Anticandidal Activity of *Quercus infectoria* "Nut Gall" Extracts on *Candida* spp.

by

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CERTIFICATE

This is to certify that the dissertation entitled "Anticandidal Activity of *Quercus infectoria* "Nut Gall" Extracts On *Candida* spp. is bona fide record of research work done by ONG JIN TING during the period from September 2012 to May 2013 under my supervision.

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ADHD	Attention-Deficit/Hyperactivity Disorder
ATTC	American Type Culture Collection
Amp B	Amphotericin B
DMSO	Dimethyl Sulfoxide
HIV	Human Immunodeficiency Virus
ICU	Intensive Care Unit
MIC	Minimum Inhibitory Concentration
NNISS	National Nosocomial Infection Surveillance System
SDA	Sabouraud Dextrose Agar

ABSTRAK

Aktiviti anticandida ekstrak organic dan akues *Quercus infectoria* atau Majakani telah dikaji pade *Candida* spp. Ekstrak organic and akues ini dilakukan secara pengekstrakan berturut-turut menggunakan kepekatan peningkatan polariti dengan kloroform, etanol, eter petroleum, dan akhirnya air. Lima strain ATCC spesies patogenik *Candida* seperti *Candida albicans*, *Candida glabrata*, *Candida tropicalis*, *Candida krusei* dan *Candida parasilopsis* telah digunakan dalam kajian ini. Untuk permulaan ujian aktiviti anticandida, kaedah difusi agar dijalankan dengan menggunakan ekstrak dari kepekatan 0.4 mg/ml, 0.8 mg/ml, 1.6 mg/ml dan 3.2 mg/ml. Untuk menentukan halangan pertumbuhan *Candida* spp kepada ekstrak adalah berdasarkan pemerhatian makroskopik pertumbuhan koloni selepas 48 jam. Keputusan kajian kami mendapati bahawa pertumbuahan semua spesies *Candida* yang diuji adalah direncat oleh amphotericin B ($\leq$ 1μg). Disamping itu, semua ekstrak yang diuji tidak menunjukkan activiti anticandida kepada kepekatan ekstrak dibawah 1.6 mg/ml. Hanya *C. albicans* dan *C. parapsilosis* yang menunjukkan kesan anticandida daripada kloroform ekstrak pada 1.6 mg/ml dan ke atas. Manakala, spesies *Candida* lain tidak menunjukkan kesan anticandida ke atas semua ekstrak lain walaupun pada kepekatan yang melebihi 3.2 mg/ml. Ekstrak chloroform *Q. infectoria* menunjukkan aktiviti anticandida yang lebih baik berbanding yang lain.

ABSTRACT

Anticandidal activity of *Quercus infectoria* "nut gall" organic and aqueous extracts were studied on *Candida* spp. The *Quercus infectoria* nut gall extracts were obtained through successive extraction with different solvents in increasing order of polarity firstly with petroleum ether, followed by chloroform, ethanol, and finally water. Five ATCC strains of pathogenic *Candida* species namely *Candida albicans, Candida glabrata, Candida tropicalis, Candida krusei* and *Candida parasilopsis* were used in this study. For preliminary anticandidal activity testing, an agar dilution method was employed by using diluted extracts with the concentration of 0.4 mg/ml, 0.8 mg/ml, 1.6 mg/ml and 3.2 mg/ml respectively. The determination of minimum inhibitory concentration of the extracts against *Candida* spp. based on the macroscopic observation of growth colony after 48 hours of incubation on SDA. Our study found that all tested *Candida* species were susceptible to amphotericin B ($\leq 1\mu g$). Besides, all extracts did not show inhibition at concentration below 1.6 mg/ml, only *C. albicans* and *C. parapsilosis* were inhibited by chloroform extract while other *Candida* species did not show growth inhibition to all other extracts at concentration of 1.6 mg/ml and above. The chloroform extract of *Q. infectoria* showed better anticandidal activity than the other organic and aqueous extracts.

CHAPTER 1

INTRODUCTION

1.1 Background of Study

Fungal infections have dramatically increased in recent years. The last few decades have seen a steady worldwide increase in the incidence of fungal infections (superficial and internal mycoses) especially by *Candida* species (Wang *et al.,* 2010). The rises in fungal infection incidence were due to prolong antibiotic therapy, invasive therapeutic procedures, radiotherapy and AIDS pandemic and to a lesser extent cytotoxic chemotherapy and in organ transplantation (Nissapatorn, *et al.,* 2003). In many cases, *Candida* species are the most common pathogens as far as fungal isolates are concerned. Candidiasis is a fungal disease with wide spectrum of clinical presentation and is caused by yeasts that belong to the genus *Candida*. The infection may be acute or chronic, superficial, deep or disseminated involving various important organs. *Candida* species are normal commensals of human anatomical sites and normally live on the skin and mucosal membranes of female genital tract, oropharygeal cavity and gastrointestinal tract without causing infection; however, overgrowth of these organisms can cause symptoms to develop. Symptoms of candidiasis vary depending on the area of the body that is infected. *Candida* spp. is an important cause of bloodstream infections, opportunistic infections and vaginal candidiasis (Pfaller *et al.,* 2007). There are over 20 species of *Candida* yeasts that can cause infection in humans, the most common of which is *C. albicans* which accounted for virtually all mucosal candidiasis and responsible for about 60% of both superficial and systemic mycoses (White *et al.,* 1998).

Nevertheless, in recent years, Candidal infections are complicated by the emergence of non-albicans *Candida* (NAC) species such as *C. glabrata, C. tropicalis, C. krusei and C. parapsilosis* which cause serious oropharygeal and esophageal candidiasis (Vazquez *et al.,* 2006). Topical, oral, esophageal and tracheobronchial candidiasis is most common in HIV-infected children (Chakraborty and Shingadia, 2007). Nevertheless, opportunistic infections continue to cause morbidity and mortality in patients with HIV infection throughout the world, with more incidence in developing countries where access to care and treatment is limited (Benson *et al.,* 2004). Patients with impaired immunity too, such as those who have AIDS or are neutropenic as a result of cancer therapy, are also at particular risk of developing *C. albicans* infections, which may become systemic (McCullough *et al.,* 1996).

The increase incidences in mycoses which is already a serious problem to medical mycology is further burden with the emergence of resistant strain and presence of limited number of antifungal agents. The available antifungals are mostly providing fungistatic but not fungicidal effects. Furthermore, continuous exposure to the antibiotics and synthetic chemical drugs results in the development of resistance in the organisms.

As at present, a most suitable first-line antifungal regimen is still an unknown fact. Therefore, it is quite normal the option of drugs used usually depends on the physician's knowledge of a drug, drug availability, patient's condition, concomitant medications and cost. Thus, in the treatment of Candidal infections, the choice of antifungal drugs is mainly of those primary drugs containing azole groups such as itraconazole, ketoconazole and fluconazole. But, there have been reports showing difficulties in complete eradication of yeasts and molds from patients although they

seem susceptible *in-vitro* to the antifungal drugs used. For instance *C. krusei* is intrinsically resistant to fluconazole but *Candida parapsilosis* may be susceptible (Chang *et al.,* 2008). Thus, with increasing ineffectiveness of the present drugs and unavailability of alternative new antifungals lead to the spread of major infectious fungal diseases in particular candidiasis.

1.2 Problem Statement

In the developed world, the condition of fungal infection is much more prevalent nowadays than it was 100 years ago, primarily because of imbalanced diets, lifestyles and over-use of antibiotics, and this situation seems to be worsening.

The incidence of Candidal infection is increasing globally. It has emerged as an important life-threatening nosocomial infection, especially to patients in the intensive care units (ICUs) through constant contacts with the health care workers. *Candida* spp. has been isolated from 15-54% of hands of health care workers in the ICU setting. The incidence was 5 to 10 fold higher than for the entire hospital and caused crude mortality rate of between 35% and 60% in patients with Candidaemia (Pfaller *et al.*, 1995).

Furthermore, it causes multiple risk factors to cancer patients admitted to the ICUs and is also a common contributor to many other immunocompromised diseases and conditions, such as AIDS, chronic fatigue syndrome, colds, flu and more (Wilks *et al.,* 2008). Diabetics too are prone to this type of problem. In addition, children with autism, Attention-Deficit/Hyperactivity Disorder (ADHD) and other developmental disorders are often found to be infected with *Candida* as well (Sipsas

et al., 2002). Thus, almost all health problems are greatly aggravated by Candidal infections.

According to findings from the National Nosocomial Infection Surveillance System (NNISS), USA, 61% of reported nosocomial fungal infections were due to *C. albicans*, followed by other *Candida* spp. A shift in the distribution of *Candida* spp. from *C. albicans* to non-*albicans Candida* spp. has been observed both in ICUs and oncology units in the last two decades. Among the *Candida* species, *C. albicans* remains the highest cause of invasive candidiasis in ICUs, followed by *C. tropicalis*, *C. glabrata* and *C. parapsilosis*. More than 90% of invasive isolates of *Candida* spp. are still remaining highly susceptible to fluconazole but among the Asia-Pacific countries, the susceptibility rate of *C. glabrata* to fluconazole varies widely from 22% to 72% (Sipsas *et al.,* 2002).

To effectively treat this *Candida* species infection, early diagnosis and prompt initiation of antifungal therapy are important. However, nowadays it is still difficult to diagnose *Candida* infections because of its non-specific clinical features and the delay in providing appropriate therapy. The delayed therapy is a major contributor to poor outcomes (Sipsas *et al.*, 2002).

Still recently, several synthetic and natural product-based drugs available for treating candidiasis are not consistently effective against pathogenic yeast infection. Furthermore, the development of resistance in fungi against most of the drugs has been reported (Darwish & Aburjai, 2011). Hence, it is of utmost importance to continually seek and to produce newer novel antifungal agents with a wide range of structural classes and selectively acting on new targets with fewer side effects. One

novel approach might be the use of plant bioactive compounds as potential sources for antifungal drug development.

Medicinal plants are well-known natural sources of remedies, used in the treatment of innumerable diseases since antiquity. Plants are invaluable sources of pharmaceutical products and the tropical rain forest of South America, Asia, and Africa has supplied an incredible array of medicinal plants that has drawn the attention of ethno-pharmacologists around the world. Many plants from various biomes (savannah, lowland and highland) have been used as natural medicines by the local population in the treatment of tropical diseases, including leismaniasis, malaria, schistosomiasis, fungal and bacterial infections (Duarte *et al.,* 2005).

After the investigations for new antifungal agent, many research on plant as antifungal has been done. A study had screened 204 crude extracts of Indonesia plants on *C. albicans* and found that there are nine plants were having antifungal effect (Cavin *et al.,* 1999*)*. Besides that, it was revealed that there was anticandidal activity in *Cassia alata* and reported that water extract of the plant was used in Ivory Coast, West Africa to treat fungal infections caused by *C. albicans* and dermatophytes (Crockett *et al.,* 1992).

In Iran, there was methanol plant extracts of 221 species was used to screen for its antifungal activities. A study also reported that plants with high antifungal activity included *Alpinia officinarum, Chrozophora verbasafalia, Cinnamomum zeylanicum, Dianthus coryphyllus, Helleborus nigra, Heracleum persicum, Myrtus communis, Terminalia chebula* and *Trachysermum copticum* which were proven to be the most effective against *C. albicans* and *Candida utilis* (Shahidi *et al.*, 2008).

In India, the investigation was carried out to evaluate the antifungal medicinal properties of *Carica papaya* and the result showed that the effects of different concentrations of alcoholic extract of *Carica papaya* (root, shoot and seed) on the radial growth of plant against *C. albicans* (Kumar *et al.*, 2013).

In Malaysia, there was a study reported that there are inhibitory effects of *Hymenocallis littoralis* methanol sonication extracts against *C. albicans*. The study has also proven that almost all part of this plant which was leaves, stem, bulb, anther, and flower and root methanol extract of the *H. littoralis* showed anticandidal activity (Sundarasekar *et al.*, 2012).

1.3 Rationale of Study

In general, treatment of Candidal infection is very difficult by using therapeutic strategies. The matter is made worst by the increase in global drug resistance of *Candida* spp. to present antifungal drugs. Besides that, the emergence of non-albicans *Candida* (NAC) species, such as *C. glabrata*, *C. tropicalis*, *C. krusei* and *C. parapsilosis*, have complicated the situation further (Vazquez *et al.,* 2006; Pfaller *et al.,* 2007). The number of available antifungal is also limited and most have undesirable side effects. The primary drugs used for the treatment of serious fungal infections were fluconazole, itraconazole, voriconazole and amphotericin B. The existing antifungals were mostly providing fungistatic rather than fungicidal effects. However, with increasing ineffectiveness and decreased susceptibility of yeasts and molds to these primary antifungal drugs is causing spread of major fungal infections worldwide. Worsening the situation further is the continuous exposure to the antibiotics and synthetic chemical drugs having led to the global development of resistance in the organisms. Thus this situation has prompted a search for new

alternative drugs that may be effective in the management and treatment of patients with mycoses especially due to the wide range of yeast pathogens.

1.4 Scope of Study

At the beginning, plant dry powder *Quercus infectoria* 'nut gall' powder was obtained. After that the plant extracts of *Quercus infectoria* "nut gall" was obtained by soxhlet extraction. *Quercus infectoria* 'nut gall' was subjected to successive extraction with different solvents in increasing order of polarity from petroleum ether (60°–80°C), to chloroform, ethanol alcohol, and finally to aqueous. In this step, several of plant active metabolite will dissolve in the different solvents. The different solvent of plant extract is test on five America Typed Cell Collection, ATCC strains of pathogenic *Candida* which are *Candida parapsilopsis* ATCC 22019, *C. krusei* ATCC 14223, *C. albicans* ATCC90028, *C. tropicalis* ATCC 70050 and *C. glabrata* ATCC 15126. Prior to testing each isolate is checked for purity and standard which yield a suspension of $1x10^6$ cells/ml to $5x10^6$ cells/ml. The agar media which used was SDA. After preparation of plant extracts, *Candida* spp. inoculums and SDA, the experiment is then preceded to the later stage.

At this stage, for preliminary anticandidal activity testing, an agar dilution method is employed by using plant extract of difference solvent which were petroleum ether, chloroform, and ethanol and aqueous with the concentration from are 0.4 mg/ml, 0.8 mg/ml, 1.6mg/ml and 3.2 mg/ml.

At the final stage, the determination of *Candida* susceptibility screening toward the extract is done by observe the presence or absence growth of *Candida* spp. on the 24 hours, 48 hours and 72 hours incubated SDA.

1.5 Objective of Study

To evaluate the anticandidal activity of organic and aqueous extracts of *Q. infectoria* "nut gall" by determination of the minimal inhibitory concentration (MICs) on *Candida* species.

CHAPTER 2

LITERATURE REVIEW

2.1 Plants and Antifungal Properties

Previously, there were several studies carried out to evaluate for antifungal activity of various plants. The researchers used different solvents to dissolve some bioactive ingredient from the plant. For example, the most commonly used solvent that being studied were ethyl acetate, methanol, ethanol, and aqueous. With different solvent used, antifungal activities of plant can be found.

Antifungal activity of ethanol extracts of five plant species that included *Syzygium jambolanum, Cassia siamea, Odina wodier, Momordica charantia* and *Melia azedarach* and two algal species, *Sargassum wightii* and *Caulerpa scalpelliformis* were tested against 25 isolated strains by disc diffusion method. Antifungal activity was observed at 100 mg/ml for *Syzygium jambolanum, Cassia siamea* and *Caulerpa scalpelliformis* and at 10 mg/ml for *Sargassum wightii* (Prabhakar *et al.,* 2008).

However, another study on antifungal activity of the lemongrass oil and citral against *Candida* spp. showed that lemongrass oil and citral have a potent in vitro activity against *Candida* spp. especially *C. albicans* (Silva *et al.*, 2008).

Beside lemongrass oil and citral, the other natural product that has been test also shown a significant anticandidal activity was garlic. Study reported that aqueous garlic extract can inhibit the growth of *Candida* spp. and minimal fungicidal concentration was found to be 14.9 mg/ml, at 24 hours of incubation time (Iwalokun *et al.*, 2004).

Besides that, a research about the antifungal activities of four traditional Chinese medicine extract, which were *Gentian, Radix et Rhizoma Rhei, and Aloe* was done and the result was showed that pseudolaric acid B had the most potent antifungal effect and possessed similar antifungal activity to all six *Candida* spp., (Yan *et al.*, 2012) which were *C. albicans, C. glabrata, C. tropicalis, Candida krusei, Candida dubliniensis,* and *Candida guilliermondii.* Their study suggested that pseudolaric acid B might be a potential therapeutic fungicidal agent in treating oral candidiasis. However the plant that being used in the present study does not contain pseudolaric acid B.

Antifungal activity was more effective in acetone and methanol extracts of *Cinnamomum tamala* against *Candida* species and it also showed fungicidal effect against the pathogen tested (Singh *et al.*, 2013).

Previous study had reported that there were no prominent antifungal activities exhibited by all crude extracts of *Piper longum* Linn. Although in this case, *Piper longum* Linn also have showed mild to moderate activities against most tested bacteria. The solvent they used were petroleum ether, ethyl acetate and chloroform (Ali *et al.*, 2007). The result showed that all of the extract, except petroleum ether showed mild activity against most of the fungi. The petroleum ether extract which was obtained from *Piper longum* stem, was found to be inactive against all the fungi tested included *C. albicans*. Beside that, from their results which tested were on *Piper longum* leave, it showed that there were mild to moderate activities against most of tested fungi. The results showed that chloroform extract of *Piper longum* leaves exhibited comparatively higher activity against most tested fungi than the other two solvents but results also showed that all of the crude *Piper longum* leaves extract displayed anticandidal activity against *C. albicans.*

In Nigeria, a study reported that the aqueous of virgin coconut oil has antifungal effect on *C. albican*, *C. tropicalis*, *C. glabrata*, *C. krusei* and *C. parapsilopsis*. Among these five *Candida* spp. strains, *C. albicans* had the highest susceptibility to coconut oil (100%) while *C. krusei* showed the highest resistance to coconut oil with an MIC of 100% (undiluted) (Ogbolu *et al.*, 2007).

In Malaysia, there are various methanolic plants extracts have been used as candidiasis treatment. Table 1.1 shows the plant species selected which are commonly used as traditional medicines in Malaysia for the treatment of candidiasis.

It is noteworthy that all plant mentioned in Table 1.1 was active against species of *Candida*. Therefore, these plants are potentially be used in the treatment of fungal infections in view of emerging drug-resistant *Candida* species.

Table 1.1 Growth inhibitions of methanolic extracts against *C. albicans*

Botanical name	Family	Used parts	*C. albicans*
Caria papaya Linn.	Caricaceae	Leaves	+
Cassia alata Linn.	Leguminosae	Leaves	+
Cinnamomun zeylanicum Blume	Lauraceae	Leaves	+
Costus specious Koenig.	Zingiberaceae	Leaves	+
Curculigo latifolia Dryand.	Hypoxidaceace	Root	+
Drymaglossum piloselloides Linn.	Polupodiaceace	Leaves	+
Elettariopsis triloba Gagneb.	Zingiberaceace	Leaves	+
Chromolaena odorata Linn.	Compositae	Leaves	+
(Eupatorium odoratum)	Araceae	Leaves	+
Homalomena rubra Hassk.	Melastomaceace	Leaves	+
Melastoma malabathricum Linn.	Rutaceae	Leaves	+
Micromelum pubescens Blume	Rutaceae	Leaves	+
Murraya koenigii Linn.	Acanthaceace	Leaves	+
Orthosiphon grandiflores Bold.	Zingiberaceae	Leaves	+
Phaemoria imperials	Piperaceae	Leaves	+
Piper caninum Blume	Piperaceae	Leaves	+
Piper nigrum Linn.	Piperaceae	Leaves	+
Piper sarmentosum Roxb.	Piperaceae	Leaves	+
Piper spPyrossia lanceolata	Cyatheaceae	Leaves	+
Rhinacanthus nasutus Linn.	Acanthaceae	Leaves	+
Thyphonium flagelliforme Lodd.	Menispermaceae	Whole plant	+
Tinospora crispa Linn.	Menispermaceae	Stem	+

+ : growth was inhibited

2.2 *Quercus infectoria*

Quercus infectoria (Fagaceae) is a small tree or shrub mainly found in Turkey, Iran, Iraq, Kurdistan, Cyprus, East Aegean Islands, Greece, Lebanon and Syria. The tree bears galls that emerge on its shoots as a result of attack by the gall-wasp called *Diploplepis gallae tinctoriae*. In Malaysia, the galls are locally known as Manjakani. The tree is occasionally cultivated for production of tanning bark and for dye production of the wood (Lim, 2012).

2.2.1 Agroecology

In its native ranges, it is usually found in semi-humid to semi-arid forests in areas with mean annual rainfall of 400-1,100 mm from 900 to 2,000 m altitude. It is intolerant of frost. It grows on a wide range of soil types from acidic to alkaline, in full to partial sun.

The seed of *Quercus infectoria* is an edible part of the plant. The seed is washed thoroughly in running water to remove the bitter tannins and cooked. Beside that, the seed can be dried and ground into powder form and used as a thickening in stew etc. or mixed with cereals for making bread. The roasted seeds can be used as a coffee substitute, while the nut gall extract or powder is used as a herbal drink or tea for health purposes (Lim, 2012).

2.2.2 Botany

A semi-evergreen, small tree or shrub, 1-4 m with grey, scaly, ridged bark. Juvenile shoots are pubescent, reddish or yellowish-brown; buds reddish-brown,

about 3 mm and pubescent. Leaves are alternate, very variable in size and color, 40-70 by 10-45 mm, leathery, glabrescent, ovate to narrowly oblong, rounded or wedge-shaped at base, margins often wavy with 4-8 crenate to saw-toothed lobes, or entire (at base of twigs): primary veins 6-11; petiole 1-15 mm. Influorescences unisexual, in axils of leaves or bud scales, usually clustered at base of new growth; staminate inflorescences lax, spicate: pistillate inflorescences usually stiff, with terminal cupule and sometimes 1-several sessile, lateral cupules. Fruit is a smooth nut, called an acorn, mucronate, ovoid elongated, 2-3.5 cm long, 1.8 cm in diameter; glabrous and is more or less enclosed in a scaly involucres called the cup or cupule. Cupules solitary or in pairs, approximately hemispherical or cyathiform, 10-18 mm in diameter with lanceolate strongly adpressed, grayish pubescent scales (Lim, 2012).

The galls or nut galls are hard, corky, resinous, and grayish-brown, nearly round excrescences formed on the young branches. The excrescences vary from the size of a large pea to that of a small hickory-nut. They are the result of a puncture made in the bark by an insect called *Diploplepis gallae tinctoriae* or *Cynips quercufolii* for the purpose of depositing its egg. A small tumour soon follows the puncture, and forms a very dense mass about the egg. The egg hatches into the fly while in these tumours, eating its way by a small opening. The nut gall has an integument with rugae-like surface interspersed by protruding blunt horn-like lumps (Lim, 2012) imparting to it a tuberculate appearance. Cross section of the gall revealed a whitish core and concentric circles of resinous materials constituting the middle layer.

2.2.3 Ethnopharmacological uses

The galls of *Quercus infectoria* are commonly used in Malay traditional medicine to treat wound infections after childbirth (Basri *et al.*, 2011). It is used in breast and vaginal firming creams. A decoction of galls is used as an enema in prolapus of rectum. However, in India, they are employed traditionally as dental applications such as that in treatment of toothache and gingivitis, and also as a gargle in nasal catarrh and sore throat (Basri *et al*, 2011). Previous study showed that this plant possessed antioxidant activity which contributed to its anti-carcinogenic effect (Shahrzard *et al.*, 2001). Flavanols as one of the major compounds from this plant was reported to inhibit cell proliferation *in vitro* (Rohana *et al.*, 2004). Besides that, galls are mainly used as a source of tannic acid, which is known as an astringent and styptic. The tannic and gallic acids extracted from the galls are often used in dysentery and diarrhea. They are a powerful astringent, used to check diarrhea. Galls are also used for tanning and dyeing, and in the manufacture of inks (Evans, 2009). It also can be externally used for the treatment of hemorrhoids, in inflammatory skin diseases such as eczema and impetigo and local treatment of mild inflammation (Indian Medicinal Plants: An Illustrated Dictionary, *Quercus infectoria*).

2.2.4 Pharmacological activities

The extract of *Quercus infectoria* was found to possess significant pharmacological activities. For example methanolic extract of *Quercus infectoria* possesses the effective antioxidant and antimicrobial substances. It is evidently shown that presence of flavonoids contributed to the antioxidant activity and promoted efficacy of drug used for treatment of various infectious disease.

Furthermore, the results of antibacterial activity were quite good, *Proteus vulgaris* showed very good results of MIC but minimum against *Enterobacter aerogenes* (Basri *et al.*, 2011).

A study of the antifungal activity of *Quercus infectoria* was performed against four fungi which were *Aspergillus fumigates, C. albicans, Aspergillus niger* and *Aspergillus flavus*. It was found that *Quercus infectoria* showed positive results against *Aspergillus fumigates* and no antifungal activity was shown against *C. albicans, Aspergillus niger* and *Aspergillus flavus* (Doughari *et al.*, 2008).

Another study was done in comparing antifungal activity of both the ethanol extract and aqueous extract of *Quercus infectoria* against the growth of *C. albicans* and *C. glabrata.* It was found that the ethanolic extracts of *Quercus infectoria* was more effective against *C. albicans* at 700 mg/ml while the aqueous extract was found to be more effective against *C. glabrata* compared to *C. albicans* of the same concentration of extracts (Heba Fadel Hassan, 2011). Normally, an alcoholic solvent is used to extract phenolics from natural sources because it is able to yield high quantities of total extract compared to other types of solvent. In the same study, aqueous extract was found to be more effective against *C. glabrata* compared with the growth of *C. albicans* at same extracts. This was probably due to the used of boiled distilled water is needed to dissolve out all these extract which may have high total tannin contents as tannin is a major compound in *Quercus infectoria* which is soluble in water.

2.2.5 Phytochemical studies

Normally, medical plants may comprise an essential source of bioactive compounds, of which the use of medicinal plants in the treatment of infectious is well known. Naturally, plants produce various types of secondary metabolites, in which many of these secondary metabolites exert antifungal properties. These compounds include flavonoids, phenolics and phenolic glycosides, unsaturated lactones, sulphur compounds, saponins, cyanogenic glycosides and glucosinolates. All these substances produced by plant as defense mechanism against predation by microorganism, insects and herbivores (Darwish *et al.,* 2011).

In this research, *Quercus infectoria* was chosen to study for its antifungal properties. *Quercus infectoria* is a round-shaped abnormal growth found arising on young brances of the oak tree as a result of attack by the gall-wasp *Adleria gallae-tinctoria*. Research showed that *Quercus infectoria* is highly rich in bioactive compounds such as tannin, vitamin A and C, calcium, iron, fiber, protein and carbohydrates and all these compounds have the ability to be antivirals (Buzzini *et al.*, 2008), antimicrobial, antibacterial, antifungal agent (Basri *et al.*, 2011) and antihelmintics (Ketzis *et al.*, 2006). Beside that, it is also the main constituents for gallotannic acid (50–70%), gallic acid (2–4%), ellagic acid, starch, and sugar (Basri *et al.*, 2011).

2.2.6 Therapeutic

Quercus infectoria galls are higly rich in tannic acid which occupied 35% of total active ingredient and 6% of gallic acid. It also contains other substances such as

ellagic acid, sugar, starch, gum, extractive, saline matter, and other subordinate ingredients.

Tannin concentration is high in gall oak with 53.1% of gallotannin, 9.55% of gallic acid and 6.9% of ellagitannin. Basically, tannin can be divided into two basic groups which are hydrolysable type and condensed type. Hydrolyzable tannins include gallic acid and ellagic acid. Both types of tannins have been used in disease treatment but hydrolysable tannins have been used more as antifungal and antibacterial agents (Heba Fadel Hassan, 2011).

Tannin is a phenolic compound which is soluble in water, alcohol and acetone and precipitates with protein. Previous study had been reported that tannin is present in high amount in the galls of *Quercus infectoria* may be the active compound which may be responsible for the antibacterial activity (Basri *et al.*, 2011).

Tannic acid is used as local astringent which has fulfilled many functions. When it is thrown in the form of a fine powder, or in the shape of gargle, spray to inhalation upon the mucous membrane of the fauces and air-passages, it reduce congestion and swelling, reduces excessive secretion, and also arrests the tendency to capillary haemorrhage. Therefore, it is normally used in the treatment of quinsy, catarrhal affections, chronic bronchitis, laryngeal phthisis, and haemoptysis (Basri *et al.*, 2011).

Tannic acid has its styptic action to a bleeding surface if no large vessel be engaged. It is also used to cure ulcer. Besides that, it is locally utilized in the shape of the glycerine of tannin to eczematous skin (Ringer), blenorrhoeal and leucorrhceal states of the urethra and vagina, sore throat, intertrigo, rectal fissures and prolapsus ani (Basri *et al.*, 2011). It also serves as an antidote to alkaloid poisoning. This was

first observed in regarding to emetin-poisoning but now the observation has now been extended to opium and its salts, digitaline, nicotine and other alkaloids (Basri *et al.,* 2011).

The second main active ingredient called gallic acid also showed astringent properties. It is often exceedingly effective in various kinds of haemorrhage especially in haemoptysis, while in heamaturia, it depends upon morbid conditions of the mucous membrane of the bladder. Beside that, it also play a role in checking the waste of valuable albuminoid matters which the system would otherwise has to support. It is shown that there will be remarkable diminishing of albuminuria in which there is usually an increase in the flow of urine (Basri *et al.,* 2011).

CHAPTER 3

MATERIALS & METHODS

The *in-vitro* anticandidal susceptibility testing was carried out by using the organic aqueous extracts of *Quercus infectoria* on *Candida* spp.

3.1 Materials and Equipments

3.1.1 Materials

- Media
    - Sabouraud Dextrose Agar, SDA pH 5.5-5.6, Merck
- Solvents
    - Petroleum ether
    - Chloroform
    - Ethanol
    - Distilled water
    - DMSO (Dimethyl sulfoxide)
- Antifungal agent
    - Amphotericin B (Amp B)

3.1.2 Apparatus

- Disposable agar plates
- Micropipette (100-200 µl)
- Micropipette (1000 µl)
- Micropipette tips and tip box

- 100 ml Duren bottle
- 100 ml Measuring cylinder
- Rounded bottom flask
- Falcon tubes
- Test tube rack
- Neubauer counting chamber

3.1.3 Equipment

- Weighing balance
- Hot plate mixer
- Rotary evaporator
- Soxhlet

3.2 Methodology

The *in-vitro* MIC of *Quercus infectoria* organic and aqueous extracts were evaluated by using the agar dilution method.

3.2.1 Fungal strain

3.2.1.1 Standard strain of *Candida* species

Candida parapsilopsis ATCC 22019, *C. krusei* ATCC 14223, *C. albicans* ATCC 90028, *C. tropicalis* ATCC 70050 and *C. glabrata* 15126 were the ATCC strains included.

3.2.1.2 Preparation of inoculums

The fungal strains were freshly subcultured onto SDA and incubated at 30°C for three days. Preparation of the test strain culture was carried out by isolating a single colony of the *Candida* spp. from the SDA and transferred into sterile distilled water. The yeast cells were suspended in sterile distilled water and counted using Neubauer chamber to obtained 10^6 cells/ml.

3.2.2 Plant

3.2.2.1 Plant source

Quercus infectoria "nut gall" was bought from the local market and was authenticated based on its morphology. The oak galls were washed with distilled water, left for drying at room temperature before the samples were crushed into

pieces and grinded into powder using a grinder before being sieved to get only a fine powder and the powder was kept in the dry container for future use.

3.2.2.2 Preparation of plant extract

In this study we obtained the plant organic and aqueous extracts of *Quercus infectoria* nut gall by soxhlet extraction. The soxhlet apparatus consists of an electric heater with a thermostat regulator upon which round bottom glass flask that fitted to an extraction unit is placed. Meanwhile the extracting unit consists of the solvent and cellulose thumble. The cellulose thumble contains the dry *Quercus infectoria* plant powder. A distiller unit is fitted on to the extraction unit. For condensation of vapor solvent , 50 g of plant powder was place inside the thumble and 500 ml of each solvents was place inside the flask and was subjected to successive extraction with different solvents in increasing order of polarity from petroleum ether (60°–80°C), to chloroform, ethanol, and finally to aqueous. The extraction was carried out for 6 hrs by heating temperature that kept the solvent at 50-60°C until a clear and colorless solvent appeared in the extracting unit. The extract was concentrated to almost dryness under reduced pressure in a rotary evaporator to yield dried plant extract. After that, the extract was transferred into a small beaker and further dried under fume hood at room temperature 27°C. After 24 hours, the beaker with the extract was weighed hourly until a consistency of mass was obtained. This step was done to confirm the complete dryness of the extract is obtained. Then, the final extract was kept frozen at -20°C until used.

3.2.2.3 Preparation of extract concentrations

A series of different *Q. infectoria* extract concentrations in which are 0.4 mg/ml, 0.8 mg/ml, 1.6 mg/ml and 3.2 mg/ml were prepared by dissolving a calculated weight of the plant extract in dimethyl sulphoxide (DMSO) which function as a solvent and kept in sterile test tube at 4°C until used.

3.2.3 Preparation of media for MIC determination

The agar media used was SDA. It is an acidic pH (pH 5.5-5.6) media which containing relatively high concentration of glucose or maltose (40%) was prepared. Firstly, 5.2 g of agar powder was weighted using analytical balance and then filled into a 100 ml Duren bottle. By using measuring cylinder, 60 ml of distilled water was measured and filled into the Duren bottle. One magnetic stirrer was put into each Duren bottle. Each Duren bottle was place on the hot plate magnetic stirrer and let it is stirred for one hour until the clear bottom zone of bottom was disappear. Then, each bottle was labeled after each extract solvent and autoclaved at 121°C, 15 minutes for sterilization.

3.2.4 Loading of sample

Duren bottles were allowed to cool to 50°C and non-solidified SDA was loaded with 1.5 ml of extracts pipetted from the stock solution. A final concentration of 0.4 mg/ml, 0.8 mg/ml, 1.6 mg/ml and 3.2 mg/ml were obtained. The content in the Duren bottle was poured immediately into a plate. The plates were allowed to solidify at room temperature. Before loading the standard inoculums, the extract solvent was allowed to evaporate by leaving the plates overnight. After that, the plates were incubated overnight for quality control. Loading of the standard