



ANTIFUNGAL AND PHYTOCHEMICAL STUDIES OF  
*ACALYPHA INDICA* LINN. EXTRACTS AND ITS SKIN  
SENSITIZATION TEST

BY

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A thesis submitted in fulfilment of the requirement for the  
degree of Master in Pharmaceutical Sciences  
(Pharmaceutical Chemistry)

Kulliyyah of Pharmacy  
International Islamic University Malaysia

AUGUST 2016

## ABSTRACT

*Acalypha indica* is a plant that has been extensively used in the traditional medicine to treat a number of illnesses. It has been used as an expectorant, to treat skin ailments, headaches and asthma. As such, this research sought to investigate the antifungal activity and active phytochemicals of *A. indica* extracts and its skin sensitivity reaction. Phytochemical screening undertaken have shown that the plant contain saponins, flavonoids, steroids, terpenoids, tannins, phenolics and alkaloids. A Kirby-Bauer disc diffusion assay was undertaken for the screening of antifungal activity against *Candida albicans*, *Microsporum gypseum* and *Tricophyton mentagrophytes*. Both chloroform and methanol extracts have shown to inhibit the growth of *C. albicans* but not *T. mentagrophytes* and *M. gypseum*. Chloroform extract produced inhibition zone against *C. albicans* at the concentration of 50 to 200 mg/mL, whereas methanol extract produced inhibition zones at all the concentration used which was 25 to 200 mg/mL. However, the petroleum ether extract did not produce any inhibition zones against all the pathogens tested. In order to obtain the minimum inhibitory concentration (MIC) value, broth microdilution test was undertaken. The result that was achieved produced MIC value against *C. albicans* for both chloroform and methanol extracts at 1.56 and 0.39 mg/mL, respectively. From the result obtained, the most active extract of *A. indica* was continued to the skin sensitivity study. The test was conducted via Organization for Economic Co-operation and Development (OECD) guideline #406 using Buehler method and erythema readings were taken via Dermalab Combo. Guinea pigs in this study were divided into four groups which were negative control, positive control, lower dose and higher dose group. It was observed that during the challenge phase, when compared to the positive control (7.21), the erythema indices of treated groups were lower (4.11 and 4.54) which were closer towards the negative control group (3.46). Both results for week 3 of induction phase and challenge were considered significant according to the p value obtained. Active extracts of *A. indica* were then undertaken for fractionation and isolation via column chromatography and chromatotron. Identification of the compounds was done via the use of gas chromatography-mass spectrometry (GC-MS) and nuclear magnetic resonance (NMR) study. Identified compounds in the *A. indica* extracts are essential oils components such as nonacosane, and pentacosane, as well as ethyl ester (ethyl palmitate, ethyl stearate), squalene, phytol and phytosterols. The major components were the phytosterols which were the combination of three major compounds of campesterol, stigmasterol and sitosterol. The compounds discovered in this extract have been reported to possess antifungal activity against *C. albicans*, thus corroborating the result obtained during the antimicrobial study. Apart from that, the compounds were also found to possess other attributes such as antioxidant and also as an emollient.

## خلاصة البحث

إستخدم نبات ال *Acalypha indica* في الطب التقليدي بكثرة في علاج العديد من الأمراض, حيث استخدم كخلاصات لعلاج الامراض الجلدية وآلام الرأس و الربو. يهدف هذا البحث لدراسة التركيب الكيميائي لنبات ال *Acalypha indica* وخواصه المضادة للفطور ضد عوامل ممرضة محددة وكذلك الحساسية الجلدية له. أظهرت دراسة التركيب الكيميائي وجود مركبات صابونينية وفلافونيدات وستيروئيدات وتيربينويدات وتانينات ومركبات فينولية وقلويدات. أجريت معايرة Kirby-Bauer على القرص لتحري الخواص المضادة للفطور ضد *Tricophyton mentagrophytes*, *Microsporum gypseum* والمبيضات البيض. أوقفت كلا الخلاصات الميتانولية الكلوروفورمية نمو المبيضات البيض ولكنها لم تؤثر على ال *Microsporum gypseum* وال *Tricophyton mentagrophytes*. أظهرت الخلاصة الكلوروفورمية فعالية ضد المبيضات البيض في التراكيز 50-200 ملغ/مل بينما كانت الخلاصة الميتانولية فعالة في التراكيز 25-200 ملغ/مل . وعلى اي حال لم تبد الخلاصة الايتيرية أي فعالية ضد اي نوع من الفطور المدروسة. تم استخدام طريقة تمديد الطور المغذي لدراسة التركيز الادنى المثبط حيث كانت التراكيز 1.65 و 0.39 للخلاصة الكلوروفورمية والميتانولية على الترتيب. تم استخدام الخلاصة الأكثر فعالية لدراسة الحساسية الجلدية طبقا لتوجيهات ال OECD رقم 406 وباستخدام طريقة بوهلر وتمت قراءة الحساسية عن طريق Dermalab Combo. استخدمت خنازير غينيا بحيث قسمت الى اربع مجموعات وهي: مجموعة الكونتروال السلبي والكونترول الايجابي ومجموعة الجرعة الدنيا والجرعة العليا. وقد لوحظ خلال مرحلة التحدي أن الكونتروال الايجابي اعطى قراءة (7.21) بالمقارنة مع المجموعات المعالجة والتي اعطت 4.11 و 4.54 والتي كانت أقرب للكونترول السلبي الذي اعطى 3.46. وكانت النتائج في الاسبوع الثالث من مرحلة الإحداث ومرحلة التحدي ملحوظة احصائيا طبقا لقيمة p. تم عزل مركبات الخلاصة الفعالة باستخدام عمود الكروماتوغرافيا وتم تحديد المركبات باستخدام تقنية الكروماتوغرافيا الغازية مع كاشف مطياف الكتلة GCMS والرنين المغناطيسي النووي NMR. تم تحديد عدد من المركبات في الخلاصة مثل الزيوت العطرية (نوناكوزان و بينتاكوزان) والاستيرات الايتيلية (نخلات الايتيل وشمعات الايتيل) والسكوالين و الفيتول والفيتوستيرولات. شكلت مجموعة الستيرولات المركبات الرئيسية في الخلاصة وكانت عبارة عن ثلاث مركبات رئيسية وهي الكامبيستيرون والسيغماستيرون والسيستوستيرون. تعرف المركبات المحددة في هذه الدراسة بفعاليتها المضادة للفطور المبيضات البيض مما يفسر النتائج التي تم الحصول عليها في هذه الدراسة. وكذلك وجد أن لدى هذه المركبات فعالية أخرى كمضادات أكسدة و عوامل استحلابية ووجود الستيرولات قد يفسر حالة البهجة التي تصيب الحيوانات وخاصة القطط عند تعرضها لهذا النبات .

## APPROVAL PAGE

I certify that I have supervised and read this study and that in my opinion, it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a thesis for the degree of Master of Pharmaceutical Sciences (Pharmaceutical Chemistry).

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## DEDICATION

*To my beloved family who have been there for me every step of the way, may they  
always be blessed by Allah S.W.T*

## ACKNOWLEDGEMENTS

Firstly, I would like to express my utmost gratitude to my main supervisor Dr. Siti Zaiton Mat So'ad for the continuous support given for my Master study and other related research, for her patience, motivation and immense knowledge that was parted during the duration of the study. Her guidance has assisted me in every aspect of the research as well as the writing of the thesis. I could not have hoped for a better supervisor and mentor for my Master study.

Besides my main supervisor, I would like to thank my co-supervisor, Dr. Wan Mohd Azizi Wan Sulaiman for his assistance and knowledge especially in the area of animal study, but also for the patience he had in teaching me in the duration of the study.

I would also like to extend my gratefulness and dedicate this work to my family for supporting me throughout the duration of the study, who have assisted me financially and spiritually in my endeavour to achieve my Master study completion. For all the behaviour that I have exhibited in the period of this study, both bad and good, I would like to express my thanks for all the patience that they have to be able to put up with it.

I would also like to give express my gratitude towards the people who have assisted me throughout the duration of the research such as science officers and lab assistants from the basic medical science and pharmaceutical chemistry department. The support and aid they provided have been greatly appreciated and their aid will not be easily forgotten.

And last but not the least; I would like to thank my friends, especially to Sr. Huda, Sr. Ain, Sr. Hawa, Sr. Fad, Sr. Akilah and my other friends in IIUM Kuantan Campus for all the memories that were created in the years of this study. These people have cheered me up throughout my study and for that; I would like to thank them.

Thank you all very much.



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## LIST OF ABBREVIATIONS

5FC	5-fluorocytosine
5FU	5-fluorouracil
ACD	Allergen Contact Dermatitis
ATCC	American Type Culture Collection
CHCl <sub>3</sub>	Chloroform
DNA	Deoxyribonucleic Acid
ESBLs	Extended Spectrum $\beta$ -lactamases
FeCl <sub>3</sub>	Ferric Chloride
FUMP	5-fluorouridylic acid
GC-MS	Gas Chromatography-Mass Spectrometry
H <sub>2</sub> SO <sub>4</sub>	Sulfuric Acid
HCl	Hydrochloric Acid
IACUC	Institutional Animal Care and Use Committee
ICD	Irritant Contact Dermatitis
ICU	Intensive Care Unit
MeOH	Methanol
MIC	Minimum Inhibitory Concentration
MS	Mass Spectrometry
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NaOH	Sodium Hydroxide
NMR	Nuclear Magnetic Resonance
OD	Optical Density
OECD	The Organization for Economic Co-operation and Development
R <sub>f</sub>	Retention factor
R <sub>t</sub>	Retention time
RNA	Ribonucleic Acid
SDA	Sabouraud Dextrose Agar
TLC	Thin Layer Chromatography
TMS	Tetramethylsilane
UV	Ultraviolet

## LIST OF SYMBOLS

$\beta$	Beta
[M+H]	molecular ions
$\mu\text{g}$	microgram
$\mu\text{L}$	microliter
$^{13}\text{C}$	Carbon NMR
$^1\text{H}$	Proton NMR
CFU	colony-forming unit
cm	centimetre
d (NMR)	doublet
g	gram
hr	hour
Hz	hertz
$J$	coupling constant
m (NMR)	multiplet
m/z	mass-to-charge ratio
mg	milligram
min	minute
mL	millilitre
mm	millimetre
ng	nanogram
nm	nanometer
$^{\circ}\text{C}$	degree celcius
$p$ value	Probability value
psi	pound per square inch
s (NMR)	singlet
sec	Second
t (NMR)	triplet
w/v	weight/volume
$\gamma$	Gamma



# **CHAPTER ONE**

## **INTRODUCTION**

### **1.1 NATURE'S ROLES IN THE WORLD OF SCIENCE**

Plants have always been considered as an important source for the discovery of novel pharmaceuticals with many vital drugs being directly or indirectly derived from plants throughout the history of human kind. The application of plants in treating diseases is referred as herbal remedies. It is a part of a larger area of medicine known as traditional medicine which has been developed before the emergence of modern medicine by various societies in order to cure a range of sickness.

It has also allowed many advance researches and drug designs for treatment of diseases. The derivation of plants to produce drugs is basically bound to a principle which describes the potential of extracting phytochemicals, isolating their active components and producing powerful drugs in standard form. Thus, to design drugs from medicinal plants of interest, it is crucial to research certain aspects of the plant itself. Researching herbal plants allow the discovery of old and new compounds that are applicable in treating diseases. This would also assist in development of new drugs to combat the increasingly resistant pathogens that are currently circulating throughout the world (Palombo, 2011)

This area of study is called pharmacognosy which can be define as “the study of the physical, chemical, biochemical and biological properties of drugs, drug substances or potential drugs or drug substances of natural origin as well as the search for new drugs from natural sources” (American Society of Pharmacognosy, 2012). Thus, research regarding up and coming natural product could become the precursor to the development of more available and affordable drugs. This area of study also

estimated that the drugs that were developed produce less adverse reactions than the conventional drug that are currently in the market.

## **1.2 FAMILY: EUPHORBIACEAE**

Amongst the world of flora, the Euphorbiaceae family is one of the most well known to have considerable economic importance. The Euphorbiaceae are usually encompassed of plants that are mostly monoecious herbs, shrubs, and trees, sometimes succulent and cactus-like. It is comprised of one of the largest families of plants with about 300 genera and 7,500 species. It can be further characterized by the frequent occurrence of milky sap which is a characteristic of the genus *Euphorbioideae* and *Crotonoideae* (Rahman and Akter, 2013). The leaves of this family are mostly alternate but may be opposite or whorled and they are simple, or compound, or sometimes highly reduced. The stipules are generally present but may be reduced to hairs, glands or spines. The flowers are unisexual and usually actinomorphic.

This family occurs mainly in the tropics, with the majority of the species are usually found in the Indo-Malayan region and followed by the tropical region of America. A large variety of this family also occurred in tropical Africa, but compared to the other two tropical regions, the variance are not as much. Nevertheless, the Euphorbiaceae can also be found in non-tropical areas such as the Mediterranean Basin, the Middle East, South Africa, and southern USA (Rahman and Akter, 2013). The plants from this family are considered economically important such as castor oil plant, *Ricinus communis*. While in medicine, some species have been proven effective against genital herpes.

It is reported that plants from this family have been traditionally used to treat cancers, tumors and warts and the application have been referenced in a number of literatures. Several studies were also conducted to validate the traditional usage of such plants. As an example, *Euphorbia heterophylla* leaf extracts was investigated for its potential wound healing properties (Omale and Emmanuel, 2010). Another example would be *Excoecaria agallocha* L. stems extract was investigated for its anti-hyperglycemic activity (Rahman et. al, 2010).

Apart from that, one of the genera that came from the said family is the *Acalypha* genus which has shown a great potential in the world of scientific advancement due to its promising biological activities. The genus *Acalypha* is composed of a great group of tropical or subtropical trees with few representatives in temperate zones and contained around 450 to 500 species of herbs and shrubs. Plants of this genus have been widely reported to have been used as traditional medicine in various ways such as to treat hypertension (Johnkennedy, Adamma and Nnedimma, 2011) hypercholesterolemia, naso-pharyngeal infection and headaches (Udobang, Nwafor and Okokon, 2010). Most common use of this genus is to treat dermatological and gastrointestinal disorders (Yusha'u, Olonntila and Aliyu, 2008).

### **1.3 PATHOGENIC FUNGI**

Fungi has not been recognized as an important pathogens to the community as it does not cause an outbreak or pandemic as well as the annual death rate that could be attributed to candidiasis has always been steady from 1950 to 1970. However, since 1970, there has been an increase in prevalence of severe systemic fungal infections due to the rise in numbers of immunocompromised patients (Ghannoum and Rice, 1999). Thus, it is to utmost importance to study alternatives for antifungal medicines

as there are always possibilities of drug resistance in pathogenic fungi and negative side effects that may come with the current antifungal medicines.

The dermatophyte is a group of closely related fungi that have the ability to cause infection of the skin in animal and human. The dermatophytoses are classified in three anamorphic genera, *Epidermophyton*, *Microsporum*, and *Trichophyton*. They invade keratinized tissue of humans and other animals thus producing an infection, dermatophytosis, commonly referred to as ringworm (Weitzman and Summerbell, 1995).

#### **1.4 SKIN SENSITIVITY**

Contact dermatitis is a term used to identify an eczematous reaction due to interaction of an external substance with the skin. These reactions can be either irritant or allergic in nature. Irritant contact dermatitis (ICD) can affect anyone when certain conditions are met such as the irritant agents are sufficiently concentrated and there was prolonged exposure to the skin. Whereas, allergen contact dermatitis (ACD) is a delayed hypersensitivity allergic reaction and have the ability to recur every time the allergen is encountered (Saint-Mezard et. al, 2003)

The skin sensitization term is also known as ACD, is an immunologically mediated cutaneous reaction to a substance. Once an individual has become sensitized, the probability for dermatitis to recur is high if the patient is exposed towards the same allergen. As the research will be focusing and in accordance to findings against *C. albicans*, the detection of potential human skin sensitizers of the active extracts are crucial in order to identify the toxicity of extract which is relevant to public health (The Organization for Economic Co-operation and Development, 1992).

## **1.5 SIGNIFICANCE OF STUDY**

The world of herbal medicine has been placed in the spotlight of the pharmaceutical world years ago with its promising results in treating diseases where sometimes the world of medicine could not reach. The discoveries of new chemical compounds have led to the emergence of new herbal-based products. Nature itself has provided us with the answers to the diseases whereas the humans try to cultivate nature in order to provide better alternative medicines.

Hence, in this study, an attempt to extract and identify compounds that could be responsible for antifungal activity is conducted. The action in which that is studied in this research in the antimicrobial as this is the initial investigation to identify the importance of this plant. Antimicrobial studies of these herbal plants have led to a number of discoveries that contribute to the pharmaceutical world. While there are an abundance of antimicrobial agents out there, it is important to identify further antimicrobial agents as microbes have been known to develop resistance to antimicrobials over time.

The present research on skin sensitization of the herbal plant may provide us with the basis of the dermal toxicity which could help with the prelude of formulation of compound for various purposes for topical application. As the research is focused onto fungi of skin diseases, the results should help as an important factor for the formulation, so that cases such as dermatitis could be avoided during the treatment of skin diseases.

## **1.6 HYPOTHESIS**

It is hypothesized that the crude extract of *A. indica* leaves has antifungal activity and would not produce skin sensitization at the regulated dose.

## **1.7 RESEARCH OBJECTIVES**

Since there is lack of current studies that focuses on the identification of compounds for antifungal activity and dermal toxicity of the plant extracts, therefore the following objectives are researched:

- 1) To screen crude extracts of *A. indica* against human pathogenic fungi of skin diseases such as *C. albicans*, *T. mentagrophytes* and *M. gypseum*.
- 2) To isolate and identify the compounds available in the active crude extracts of *A. indica*.
- 3) To examine the skin sensitization effects of the extract against guinea pigs.

## CHAPTER TWO

### LITERATURE REVIEW

#### 2.1 NATURE'S GIFT OF HERBAL MEDICINE

The world of flora and fauna is a vast place and the abundance of it has contributed to a number of sectors in the world such as agriculture, as well export and import industries. However, the most important of this review would be the application of this wonder of nature in the world of ethnomedicine, which is also called the traditional medicine.

The precious gifts have assisted human in older times and place where medicine are scarce, to treat various account of diseases recorded. It is a vast area to study in which discoveries may lead to the development of novel compound and innovative drug. It would serve as a reminder however, prior to the invention of drugs; these natures's medicine has provided a number of solutions for treatment of disease.

Ethnomedicine have been practiced all over the world, no matter how big or small a community is. In India, there is an ethnomedicine practice called *Ayurveda*. Their remedies and recipes are very specific. The application of a treatment for arthritis called *kizhi*, in which gauze-wrapped rice powder soaked with herbal oil were applied transdermal and massaged aggressively onto the patient's body, is an example of Ayurvedic medicine (Swerdlow, 2000).

While in China, their ethnomedicine is highly focused on the use of herbs to treat diseases. Thin shavings of *hou po*, or magnolia bark, in which when grounded will become reddish powder is popularly utilized to treat gastrointestinal problem such as indigestion, vomiting and diarrhoea.

### 2.1.1 *Acalypha* sp.

In the Euphorbiaceae family, the *Acalypha* is fourth largest genus, which is composed of a great group of tropical or subtropical trees with few representatives in temperate zones and contained around 460 to 570 species of herbs and shrubs (Schmelzer, 2007(a); Ikewuchi, Onyeike, Uwakwe and Ikewuchi, 2011). This genus is commonly found from the tropical to subtropical regions mainly in the tropics of Africa, America and Asia (Seebaluck, Gurib-Fakim and Mahomoodally, 2015). As an example, *Acalypha dictyoneura* can be found in Ecuador and its habitat is subtropical or tropical moist montanes (Sanz and Rodríguez, 2012).

Plants of this genus are more commonly known as copperleaves or three-seeded mercuries. The leaves of *Acalypha* are succulent with sappy stalks which tend to lose sappiness as it aged. They are alternate, stipulate and identified with serrated edges, obvious mid-ribs and veins (Soladoye, Sonibare and Rosanwo, 2008). Most of the *Acalypha* species were used as medicinal plants in West and East Africa, prevalently in Nigeria (Emeka, Badger-Emeka and Fateru, 2012). Every part of the plant including the leaf, stem and roots are used in making mixtures and decoctions to treat various ailments.

*Acalypha communis* and *Acalypha ornata* are examples of this genus that were used as folk medicine to treat diuretic, anthelmintic as well as for respiratory problem such as bronchitis, asthma and pneumonia (Emeka et al., 2012). Other type of plant from this genus such as *Acalypha wilkesiana* were utilized as local therapies due to its antioxidant, antiepileptic, possible analgesic and anti-inflammatory activities.