ANTIFUNGAL EFFECT OF MALAYSIAN ALOE VERA LEAF EXTRACT ON SELECTED FUNGAL SPECIES OF PATHOGENIC OTOMYCOSIS IN IN-VITRO CULTURE MEDIUM

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ABSTRAK

Pengenalan: *Aloe barbadensis miller* ataupun lebih dikenali sebagai Aloe vera atau daun lidah buaya telah digunakan sebagai ubatan tradisional selama betahun-tahun. Tujuan kajian ini adalah untuk mengenalpasti kesan antikulat ekstrak daun Aloe vera (lidah buaya) di Malaysia terhadap dua kulat patogenik yang meyebabkan kulat telinga ataupun otomikosis iaitu *Aspergillus niger* dan *Candida albicans*. Tujuan kajian ini adalah untuk mengkaji kesan antikulat ekstrak daun lidah buaya Malaysia terhadap *Aspergillus niger* dan *Candida albicans*. Selain daripada itu, kajian ini juga membandingkan aktiviti antikulat antara ekstrak lidah buaya air dan alkohol terhadap *Aspergillus niger* dan *Candida albicans.*

Metodologi: Kajian ini adalah satu kajian perintis yang dijalankan sepenuhnya di makmal mikrobiologi perubatan dan farmakologi di Pusat Pengajian Sains Perubatan, Universiti Sains Malaysia, dijalankan dalam persekitaran yang terkawal. Pertamanya, daun lidah buaya yang bakal digunakan diidentifikasikan oleh pakar botani di Herbarium Universiti Sains Malaysia. Daun lidah buaya Malaysia dari satu kawasan yang sama dibasuh, dikeringkan dan kemudian dikisar sehingga menjadi serbuk. Seterusnya, serbuk daun lidah buaya Malaysia diekstrak menggunakan larutan etanol dan air melalui proses pengekstrakan Soxhlet.

Selepas pengekstrakan, proses pembekuan kering dilakukan untuk memperolehi serbuk. Seterusnya, serbuk ekstrak lidah buaya telah dicairkan dengan air steril untuk mewujudkan 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 mempunyai *Candida albicans* dan *Aspergillus niger*. Kaedah penyebaran agar telah digunakan dan zon

perencatan telah diukur. Lima replikasi dilakukan dengan menggunakan lima kepekatan berbeza. Keputusan dicatatkan dan dibandingkan dengan statistik menggunakan 'one-way ANOVA' dan ujian 'independent-t'.

Keputusan: Bagi *Aspergillus niger*, didapati kedua-dua ekstrak air dan alkohol daun lidah buaya merencatkan pertumbuhan kulat tersebut bagi kesemua konsentrasi kecuali 3.125 g/ml. Juga didapati bahawa zon perencatan adalah lebih tinggi dengan kepekatan yang lebih tinggi bagi kedua-dua ekstrak air dan alkohol. Untuk kulat *Candida albicans*, didapati bahawa untuk kedua-dua ekstrak air dan alkohol daun lidah buaya, tiada zon perencatan.

Kepekatan perencatan minimum bagi kulat *Aspergillus niger* adalah 5.125 g/ml bagi ekstrak air dan 4.35 g/ml bagi ekstrak alkohol. Kepekatan perencatan minimum bagi kulat *Candida albicans* tidak diperolehi berikutan tiada zon perencatan untuk kedua-dua ekstrak air dan alkohol.

Kesimpulan: Didapati bahawa, ekstrak alkohol daun lidah buaya Malaysia mempunyai kesan antikulat yang lebih ketara berbanding ekstrak air bagi kulat *Aspergillus niger* ($p<0.001$). Daun lidah buaya Malaysia memiliki kesan antikulat yang ketara. Bagi kulat *Candida albicans*, daun lidah buaya Malaysia didapati tiada kesan antikulat untuk kedua-dua ekstrak air dan alkohol.

ABSTRACT

Introduction: *Aloe barbadensis miller* or Aloe vera has been used for therapeutic purposes since ancient time. Antifungal activity is amongst the medicinal properties owned by Aloe vera. This study proposes antifungal effect of Malaysian Aloe vera leaf extract on pathogenic otomycosis species, *Aspergillus niger* and *Candida albicans.*

Methodology: This pilot study is a laboratory-controlled prospective study conducted in Universiti Sains Malaysia. Powdered form of Malaysian Aloe vera leaf was extracted with 70% ethanol and aqueous via Soxhlet extraction method, after being evaporated using rotary evaporator to a thicker compound. The concentrated extract is then freeze dried to obtain powdered form which is diluted into establish five different concentrations of 50 g/ml, 25 g/ml, 12.5 g/ml, 6.25 g/ml and 3.125 g/ml. Five replicates were performed for five different concentration. Results were statistically analysed using one-way ANOVA and independant-t test. Sabaroud Dextrose Agar lawned with tested fungal isolates were inoculated with the extracts using well-diffusion method. Zone of inhibition was measured followed by minimum inhibitory concentration.

Results: For *Aspergillus niger,* zone of inhibition for alcohol and aqueous extract were obtained for all concentrations except 3.125g/ml. As for *Candida albicans,* there were no zone of inhibition for both alcohol and aqueous extract of Aloe vera leaf. The minimum inhibitory concentration value of aqueous and alcohol extracts were 5.129g/ml and 4.35g/ml for *Aspergillus niger* and as for *Candida albicans,* it was not determined as no zone of inhibition was obtained.

Conclusion: Antifungal effect of alcohol extract of Malaysian Aloe vera leaf is better as compared to aqueous extract for *Aspergillus niger* ($p<0.001$). Malaysian Aloe vera has significant antifungal effect towards *Aspergillus niger*. Malaysian Aloe vera leaf extract has no antifungal effect against *Candida albicans.*

CHAPTER 1

INTRODUCTION

1.1 INTRODUCTION

1.1.1 Background of study

Throughout the globe, many plants have been utilised for their medicinal properties. According to Mukherjee and Wahile, World Health Organization (WHO) considered that 80% of the world's population is dependent on ancestral medicines.[1] The remaining 20% of the world population on the other hand are considered to be the occupants of developed countries, therapeutic product of plants plays an important role. Of the medicinal plants, Aloe species has been used for therapeutic purposes since ancient time.[2]

Aloe barbadensis miller, commonly referred as Aloe vera is one of more than 400 species of Aloe which belongs to family Liliacaea.[3] Aloe vera is considered to be the most potent and popular plant among the Aloe species.[4] For over 2000 years, Aloe vera species has been used in folk medicine and till date has remained an important component in the traditional medicine of many countries including: China, India, West Indies and Japan. Common names of the plant include *Aloe, Aloe capensis, Aloe spicata, Aloe vera, Barbados loe, Cape aloe, Chirukattali* (India), *Curacao aloe, Ghai Kunwar* (India), *Ghikumar* (India), *Indian aloes, Kumari* (Sanskrit), *Laloi* (Haiti), *Lohoi* (Vietnam), *Luhui* (Chinese), *Nohwa* (Korean), *Rokai* (Japanese), *Sabilla* (Cuba), *Socotrine aloe, Subr* (Arabic), and *Zanzibar aloe.*

Aloe vera is a succulent plant, hence it adapts to living in areas of lower water availability and has a large water storage tissue. It is said that Aloe vera's prominent feature is its high water content which may range from 99-99.5% with the remaining 1.0-1.0% is reported to contain over 75 different potentially active compounds including water-soluble and fat-soluble

vitamins, minerals, enzymes, simple or complex polysaccharides, phenolic compounds and organic acid.

Chemical analysis of the Aloe vera plant by Prof Tom D. Rowe in 1941 reported that the Aloe vera has 75 nutrients and 200 active compounds which includes: sugar, anthraquinones, saponins, vitamins, enzymes, minerals, lignin, salicylic acid and amino acids. Each of the 75 active compounds has a remedial property (Table 1). This includes; lignin which has capacity of penetrating human skin, saponins with its antiseptic property and as a foaming agent.[2] Based on study by Sandeep Kumar et al, active phytochemical composition within the Aloe vera which contributes to the antifungal property is aloe-emodin.[4]

Aloe vera leaf can be divided into: outer rind, inner gel and intermediate pulp layer (Fig.1). Each leaf is composed of: outer thick layer or rind which consists of 15-20 cells which contains hydroxyanthracene, anthraquinone and glycosides aloin A and B, hydroxyanthrone, aloe-emodin-anthrone 10-C-glucoside and chrones. Middle layer of latex which contains-Antraquinones and Glycosides and inner clear gel which contains 99% water and rest is of glucomannans, amino acids, enzymes, lipids, sterols and vitamins.

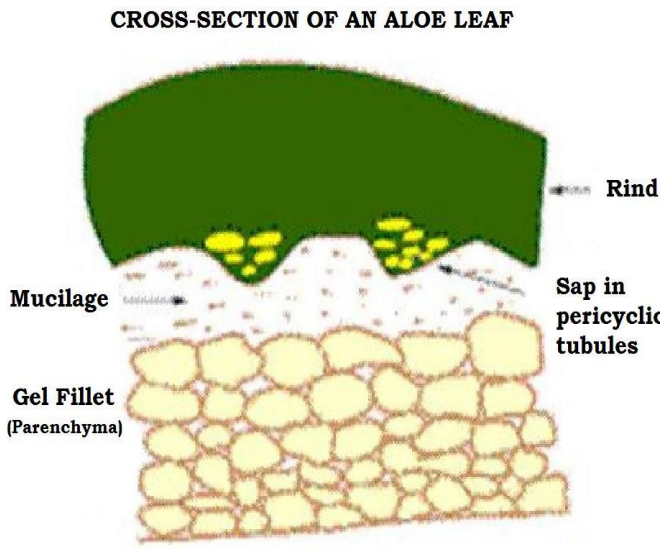

Fig. 1: Components of Aloe vera

http://www.positivehealth.com/img//img/image-article/e02920/athertn2.gif

Table 1: Composition of Aloe vera

http://pubs.sciepub.com/ajfst/2/3/3/image/equ1.png

Class	Compounds
Anthraquinones	Aloin/Barb-aloin, Isobarba-aloin, Aloe-emodin, Emodin, Aloetic acid, Ester of cinnamic acid, Anthranol, Chrysophanic acid, Resistannol Anthracene, Ethereal oil.
Vitamins	B1, B2,B6, A-Tocopherol, β–Carotene, Choline, Folic acid, Ascorbic acid
Enzymes	Cyclo-oxygenase, Oxidase, Amylase, Catalase, Lipase, Alkaline- phospahatase, Carboxy-peptidase
Miscellaneous	Cholesterol, Steroids, Tricylglycerides, β – Sitosterol, Lignins, Uric Acid, Gibberellin, Lectin like substances, Salicylic Acid, Arachidonic Acid
Saccharides	Mannose, Glucose, L-Rhamnose, Aldo-pentose
Carbohydrates	Cellulose, acetylated mannan, Arabinogalactan, Xylan, Pure mannan, pectic substance, glucomannan, Glucogalc-tomannan, Galactan
Inorganic Compunds	Calcium, Sodium, Chlorine, Manganese, Zinc, Chromium, Copper, Magnesium, Iron
Non-essential Amino acids	Histidine, Arginine, Hydroxyproline, Aspartic Acid, Glutamic Acid, Proline, Glycine, Alanine
Essential Amino acids	Lysine, Threonine, Valine, Leucine, Iso-leucine, Phenyl-alanine, Methionine

1.12 Otomycosis

Otomycosis is a fungal infection of the external auditory canal which is also known as fungal otitis externa. Otomycosis are more common amongst male.[5] Otomycosis incidence are contributed by the degree of humidity and heat, dusty environment as well as amongst low socioeconomic group. Recent wide use of medications including; broad-spectrum antibiotics, steroids and also chemotherapeutic agents has led to its emergence. Usage of topical antifungal agents or at times oral medications has been the choice of treatment. Despite the usage of various topical antifungal agents, eradication of otomycosis is still a challenge, not to mention the cost of the medication and the devastating complications of antifungal agents. With the emergence of resistance to antifungal agents yearns need of better understanding of the molecular mechanisms responsible of the development of drug resistance.[6]

This dilemma of resistance to antifungal agents gives way to traditional medicine including herbal medicine as an alternative to curb otomycosis. In the ancient age, plant was the main source of medication however there are limited studies on usage of plants as an antifungal agent. Aloe vera extracts has been widely used over the years to cure arthritis, skin cancer, burns, eczema, psoriasis, digestive problems high blood pressure and diabetes.[7] Aloe vera extract has been proven to have antibacterial and antifungal property. Based on the data collected by Chin et al in a study done in Malaysia on fungal isolates in otomycosis, *Aspergillus sp.* was the commonest isolated (71%) followed by *Candida sp.* (23.4%).[8] Study by Kedarnath et al has proven antifungal effect of Indian Aloe vera extract on *Aspergillus spp.* and *Candida spp*.[9] However, there are limited publications on antifungal effect of Malaysian Aloe vera leaf extract to *Aspergillus spp.* and *Candida spp.* Different Aloe species would have interspecies

variation and varying climate and soil conditions thus, whether Malaysian Aloe vera has similar antifungal properties as Indian Aloe vera has not been explored till date. This is a pilot study on antifungal effect of Malaysian Aloe vera leaf extract towards *Aspergillus niger* and *Candida albicans*.

Diagnosis of otomycosis

Diagnosis is usually made based on patient's presenting complaint and history, physical examination, and also otoscopic examination. Diagnosis is usually confirmed by microbiological investigation. Imaging studies is usually reserved for invasive type of otomycosis. Otoscopic examination is prudent to establish otomycosis diagnosis albeit, different findings between the various fungal infection.

Otoscopic features of *Aspergillus niger* includes blackish hyphae, mycelia and wet paper appearance. As for *Candida albicans*, the classical otoscopic appearance is cotton-wool like features or debris assembling white blanket.

Treatment of otomycosis

Till date there has been no definitive treatment for otomycosis. Current management of otomycosis includes usage of topical antifungal agents with aural toileting, although recurrence is fairly common. An ideal antifungal agent should have low risk of ototoxicity, water soluble, low allergic effect, broad-spectrum antifungal effects, safe for paediatrics patients and cheap.[10]

Antifungals available today can be grouped into three classes based on their site of action: azoles, polyenes and 5-fluorocysteine. Azoles inhibits the synthesis of ergosterol (the main fungal sterol) while polyenes interacts with fungal membrane sterols physicochemically and 5-fluorocytosine inhibits macromolecular synthesis.

Azoles or triazoles family includes: clotrimazole, fluconazole and miconazole. Polyenes group of drugs are nystatin and amphotericin B. Study by Munguia and Daniel revealed that clotrimazole and fluconazole are effective antifungal agents for otomycosis.[11] Clotrimazole has been proposed as the most effective antifungal agents for otomycosis.[12] However, albeit being a broad-spectrum antifungal, clotrimazole has its fair share of side-effects including signs of skin irritation: skin rash, hives, blister formation, burn, itchiness, erythema and oedema.

With regards to the ideal antifungal properties, there has yet any antifungal agents commercially available. This is also coupled by the development of resistance to antifungal agents. Various types of mechanisms contribute to the development of resistance to antifungals including alteration to the drug target, alteration in sterol biosynthesis, reduction in the intercellular concentration of target enzyme, and overexpression of the antifungal drug target.

The significant clinical implication and urge to discover an ideal antifungal drug has led to heightened interest in the study of various alternatives for treating otomycosis. The past decade has witnessed a significant increase in the number of unresolved otomycosis which has implications for morbidity, mortality and health care costs. Thus, substantial attention has been focused on alternative treatment for treating otomycosis.

Treatment of otomycosis in alternative medicine

Various studies using plants has showed promising results for treating otomycosis. *Plumeria acuminata ait* (Kalachuchi) has one of the highest antifungal activity against *Candida spp.* Preliminary study done on the efficacy of *Plumeria acuminata* bark extract versus clotrimazole cream in treating otomycosis demonstrated a cure rate of 75% as compared to 87.5% for clotrimazole cream.

Other plants exhibiting antifungal properties includes: neem leaf extract, garlic, black walnut, oregano oil and cloves.

1.13 C*andida albicans* & its features

Candida spp. are ubiquitous fungi and is considered among the common fungal pathogens which affects humans. *Candida albicans* comprises of microorganism which lives in the oral cavity and the gastrointestinal system. Its existence is usually linked to compromised immune system or weakened balance between normal flora. *Candida albicans* is a dimorphic fungus as it has two different phenotypic form, an oval shaped yeast form and a branching hyphal form. It grows best under aerobic condition although it exhibits a limited degree on growth under anaerobic condition.

Candida albicans can grow on a simple media containing carbon (glucose), nitrogen (ammonium salts) and phosphate. It can grow well in the temperature range of 20-40 degree celcius and a pH below 2 albeit growth rate on synthetic medium is usually 30 degree celcius. On a Sabaroud Dextrose Agar (SDA) plate its colonies are white to cream coloured, smooth and yeast like.

Candida albicans has the capability to form biofilm. This characteristic feature reduces its susceptibility to host immune system and thus resistance to antifungal agents.

1.14 *Aspergillus niger* & its features

Aspergillus niger is a ubiquitous fungus that grows very quickly. Its strains can be isolated from many different ecological habitats such as soil, plant debris, rotting fruit, and even indoor air environments. *Aspergillus niger* macroscopically can be identified growing on substrates producing colonies of felt like yellow to white hyphae, turning black with the formation of conidia. Microscopically, this fungal species can be identified by its hyaline, septate hyphae. Asexual conidiophores can be identified by being long and globose at the tip, with what appears to be a hymenial layer of structures, each "ejecting" its own spore.

Some strains of *A. niger* have been reported to produce potent mycotoxins called ochratoxins other sources disagree, claiming this report is based upon misidentification of the fungal species. Recent evidence suggests some true *Aspergillus niger* strains do produce ochratoxin A.[13] It also produces the isoflavone orobol.

1.14 Therapeutic Indications

Burn wound and healing effect

Aloe vera's in-vitro extract has been proven to stimulate proliferation of several cell types. Treatment with whole Aloe vera gel extracts results in faster healing of wounds. Aloe vera's wound healing effect is via its property that increases the rate of wound contraction. Mannose-6-phosphate carries out this by not only increasing in wound contraction formation but also by collagen synthesis. Apart from that, another important property of Aloe vera is its ability to promote fibroblast proliferation and the production of hyaluronic acid and hydroxyproline in fibroblasts which has significant contribution to wound healing as it causes extracellular metrix remodelling.[14]

In-vitro extract of Aloe vera has shown to stimulate proliferation of several cell types. Treatment with whole Aloe vera gel extract results in faster healing wounds.[15] Direct effect of wound healing process by Aloe vera extract is manifested by increase rate of contraction of wound area.[16] Increase in wound contraction and collagen synthesis is made possible due to the presence of mannose-6-phosphate in the Aloe vera gel.[17]

Immunomodulatory effect

Immunomodulatory effect is another prominent property of Aloe vera whereby in human macrophages it downregulates lipopolysaccharide-induced inflammatory cytokine production and expression on NLRP3 inflammasome. Reduction of leucocyte adhesion and proinflammatory cytokines are noted following burn injury as Aloe vera could inhibit the inflammatory process. Based on a study by Liu et al, on rats with severe traumatic-haemorrhagic, Aloe polysaccharides pre-treatment have shown to reduce cerebral ischemia and reperfusion injury through inhibiting systemic inflammatory response and leucocytic aggregation.[17] Aloe vera administration has been proven to increase phagocytic and proliferative activity of reticuloendothelial system. Aloe vera's important role of inflammation is via the inhibitory action of cyclooxygenase pathway and reduction of prostaglandin E2.

Effect on skin penetration enhancement

Aloe vera increases the in-vitro skin penetration of compounds. The effect of skin penetration enhancement is based on the pull effect of complexes formed between the compound and the enhancing agent within the Aloe vera.[14]

Antimicrobial effect

Aloe vera antimicrobial property is due to its active compound. For example anthraquinone property of Aloe vera is a structural analogue of tetracycline. This enables the inhibition of bacterial protein synthesis by blocking the ribosomal A site which is similar to mechanism of action of tetracycline. So, bacteria cannot grow in a media containing Aloe vera extract.

Another active compound of Aloe vera which exhibits antibacterial property is polysaccharide. Polysacchharide which is contained within the gel layer of the Aloe vera leaf has a direct antibacterial activity through the stimulation of phagocytic leucocytes to destroy bacteria.

Pyrocathecol which is a hydroxylated phenol in the Aloe vera leaf is known to have a toxic effect on microorganisms.[14]

Antiviral effect

Aloe vera gel has been proven to have antiviral activity which prevents virus absorption, attachment or entry to the host cell. The antiviral property is due to the content of its active composition-anthraquinone derivatives such as *Aloe-emodin*, emodin and chrysophanol. Whereby it has inhibitory mechanism and effect against influenza A virus which reduces virus induced cytopathic effect and inhibits replication of influenza A.[14]

Antiulcer effect

Aloe vera leaf extract has been used for digestion and also in the treatment of peptic ulcer as it exhibits cytoprotective action. This is due to the property of the Aloe vera gel which has antibacterial properties against both susceptible and resistant *H.pylori* strains.[14]

Hepatoprotective effect

Iophenol and cycloartanol components of physosterol are able to downregulate fatty acid synthesis along with upregulation of fatty acid oxidation in the liver. This action actually lead to reduction of the intraabdominal fat and further improvement of hyperlipidaemia. Obesity-induced inflammatory response is also suppressed by reducing levels of proinflammatory cytokines. Based on a study by Satio et al, A.vera gel extracts prevents ethanol-induced fatty liver. This is via suppressing the lipogenic genes in the liver.[14]

Effect on estrogen status

Aloe-emodin has been shown to suppress breast cancer cell proliferation via distinct route by specifically targeting esrogen receptor-alpha protein stability. A.vera gel as well maintsins ovarian steroid status in conditions like Polycystic Ovarian Syndrome (PCOS).[14]

1.16 Literature review

Aloe vera plant grown in Malaysian soil may have different phytochemical composition or active composition as the soil in Malaysia is different as compared to the other countries. Aloe vera can grow in all types of environment however, the quality and quantity of particular constituent warrants certain condition. The antifungal activity of plants differs based on the type of solvents and various methods of preparation. Till date, there is yet to be any research done on antifungal effect on Malaysian Aloe vera as compared to other Asian countries. This chapter reviews few of the various literatures published on antifungal properties on Aloe vera extract.

Role of climatic conditions and geographical locations on variations of component in Aloe vera

Based on literature review on antifungal effect of Aloe vera leaf extract, study done by Sandeep Kumar et al. showed role of climatic condition and geographical locations on variations in amount of aloe-emodin which is 1,8-dihyydroxy-3-(hydroxymethyl)anthracene-9,10-dione an antimicrobial compound derived from the leaves of Aloe vera, hence the variable antimicrobial effect.[19] Antimicrobial activity of methanolic extracts of Aloe vera leaf was taken from six different agro-climatic regions of India from 12 different accessions, where nine bacteria and two fungal stains (*C.albicans* and *A.niger*) were used for this study. nine bacterial strains includes; seven gram negative bacteria – *Shigella flexneri, Proteus mirabilis, Salmonella typhi, Serratia marcescens, Klebsiella pneumonia, Escherichia coli, Pseudomonas aeruginosa* and two gram positive bacteria including *Enterococcus faecalis* and *Staphylococcus aureus.* The

12 geographical locations and its agro-climatic zones includes: Jammu and Himanchal Pradesh (highland), Punjab and Haryana (semi-arid), Rajasthan and Gujarat (arid), Uttar Pradesh and Madhya Pradesh (humid subtropical), West Bengal and Andhra Pradesh (tropical wet and dry), finally, Goa and Kerala (tropical wets). Overall methanolic extract of all 12 accessions exhibited good antimicrobial and antifungal activity. The antifungal and antibacterial activity was checked using agar well diffusion method. The alcohol extracts were more active against fungus *C.albicans* than *A.niger.* Variations in inhibition zone may be due to difference in phytochemical composition in different climatic condition. Hence, climatic role and geographical location may affect the composition of Aloe vera hence the antifungal capacity.[19]

Effect of Aloe Arborescens Miller on Tinea pedis (Athlete's foot) on guinea pig

Tinea pedis is a skin fungal infection which occurs among humans. Study was done by Kawai et al on *Tricophyton mentagrophytes* which causes Tricophytosis or also known as *Tinea pedis* among humans. Tricophytosis was induced in guinea-pigs by the inoculation of arthrospores of *Tricophyton mentagrophytes* cephalic strain over the plantar part of guinea-pig feet and antifungal effects of Aloe arborescens Miller (*Kidachi aloe*) were evaluated. Fresh whole leaves of *Kidachi aloe* was freeze dried with average yield of 35g per 1kg. Sabouraud dextrose broth medium was used for the fungal inoculation. Culture studies after application of 30% freeze dried Aloe arborescens miller for 10 days showed a 70% growth inhibition. Antifungal effect was shown to be due to action of Carboxypeptidase and Barbaloin (Alloin) composition of Kidachi aloe. Alloin and carboxypeptidase are also the main composition of Aloe vera thus may exhibit antifungal effect on otomycosis species as well.[20]

Effect of solvent for exraction of Aloe vera

Study done by Mbajiuka et al was done using crude Aloe vera gel to investigate the microbial activity on selected human pathogens, best solvent for extraction and susceptibility of *E.coli, S.aureus* and *C.albicans.*[21] Ethanol, methanol and aqueous extracts as solvents were used for extraction. Aqueous extracts has the highest yield with 19.0gm, followed by ethanol extracts with 18.4gm, finally methanol with 18.0gm. However, ethanol was regarded as the best solvent for extraction. Agar well diffusion method was used to determine the susceptibility of *Escherichia coli, Staphyococcus aureus* and *Candida albicans* to the crude extract of Aloe vera gel. Negative control used was dimethylsulphoxide (DMSO). As for positive control, gentamicin was the option used. Growth of *Escherichia coli, Staphylococcus aureus* and *Candida albicans* was inhibited by ethanol extract with zones of inhibition of 6, 5 and 4 mm. As for aqueous extract, the zones of inhibition was 6, 4 and 3mm respectively. The ethanol extract had the best minimum inhibitory concentration (MIC) of 0.125, 0.125 and 0.40mg/ml than aqueous extract which is 0.25, 0.25 and 0.25mg/ml followed by methanol extract of 0.50, 0.00 and 0.00mg/ml on *Escherichia coli, Staphylococcus aureus* and *Candida albicans.* Conclusion of this study revealed that ethanol and aqueous extracts of Aloe vera gel was susceptible to all the three pathogens investigated subsequently proved the acceptability of these crude extracts for therapeutic purposes.[21]

Soxhlet extraction method as a method of extraction of Aloe vera

Based on study by Kedarnath et al, the antifungal and antimicrobial activity of Aloe vera was tested against pathogenic bacteria like *Staphylococcus aureus, Klebsiella pneumonia* and *E.coli*

and fungi including *A.niger* and *C.albicans* with various solvents such as petroleum ether, chloroform and methanol extracts.[9] Whole leaf Aloe vera leaves were used for this study. Leaves of Aloe vera were air dried and crushed into small pieces and then powdered using an electric grinder. It was later subjected for Soxhlet extraction using a Soxhlet apparatus. Solvents includes: petroleum ether, chloroform and methanol. The extracts were later concentrated to dryness in a flask evaporator under reduced pressure and controlled temperature. The extracts were later collected and stored in a sterile vial. The antifungal and antibacterial activity were carried out by disc diffusion technique. Sterile nutrient agar plate and potato dextrose agar plate were used for this study. The microbial organism were spread over the nutrient agar plates by using sterile cotton bud, while potato dextrose agar plates were used to spread the fungal test organism. The fungal plates were incubated at 24 degrees for 72 hours and the bacterial plates were incubated at 27 degrees for 24 hours. Minimum zone of inhibition diameter were measured in mm and 3 replicates were performed for each test. ANOVA test was used to determine the difference between extracts used and length of incubation using the data obtained. Aloe vera extracts was found to show moderate to high activity against both gram positive and gram negative bacteria. Methanol extract showed significant activity against *A.niger* and *C.albicans*. Methanol and petroleum ether showed significant activity against *K.pneumonia* and *E.coli.* Alcohol has better outcome as solvent for Aloe vera.[9]

Effect of antifungal activity Aloe vera leaf extract against *Aspergillus spp.*

Arunkumar and Muthuselvam published a study on Analysis of phytochemical constituents and antimicrobial activities of Aloe vera leaf against clinical pathogens whereby 26 bioactive

phytochemical compounds were identified using aqueous, ethanol and acetone to screen antimicrobial activity of selected human clinical pathogens by agar diffusion method.[22] Antifungal activity of *Aspergillus niger* and *Aspergillus flavus* was analysed. Maximum antifungal activity was observed in acetone extract with maximum growth suppression was observed in *Aspergillus flavus.* Antifungal activity was carried out by disc diffusion method and alcohol exract has better outcome as solvent for Aloe vera.[22]

CHAPTER 2

OBJECTIVES OF STUDY

2.1 General objective

To study antifungal effect of Malaysian Aloe vera (*Aloe barbadensis miller*) leaf extracts on *Aspergillus niger* and *Candida albicans*

2.2 Specific objectives

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

CHAPTER 3

MANUSCRIPT

3.1 Title page

ANTIFUNGAL EFFECT OF MALAYSIAN ALOE VERA LEAF EXTRACT ON SELECTED FUNGAL SPECIES OF PATHOGENIC OTOMYCOSIS SPECIES IN IN-VITRO CULTURE MEDIUM

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3.2 ABSTRACT

Objective: *Aloe barbadensis miller* or Aloe vera has been used for therapeutic purposes since ancient time. Antifungal activity is amongst the medicinal properties owned by Aloe vera. This study proposes antifungal effect of Malaysian Aloe vera leaf extract on pathogenic otomycosis species, *Aspergillus niger* and *Candida albicans.* **Methods:** This pilot study is a laboratory-controlled prospective study conducted in Universiti Sains Malaysia. Powdered form of Malaysian Aloe vera leaf was extracted with 70% ethanol and aqueous via Soxhlet extraction method, after being evaporated using rotary evaporator to a thicker compound. The concentrated extract is then freeze dried to obtain powdered form which is diluted into establish five different concentrations of 50 g/ml, 25 g/ml, 12.5 g/ml, 6.25 g/ml and 3.125 g/ml. Sabaroud Dextrose Agar lawned with tested fungal isolates were inoculated with the extracts using well-diffusion method. Zone of inhibition was measured followed by minimum inhibitory concentration. **Results:** For *Aspergillus niger,* zone of inhibition for alcohol and aqueous extract were obtained for all concentrations except 3.125g/ml. As for *Candida albicans,* there were no zone of inhibition for both alcohol and aqueous extract of Aloe vera leaf. The minimum inhibitory concentration value of aqueous and alcohol extracts were 5.129g/ml and 4.35g/ml for *Aspergillus niger* and as for *Candida albicans,* it was not determined as no zone of inhibition was obtained. **Conclusion:** Antifungal effect of alcohol extract of Malaysian Aloe vera leaf is better as compared to aqueous extract for *Aspergillus niger* ($p<0.001$). Malaysian Aloe vera has significant antifungal effect towards *Aspergillus niger.*

KEYWORDS

Otomycosis, Aloe vera, *Aspergillus niger*, *Candida albicans*

3.3 INTRODUCTION

Otomycosis also known as fungal otitis externa are fungal infections of external auditory canal seldom involving the middle ear. Albeit a benign condition, eradication of this entity remains a challenge to medical practitioners especially the otorhinolaryngologists. *Aspergillus sp.* and *Candida sp.* are the most frequent isolated fungi in otomycosis. Till date there has been no standardised treatment regimen for otomycosis which opens up to new horizons including herbal medicine.

Throughout the globe, many plants have been utilised for their medicinal properties. Aloe vera species has been used in folk medicine for over 2000 years and has remained an important component in the traditional medicine of many countries till today. *Aloe barbadensis miller* also known as Aloe vera is one of more than 400 species of Aloe which belongs to family Liliacaea.[1] Aloe vera's prominent feature is its high water content which may range from 99-99.5% with the remaining 1.0-1.0% is reported to contain over 75 nutrients and 200 active compounds including sugar, anthraquinones, saponins, vitamins, enzymes, minerals, lignin, salicylic acid and amino acids different potentially active compounds including water-soluble and fat-soluble vitamins, minerals, enzymes, simple or complex polysaccharides, phenolic compounds and organic acid.[2] Aloe vera has two parts, the outer rind and the inner colourless parenchyma; aloe gel. Both parts of Aloe vera has been shown to have medicinal values. Based on in-vitro and animal studies which uses total leaf extract, Aloe vera exhibits anti-inflammatory, anti-arthritic, antibacterial and hypoglycemic properties.[3] Several studies have proven on antifungal properties of Aloe vera extract.[4] This is a pilot study to determine

antifungal properties of Malaysian Aloe vera leaf extract on otomycosis species including *Aspergillus niger* and *Candida albicans.*

3.4 METHODOLOGY

This is a laboratory-controlled prospective study fully conducted in microbiology and pharmacology laboratories in Universiti Sains Malaysia. This study has been approved by the Ethics Committee of Universiti Sains Malaysia. Aloe vera leaves collected from a single area were washed with distilled water prior to being oven dried at 45 ºC for three to five days. (Figure 1) Dried Aloe vera leaves were grinded to achieve powder form and stored in a tightly sealed container.

Albeit myriad techniques present till date for extraction, Soxhlet extraction method using Soxhlet apparatus is the most sought out for.[5] For this research, two forms of solvents were used: aqueous and ethanol 70%, as it confines to our objectives. Soxhlet thimble filled with tested dried powdered form of Aloe vera leaf extract is inserted into the Soxhlet main chamber and closed. One litre of ethanol 70% is filled into the Soxhlet main chamber and attached to the Soxhlet apparatus. It is then heated with suitable temperature till the solvent vapour travels up a distillation arm and fills into the main Soxhlet chamber. The solvent vapour which is condensed then drips back down into the chamber which contains the tested Aloe vera leaf extract. (Figure 2)

The Aloe vera leaf extract which is extracted using alcohol solvent is then evaporated with rotary evaporator at 30 ^{o}C and concentrated till 50 ml prior to being freeze dried. The freeze dried extracts are in powdered form and kept in freezer to maintain its compound. The same extraction technique was carried out for the Aloe vera leaf extract using water as a solvent by replacing the 70% ethanol with distilled water. The powdered form of Aloe vera leaf extracts were then used to establish 5 different concentrations as per required in this research. The concentration which were required are 50 g/ml, 25 g/ml, 12.5 g/ml, 6.25 g/ml and 3.125 g/ml. Serial dilution technique was used using initial 50 g/ml concentration. This is achieved by diluting 100 grams of the powdered Aloe vera leaf extract with two mls of distilled water for the water extract and DMSO (Di-methyl sulphoxide) a universal solvent for alcohol extract to produce 50 g/ml concentration. One ml from the 50 g/ml concentration is added to distilled water to achieve two ml level to make 25g/ml concentration. This is then followed by taking one ml from 25 g/ml concentration to make up 12.5 g/ml. Similarly, one ml from 12.5g/ml concentration is taken and dilution is continued till 3.125 g/ml concentration. The remaining powdered Aloe vera leaf extracts are discarded. A new set of preparation is carried out for new replicates using the powdered Aloe vera lead extracts.

As for preparation of in-vitro culture medium, tested fungal isolates used in this study includes *Candida albicans* and *Aspergillus niger* from otomycosis which were obtained from the archives of Microbiology Laboratory of the School of Medical Sciences, Universiti Sains Malaysia (Figure 3). *Aspergillus niger* and *Candida albicans* from the Sabaroud Dextrose Agar (SDA) plates were suspended in sterile distilled water and adjusted to 10^6 cells with colony forming units (CFU)/ml[9] (0.5 McFarland standard) using nephelometer. The standardised fungal isolates were used to lawn the SDA plates using sterile swabs after diluting the organisms for 15 minutes. The SDA plates lawned with fungal isolates were then divided into

four quadrants equally. With the help of a sterile glass pipette, four wells were created equally. The Aloe vera leaf extracts based on its concentration were transferred into the well using micropipette. For this, 100 microlitre of the extracts was measured and transferred into the well. The aqueous Aloe vera extract was transferred to the upper quadrant of the well with its control aqueous solution at the opposite site. As for the alcohol extract of the Aloe vera it was transferred to the lower quadrant with its control in the opposite site. The SDA plates were then subsequently kept lid side up in a 30 ºC incubator. This process was continued with five different replicates.

The SDA plates placed in the incubator were checked daily in order to make sure that there is no spillage or growth of other organism. Measurement of the inhibition zone was done on the third day as the margin of inhibition were clearly visible. The zones of complete inhibition was measured using a Vernier calliper in millimetres by gross visual inspection and it was measured over the back of the inverted SDA plate over a non-reflective surface from above.

For the determination of minimum inhibitory concentration (MIC) of each tested Malaysian Aloe vera leaf extracts, agar diffusion method was used.[5] After measurement of the zone of inhibition, a scattered plot graph X^2 Versus Log Concentration is plotted. A linear line which represent the mean value drawn and the area where X^2 equal to zero was taken for the MIC level of the Aloe vera extract. Antilog of the selected value was mathematically identified as MIC. The X value represent zone of inhibition diameter subtract the well diameter and divided by two.