

**ANTIFUNGAL ACTIVITY OF THREE LOCAL MALAYSIAN
HONEYES ON SELECTED PATHOGENIC FUNGI OF
OTOMYCOSIS; AN IN VITRO EVALUATION**

By

DR. ZUBAIDAH BINTI HAMID

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List of Abbreviations

cfu	=	Colony forming unit
CONS	=	<i>Coagulase-negative staphylococci</i>
FAMA	=	Federal Agriculture Marketing Authority
FDA	=	Food and Drug Administration
HEPA	=	High-efficiency particulate air
HUSM	=	Hospital Universiti Sains Malaysia
kGy	=	Kilo Gray
SDA	=	Saboraud Dextrose Agar
USM	=	Universiti Sains Malaysia
USM-BJIM	=	Universiti Sains Malaysia - Medicinal Trigonal Bee Rearing
w/v	=	Water per volume

ABSTRAK

Tajuk

Aktiviti antikulat tiga madu tempatan Malaysia ke atas kulat patogen terpilih penyebab penyakit otomikosis; kajian di dalam makmal.

Pengenalan

Madu telah digunakan sebagai makanan dan produk perubatan sejak zaman purba. Ciri antibakteria madu mendominasi dan didokumenkan secara meluas. Sebaliknya pula, keberkesanan antikulat terhad digambarkan. Tambahan pula, kebelakangan ini juga, terdapat peningkatan kekebalan terhadap ubat antikulat. Ini mendorong keperluan untuk menyelidik bahan semula jadi yang lebih berkesan dan selamat. Madu sejenis bahan semulajadi, berjaya menarik minat potensi antikulat. Terdapat pelbagai jenis madu tempatan di Malaysia. Walau bagaimanapun, sehingga kini, tiada kajian yang menyeluruh kesan antikulat madu-madu tersebut dilaksanakan. Oleh itu, kajian ini menilai kesan antikulat tiga jenis madu Malaysia terhadap dua kulat penyebab utama penyakit otomikosis. Ketiga-tiga madu yang digunakan dalam kajian ini adalah unik. Tualang adalah sejenis madu multiflora. Sementara, Akasia adalah sejenis madu diantara bebunga dan Kelulut pula adalah madu dari lebah tanpa sengat.

Objektif

Untuk menyiasat kesan antikulat tiga jenis madu Malaysia (Akasia, Kelulut dan Tualang) terhadap *Aspergillus niger* dan *Candida albicans*. Kajian ini juga menunjukkan madu dengan kesan antikulat terkuat.

Metodologi

Ini adalah kajian prospektif makmal yang dikawal. Tiga jenis madu Malaysia dalam beberapa kepekatan yang berbeza, dikaji dalam makmal untuk aktiviti anti kulat terhadap *Aspergillus niger* dan *Candida albicans*. SDA disediakan bersama madu dalam empat kepekatan yang berbeza; 5 peratus (w/v), 10 peratus (w/v), 15 peratus (w/v) dan 25 peratus (w/v), yang bertindak sebagai media. Bilangan koloni diatas media SDA sahaja dibandingkan dengan media SDA bersama madu. Ujian statistik “Paired t-Test” dan “Wilcoxon Signed Rank Test” dijalankan.

Keputusan

Di dalam makmal, ketiga-tiga madu Malaysia mempunyai kesan antikulat yang signifikan ke atas dua kulat patogen utama penyakit otomikosis. Tualang dan Akasia mempunyai kepekatan yang sama untuk aktiviti antikulat terhadap kedua-dua kulat iaitu pada kepekatan 10 peratus (w/v). Sementara itu Kelulut telah mula menunjukkan kesan antikulat pada kepekatan serendah 5 peratus (w/v). Keberkesanan antikulat bagi ketiga-tiga madu adalah berkadar terus dengan kepekatan, sehingga tahap perencatan penuh terhasil. Kepekatan perencatan penuh bagi kedua-dua kulat adalah sama bagi setiap madu berasingan kecuali madu Akasia. Akasia memerlukan kepekatan yang lebih tinggi untuk perencatan *Aspergillus niger* iaitu 15 peratus (w/v) berbanding hanya 10 peratus (w/v) bagi *Candida albicans*. Perencatan sepenuhnya bagi Kelulut adalah 10 peratus (w/v) sementara Tualang adalah pada 25 peratus (w/v). Antara ketiga-tiga madu yang diuji, Kelulut menunjukkan kesan antikulat paling signifikan terhadap kedua-dua kulat.

Kesimpulan

Kajian ini menunjukkan, di dalam makmal tiga madu Malaysia mempunyai kesan antikulat terhadap kulat patogen utama penyakit otomikosis. Oleh itu, ia berpotensi sebagai sumber alternatif dalam rawatan otomikosis.

Kata kunci : Madu Malaysia, Otomikosis, *Aspergillus niger*, *Candida albicans*, Tualang, Kelulut, Akasia

ABSTRACT

Title

Antifungal activity of three local Malaysian honeys on selected pathogenic fungi of otomycosis; an in vitro evaluation.

Introduction

Honey has been used as foods and medicinal products since ancient times. Its antibacterial properties has extensively documented. Contrarily, limited antifungal efficacy of honey was illustrated. Furthermore, in recent years, there are increasing in the antifungal drugs resistance. This drives the need to pursuit for natural solution which is more effective and safe. Honey, a natural solution attracted interest of its potential antifungal effect. They are various types of Malaysian honey. However, to date, no extensive studies on it antifungal properties have been conducted. Hence, this study evaluates antifungal effect of three Malaysian honeys against two most common pathogenic fungi of otomycosis. All honeys used in this study are unique. Tualang is a multifloral honey. Meanwhile Akasia are extrafloral honey and Kelulut are honey from stingless bee.

Objectives

To investigate the antifungal action of three types of Malaysia honeys (Akasia, Kelulut and Tualang) against *Aspergillus niger* and *Candida albicans*. This study also investigates honey with strongest antifungal effect.

Methodology and study design

This is a laboratory-controlled prospective study. Different concentrations of three types of Malaysian honey studied in vitro for their anti fungal activity on fungal isolates of *Aspergillus niger* and *Candida albicans*. Four different concentrations of SDA media incorporated with honey; 5 percent (w/v), 10 percent (w/v), 15 percent (w/v) and 25 percent (w/v) act as media. The numbers of colonies on SDA media alone were compared with SDA with honey. Statistical test of Paired t-Test and Wilcoxon Signed Ranks Test applied.

Results

In vitro, all three Malaysia honeys used, has significant antifungal effects on two most common pathogenic fungi of otomycosis. Tualang and Akasia have equal concentration for antifungal effect towards both fungi at 10 percent (w/v). Meanwhile Kelulut already demonstrate the effect at 5 percent (w/v). The antifungal efficacy is proportionate to concentration of all three honeys proceeding to total inhibition. Total inhibition noted to be equal for both *Candida albicans* and *Aspergillus niger*, for each individual honey except Akasia. Akasia need higher concentration for total inhibition of *Aspergillus niger*; 15 percent (w/v) compare to *Candida albicans* at 10 percent (w/v). For Kelulut it was at 10 percent (w/v) and Tualang at 25 percent (w/v). Among three honeys tested, Kelulut demonstrates the strongest antifungal effect towards both fungi.

Conclusion

This study demonstrates in vitro antifungal activity of three Malaysian honeys against two known pathogenic fungi of otomycosis. Hence, it has potentials as alternative agents in the treatment of otomycosis.

Keywords : Madu Malaysia, Otomikosis, *Aspergillus niger*, *Candida albicans*, Tualang, Kelulut, Akasia

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 OTOMYCOSIS

1.1.1 DEFINITION

Otomycosis or otherwise known as otitis externa of fungal etiology is a chronic or subacute infection of the pinna, the external auditory meatus and the ear canal (Chin and Jegathesan, 1982). It was defined as superficial mycotic (fungal) infection of the outer ear canal (Dorland's, 2012). Gil-Carcedo *et al.* (2004) defined it more precisely as subacute or chronic infections produced by yeast and filamentous fungi that affect the squamous epithelium of the external auditory canal.

1.1.2 EPIDEMIOLOGY

In the past there were controversies regarding the prevalence and even existence of otomycosis. The debate was whether the fungi are true infective agents. Some believe it was mere fungal colonization due to compromised local host immunity secondary to bacterial infection but are of no etiological association (Kingery, 1965) and others doubt fungi are capable of producing inflammatory process, and only accept them as secondary invaders (Youssef and Abdou, 1967). Conant *et al.* (1954) proposed that not more than 15 to 20 percent of the ear infections are true otomycosis. Despite the widely divergent opinions, at this current era, the weight of the evidence in the literature, based on clinical and laboratory study, supports otomycosis as a true pathologic entity (Chin and Jegathesan, 1982; Ho *et al.*, 2006; Paulose *et al.*, 1989; Zaror *et al.*, 1991).

Many different data published regarding magnitude of otomycosis. Carney (2008) documented otomycosis affect 10 percent of the population in their lifetime. It accounts for approximately 9 to 10 percent of all cases of otitis externa, (Bojrab *et al.*, 1996; Ho *et al.*, 2006; Stern *et al.*, 1988) and as high as 30.4 percent in patient with inflammatory conditions of the external auditory meatus (Kurnatowski and Filipiak, 2001).

Otomycosis has a world-wide distribution, with higher prevalence at tropical and sub-tropical regions that has hot, humid and dusty climate (Carney, 2008; Kumar, 2005; Munguia and Daniel, 2008; Paulose *et al.*, 1989) between second and third decade of life (Kumar, 2005; Paulose *et al.*, 1989). However studies pertaining prevalence of otomycosis remains scant, despite of its high occurrences especially from those countries in the tropical region. In Malaysia, way back in 1982, Chin and Jegathesan (1982) had published fungal isolates from suspected cases of otomycosis over a period of twelve years; 1969 till 1981. However their study do not reveal incidence of otomycosis during this period. Meanwhile in Singapore, Ong and Chee (2005) has published an article on infections of the external ear, also did not document magnitude of otomycosis either. The study that was conducted by Zaror *et al.* (1991) in Brazil, highlights several facts namely, limited prior studies in this area at Brazil, despite being a country with tropical climate and secondly, 90 percent from clinically suspected case were confirmed to have otomycosis. In Bahrain, Paulose *et al.*(1989) found 6 percent of patient with symptoms of ear disease seen at their outpatient clinics has otomycosis. Clearly, the incidence of otomycosis remains limited and their finding varies across countries.

There were very scanty researches that look into gender preponderance. Different results were reported. Kumar (2005) reported males and females were almost equally affected. However Paulose *et al.* (1989) and Ho *et al.* (2006) documented very slim preponderance for male. Contrarily Zaror *et al.* (1991) report higher incidence (65%) in female.

Otomycosis is predominantly a unilateral disease, although small percentages (7-20.8%) of bilateral cases were reported (Ho *et al.*, 2006; Kurnatowski and Filipiak, 2001; Mugliston and O' Donoghue, 1985; Paulose *et al.*, 1989; Zaror *et al.*, 1991).

1.1.3 ANATOMY, PHYSIOLOGY AND MICROBIOLOGY OF EXTERNAL EAR CANAL IN RELATION WITH OTOMYCOSIS.

The external auditory canal extends from concha of the auricle to the tympanic membrane and is approximately 2.4 cm long. The supporting framework of the canal wall is cartilage in the lateral one third (8 mm) and bony in the medial two thirds (16 mm). There are two intrusions into the canal, formed by suture lines. This two suture

lines are tympanoquamous anteriorly and tympanomastoid posteriorly which project into the canal making ear toilet in a patient with otomycosis a challenge.

Apart from intrusions, there are also two constrictions; at the bony cartilaginous junction and isthmus. The isthmus is a constriction located 5mm from tympanic membrane and beyond the isthmus presents a recess called anterior recess. Anterior recess is the anteroinferior portion of the canal, dips in between the tympanic membrane and canal, forming a wedge-shaped recess. The anterior recess acts as cesspool for discharge, keratin debris and cerumen. Furthermore this recess is a difficult spot for access either for procedure in the office setting or during surgery because it is a hidden area due to the presence of constriction (isthmus), making it difficult for cleaning, thus favour colonization by fungus.

The skin of external segment is hairy and has sebaceous and ceruminous gland. It is also more adherent than skin of the internal segment. The glandular secretions mixed with flaked cellular epithelial elements to form an acidic ceruminous substance which is hydrophobic, making it capable of repelling water. As a result, the canal is impermeable and thus avoiding maceration and epithelial damage. Cerumen consists of lipids, proteins, free amino acids and mineral ions. It also contains lysozyme, immunoglobulins and fatty polyunsaturated acids. The long-chain fatty acids present in unbroken skin inhibit microorganism growth. Thus, the areas of skin that take part in cerumen production have all the components of an active local immune system and protect the canal by an antibody-mediated local immune response (Mugliston and O' Donoghue, 1985; Sirigu *et al.*, 1997). Basically, cerumen has both antimycotic and bacteriostatic properties. Otomycosis occurs because the protective lipid/acid balance of the ear is lost, as once the ear becomes inflamed, healthy cerumen rapidly removed from the ear and is no longer produced (Carney, 2008; Paulose *et al.*, 1989; Zaror *et al.*, 1991). Contrarily, some authors advocate that with the presence of cerumen, acting as a support for fungal growth (Mugliston and O' Donoghue, 1985; Munguia and Daniel, 2008).

The microbial flora of the external auditory canal is usually commensal or non-pathogenic comprising of *Staphylococcus epidermis*, *Corynebacterium spp*, *Bacillus spp*, Gram-positive cocci (*Staphylococcus aureus*, *Streptococcus spp* and non-

pathogenic micrococci), Gram-negative bacilli (*Pseudomonas aeruginosa*, *Eschericia coli*, *Haemophilus influenza*, *Moraxella cararralis*) and mycelial fungi of the *Aspergillus* genus or yeast-like fungi, particularly *Candida spp.* As long as the balance between bacteria and fungi is maintained, this commensal flora remains non-pathogenic (Murray, 1995). The fungal colonization of the external auditory canal is not permanent but temporary probably related to environmental changes as was noted when healthy people and patients were studied over time (Hueso *et al.*, 2005). Local lesion observed in otitis, leads to inflammation process, resulting in edema which entrap water and moisture in the ear canal. Thus cerumen is no longer produced. Water and moisture also lead to shift from a predominantly Gram-positive skin flora to a Gram-negative one. The balance is disturbed. With further edema and scratching, there is disruption of the epithelial layer and invasion of resident or introduced organism occur (Carney, 2008).

The pH of the normal ear canal is on the acidic site of neutrality (5-7) and *Aspergilli* have optimum growth in pH within this range (Yassin *et al.*, 1964). Maximum growth rates for *Aspergilli* were at 37⁰C. All this facts clinically support the predilection of the fungi to grow at inner one third of the ear canal which has temperature near to core body temperature (Yassin *et al.*, 1964).

In summary, Conley (1948) beautifully concluded that the external ear canal fulfills many of the requirements for fungal growth; where the environmental factor being essentially warmth, moist with optimum pH.

1.1.4 CAUSATIVE AGENTS

Many species of fungi have been identified as a cause of otomycosis (Munguia and Daniel, 2008; Stern *et al.*, 1988). However the most implicated fungi in otomycosis is *Aspergillus* species and secondly by *Candida* species (Bojrab *et al.*, 1996; Carney, 2008; Chin and Jegathesan, 1982; Ho *et al.*, 2006; Kiakojuri *et al.*, 2007; Munguia and Daniel, 2008; Ong and Chee, 2005; Paulose *et al.*, 1989; Stern *et al.*, 1988; Zaror *et al.*, 1991). Munguia and Daniel (2008) have done extensive literature review on otomycosis from 1951 to 2007. They concluded *Aspergillus niger* is the most commonly described agent in the literature, followed by *Candida albicans* (Bojrab *et al.*, 1996; Carney, 2008; Ho *et al.*, 2006; Kiakojuri *et al.*, 2007; Munguia and Daniel, 2008; Ong and Chee, 2005; Paulose *et al.*, 1989; Zaror *et al.*, 1991). However Stern *et al.* (1988) describes

predominant fungal based on climate; where *Aspergillus niger* is the major isolate in tropical zones where else *Candida albicans* is more common at temperate zones.

Aspergillus niger belongs to the molds are saprobes which grow as long tangled strands of cells that give rise to visible colonies (Figure 1.1). Contrarily *Candida albicans* belongs to the yeast sub-division and which are unicellular. The colonies resemble bacterial colonies (Figure 1.2). Otomycosis attributed to *Candida* is often identified by culture data, as it is lack of characteristic appearances clinically, unlike *Aspergillus* (Ho *et al.*, 2006).



Figure 1.1: Gross appearance of *Aspergillus niger* on Saboraud Dextrose Agar (SDA) plate showing black colonies.

(Source: www.pgodoy.com/aspergillus_niger)



Figure 1.2: Gross appearance of *Candida albicans* on Saboraud Dextrose Agar (SDA) plate showing whitish colonies.

(Source : www.static.ddmcdn.com/candida_albicans)

In Malaysia, little has been done in the way of characterization of the etiological agents, thus Chin and Jegathesan (1982) performed mycological study for 12 years duration, on clinically suspected cases of otomycosis. It showed 74.6 percent positive fungal isolation. *Aspergillus* (78.4%) was the most predominant genus isolated and *Candida*, the next.

Various incidence of mixed fungal infection were also reported; ranging from as low as 7.3 per cent (Chin and Jegathesan, 1982) to 28 per cent (Youssef and Abdou, 1967). Bacterial co-infection was also discussed (Kumar, 2005). Among commonly isolated bacteria includes *coagulase-negative staphylococci* (CONS), *Pseudomonas* sp., *Staphylococcus aureus*, *E. coli* and *Klebsiella* sp. (Kumar, 2005).

The importance of fungal culture cannot be understated (Stern *et al.*, 1988). Chin and Jegathesan (1982) and Kurnatowski and Filipiak (2001) advocate cultural confirmation of all cases. This is to apply most adequate treatment, prevent mismanagement, relapses and for better knowledge on otomycosis and the causative agents. Therapeutically, most authors believe that it is important to identify causal agent of otomycosis in order to use appropriate treatment, where antimycotic treatment chosen should be based on susceptibility of the identified species (Arthur *et al.*, 2004; Bassiouny *et al.*, 1986; Munguia and Daniel, 2008; Stern *et al.*, 1988). Contrarily, others proposed regardless of the causative agents, important strategy is to select specific treatment based on efficacy of the drugs (Araiza *et al.*, 2006; Blyth *et al.*, 2007; Munguia and Daniel, 2008).

1.1.5 RISK FACTORS

Very often otomycosis manifest only in the presence of predisposing factors. Some identified culprits include hot, humid climates and is often secondary to prolonged treatment with topical antimicrobials (Carney, 2008; Ho *et al.*, 2006; Kumar, 2005; Munguia and Daniel, 2008). However, no specific ototopical antimicrobial preparation appears to link with higher development of otomycosis (Ho *et al.*, 2006). There appear to be little consensus with respect to the predisposing factors. For instance, when most of the researches blame widespread usage of antibiotics, some authors suggest higher incidence of otomycosis is not related with usage of topical or systemic antibiotics and steroids (Mugliston and O' Donoghue, 1985; Paulose *et al.*,

1989). Several recent articles also established the potential risk of autoinoculation of the ear canal by patients suffering dermatomycosis, while other disagrees (Ho *et al.*, 2006; Kumar, 2005).

Diabetes and immune-compromised state also increase the risk of developing otomycosis (Carney, 2008; Ho *et al.*, 2006; Kumar, 2005; Munguia and Daniel, 2008; Ong and Che, 2005). Hormonal changes as seen during menstruation or pregnancies precipitate infection (Kumar, 2005; Munguia and Daniel, 2007).

Other factors include presence of mastoid cavities, hearing aids with occlusive molds, fungal infections elsewhere in the body like dermatomycosis or vaginitis, trauma and bacterial infections (Carney, 2008; Kumar, 2005).

Prior otologic procedures also appear to increase the risk (Ho *et al.*, 2006). Several factors contribute to this. Recurrent drainage or subsequent topical antimicrobial or antiseptic may alter the local environment and microbial flora; thus allow superinfection by nosocomial fungi. Surgical procedures also alter the anatomy of ear canal that affect cerumen production or relative humidity that favor fungal growth (Ho *et al.*, 2006).

1.1.6 CLINICAL FEATURES

Clinically, the most common presenting complaints were otalgia and otorrhea, followed by hearing loss, aural fullness and pruritus (Ho *et al.*, 2006; Munguia and Daniel, 2007). Aural fullness and hearing loss occurs secondary to accumulation of fungal debris in the canal. Pruritus has been frequently reported as one of the hallmark symptom in otomycosis (Kiakojuri *et al.*, 2007; Kumar, 2005; Kurnatowski and Filipiak, 2001; Ong and Chee, 2005).

On otoscopic examination, the most common finding is a black, green, grey, yellow or white discharge with musty odor and debris, that is often said resemble wet newspaper (Carney, 2008). Sometimes the debris is seen with visible fungal hyphae. *Aspergillus niger* appears as black-headed filamentous growth (Figure 1.3), where else *Candida albicans* as white or creamy deposits (Figure 1.4).

Otomycosis can usually be diagnosed by clinical examination and often occurs in persistent otorrhea (Ho *et al.*, 2006). Chin and Jegathesan (1982) concluded that clinical presentation often allows diagnosis to be made with fairly good accuracy when they found 74.6 percent yield fungal growth. However diagnosis of otomycosis caused by *Candida albicans* can be very difficult because of lack of its characteristic appearance like *Aspergillus*. It can present as otorrhea not responding to aural antimicrobials and is often identified by laboratory culture study (Ho *et al.*, 2006). Basically, high index of suspicion is required for correct diagnosis of otomycosis as the presenting complaints are relatively non-specific.

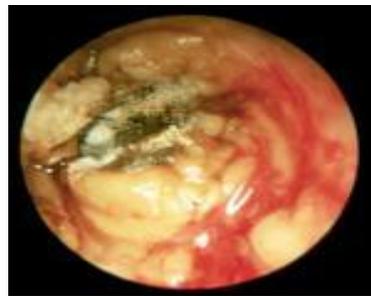


Figure 1.3: Otomycosis caused by *Aspergillus niger*
(Source:www.eac.hawkelibrary.com/otomycosis)



Figure 1.4: Otomycosis caused by *Candida albicans*
(Source:www.eac.hawkelibrary.com/otomycosis)

1.1.7 MANAGEMENT

Treatment with antifungal agents is not enough to ensure complete treatment and should aim at restoring the physiology of the external auditory canal (Hueso *et al.*, 2005). Ear toilet with removal of the debris and topical antifungal is the customary treatment (Carney, 2008).

Reviewed literature emphasized on ear toilet and aural hygiene. It can be done either by suctioning or mopping. Ear toilet removes all the discharges and epithelial debris which are conducive for the growth of fungus. Otological medications also only work best following removal of secretions or debris (Ho *et al.*, 2006; Munguia and Daniel, 2007).

To date, there are four main classes of drugs for the treatment of fungal infections: polyenes, triazoles, nucleoside analogues, and echinocandins. The polyenes family includes amphotericin B and nystatin. The triazoles family, better known as azoles includes clotrimazole, miconazole, and fluconazole. Meanwhile flucytosine is an example of nucleotide synthesis. Echinocandins are a novel class of antifungal agent, however their usage in otomycosis has not been reported (Munguia and Daniel, 2008). Munguia and Daniel (2008), based on their literature review for period of 56 years also found antifungal from the azole class seem to be the most effective for otomycosis, followed by nystatin and tolnaftate.

Many agents have been recommended, but no preparation has been widely accepted and there seems to be no consensus as to which medications are the most effective. To date there is no FDA-approved antifungal otic preparation for the treatment of otomycosis. Clinicians have struggled to identify the most effective agent. However use of few topical antifungal has persisted throughout time, including nystatin and the azoles family (Bassiouny *et al.*, 1986; Ho *et al.*, 2006; Kiakojuri *et al.*, 2007; Paulose *et al.*, 1989).

Treatment of otomycosis varies among different authors. For an instance, one study showed that clotrimazole and econazole had broad-spectrum antifungal activity; inhibiting both yeasts and moulds, and therefore should be the treatment of choice for otomycosis. It may be safely used as otic drops (Bassiouny *et al.*, 1986). Meanwhile, Stern *et al.* (1988) claimed among topical antifungal agents, clotrimazole and nystatin have the widest spectrum of activity.

As a conclusion, there is no consensus on the most effective antifungal agent, although multiple in vitro studies have examined the efficacy of various antifungal

agents (Kurnatowski and Filipiak, 2001). However, various agents have been used clinically with variable success rates (Paulose *et al.*, 1989).

Oral antifungal is reserved for cases with severe disease and poor response to therapy, though rarely necessary. Oral antifungal are unlikely to succeed in the absence of adequate local care of ear canal (Ho *et al.*, 2006).

In cases of resistant otomycosis, it is prime important to exclude fungal infection elsewhere, including athlete's foot (Carney, 2008). The 'foot and ear' reaction can cause recurrent otomycosis if the primary fungal infection elsewhere remains untreated (Busch, 1998). Ho *et al.* (2006) also reported higher recurrent and residual disease rates in the presence of mastoid cavity.

In immune-compromised patients, rarely fungi can cause invasive otitis externa. There's often high mortality rate from this condition (Harley *et al.*, 1995). In such patients, aggressive systemic antifungal therapy is required (Cunningham *et al.*, 1988).

1.1.8 THE CHALLENGE

Otomycosis is not a life threatening ailment, but can be a very frustrating disease for both patient and physician. Although rarely life threatening, patients frequently requires long term treatment and follow-up, yet recurrence rate remains high (15%) (Ho *et al.*, 2006). These pose challenges and frustration for both patients and otolaryngologists.

The literature indicates that antifungal medications vary widely and there seems to be no consensus as to which medications are most effective in otomycosis (Kurnatowski and Filipiak, 2001; Lawrence *et al.*, 1978). This is due to the resistance pattern of the implicated fungi to the various available anti-fungal agents. It was shown that antifungal solutions, such as clotrimazole or nystatin may be effective against *Candida* but not *Aspergillus*. Ruckenstein (2005) recommended treatment of with oral itraconazole. Where else Bassiouny *et al.* (1986) strongly recommend both econazole and clotrimazole otic drop should be used worldwide. This was based on broad-spectrum antifungal activity, well tolerated and with no side effects. Both of these drugs also possess the advantage of having an antibacterial effect in addition to their

antifungal action (Lawrence *et al.*, 1978). This added advantage when treating mixed infection. Munguia and Daniel (2008), in their extensive literature review from January 1951 till March 2007, found clotrimazole is the most widely used and effective topical medication with effectiveness from 95 percent to 100 percent in most studies.

It is remarkable how many medications have been used for this stubborn condition (Kurnatowski and Filipiak, 2001; Munguia and Daniel, 2008; Paulose *et al.*, 1989). Nevertheless, treatment with antifungal agents alone is not enough to ensure complete treatment. It should be coupled with mechanical debridement, which aims at restoring the physiology of the external auditory canal (Ho *et al.*, 2006; Hueso *et al.*, 2005).

It is generally understood, that fungus grows into the squamous epithelium of the external auditory canal (Gil-Carcedo *et al.*, 2004). Keratolytic or antiseptic/acid ear drops removes superficial layers of dermis, and along with that, the fungal mycelia growing into them. Thus research that looks into role of this agents, have also been done (Kiakojuri *et al.*, 2007; Munguia and Daniel, 2008). Ozcan *et al.* (2004) concluded that administration of 4 per cent boric acid solution in alcohol and frequent suction cleaning of the ear canal might be a cost-effective treatment for otomycosis as 77 per cent of the patients were treated effectively. While Kiakojuri *et al.* (2007) reveal no significance difference between group that undergoes suction clearance and application of topical miconazole compared with another group that received same combination with 3 per cent acetic acid drops.

1.2 HONEY

1.2.1 Honey and origin

Honey has been used as food and medicinal products since the earliest time. It is the natural substance produced by the honey bees *Apis spp.*, from the nectar or blossom or exudates of trees and plants giving nectar honey or honeydew respectively (Alvarez-Suarez *et al.*, 2010; Molan, 1992).

As the only available natural sweetener, honey was an important food for *Homo sapiens* from the very beginning. It was an important source of carbohydrates and the

only widely available sweetener, until the production of industrial sugar began to replace it after 1800 (Crane, 1975). In the long human tradition, honey has been used not only as nutrient but also as a medicine. Indeed, the relationship between bees and humans started as early as Stone Age. The first written reference to honey was found on a Sumerian tablet dating back 2100-2000 BC, mentioning the use of honey as drugs and ointment (Crane,1975). As stated in the Holy Quran verses 16:68-69, “And your Lord inspired the bee, saying “take your habitations in the mountains and the trees, and in what they erect. Then, eat of all fruits, and follow the ways of your Lord made easy (for you)”. There comes forth from their bellies, a drink of varying colours wherein is healing for men. Verily, in this indeed a sign for people who think.” (Holy Quran, An-Nahl, 16 : 68-69).

Honey has been used in many cultures since ancient time for its medicinal properties, including as a remedy for burns, cataracts, ulcers and wound healing (Alvarez-Suarez *et al.*, 2010; Coulston, 2000). Aristotle, 350 BC and Dioscorides, AD 50 recommended that honey collected in specific regions and seasons to be used for the treatment of different ailments (Molan, 1992). Ibne Sina, the prince among Muslim physicians, in his work, “The Canon of Medicine”, listed several beneficial uses of honey (Crane, 1975). Such considerations have continued into present day. The strawberry tree honey of Sardinia is valued for its therapeutic properties while in India lotus honey is used for eye diseases (Molan, 1992).

1.2.2 Properties of honey

The quality of honey depends on the composition of nectar and floral origin. The active component in plants depends on various factors too; plant-bio, chemotype and climatic conditions. Consequently, honey properties from different locations should be different (Alvarez-Suarez *et al.*, 2010; Cushnie and Lamb, 2005; Koc *et al.*, 2009).

Honey is mainly made up of carbohydrates which constitute about 95% of its dry weight. It contains at least 181 substances (Chow, 2002). It is a supersaturated solution of sugars, mainly composed of fructose (38%) and glucose (31%), containing also minerals, proteins, free amino acids, enzymes and vitamins (Perez *et al.*, 2002; Terrab *et al.*, 2003). A wide range of minor constituents are also present in honey.

These include phenolic acids and flavanoids, certain enzymes (glucose oxidase, catalase) and amino acids.

The concentration and type of polyphenolic substances depend on the floral origin of honey, and are major factors responsible for producing numerous nutritional and biological effects. That includes antifungal, antiviral, antibacterial, antioxidant, anti-inflammatory, anti mutagenic and immunosuppressive activities (Ahmed and Othman, 2013; Alvarez-Suarez *et al.*, 2010; Cushnie and Lamb, 2005; Koc *et al.*, 2009). Benzoic acid, cinnamid and flavanoid are mains polyphenolic.

1.2.3 HONEY IN MEDICINE

The belief that honey could be used as nutrient, a drug and an ointment has continued to the present time. Currently, information on the use of honey for the treatment of many human diseases can be found in general magazines, bee-keeping journals and natural products leaflets, suggesting a wide variety of unfounded properties. An alternative medicine branch, called apitherapy has developed in recent years. Apitherapy or therapy with bee products is an age-old therapeutic practice as recorded by several ancient civilizations. Indeed, medicinal importance of honey has been documented in the world's oldest medical literatures (Crane, 1975; Maryann 2000). In light of modern science, several important therapeutic effects of honey have been elucidated. Modern science has made it possible to specify their medical significance as bactericidal, bacteriostatic, antiviral, antifungal, antioxidant, anti-inflammatory, and antitumoral (Ahmed and Othman, 2013; Alvarez-Suarez *et al.*, 2010; Cushnie and Lamb, 2005; Koc *et al.*, 2009; Tan *et al.*, 2009).

The antibacterial activity of honey was first reported by Van Ketel in 1892 and the next report was by Sackett in 1919 (Molan, 1992). They also reported that the antibacterial potency was increased by limited dilution of honey, an observation that was hard to explain. Its antibacterial action is effective against a very broad spectrum of species. It is postulated osmotic action secondary to high sugar content of honey, hydrogen peroxide action by enzymatic activity in honey and acidity pH of honey are responsible for this effect (Molan, 1992; Tan *et al.*, 2009). However mechanism of antifungal activity is still not clearly understood yet as fungi are inhibited under conditions where the sugar content of the honey is clearly not responsible unlike

bacteria (Molan, 1992). To date, very few attempts have been made to assess the antifungal properties of honey, compared to the large volume of published literature which has established that honey has significant antibacterial activity (Alvarez-Suarez *et al.*, 2010; Aween *et al.*, 2012a; Aween *et al.*, 2012b; Mohamed *et al.*, 2010; Molan, 1992; Tan *et al.*, 2009; Tumin *et al.*, 2005).

There are different types of honey available globally with potential beneficial medicinal products. There are countries with different types of honey bearing different properties such as Manuka honey of New Zealand, Brazilian Acasia honey and locally available Malaysian honey such as Tualang, Kelulut, Gelang emas, Akasia and Pucuk Daun. To date, most of the study in Malaysia concentrates on various properties and benefit of Tualang and its other effect such as antibacterial and antitumour (Ahmed and Othman, 2013; Aween *et al.*, 2012a; Aween *et al.*, 2012b; Fauzi *et al.*, 2011; Ghashm *et al.*, 2010; Halima *et al.*, 2010; Kannan *et al.*, 2009; Khalil *et al.*, 2011a; Khalil *et al.*, 2011b; KirnpalKaur *et al.*, 2011; Kishore *et al.*, 2011; Mohamed *et al.*, 2010; Nasir *et al.*, 2010; Tan *et al.*, 2009; Tumin *et al.*, 2005).

1.2.4 MALAYSIAN HONEY

Honey is a prized delicacy to the Malaysians, particularly among the Malays. For centuries honey is known to be the enemy of diseases. There is a special Malay recipe for treatment of asthma using egg, lime juice and honey. It is also used in the preparation of the famous traditional Malay herbal remedy “ubat periuk” for post natal care. Honey also found to be effective in curing burns, carbuncle, boils and diabetic wound.

They are long list of Malaysian honey available, examples are “Tualang”, “Hutan”, “Gelang”, “Pucuk Daun”, “Ee Feng Gu”, “Kelulut” and “Akasia”.

Tualang honey is extracted from honeycombs from the Asian Rock bees (*Apis dorsata*) which build their hives high up in the Tualang tree (*Koompassia excelsa*). Tualang tree is Malaysia’s tallest tree, which grows to an astonishing height of more than 250m (about 30 storeys) and found in East Asian rainforest and is most found in low land rain forest of Peninsular Malaysia, Southern Thailand, North Eastern Sumatera and Borneo. The sources of nectars are multifloral.

Kelulut honey is extracted from honeycombs of Kelulut bees (*Trigona spp*). Kelulut bees are the smallest bees and are more of a 'house bee'. This bee is unique as it is the only bee which stingless. The Malays considered these small bees have more medicinal powers.

Akasia honey (honey-dew honey) is extracted from honeycombs of *Apis mellifera*. The source of nectar is extrafloral; in between leaf of Acacia tree (*Acacia mangium*). Acacia tree is tree native to Australia and were brought to Malaysia for timber and pulp production to be used for paper production.

Among variety of local honey available, this study will focus on three (3) types of honey, namely Tualang, Akasia and Kelulut for few reasons. Tualang honey has been well-known for its healing and therapeutic effect. Most of the study in Malaysia, incorporated Tualang as the major honey in their research (Ahmed and Othman, 2013; Fauzi *et al.*, 2011; Ghashm *et al.*, 2010; Halima *et al.*, 2010; Kannan *et al.*, 2009; Khalil *et al.*, 2010; Khalil *et al.*, 2011a; Khalil *et al.*, 2011b; KirnPalKaur *et al.*, 2011; Kishore *et al.*, 2011; Mohamed *et al.*, 2010; Nasir *et al.*, 2010; Tan *et al.*, 2009; Tumin *et al.*, 2005). Meanwhile as for Akasia, source of nectar is extrafloral. Thus it is continuously available. Comparing with Tualang in which the source of nectar is multifloral. As a result, its availability depends on flowering season. In addition, source of nectar are also available in large scale as availability of Acacia tree in large magnitude for manufacture of paper. Furthermore *Apis mellifera* is one of the bee-rearing projects in USM which is commercialised. As for Kelulut, it is unique as it is the only stingless bee. It is also under USM bee-rearing project. Thus availability of honey is not an issue.

1.3 HONEY AND OTOMYCOSIS

Very limited studies done overseas, demonstrate antifungal activity of honey. They used their local honey (Boukraa and Bouchegrane, 2007; Candiracci *et al.*, 2012; DeMera and Angert, 2004; Estevinho *et al.*, 2011; Feas and Estevinho, 2011; Irish *et al.*, 2006; Koc *et al.*, 2009; Moussa *et al.*, 2011; Moussa *et al.*, 2012a; Moussa *et al.*, 2012b; Ngatu *et al.*, 2011; Obaseiki-Ebor and Afonya, 1984; Theunissen *et al.*, 2001). On the other hand, there were studies that document selected fungal resistant on honey (Kuncic *et al.*, 2012; Lusby *et al.*, 2005).

To best of our knowledge, based on research and English literature review, this will be the first study to reveal the antifungal properties of different types of Malaysian local honey to pathogenic fungi of otomycosis, specifically to *A. niger* and *C. Albicans*. Both are the most common cause for otomycosis. We hope with this effort we will be able to determine the antifungal effect of our local honey on pathogenic fungi of otomycosis, which are easily available and will subsequently be useful in the management of otomycosis, with added value to the international effort in this field.

CHAPTER 2

OBJECTIVES

2.1 General Objective:

To study the antifungal effect of three types of Malaysian local honey (Tualang, Kelulut and Akasia) on *Aspergillus niger* and *Candida albicans*

2.2 Specific Objectives:

- 1) To determine the antifungal effect of three different honeys on *Aspergillus niger*
- 2) To determine the antifungal effect of three different honeys on *Candida albicans*
- 3) To determine the lowest concentration with antifungal effect and total inhibitory concentration of each honey on *Aspergillus niger*
- 4) To determine the lowest concentration with antifungal effect and total inhibitory concentration of each honey on *Candida albicans*
- 5) To establish the type of honey with strongest antifungal effect

2.3 Research Hypothesis

Different type of Malaysian honey has antifungal effect at different concentrations against *Aspergillus niger* and *Candida albicans*, which are known to be the common causative organisms for otomycosis.

CHAPTER 3

METHODOLOGY

3.1 Study design: This is a laboratory-controlled prospective study. In this study different concentrations of each honey will be studied in vitro, for their anti fungal activity on fungal isolates of *Aspergillus niger* and *Candida albicans*.

3.2 Time and setting

Duration of study: 7 months (From December 2013 till June 2014).

Setting: This study conducted under well-controlled environment in the Medical Microbiology and Pharmacology laboratories in the School of Medical Sciences, Universiti Sains Malaysia Health Campus, Kubang Kerian, Malaysia. It was carried out after obtaining approval from the University Human Ethical Committee.

3.3 Materials

The materials used in this study include:

1. Three different types of honey – Tualang, Acasia and Kelulut
2. Fungal isolates (*Aspergillus niger* and *Candida albicans*)
3. Sabouraud Dextrose Agar (SDA) plate 90mm (Oxoid-CM 0041^(R)) and Sabouraud Dextrose Agar (SDA) powder
4. Plate
5. BD PhoenixSpec^(R) Nephelometer and Vortex
6. Pipette and tip
7. Distilled sterile water
8. Sterile tube
9. Hockey stick and Bunsen burner
10. Alcohol 70%
11. Sterile gloves

3.4 Method

3.4.1 Honey preparation

The Tualang Honey used in this study was obtained from Federal Agriculture Marketing Authority (FAMA), Kedah, Malaysia. Meanwhile, for Acasia honey and Kelulut honey the supply were from USM-BJIM Project (Medicinal Trigonal Bee Rearing project). All this honeys were subjected for sterilization with gamma-irradiation at a dose of 25kGy followed by sterility test. This radiation dose was sufficient to achieve sterility while preserving its antimicrobial properties which is heat-labile (Molan and Allen, 1996).



Figure 3.1: Three types of honey (Tualang, Kelulut, Acasia) used in this study were subjected to gamma-irradiation and kept in the glass bottle and dark place.

Honeys were kept in glass bottles and stored in the dark at room temperature because both hydrogen peroxide and glucose oxidase are light sensitive (Mousaa *et al.*, 2012a; White *et al.*, 1963).

3.4.1.1 Inclusion criteria

- 1) Honeys supplied from either FAMA or USM-BJIM Project through Pharmacology department were only used.
- 2) Honeys are kept in glass bottles and dark room.
- 3) Honeys are sterilized with gamma-irradiation. Subsequently, it passes sterility test when there is no growth on SDA (control) when honey incorporated.
- 4) Sterility of the equipment maintained throughout research.
- 5) Consistent temperature in laboratory setting.

3.4.1.2 Exclusion criteria:

- 1) Honey from other sources.
- 2) Honey which does not undergo sterilisation, were not used to avoid contamination.
- 3) Honey which has fungal growth on SDA, were not used because of the contamination with fungal.
- 4) Honey which are exposed to sunlight/ not kept properly in dark room, were not used. As it may already lost its antifungal ability.

3.4.2 Fungal strains

The fungal isolates of *Aspergillus niger* and *Candida albicans* used in this study were obtained from patients attending Otorhinolaryngology – Head and Neck Surgery out-patient clinic of Hospital Universiti Sains Malaysia (HUSM). These are patients with symptoms and signs of otomycosis, without prior installation of antibiotic/antifungal ear drop. Swabs were taken from ear and spread on SDA for culture. Ready made SDA plates were used to avoid contamination and to maintain standard. Plates were wrapped in parafilm to ensure secure closure, thus avoiding contamination.



Figure 3.2: Sterile swab stick used to get ear swab from ear (left). Note the tip is slim for narrow ear canal. The stick then was lawn on petri dish (right) with Sabouraud Dextrose Agar (Oxoid-CM 0041^(R)). It is used for the cultivation of fungi. The parafilm (white arrow) was used to wrap the plate.

It was then sent immediately to Microbiology Department. Each of isolates was then evaluated. Any SDA which grew *Aspergillus niger* or *Candida albicans* were then maintained and used for this study. In total, cultures were taken from 20 patients, with 10 patients for each fungi.

For SDA which grew *Candida albicans*, it is kept in refrigerator (2⁰C - 8 ⁰C) until testing was performed (Figure 3.3). Meanwhile for SDA which grew *Aspergillus niger*, part of the agar (approximately 1x1cm) were cut and maintained in sterile distilled water in room temperature until testing was performed (Figure 3.4). This is to avoid overgrowth and spillage of *Aspergillus niger* that grows rapidly when maintained in media that kept in refrigerator.



Figure 3.3: *Candida albicans* growth in SDA (left). The plates were kept in refrigerator (right) until testing was performed.

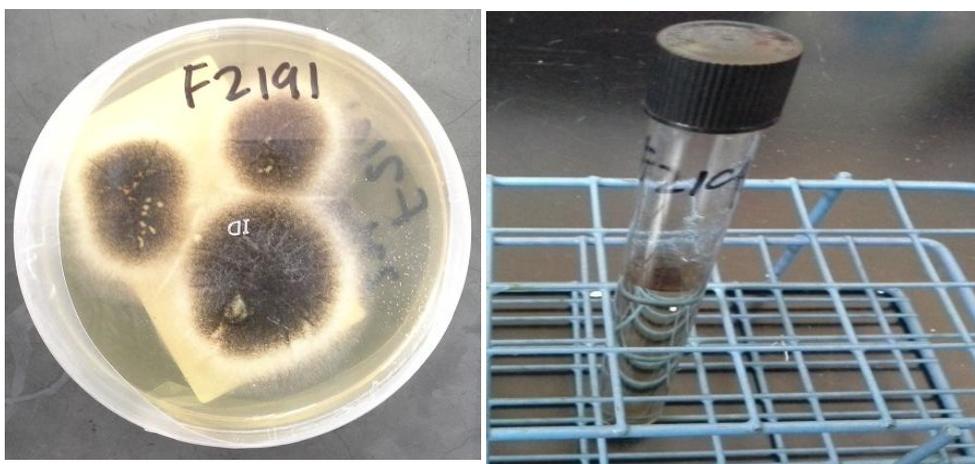


Figure 3.4: *Aspergillus niger* growth in SDA (left). Part of the agar maintained in sterile distilled water in room temperature until testing was performed (right).

Both were then sub cultured before testing was performed. Each individual fungal was coded for easier labelling on petri dish later. Examples *Aspergillus niger*, lab code F2159, code used A. Meanwhile for *Candida albicans*, lab code C673, code used B.

3.4.3 Inoculums preparation and standardization

Preparations of inoculums were done in Biohazard Safety Cabinet (Figure 3.5). This will prevent any hazardous material from spreading to the laboratory spaces. Its HEPA (*High-efficiency particulate air*) filter will remove harmful bacteria and viruses. It has warning alarm if the protective glass opened beyond safety range.



Figure 3.5: NuAire^(R) Class II, Type A2 Biosafety cabinet (NuAire, USA).

Fungi needed for this study were grown by subcultures in SDA for three days in incubator. The incubator maintains optimal temperature, humidity, oxygen and carbon dioxide content inside. Therefore this will provide conducive environment for fungal growth.



Figure 3.6: Memmert^(R) Standard Incubator (Memmert, Germany) with temperature 30 - 35⁰C.

Stock suspensions of *Aspergillus niger* were prepared from sporulating 3-day-old cultures. Colonies were covered with 5 ml sterile distilled water and the surface was scraped with sterile cotton swab. While doing this the plate cover was open slightly to avoid contamination. The mixture of conidia and hyphal fragments were filtered with sterile gauze and collected in sterile specimen container (Figure 3.7). This procedure removed the majority of the hyphae, producing inocula composed mainly of spores (Petrikkou *et al.*, 2001).



Figure 3.7 : Subcultured *Aspergillus niger* bathed with sterile distilled water (left) to produce suspension which was filtered with sterile gauze and collected in sterile specimen container (right).

Subsequently, the inoculum suspensions were transferred adequately into a sterile tube containing 3 ml of distilled water and were mixed well using Vortex. The tubes have been sterilised by autoclave. Sterile tubes are important to avoid contaminations of other organism which will interfere with the results.



Figure 3.8: Sterile specimen container (left), autoclaved sterilized tubes (centre) and sterile cotton swab (right) used in this study.

The final inoculums suspension density was adjusted to the turbidity of 0.5 McFarland standard using Nephelometer, which is equivalent to $0.5-5 \times 10^6$ cfu/ml.



Figure 3.9: BD PhoenixSpec^(R) Nephelometer 440910 (Becton Dickson & Company, USA), on left used to measure turbidity and Vortex, on right to ensure proper mixture of suspension.

Meanwhile for *Candida albicans*, it was also prepared from sporulating 3-day-old cultures. The colony was scraped with sterile cotton swab. This were also done with the upper lids of plates were slightly lifted to avoid contamination. Following this, the cotton swab was dipped into a sterile tube containing 3 ml of distilled water and firmly rotated to mix well the colony and water. The swab was removed then, while the tube