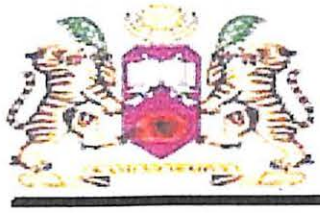
UNIVERSITY SCIENCE OF MALAYSIA



UNIVERSITI SAINS MALAYSIA

Studies on the antifungal effect of *Cymbopogon nardus* (serai wangi) on several fungi and yeasts

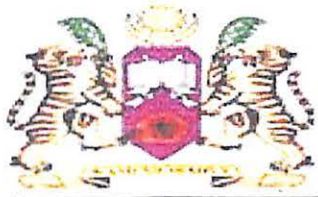
Dissertation submitted in partial fulfillment for the Degree of Bachelor of Science (Health) in Biomedicine

Nik Aida Diana binti Nik Zainuddin

School of Health Sciences
Universiti Sains Malaysia
Health Campus
16150 Kubang Kerian, Kelantan
Malaysia

2004

UNIVERSITY SCIENCE OF MALAYSIA



UNIVERSITI SAINS MALAYSIA

Studies on the antifungal effect of *Cymbopogon nardus* (serai wangi) on several fungi and yeasts

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

Nik Aida Diana binti Nik Zainuddin

School of Health Sciences
Universiti Sains Malaysia
Health Campus
16150 Kubang Kerian, Kelantan
Malaysia

2004

CERTIFICATE

This is to certify that the dissertation entitled

"Studies on the antifungal effect of *Cymbopogon nardus* (serai wangi) on several fungi and yeasts"

is the bonafide record of research work done by

Ms Nik Aida Diana binti Nik Zainuddin

during the period of August 2003 to February 2004

under our supervision.

Signature of Supervisor :

Name and address of Supervisor : Dr. Noor Izani bin Noor Jamil
School of Health Sciences
Universiti Sains Malaysia
16150 Kubang Kerian
Kelantan, Malaysia

Date : 14/4/2004

Signature of Co-Supervisor :

Name and address of Co-Supervisor : Dr. M. Shahjahan
School of Health Sciences
Universiti Sains Malaysia
16150 Kubang Kerian
Kelantan, Malaysia

Date : 14/4/2004

Acknowledgements

First and foremost, I would like to express my highest and deepest gratitude to All-Mighty; Allah who gives me enough strength and time to accomplish this thesis. I would also like to thank my supervisor; Dr. Noor Izani bin Noor Jamil and my co-supervisor; Dr. M. Shahjahan for supporting me in completing this thesis.

My heartiest appreaciation to all the staff of Pharmacology Department especially Dr. Siti Amrah Sulaiman, Puan Halijah, Encik Norman, Kak Chik (Microbiology Laboratory), and the staffs of Biomedicine Laboratory and Laboratory Facility Unit (UKM) whom have made possible the completion of my thesis. I would also like to express my dedication to my whole family especially my lovely mom whom gave me continuous encouragement and full support in order to complete this thesis. Not forgetting, thanks to all my friends whom willingly helped me finished this thesis.

Last but not least, my sincere thanks to the School Of Health Sciences and the library in assisting my needs in completing my work.

Nik Aida Diana binti Nik Zainuddin

February, 2004

CONTENTS

LIST OF TABLES

PAGE NUMBERS

LIST OF FIGURES

Abstract

Antifungal studies of *Cymbopogon nardus* were carried out against several different fungi and yeast. Cold extraction method employing organic solvents was used in this study. The filtered aqueous methanolic extract of the leaves was sequentially extracted with n-hexane, chloroform and ethanol respectively. *Candida albicans, Candida glabrata, Candida tropicalis* and *Cryptococcus neoformans* were selected as test organisms. The chloroform extracts of *Cymbopogon nardus* was active against *Candida albicans*, *Candida glabrata* and *Candida tropicalis* giving a clear zone of 0.7-0.8 cm at different concentrations (0.01 mg/ml, 0.1 mg/ml and 1.0 mg/ml). The n-hexane, ethanol and methanol extract did not show any inhibitory activity against the microorganisms tested except for *Candida glabrata*, where a clearing zone of 0.6-0.9 cm was noted for the ethanol and methanol extracts at different concentrations. n-Hexane, chloroform, ethanol and methanol extracts were active against *Cryptococcus neoformans* at all the concentration tested. However, the growth inhibitory effect of all the extracts are insignificant compared to the antibiotic Amphotericin B.

Introduction

Malaysia is one of the twelve countries in the world with vast diversity in bioresources. Hence, it is an important center rich in biodiversity with unsurpassed source of molecular structural biodiversity. From 15 000 flowering plants known, about 1200 species were reported to have some kind of medicinal properties. But only 15% of these plants were phytochemically studied to some extent for their medicinal potential. In Malaysia, medicinal plants and their extracts are available in the market and sidewalks, mostly in crude form sold by the traditional medical practitioner or home grown for self use. These medicinal plants are used by various ethnic groups residing in this country and normally obtained from the wild or imported mostly from China, Indonesia, and India. Besides Kacip Fatimah, Tongkat Ali, Pegaga, Gelenggang, Mengkudu to name a few, Serai wangi is also an important medicinal plant used by the locals. In this study, Serai wangi is the plant of choice not only due to its extensive used and availability but because it is believed to have an anti-fungal properties. The term antifungal can be defined as antimycotic; which means destructive to fungi or suppressing their growth or reproduction, effective against fungal infections and an agent that so acts. Meanwhile, fungus is interpreted as any organism belonging to the fungi and anything which resembles such an organism. Yeast is a general term including single-celled, usually rounded fungi that produce by budding, some yeast transform to a mycelial stage under certain environmental conditions, while others remain single-celled. They are fermenters of carbohydrates and a few are pathogenic for humans. (Dorland's Pocket Medical

Dictionary 26th Edition, 2001). Some examples of fungi and yeasts; which have been used in this study include *Candida albicans, Candida glabrata, Candida tropicalis* and *Cryptococcus neoformans.*

Ethnobotanical

Cymbopogon nardus or 'serai wangi' in Malay language; belongs to Graminae or Poaceae family (grass family). Common names for this plant are citronella grass in English, nardus, nard grass, mana grass, andropogon nardus, Sri Lanka citronella and lenabatu citronella. It is native to South East Asia and it is also grown commercially in Sri Lanka, India, Burma, Indonesia and Jawa. (http://www.floridata.com/ref/C/cymb_nar.cfm).

Taxonomy

Citronella grass is a coarse, clump-forming tropical grass that can grow 5-6 feet (1.5-1.8 meter) tall. The leaves are sessile, simple, green, linear, equitantly arranged and can grow to an average size of 60 cm long and 2.5 cm wide. The leaf is glabrous, venation parallel with an acuminate apice and sheathing base. There are no true stems. The leaf sheath is tubular and acts as a pseudo-stem (http://www.HerbaMalaysia_net Your One-Stop Herbal Portal.htm). The stems are cane like and the leaves are grayish green, flat, about 3 feet (0.9 m) long and 1 in (2.5 cm) or so wide. It does not spread by runners, as some grasses do, but the

clump increases in size as the plant matures. It takes about two and a half to three months to mature.

Ethnopharmacological

The leaves have been used for their fragrance and value as a medicine for centuries. Citronella is renowned for it's use as an excellent insect repellent. Citronella oil's most useful quality is that of insect repellent. It is best used in a spray, a diffuser or on a cotton ball amongst the linen.

The Chinese use the leaves for treating rheumatism. Other traditional uses include fevers, intestinal parasites, digestive (stomach complaints) and menstrual problems. (http://www.naturedirect2u.com/Essential%20oils/citronella.html). The essential oil is rubbed topically on the stomach for comfort. Nevertheless, it is also the source of commercial citronella oil. The perfume and soap industry often used the essential oils from *Cymbopogon nardus* due to its powerful, slightly sweet and lemony aroma. Inexpensive soaps sold in Asian markets are scented with citronella oil. Known as 'citrus' oil, it is a yellow brown liquid with a powerful, fresh, lemon scent. The Jawa Citronella oil is colorless to a pale yellow and its scent is more woody and sweet. It is considered superior in perfumery work. Both blend well with geranium, lemon, bergamot, orange, cedarwood, and pine. The oil is also mixed with other vegetable oils and used in massage. It is also useful in ridding cats and dogs of fleas.

Citronella oil plays a roll in clearing the mind and has a toning effect on the body. It can be helpful with colds, flu and minor infections and also has deodorizing qualities. (http://www.essentialoils.co.za/essential_oils/citronella.html). Citronella candles and incense, however, are less effective in keeping the mosquitoes away.

In the traditional preparation of serai wangi, the fresh plant (especially leaves) is deep fried in the cooking oil (especially coconut oil) until the leaves turned brown and crispy. The oil is then separated from the leaves and used. As we all know, the oil has a very strong lemon fragrant. Besides that, the whole fresh plant can also be steamed sterilized and the essential oil is collected through the distillates. Excess water is then removed by using salt before the concentrated oil is used. (personnel communion)

Figure 1 : *Cymbopogon nardus* (serai wangi) plant.

Literature Review

There are some literatures written on *Cymbopogon nardus* (serai wangi), it's antifungal and antibacterial properties. According to Virginia *et al* (2001), the mycelium growth of *Aspergillus niger* (Van Tiegham) was completely inhibited at 800 mg/L by *Cymbopogon nardus* (L.) Var. W. Watson var. essential oil. It was determined on agar medium and the concentration (800 mg/L) was found to be lethal under the test conditions.

Inouye *et al* (2000) have shown an inhibitory effect on the apical growth of *Aspergillus fumigatus* hyphae by vapor contact from *Cymbopogon nardus* extracts. It was able to inhibit the growth by stopping the apical growth of the fungus. Puatanachokchai *et al* (2002) also found that *Cymbopogon* extracts were able to inhibit hepatocarcinogenesis on rats. After initiation with diethynitrosamine, the male fisher 344 rats have shown an inhibitory effect on the early phase of hepatocarcinogenesis. According to Inouye *et al* (2001), the antibacterial activity of 14 essential oils and their major constituents in the gaseous state was determined against several bacteria. The most effective antibacterial action was from the essential oils of *Cymbopogon nardus* that was used at high vapor concentration for a short time. It was tested on *Haemophilus influenza, Streptococcus pneumoniae, Streptococcus pyogenes* and *Staphylococcus aureus.*

Friedman *et al* (2002) have reported that 80% ethanol extract of *Cymbopogon spp.* was found to be anti mutagenic against various known mutagens in the *Salmonella* assay. Pattnaik *et al (*1996) also found that several essential oils show some reactions on antimicrobial activity such as the essential oils of aegle, ageratum, citronella, eucalyptus, geranium, lemongrass, orange, palmarosa, patchouli and peppermint.

A study done by Hanina *et al* (2002) showed that some components of *Cymbopogon nardus* extract was active against *Staphylococcus aureus.* Further study done by Ahmad *et al* (2000) also showed that several fractions from the *Cymbopogon nardus* extract possess anti-bacterial activity against few bacteria including *Staphylococcus aureus.*

According to Ahmad *et al* (1992), the in vitro antiviral activity of Cymbopogon nardus crude extract protects *Datura Stramonium* from viral infections. A study done by A.M Marini *et al* (1998) also found that the cells used in optimizing the parameters of a vitro cytotoxicity assay treated with methanolic extracts of *Cymbopogon nardus* (L.) Rendle have a lower CD_{50} value.

Lacunae

Studies on the antifungal effects of this plant extracts are not well documented. Therefore, the effects of the plant extracts on clinically important yeasts and fungi will be investigated.

Objectives Of The Study

Since not much work has been done on the antifungal properties of *Cymbopogon nardus*, thus on aims are to :

1. To isolate and fractionate the crude extracts for anti-fungal studies.
2. To determine the anti-fungal effects of *Cymbopogon nardus* (serai wangi) fractionates on several fungi and yeasts.

Materials and methods

Plant

Cymbopogon nardus was obtained from Pengkalan Chepa, Kota Bharu, Kelantan. The fresh plant (5 kilogram) was then washed with distilled water to remove unwanted materials. The leaves were used in this study.

Microorganisms

The fungi and yeast used in this research were *Candida albicans, Candida tropicalis, Candida glabrata* and *Cryptococcus neoformans.* They were obtained from Department of Microbiology, School of Medical Sciences, University Science of Malaysia (USM) Health Campus, Kubang Kerian, Kelantan.

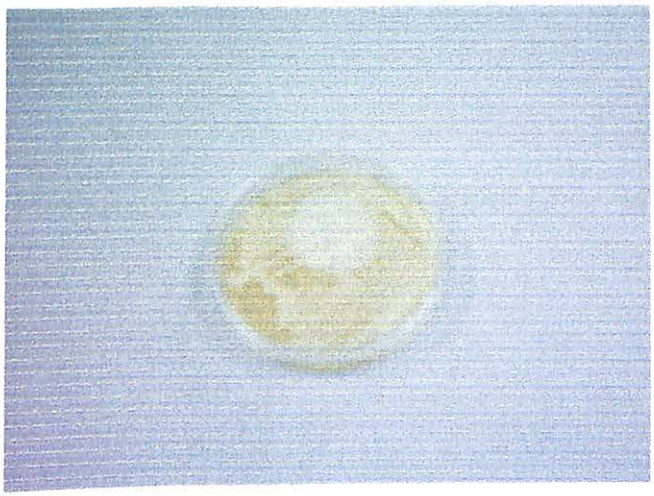


Figure 2 : Culture of *Cryptococcus neoformans* on the SDA agar.

Figure 3 : Subculture of several yeasts and fungi on slant agar.

Figure 4 : Petri dish that containing subculture of *Candida albicans* and *Candida tropicalis* on SDA.

Disc diffusion and spread plate method

The disc diffusion method for susceptibility testing (also known as the Kirby Bauer (KB) method) was standardized primarily for testing of rapidly growing fungus. To perform the test, filter paper discs impregnated with a specific amount of antifungal agents were applied to the surface of an agar medium that was inoculated with a known amount of the test organism. The drug in the disc diffused through the agar. As the distance from the disc increased, the concentration of the antifungal agent continues decreased creating a gradient of drug concentrations in the agar medium. Concomitant with diffusion of the drug, the fungi that were inoculated and that were not inhibited by the concentration of the antifungal multiplied until a lawn of growth was visible. In areas where the concentration of drug was inhibited, no growth occurred, formed a clear zone of inhibition around each disc.

Extraction method

For the extraction method, only one method was used in this study; which was cold extraction method. In this method, first and foremost the plant was washed with sterile distilled water. Next, it was cut into small pieces to make it easier to blend it in the blender (Khind, BL 310N). The concentrated juice was added with methanol and later extracted to get the methanolic extract. The extraction procedure is as shown in figure 8.

Equipments and apparatus

The equipments and apparatus used in this study includes sterilized water filter, rotary evaporator (Heidolph VV 2011 and Heidolph WB 2001), shaker (Bigger Bill, Thermolyne), freeze drier (FTS Systems INC; Multi tainer^{TM} and Flexi-dry^{TM}), blender (Khind, BL 310N), petri dishes, separating funnel, sterile swab and etc.

Figure 5 : Rotary evaporator.

Figure 6 : Shaker used to mix the extracts.

Chemicals

The chemicals used were of analytical grade. The chemicals wer methanol, n-Hexane, chloroform and ethanol (Merck, Germany).These organic solvents with different polarity were used in the extraction methods.

Preparation of agar media

Sabouraud's dextrose agar (SDA); Oxoid LTD, Basingstoke, Hampshire, England was used in this study. Suspended 65g in 1 liter of distilled water. Boiled to dissolve completely. Sterilized by autoclaving at 121^{0}C for 15 minutes.

The sterilized Sabouraud's dextrose agar was poured into petri dish and allowed to cool at room temperature for 4-5 hours. The petri dish containing SDA agar was kept in the incubator overnight at 37^0C. The agar media was stored in the cold room at 4^0C prior to use.

Figure 7 : Preparation of Sabouraud's dextrose agar (SDA) was done (pour plate).

Determination of dilution test method

For the dilution test method, first of all to achieve concentration of 0.1 mg/ml, the extract was taken from a sterilized universal bottle and was weighed for approximately 0.1g by using the weighing machine (AND, GR-200). Then, it was added to 10 ml of organic solvent and mixed before transferred to a sterilized universal bottle. To obtain the concentration of 0.01 mg/ml dilution, the second mixture or dilution (0.1 mg/ml) was measured for 1 ml before poured it into a sterilized universal bottle. Then, measured 9 ml of the same organic solvent and

mixed them well in the universal bottle. Therefore, there are three types of concentration of dilution for each organic solvent used in this study.

Dilution of fungus and yeast

Streaked a single colony from a culture of microorganism in the petri dish using a sterilized wire loop. Before streaking was done, approximately 10 ml of sterilized filter water poured into a 100 ml test tube. Next, the sterilized wire loop was dipped into the sterilized filter water in the test tube and mixed them well. All of these steps were done in the Purifier Class II Biosafety Cabinet (LabConeo, Kansas City, Missouri) to avoid contamination of the yeast and fungi to the surrounding environment. After that, the test tube was examined to achieve a suitable concentration of each dilution using Mc Farland's reader. This concentration of dilution was then compared to Mc Farland's 1.0 dilution that acts as a control for this determination. The best selection was done if the correct concentration or the approximate value of the dilution was achieved.

Bioassay test method

A spread plate method was carried out in this study; whereby the dilution of each fungus and yeast was spread and lawn on Sabouraud's Dextrose agar (SDA) using a sterile swab. The petri dish was divided and marked into four columns; which includes three types of concentration of dilution for each organic solvent and the standard antifungal or the drug (50mg Fungizone Intravenous Ampotericin B for infection USP, Bristol-Myers Squibb).

A filter paper disk (Whatman^{R}, Cat No 1001 125, 125mm X 100 circles) was placed onto each column in the petri dish before the dilution for each types of concentration from the dilution test method and the standard antifungal or the drug (50mg) were pipetted down using Appendorf pipette (France) for 2.5 µl. The pipetting step was done separately in other petri dish to allow the organic solvent used had been dried before the filter paper disk was applied onto the petri dish. All of the steps mentioned above were done in the Purifier Class II Biosafety Cabinet.

Next, the petri dish was kept in the incubator for 48 hours (2 days) at 37^{0}C. The result for this bioassay method was obtained by measuring the diameter from the clearing zone produced; which occurred around the disk and observed easily by naked eyes from the back of the petri dish. From this measurement, the antifungal activity was determined and were categorized as resistant or sensitive.

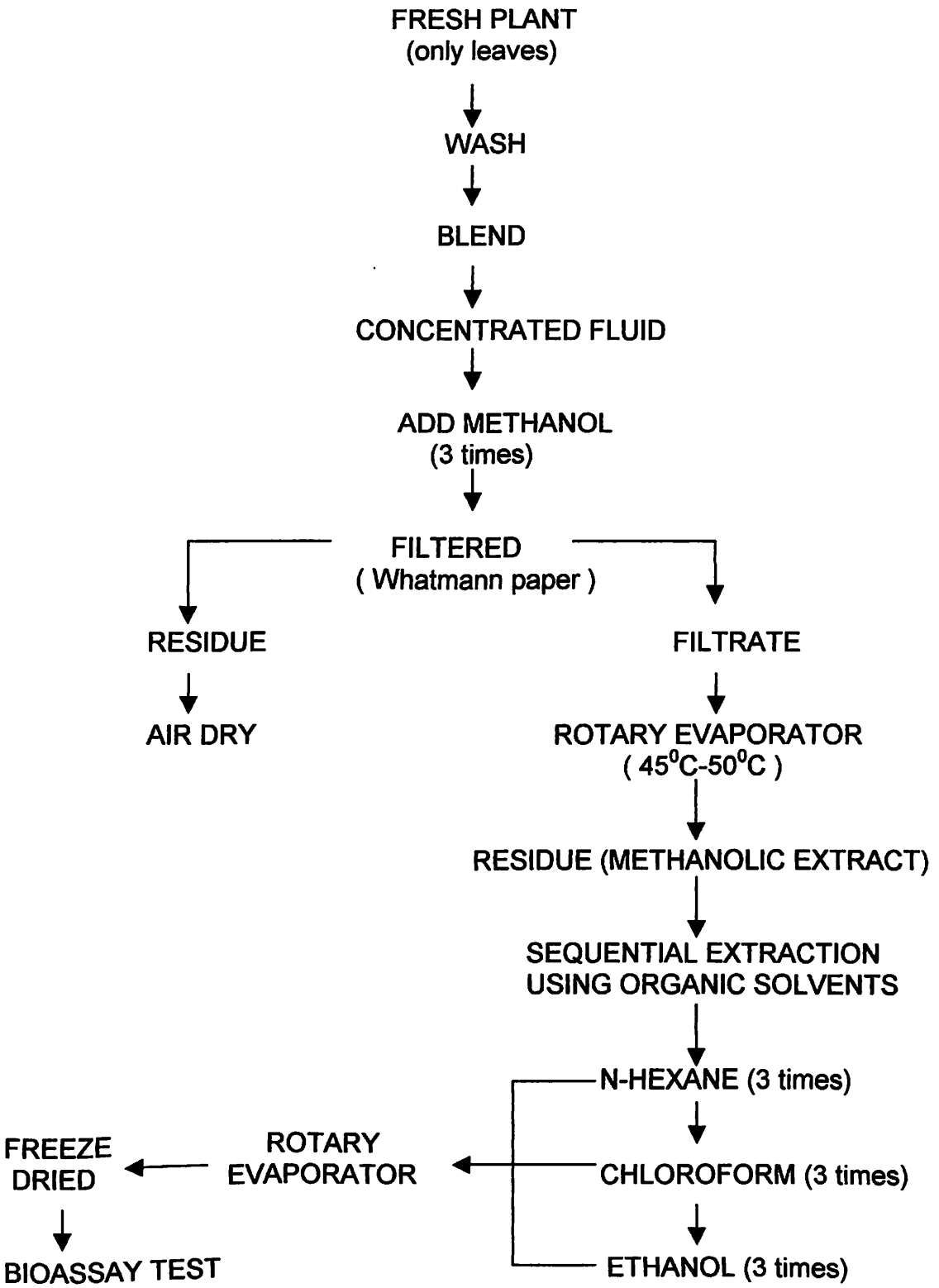


Figure 8 : Flow chart of organic solvent extraction method.

Results and Discussion

A study on the antifungal effects of *Cymbopogon nardus* (serai wangi) against several types of fungi and yeasts were done, and the results achieved were as shown in the tables and figures below.

Antifungal effects of various organic solvents extract against *Candida albicans*.

Table 1 showed the antifungal effect of n-hexane, chloroform, ethanol and methanol extracts on *Candida albicans* at different concentration. There was no effect of n-hexane, ethanol and methanol extract at different concentration used in this study. Nevertheless for the chloroform extract, there was a small diameter of clearing zone marked on the SDA medium which ranges from 0.7 cm and 0.8 cm. This maybe due to the characteristics owned by chloroform itself; which was easily oxidized to the surrounding environment. Besides that, it also showed the maximum effect of chloroform extract at the concentration of 0.1 mg/ml against *Candida albicans*.

	Clear zone of inhibition produced by			
	n-hexane extract	**Chloroform extract**	**Ethanol extract**	**Methanol extract**
Amphotericin B	1.4 cm	1.5 cm	1.4 cm	1.5 cm
1.0 mg/ml	-	0.7 cm	-	-
0.1 mg/ml	-	0.8 cm	-	-
0.01 mg/ml	-	0.7 cm	-	-

Table 1 : Antifungal effects of various organic solvents extract against *Candida albicans*.

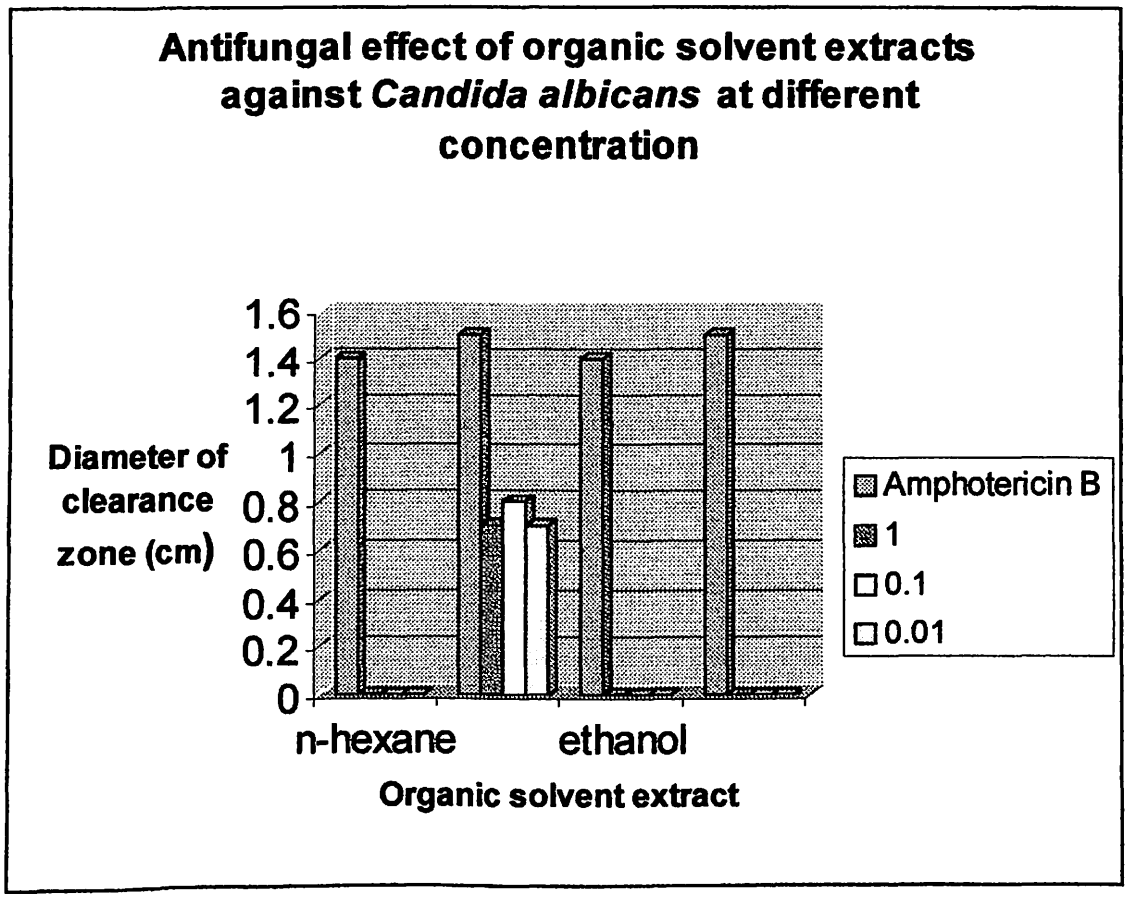


Figure 9 : Graph shows the antifungal effect of organic solvent extracts against *Candida albicans* at different concentration.

Figure 9 above showed the antifungal effect of organic solvent extracts against *Candida albicans* at different concentration. Only chloroform extract showed inhibitory reaction for each concentration used in the study. The diameter of clearance zone obtained did not differ much.

Antifungal effects of various organic solvents extract against *Candida glabrata.*

Table 2 showed the antifungal effects of n-hexane, chloroform, ethanol and methanol extraction against *Candida glabrata*. It showed the same diameter clearing zone for all types of concentration in chloroform extraction. The maximum antifungal properties of ethanol extraction was observed at 0.1 mg/ml while for the methanol extraction, it was observed at the 0.01 mg/ml.

	Clear zone of inhibition produced by			
	n-hexane extract	**Chloroform extract**	**Ethanol extract**	**Methanol extract**
Amphotericin B	1.8 cm	1.5 cm	1.4 cm	1.4 cm
1.0 mg/ml	-	0.7 cm	0.8 cm	0.6 cm
0.1 mg/ml	-	0.7 cm	0.9 cm	0.7 cm
0.01 mg/ml	-	0.7 cm	0.8 cm	0.8 cm

Table 2 : Antifungal effects of various organic solvents extract against *Candida glabrata*.