm

Candida tropicalis (table 1b)

T° / [Extract]	28°C	30°C	37°C
1.00 mg/ml	-	-	-
0.10 mg/ml	-	-	-
0.01 mg/ml	-	-	-
Control	1.7 cm	1.8 cm	1.5 cm

Cryptococcus neoforman (table 1c)

T° / [Extract]	28°C	30°C	37°C
1.00 mg/ml	-	-	-
0.10 mg/ml	-	-	-
0.01 mg/ml	-	-	-
Control	2.3 cm	2.2 cm	3.2 cm

Water extract with lime

Candida albican (table 2a)

T° / [Extract]	28°C	30°C	37°C
1.00 mg/ml	-	-	-
0.10 mg/ml	-	-	-
0.01 mg/ml	-	-	-
Control	1.4 cm	1.2 cm	1.3 cm

Candida tropicalis (table 2b)

T° / [Extract]	28°C	30°C	37°C
1.00 mg/ml	-	-	-
0.10 mg/ml	-	-	-
0.01 mg/ml	-	-	1.3 cm
Control	1.5 cm	1.4 cm	1.5 cm

Cryptococcus neoforman (table 2c)

T° / [Extract]	28°C	30°C	37°C
1.00 mg/ml	-	-	Fully inhibited by drug and extract.
0.10 mg/ml	-	-	
0.01 mg/ml	-	-	
Control	2.4 cm	2.5 cm	

Ethanol extract

Candida albican (table 3a)

T° / [Extract]	28°C	30°C	37°C
1.00 mg/ml	-	-	-
0.10 mg/ml	-	-	0.9 cm
0.01 mg/ml	-	-	0.9 cm
Control	1.4 cm	1.8 cm	1.4 cm

Candida tropicalis (table 3b)

T° / [Extract]	28°C	30°C	37°C
1.00 mg/ml	-	-	-
0.10 mg/ml	-	-	-
0.01 mg/ml	-	-	-
Control	1.7 cm	1.8 cm	1.5 cm

Cryptococcus neoforman (table 3c)

T° / [Extract]	28°C	30°C	37°C
1.00 mg/ml	0.7 cm	0.7 cm	1.5 cm
0.10 mg/ml	0.8 cm	0.8 cm	1.5 cm
0.01 mg/ml	0.8 cm	0.9 cm	1.8 cm
Control	2.0 cm	2.2 cm	2.2 cm

Ethanol extract with lime

Candida albican (table 4a)

T° / [Extract]	28°C	30°C	37°C
1.00 mg/ml	-	-	-
0.10 mg/ml	-	-	-
0.01 mg/ml	-	-	-
Control	1.3 cm	1.2 cm	1.3 cm

Candida tropicalis (table 4b)

T° / [Extract]	28°C	30°C	37°C
1.00 mg/ml	-	-	-
0.10 mg/ml	-	-	-
0.01 mg/ml	-	-	-
Control	1.3 cm	1.2 cm	1.3 cm

Cryptococcus neoforman (table 4c)

T° / [Extract]	28°C	30°C	37°C
1.00 mg/ml	-	-	Fully inhibited by drug and extract.
0.10 mg/ml	-	-	
0.01 mg/ml	-	-	
Control	2.4 cm	2.5 cm	

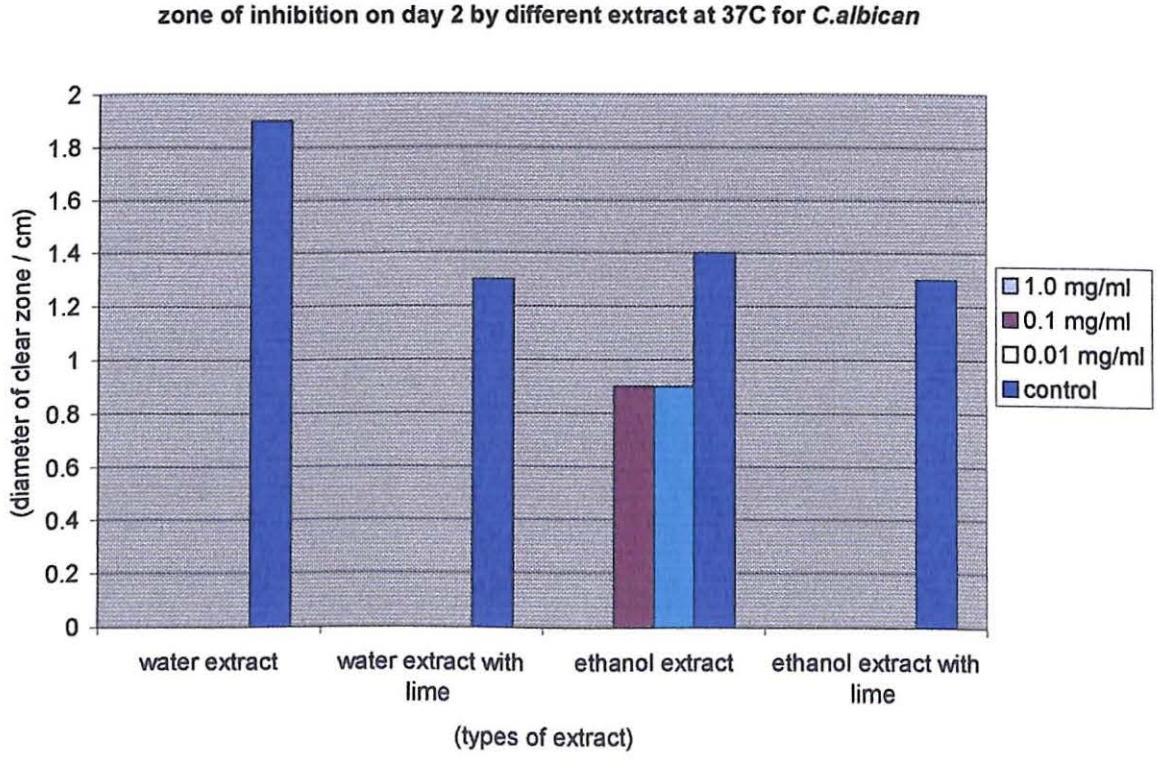

Figure 2

From the graph (figure 2), it is note that ethanol extract show antifungal activity on *C.albican* by performing clear zone. The extract showed their activity at concentration of 0.1 mg/ml and 0.01 mg/ml with same diameter of clear zone. 37°C is the most effective temperature for antifungal reaction of ethanol extract on *C.albican*.

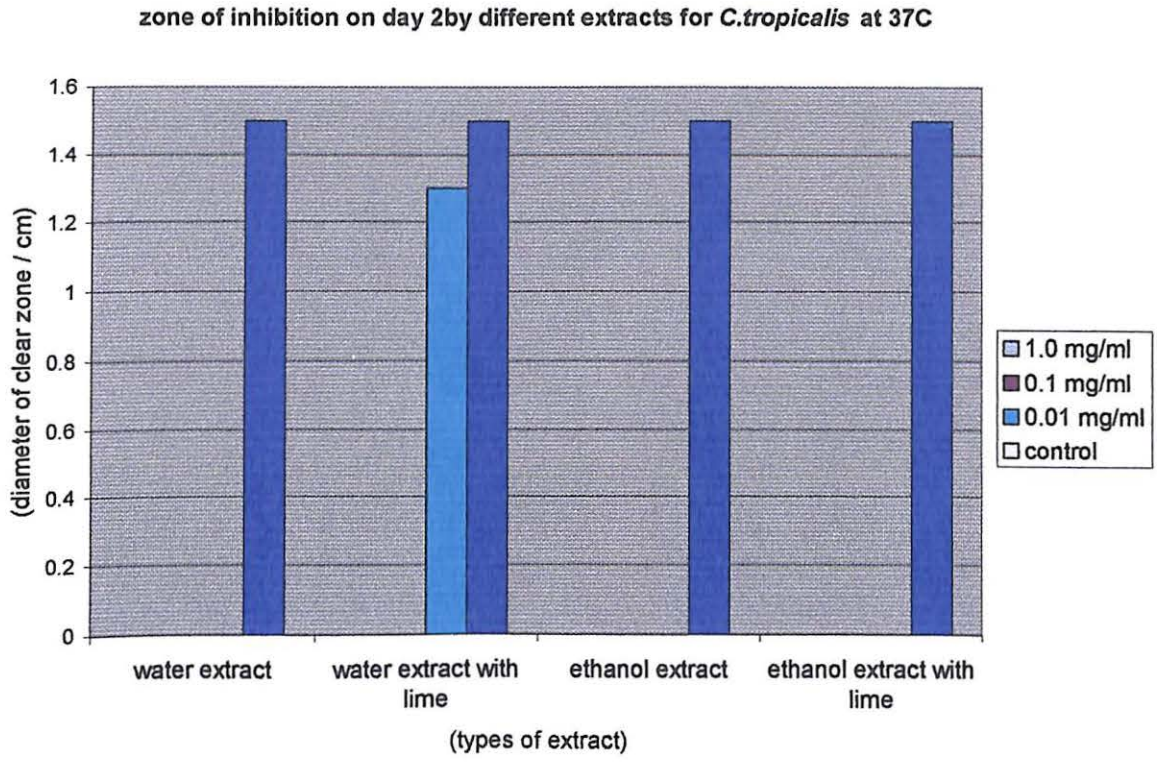

Figure 3

As show on Figure 3, the growth of *C.tropicalis* was inhibited by water extract mixed with lime. No reaction was showed by other extract that inhibit the growth of *C.tropicalis* on day 2 except for the control.

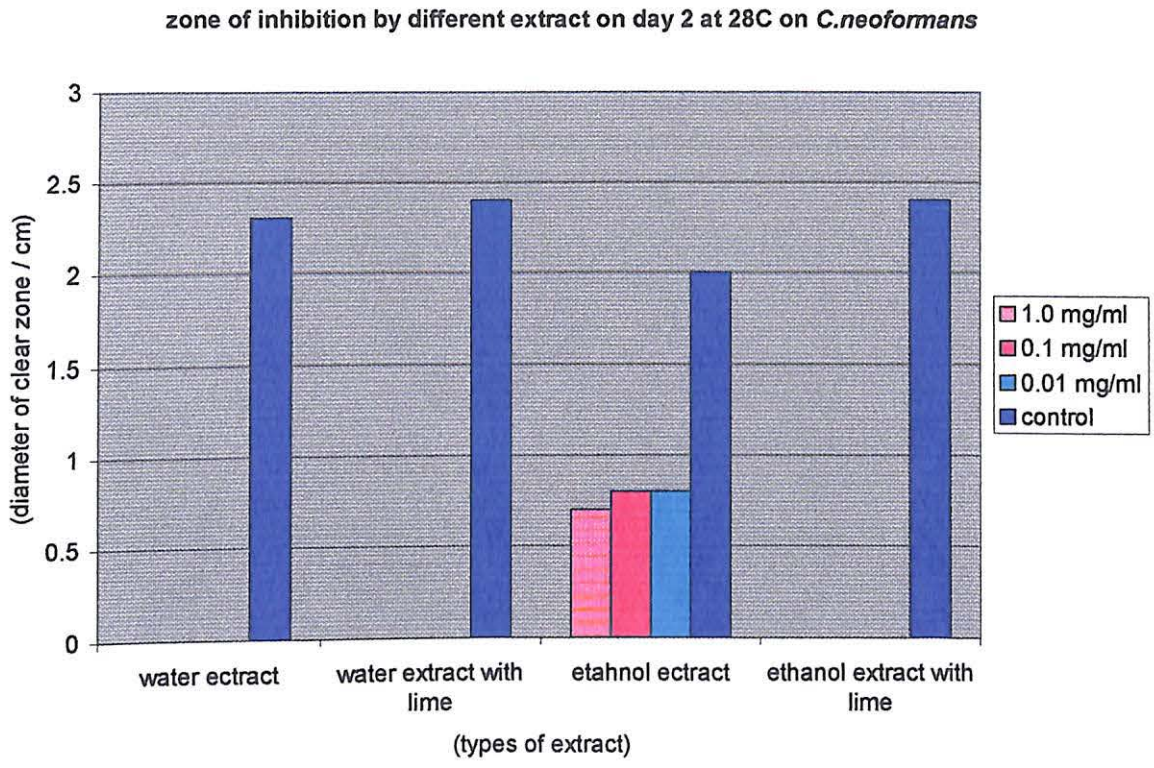

Figure 4

No zones were noted on *C.neoformans* except for ethanol extract as showed on Figure 4. All three concentration of ethanol extract showed their activity on day 2 at 28°C. 1.0 mg/ml extract performed less activity compared to the other two.