EVALUATION OF *Andrographis paniculata* (HEMPEDU BUMI) EXTRACT FORMULATION AS AN ANTIFUNGAL AGENT AGAINST SUPERFICIAL MYCOSES

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by

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LIST OF SYMBOLS, ABBREVIATIONS AND ACRONYMS

AECUSM	Animal Ethics Committee Universiti Sains Malaysia
ANOVA	Analysis of variance
AP	*Andrographis paniculata*
ARASC	Animal Research and Service Centre
ATCC	American Type Culture Collection
ATR	Attenuated Total Reflectance
ATR-FTIR	Attenuated Total Reflectance - Fourier Transform Infrared Spectroscopy
CFU	Colony forming unit
C-H	Carbon-hydrogen bond
$C_{20}H_{30}O_5$	Andrographolide
C=O	Carbon–oxygen bond
CO_2	Carbon dioxide
DAD	Diode array detector
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
DPX	Distyrene plasticiser xylene
EDTA	Ethylenediaminetetraacetic acid
EMA	European Medicines Agency
FBS	Fetal Bovine Serum
G	Growth
g	Gram

GMS	Grocott-Gomori's Methanamine Silver
GST	Glutathione-S-transferase
H&E	Haematoxylin & Eosin
HCl	Hydrochloric acid
HIV	Human Immunodificiency Virus
HPLC	High Performance Liquid Chromatography
HPTLC	High Performance Thin Layer Chromatography
H_2SO_4	Sulphuric acid
IC	Inhibitory Concentration
IR	Infrared
IUPAC	International Union of Pure and Applied Chemistry
IVC	Individually ventilated cages
kg	Kilogram
LC	Lethal Concentration
LPCB	Lactophenol cotton blue
M	Molar
MARDI	Malaysian Agricultural Research and Development Institute
mg	Milligram
Ml	Millilitre
mm^2	square millimeters
NA	Nutrient agar
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NG	No growth
OD	Optical density

OECD	Organization for Economic Cooperation and Development
O-H	Oxygen-hydrogen bond
PBS	Phosphate Buffered Saline
PDA	Potato Dextrose Agar
PDII	Primary Dermal Irritation Index
pH	Potential of hydrogen
PPARs	Peroxisome proliferator-activated receptors
R_f	Retention factor
RT	Room temperature
SAIP	Sabah Agro-Industrial Precinct
SEM	Scanning Electron Microscope
TCM	Traditional Chinese Medicine
TLC	Thin Layer Chromatography
UK	United Kingdom
USA	United State of America
USM	Universiti Sains Malaysia
UV	Ultra violet
v/v	volume per volume
WHO	World Health Organization
w/v	weight per volume
w/w	weight per weight
ZnSe	Zinc selenide
µg	Microgram

PENILAIAN FORMULASI EKSTRAK *Andrographis paniculata* (HEMPEDU BUMI) SEBAGAI AGEN ANTIKULAT TERHADAP MIKOSIS SUPERFISIAL

ABSTRAK

Jangkitan kulat superfisial biasanya muncul sebagai lesi pada kulit, kegatalan, kerosakan kuku dan keguguran rambut. Walaupun terdapat pelbagai agen antikulat, pembangunan kelas baru agen antikulat sangat terhad. Keperluan terhadap terapi antikulat dan penemuan agen novel bagi rawatan jangkitan kulat sangat diperlukan memandangkan wujudnya rintangan terhadap antikulat. Penggunaan ubat antikulat tanpa kawalan menyebabkan terjadinya rintangan terhadap antikulat. *Andrographis paniculata* (AP), sejenis herba perubatan purba, didapati mempunyai pelbagai aktiviti farmakologi termasuklah sebagai agen antimikrob. Oleh itu, kajian ini dijalankan untuk menilai kesan antikulat AP dan formulasinya terhadap kulat patogen terpilih yang menyebabkan jangkitan superfisial. Pada kajian awal, ekstrak methanol, etanol dan akues AP diuji kesannya terhadap pertumbuhan *Trichophyton mentagrophyte, T. rubrum, T. interdigitale, Microsporum fulvum, M. nanum, M. gypseum, M. canis, Fusarium solani* dan *Aspergillus fumigatus* menggunakan kaedah penyebaran cakera dan kaedah keracunan makanan di atas agar dekstros kentang. Ekstrak yang menunjukkan kesan antikulat terbaik diformulasikan kepada gel dan krim. Kesan toksik ekstrak AP dinilai menggunakan kultur sel fibroblas (L929), udang air tawar dan Arnab Putih New Zealand. Ujian kestabilan formulasi gel dan krim juga turut dijalankan. Pada kajian haiwan, jangkitan kulat superfisial pada Tikus Sprague Dawley telah dilakukan dan kesan antikulat *in vivo* formulasi AP terhadap *T. mentagrophyte, T. rubrum, T. interdigitale, M. gypseum* dan *M. canis* telah dikaji.

Sebatian kimia ekstrak AP juga telah dikenalpasti dengan menggunakan kaedah ujian saringan fitokimia, HPLC dan ATR-FTIR. Ekstrak etanol AP dalam bentuk formulasi gel menghasilkan aktiviti antikulat terbaik. Ujian kestabilan formulasi gel dan krim menunjukkan kehebatan formulasi AP gel. Ujian penilaian ketoksikan menunjukkan semua bentuk AP yang dikaji adalah selamat. Keputusan kajian kesan antikulat *in vivo* menunjukkan kesan antikulat yang signifikan pada rawatan gel ekstrak etanol AP berbanding kawalan negatif (tidak dirawat), dari segi peratus penyembuhan luka, penurunan saiz luka dan skor keadaan luka. Kajian histopatologi pada seksyen luka menggunakan pewarnaan haematoxylin-eosin (H&E) dan Grocott-Gomori's Methanamine Silver (GMS) menunjukkan pembasmian elemen kulat pada bahagian yang dirawat dengan gel ekstrak etanol AP berbanding bahagian yang tidak dirawat. Penilaian kimia ekstrak AP mendedahkan kewujudan kandungan andrographolide dan neo-andrographolide di dalam ekstrak methanol dan etanol, manakala ekstrak etanol menunjukkan kewujudan saponin, flavonoid, dan tanin. AP mempunyai aktiviti antikulat yang signifikan di mana ia menunjukkan kesan antikulat dalam kajian *in vitro* dan *in vivo*. Oleh itu, penemuan ini menunjukkan bukti terhadap potensi kegunaan AP sebagai agen antikulat novel. Kajian makmal dan klinikal lanjut masih diperlukan untuk membangunkan penggunaan formulasi baru AP sebagai rawatan alternatif bagi jangkitan mikosis superfisial.

EVALUATION OF *Andrographis paniculata* (HEMPEDU BUMI) EXTRACT FORMULATION AS AN ANTIFUNGAL AGENT AGAINST SUPERFICIAL MYCOSES

ABSTRACT

Superficial mycoses are commonly encountered fungal infections, which often present as skin lesion, itchiness, nail damage and hair loss. Although a number of antifungal agents are available at present, development of new classes of antifungal agents is limited. The increasing need for antifungal therapy and the emergence of antifungal resistance warranted the need for discovery of novel antifungal agents. *Andrographis paniculata* (AP), an ancient medicinal herb, is claimed to possess various pharmacological activities including antimicrobial activities. Therefore, the present study was embarked to evaluate the antifungal effect of AP extract and its formulations against selected fungal pathogens causing superficial mycoses. In the initial part of the study methanol, ethanol and aqueous extracts of AP, were tested for their effects on the growth of *Trichophyton mentagrophyte, T. rubrum, T. interdigitale, Microsporum fulvum, M. nanum, M. gypseum, M. canis, Fusarium solani* and *Aspergillus fumigatus* using disc diffusion and food poisoning method on potato dextrose agar. The extract which exhibited the best antifungal effect was formulated into cream and gel. The toxicity effects of AP extract were evaluated using mouse skin fibroblast cell lines (L929), brine shrimp and New Zealand White Rabbit. The stability of the gel and cream formulations were also evaluated. In an animal study, superficial fungal infection in a Sprague-Dawley rat model was established and the *in vivo* antifungal and wound healing effects of AP formulations against *T. mentagrophyte, T. rubrum, T. interdigitale, M. gypseum* and

M. canis infections was examined. The chemical compounds of AP extract were also identified using phytochemical screening, HPLC and ATR-FTIR methods. AP crude extract showed activity against *T. mentagrophyte, T. rubrum, T. interdigitale, M. fulvum, M. nanum, M. gypseum,* and *M. canis.* Ethanol extracts of AP in gel formulation showed the best antifungal activity. The stability evaluation of the gel and cream formulations demonstrated the superiority of AP gel formulation. The toxicity evaluation demonstrated adequate safety of all forms of AP tested. The results of *in vivo* antifungal effect showed significant antifungal effects ($p<0.01$) in AP ethanol extract gel compared to negative control (untreated), in terms of the percentage of wound healing, wound size reduction and wound conditions scoring. Histopathological examination of the wound sections using haematoxylin-eosin (H&E) and Grocott-Gomori's Methanamine Silver (GMS) staining showed eradication of fungal elements following AP ethanol extract gel treatment compared to untreated control. The chemical evaluation of AP extracts revealed the presence of andrographolide and neo-andrographolide content in methanol and ethanol extract, while ethanol extract showed the presence of saponins, flavonoids and tannins. AP has pronounced antifungal activity based on the demonstration of its effects in both *in vitro* and *in vivo* studies. Therefore, the findings in this study have provided the evidence for potential use of AP as a novel antifungal agent for superficial mycoses. Further laboratory and clinical studies are needed to ascertain the use of new AP formulation as an alternative treatment for superficial mycoses.

CHAPTER 1

INTRODUCTION

1.1 Herbal Medicine

Herbs have been treasured for their qualities, be it medicinal, aromatic, or savoury. Herbs can be viewed as biosynthetic chemical laboratories, which generate a number of chemical compounds. As medicine, they are eaten, drunk, or applied topically. Herbal products normally have various naturally-occurring biochemicals from plants that many of them can add to the plant's medicinal benefits. Chemicals that have these medicinal benefits are referred to as 'active ingredient' or 'active constituent' and their presence depends on several factors like plant species, harvesting time and season, soil-type and the herb's method of preparation. Many people are primarily attracted to herbal therapies for the belief that they will help them live more healthily (Kunle *et al*., 2012).

The variability of the constituents in herbs or herbal preparations depends on their genetic, cultural and environment factors. The challenge includes the difficulties in controlling the quality of different batches of herbal preparation.

In some countries, herbal products have made their way into the market without adequate scientific evaluation, and with the absence of any mandatory safety and toxicological studies. Consumer can purchase herbal products without being prescribed with them and subsequently, they might not be aware of the potential hazards in an inferior product. A well-defined and constant composition of the drug is therefore vital to render a drug of high quality.

1.2 The Development of World Herbal Industry

For the past ten years, there has been a renewed interest in traditional medicine and the world has given it the much-deserved respect and acknowledgment, further leading to the rebirth of green medicine. The World Health Organization (WHO) is trying to incorporate knowledge on traditional medicine into health systems especially in developing countries, since allopathic methods are expensive, and often inappropriate (http://www.who.int/bulletin/volumes/86/8/07-042820/en). Even though WHO has recognized that it will simply not be possible to replace traditional medicine with western health care for all people, however WHO seeks to promote plant medicine as chemistry by applying scientific analysis to the herbal treatments used (Anthony, 1984).

Consumers' preferences now are moving towards the product that is free from chemicals and away from synthetics. These provide better and increased opportunities for trade in herbal product. The herbal industry can also be an important source of income for rural and smallholder agriculture. The Global Herbal Medicine markets mainly include western herbalism, traditional Chinese medicine, homeopathy and Ayurveda. Most of them (50.9%) are used for western herbalism (Figure 1.1).

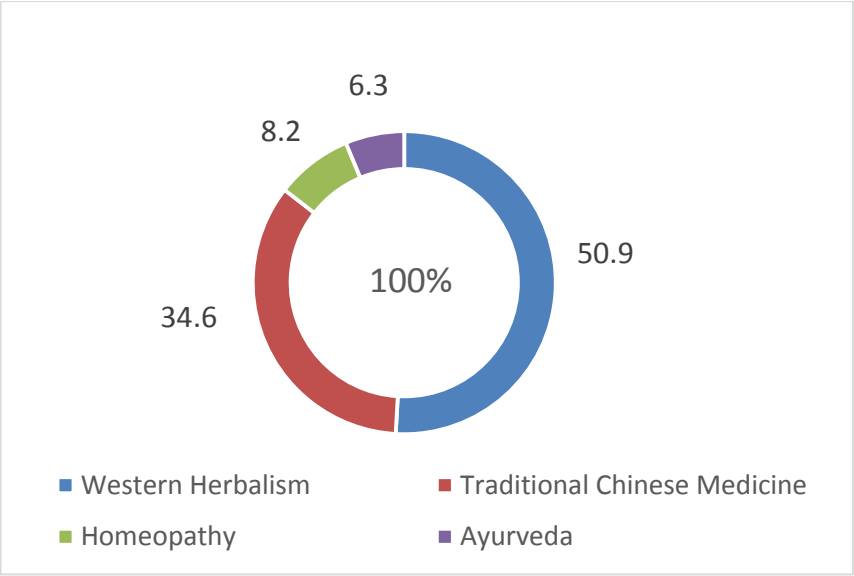


Figure 1.1 Global Herbal Medicine Market by Segment
Source: Global Industry Analysts, Inc., 2014.

Medicinal plants are known to be an integral part of the rainforest biodiversity in Malaysia. From the several thousands of species of higher plants discovered in Malaysia, an estimated figure of 1200 of these plant species have been found to have potential pharmaceutical value and some served as herbal medicine (Soepadmo *et al.*, 1991).

The domestic market for herbs and medicinal plants in Malaysia are mainly used for flavor and fragrance, pharmaceutical, nutraceutical and herbal remedies (Table 1.1).

Table 1.1 Industrial use of herbs and medicinal plants in Malaysia per year.

Type	Value (RM)
Flavor and Fragrance	**1.6 billion**
Pharmaceuticals/ Nutraceuticals	**950 million**
Herbal Remedies	**2 billion**
Total	4.55 billion

Source: Herbs-the industrial crop in Malaysian agriculture (Sharif, 1999).

The local sales turnover of herbal-based products in Malaysia had touched RM3 billion in year 2000 and was fast increasing every year. With the alternative medicines being the latest trend, the export of local herbs has become very promising. Some 8,500 local herbal medicines have been registered with the government, with about 135 factories manufacturing and selling them (Indu and Lean, 2000). One of the plans of Nasuha Enterprise Sdn. Bhd., one of Malaysian largest producer of herbs, is to extract essences from herbs and have them sold abroad. It is currently planting, manufacturing, marketing and planting herbs (Indu and Lean, 2000).

Following the success of Nasuha, and the demand for herbal products, this has led to the establishment of other herb gardens in Malaysia include: Pharmaceutical Park at Bandar Sri Iskandar, Perak; MARDI agroforestry at Sungai Buloh; Sabah Agro-Industrial Precinct (SAIP); Konsortium Pasifik SAMA Sdn. Bhd.; Herbs industry in Sungkai, Perak and MARDI station at Telong, Kelantan.

It should be made clear that although the global herbs industry, currently worth RM900 billion, involves the manufacturing of health care products, health foodstuff and personal care items, the Malaysian herbs industry is still lagging behind. Having imported more that RM1 billion worth of herbal products every year, Malaysia could play a major role in the international herbs market because of its strong foundation (Sabah Times, 1999).

Malaysia's vision is to be a leading exporter of herbal products by the next decade. Malaysia has a number of strong positive factors that can favor the development and expansion of the herbal industry. These include a good resource base, strong government commitment, available infrastructure, ready institution to

support the industry, capable human resource and enterprising private sector (Sharif, 1999).

1.3 Phytochemicals of herbal plant

Humans tend to use a variety of foods, drugs, and dietary supplements derived from plants and advantageous for the medicinal properties. The active properties of these substances can be explained by the plants' secondary metabolites. These chemicals are not required for the immediate survival of the plant but would work in a synthesis to improve the fitness of the plant to survive by allowing its interaction with its environment.

Many of the phytochemicals can be grouped according to their potentially active secondary metabolite constituents.

1.3.1 Medicinal properties of phytochemicals

Herbal plant is an important source of chemical compounds for medicinal properties. Some, such as carbohydrates, amino acids and proteins, are classified as primary metabolites, while others, such as alkaloids, saponins, tannins, terpenoids, flavonoids and phenolics, are classified as secondary metabolites.

Saponins are glycosides that have some unique characteristics. Tannins are classified as the flavan-3-ol class of flavonoids, and thus it has excellent antioxidant activities. Tannins are astrigent, bitter plant polyphenolic compound that can bind, precipitate or shrink proteins and many other organic compounds. Tannins have been reported to play an important role in the protective activity against protein oxidation

and glycation. Tannins are plant metabolites well known for their antimicrobial properties (Nakagawa *et al*., 2002).

Terpenoids (also called "isoprenoids") is one of the largest families of natural products which contain more than 40,000 individual compounds of both primary and secondary metabolisms. Most originate from plants, and annually, hundreds of new structures are recorded. Several bioactive terpenoids contained in herbal compounds can modulate ligand dependent transcription factors activities, such as peroxisome proliferator-activated receptors (PPARs) (Tsuyoshi *et al*., 2010).

As polyphenolic compounds flavonoids can be categorised into flavonols, flavones, catechins, flavanones, anthocyanidins and isoflavonoids. To analyse this, they are classified into three basic types namely flavonoids glycoside, non-polar flavonoids (aglycones, methylated or alkylated) and anthocyanins (Sarker and Nahar, 2007).

Flavonoids have high antioxidant activities and they are regarded as free radical scavengers and potent metal chelators (Lukacinova *et al*., 2008) and they also have both antifungal and antibacterial activities, other than possessing anti-inflamatory activity (Vessal *et al*., 2003).

1.3.2 Safety of herbal medicine

It is unfortunate to see that basic pharmaceutical and modern healthcare facilities are often unavailable and distant from the rural villages. Although some of the basic pharmaceutical drugs are physically available, it is still costly and thus, the villagers from remote areas cannot afford to have proper treatments. This makes them still continue to depend on herbal medicine and traditional herbalists for their

health and wellbeing. In Uganda for example, most people still turn to the traditional healers (Hamill *et al.*, 2000).

With alternative herbal medicine, an individual patient often performs self-treatment and it goes without high-quality professional advice. To be safe, the patients need to make sure that herbal therapies are safely used by deciding on health goals such as; informing themselves on the medicine efficacy, safety, drug interactions, and usage; selecting therapies that can meet their goals; having a correct diagnosis prior to the therapy; asking for advice from reputable practitioners; informing the practitioners about all the remedies used; and monitoring their consequences (Dunning, 2003).

The important information on product-labeling includes the product's name and composition, parts of the plant and quantity of raw material, daily and timing of dosages, allergy and other warning statements, quality and safety testing, expiry date, manufacturer, country of manufacture, claims and indications of the products and details on product storing (Kron, 2002).

McGuffin *et al*. (1997) in his book, *Botanical Safety Handbook* had categorized the herbs into four classes. Class 1 which is safe to consume, Class 2 safe to consume with restrictions (2a only for external use only, 2b not suitable for use in pregnancy, 2c not suitable for use while nursing, and 2d indicating other specific restrictions), Class 3 restricted to use only with expert supervision, and Class 4 herbs which do not have enough data to classify its safety. *Andrographis paniculata* falls in class 2b, which is not suitable in months of pregnancy.

In Germany, the regulatory authority Commission E oversees herbal preparations and their uses among the public. Currently, the United States only categorizes herbal preparations as dietary supplements. There is no standardization or

regulations regarding its active ingredients, purity, or concentration, and types of herbs which can be commercialized (Shenefelt, 2011).

In Malaysia, plants have been widely used because of their wonderful aromas and tastes, which add variety and flavor to the food we eat. Traditionally, many of these plants are also used for various human ailments. On the other hand, research has proven that some of the plants are harmful to humans and animals due to the presence of certain compounds proven to be toxic to them (Orech, 2005). Investigating the plant extracts for potential toxicity is considered an important step in evaluating whether they are suitable for commercial applications.

1.4 Fungal infections

Fungal infections are acquired from the environment or sometimes are endogenously caused by normal flora. Common routes of infections include the inhalation of infectious conidia generated from molds which grow in the environment. Some environmental fungi can inflict disease after they are accidentally injected past the skin barrier (Kenneth *et al.*, 2010).

The fungi which may cause human disease include the dermatophytes and the yeast-like fungus *Candida albicans*. These fungi are accountable for superficial infections on the skin, hair and nails, and mucous membranes. Other fungi can cause even deeper infections, which may remain localized (e.g. mycetoma) or cause systemic disease (e.g. histoplasmosis).

Dermatophytosis are superficial infections of skin and its appendages. Dermatophytosis is commonly known as ringworm, and is commonly caused by *Trichophyton*, *Epidermophyton* and *Microsporum*. There are many other filamentous

fungi which are associated with skin and mucocutaneous infections (Kenneth *et al.*, 2010).

Aspergillus species are widely distributed in nature and discovered in all over the world. The conidia thrive well in the environment with inhalation being the mode of infection. Occasionally, construction, or other forms of environmental intervention have been associated with increased frequency of *Aspergillus* contamination, colonization, or infection.

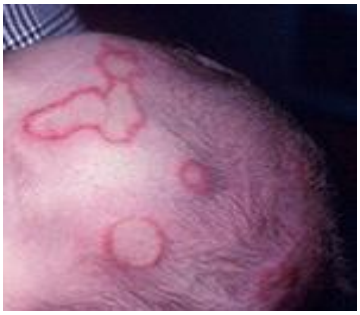
In usual cases, cutaneous aspergillosis is a cutaneous manifestation of disseminated infection with *Aspergillus. A. fumigatus* and *A. flavus* commonly cause primary cutaneous disease. Besides that, *A. terreus* and *A. ustus* were also the causes of cutaneous infections, however the cases are rare (Chiu, 2016).

Fusarium species are ubiquitous as their habitat is in the soil, air and on plants. The skin lesion can provide a significant clue to diagnose the initial phase of the *Fusarium* infection. Typical skin lesions are characterized by painful red or violaceous nodules, the center of which is often ulcerated and covered by a black eschar. Multiple necrotizing lesions can be noted on the trunk and the extremities. Other disease such as onychomycosis is most commonly due to *F. oxysporum* or *F. solani* (Gupta *et al.,* 1999).

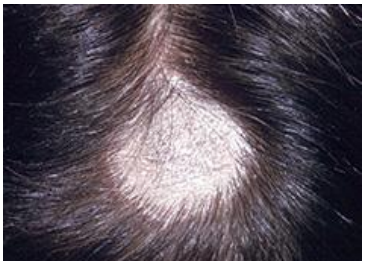
1.4.1 Dermatophyte infection

The dermatophytes are a group of closely related fungi that invade keratinized tissues. The infection is generally cutaneous and restricted to the non-living cornified layers because the fungi cannot penetrate into the deeper tissues or organs of immunocompetent host (Kenneth *et al.*, 2010).

Some fungi are confined to human (anthropophilic), others principally affect animals (zoophilic) but occasionally infect humans. When animal fungi cause human skin lesions their presence often provokes severe inflammatory reactions (eg. cattle ringworm). Dermatophytes grow only in keratin (the stratum corneum of the skin), hair and nails (Figure 1.2). It is common for infection to be acquired by contact with keratin debris that has fungal hyphae (Robin *et al.*, 2015).



(a)



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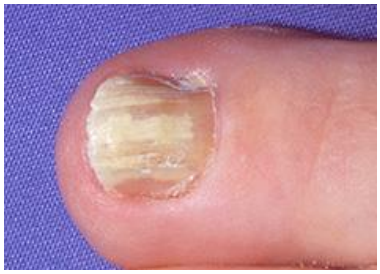

(c)

Figure 1.2 Dermatophytes grow on skin (a), hair (b) and nail (c)

Source: sahealth.sa.gov.au

The important medical dermatophytes are largely separated by their macroconidia morphology and the existence of microconidia. The growth

characteristics of fungal pathogens causing dermatophyte infections, subcutaneous fungi and other superficial fungi, and are shown in Table 1.2.

Table 1.2 The characteristics of fungal growth (Kenneth *et al.*, 2010).

Fungus	**Fungal growth**			
	In lesion	**In culture ($25^{o}C$)**	**Infection site**	**Disease**
Dermatophytes				
Microsporum canis	Septate hyphae	Mold	Hair, skin	Ringworm
Microsporum audouini	Septate hyphae	Mold	Hair	Ringworm
Microsporum gypseum	Septate hyphae	Mold	Hair, skin	Ringworm
Trichophyton tonsurans	Septate hyphae	Mold	Hair, skin, nails	Ringworm
Trichophyton rubrum	Septate hyphae	Mold	Hair, skin, nails	Ringworm
Trichophyton mentagrophytes	Septate hyphae	Mold	Hair, skin	Ringworm
Trichophyton violaceum	Septate hyphae	Mold	Hair, skin, nails	Ringworm
Epidermophyton floccosum	Septate hyphae	Mold	Skin	Ringworm
Other superficial fungi				
Malassezia furfur	Yeast (mycelia)	Yeast	Skin (pink to brown)	Pityriasis (tinea) versicolor
Hortaea werneckii	Septate hyphae, ellipsoidal cells	Yeast (mold)	Skin (brown-black)	Tinea nigra
Trichosporon cutaneum	Septate hyphae	Mold	Hair (white)	White piedra
Piedraia hortae	Septate hyphae	Mold, ascospores	Hair (black)	Black piedra
Subcutaneous fungi				
Sporothrix schenckii	Cigar-shaped yeast (rare)	Mold	Subcutaneous, lymphatic spread	Sporotrichosis
Fonsecaea pedrosi	Muriform body	Mold	Wart-like foot lesions	Chloromoblastomycosis
Phialophora verrucosa	Muriform body	Mold	Wart-like foot lesions	Chloromoblastomycosis

Cladophialophora (Cladosporium) carrionii	Muriform body	Mold	Wart-like foot lesions	Chloromobl astomycosis

Dermatophyte infections can come in the form of unapparent colonization to chronic progressive eruptions that take months or even years, leading to discomfort and disfiguration. Each infection would tend to be given its own "disease" name, for example, tinea capitis (scalp), tinea pedis (feet, athlete's foot), and a few others. The clinical, etiologic, and epidemiologic differences are broad across these syndromes, but the bottom line is that they are all the same disease in varying locations (Kenneth *et al.*, 2010).

1.4.2 Antifungal treatment

There are some topical antifungal agents available for dermatophyte infection treatments, such as miconazole, clotrimazole and terbinafine (Figure 1.3). These treatments are useful when small areas of the skin are affected, but in cases where the fungal infection is extensive it would be wiser to use an oral agent such as griseofulvin or itraconazole (Figure 1.4). When topical agents is not working in scalp ringworm, so treatment with griseofulvin is highly recommended (Robin and Tony, 2005).

For skin and hair infections, griseofulvin should be given for a period of 4 – 6 weeks. In children, the dosage is calculated according to the child's weight (10 mg/kg), while in adults the usual daily dosage is 500 mg (Robin and Tony, 2005). It is also possible to treat skin infections with terbinafine 250 mg daily for 2 – 4 weeks or itraconazole 100 mg on a daily basis for the duration of 15 – 30 days.The treatment of choice for nail infections is oral terbinafine (250 mg/daily) for six weeks

in fingernail infections and three months in toenail infections. An alternative is itraconazole pulse therapy (200 mg twice daily) for fingernails, repeat once after a 3-weeks drug-free interval; for toenails, repeat twice with a 3-week drug-free interval between each course (Robin and Tony, 2005).

Figure 1.3 Various topical antifungal medicine

Figure 1.4 Various oral antifungal medicine

1.4.3 Side effect and resistance of antifungal agents

Relatively only few antimicrobials can be used to treat fungal infections, compared with antibacterial agents. There is proof that many substances with antifungal activity are unstable, poisonous to human, as well as having undesirable pharmacologic characteristics (Kenneth *et al.*, 2010).

Superficial mycoses often require treatment, but topical therapy would be sufficient, thus it restricts toxicity to the host. The rest of the small groups of deep mycoses which are uncontrolled by the host's immune system, require a continuous use of antifungals. Patients with underlying immunosuppression often presented with complicated fungal infections and often require prolonged systemic antifungal therapy (Kenneth *et al.*, 2010).

The resistance mechanisms seen in bacteria are also found in fungi. A major addition is the much greater use of metabolic means such as efflux pumps and changes in synthetic pathways by fungi. The most glaring difference is the complete absence of enzymatic inactivation of antifungals as resistance mechanism (Kenneth *et al.*, 2010). The features of currently used antifungal agents, mechanism of action and resistance of dermatophytes and dimorphic fungi are summarized in Table 1.3.

Table 1.3 Mechanism of action and resistance of antifungal agents.

Agent	Mechanism of action	Mechanism of resistance	Route	Clinical use
Azoles				
Ketoconazole	Ergosterol synthesis (demethylase)	Efflux, demethylase alteration, bypass, overproduction	Oral	*Candida*, dermatophytes, dimorphic fungi
Fluconazole	Ergosterol synthesis (demethylase)	Efflux, demethylase alteration, bypass, overproduction	Oral, intavenous	*Candida*, *Cryptococcus*, dimorphic fungi
Itraconazole	Ergosterol synthesis (demethylase)	Efflux, demethylase alteration, bypass, overproduction	Oral, intavenous	*Aspergillus*, *Sporothrix*, *Candida*, dimorphic fungi
Clotrimazole	Ergosterol synthesis (demethylase)	Unknown	Topical	*Candida*, dermatophytes
Miconazole	Ergosterol synthesis (demethylase)	Unknown	Topical	*Candida*, dermatophytes
Allylamines				
Terbinafine	Ergosterol synthesis (squalene epoxidase)	Efflux	Oral	Dermatophytes, combined with azoles for *Candida*, *Aspergillus*
Naftifine	Ergosterol synthesis (squalene epoxidase)	Unknown	Topical	Dermatophytes
Griseofulvin	Microtubule disruption	Unknown	Oral	Dermatophytes
Tolnaftate	Unknown	Unknown	Oral	Dermatophytes

(Source: Sherris Medical Microbiology, Fifth Edition)

Naturally, some species of fungi are resistant to certain types of antifungal medications. Other species may normally be sensitive to a certain type of medication, but develop resistance over time due to poor antifungal use, for example, too low dosages or short treatment (Shah *et al.*, 2012).

Although most antifungal resistance can be seen in *Candida* species, resistance in other types of mold fungi is also an issue of concern. We have yet to know the full extent of the problem, but the prevalence of azole resistance in *Aspergillus* worldwide is estimated to be roughly 3 to 6 percent (Arendrup, 2014).

Similar to *Candida*, *Aspergillus* infections have been reported to be associated with high mortality, and resistant infections can develop in people who were exposed to certain antifungal medications. Furthermore, some studies recommend that resistance in *Aspergillus* may be partially driven by the use of agricultural azoles, which shelter the crops from fungi (Mortensen *et al.*, 2010).

The dissemination of multidrug-resistant strains of fungus and the smaller amount of drugs available make the discovery of new classes of antifungals and compounds that inhibit these resistant mechanisms, important. This pushes for the pursuit of therapeutic alternatives, particularly among medicinal plants and compounds isolated for their empirically antifungal properties. Among natural sources, some molecules with antifungal activity against different strains of fungus have been discovered, which is very important to the living beings (Vengurlekar *et al.*, 2012).

Other than antifungal resistance, antifungal medicines can also bring about side effects, depending on the type of antifungal medicine adopted. Side effects of topical antifungals medicines, such as creams, include itching, mild burning sensation, and redness onto the skin.

Some side effects can occur from oral antifungals, which examples include; feeling sick, abdominal pain, diarrhoea and indigestion. Other than being mild, the side effects only last for a short period of time.

However some antifungals can also cause serious reactions, such as allergic reaction and severe skin reaction. The worst side effect is not common, but cases of liver damage associated with antifungal therapy have been reported. The signs of liver damage are usually linked with symptoms such as the lack of appetite, vomiting, feeling sick for a period of time, jaundice, dark urine or pale faeces and fatigue (http://www.nhs.uk/Conditions/Antifungal-drugs/Pages/Side-effects.aspx).

1.4.4 Herbal medicine for the treatment of superficial mycoses

Herbal therapy for skin disorders has been adopted for thousands of years, where specific herbs and their uses were developed by region, based on what is available locally and the kinds of trade in ethnobotanical remedies. Most common dermatologic disorders have a lot of available beneficial herbal treatments. Some simple herbal remedies can destroy the fungi that cause the infection, and also reduce the symptoms' intensity (Shenefelt, 2011).

Herbal remedies have steadily gained popularity among patients and physicians. In Asia, especially in China and India, at the moment, herbal treatments that have been used for centuries are scientifically studied (Shenefelt, 2011).

A lot of metabolites from marine invertebrates, from plants and various other natural sources are said to curb pathogenic fungi. These compounds depict many structural classes ranging from cinnamodial sterols to terpenoids in natural origin category (Vengurlekar *et al.*, 2012).

Garlic (*Allium sativum*) has been shown to have antifungal activity (Ledezma *et al.*, 1996). Tea tree oil is applied topically to treat bacterial and fungal infections. Tea tree oil has shown *in vitro* activity against an array of microorganisms,

including *Propionibacterium acnes, Staphylococcus aureus* and *Trichophyton rubrum* (Williams *et al.*, 1988). Tea tree oil may thus play a role in the symptomatic treatment of tinea pedis, onychomycosis, and other superficial wounds. However, it must be stated that it should not be used on burns because it has a cytolytic effect on epithelial cells and fibroblasts (Faoagali *et al.*, 1997).

Thyme oil from thyme (*Thymus vulgaris*) has been used topically both as an antibacterial and an anticandidal agent (van Wyk *et al.*, 2004). Besides that, the traditional Korean antifungal herb *Galla rhois* methanol extract was active against *C. albicans* (Seong, 2007).

Fructus psoraleae (Buguzhi) and *Folium eucalypti globuli* (gum tree) have long served as Chinese medicines to solve the issue of dermatomycosis. Both plants inhibit the *in vitro* growth of *T. mentagrophytes* and *T. rubrum* effectively (Lau *et al.*, 2010).

Ponnusamy *et al.* (2010) studied the medicinal plant *Wrightia tinctoria* (Pala indigo plant) activity against dermatophytic and non-dermatophytic fungi. Its leaf chloroform extract showed activity against *T. rubrum, E. floccosum, A. niger* and *Scopulariopsis brevicaulis*.

The study of the turmeric (*Curcuma longa*) methanol extract established that there is an antifungal activity against *Cryptococcus neoformans* and *C. albicans* (Ungphaiboon *et al.*, 2005). The study of hexane extract of *C. longa* pointed to the antifungal effect against *Rhizoctonia solani, Phytophthora infestans,* and *Erysiphe graminis*. It was also illustrated that ethyl acetate extract of *C. longa* showed an inhibitory effect against *R. solani, P. infestans, Puccinia recondita,* and *Botrytis cinerea* (Kim *et al.*, 2003).

Fusarium solani and *Helminthosporium oryzae* are two phytophagous fungi which were inhibited by exposure to curcumin and turmeric oil. Some dermatophytes were also inhibited when they were tested using crude methanol extract of *C. longa*. It was demonstrated that distilled oil from *C. longa* rhizome showed the best antifungal effect against *T. rubrum, T. mentagrophytes, E. floccosum,* and *M. gypseum* (Wuthi-udomlert *et al.,* 2005). An animal study on infected guinea pigs with *T. rubrum* has provided evidence that the dermal application of turmeric oil led to an improved healing of the lesions (Apisariyakul *et al.*, 1995).

1.5 *Andrographis paniculata* (AP)

One of the herbs used for superficial mycoses treatment is AP. Locally named as *hempedu bumi,* it is widely planted in Southern and Southeastern Asia, where traditionally it was used to treat infections and some other diseases. The leaves and roots have been used for medicinal purposes. The taxonomic hierarchies of AP are shown in Table 1.4.

Table 1.4 Taxonomic hierarchies of AP (adopted from Mishra *et al.*, 2007)

Domain	Eukaryota
Kingdom	Plantae
Subkingdom	Tracheobionta
Superdivision	Spermatophyta
Division	Angiosperma
Class	Dicotyledonae
Subclass	Gamopetalae
Series	Bicarpellatae

Order	Personales
Family	Acanthaceae
Subfamily	Acanthoideae
Tribe	Justiciae
Subtribe	Andrographideae
Genus	*Andrographis*
Species	*A. paniculata* (Burm. f.) Nees

1.5.1 Occurrence, botany and physiology of AP

Andrographis is comprised of 18 species which are commonly found throughout India to China. Belonging to the small tribe *Andrographideae* of the subfamily *Acanthoideae*, AP is characterized by its articulated shoots and epidermis with cystoliths in combination with a 2-lipped corolla with ascending lobes and many ovules per locule. The pollen shows a unique structure as well (Benoy *et al.*, 2012).

Within various populations of AP in India and Bangladesh 9 different cytotypes have been identified. These proved to be related to the environment, particularly to soil conditions (Benoy *et al.*, 2012). Although *Andrographis* is found commonly in India, it is also distributed over tropical Asia including Malaysia (Ricky *et al.*, 2002).

AP is commonly found growing wildly either beside the drain or road, or house lawn. This plant grows better under open sunlight. Besides that, it is mostly grown on sandy soil (Hillary, 2002).

There are several varieties of AP, some can grow to a height of 60-70 cm (Indu and Lean, 2000), while other types can reach 80 cm height (Figure 1.5) (Sharma, 2002). To shed light on the physical appearance of AP, it is generally a slender upright plant that varies in height from 30 to 100 cm (1 to 3 feet), with a square stem and lanceolate leaves. The stems are quadrangular and branches are profuse. The leaves are green, glabrous, decussately arranged with entire margins, measuring 8 x 2-4 cm. The shape of the leaves is lanceolate, acuminate at the apice and attenuate at the base. When it matures, the leaf size is reduced. Most of the leaves also become dark green (Ng and Jaganath, 2002).

The flowers length are borne on terminal and axillary panicles of 2-5 cm. Bracts are about 2.5 cm long, green in colour and glabrous. The flowers are bisexual, about 1.2 cm long, and they have no specific odour and very irregular in shape. They have five sepals with glandular hairs, and partially gamosepalous. The corolla or petals are bilabiate and covered with minute hairs. The color are white or purple, and the length are about 1.1 cm. There are two stamens, notably united at the anthers. The filaments are flat, hairy and they arise from midway down the corolla tube. There is a single central style which shape is filliform and with a terete stigma. This plant carries simple fruits (capsules) which shape is linear-oblong, about 20 mm long and yellowish brown in colour (Figure 1.6) (Indu and Lean, 2000).

Figure 1.5 *Andrographis paniculata* plant (adopted from Sharma *et al.*, 2002)

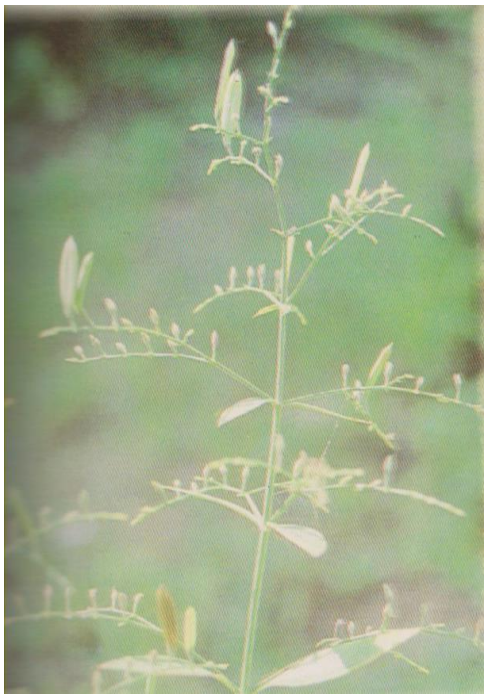

Figure 1.6 *Andrographis paniculata* bears simple fruits (capsules) which are linear-oblong in shape (adopted from Prakash, 2005)

1.5.2 Chemical properties of AP

For so long, AP has been considered as a wonder drug in traditional Ayurvedic systems of medicine as well as in tribal medicine in India and some other countries for a wide range of clinical applications. The therapeutic value of AP can be put down to its mechanism of action probably via enzyme induction. The plant extract exhibits anti-typhoid and anti-fungal activities. AP also has anti-hepatotoxic, antibiotic, anti-malarial, anti-hepatitic, anti-thrombogenic, anti-inflammatory, anti-snakevenom, and anti-pyretic properties to mention a few, besides its use as an immunostimulant agent (Benoy *et al.*, 2012; Shen *et al.*, 2002; Dua *et al.*, 2000).

The plant's juice and the leaves with other herbal combinations has shown medicinal potential in traditional Asian medicines. The plant therapeutic activities are attributed to the presence of diterpene andrographolide (Figure 1.7) and neo-andrographolide (Figure 1.8). Andrographolide was found to be the major chemical of the plant. *In vitro* studies on andrographolide showed cytostatic and cytotoxic activity in neoplastic cells (Siripong *et al.*, 1992; Ma *et al.*, 1997), and anti-inflammatory (Shen *et al.*, 2000) and hepatoprotective activity against galactosamine and paracetamol intoxication (Clander *et al.*, 1995).