ANTIFUNGAL EFFECT OF PIPER BETLE (SIREH) LEAF EXTRACT ON SELECTED FUNGAL SPECIES OF PATHOGENIC OTOMYCOSIS IN IN-VITRO CULTURE MEDIUM

DR ARDHI BIN ABDULLAH

DISSERTATION SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF MEDICINE (OTORHINOLARYNGOLOGY-HEAD AND NECK SURGERY)

SCHOOL OF MEDICAL SCIENCES
UNIVERSITI SAINS MALAYSIA
2018

ACKNOWLEDGEMENT

I am grateful to Allah for giving me the opportunity and strength to complete this study. My utmost gratitude goes to Assoc. Prof. Dr. Irfan bin Mohamad for the continuous effort, motivation and tireless guidance, endless optimism and smile in which has efficiently guided me along the process of finishing this study. My heartfelt thanks go to my co-supervisor, Assoc. Prof. Dr. Rosdan bin Salim for his endless support. My sincere appreciation to Prof. Dr. Mohd Khairi bin Md Daud, Head of Department in Otorhinolaryngology-Head and Neck Surgery, School of Medical Sciences, Universiti Sains Malaysia for his continuous support and encouragement.

I would like to thank other co-supervisors namely Prof. Madya Dr. Azian Harun for revealing to me the beauty of microbiology and teaching me valuable scientific techniques and thorough understanding on conducting laboratory-based research. Furthermore, I am also thankful to the statistician, Nurzulaikha bt Mahd Ab.lah for helping me in analysing the results. I hope that this study will promote greater interest among the readers for a new knowledge on the *Piper betle* leaf.

Moreover, I am also indebted to all my teachers, colleagues and the staffs of Otorhinolaryngology-Head and Neck Surgery for rendering generous support and willing assistance in contributing to this study.

Nevertheless, after saying all this, certainly I am thankful to my beloved wife, Nor Ilyani Abdullah, our lovely daughter Maryam Sakinah and also our sons Yusuf Muslim and Ibrahim Adham, not forgetting my parents for their fullest love and unconditional support, understanding and co-operation throughout my postgraduate study.

TABLE OF CONTENTS

ABSTRACT

ABSTRAK

Pendahuluan: Otomikosis ialah sejenis jangkitan pada telinga yang berpunca daripada kulat yang semakin meningkat berikutan penggunaan meluas antibiotik, steroid dan agen kemoterapi, daun sireh *(Piper betle)* telah lama digunakan dalam perubatan tradisional oleh masyarakat terdahulu. Ekstrak daun sireh berpotensi sebagai rawatan anti kulat namun kajian mengenainya masih belum dilakukan secara meluas. Kajian ini bertujuan menilai kesan antikulat daun sireh terhadap dua kulat patogenik otomikosis iaitu *Aspergillus niger* dan *Candida albicans*.

Objektif: Mengkaji kesan antikulat ekstrak daun sireh ke atas *Aspergillus niger* dan *Candida albicans* dalam jenis dan kepekatan larutan yang berbeza (air dan alkohol).

Metodologi: Kajian ini merupakan kajian makmal kawalan prospektif yang dijalankan di Universiti Sains Malaysia. Daun sireh diproses menggunakan kaedah pengekstrakan Soxhlet dengan larutan 70% etanol dan air, kemudian dikeringkan untuk memperoleh serbuk yang pekat. Seterusnya, serbuk ekstrak tersebut telah dilarutkan dengan air steril untuk mendapatkan lima kepekatan berbeza iaitu 50 g/ml, 25 g/ml, 12.5 g/ml, 6.25 g/ml dan 3.125 g/ml. Kemudian ekstrak tersebut telah diuji berulang kali pada agar Sabouraud Dektrose yang telah disapukan kulat. Kaedah penyebaran agar telah digunakan dan zon perencatan diukur.Kepekatan perencatan minima telah diukur. Keputusan dicatatkan dan dianalisa menggunakan kaedah statistik.

Keputusan: Kedua-dua larutan air dan alkohol ekstrak daun sireh menunjukkan perencatan pada pertumbuhan *Aspergillus niger* dan *Candida albicans*. *Candida*

albicans menunjukkan kesan antikulat pada larutan ekstrak berkepekatan 50 g/ml, 25 g/ml, 12.5 g/ml dan 6.25 g/ml untuk larutan air dan manakala kesan antikulat pada larutan ekstrak 50 g/ml, 25 g/ml dan 12.5 g/ml untuk larutan alkohol. Bagi kadar perencatan pada pertumbuhan *Aspergillus niger* berlaku pada larutan ekstrak berkepekatan 50 g/ml, 25 g/ml dan 12.5 g/ml untuk larutan air dan 50 g/ml dan 25 g/ml bagi larutan ekstrak alkohol. Kesan antikulat yang ditunjukkan oleh *Aspergillus niger* adalah lebih baik dalam larutan air jika dibandingkan dengan larutan alkohol dengan signifikasi $p<0.005$. Begitu juga kesan antikulat yang ditunjukkan oleh *Candida albicans* adalah lebih baik dalam larutan air jika dibandingkan dengan larutan alkohol dengan signifikasi $p<0.005$. Nilai kepekatan perencatan minima (Minimum inhibitory concentration, MIC) untuk larutan air terhadap *Candida albicans* ialah 5.01 g/ml, larutan alkohol terhadap *Candida albicans* ialah 11.48 g/ml, larutan air terhadap *Aspergillus niger* ialah 12.88 g/ml, dan larutan alkohol terhadap *Aspergillus niger* ialah 23.98 g/ml.

Kesimpulan: Daun sireh terbukti mempunyai kesan antikulat terhadap *Aspergillus niger* dan *Candida albicans.* Perbandingan menunjukkan bahawa kesan antikulat adalah lebih baik dalam larutan air berbanding larutan alkohol.

ABSTRACT

Introduction: Otomycosis is an infection of the ear by the fungal species. The prevalence keeps increasing due to the widespread use of broad-spectrum antibiotics, steroids and other chemotherapeutic agents. *Piper betle* leaf has been used extensively as a traditional healing by old folk. *Piper betle* leaves extracts has potential to act as an antifungal medication but not been studied. This is a pilot study which analyses antifungal properties of *Piper betle* leaves towards pathogenic otomycosis, particularly *Candida albicans* and *Aspergillus niger*.

Objective: To study the antifungal effect of *Piper betle* leaf extracts on *Aspergillus niger* and *Candida albicans* by determining inhibitory efficacy and comparing antifungal activity of different concentration of *Piper betle* leaf aqueous and alcohol extracts on *Aspergillus niger* and *Candida albicans*

Methodology: This is a laboratory-controlled prospective study.

Results: Both *Aspergillus niger* and *Candida albicans* showed sensitivity to aqueous and alcohol *Piper betle* leaf extracts. Mean zone of inhibition increase in proportion to extract concentration. Statistically, one-way ANOVA showed significant mean differences between groups of concentration for both aqueous and alcohol extracts. Independent t-test was applied to compare mean between two groups.

Conclusion:.*Piper betle* leaf extract has potent antifungal activities on *Aspergillus niger* and *Candida albicans.* The antifungal efficacy in aqueous solution is better than alcohol solution.

CHAPTER 1
INTRODUCTION

CHAPTER 1

1.1 INTRODUCTION

Traditional herbal medicine is widely practiced worldwide. *Piper betle* leaf (PBL) is a herbal plant which has been used for a long time in agriculture and medicine. This green, heart-shaped leaves is originated in South and South East Asia[1]. Numerous studies suggest that PBL have a tremendous potential as a potent source for novel therapeutic usage. PBL has been reported to contain various important chemical constituents such as hydroxychavicol, chavibetol and hydroxybenzoic acid[2,3,4]. These components are valued for its medicinal properties like antifungal[2], antibacterial[3,4] and antioxidant[7].

Otomycosis or fungal infection of the ear canal and sometimes can be involved in the middle ear. The usual presentation is aural pruritus and ear discharge[18]. The prevalence of otomycosis ranges from 9%[19], to 30.4%[18] and has been reported to be increasing in recent years. Increased use of topical antibiotics or steroid preparations, instrumentation of the ear, and immunocompromised host were identified as among the predisposing factors to develop otomycosis[20]. Recent clinical mycological study stated that the most common fungal species isolated in otomycosis belonged to species of *Aspergillus species* (64.28%) with *Aspergillus niger* was the most common isolated (55.36%), followed by *Candida spp*.(19.64%)[10]. Malaysian data on fungal isolates in otomycosis was in accordance with other studies, with *Aspergillus niger* the commonest isolated fungi (71%), followed by *Candida albicans* (23.4%)[11].

Aspergillus niger colonies is a filamentous fungus that consists of a compact white or yellow basal felt covered by a dense layer of dark-brown to black conidial heads. It

produces several secondary toxic metabolites such as 3-nitropropionic acid and ochratoxin A[21]. In contrast, *Candida albicans* has the capability to form biofilm, which made it less susceptible to the host immune system and to conventional antifungal drug therapy[22].

The diagnosis of otomycosis is clinical, accompanied by microbiological confirmation. On otoscopic examination, sometimes the debris is seen with visible fungal hyphae, where *Aspergillus niger* appears as black headed filamentous growth, while *Candida albicans* as white or creamy deposits.

A standard treatment regime for otomycosis has not yet established. Thus, eradication of otomycosis remains a challenge to medical practitioners especially the otorhinolaryngologist. Articles reviewed on ototopical antifungal and otomycosis had limited data regarding safety of use of ototopical medication, especially in presence of tympanic perforation[16]. In a case of perforated tympanic membrane, the ototopical antifungal use should be avoided because it can cause inflammation and formation of granulation tissue in the middle ear. Besides that, the topical antifungal can readily reach cochlea by diffusion through the round window. Temporary and permanent electrophysiology changes within inner ear or morphological injury to stria vascularis, hair cells and supporting cells of organ of corti may occur if the agent has ototoxic property, which lead to ototoxicity and sensory neural hearing loss later[16,17].

Certain factors should be considered when choosing topical antifungal drugs, such as water soluble, low risk of ototoxicity, low allergic effect, a broad spectrum antimycotic drug, suitable for application on paediatric patients, and commercially available[16]. However, to date, there is no topical antifungal that can provide all the mentioned factors. These opens door for the option of safer alternative treatment such as herbal

and plant based medication probably derived from PBL. There were limited study conducted on antifungal effect of PBL especially on *Aspergillus niger* and *Candida albicans*. Hence, in this study, we are analysing the antifungal properties of PBL extracts towards pathogenic otomycosis, particularly *Aspergillus niger* and *Candida albicans*.

CHAPTER 2
OBJECTIVES

CHAPTER 2

2.1 General Objective

To study the antifungal effect of *Piper betle* leaf extracts on *Aspergillus niger* and *Candida albicans*

2.2 Specific Objectives

1. To determine inhibitory efficacy of different concentration of *Piper betle* leaf in water and alcohol extracts on *Aspergillus niger*
2. To determine inhibitory efficacy of different concentration of *Piper betle* leaf in water and alcohol extracts on *Candida albicans*
3. To compare antifungal activity between water and alcohol *Piper betle* leaf extract against *Aspergillus niger*
4. To compare antifungal activity between water and alcohol *Piper betle* extract against *Candida albicans*

2.3 Research Hypothesis

Piper betle leaf extracts has antifungal effect against *Aspergillus* niger and *Candida albicans* at different concentrations.

CHAPTER 3
MANUSCRIPT

CHAPTER 3

3.1 TITLE: ANTIFUNGAL EFFECT OF PIPER BETLE (SIREH) LEAF EXTRACT ON SELECTED FUNGAL SPECIES OF PATHOGENIC OTOMYCOSIS IN IN-VITRO CULTURE MEDIUM

Corresponding Author:

ARDHI BIN ABDULLAH

Department of Otorhinolaryngology-Head and Neck Surgery

School of Medical Sciences, Universiti Sains Malaysia

16150 Kota Bharu, Kelantan.

Email :ardhi_usm@yahoo.com

Office : 09-7676430, Fax : 09-7676424

3.2 ABSTRACT

Background: *Piper betle* (Sireh) leaf has been used traditionally for a long time in agricultural and alternative medicine. *Piper betle* had been proven effective against certain fungi that could infect human body.

Objective: To study the antifungal effect of *Piper betle* extracts on *Aspergillus niger* and *Candida albicans*.

Methods: This is a laboratory-controlled prospective study conducted in Universiti Sains Malaysia. *Piper betle* was extracted with 70% ethanol and aqueous using Soxhlet extraction method. The concentrated extract then freeze dried to obtain powdered form which was diluted to establish five different concentrations of 50 g/ml, 25 g/ml, 12.5 g/ml, 6.25 g/ml and 3.125 g/ml. Sabouraud Dextrose Agar (SDA) lawned with tested fungal isolates were inoculated with the extracts using well-diffusion method. Zone of inhibition was measured followed by minimum inhibitory concentration (MIC).

Results: There were presences of zone of inhibition for both aqueous and alcohol *Piper betle* extracts on *Aspergillus niger* and *Candida albicans* growth. *Piper betle* aqueous extract have bigger mean of inhibition as compare to alcohol extract with significance $p<0.005$ against *Aspergillus niger*. In contrast, *Piper betle* alcohol extract have bigger mean of inhibition as compare to aqueous extract with significance $p<0.005$ against *Candida albicans.* The MIC of *Piper betle* aqueous extract against *Candida albicans* was 5.01 g/ml, alcohol extract against *Candida albicans* was 11.48 g/ml, *Piper betle* aqueous extract against *Aspergillus niger* was 12.88 g/ml and alcohol extract against *Aspergillus niger* was 23.98 g/ml.

Conclusion: *Piper betle* has significant antifungal effect towards pathogenic fungi causing otomycosis, particularly *Candida albicans* and *Aspergillus niger.* Statistically antifungal activity of *Candida albicans* is better in aqueous extracts as compared to alcohol extract with significance $p<0.001$. For the antifungal activity of *Aspergillus niger* is also better in aqueous extracts as compared to alcohol extract with significance $p<0.001$. *Candida albicans* was inhibited better as compared to *Aspergillus niger* in both aqueous and alcohol *Piper betle* extract.

KEYWORDS

Otomycosis, *Piper betle*, Hydroxychavicol, *Aspergillus niger*, *Candida albicans*

3.3 INTRODUCTION

Piper betle leaf has been used for a long time in folk medicine[1]. Various studies have shown that the phytochemicals compound of *Piper betle* have antifungal[2] antibacterial[3,4], anti-inflammatory[5], anti-allergy[6], antioxidant[7] and anticarcinogenic activity[8].

Phytochemical study have shown that hydroxychavicol is the main compound in *Piper betle* [2]. The other chemical compound found in *Piper betle* are chavibetol, hydroxybenzoic acid, isomethyl eugenol, dihydrochalcone, derivative of safrole, piperolide, ethylsitosterol and diethylphenylheptanediol[3,9]. Research shows that hydroxychavicol has strong antifungal properties[2].

Otomycosis or fungal infection of the ear canal can be caused by *Aspergillus niger*, *Aspergillus fumigatus*, *Aspergillus flavus*, *Candida albicans*, and *Penicillium spp*.[10]. Malaysian data on fungal isolates in otomycosis revealed that *Aspergillus niger* is the commonest isolated fungi (71%), followed by *Candida albicans* (23.4%)[11].

Aspergillus niger able to produce several secondary toxic metabolites such as 3-nitropropionic acid and orchratoxin A[12, 13, 14]. In contrast, cell surface hydrophobicity, biofilm formation and adhesion composite resin process are the important factors in defense mechanism of *Candida albicans*[15]. The significance of this character is a reduced susceptibility to the host immune system and to conventional antifungal drug therapy[15].

Eradication of otomycosis remains a challenge to medical practitioners especially the otorhinolaryngologist. Articles reviewed on ototopical antifungal and otomycosis had limited data regarding safety of use of ototopical medication, especially in presence of tympanic perforation[16]. In a case of perforated tympanic membrane, the ototopical

antifungal use should be avoided because it can cause inflammation and formation of granulation tissue in the middle ear. Besides that, the topical antifungal can readily reach cochlea by diffusion through the round window. Temporary and permanent electrophysiology changes within inner ear or morphological injury to stria vascularis, hair cells and supporting cells of organ of corti may occur if the agent has ototoxic property, which lead to ototoxicity and sensory neural hearing loss later[16,17].

Certain factors should be considered when choosing topical antifungal drugs, such as water soluble, low risk of ototoxicity, low allergic effect, a broad spectrum antimycotic drug, suitable for application on paediatric patients, and commercially available[16]. However, to date, there is no topical antifungal that can provide all the mentioned factors. These opens door for the option of safer alternative treatment such as herbal and plant based medication probably derived from PBL. There were limited study conducted on antifungal effect of PBL especially on *Aspergillus niger* and *Candida albicans*. Hence, in this study, we are analysing the antifungal properties of PBL extracts towards pathogenic otomycosis, particularly *Aspergillus niger* and *Candida albicans*.

3.4 METHODOLOGY

Study design

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

Extracts from *Piper betle* was isolated using two different solvents which is aqueous and alcohol. Each extracts were diluted into five different concentrations and test to five replicates of fungal cultures. Thus, one type of fungal culture was used for five separate concentrations and one fungal culture test with two types of solvents which are ten samples. Therefore, 50 samples are tested for five replicates in standard laboratory settings. Total sample for two types of fungal culture was 100 samples[21].

Sample preparation method

Piper betle were collected from a single area from Kampung Panchor, Kemumin Pengkalan Chepa, prepared for USM herbarium identification (voucher reference number 11627). The remaining *Piper betle* were washed with distilled water and dried in oven at 50^{o}C for two days. Dried *Piper betle* leaves should maintain the original green colour. Then, the dried leaves were grinded using leaf grinder machine to smaller course powder form and stored in tightly sealed glass container.

Sample extraction method

The method of choice for extraction was the Soxhlet extraction by using Soxhlet apparatus. This uses a solvent for extraction and at completion the solvent will be fully removed. We used two types of solvent, which was aqueous, and ethanol 70%. The dried powder form of the tested leaves was inserted into the Soxhlet thimble and closed with white thin gauze. The thimble was inserted into the Soxhlet main chamber and closed. The solvent chamber was filled with ethanol 70% 1 litre, and attached to Soxhlet apparatus. Solvent chamber should not be overfilled and the volume of solvent in the vessel should be 3 to 4 times the volume of the Soxhlet chamber. The solvent chamber was heated and solvent vapour travels up a distillation arm, and fills into the main Soxhlet chamber. The condenser ensures that any solvent vapour cools, and drips back down into the chamber housing the powder. Some of the desired compound was then dissolved in the warm solvent. When the Soxhlet chamber was almost full, the chamber was automatically emptied by a siphon side arm, with the solvent running back down to the distillation flask. This extraction allowed for 4-6 cycles. By 6^{th} cycle, the solvent chamber was dark in colour and Soxhlet chamber was clearer. The solvent with desired compound was extracted and in solvent chamber. The extracted leaves in alcohol solvent then evaporated with rotary evaporator. The volume of extract was concentrated till 50 ml and inserted into multiple sterile containers. It was freeze dried to avoid further heat damage on a freeze dryer machine. For water solvent, same process done whereby the ethanol was replaced with distilled water in solvent chamber.

This powder form extracts were used to establish different concentrations as required in this study. The concentrations required were 50 g/ml, 25 g/ml, 12.5 g/ml, 6.25 g/ml and

3.125 g/ml. Initial 100 g mixed with two ml of distilled water to produce 50 g/ml concentration. Then one ml from the 50 g/ml concentration taken and distilled water added till two ml level to make 25g/ml concentration. Similarly, one ml was taken from 25 g/ml concentration to make up 12.5 g/ml. The dilution continued till 3.125 g/ml concentration. The left over extracts was discarded and a new preparation done for new replicates.

Preparation of *in vitro* culture medium

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

The suspended sterile fungal organism labelled and used for the next step to lawn and prepare for testing in SDA plates. Within 15 minutes of diluting the organism, sterile swab was dipped into the properly adjusted inoculums of tested fungal organism. The sterile swab was slightly lifted up, and then the swab was firmly rotated several times against the upper inside wall of the tube to express excess fluid. The plate was open slightly to lawn the fungal organism. Later, by using glass pipette, four wells were created in four quadrants.

Initiation of *in vitro* test and data collection

The SDA plate was kept lid side up in a 30°C incubator. Before inserting in an incubator, micropipette was used to drop 100 microliter of extracts into wells. The upper quadrant well, the aqueous *Piper betle* leaf (PBL) extract was inserted with its aqueous control at opposite site. The lower quadrant well, the alcohol extracts was inserted with its alcohol control at opposite site. This was done using different concentration which was diluted prior to this. Five different replicates were done. The plates were examined every day to make sure no spillage or growth of other organism. The measurement was done on third day whereby this was the perfect time to visualize the margin of inhibition. After the third day, there was overgrowth especially the *Aspergillus niger* which may jeopardize the safety of the staff. Measurement was done of zones showing complete inhibition by gross visual inspection.

Determination of minimum inhibitory concentration (MIC)

Minimum inhibitory concentration measured by agar diffusion method to determine MIC of each tested PBL extracts. After measurement of all the result, a scattered plot graph X^2 Versus Log Concentration was plotted. A linear line represent the mean value was drawn and the area where X^2 equal to zero was taken for the MIC level of the extract. Antilog of the selected value was mathematically identified as MIC. The X value represent the zone of inhibition diameter subtract the well diameter and divided by two[8].