# IN VITRO ANTIBACTERIAL, ANTIFUNGAL AND SKIN CYTOTOXIC ACTIVITIES OF *ALPINIA JAVANICA*, *A. GALANGA* AND *A. CONCHIGERA*

BY

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# ABSTRACT

The genus *Alpinia* belongs to the family of Zingiberaceae. It is distributed in tropical Africa, Asia, and particularly in Southeast Asia. The study was conducted to evaluate the antimicrobial and skin cytotoxic activities of *Alpinia javanica, Alpinia galanga* and *Alpinia conchigera* extracts. The crude extracts of *A. javanica, A. galanga* and *A. conchigera* were evaluated for their antimicrobial activity against human dermatophytes, gram positive and gram negative bacteria, and multidrug/methicillin resistant *Staphylococcus aureus* (MRSA) by using Disk Diffusion assay. Minimum inhibitory concentrations (MICs), minimum bactericidal and fungicidal concentrations (MBC and MFC) of *A. javanica, A. galanga* and *A. conchigera* crude extracts were determined based on a broth micro-dilution method in 96-microwell plates. The acetoxychavicol acetate (ACA), which is the major compound in *Alpinia*, was analysed and identified by using thin layer chromatography (TLC) and high performance liquid chromatography (HPLC). The toxic or hazardous effects of *Alpinia* extracts on normal mouse fibroblast (NIH/3T3) and normal human epidermal keratinocytes - adult (NHEK-a) cell lines were assessed through MTT assay. Overall, all extracts of *A. javanica, A. galanga* and *A. conchigera* showed good antibacterial and antifungal activities. However, hexane extract from *A. javanica* rhizome demonstrated strongest antibacterial activity with inhibition zone of 26 ± 0 mm against *Staphylococcus aureus*. In general, rhizome extracts of *A. galanga* showed strongest antibacterial effect as compared to leaf extracts. Overall, all *Alpinia* extracts showed good antibacterial activities against multidrug/methicillin-resistant *Staphylococcus aureus* (MRSA), however hexane extract from rhizome of *A. javanica* demonstrated strongest antibacterial activity against all strains of MRSA, followed by dichloromethane (DCM) and methanol extracts. The antibacterial activity of hexane extract was 29 ± 0.60 mm against MRSA. Similarly, *A. javanica* rhizome extracts demonstrated strongest antifungal activity against *Microsporum canis*. Antifungal activities of hexane extracts were 20 ± 0 mm and 21 ± 1.5 mm, against *Trichophyton rubrum* and *M. canis* respectively. All extracts of *A. conchigera* showed significant antifungal activities; however, compared to the *A. javanica* and *A. galanga* extracts, the inhibition is weaker. In MIC, MBC and MFC study, hexane and methanol extracts showed significant inhibitory effects as compared to DCM extract. According to TLC analysis, all hexane and methanol extracts of *A. javanica*, *A. galanga* and *A. conchigera* rhizome showed the presence of ACA at the $R_f$ value of 0.61. HPLC analysis also confirmed the presence of ACA compound in all rhizomes extracts (except DCM) of *A. javanica*, *A. galanga* and *A. conchigera*. HPLC fingerprint showed identical ACA peak at retention time of 7.66 min (standard) and 7.66 min for hexane and methanol extracts of *A. javanica, A. galanga* and *A. conchigera* rhizome extracts. The present study showed that all extracts of *A. javanica, A. galanga* and *A. conchigera* were not toxic towards both NIH/3T3 and NHEK-a cell lines. In conclusion, the findings demonstrated that *Alpinia* could be a cheaper and feasible source to fight against pathogens that causes fungal skin infections such as tinea and MRSA infections but further research should be carried out on the isolation of specific bioactive compounds and their characterizations.

# خلاصة البحث

ينتمي جنس ال *Alpinia* لعائلة الزنجباريات ويوجد في أفريقيا وآسيا وبشكل خاص في جنوب شرقي آسيا. أجريت هذه الدراسة لتقييم فعالية خلاصة كل من *Alpinia javanica, Alpinia galanga, Alpinia conchigera* كمضادة للبكتيريا والسمية الجلدية. لقد تم تقييم فعالية الخلاصات ضدبكتيريا موجبة وسالبة الغرام وو ضد المكورات العنقودية الذهبية المقاومة للميثيسلين باستخدام المعايرة على طبق بيتري. وقد تم تحديد التراكيز الدنيا المثبطةو التراكيز الدنيا القاتلة للبكتيريا والفطريات باستخدام تقنية تمديد الوسط المغذي على طبق ب 96 خلية. وقد تم تحديد مركب الاسيتوكسيكايكول اسيتات وهو المركب الرئيسي في خلاصة ال*Alpinia* باستخدام تقنيتي الاستشراب اللوني على الطبقة الرقيقة والاستشراب اللوني السائل عالي الانجاز. درست سمية الخلاصات على خطين خلويين وهماخلايا الفئران الليفية الطبيعية (NIH/3T3) والخلايا الجلدية المولدة للكيراتين البشرية (NHEK-a)باستخدام معايرة ال MTT. بالنتيجة أظهرت كل الخلاصات فعالية مضدة للبكتيريا والفطور وكانت خلاصة *A. conchigera*الهكسانية الاقوى من حيث الفعالية المضادة للمكورات العنقودية الذهبية بمساحة تثبيط بلغت 26 ملم.و كانت فعالية خلاصة جذور ال *A. galanga*المضادة للبكتيريا أقوى من خلاصة الاوراق. وكذلك أظهرت كل خلاصات ال*Alpinia* فعالية جيدة مضادة ضد المكورات العنقودية الذهبية و كانت خلاصة جذور ال *A. javanica*الهكسانية هي الاقوى من حيث الفعالية المضادة للبكتيريا ضد جميع سلالات ال MRSA متبوعة بخلاصة ثنائي كلورو الميتان DCM والخلاصة الميتانولية حيث ابدت الاولى فعالية تقدر ب 0.60 ± 29 ملم. و كذلك ابدت خلاصة جذور ال *A. javanica* فعالية قوية ضد البويغاء الكلبية بالمقارنة مع النستاتين حيث كانت الفعالية المضادة للفطور للخلاصة الهكسانية 0 ± 20 ملم و 1.5 ± 21 ملم ضد البويغاء الكلبية والشعروية الحمراء على الترتيب. أبدت جميع خلاصات ال *A. conchigera* *فعالية مضادة للفطور ولكنها اقل من خلاصات* ال *A. javanica*و*A. galanga*.أما بالنسبة للتراكيز الدنيا القاتلة والمثبطة للجراثيم والفطور فقد ابدت الخلاصات الهكسانية والميتانولية فعالية ملحوظة بالمقارنة مع خلاصة ال DCM. وقد ظهر مركب ال ACA في جميع الخلاصات بعد فحصها بتقنية TLC بعامل احتفاظ قدره 0.61. وقد تم تأكيد هذه النتيجة بتقنية HPLC حيث وجد مركب ال ACA في جميع الخلاصات عدا خلاصة ال DCM. أظهرت الدراسة HPLC مركب ال ACA في وقت احتفاظ يقدر ب 7.66 دقيقة بالنسبة للمركب المعياري والخلاصات الهكسانية والميتانولية. أظهرت الدراسة الحالية أن كل خلاصات ال *A. javanica*و ال *A. galanga* و *A. conchigera* ليس لها أي سمية تجاه الخطين الخلويين NIH/3T3 و NHEK-a. في الخلاصة: إن نبات*Alpinia* هو مرشح محتمل للقضاء على العوامل الممرضة المسببة للالتهابات الجلدية مثل القوباء الحلقية وال MRSA ولكن لازلنا بحاجة للكثير من الابحاث لعزل وتحديد المركبات الفعالة حيويا.

# 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 Science (Pharmaceutical Technology)

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# LIST OF SYMBOLS/ABBREVIATIONS

| | |
|---|---|
| °C | Degree Celsius |
| G | Gram |
| H | Hour |
| kg | Kilogram |
| L | Liter |
| mg | Milligram |
| mL | Mililiter |
| mm | Millimeter |
| µg | Microgram |
| µg/ µL | Microgram / microliter |
| µg/ µL | Microgram / microliter |
| µg/mL | Microgram / mililiter |
| µL | Microliter |
| *A. conchigera* | *Alpinia conchigera* |
| *A. galanga* | *Alpinia galanga* |
| *A. javanica* | *Alpinia javanica* |
| ACA | Acetoxychavicol acetate |
| ATCC | American Type Culture Collection |
| *C. albicans* | *Candida albicans* |
| DCM | Dichloromethane |
| DMSO | Dimethyl sulfoxide |
| *E. coli* | *Escherichia coli* |
| HPLC | High Performance Liquid Chromatography |
| *M. canis* | *Microsporum canis* |
| MBC | Minimum bactericidal concentration |
| MeOH | Methanol |
| MFC | Minimum fungicidal concentration |
| MHA | Mueller Hinton agar |
| MHB | Mueller Hinton broth |
| MIC | Minimum Inhibitory Concentration |
| MRSA | Multidrug/Methicillin Resistant *Staphylococcus aureus* |
| MRSA-G | Gentamicin / Methicillin Resistant *Staphylococcus aureus* |
| MRSA –V | Reduced Vancomycin susceptibility/Methicillin Resistant *Staphylococcus aureus* |
| MTT | Methyl tetrazolium |
| NAM | Nutrient agar media |
| NBM | Nutrient broth media |
| NHEK-a | Normal Human Epidermal Keratinocytes – adult |
| *P. aeruginosa* | *Pseudomonas aeruginosa* |
| PBS | Phosphate buffered saline |

| | |
|---|---|
| $R_f$ | Retention factor |
| Rt | Retention time |
| *S. aureus* | *Staphylococcus aureus* |
| *T. rubrum* | *Trichophyton rubrum* |
| TLC | Thin layer chromatography |
| v/v | Volume per volume |

# CHAPTER ONE

# INTRODUCTION

## 1.1 ROLE OF MEDICINAL PLANTS

Herbal medicines represent one of the most important fields of traditional medicine throughout the world. It plays a significant role in the management of medical illness, such as digitalis, which contains cardiac glycosides and cardiac glycoside is commonly used for the treatment of heart failure. Modern physicians are still prescribing many drugs of botanical origins. Local communities in the world are using about 10% of medicinal plants for the treatment of various infections, out of which only 1% have been recognized by scientists. Different types of medicinal plants are used for treatment of infectious diseases due to its less side effects and low toxicity (Robert et al., 2008).

The genus *Alpinia* belongs to the family Zingiberaceae distributed in the tropical Africa and particularly in Southeast Asia. There are more than 1300 species of Zingiberaceae, many species are used as medicinal plants (Lin-Chun et al., 2011). *Alpinia conchigera* is used in Thai and Malaysian folk medicine to treats various types of diseases. The rhizomes of *Alpinia conchigera* and ginger (*Zingiber officinale)* have been extensively used as condiment for flavoring and in local medicines for the stomachache, carminative and treating diarrhea.

*Alpinia conchigera*, which is locally known as "Lengkuas ranting", is native to Peninsular Malaysia. In Malaysian folk medicine, various species of *Alpinia* are used by Malays to treat fungal infections and rashes (Ibrahim et al., 2000). Some research showed that *A. conchigera* has important cytotoxic activities (Lee et al., 2005).

## 1.2 PROBLEM STATEMENTS

In recent years, various reports have been published concerning the composition and biological properties such as antimicrobial, anticancer and antioxidant of Zingiberaceae extracts. Even though a number of antimicrobials have been isolated and studied, and pharmaceutical industries have produced a number of new antimicrobial drugs in the last few years, but resistance to these drugs by microorganisms has increased. So, there is a growing need for the discovery of new antibacterial and antifungal from medicinal plants as there is an ever increasing crisis of bacterial resistance towards the existing drugs (Antonio et al., 2014). Plants and their constituents (crude extract and essential oils) are a rich source of biologically active compounds. In light of these facts, the problem of antibiotics resistant can be minimized as *Alpinia* species may potentially offer a novel natural product to fight against the resistant strains which indirectly combat the widespread of infectious diseases caused by pathogenic microorganisms.

## 1.3 SIGNIFICANCE OF STUDY

*Alpinia* belongs to the family Zingiberaceae, which is distributed in the tropical Africa, Asia and particularly in Southeast Asia. *A. conchigera* and *A. galanga* are used in Thai and Malaysian folk medicine to treats various types of diseases. Previous study showed that the extract from *A. galanga* has antimicrobial activity against many microorganisms such as *S. typhimurium*, *S. aureus* and *E. coli*. It was reported that diethyl ether extract of *A. galanga* has antifungal activities and was active against *Trichophyton* species (Rao et al., 2010). *A. conchigera* also have good antimicrobial and anticancer activities. Ito et al., (2005) reported that ACA obtained from *Alpinia* species has very good antioxidant and

anti-inflammatory activities. Previous studies on the antimicrobial activities of *A. galanga* and *A. conchigera* showed inhibitory activity against a wide spectrum of microorganisms including MRSA. But, this is the first antimicrobial study on the Malaysian indigenous species of *A. javanica*. The present study was conducted to ascertain that the crude extracts from rhizome and leaf of the selected *Alpinia* species can act against those pathogens that cause fungal skin infections such as tinea and MRSA infections.

## 1.4 RESEARCH OBJECTIVES

The present study was undertaken to scientifically investigate the bioactivity of hexane, dichloromethane (DCM), and methanol extracts of *Alpinia javanica, A. galanga* and *A. conchigera*. The specific objectives of the present study are as follows:

1. To determine antibacterial activities of crude extract of *A. javanica, A. galanga* and *A. conchigera* against selected gram positive and gram negative bacteria.
2. To determine antibacterial activities of crude extracts of the selected *Alpinia* species against Multidrug/Methicillin-resistant *Staphylococcus aureus* (MRSA).
3. To determine antifungal activities of crude extracts of the selected *Alpinia* species against human dermatophytes and *Candida albicans.*
4. To analyse the presence of acetoxychavicol acetate (ACA) in the crude extracts of selected *Alpinia* species by the use of Thin-layer

chromatography (TLC) and High Performance Liquid Chromatography (HPLC).

5. To investigate the skin cytotoxic properties of crude extracts of the selected *Alpinia* species against normal mouse fibroblast (NIH 3T3) and normal human epidermal keratinocytes - adult (NHEK-a) cells.

# CHAPTER TWO

# LITERATURE REVIEW

## 2.1 HERBAL MEDICINE

Medicinal plants are the most exclusive source of life saving drugs for the majority of the world's population. Bioactive compounds currently extracted from plants are used as antimicrobials, anticancer, food additives, insecticides, cosmetics, perfumes, fine chemicals and for the treatment of various diseases (Khan and Khan, 2007).

Malaysia is a tropical country that is rich in natural products, and the herbs have been used in the local folk medicine to treat various types of illnesses (e.g. pain and inflammation). The traditional medicinal herbs are still used by most Malaysians extensively as an alternative to the modern medicine. Different types of medicinal plants are used for treatment of infectious diseases due to its less side effects and low toxicity (Gamboe et al., 2008).

The history of herbal medicine is too old, in many countries of the world, particularly in Asian and African countries more than 80% percent of population relies on traditional medicine for the treatment of various diseases. In modern medicine, herbal medicine is recognize as a form of alternative medicine and still widely practiced today. Many of the pharmaceuticals currently available to physicians have a long history of use as herbal remedies, including opium, aspirin, digitalis, and quinine. According to the WHO (World Health Organization) only in United States of America, more than 25% of modern drugs used by community have been derived from various plants (Baydoun et al.,

2015). Bioactive compounds extracted from the plants are widely used in modern medicine for the treatment of various diseases. In the modern pharmacopoeia more than 7,000 compounds are derived from various plants. Much of today's modern medicine though is previously based on plants that had been long used in traditional medicine (Cravotto et al., 2010).

## 2.2 ZINGIBERACEAE

Zingiberaceae is a family of flowering plants consisting of aromatic perennial herbs with creeping horizontal or tuberous rhizomes, comprising about 52 genera distributed throughout the tropical Africa, Asia, America, and particularly in South-East Asia. There are more than 1300 species of Zingiberaceae, many species are used as medicinal plants. The rhizome of Zingiberaceae is usually at the surface or just below the surface of the ground. In some genera it is often more deeply buried (eg. *Achasma*), while in others species such as *Homstedia* and *Geostachhys* stout unbranched stilt-roots, which may be long in some cases, support it above the ground. In many plants of other families, the rhizomes function as resting organs, persisting below ground during unfavourable seasons for growth, while the leafy shoots wither. In South-East Asian countries like Malaysia, Thailand and Indonesia, there is no such unfavourable season is encountered and growth is possible at almost all year. So, the rhizome of Zingiberaceae does not serve as a resting organ in South-East Asian countries (Baydoun et al., 2015).

In general, the leaf of the Zingiberaceae family are thinly membranous or rather fleshy, large ovate or lanceolate, usually petioled shortly, and ligulate, sheating below. The leaf-shoot is generally erect about 1- 5 meter tall with the apex curving over a little

and unbranched. The sizes of the leaf of the Zingiberaceae family vary with different genera, some leaf are quite huge around 1 meter long, however, in many species the length is about a few centimeters long (Kress et al. 2010).

The flowers of Zingiberaceae are bisexual, usually strongly zygomorphic, and often are associated with conspicuous floral bracts in a spike or raceme, ranging from medium to large in size. The flowers are all tubular and contain nectar. The form of the flower is usually constant throughout the Zingiberaceae family so it is quite difficult to divide the family into groups on the basis of floral. However it is the character which separates Zingiberaceae from all other plants. The classification of Zingiberaceae work is carried out by various notable scientists and botanist, such as Roscoe, Holttum and W. John Kress. Figure 2.1 and Figure 2.2 illustrate the classification of Zingiberaceae by W. John Kress.

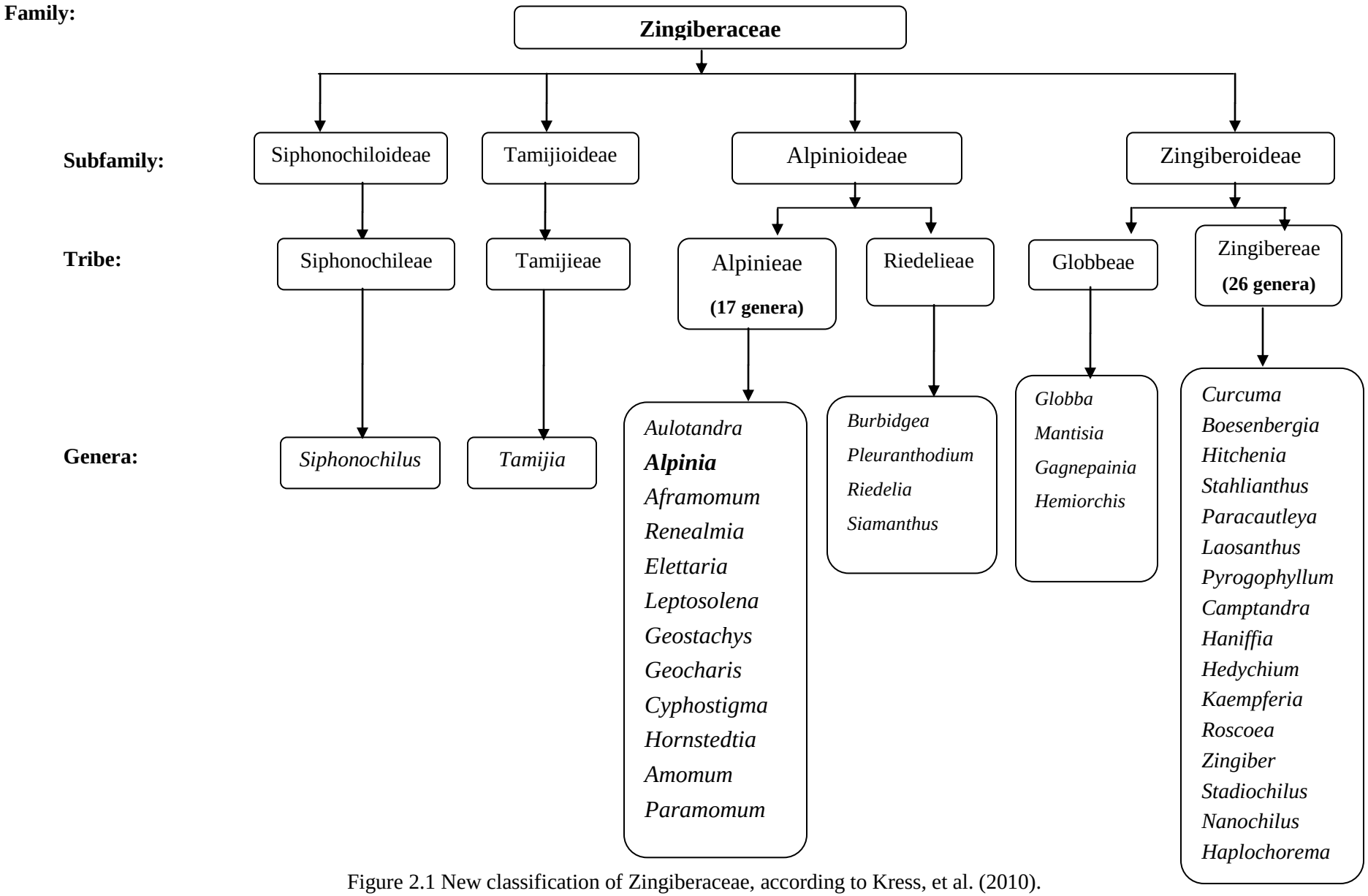


Figure 2.1 New classification of Zingiberaceae, according to Kress, et al. (2010).

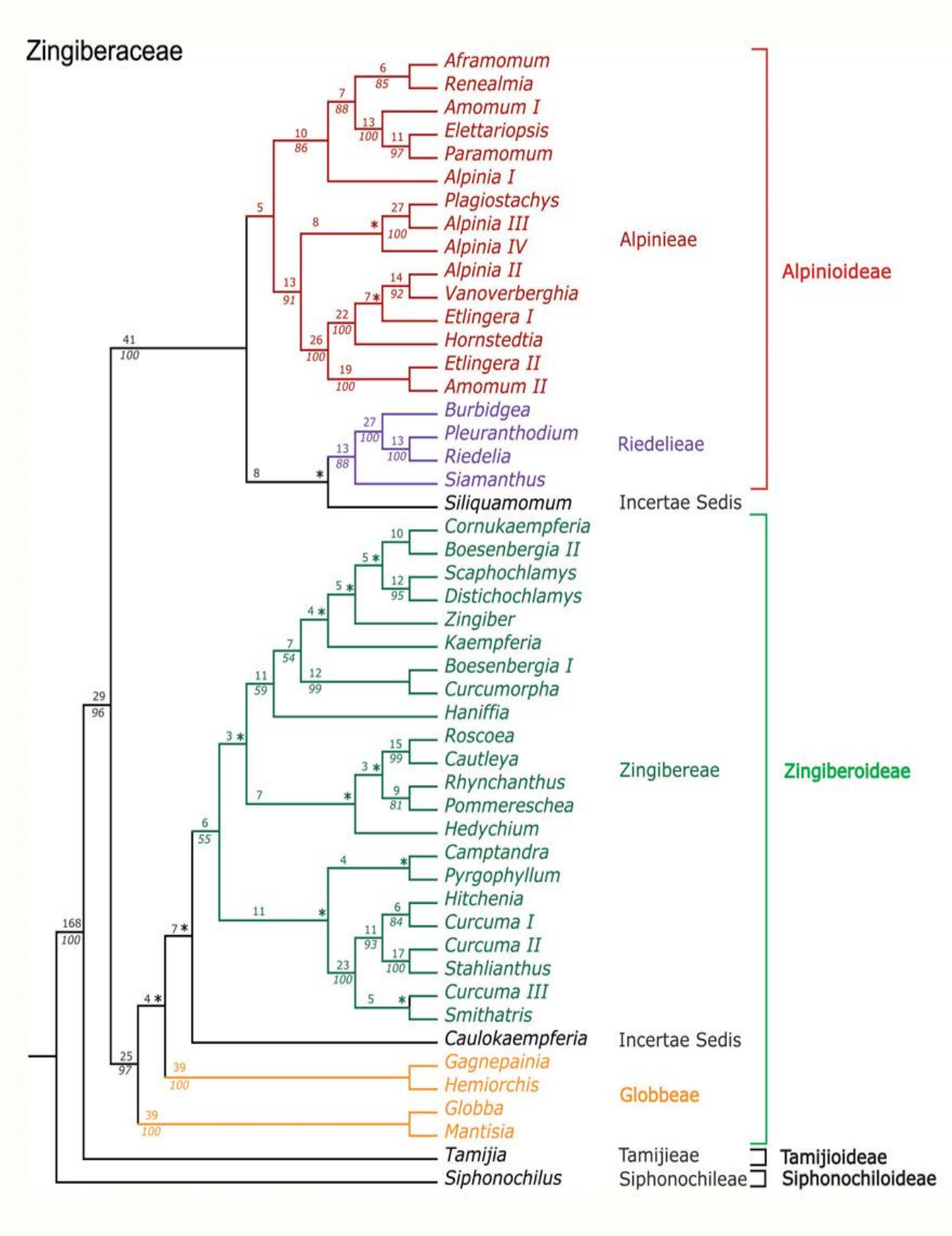


Figure 2.2 Detail classification of Zingiberaceae by John Kress. (Source: PhytoKeys, 2010).