UTILIZATION OF OKINAWA PLANTS FOR PESTICIDAL AND MEDICINAL APPLICABILITIES

殺虫及び医薬への応用を目的とした
沖縄産植物の利用

NGUYEN CAO QUAN BINH

2016

UTILIZATION OF OKINAWA PLANTS FOR PESTICIDAL AND MEDICINAL APPLICABILITIES

A DISSERTATION SUBMITTED TO

THE UNITED GRADUATE SCHOOL OF AGRICULTURE SCIENCES

KAGOSHIMA UNIVERSITY, KAGOSHIMA, JAPAN,

FOR THE REQUIREMENT OF THE DEGREE OF DOCTOR OF PHILOSOPHY

IN AGRICULTURAL SCIENCES

BY

NGUYEN CAO QUAN BINH

MARCH, 2016

ACKNOWLEDGEMENTS

Foremost, at bottom of my heart, I would like to express my deepest gratitude to Prof. Shinkichi Tawata at University of the Ryukyus for giving me the wonderful opportunity to complete my PhD thesis under his supervision, it is truly an honor. I am profound gratitude for his invaluable help and constant encouragement, and moral patience in guiding me through the course of this research. His wealth of knowledge in the field of organic chemistry in particular is inspiring. Thank you for giving me the chances to grow in this field of research. I am also thankful for the excellent example he has provided as a successful researcher and above all, as the most humble human being I have ever met. Without his support and guidance, I would not have achieved this far and this thesis would not have been accomplished.

I owe a debt of great gratitude and the deepest sense of appreciation to my thesis committee, Prof. Ryo Takano (University of the Ryukyus), Assoc. Prof. Masakazu Fukuta (University of the Ryukyus), Prof. Hisanori Tamaki (Kagoshima University), Assoc. Prof. Takeshi Miyata (Kagoshima University), Assoc. Prof. Md. Amzad Hossain (University of the Ryukyus), Prof. Kohzo Kanda (Saga University) for their invaluable suggestions and the constructive comments. I am greatly indebted to Prof. Hiroshi Maruta (PAK Research Center, Okinawa) for providing useful scientific hints, and for his advices and help.

I am very grateful to my supervisors at Cuu Long Delta Rice Research Institute (CLRRI),Viet Nam, Prof. Nguyen Thi Lang, Prof. Bui Chi Buu, Assoc. Prof. Duong Van Chin, Dr. Le Van Banh, and Assoc. Prof. Tran Dang Xuan (Hiroshima University) for things they taught and guided me, and gave me chance to take a leave to study.

I also extend my gratitude to members of the Tawata's laboratory, Mr. Tuneaki Toyama, Mr. Masanao Chinen, Mr. Mondo Mochizuki, Mr. Shota Shiroma, Mr. Ashitomi Hiroto, Mrs. Pham Thi Be Tu, Ms. Hana Oshiro, Ms. Wakana Hishada, Mr. Shunya Taira, Mr. Sentaro Kokubu, Dr. Takahiro Ishii, and many other labmates for good advices and collaboration, and for joyful moments that we had in the laboratory. I would like to express special thanks to Mr. Nozomi Taira and Mr. Takuto Yamashiro for their unlimited support, introducing me beautiful places and nice foods in Okinawa, and for always being ready to lend me their helping hands. Above all, I hope they understand how deeply I cherish these memories.

Special thanks should also be given to the staff of the Agriculture Faculty in Kagoshima University and University of the Ryukyus for their kind help and support during my journey. I also thank students in Vietnamese Student Association in University of the Ryukyus for their useful shares.

Finally, I thank to my brother and sister for their unwavering support and encouragement. I am specially grateful to my lovely wife Tran Thu Dong and son Nguyen Duc Khiem, for being sources of spiritual strength, for their great sacrifices, and companions in all my times of difficulties, happy in my humble achievements. Words and pages are not enough to express my feelings of deep gratitude for the tremendous help that my parents made to ensure that I had an good education. For this and much more, I am forever in their debt. It is to them that I dedicate this dissertation.

Thank you.

NGUYEN CAO QUAN BINH

March, 2016

LIST OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

AChE:	acetylcholinesterase
alpinia:	*Alpinia zerumbet*
ARC:	artepillin C
cAMP:	cyclic adenosine monophosphate
CAPE:	caffeic acid phenethyl ester
CBI:	cucurbitacin I
COX:	cyclooxygenase
CREB:	cAMP response element (CRE) binding protein
DDK:	dihydro-5,6-dehydrokawain
DK:	5,6-dehydrokawain
EtOAc:	ethyl acetate
FDA:	Food and Drug Administration
HFDPC:	human follicle dermal papilla cell
HPLC:	high performance liquid chromatography
IBMX:	3-isobutyl-1-methyl xanthine
IC_{50}:	50% inhibitory concentration
IP:	immunoprecipitation
LC-MS:	liquid chromatography-mass spectrometry
LD_{50}:	50% lethal dose
leucaena:	*Leucaena leucocephala*
LIMK:	LIM kinase

MFFY:	mimosine-phenylalanine-phenylalanine-tyrosine
MFWY:	mimosine-phenylalanine-tryptophan-tyrosine
MFYY:	mimosine-phenylalanine-tyrosine-tyrosine
MITF:	microphthalmia-associated transcription factor
NMR:	nuclear magnetic resonance
NSAIDs:	nonsteroidal anti-inflammatory drugs
PAK:	p21-activated kinase
pilosa:	*Bidens pilosa* L. var. *radiata* Scherff
PKA:	protein kinase A
α-MSH:	α-melanocyte-stimulating hormone

ABSTRACT

Bioactive compounds from plants have been recognized as an important source for lead discovery in medicine as well as in agrochemical in recent years. However, only a few identified components are used directly as active ingredient in crop protection and medicine due to mainly low biological potency. This drawback gives the opportunity and inspiration for design and development of novel compounds with improved bioactivities. Alpinia, leucaena, pilosa, and bitter melon are popular plants in Okinawa, and contain the interesting bioactive constituents such as DK, DDK, mimosine, and cucurbitacin I. These compounds have been known as promising structural units in the field of medicinal and agricultural chemistry. Therefore, in this study, novel biological properties of four above isolated compounds were explored in order to develop more potent derivatives for pesticidal activities, and for PAK1 blocking, anticancer, skin-brightening, and hair loss treatment as well.

Firstly, I found that mimosine phosphoramidothionate derivatives had promising potential effects as insecticide and nematicide. Their activities were 30-100 times more active than those of starting material mimosine. Furthermore, a small library of ten mimosine dipeptides, mimosinol, and deuterated mimosinol (D-mimosinol) were effective melanogenesis inhibitors in B16F10 melanoma cells by suppressing the cellular tyrosinase without undesirable cytotoxicity. Thus, it could be used as potential compounds in skin brightening. In addition, mimosine dipeptides also inhibited strongly cyclooxygenases (COX-1/2) which are major targets for anti-inflammatory drugs. More interestingly, mimosinol and D-mimosinol showed an outstanding activity for hair cell growth promotion; therefore, it would also be useful for hair loss treatment.

Secondly, since PAK1 is essential for a variety of diseases, the search for effective PAK1 inhibitors is at the center of very competitive efforts. I introduced a new method coined “Macaroni-Western” assay, by combination of immunoprecipitation (IP) of PAK1 from cell lysates and in vitro kinase assay based on ATP-dependent luciferin-luciferase system, for the assessment of potent and safe PAK1 blockers in cell culture. This universal system allowed to monitor any change in the kinase activity of PAK1 in cells directly, independent of its auto-phosphorylation sites, without SDS-PAGE. I also displayed new findings that DDK, DK, hispidin, and cucurbitacin I directly inhibited PAK1 in vitro. Three hispidin derivatives inhibited PAK1 at a low micromolar level. Interestingly, mimosine tetrapeptides such as MFFY and MFWY suppressed PAK1 at a nanomolar level, and thus could be used as drug candidates for the treatment of various diseases in the future. In particular, PAK1 is responsible for the growth of cancer, melanogenesis, and hair loss. Based on the results, some of the above PAK1 blockers, in particular cucurbitacin I, could be a good candidate for treatment of these diseases.

In conclusion, this study demonstrated that DK, DDK, mimosine, and cucurbitacin I are potential leads for the development of more potent novel drugs and pesticides. The highlight I found was several herbal compounds from Okinawa plants were effective PAK1 blockers which were good candidates for the treatment of cancer, hair loss, and skin brightening. Moreover, new “Macaroni-Western” PAK1 assay system I developed, make advantages to select only highly cell-permeable PAK1 blockers useful for potential clinical application.

要約

近年、植物由来の生物活性物質は、農薬のみならず医薬開発におけるリード化合物探索のための重要な供給源であると認識されてきている。しかしながら、主に生物活性が低いという理由のために、作物防御や医薬においてほんの少数の同定化合物だけが活性成分として直接利用されているに過ぎない。これらの事柄より、生物活性を改善するための新規化合物のデザイン開発や発想転換の好機がもたらされている。月桃、ギンネム、コセンダングサ、ゴーヤは沖縄ではよく見られる植物であり、DK、DDK、 ミモシン、CBI のような興味深い生物活性成分が含まれている。これらの化合物は、医薬や農薬の分野において有望な化学構造を有するとして知られている。それゆえ、本研究においては、上記 4 種から単離した化合物の新規生物学的特性を調査し、殺虫活性、PAK1 阻害、抗癌性、美白、脱毛処理についても同様に、より強力な誘導体の開発を行うために綿密に探索された。

第一に、私はミモシンのアミドチオリン酸誘導体が、強力な殺虫及び殺線虫活性を持つことを見出した。それらの活性は、出発物質であるミモシンの 30-100 倍であった。さらに、ミモシンのジペプチド、ミモシノール、D-ミモシノールの小ライブラリーが、B16F10 メラノーマ細胞において、不要な細胞毒性を示さない効果的なメラニン形成阻害剤であり、美白剤として有望な化合物になりうることが明らかになった。また、ミモシンジペプチドは、抗炎症剤の主たる標的である、シクロオキシゲナーゼを強く阻害した。さらに興味深いことに、ミモシノールと D-ミモシノールは、毛細胞の成長を著しく促進し、脱毛症に対して有用である可能性が示された。

第二に、PAK1 は癌や脱毛症、糖尿病など様々な病気において重要な側面に関わっていることから、効果的な PAK1 阻害物質の探索が新薬開発において競争的研究の中心と

なってきている。そのためにマカロニウエスタン法と呼ばれる新しい手法が導入された。本方法では、細胞抽出液中の PAK1 の免疫沈降と ATP 依存性ルシフェリン-ルシフェラーゼ系に基づいた試験管内キナーゼアッセイを組み合わせ、培養細胞中での効果的で安全な PAK1 ブロッカーの評価を行う事ができる。この体系により、SDS-PAGE を行わずに、自己リン酸化サイト非依存的に、細胞中の PAK1 のリン酸化酵素活性のあらゆる変化をモニターすることが可能になった。また、DK、DDK、ヒスピジン、CBI が、試験管内において PAK1 を直接阻害するという新しい知見も得られた。3 種類のヒスピジン誘導体が、μM レベルという低い値で PAK1 を阻害することも見出された。興味深いことに、MFFY、MFWY のようなミモシンテトラペプチドは、nM レベルで PAK1 を抑制し、将来的には PAK1 依存性の様々な病気の治療へ向けて、有望な薬の候補となりうると考えられる。特に、PAK1 は癌の増殖、メラニン形成、脱毛に関わっていることから、これらの結果に基づき、上記 PAK1 ブロッカーのいくつか、特に CBI が、これらの病気の治療薬の候補になりうると思われる。

結論としては、本研究において、DK、DDK、ミモシン、CBI が有効な新規医薬や殺虫剤の開発に向けてリード化合物になりうることが示された。今回の発見で最も重要なことは、沖縄の植物由来のいくつかのハーブ化合物は、有効な PAK1 ブロッカーであり、癌、脱毛、美白剤としての良い候補物質になりうることが示されたことである。さらには、将来の臨床応用に向けて有用な細胞透過性 PAK1 ブロッカーのみを選別できるという利点を持つ、マカロニウエスタン PAK1 アッセイ法が開発されたことが研究のハイライトとしてあげられる。

CHAPTER 1

GENERAL INTRODUCTION

INTRODUCTION

The great majority of secondary metabolites do not participate directly in plant growth and development. These substances have been shown to be an important source of novel compounds for development of new drugs and pesticides. However, many biologically active natural compounds have the structural complexity, low biological activity, and contain reactive functionalities that make them to be toxic and useless. The chemical derivation or modification from these natural compounds is the best practical way to explore structure-activity relationships and to produce analogs with improved biological properties. Moreover, the chemical transformations are also possible to introduce new functions associated with certain biological activities. This approach gives chances to make natural-like new compounds which are more selective and desirable.

Alpinia zerumbet (alpinia), *Leucaena leucocephala* (leucaena), *Bidens pilosa* L. var. *radiata* Scherff (pilosa), and *Momordica charantia* (bitter melon) are plants growing widely in Okinawa, Japan. It has been shown to have the interesting biological activities which are associated with their major bioactive constituents such as DK, DDK, mimosine, and cucurbitacin I (CBI). These compounds were chosen to explore as promising structural templates for developing more potent derivatives for pesticidal activities, and for anticancer, skin-brightening, and hair loss treatment. On the other hand, PAK1 is the major oncogenic/aging kinase which is known to be responsible for many distinct diseases such as cancer, HIV-infection, Alzheimer's and Huntington's, inflammation, epilepsy, malaria, and lifespan. Since PAK1 is not essential for the

growth and development of the normal cells, PAK1 blockers would be potentially useful for medicinal treatments in the future.

In this thesis, Chapter 1 provides a general introduction. The main literature review is described in Chapter 2. Chapter 3 reports insecticidal and herbicidal activities of leucaena mimosine, pilosa and alpinia extracts. A series of mimosine derivatives are synthesized and evaluated for insecticidal and nematicidal activities in Chapter 4, and for cyclooxygenase and melanogenesis inhibition in Chapter 5. Chapter 6 describes anti-melanogenic and hair cell growth promoting activities in cell culture of two mimosine amino alcohols. In Chapter 7, a new "Macaroni-Western" system is introduced for the assessment of potent and safe PAK1 inhibitors in cell culture. Chapter 8 reports anti-oncogenic, anti-melanogenic and anti-alopecia activities of several PAK1-blocking compounds. Chapter 9 summarizes significant findings and shows the highlights of the study.

CHAPTER 2

LITERATURE REVIEWS

2.1 Okinawa plants

The Okinawa area is located at the southern region of Japan with more than 100 islands (Kumazawa et al., 2007). Okinawa is the only region in Japan that has a subtropical climate, allowing the growth and cultivation of both tropical and temperate plants. The local Okinawa people have utilized traditionally these plants as medicinal herbs and foods (Takahashi et al., 2012) to cure diseases or to improve health (Taba et al., 2008). Some researchers indicated that the intake of typical Okinawan medicinal and edible plants contributes to the longevity of the local people (Hokawa, 1999; Shinjo and Yamamoto, 1999). With the abundance of antioxidant compounds in nature, Okinawa plants have been shown to protect liver from inflammation and to prevent diabetes complications (Aniya et al., 2002a; Aniya et al., 2002b). Recently, we have shown one Okinawa plant, *Alpinia zerumbet*, could hold promises for the longevity (Upadhyay et al., 2013). Moreover, many of Okinawa plants have also pesticidal activities such as herbicidal, insecticidal, nematicidal and fungicidal activities (Deba et al., 2007, 2008; Taba et al., 2008).

In this study, four Okinawa plants, *Alpinia zerumbet* (alpinia), *Leucaena leucocephala* (leucaena), *Bidens pilosa* L. var. *radiata* Scherff (pilosa), and *Momordica charantia* (bitter melon) were utilized to develop new compounds for various functional properties. Alpinia (Family: Zingiberacae) grows abundantly in the Ryukyu Islands, and has been a traditionally important and economic plant in Okinawa. Alpinia essential oils are used as cosmetics, perfumes, and soaps (Tawata et al., 2008). Pharmacological effects of alpinia have been reported (Zoghbi et al., 1999; Liao et al., 2000; Bezerra et al., 2000; Lahlou et al., 2003; Chompoo et al., 2012a,b; Upadhyay et al., 2011a).

Biochemical analyses have shown that alpinia leaves are rich in flavonoids (quercetin, epicatechin, rutin, kaempferol 3-*O*-rutinoside, catechin, kaempferol 3-*O*-glucuronide) (Mpalantinos et al., 1998), and kava pyrone (dihydro-5,6-dehydrokawain (DDK) and 5,6-dehydrokawain (DK)) (Elzaawely et al., 2007). Leucaena, a leguminous tree, is tolerant to drought, and is grown for soil erosion prevention and soil improvement. With high annual yield and rich palatable protein in foliage, leucaena may help to resolve the shortage of animal feeds in developing countries (Tawata et al., 2008). Moreover, it can serve other many purposes as vitamin K and β-carotene supplements, as fuel wood, as green manure (Lalitha et al., 1993). The large amount of mimosine isolated from leucaena has high value as a raw material for medicine and agricultural chemicals (Tawata et al., 2008). Pilosa is well-known as "hairy beggar ticks", "sticks tights" or "Spanish needles" and is reported to be a weed of 31 crops in > 40 countries (Holm et al., 1991; Taba et al., 2008). The whole plant or its aerial parts are used in various folk medicines and as a popular ingredient in herbal tea for its antiseptic, liver-protective, blood-pressure lowering, and hypoglycemic effects. Topical application of this herbal extract is also used for skin disorders (Wu et al., 2004). Moreover, the essential oil and phytotoxin compounds from pilosa have been shown for herbicidal activity (Deba et al., 2007, 2008). Bitter melon, a climber belonging to family Cucurbitaceae, is one such plant that has been used frequently as medicine (Grover and Yadav, 2004). Okinawan centenarians demonstrate normal cognitive function such as attention span and memory, and delayed onset of dementia, which was associated with various life-style factors including consumption of bitter functional foods (Willcox et al., 2007, 2009). Bitter melon is one of the factors supporting the longevity of Okinawa people. It has also been known to increase appetite, to prevent susceptibility to summer heat (Miyasaki et al.,

2012), and to ameliorate obesity-associated neuroinflammation and stress (Nerurkar et al., 2011). To sum up, this study will utilize four Okinawa plants for developing new compounds for medicinal and pesticidal activities.

2.2 Pesticides

One of the major problems with agriculture is demanding the production of more and more in order to provide food for the population which is in permanent augmentation. In realizing this, one of the stumbling blocks seems to be the yield losses due to pests (Dubey et al., 2008). Modern crop production needs chemical agents for pest control, which are referred to as pesticides (Feng et al., 2010). Due to the ability of pests to rapidly develop resistance and the desire to have compounds with less mammalian and environmental toxicity, the discovery of novel active molecules with ideal properties has been demanded. It is well-known that plant secondary metabolites result from the interaction between plants and the environment during the long period of evolution in plants (Isman et al., 2006). Pesticides produced from these compounds may result in less or slower resistance development and lower pollution (Wang et al., 2013a). The discovery of new pesticidal compounds from plants, and their use as lead structures for further modification have been important approaches for the research and development of new pesticides (Guo et al., 2012).

2.3 Acetylcholinesterase (AChE) as target for neuroactive insecticides

Neuroactive insecticides are the principal means of protecting crops, people, livestock, and pets from pest insect attack and disease transmission. Currently, the four major nerve targets are acetylcholinesterase, the nicotinic acetylcholine receptor, the γ-aminobutyric acid receptor or chloride channel, and the voltage-gated sodium channel (Casida and Durkin, 2013).

Acetylcholine-mediated neurotransmission is fundamental for nervous system function, and its abrupt blockade is lethal (Soreq and Seidman, 2001). Acetylcholinesterase (AChE, EC 3.1.1.7) is found in many types of various tissues including nerve, muscle, central and peripheral tissues, motor and sensory fibers, and cholinergic and noncholinergic fibers. AChE is virtually a ubiquitous enzyme in vertebrates and invertebrates (Koelle, 1954; Chacho and Cerf, 1960; Massoulie et al., 1993). It is localized in areas of central nervous system and in organs and glands that are controlled by the parasympathetic division of the autonomic nervous system (Fukuto, 1990). It regulates the neurotransmitter acetylcholine in mammals, birds, and insects. Mechanistically, AChE is a serine hydrolase. Its catalytic site contains a catalytic triad, serine, histidine, and an acidic residue (Soreq and Seidman, 2001). Moreover, AChE is one of the fastest enzymes (Nair et al., 1994). Due to its critical role in acetylcholine-mediated neurotransmission, AChE is a sensitive target for both natural and synthetic cholinergic toxins. Anti-AChEs compounds such as organophosphate and carbamate are first studied and manufactured as highly poisonous insecticides (Wilson, 1959). Their toxicity to insects and mammals is attributable to inhibition of AChE, which is responsible for the hydrolysis of acetylcholine (Ach) at synaptic regions of cholinergic

nerve endings (Casida, 1956; Eto, 1974; Koelle, 1963). Specificity of insecticides between the AChE of insects and mammals, and between susceptible and tolerant strains of insects contributes to selective toxicity (Casida, 1956; Eto, 1974).

2.4 Cyclooxygenase (COX)

Nonsteroidal anti-inflammatory drugs (NSAIDs) are of huge therapeutic benefit in the treatment of rheumatoid arthritis and various types of inflammatory conditions. The target for these drugs is cyclooxygenase (COX) enzymes (Vane and Botting, 1998; Sanchez and Moreno, 1999; Blobaum and Marnett, 2007). Cyclooxygenase (COX) catalyzes the conversion of arachidonic acid (AA) to prostaglandin H_2 (PGH_2), the common biosynthetic precursor to prostaglandins and thromboxane. These bioactive lipids mediate numerous physiological and pathological effects, including pain, fever, inflammation, homeostasis, regulation of renal function, and maintenance of mucosal integrity in the stomach (Marnett and Kalgutkar, 1999). COX exists in two distinct isoforms, COX-1 and COX-2. COX-1 is known as a housekeeping enzyme that is constitutively expressed in all tissues (Smith et al., 2009). It plays a role in several pathological conditions such as thrombosis, atherosclerosis, and tumorigenesis. Importantly, platelet COX-1 is the target of one of the most efficacious antithrombotic agents used for prevention of vascular occlusive event (Vitale et al., 2013). In contrast, COX-2 is constitutively expressed only in kidney, brain and ovaries. COX-2 is induced by stimuli such as mitogenes and oncogenes, growth factors, hormones. It is also induced by disorders of water-electrolyte homeostasis linking its involvement to pathological processes such as inflammation and various cancer types (Herschman,

1996; Kawamori et al., 1998; Kanaoka et al., 2007). The gastrointestinal side effects associated with NSAIDs are due to the inhibition of gastroprotective PGs synthesized through the COX-1 pathway (Allison et al., 1992; Eberhart and DuBois, 1995). Thus, selective inhibitors of COX-2 over COX-1 are useful for the treatment of inflammation and inflammation-associated disorders with reduced gastrointestinal toxicities. COX-2 plays an important role in multiple diseases by involving in the pathogenesis of inflammation. Recently, it is indicated that COX-2 becomes a potent molecular target for prevention and therapy of oral cancer (Urade, 2008). COX-2 inhibitors also are new therapeutic targets for Alzheimer's disease (Jain et al., 2006).

2.5 Melanogenesis

Skin pigmentation is produced by the dermal melanocytes. Melanogenesis has been defined as a process leading to the formation of the dark macromolecular pigment melanin (Chang, 2009). Melanin is synthesized in the melanosomes of melanocytes, which lie at the stratum basal of the epidermis (Schwahn et al., 2001). Melanin-containing melanosomes are then transferred to keratinocytes, resulting in the distribution of melanin throughout the epidermis (Cardinali et al., 2005). Although melanin plays an important role to protect the skin against damage induced by UV radiation (Gillbro and Olsson, 2011), the excessive accumulation of melanin can cause hyperpigmentation, leading to the aesthetic problems of melasma and age spots (Picardo et al., 1999; Brenner and Hearing, 2008), and can increase the risk of malignant melanoma (Jo et al., 2014).

Melanin synthesis is stimulated by a large number of effectors including ultraviolet-B radiation (Friedman and Gilchrest, 1987), cAMP-elevating agents as forskolin, IBMX, α-MSH, glycyrrhizin (Lee et al., 2005), and the placental total lipid fraction (Singh et al., 2005). To date, two major signaling pathways have been found to induce melanogenesis, including the protein kinase C-mediated pathway and the cAMP-mediated pathway. While the role of protein kinase C in the induction of melanogenesis is still unknown, compelling data have shown that the cAMP-mediated pathway plays a key role in regulation of melanogenesis (Maeda et al., 1997; Lee et al., 2005). Through the activation of PKA and CREB transcription factors, cAMP promotes an increase in the expression of microphthalmia-associated transcription factor (MITF) (Bertolotto et al., 1998), a melanocyte-specific transcription factor, that is essential for the development and differentiation of melanocytes. As a result, MITF binds to and activates tyrosinase, tyrosinase-related protein 1 (TRP-1), and tyrosinase-related protein 2 (TRP-2), leading to the stimulation of melanogenesis (Steingrimsson et al., 1994). Among these enzymes, tyrosinase is a multi-functional, glycosylated, and copper-containing oxidase that catalyzes the hydroxylation of tyrosine to dopa and the subsequent oxidation of dopa to dopa quinine, the two initial rate-limiting steps in melanogenesis (Chang 2009; Gillbro and Olsson, 2011). Despite the fact that several enzymes and melanogenic factors modulate melanin production in either a positive or negative manner, tyrosinase is the primary target protein for depigmentation of skin (Chang, 2009).

2.6 Hair loss

Hair loss (alopecia) is a common and distressing problem that can cause such as nutritional deficiency, aging, hormone imbalance, and stress (Finn et al., 2006; Naito et al., 2008). In particular, the occurrence of androgenetic alopecia has increased to comprise 70% of all alopecia cases (Andl et al., 2002).

Hair follicles are tubular pouches in the skin in which active proliferation of hair epithelial cells rises to hair growth (Muller-Rover et al., 2001). Hair follicles undergo a cyclic process of growth, regression and resting phases (anagen, catagen, and telogen, respectively), and it is composed of both dermal and epidermal compartments (Plikus et al., 2008). The most important element of hair follicles and skin is the dermal papilla. Dermal papilla cells are embedded at the bottom of each hair follicle. Although most hair follicle cell lines are differentiated from the ectoderm, the dermal papilla is differentiated from mesoderm. The most important role of the dermal papilla is the regulation of hair growth. The useful and rapid method for evaluating hair growth promotion is by determining the dermal papilla growth rate as influenced by various samples (Kawano et al., 2009). Mechanisms of hair growth promotion have been suggested by the inhibition of 5α-reductase (Kumar et al., 2012), and the opening of ATP-sensitive K^+-channel, the upregulation of vascular endothelial growth factor and the activation of the β-catenin pathway in dermal papilla cells (Kang et al., 2012). Currently, there are several treatment options for alopecia patients, such as wearing a wig, using oral or topical medicines such as minoxidil and finasteride, or surgical management. Drug treatment provides only temporary relief, and the discontinuation of medication may results in immediate depilation. For hair transplantation, the limitation

comes from the restricted transplantable hair and cost of the treatment. Therefore, the development of new drugs are urgently needed for hair loss treatment (Sawaya and Shapiro, 2000; Dong et al., 2014).

2.7 p21-Activated Kinase 1 (PAK1)

The family of p21-activated protein kinases (PAKs) belongs to Ras-related C_3 botulinum toxin substrate/cell division control protein 42 (RAC/CDC42)-dependent serine/threonine kinases (Dummler et al., 2009). They act as downstream nodes for various oncogenic signalling pathways. PAKs are well-known regulators of cytoskeletal remodeling and cell motility, but have recently also been shown to promote cell proliferation, regulate apoptosis and accelerate mitotic abnormalities, which result in tumour formation and cell invasiveness (Kumar et al., 2006). The PAK isoforms identified in mammalian cells are characterized into group I (PAK1-3) and group II (PAK4-6). The first group PAKs share a high sequence homology which are highly evolutionarily conserved (Jaffer and Chernoff, 2002). PAK family kinases are found in all eukaryotes from yeast to human, but no PAK homologue is present in either bacteria or plants (Maruta, 2014). PAK1 is expressed highly in brain, muscle, and spleen in normal adult animals. PAK2 and PAK4 expression is ubiquitous relatively, whereas PAK3, PAK5, PAK6 expression is enriched in neuronal tissues (Arias-Romero and Chernoff, 2008). Among PAKs members, research centered on PAK1 is a subject of great interest due to strong implication in human health (Parrini et al., 2012; Rudolph et al., 2014) (**Table 2.1**)

PAK1, the first of the PAK family members to be discovered, is originally cloned from brain tissue as a p21-interacting kinase that is homologous to yeast Ste20 (Manser et al., 1994). PAK1 is a downstream effector of the Rho-family GTPases, Rac1 and Cdc42. As all PAK proteins, PAK1 consists of a highly conserved C-terminal catalytic kinase domain and an N-terminal region with a regulatory role. The PAK1 regulatory domain contains a GTPase-binding domain (GBD), an auto-inhibitory switch domain (IS) and several proline-rich motifs that bind to SH3 domains of Nck and Grb2 adapters or of the PIX α/β exchange factors. Inactive PAK1 has a homodimeric conformation (Parrini et al., 2012). PAK1 has been well known to be responsible for a variety of diseases such as cancer (mainly solid tumors), Alzheimer's disease, acquired immune deficiency syndrome and other viral/bacterial infections, inflammatory diseases (asthma and arthritis), diabetes (type 2), neurofibromatosis, tuberous sclerosis, epilepsy, depression, schizophrenia, learning, disability, autism, etc. (**Figure 2.1**). Since PAK1 is not essential for growth of normal cells, these PAK1 blockers would not cause any side effects (Maruta, 2014). Selective small molecule PAK1-blockers would have a potentially huge market value for the treatment of a variety of PAK1-dependent disorders/diseases. Although several distinct synthetic PAK1-blockers have been recently developed, no FDA-approved PAK1 blockers are available on the market as yet (Maruta, 2014). There is sufficient evidence to implicate PAK1 as attractive targets for the therapy of various diseases/disorders. Development of compounds with improved pharmacological properties, and an understanding of the mechanism that could circumvent inactivation of PAK1 are the research area of critical importance for the further clinical advancement of PAK1-oriented therapy (Kichina et al., 2010).

Table 2.1

Tissue expression, mouse knockout phenotypes, and species conservation of the six PAK isoforms

Isoform	Tissue expression	Mouse knockout phenotype	Species conservation (%)[a]
PAK1	Wide expression; high in brain, muscle, and spleen	Viable, fertile, and normal life span. Immune defects, glucose homeostasis defects, neuronal defects, compromised cardiac function upon challenges	M 99.26 R 99.08 D 96.87
PAK2	Ubiquitous; high in endothelial cells	Embryonic lethal; multiple developmental abnormalities, most prominently involving defective vascularization	M 97.14 R 96.56 D 95.80
PAK3	Mostly neuronal tissues	Viable, fertile, impaired synaptic plasticity, defects in learning and memory[b]	M 98.53 R 98.71 D 95.29
PAK4	Ubiquitous	Embryonic lethal (heart defect); neuronal defects, improperly formed neural tube	M 93.22 R 93.22 D 95.59
PAK5	Mostly neuronal tissues	Viable, fertile, deficits in locomotion, learning and memory	M 93.88 R 94.57 D 96.94
PAK6	Mostly neuronal tissues	Viable, fertile, deficits in locomotion, learning and memory	M 92.66 R 92.95 D 94.42

[a]Percent homology to human isoform: M: mouse; R: rat; D: dog. [b]Loss-of-function mutations in PAK3 are associated with familial cognitive disorders in humans (Rudolph et al., 2014).

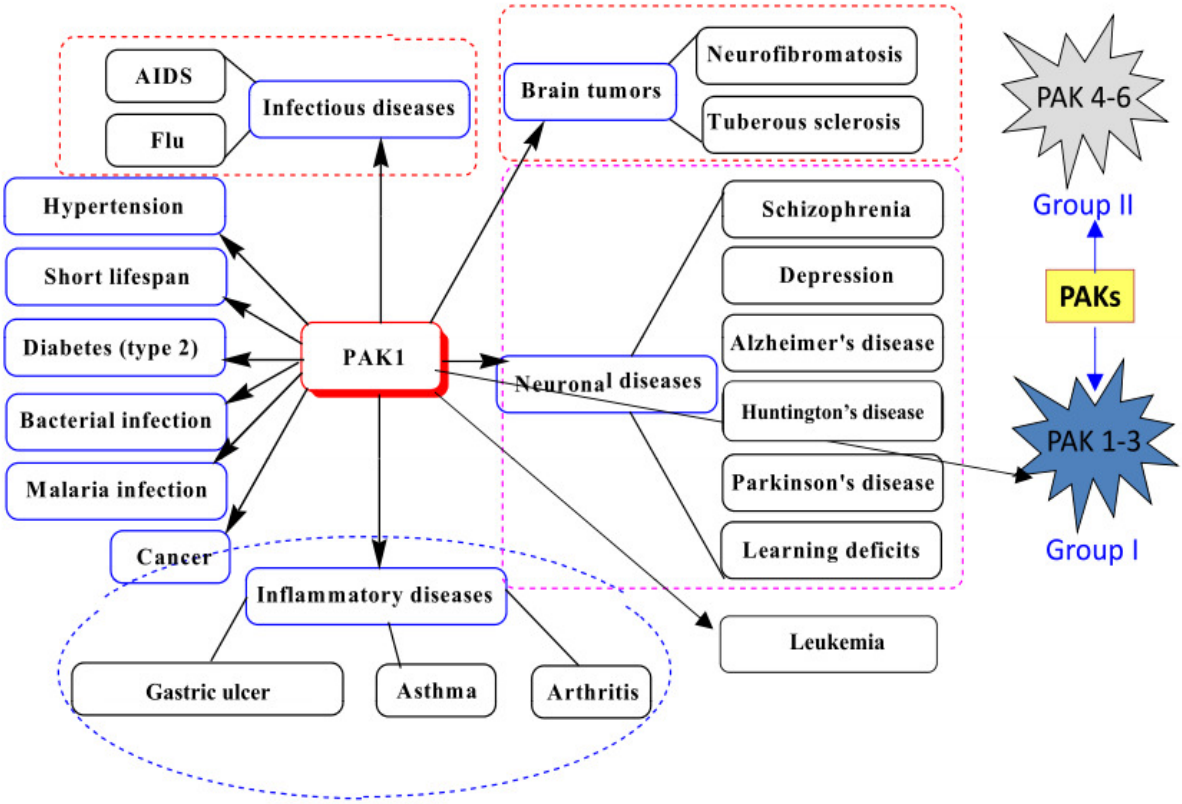

Figure 2.1. PAK1-dependent diseases/disorders**.** PAK1 belongs to group I of PAKs family (PAK1-3). PAK1 is as central node to be responsible for a variety of various diseases/disorders (Maruta, 2014).

2.8 LIM kinase (LIMK)

LIM kinase (LIMK) is a serine-threonine protein kinase (Bernard et al., 1994). It is regulated by several upstream signalling pathways, principally acting downstream of Rho GTPases to influence the architecture of the actin cytoskeleton by regulating the activity of the cofilin family proteins (cofilin 1, cofilin 2, and destrin) (Scott and Olson, 2007). The LIM kinase family consists of two members; LIM kinase 1 (LIMK1) and

LIM kinase 2 (LIMK2), which have 50% overall identity and 70% identity in their kinase domains (Bernard, 2007). To date, published data suggest that the function of LIMK1 and LIMK2 is very similar because both proteins phosphorylate cofilin resulting in its activation, suggesting that the main difference between these two proteins may be their cell type-specific expression and a different subcellular localization (Acevedo et al., 2006). The majority of research reported in the literature has focused on LIMK1, likely due to the fact that it was isolated first; in fact, many reports use the generic term "LIMK" without defining the kinase to which it refers (Scott and Olson, 2007). LIMK1 is found to be expressed widely in embryonic and adult tissues, with notably high expression in the brain, kidney, lung, stomach, and testis. LIMK2 is found to be expressed in almost all embryonic and adult tissues examined with the exceptions of glial cell, the testis, and kidney glomeruli. LIMK activity is associated with a variety of diseases including Williams syndrome, Alzheimer's disease, psoriatic epidermal lesions, primary pulmonary hypertension, intracranial aneurysms, ocular hypertension/glaucoma, HIV and other viral infections, and cancers and cancer cell migration/invasion (Yin et al., 2014). Therefore, LIMK inhibitors could find wide applications. Recently, it is suggested LIMK inhibition is supposed to have high potentials as therapeutics in anti-HIV infection applications (Vorster et al., 2011). The observation data suggest that the possibility of up-regulated LIMK1 as a cellular oncogene, and inhibition of LIMK1 activity in cancerous prostate cells and tissues could lead to reduction of phosphorylated cofilin and decrease of the cell's motility and thus the invasiveness of tumor cells and their evolution to metastasis. Therefore, small molecules inhibitors of LIMK1 could be potential therapeutic agents for prostate cancers. It is also suggested that the use of LIMK inhibitors may provide a novel way to

target the invasive machinery in glioblastoma multiforme (Park et al., 2014; Rak, 2014; Mashiach-Farkash et al., 2012).

2.9 Cancer

Cancer is now considered as one of the most serious health problems all over the world and also one of the leading causes of death (Gao et al., 2013, Lee et al., 2013). Deaths from cancer worldwide are predicted to continue to rise to over 13.1 million in 2030 (El-Ansary et al., 2014). Lung cancer is now the most common cause of cancer-related mortality among both men and women. This exceeds the sum of the next three leading causes of death due to breast, colon, and prostate cancer. There are over one million deaths worldwide due to malignant tumors of the lung, making it an epidemic disease (Jemal et al., 2002). Lung cancer is classified into non-small cell (NSCLC) and small cell lung cancer (SCLC); 80% are NSCLCs, including adenocarcinomas, squamous cell (epidermoid), and large cell carcinomas, and 20% are SCLCs (Rom et al., 2000). A large number of novel targets and approaches to anticancer therapy have emerged from the literature. Approaches to anticancer therapy include targeting the growth signal transduction mechanisms within cancer cells, processes involved in cell invasion and metastatic spread, apoptosis and the cell cycle, and tumor-related angiogenesis. Other approaches focus on tumor-specific antigens, targeted poisons, stimulation of the immune system, gene therapy and use of antisense molecules as drugs, and anti-hormones, among others (Faivre et al., 2006). For target molecules, serine/threonine kinases are the interesting targets, and involved in growth, pro-apoptotic signaling, and angiogenesis, and are well-known targets for cancer and a

variety of other diseases and disorders (Wang et al., 2013b). PAK1 belongs to a family of serine/threonine protein kinases which is Achilles's heel for subset of cancer cells including breast and lung cancer that require PAK1 for their growth and progress (Ong et al., 2011, 2013; Maruta, 2014). Presently, despite the mass research are devoted and rapid progress has been achieved; unfortunately, no currently available anticancer drugs would eradicate cancer cells without harming normal tissues (Zhang et al., 2013; El-Ansary et al., 2014).

2.10 Objectives of the study

Alpinia, leucaena, pilosa, and bitter melon are popular plants in Okinawa. It has been shown to have the interesting biological properties which are associated with their major bioactive constituents such as DK, DDK, mimosine, and CBI. Thus, these compounds were chosen for the study. Novel biological properties of four above isolated compounds were explored in order to develop more potent derivatives for pesticidal activities, and for PAK1 blocking, anticancer, skin-brightening, and hair loss treatment as well. Because PAK1 is not essential for the growth of normal cells, anti-PAK1 drugs derived from these lead compounds could have therapeutic values to cure various diseases/disorders without any unwanted effects. Therefore, this study focused on following aspects:

i) To explore the novel bioactivities of aforementioned four compounds in order to develop potent compounds for insecticidal, herbicidal, and nematicidal activities.

ii) To develop more potent melanogenesis and cyclooxygenase inhibitors from the identifies ones.

iii) To develop a new approach for the assessment of potent and safe PAK1 blockers in cell culture.

iv) To study the effect of PAK1 blockers on melanogenesis inhibition, anticancer and hair growth-promoting activities in B16F10 melanoma, lung cancer (A549), human follicle dermal papilla (HFDPC) cells, respectively.

CHAPTER 3

INSECTICIDAL AND HERBICIDAL ACTIVITIES OF LEUCAENA MIMOSINE AND *BIDENS PILOSA* L., *ALPINIA ZERUMBET* EXTRACTS

3.1 SUMMARY

Tyrosinase, regarding to regulation of normal developmental processes in insects, is being emerged as potential target for development of novel insecticides. Interestingly, the results showed that mimosine demonstrated insecticidal activity which could be due to effective inhibition on tyrosinase. On the other hand, EtOAc and butanol extracts from *B. pilosa* flowers exhibited high insecticidal activity with LD_{50} of 5.52 and 2.93 µg/insect, respectively. The acetone extract of alpinia seeds was found at lower activity (LD_{50} = 27.24 µg/insect). The insecticidal activity of EtOAc and acetone extracts also may be due to tyrosinase inhibition. Herbicidal activity of extracts and mimosine was also evaluated. Mimosine displayed strong herbicidal activity. These findings provide evidence for utilization of pilosa for questing lead compounds for insecticidal activity with their biological nature, and also suggest the possibility in the design of new herbicides and insecticides from mimosine.

3.2 INTRODUCTION

Due to increasing human health and environmental risks from the use of synthetic pesticides, natural products stemmed from plants are considered as a promising strategy for fighting insect pests since they are environmental friendly, biodegradable and less toxic to nontarget organisms (Rattan, 2010; Tsao et al., 2002; Isman, 2006). Acetylcholinesterase (AChE) plays roles in termination of neurotransmission by degradation of the neurotransmitter acetylcholine at the nerve synapse (Soreq and

Seidman, 2001), while tyrosinase is a copper containing enzyme which plays key roles in normal developmental processes in insects (Pan et al., 2011). Inhibition of both these enzymes serves as major target for development of novel insecticides (Pan et al., 2011; Lu et al., 2012).

*Leucaena leucocephala (*leucaena)*, Bidens pilosa* L. (pilosa) and *Alpinia zerumbet* (alpinia) are grown widely in tropical and subtropical regions (Tawata et al., 2008; Yang et al., 2006). Leucaena, a multipurpose leguminous plant, is protected from pathogen attacks by the presence of mimosine – a nonprotein aromatic amino acid. Mimosine isolated from leucaena has been reported for allelopathic effects, and could be used as raw material with high value for medicine and agricultural chemicals. It has also exhibited antityrosinase activity and therapeutic benefits (Cabanes et al., 1987; Upadhyay et al., 2011). The previous study indicates that a small library of mimosine tetrapeptides may be potential candidates in the design of novel drugs against neuraminidase and tyrosinase. On the other hand, evidences from literature data show that pilosa and alpinia possess many significantly biological activities. The extracts of pilosa have immunosuppressive and anti-inflammatory (Pereira et al., 199), antihypertensive (Dimo et al., 2002), antimalarial (Oliveira et al., 2004), antihyperglycermic (Hsu et al., 2009), antidiabetic activities (Chien et al., 2009), while alpinia is responsible for antiinflammatory, bacteriostatic and fungistatic effects (Zoghbi et al., 1999). Furthermore, in recently reported studies, alpinia has been identified as source of bioactive compounds against HIV-1 integrase and neuraminidase, advanced glycation end products, and skin disease-related enzymes (Upadhyay et al., 2011; Chompoo et al., 2011, 2012a).

Given significantly biological activities in these three species reveal that there is still limited scientific data about efficiency for insecticidal activity. Therefore, the present study was designed to explore insecticidal activity of pilosa and alpinia extracts as well as mimosine isolated from leucaena, and to investigate their mode of action against insects on AChE and tyrosinase inhibition. Herbicidal activity was also evaluated.

3.3 MATERIALS AND METHODS

3.3.1 Chemicals and reagents

Acetylcholinesterase from *Electrophorus electricus* (electric eel), 5,5'-dithiobis-(2-nitrobenzoic acid), acetylthiocholine iodide, bovine serum albumin were purchased from Sigma-Aldrich K.K., Tokyo, Japan. L-tyrosine, 1,8 cineole were from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Mushroom tyrosinase was obtained from Sigma-Aldrich, Inc. (Missouri, USA). Kojic acid was bought from Kanto Chemical Co., Inc. (Tokyo, Japan).

3.3.2 Insect collection and culture

Termites were hand-collected with forceps around University of the Ryukyus campus, and were held in 100 L (38.5 x 24.5 cm) plastic trays. Insects were reared by providing pine and newspaper as food source, and kept at 24-26 ^{o}C, 75-80 % humidity.

3.3.3 Preparation of extracts and mimosine isolation

Fresh flowers of *B. pilosa* were collected around Faculty of Agriculture, University of the Ryukyus, located at 26^{o} N, 127^{o} E. Samples were rinsed, and air-dried for 3 days at room temperature. Two hundred-fifty grams of dried-flowers sample were

extracted with 2 L chloroform:MeOH (1:1) by shaking at room temperature for 4 days. The extract was filtered through Whatmann paper, and the filtrate was evaporated to dryness under reduced pressure at 40 ^{o}C. Each of 2 g chloroform:MeOH extract (FB) was successively reextracted with EtOAc, CH_2Cl_2, *n*-BuOH and water to give FBE (53.3 mg), FBC (200.4 mg), FBB (193.1 mg) and FBH (1.403 g) crude extracts, respectively. Because the acetone extract of alpinia seeds and mimosine had antityrosinase activity, they are chosen to investigate insecticidal activity. Seed extract of alpinia was prepared with 70 % acetone to obtain crude extract SAA (3 g) (Upadhyay et al., 2011b; Chompoo et al., 2012b). For mimosine isolation from leucaena, fresh leaves (1.5 kg) were boiled in 5 L water for 10 min. The cooled liquid extract was filtered, and the filtrate was subjected to the cation-exchange resin (2 kg). The mixture was left for overnight, and the filtrate was removed. The resin was rinsed with distilled water for 5-6 times and 80 % ethanol (5 L), and added 6 L of 2 N NH_4OH. The liquid extract was concentrated to final volume (200 mL) at 40 ^{o}C under reduced pressure. The pure mimosine was obtained when the solution was adjusted to pH 4.5-5.0 with 6 N HCl (Tawata, 1990). All crude extracts and mimosine were stored at 4 ^{o}C until to be used further.

3.3.4 Acute toxicity bioassay against termite

Acute toxicity bioassay was performed by topical application to worker termites (Zhu et al., 2003). A series of six different doses as 3, 5, 15, 25, 35 and 50 μg/termite of each test sample was prepared in acetone solution. The aliquots (1 μL) of diluted samples were applied topically to the abdomens of worker termites. Controls were treated with 1 μL of acetone only. Termites were transferred into Petri dishes (4.2 cm diameter) lined with filter papers, and kept in an incubator at 25 ^{o}C. A few drops of distilled water were

supplied daily to the bottom edge of Petri dishes to maintain moisture during the experiment. Three replicates of 10 termites each were used per treatment. Mortality of insects was observed during 4 days after treatment. Insects were evaluated as dead when they become immobilize, and not express with external stimuli.

3.3.5 Bioassay for acetylcholinesterase (AChE) inhibition

AChE enzyme inhibitory assay was followed by Ellman's method with several modifications (Dohi et al., 2009). In brief, twenty-five microliters of samples dissolved in methanol was mixed with 50 µL of 50 mM Tris-HCl buffer (pH 8.0), 25 µL of 0.22 U/mL AChE. After incubation at 37 ^{o}C for 15 min, the mixture was added to 125 µL of 3mM DTNB and 25 µL of 15 mM ATCI followed by incubation at room temperature for 30 min. The absorbance was measured at 412 nm using microplate spectrophotometer (Benchmark Plus, Biorad, UK). Methanol was used as negative control while 1,8 cineole was used as positive control. The percentage of inhibition was calculated by using the formula:

AChE inhibition (%) = ($A_{control}$-A_{sample}/$A_{control}$) x 100 where $A_{control}$ is the absorbance of the control (sample solution containing methanol instead of test compound) whereas A_{sample} is the absorbance of test compound. The IC_{50} value shows sample concentration required to inhibit 50 % AChE activity.

3.3.6 Bioassay for tyrosinase inhibition

Tyrosinase inhibitory assay was done using microplate method (Chompoo et al., 2012b). The amount of samples (20 µL) with various concentrations was transferred into each of 96-well plate followed by adding 120 µL of 20 mM sodium phosphate buffer (pH 6.8), 40 µL of 500 U/mL mushroom tyrosinase enzyme dissolved in buffer. The mixture

was incubated at 25 ^{o}C for 20 min, and then added 20 µL of 0.85 mM L-tyrosine solution. After 10 min incubation at room temperature, the absorbance was recorded at 470 nm by using microplate reader (Benchmark plus, Biorad, UK). Kojic acid was used as positive control. The percentage of inhibition was calculated as the following:

Inhibition (%) = $[(C_E-C_o)-(S_E-S_o)]/(C_E-C_o)$ x 100 where C_E is the absorbance of control with enzyme; C_o is the absorbance of control without enzyme; S_E is the absorbance of tested sample with enzyme; S_o is the absorbance of tested sample without enzyme

3.3.7 Herbicidal activity assay

Seeds of lettuce (*Lactuca sativa*) were sterilized on surface in 2 % sodium hypochlorite (v/v) solution for 20 min and rinsed with distilled water for six times to avoid fungal contamination. To stimulate germination, seeds were incubated in oven at 80 ^{o}C for 2 min. All samples were dissolved in sterile distilled water which was used as control. Ten lettuce seeds were sown on 0.8 % agar medium, and 1 mL of sample at 1000 µg/mL was added into glass tube. Samples are incubated at 25 ^{o}C in light : dark (13 : 11 hours) condition. The experiment was conducted with four replicates for each treatment. Data was measured at 7-day old seedlings.

3.3.8 Data analysis

Mortality rate was corrected using Abbott's formula (Abbott, 1925). Probit analysis was conducted according to Finney's method (Finney, 1952). The IC_{50}, LD_{50} values are calculated graphically using Microsoft Excel 2003. The data were analyzed by one-way ANOVA, and the means were separated using Turkey's HSD range test at P = 0.01. All statistical analyses were conducted using SPSS version 16.0.

3.4 RESULTS AND DISCUSSION

3.4.1 Insecticidal activity by topical application

For this assay, various dosages of samples were applied to the abdomens of termites, and the results were evaluated at 4 days after treatment (DAT). As shown in **Table 3.1**, five of six investigated extracts were found to have insecticidal activity. Both FBB and FBE extracts exhibited the strongest activity against termites with LD_{50} of 2.93 and 5.52 µg/insect, respectively. Lower activity was found in FB (LD_{50} = 12.20 µg/insect) and FBC (LD_{50} = 13.89 µg/insect). The isolated mimosine also exhibited insecticidal activity (LD_{50} = 54.09 µg/insect) whereas LD_{50} value of SAA extract was at 27.24 µg/insect. The insecticidal activity of water extract (FBH) was less significant than others with 30 % mortality at dose of 50 µg/insect.

3.4.2 Inhibition of acetylcholinesterase (AChE) activity

For investigating mode of action for acute toxicity, AChE inhibitory bioassay was performed by using microplate assay method. Acetylthiocholine iodide (ATCI) was used as the substrate. The results were shown in **Figure 3.1**. All tested samples presented a wide range of AChE inhibitory activity by 96.40 - 2508.40 µg/mL. Although FBB extract had high insecticidal activity, it exhibited weak AChE inhibitory activity with value IC_{50} = 326.02 µg/mL. Mimosine isolated exhibited poor inhibitory activity (IC_{50} = 950.21 µg/mL). The similar result was also found in SAA extract (IC_{50} = 722.35 µg/mL). However, it is worth for reporting in pilosa that AChE inhibitory activity of FBE extract was recorded at value IC_{50} = 96.40 µg/mL.

3.4.3 Mushroom tyrosinase inhibition

Due to less efficient activity on AChE inhibition of the extracts and mimosine, whether they act on tyrosinase inhibition. To test this hypothesis, the inhibitory assay was performed for mushroom tyrosinase. The results indicated the potent insecticidal extract (FBE) had high antityrosinase activity (IC_{50} = 2.35 μg/mL) whereas the other one (FBB) was no inhibitory activity on this enzyme (see **Table 3.2**). Mimosine and SAA extract had strong tyrosinase inhibitory activity with their IC_{50} values of 13.29 μg/mL and 3.02 μg/mL, respectively.

3.4.4 Herbicidal activity

All extracts had herbicidal activity at 1000 μg/mL with 63-91% shoot growth inhibition and 81-100% for root growth inhibition while herbicidal activity of mimosine for shoot and root growth inhibition was 92 and 100%, respectively (**Figure 3.2**).

3.4.5 Discussion

Termites are chosen as representative insect in this study because of their significance worldwide in general and in Japan in particular (Verma et al., 2009). The present experiment was designed to explore insecticidal activity of pilosa and alpinia extracts as well as leucaena-derived mimosine. To the best knowledge, this is the first report for investigating mode of action against insects in these two medical plants.

In this report, all extracts and mimosine exhibited insecticidal activity except for FBH extract. Among these, two extracts FBE, FBB from pilosa flowers revealed the strongest activity against insects (see **Table 3.1**). Because the tested extracts and mimosine were less active on AChE inhibition, the inhibitory effect on tyrosinase enzyme were evaluated. Tyrosinase involves to regulation of normal developmental processes in

insects. Inhibition of this enzyme is emerging as new potential target for biological insect control (Pan et al., 2011). Although FBB extract had high insecticidal activity, this one may act rather than on that of AChE and tyrosinase enzyme inhibition. On the other hand, FBE extract was identified for strongly killing insects which may be due to inhibition of tyrosinase activity (see **Table 3.2**). The SAA extract having high tyrosinase inhibitory activity was also found for insecticidal activity. It may be attributed to the presence of cholest-4-ene-3,6-dione which is major compound in this extract (Chompoo et al., 2012b). However, evidence to support for this need to be investigated further. It is interesting in this report that mimosine demonstrated insecticidal activity which can be due to tyrosinase inhibition (IC_{50} = 13.29 μg/mL). The toxic characteristic of mimosine against insects might be governed by the 3-hydroxy group of the pyridine ring (Tawata et al., 2008). The effect of mimosine on inhibition of development and growth of *Tribolium castaneum* larvae is reported (Ishaaya et al., 1991). Previous result plus this result suggest the possibility for development of new insecticidal compounds from mimosine. In particular, the progress of termite biological control is still rather slow after 50 years of research due to poor biological understanding for skipping off termite defense mechanisms (Chouvenc et al., 2011). This study indicates that the inhibition of tyrosinase also could be effective way to go for combating termites biologically. All extracts had relative herbicidal activity while mimosine had strong inhibition.

3.5 CONCLUSION

The present experiment provided evidence about efficiency for insecticidal activity of *B. pilosa*, and this activity may involve to inhibition of tyrosinase enzyme. On the other hand, the results also indicated that mimosine, a tyrosinase inhibitor, could be candidate for insecticidal and herbicidal activities. The results also suggest the utilization of *B. pilosa* and alpinia in searching for new compounds for insecticidal activity, and development of mimosine-based new derivatives for insect and weed control.

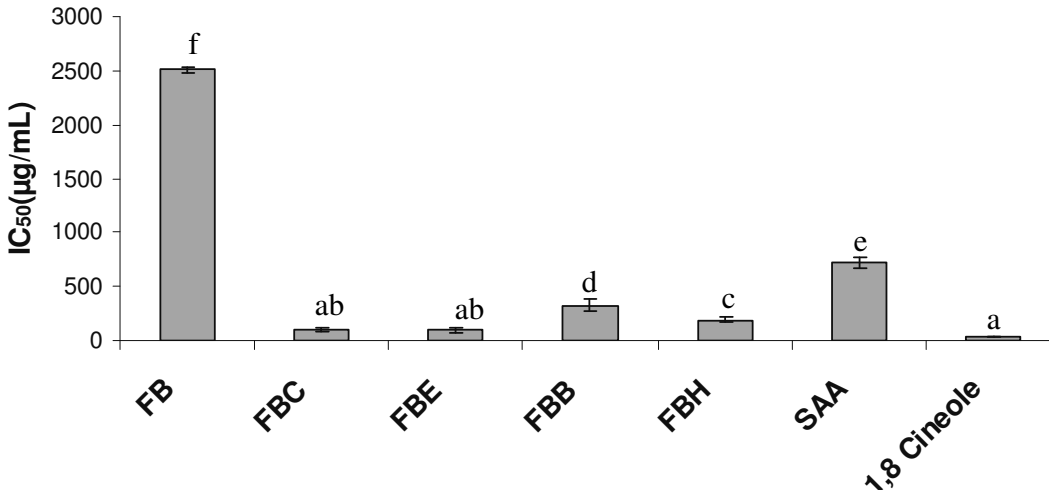

Figure 3.1. Effect of extracts on AChE inhibitory activity. 1,8 Cineole was used as positive control. IC_{50} value represented for 50% AChE inhibition. Values are presented as mean ± SE of two repeated times.

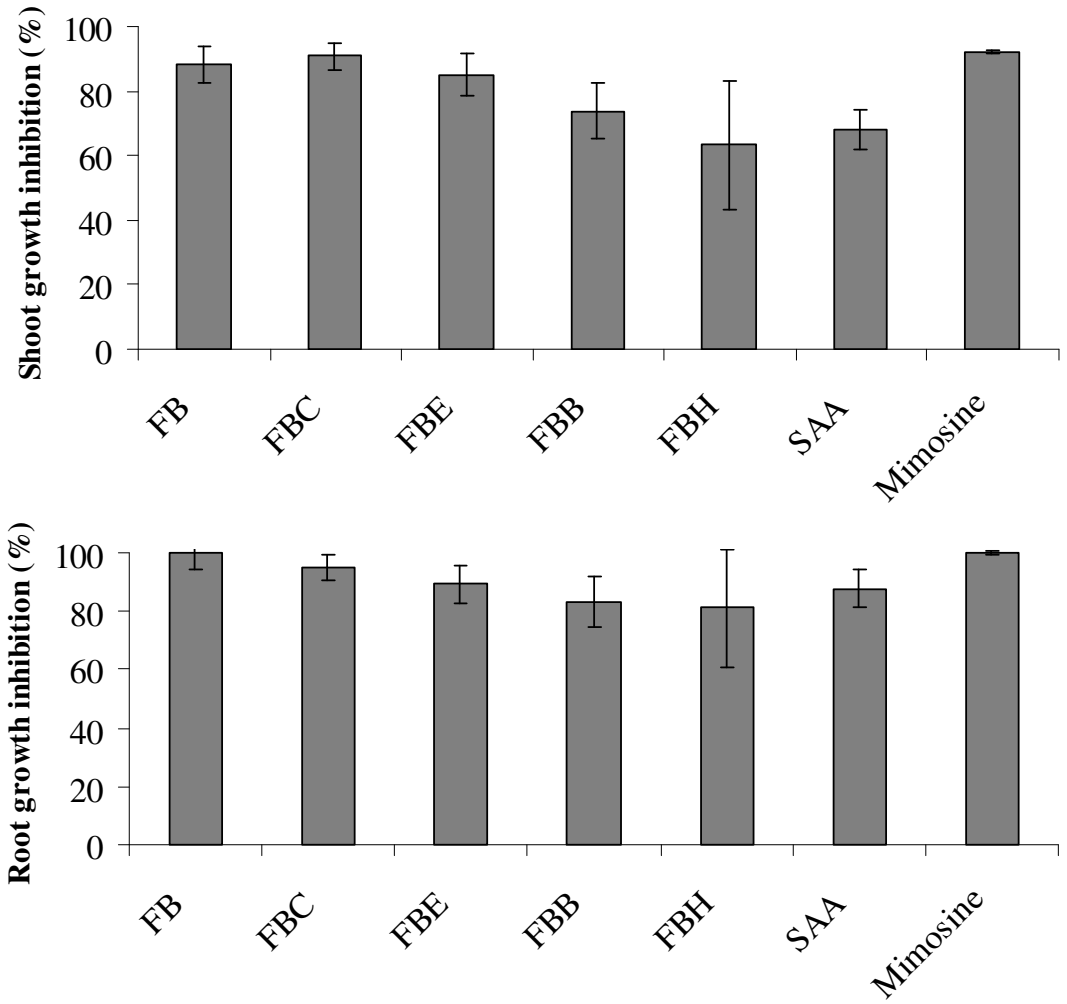

Figure 3.2. Herbicidal activity of extracts and mimosine on lettuce. FB: *B. pilosa* flower extract, FBC: dichloromethane fraction, FBE: ethyl acetate fraction, FBB: n-butanol fraction, FBH: water fraction, SAA: acetone extract of *A. zerumbet* seeds. The experiment was conducted with four replicates, 10 lettuce seeds per glass tube as one replicate. Lettuce is used as indicator plant. Shoot and root growth were evaluated at 7 days post-treatment with extracts and mimosine at 1000 µg/mL. Values are presented as means ± SE.

Table 3.1

Topical toxicity of extracts and mimosine against termites

	Mortality (%) at doses (µg/insect)[i]							LD_{50}[ii] (µg/insect)
	0	3	5	15	25	35	50	
FB	0	7	31	59	66	80	90	12.20 a
FBC	0	0	20	43	77	90	93	13.89 a
FBE	0	30	47	93	100	100	100	5.52 a
FBB	0	50	93	100	100	100	100	2.93 a
FBH	0	0	0	7	10	20	30	>50 b
SAA	0	0	0	23	40	67	73	27.24 a
Mimosine	0	0	33	53	57	63	100	54.09 b

[i]Applied dosages. [ii] LD_{50} value was determined by log-probit analysis and represented as mean ± SE of three replicates. Different letters in the same column indicate statistical significant differences

Table 3.2

Tyrosinase inhibition of *B. pilosa* and *A. zerumbet* extracts

	Tyrosinase inhibition (IC_{50} = µg/mL)
FB	18.62 ± 4.87 b
FBC	N/A
FBE	2.35 ± 0.01 a
FBB	19.04 ± 3.91 b
FBH	4.45 ± 0.59 a
SAA	3.02 ± 0.42 a
Mimosine	13.29 ± 1.59 b
Kojic acid	1.89 ± 0.28 a

Different letters in the same column indicate statistical significant differences. N/A: not active.

CHAPTER 4

INSECTICIDAL AND NEMATICIDAL ACTIVITIES OF NOVEL MIMOSINE DERIVATIVES

4.1 SUMMARY

Mimosine, a non-protein amino acid, is found in several tropical and subtropical plants, which has high value for medicine and agricultural chemicals. Here, in continuation of works aimed to development of natural product-based pesticidal agents, the first significant findings for insecticidal and nematicidal activities of novel mimosine derivatives were reported. Interestingly, mimosinol and deuterated mimosinol (D-mimosinol) from mimosine had strong insecticidal activity which could be a result of tyrosinase inhibition (IC_{50} = 31.4 and 46.1 μM, respectively). Of synthesized phosphoramidothionate derivatives from two these amino alcohols, two compounds (**1a** and **1b**) showed high insecticidal activity (LD_{50} = 0.5 and 0.7 μg/insect, respectively) with 50%–60% mortality at 50 μg/mL which may be attributed to acetylcholinesterase inhibition. Compounds **1a** and **1b** also had strong nematicidal activity with IC_{50} = 31.8 and 50.2 μM, respectively. These results suggest that the length of the alkyl chain and the functional group at the C_5-position of phosphoramidothionates derived from mimosinol and D-mimosinol are essential for the insecticidal and nematicidal activities. These results reveal an unexplored scaffold as new insecticide and nematicide.

4.2 INTRODUCTION

Organophosphorus compounds are a highly diverse class of organic chemicals with many uses (Gungordu et al., 2013), and form one of the most important groups of modern pesticides (Pandey et al., 2008). The advantageous properties of phosphorus

compounds are a result of their relatively low stability, their decomposition to products that are not toxic to humans and animals, and their low rate of use per unit treated area (Jaiswal et al., 1998). Another important feature of these compounds is that their action is highly selective (Chandra et al., 2005). Designed compounds containing phosphorus may be less dangerous in use without losing their values as effective pesticides (Jaiswal et al., 1998). Utilization of organophosphorus pesticides is favored over organochlorine compounds because of their ability to degrade more readily in the environment (Kolosova et al., 2003). However, overuse of these compounds can lead to the development of resistance in pests (Carvalho et al., 2013). Pest resistance has led to the need for development of new non-persistent and non-toxic pesticides with selective activity to help maintain stable food production.

Acetylcholinesterase (AChE) functions in termination of the neurotransmission by breaking down the neurotransmitter acetylcholine at cholinergic synapses in all animals. In insects, it serves as a major target for anti-cholinesterase insecticides, and constitutes a common mechanism of insecticide resistance through its reduced sensitivity to the insecticides (Lu et al., 2012). Tyrosinase plays important roles in normal developmental processes in insects; inactivation of tyrosinase activity can lead to the loss of insect defense mechanisms or to abnormal body softening which could lead to pest control (Pan et al., 2011). Thus, these two enzymes serve as targets for the development of novel anti-cholinesterase insecticides and bioinsecticides (Pan et al., 2011; Lu et al., 2012).

Mimosine [*β*-[*N*-(3-hydroxy-4-oxypyridyl)]-α-aminopropionic acid] is a non-protein amino acid containing an alanine side chain bound to the nitrogen atom of a hydroxypyridone ring. It is found in several tropical and subtropical plants, which

possesses a wide range of biological activities and has strong medicinal properties (Tawata et al., 2008) including anti-viral (Dai et al., 1994), anti-inflammation (Conti et al., 2002), and anti-cancer effects (Chang et al., 1999). Mimosine is also responsible for herbicidal activity (Tawata, 1990), and can suppress insect growth along with its effects on the activity of trehalase, invertase, and amylase in a dose-dependent manner (Ishaaya et al., 1991). However, a review of the literature reveals mimosine derivatives with insecticidal and nematicidal activities have not been thoroughly reported yet.

Cyclic phosphorus compounds such as salithion, 4-isobutyl-2-methoxy-1,3,2-oxazaphospholidine 2-sulfide and 2-methoxy-5-phenyl-1,3,2-oxazaphospholidine 2-sulfide have been shown in connection with strong insecticidal activity (Hirashima et al., 1992). The insecticidal activity has also been shown in five-membered cyclic phosphoramidates and phosphoramidothiolates (Tawata et al., 1978). Moreover, Eto et al. (1978) have reported that five-membered cyclic phosphoramidothionates from amino alcohol of L-leucine and other amino acids have insecticidal activity by inhibiting acetylcholinesterase. Taken together, in this study, using mimosine (non-protein amino acid) as starting material, the insecticidal activity in amino alcohols derived from mimosine was identified, and thus a family of five-membered cyclic phosphoramidothionate derivatives from these alcohols to assess their insecticidal and nematicidal activities were synthesized. Their insecticidal mode of action was also tested on acetylcholinesterase and tyrosinase inhibition. To the best knowledge, this is the first report on the synthesis of novel amino alcohols and phosphoramidothionate derivatives from mimosine, and the investigation of their insecticidal and nematicidal activities.

4.3 MATERIALS AND METHODS

4.3.1 Chemicals and reagents

Tris(triethylsilyl)silane, rotenone, 5,5′-dithiobis-(2-nitrobenzoic acid), acetylthiocholine iodide, and sodium borodeuteride were obtained from Sigma-Aldrich (Tokyo, Japan). L-tyrosine and sodium borohydride were purchased from Wako Pure Chemical Industries (Osaka, Japan). Trifluoromethanesulfonic acid was obtained from Nacalai Tesque (Kyoto, Japan), and thiophosphoryl chloride was obtained from Tokyo Chemical Industry Co. (Tokyo, Japan). Fenitrothion was obtained from Dr. Ehrenstorfer GmbH (Augsburg, Germany). Mushroom tyrosinase was obtained from Sigma-Aldrich (Saint Louis, MO, USA), and kojic acid was purchased from Kanto Chemical Co. (Tokyo, Japan). Unless otherwise mentioned, all reagents were of analytical grade and were obtained from Wako Pure Chemical Industries and Kanto Chemical Co., Japan. The ^{1}H- and ^{13}C-NMR spectra were recorded on a JEOL JNM-ECA400 spectrometer (JEOL Resonance, Tokyo, Japan) in $CDCl_3$. Chemical shifts were expressed in parts per million (δ) relative to tetramethylsilane (TMS).

4.3.2 Mimosine isolation from Leucaena leucocephala leaves

Samples of *Leucaena leucocephala* leaves were collected at the Faculty of Agriculture, University of the Ryukyus, Okinawa, Japan (lat 26 ºN, long 127 ºE). Fresh leaves (1.5 kg) were boiled in 5 L water for 10 min. The cooled liquid extract was sieved by suction filtration in a shaking bath (SB-20; As One, Osaka, Japan), and the filtrate was mixed with ion-exchange resin (2 kg) (Mitsubishi Chemical, Tokyo, Japan), stirred for 30 min, and left overnight. The resin was rinsed with distilled water 5–6

times, and 5 L of 80% ethanol was added dropwise to remove chlorophyll. Mimosine was dissolved from the resin with dropwise addition of 6 L of 2 N NH_4OH. The liquid extract was concentrated to a final volume of 300 mL at 40 °C under reduced pressure. The solution was adjusted to pH 4.5–5.0 with 6 N HCl, and mimosine was precipitated at 4 °C overnight. The precipitate was recrystallized from 5 N NaOH (pH 9.0) and 6 N HCl (pH 4.5–5.0) and then allowed to stand at 4 °C to give pure mimosine. Mimosine was stored at –20 °C until further use. Mimosine was identified by LC-MS (*ESI*): *m/z* $[M + H]^+$ 199.1 (Tawata, 1990).

4.3.3 Synthesis of mimosinol and deuterated mimosinol

Trifluoromethanesulfonic acid (187 μL, 2 mmol) was added to a 25-mL round-bottom flask containing 3.4 mL dichloromethane (CH_2Cl_2). After stirring at room temperature, tris(triethylsilyl)silane solution (618 μL, 2 mmol) was added dropwise, and the mixture was stirred at room temperature for 3 h until the solution became clear. Mimosine (0.4 g, 2 mmol) was placed in a round-bottom flask, to which imidazole (0.15 g, 2.2 mmol) and DMF: CH_2Cl_2 (3.4 mL, 1:1) were then added. The reaction flask was cooled to 0 °C, and tris(triethylsilyl)silyl triflate was added dropwise. After the addition was completed, the reaction was stirred at room temperature for 2 h. Mimosine ester was obtained from the filtrate by evaporation. A solution of sodium borohydride (0.28 g, 7.2 mmol) or sodium borodeuteride (0.3 g, 7.2 mmol) in 3 mL 50% ethanol was added to solution of mimosine ester in 3 mL of 50% ethanol. The resulting mixture was refluxed at room temperature for 5.5 h, and the solvent ethanol was evaporated *in vacuo*. The aqueous solution thus obtained was extracted with ethyl acetate (3 × 20 mL); the combined extracts were washed with saturated sodium chloride, dried over anhydrous sodium sulfate, and evaporated to give mimosinol (**1**) as a colorless crystal (352 mg,

95% yield) and deuterated mimosinol (D-mimosinol) (**2**) as a colorless liquid (274 mg, 73.5% yield). The NMR spectral data were in agreement with reported values for mimosinol and D-mimosinol described previously by Nguyen et al. (2014). LC-MS (*ESI*): *m*/*z* $[M]^+$ 184.1, 186.1 for mimosinol and D-mimosinol, respectively.

4.3.4 General procedure for synthesis of mimosinol (**1a**-**c**) *derivatives*

The derivatives were prepared following procedure described by Tawata et al. (Tawata et al., 1980) with modifications. A solution of mimosinol (1 mmol) and triethylamine (2 mmol) in 5 mL dioxane was cooled in an ice bath; thiophosphoryl chloride (1 mmol) in 5 mL dioxane was added dropwise, and the reaction mixture was stirred for 1 h. The resulting triethylammonium chloride was filtered off, and the filtrate was washed twice with dioxane. Sodium methoxide (1 mmol) in alcohol solution was added slowly to the filtrate. After stirring for 10 min, the solvents were evaporated, and the oily residue was dissolved in chloroform and washed twice with saturated sodium chloride solution. The organic layer was dried over anhydrous sodium sulfate and distilled to afford mimosinol derivatives (**1a**–**c**).

4.3.5 General procedure for synthesis of deuterated mimosinol (**2a**–**c**) *derivatives*

The derivatives were synthesized following procedure described by Tawata et al. (Tawata et al., 1980) with modifications. A solution of D-mimosinol (1 mmol) and triethylamine (2 mmol) in 10 mL dioxane was cooled in an ice bath, and thiophosphoryl chloride (1 mmol) in 10 mL dioxane was added dropwise. The reaction mixture was stirred for 10 min at a temperature <10 °C. After completion of the reaction, the triethylamine hydrochloride was filtered off, and the filtrate was obtained. A solution of alcohol (1.4 mmol), triethylamine (1 mmol) in 10mL dioxane was added slowly to the filtrate. Approximately 100 min was required for displacement of a chlorine atom with a

bulky alkyl group. The formed triethylamine hydrochloride was removed by filtration, and the filtrate was concentrated. Chloroform was added to the residue solution, and the chloroform solution was washed twice with saturated sodium chloride solution. The separated organic layer was dried over anhydrous sodium sulfate, and distilled to afford D-mimosinol derivatives (**2a**–**c**).

3-Hydroxy-1-(((4S)-2-methoxy-2-sulfido-1,3,2-oxazaphospholidin-4-yl)methyl)pyridin-4(1H)-one (**1a**): Pale yellow crystals, yield 34%. ^{1}H-NMR ($CDCl_3$, 400 MHz) δ: 1.29 (m, 2H, CH_2CHNH), 3.29 (d, 3H, OCH_3), 3.82 (m, 1H, CHNH), 4.06–4.13 (m, 2H, OCH_2), 7.54 (s, 1H, CH), 8.20 (s, 1H, CH), 9.21 (s, 1H, CH). ^{13}C-NMR ($CDCl_3$) δ: 49.7 (s, CH_2CHNH), 55.1 (d, OCH_3), 58.3 (d, CHNH), 60.8 (d, CHNH), 63.3 (d, OCH_2), 115.4 (s, CH), 120.4 (s, CH), 138.5 (s, CH), 166.3 (s, C), 181.8 (s, C). LC-MS (*ESI*): *m*/*z* $[M]^+$ 276.1.

1-(((4S)-2-ethoxy-2-sulfido-1,3,2-oxazaphospholidin-4-yl)methyl)-3-hydroxypyridin-4(1H)-one (**1b**): Pale yellow crystals, yield 39.6%. ^{1}H-NMR ($CDCl_3$, 400 MHz) δ: 1.30 (m, 2H, CH_2CHNH), 3.29 (d, 3H, CH_2CH_3), 3.48 (s, 2H, OCH_2), 3.78 (m, 1H, CHNH), 4.01–4.15 (m, 2H, OCH_2), 7.54 (s, 1H, CH), 8.18 (s, 1H, CH), 9.22 (s, 1H, CH). ^{13}C-NMR ($CDCl_3$) δ: 16.0 (d, CH_3), 50.6 (s, CH_2CHNH), 56.3 (d, OCH2), 58.4 (d, CHNH), 59.4 (d, CHNH), 63.4 (d, OCH_2), 116.4 (s, CH), 121.3 (s, CH), 138.1 (s, CH), 166.4 (s, C), 181.8 (s, C). LC-MS (*ESI*): *m*/*z* $[M]^+$ 290.1.

3-Hydroxy-1-(((4S)-2-propoxy-2-sulfido-1,3,2-oxazaphospholidin-4-yl)methyl)pyridin-4(1H)-one (**1c**): Pale yellow crystals, yield 28.9%. ^{1}H-NMR ($CDCl_3$, 400 MHz) δ: 0.91 (m, 3H, CH_2CH_3), 1.29 (m, 2H, CH_2CHNH), 1.70 (m, 2H,CH_2CH_2), 3.29 (dd, 2H, OCH_2), 3.78 (m, 1H, CHNH), 4.05–4.12 (m, 2H, OCH_2), 7.55 (s, 1H, CH), 8.17 (s, 1H, CH), 9.20 (s, 1H, CH). ^{13}C-NMR ($CDCl_3$) δ: 10.1 (d, CH_3), 23.5 (m, CH_2), 46.0 (s,

CH_2CHNH), 55.4 (d, OCH_2), 56.8 (d, CHNH), 58.3 (d, CHNH), 63.3 (d, OCH_2), 116.7 (s, CH), 121.2 (s, CH), 138.7 (s, CH), 166.3 (s, C), 181.1 (s, C). LC-MS (*ESI*): *m/z* $[M]^+$ 304.5.

3-Hydroxy-1-(((4S)-2-methoxy-2-sulfido-1,3,2-oxazaphospholidin-4-yl)methyl)pyridin-4(1H)-one (**2a**): Pale yellow crystals, yield 15%. ^{1}H-NMR ($CDCl_3$, 400 MHz) δ: 1.30 (m, 2H, CH_2CHNH), 3.31 (d, 3H, OCH_3), 3.83 (m, 1H, CHNH), 7.54 (s, 1H, CH), 8.19 (s, 1H, CH), 9.22 (s, 1H, CH). ^{13}C-NMR ($CDCl_3$) δ: 49.4 (s, CH_2CHNH), 54.9 (d, OCH_3), 58.0 (d, CHNH), 60.2 (d, CHNH), 63.1 (d, OCH_2), 115.7 (s, CH), 120.2 (s, CH), 137.7 (s, CH), 166.0 (s, C), 180.9 (s, C). LC-MS (*ESI*): *m/z* $[M]^+$ 278.1.

1-(((4S)-2-ethoxy-2-sulfido-1,3,2-oxazaphospholidin-4-yl)methyl)-3-hydroxypyridin-4(1H)-one (**2b**): Pale yellow crystals, yield 12%. ^{1}H-NMR ($CDCl_3$, 400 MHz) δ: 1.31 (m, 2H, CH_2CHNH), 3.32 (d, 3H, CH_2CH_3), 3.50 (m, 2H, OCH_2), 3.77 (m, 1H, CHNH), 7.54 (s, 1H, CH), 8.18 (s, 1H, CH), 9.23 (s, 1H, CH). ^{13}C-NMR ($CDCl_3$) δ: 15.8 (d, CH_3), 50.3 (s, CH_2CHNH), 56.1 (d, OCH2), 58.4 (d, CHNH), 59.2 (d, CHNH), 63.2 (d, OCH_2), 116.1 (s, CH), 121.0 (s, CH), 137.9 (s, CH), 166.1 (s, C), 181.5 (s, C). LC-MS (*ESI*): *m/z* $[M]^+$ 292.4.

3-Hydroxy-1-(((4S)-2-propoxy-2-sulfido-1,3,2-oxazaphospholidin-4-yl)methyl)pyridin-4(1H)-one (**2c**): Pale yellow crystals, yield 10%. ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 0.90 (m, 3H, CH_2CH_3), 1.30 (m, 2H, CH_2CHNH), 1.68 (m, 2H,CH_2CH_2), 3.28 (dd, 2H, OCH_2), 3.77 (m, 1H, CHNH), 7.56 (s, 1H, CH), 8.20 (s, 1H, CH), 9.19 (s, 1H, CH). ^{13}C NMR ($CDCl_3$) δ: 10.0 (d, CH_3), 23.3 (m, CH_2), 45.8 (s, CH_2CHNH), 55.2 (d, OCH_2), 56.8 (d, CHNH), 58.1 (d, CHNH), 63.1 (d, OCH_2), 117.7 (s, CH), 121.2 (s, CH), 138.2 (s, CH), 165.9 (s, C), 181.4 (s, C). LC-MS (*ESI*): *m/z* $[M]^+$ 306.5.

4.3.6 Topical assay against termites

An acute toxicity bioassay was performed by topical application to worker termites. A series of seven different doses (0.25, 0.5, 1.5, 2.5, 7.5, 12.5, and 25 μg/termite) was prepared in ethanol solution for each tested sample. Aliquots (0.5 μL) of diluted samples were applied topically to the abdomens of worker termites. Controls were treated with 0.5 μL ethanol only. Termites were transferred into petri dishes (4.2-cm diameter) lined with filter paper and kept in an incubator at 23–25 °C. A few drops of distilled water were supplied daily to the bottom edge of each dish to maintain moisture during the experiment. Four replicates of 20 termites each were used per treatment. Fenitrothion and rotenone were used as positive controls. Mortality of insects was evaluated after 48 h treatment. Insects were considered dead when they became immobilized and did not respond to external stimuli. LD_{50} values were calculated by probit analysis using Graphpad Prism 6.01 (GraphPad, La Jolla, CA, USA).

4.3.7 No-choice assay against termites

The no-choice bioassay method as described by Tawata et al. (1996) was used to evaluate insecticidal activity. The tested compounds were dissolved in ethanol and acetone to three concentrations (50, 100, 250 μg/mL), and applied to filter paper that was placed in petri dishes (8.5-cm diameter). Filter paper treated with ethanol and acetone was used as the control. After removing the solvent from treated filter paper by air drying at ambient temperature for 24 h, 20 termites were placed onto each paper. The dishes were covered and placed in incubator at ±23 °C. A few drops of distilled water were supplied daily to the bottom edge of each petri dish to maintain moisture during the experiment. The experiment was performed using four replicates of each

treatment and was repeated two times. Mortality was evaluated after seven days treatment. Insects were considered dead when they became immobilized and did not respond to external stimuli. Insect mortality rate was calculated as a percentage in comparison with the control group. Rotenone and fenitrothion were used as positive controls.

4.3.8 Nematicidal activity assay against Caenorhabditis elegans

The nematicidal activity assay was conducted according to previously described procedures with minor modifications (Jang et al., 2004; Solis and Petrascheck, 2011). The nematode *Caenorhabditis elegans* was cultivated on nematode growth medium (NGM) [3 g NaCl, 15 g agar, 2.5 g polypeptone, 136.1 g KH_2OP_4, 17.9 g KOH, 1 mL 1 M $MgSO_4$, 1 mL 1 M $CaCl_2$, 1 mL cholesterol (5 mg/mL), and 500 μL ampicillin (100 μg/mL)] plate covered with *Escherichia coli* strain OP50. The ampicillin-resistant OP50 was used as a feed source to prevent cross-contamination of the nematode culture. The NGM plates contained high densities of worms after 4 days incubation at 20 °C. For preparation of synchronous worm culture, worms were rinsed from the agar plate with S-basal buffer (5.85 g NaCl, 1 g K_2HPO_4, 6 g KH_2PO_4, and 1 mL cholesterol [5 mg/mL ethanol]/L), washed twice with S-basal buffer, and added to household bleach/10 N NaOH solution. The supernatant was shaken for 10–15 min, washed three times by centrifugation for 1 min at 3000 rpm, and then resuspended in S-basal buffer. One hundred microliters of worm/OP50 (1.2×10^9 bacteria/mL) solution was transferred into each well of a 96-well plate, and 20 μL sample stock solution was added to give final concentrations 25, 50, 100, 250, and 500 μM. The plate was shaken thoroughly for 2–3 min and incubated at 20 °C. Water was used as a negative control. The experiment was performed using four replicates of each treatment and was repeated two times. Dead and

active nematodes were recorded after 48 h treatment. Nematode mortality rate was calculated as a percentage in comparison with the control group.

4.3.9 Assay for acetylcholinesterase (AChE) inhibition

Whole bodies of termites (20 mg) were homogenized in 1 mL of 0.1 M phosphate buffer (pH 8.0), and the homogenate was centrifuged at 12,000 rpm for 20 min. The supernatant was used as the enzyme source. All procedural steps for preparing the crude enzyme were performed at 4 °C (Siramon et al., 2009). The AChE activity was measured using the modified Ellman's method (Yeom et al., 2012). Twenty-five microliters of sample was transferred into each well of a 96-well microplate containing 125 μL 0.1 M phosphate buffer (pH 8.0), and 30 μL of the enzyme solution was added to each well. The mixture was incubated for 10 min at 25 °C, after which 50 μL of 5,5′-dithiobis-(2-nitrobenzoic acid) (final concentration 0.4 mM) and 25 μL of acetylthiocholine iodide (final concentration 1 mM) were added. The control was treated by adding 25 μL water. Enzyme activity was measured for 20 min at 412 nm. The AChE inhibition assay was performed using four replicates of each treatment and was repeated two times. The inhibition rate of AChE activity was calculated as a percentage as follows:

$$\text{Inhibition } (\%) = (A_o - A_E)/A_o \times 100 \quad (1)$$

where A_o is the absorbance of the control, and A_E is the absorbance of the tested sample.

4.3.10 Tyrosinase inhibition assay

A microplate assay for tyrosinase inhibitory activity was performed following a previously described procedure (Tadtong et al., 2009). Samples (20 μL) with various concentrations were transferred into each well of a 96-well plate; 120 μL of 20 mM

sodium phosphate buffer (pH 6.8) and 20 μL of 500 U/mL mushroom tyrosinase enzyme dissolved in buffer were then added to each well. The mixture was incubated at 25 °C for 15 min, after which 20 μL of 0.85 mM L-tyrosine solution was added. Absorbance was recorded at 470 nm using a microplate reader (Benchmark plus; Biorad, Hertfordshire, UK). Mimosine and kojic acid were used as a positive controls. The percentage of inhibition was calculated as follows:

$$\text{Inhibition } (\%) = [(C_E - C_o) - (S_E - S_o)]/(C_E - C_o) \times 100 \quad (2)$$

Where C_E is the absorbance of the control with enzyme, C_o is the absorbance of the control without enzyme, S_E is the absorbance of the tested sample with enzyme, and S_o is the absorbance of the tested sample without enzyme.

4.3.11 Data analysis

Statistical analyses were performed using statistical analysis system (SAS) software, version 9.1.3 (SAS Institute Inc., Cary, NC, USA). Significance was assessed by one-way ANOVA analysis, and means were separated using Duncan's test at $p \leq 0.01$. All calculations were conducted in Microsoft Excel 2003. The IC_{50} values were determined graphically as the concentration of each compound that showed 50% inhibitory activity.

4.4 RESULTS AND DISCUSSION

4.4.1 Synthesis

In this study, a route for synthesis of amino alcohols and their phosphoramidothionates from mimosine was described. Mimosinol and D-mimosinol were prepared using a three-step procedure. First, tris(triethylsilyl)silyl triflate was

generated *in situ* by mixing tris(triethylsilyl)silane and triflic acid. Second, installation of the supersilyl group (tris[triethylsilyl]silyl) was achieved by treatment of mimosine with tris(triethylsilyl)silyl triflate in the presence of imidazole and DMF:CH_2Cl_2 (1:1). Finally, mimosine supersilyl ester was reduced to mimosinol and D-mimosinol using sodium borohydride or sodium borodeuteride with yields of 95% and 73.5%, respectively (**Figure 4.1**). Phosphoramidothionate derivatives were produced by the reaction of mimosinol and D-mimosinol with thiophosphoryl chloride (**Figure 4.2**). The chloride atom of intermediates was displaced by bulky alkyl group under the triethylamine catalyst to afford compounds **1a–c** (28.9%–39.6% yield) from mimosinol and compounds **2a–c** (10%–15% yield) from D-mimosinol.

4.4.2 Insecticidal activity

Insect mortality rate was evaluated by topical application and a no-choice contact method. Mimosinol and its derivatives (**1a–c**) had strong insecticidal activity with LD_{50} values in the ranges from 0.5 to 1.2 μg/insect, whereas D-mimosinol and its derivatives (**2a–c**) exhibited lower insecticidal activity (LD_{50} from 1.2 to 3.1 μg/insect) (**Table 4.1**). Among all tested compounds, compounds **1b** and **1a** showed promising activity against insects (52%–62% and 71%–88% mortality at 50 and 100 μg/mL after seven days treatment, respectively; **Table 4.2**), which is comparable to the commercial insecticide rotenone (**Tables 4.1 and 4.2**).

4.4.3 Tyrosinase and AChE inhibition

To determine insecticidal mode of action, AChE and tyrosinase inhibition was investigated. Mimosinol and its derivatives (**1a–c**) had strong AChE inhibitory activity that was significantly better than that of D-mimosinol and its derivatives (**2a–c**) (**Figure 4.3**). The IC_{50} values against AChE of compounds **1a** and **1b** were 95.9 and 104.0 μM,

respectively. Fenitrothion showed AChE inhibitory activity, with IC_{50} of 181.5 μM. Interestingly, mimosinol and D-mimosinol showed strong inhibitory activity against tyrosinase, with IC_{50} values of 31.4 and 46.1 μM, respectively. The other synthesized derivatives displayed low tyrosinase inhibitory activity (**Figure 4.4**).

4.4.4 Nematicidal activity of mimosine derivatives

The assay for nematicidal activity against *C. elegans* was conducted using the 96-well microplate method. Interestingly, mimosine had high nematicidal activity, with an IC_{50} value of 16.8 μM (**Figure 4.5**). Mimosinol and D-mimosinol showed moderate nematicidal activity, with IC_{50} values of 376.2 and 390.0 μM, respectively (**Figures 4.5 and 4.6**). Nematicidal activity differed among functional groups. Phosphoamidothionate derivatives derived from mimosinol had activity better than those derived from D-mimosinol. Compounds **1a** and **1b** exhibited particularly strong nematicidal activity, with IC_{50} values of 31.8 μM and 50.2 μM, respectively. The other derivatives had pronounced nematicidal activity.

4.4.5 Discussion

The chemical modification of amino acid has been attempted for searching a new lead compound for insecticides. Since discovery of L-leucine amino acid as a neuroactive substance in the blood of DDT-treated silk-worm larvae, a new series of insecticidal five-membered cyclic phosphoramidothionates from this amino acid and other natural ones have been developed (Eto et al., 1978). Therefore, in this study, mimosine, a natural non-protein amino acid, was used as starting material for developing new five-membered cyclic phosphoramidothionates for insecticidal and nematicidal activities. Termites were chosen as a representative insect because of their significance worldwide and particularly in Japan (Verma et al., 2009). As shown in

Table 4.1, mimosinol and D-mimosinol from mimosine had much higher insecticidal activity (> 30 times) than mimosine, suggesting that the importance of modification of –COOH group of mimosine for increasing insecticidal activity. For the mimosinol- and D-mimosinol-derived phosphoramidothionates, the alkyl group in –OR affects insecticidal activity of these compounds. The increase of alkyl side chain will lead to the reduction of insecticidal activity (**Tables 4.1 and 4.2**).

Development of insecticides requires the full understanding of molecular mechanism of the insecticides that we used into the target insects. Many organophosphate insecticides including five-membered cyclic phosphorous insecticide inhibit AChE, which results in the prolonged binding of acetylcholine to its postsynaptic receptor, leading to the death of target insects by a prolonged neuroexcitation (Tawata et al., 1978; Eto et al., 1978; Hirashima et al., 1992; Rajashekar et al., 2014). Tyrosinase, a copper-containing enzyme, is also a potential candidate for novel bioinsecticide development (Pan et al., 2011). Tyrosinase catalyzes the hydroxylation of monophenols and oxidation of *o*-diphenols to *o*-quinones which polymerise spontaneously to form dark macromolecular pigments, such as insoluble melanin. Moreover, *o*-quinones cross link with cuticular structural proteins and chitin, which result in hardening of the cuticle. Therefore, inhibition of tyrosinase could cause abnormal body softening (Bai et al., 2014). Interestingly, the new findings indicate that the insecticidal activity of mimosinol and D-mimosinol could be due to tyrosinase inhibition; therefore, mimosinol and D-mimosinol could inhibit the development of insects. It is interesting to test whether mimosinol and D-mimosinol inhibit tyrosinase expression in vivo or not. Since the insect tyrosinase was labile and easy to inactivate during purification, its inactivate precursor, prophenoloxidase (PPO), was more stable (Pan et al., 2011). Thus, it is

interesting to test whether these two compounds inhibit PPO expression in insects or not. On the other hand, in contrast, the insecticidal activity of phosphoramidothionate derivatives of mimosinol and D-mimosinol, including compounds **1a** and **1b**, could be attributed to AChE inhibition when compared to tyrosinase inhibition (**Figures 4.3 and 4.4**). **Figure 4.3** showed that AChE inhibitory activity was influenced by replacing two hydrogens at the C_5-position. One plausible explanation is that the functional group at C_5-position may contribute to the interaction with the insect nerve.

Nematodes attack a wide variety of organisms and present a major challenge to humans and agriculture (Tawata et al., 1980). The results showed that mimosine and compounds **1a** and **1b** are promising compounds for nematicide. The nematicidal activity was influenced by substituent of C_5-position rather than the alkyl group (-OR) (**Figures 4.2 and 4.6**).

In fact, the biological activities of cyclic phosphoramidates in general are greatly affected by the ring size, the exocyclic ester group and alkyl group at C_4-position including the configuration there. The five-membered ring is necessitated for the biological activities (Eto et al., 1978). The investigation on structure-activity relationship has seen that other interesting factors affect considerably the activity of five-membered cyclic phosphoramidothionates. It was found that the length of the alkyl chain (-OR) was inversely proportional to insecticidal and nematicidal activities. Substitution of two hydrogens at the C_5-position on five-membered ring would also lead to reduced insecticidal and nematicidal activities.

4.5 CONCLUSION

In conclusions, this report provides the first evidence for insecticidal and nematicidal activities of novel mimosine derivatives. Mimosinol and D-mimosinol could be potential bioinsecticides which had strong insecticidal activity by inhibiting tyrosinase. Among phosphoramidothionate derivatives prepared, compounds **1a** and **1b** showed promise as having both of these activities. These findings also indicate that mimosine was a good nematicidal compound. Appropriate length of the alkyl chain and the functional group at the C_5-position of phosphoramidothionates derived from mimosinol and D-mimosinol are important for conferring insecticidal and nematicidal activities. These results introduce mimosine as previously unexplored scaffold of insecticide and nematicide.

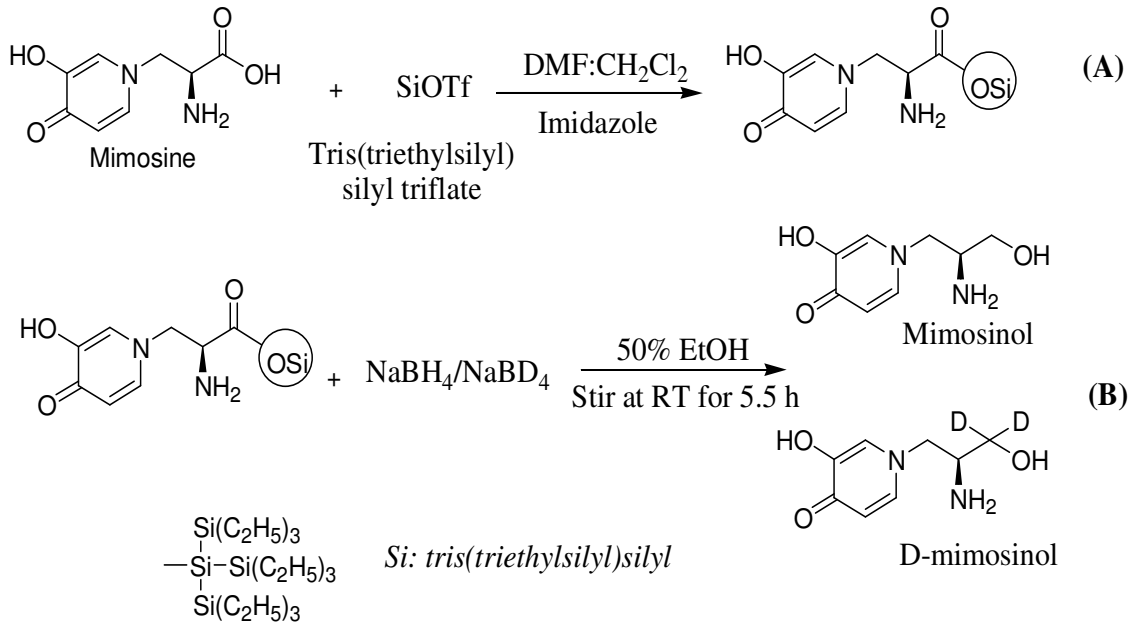

Figure 4.1. Scheme for reduction of mimosine into mimosinol and D-mimosinol. (A) Installation of tris(triethylsilyl)silyl group into mimosine, and (B) reduction of mimosine ester to mimosinol and D-mimosinol.

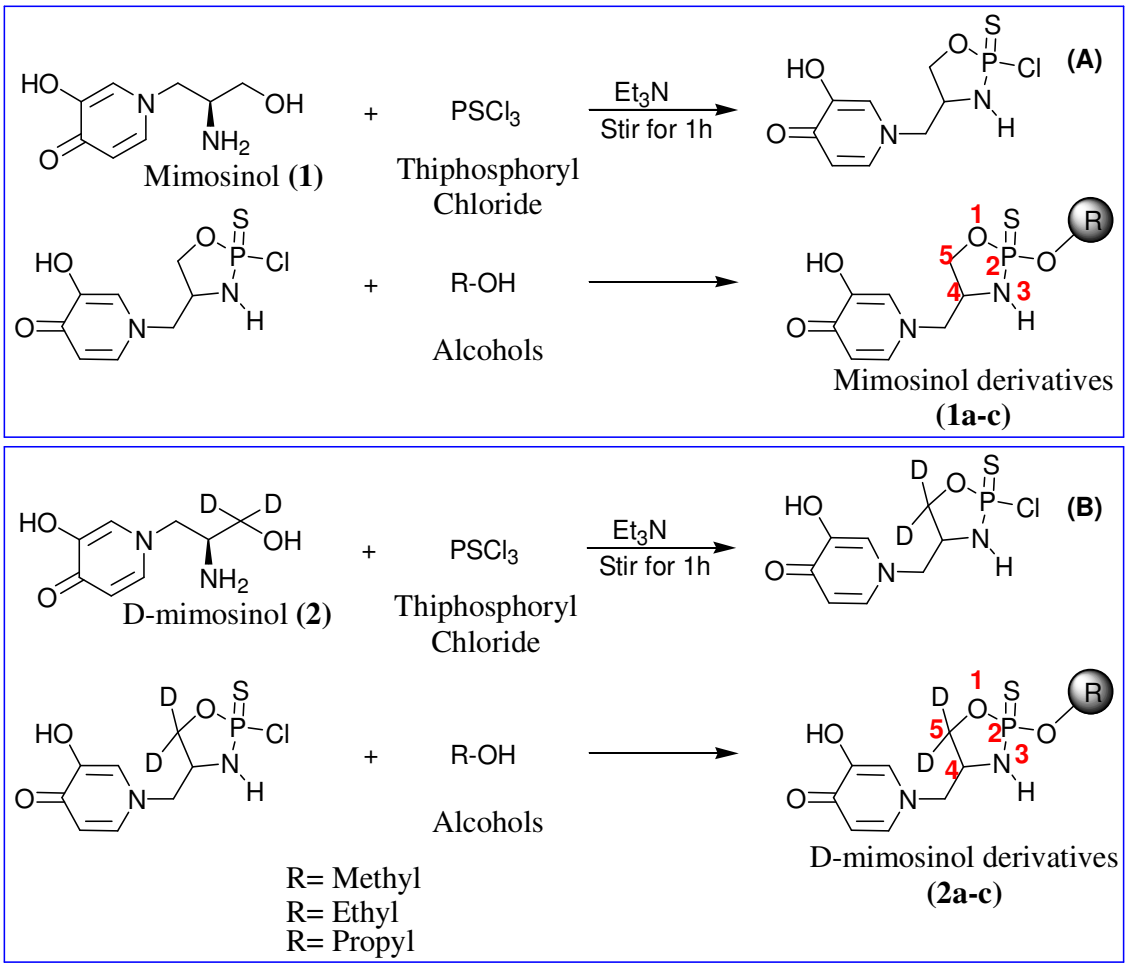

Figure 4.2. General route for synthesis of phosphoramidothionate derivatives. (A) Scheme for synthesis of derivatives from mimosinol. (B) Preparation of derivatives from deuterated mimosinol (D-mimosinol).

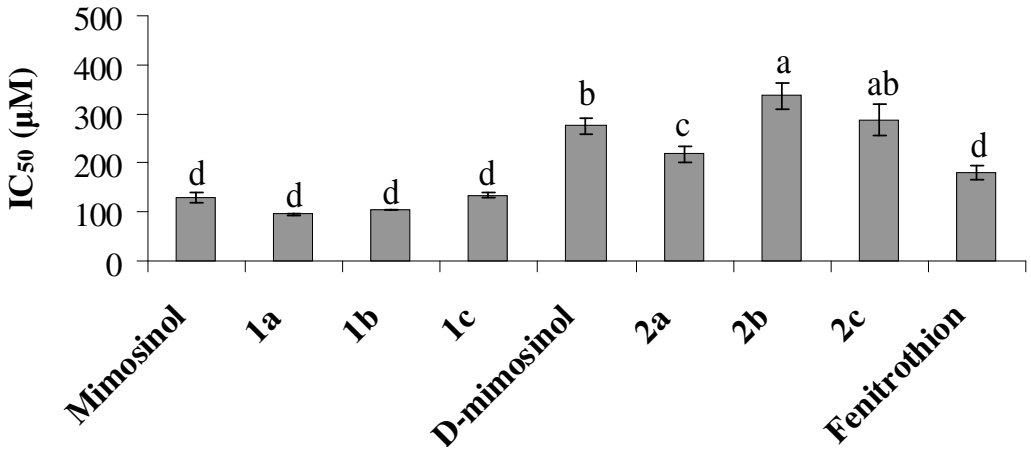

Figure 4.3. Inhibition of acetylcholinesterase (AChE) activity from termite whole bodies of mimosinol, deuterated mimosinol (D-mimosinol) and their derivatives (**1a**–**c**, **2a**–**c**). Values are means of two independent experiments ± SE. IC_{50} value represents 50% inhibition of AChE activity of tested compounds. The different letters above bars represent significant differences statistically at $p \leq 0.01$. Mimosine and kojic acid were not active for AChE inhibition with IC_{50} of 1528 and 5477 µM, respectively.

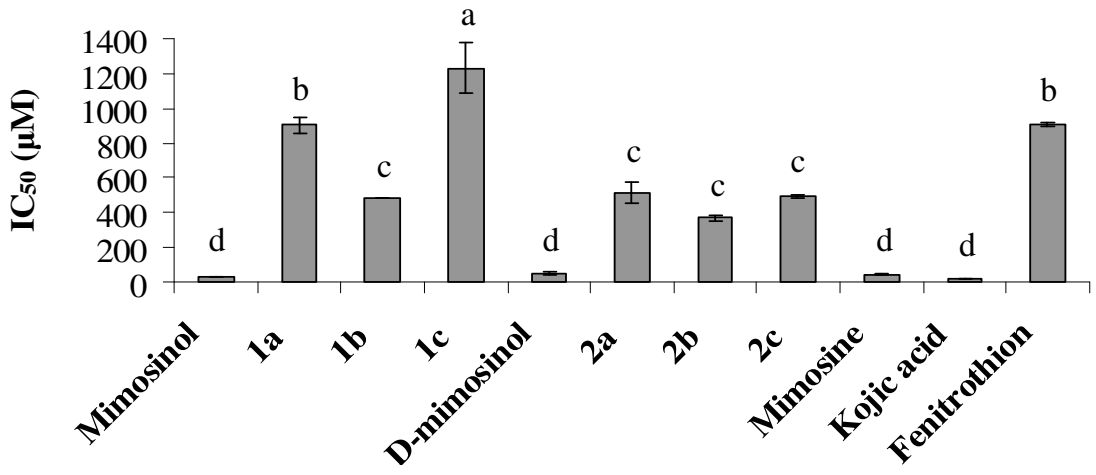

Figure 4.4. Inhibition of mimosinol, deuterated mimosinol, and their derivatives (**1a**–**c**, **2a**–**c**) against tyrosinase. Values are means of two independent experiments ± SE. IC_{50} value represents 50% inhibition. The different letters above bars represent significant differences statistically at $p \leq 0.01$.

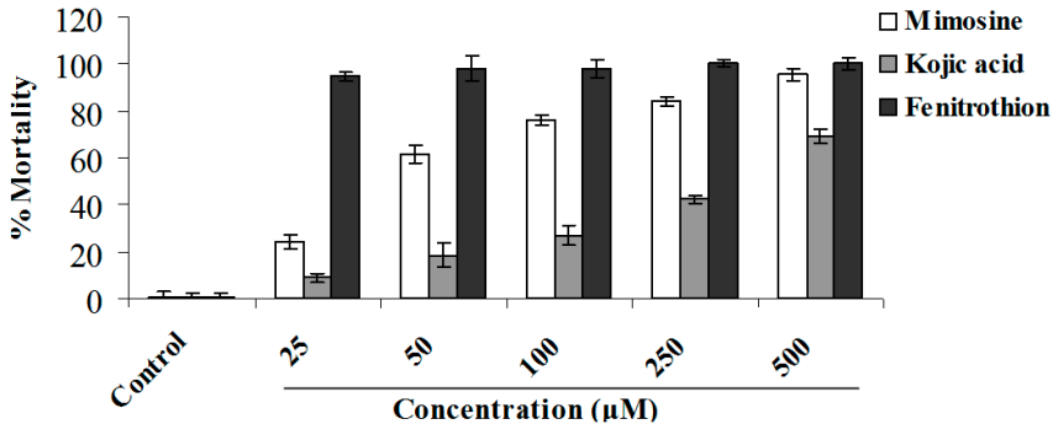

Figure 4.5. Nematicidal activity of mimosine against *Caenorhabditis elegans*. Dead and active nematodes were recorded after 48 h treatment. Nematode mortality rate was calculated as a percentage relative to that recorded in the control group. Values are means of two independent experiments ± SE. Mimosine and kojic acid had the IC_{50} values of 16.8 and 327.9 µM, respectively.

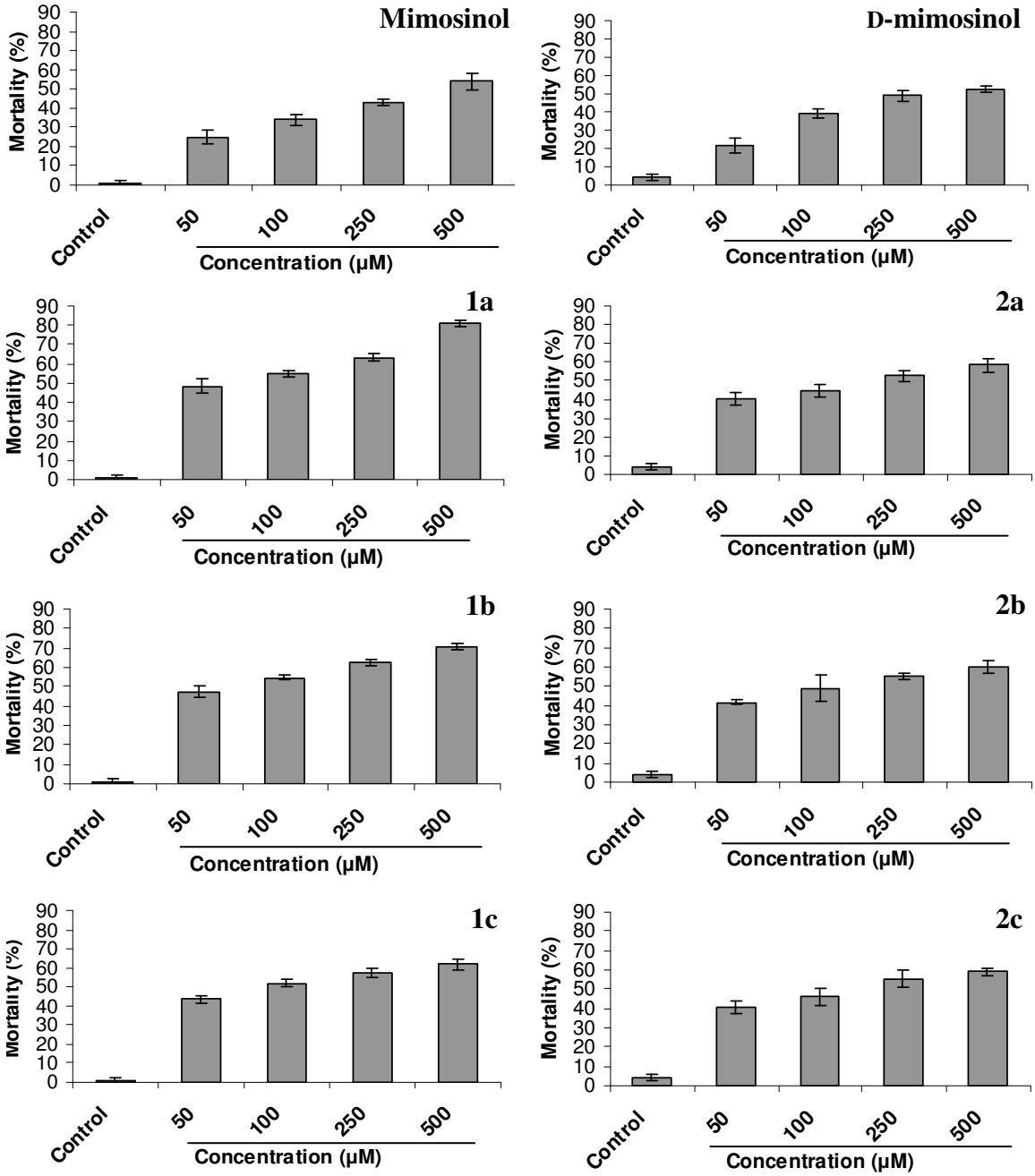

Figure 4.6. Nematicidal activity of mimosinol and its derivatives (**1a**, **1b**, **1c**), deuterated mimosinol (D-mimosinol) and its derivatives (**2a**, **2b**, **2c**) against *Caenorhabditis elegans*. Dead and active nematodes were evaluated after 48 h treatment. Nematode mortality rate was calculated as a percentage relative to that recorded in the control group. Values are means of two independent experiments ± SE. The IC_{50} values of mimosinol (376.2 μM), **1a** (31.8 μM), **1b** (50.2 μM), and **1c** (116.1 μM). The IC_{50} values of D-mimosinol (390.0 μM), **2a** (251.6 μM), **2b** (186.7 μM), and **2c** (218.1 μM).

Table 4.1

LD_{50} values of mimosine derivatives against termites by topical application

Compound	LD_{50} (μg/insect)[i]
Mimosinol	1.2 ± 0.2 de
1a	0.5 ± 0.1 fg
1b	0.7 ± 0.1 ef
1c	0.8 ± 0.1 ef
D-mimosinol	1.8 ± 0.3 d
2a	1.2 ± 0.2 de
2b	2.1 ± 0.4 c
2c	3.1 ± 0.5 b
Mimosine	54.1 ± 5.9 a
Rotenone	0.8 ± 0.2 ef
Fenitrothion	0.025 ± 0.0 g

[i] Different letters in the same column indicate the existence of significant difference statistically. Data have statistical significance at $p \leq 0.01$. The results are means ± SE of four replications for each treatment.

Table 4.2

Insecticidal activity of mimosine derivatives using a no-choice contact method

Compound	Mortality (%)		
	250 μg/mL[ii]	100 μg/mL	50 μg/mL
Mimosinol	68.5 ± 0.6 c	52.5 ± 2.7 de	38.1 ± 1.3 cde
1a	90.6 ± 1.3 a	88.8 ± 0.9 a	62.5 ± 1.8 a
1b	84.4 ± 2.2 ab	71.9 ± 1.3 b	52.5 ± 3.5 ab
1c	77.5 ± 0.9 b	59.4 ± 1.3 cd	45.6 ± 0.4 bc
D-mimosinol	60.6 ± 0.6 cd	53.8 ± 3.8 de	33.1 ± 1.9 de
2a	82.5 ± 1.3 ab	70.6 ± 0.6 b	48.8 ± 2.5 bc
2b	63.8 ± 0.0 c	52.5 ± 2.5 de	42.5 ± 2.5 bcd
2c	52.5 ± 3.8 d	44.4 ± 1.9 e	29.4 ± 1.9 e
Mimosine	9.1 ± 2.0 e	7.5 ± 1.0 f	2.5 ± 1.1 f
Rotenone	80.0 ± 0.9 b	68.1 ± 1.3 bc	48.8 ± 0.9 bc
Compound	Mortality (%)		
	15 μg/mL	10 μg/mL	5 μg/mL
Fenitrothion	83.8 ± 1.9	69.4 ± 0.6	26.9 ± 1.3

[ii] Compound dose. Data have statistical significance at $p \leq 0.01$. Different letters in the same column indicate the existence of significant difference statistically. The results are means ± SE of two independent experiments with four replications for each treatment.

CHAPTER 5

MIMOSINE DIPEPTIDE ENANTIOMERS: IMPROVED INHIBITORS AGAINST MELANOGENESIS AND CYCLOOXYGENASE

5.1 SUMMARY

Melanogenesis plays an important role in the protection of skin against UV through production of melanin pigments, but abnormal accumulation of this pigment causes unaesthetic hyperpigmentation. Much effort is being made to develop effective depigmenting agents. Here, it is the first time to indicate that a small library of mimosine dipeptide enantiomers (Mi-L/D-amino acid) inhibit the melanogenesis in B16F10 melanoma cells by down-regulating the cellular tyrosinase with little effect on their growth or viability. Two of them, Mi-D-Trp and Mi-D-Val, turned out to be the most potent inhibitors on melanin content and cellular tyrosinase in B16F10 melanoma cells. In addition, most of the mimosine dipeptides were more potent than mimosine for inhibiting cyclooxygenase 1 (COX-1) with IC_{50} of 18–26 μM. Among them, Mi-L-Val and Mi-L-Trp inhibited cyclooxygenase 2 (COX-2) more potently than indomethacin, with IC_{50} values of 22 and 19 μM, respectively. Taken together, these results suggest the possibility that mimosine dipeptides could be better candidates (than mimosine) for anti-melanogenic (skin hyperpigmentation treatment) and cyclooxygenase (COX) inhibition.

5.2 INTRODUCTION

Melanogenesis is a physiological process that results in the synthesis of melanin pigment and has many functions in living systems (Jiang et al., 2011). Melanin is an important factor for protection of skin against deleterious UV irradiation. However, the excessive production of melanin causes hyperpigmentation (Lee et al., 2013) that is a skin

pigmentation disorder in which skin becomes darker in color compared to the normal surrounding skin (Seo et al., 2012). Tyrosinase is a key enzyme in melanogenesis in melanocytes (Kamei et al., 2009). It acts as the catalyst for two rate-limiting steps of melanogenesis, the hydroxylation of tyrosine to 3,4-dihydroxyphenylalanine (DOPA) and oxidation of DOPA to dopaquinone (Hearing and Tsukamoto, 1991). Therefore, the inhibition of tyrosinase activity is capitalized for inhibition of melanogenesis and the treatment of skin hyperpigmentation and whitening (Lee et al., 2013).

Nonsteroidal anti-inflammatory drugs (NSAIDs) have many therapeutic benefits in the treatment of rheumatoid arthritis and other inflammatory conditions (Gautam et al., 2011). The major action of NSAIDs is based on inhibition of cyclooxygenase (COX), which is the rate limiting enzyme in the pathway facilitating the conversion of arachidonic acid to inflammatory prostaglandins (Ulbrich et al., 2002). There are two different isoforms of COX, COX-1 and COX-2 (Jain et al., 2006). COX-1 is expressed in essentially all mammalian tissues, and is responsible for prostaglandin production that maintains homeostasis. In contrast, COX-2 is manifested constitutively in brain, kidneys, and ovaries, and is activated in cells in response to inflammatory stimuli (Vitale et al., 2013; Gierse et al., 2008).

Mimosine [β-[*N*-(3-hydroxy-4-oxypyridyl)]-α-aminopropionic acid] is a non-protein amino acid containing an alanine side chain bound to the nitrogen atom of a hydroxy pyridone ring. It is found in several tropical and subtropical plants, which possesses a wide range of biological activities (Tawata et al., 2008; Upadhyay et al., 2011). It is hypothesized that mimosine could be used as a source of bioactive compounds and could be useful in the design of novel drugs. Recently, a series of mimosine tetrapeptides are more effective than mimosine to inhibit neuraminidase and

tyrosinase as well as the oncogenic/ageing kinase PAK1 (Upadhyay et al., 2011b; Nguyen et al., 2014). Interestingly, melanogenesis and COX activation are among a variety of PAK1-dependent phenomena (Nguyen et al., 2014). Thus, in continuation of earlier work, a small library of enantiomeric mimosine dipeptides were synthesized, and found that they inhibit at least COX and melanogenesis in B16F10 melanoma cells more effectively than mimosine.

5.3 MATERIALS AND METHODS

5.3.1 Chemicals and reagents

Fmoc-L-amino acids were purchased from Hipep Laboratories (Kyoto, Japan) whereas Fmoc-D-amino acids were obtained from Sigma-Aldrich (Tokyo, Japan). *N*-[9-fluorenylmethoxycarbonyloxy]succinimide (Fmoc-Osu), 2-[1*H*-benzotriazole-1-yl]-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) were from Novabiochem (Hohenbrunn, Germany). Wang resin (1% DVB), *N*,*N*-diisopropylcarbodiimide (DIC), *N*,*N*-diisopropylethylamine (DIPEA), and 1-hydroxy-1*H*-benzotriazole (HOBt), Dulbecco's modified minimum essential medium (D-MEM) were purchased from Wako Pure Chemical Industries (Osaka, Japan). Thiazolyl blue tetrazolium bromide (MTT) were obtained from Sigma-Aldrich (Tokyo, Japan). Fetal bovine serum (FBS) was purchased from HyClone Laboratories Inc. (Victoria, Australia). Penicillin/streptomycin was obtained from Lonza Walkersville, Inc. (City (Walkersville), MD, USA). Mushroom tyrosinase was obtained from Sigma-Aldrich (City (St. Louis), MO, USA). Unless otherwise mentioned, all reagents used were of analytical grade and were obtained from Wako Pure Chemical Industries, Ltd. and

Kanto Chemical Co., Inc., Japan. The ^{1}H- and ^{13}C-NMR spectra were recorded on a JEOL JNM-ECA400 (JEOL, Tokyo, Japan). Chemical shifts are expressed in parts per million (δ) relative to tetramethylsilane (TMS).

5.3.2 Mimosine isolation from Leucaena leucocephala leaves

Samples of *Leucaena leucocephala* leaves were collected near the Faculty of Agriculture, University of the Ryukyus, located at 26°N, 127°E. Fresh leaves (1.5 kg) were boiled in 5 L of water for 10 min. The cooled liquid extract was sieved by suction filtration (Shaking Baths SB-20, As One, Osaka, Japan), and the filtrate was mixed with ion-exchange resin (2 kg). After stirring for 30 min, the mixture was incubated overnight. The resin was rinsed with distilled water 5–6 times and added dropwise to 5 L of 80% ethanol to remove the chlorophyll. Mimosine was removed from the resin by dropwise addition of 6 L of 2 N NH_4OH. The liquid extract was concentrated to a final volume of 300 mL at 40 °C under reduced pressure. The solution was adjusted to pH 4.5–5.0 with 6 N HCl, and mimosine was precipitated at 4 °C overnight. The resulting mimosine was recrystallized using 5 N NaOH (pH 9.0) and 6 N HCl (pH 4.5–5.0), then allowed to stand at 4 °C to form pure mimosine. Mimosine was stored at −20 °C for further analysis (Tawata, 1990). Mimosine was identified by LC-MS (ESI-): *m/z* $[M + H]^+$ 199.1.

5.3.3 Preparation of Fmoc-Mimosine

Mimosine (2.5 g) and sodium carbonate (Na_2CO_3) (2.75 g) were dissolved in distilled water (37.5 mL). Fmoc-Osu (6.25 g) dissolved in 37.5 mL of 1,4-dioxane was added dropwise to the solution and stirred for 20 h at room temperature. Next, 150 mL of Na_2CO_3 (0.1 M) was added. The mixture was stirred for 7 h at 26 °C and was then filtered and washed with 75 mL of ethyl acetate to remove excess Fmoc-Osu and by-

products. The water fraction was kept in an ice bath and adjusted to pH 4.0 using 6 N HCl and incubated overnight at 4 °C. The resulting precipitate was filtered, washed with distilled water, and dried under reduced pressure to give Fmoc-mimosine (Upadhyay et al., 2011b).

5.3.4 General procedure for synthesis of mimosine dipeptides

Fmoc-amino acids (L- or D-form) (2.35 mmol) were dissolved in a solution of HOBt (2.35 mmol, 360 mg) and DIC (2.35 mmol, 365 μL), followed by preactivation in 5 mL of dimethylformamide (DMF). The solution was then added to Wang resin (0.5 g) which was swollen in 2 mL of dichloromethane (CH_2Cl_2) for 30 min. 4-Dimethylaminopyridine (DMAP) (0.235 mmol, 29 mg) was then suspended in 500 μL of DMF and added to the mixture prior to stirring for 3 h at room temperature. This procedure was repeated twice, and the resin was washed with DMF, CH_2Cl_2, methanol (MeOH), and dried under reduced pressure. A small portion of the resin (5 mg) was used for analysis of Fmoc-group content. Fmoc protection was removed by adding 25% piperidine and shaking for 45 min at room temperature. To couple the mimosine to the amino acids, a solution of Fmoc-mimosine (4 equiv relative to amino acid on resin), HOBt (4 equiv.), HBTU (3.6 equiv.), and DIPEA (8 equiv.) was added to the amino acid-resin suspended in DMF, and the mixture was stirred at room temperature for 1 h. The Kaiser's test was used to assess the completeness of the coupling reaction. The final cleavage was performed by shaking the resin vigorously in 95% trifluoroacetic acid (TFA) for 90 min and then filtering and washing with TFA. Ice-cold diethyl ether was used to precipitate the combined washings. The resulting precipitate was filtered, washed with diethyl ether, and dried under a vacuum to obtain the desired mimosine dipeptides. All crude peptides were a white solid, and their yields are presented in **Table**

5.4. All samples were purified using high-performance liquid chromatography (HPLC) (Shimadzu, Kyoto, Japan) before being used in enzyme inhibition assays. The major peaks were collected using a Phenomenex (150 mm × 4.6 mm; 4 μm) column with mobile phase of solvent A (0.1% TFA in water) and solvent B (0.1% TFA in acetonitrile). The flow rate and absorbance wavelength were set at 1 mL/min and 210 nm, respectively. The purified compounds were identified using LC-MS (ESI-): *m*/*z* [M – H]$^+$ 344.2, 344.3, 268.1, 268.3, 383.2, 382.3, 296.2, 296.4, 294.1, 294.4 for Mi-L-Phe, Mi-D-Phe, Mi-L-Ala, Mi-D-Ala, Mi-L-Trp, Mi-D-Trp, Mi-L-Val, Mi-D-Val, Mi-L-Pro, and Mi-D-Pro, respectively. Representative data for some of the synthesized mimosine dipeptides are as follows.

Data for Mi-L-Phe. ^{1}H-NMR (400 MHz, D_2O) δ: 3.27–3.14 (dd, 2H, CH_2), 3.80 (s, 1H, CH-NH_2), 3.99 (q, 1H, CH-NH_2), 4.17 (q, 1H, CH), 6.59 (d, 1H, CH), 7.33 (s, 1H, CH), 7.38 (s, 1H, CH), 7.43 (s, 1H, CH), 7.65 (s, 1H, CH). ^{13}C-NMR δ: 36.30, 55.99, 71.12, 97.49, 114.79, 127.65, 129.07, 129.32, 135.05, 140.2, 148.2, 173.89, 181.52. LC-MS (ESI): m/z 344.2 [M – H]$^+$.

Data for Mi-L-Ala. ^{1}H-NMR (400 MHz, D_2O) δ: 1.48 (d, 3H, CH_3), 3.78 (dd, 1H, CH-NH_2), 4.20–4.17 (q, 1H, CH-NH_2), 4.49–4.45 (q, 1H, CH_2), 4.50–4.64 (q, 1H, CH), 6.60 (d, 1H, CH), 7.67 (s, 1H, CH). ^{13}C-NMR δ: 16.08, 42.80, 50.47, 97.79, 114.82, 127.65, 129.07, 130.70, 138.52, 140.2, 148.2, 175.75, 181.55. LC-MS (ESI): m/z 268.1 [M – H]$^+$.

Data for Mi-L-Trp. ^{1}H-NMR (400 MHz, D_2O) δ: 3.27 (d, 2H, CH_2), 3.32 (d, 2H, CH_2), 3.50 (q, 1H, CH-NH_2), 4.05 (q, 1H, CH-NH_2), 4.15 (q, 1H, CH), 4.45 (q, 1H, CH), 6.57 (d, 1H, CH), 7.17 (s, 1H, CH), 7.26 (d, 1H, CH), 7.74–7.30 (m, 4H, benzene). ^{13}C-NMR δ: 26.32, 48.07, 55.00, 68.44, 90.77, 98.99, 107.04, 111.89, 118.39, 119.41,

122.08, 124.97, 132.77, 136.34, 138.31, 148.2, 174.43, 181.52. LC-MS (ESI): m/z 383.2 $[M - H]^+$.

5.3.5 Tyrosinase inhibition assay

A microplate assay was used to assess tyrosinase inhibition, as previously described (Tadtong et al., 2009). Samples (20 μL) with various concentrations were transferred into each well of 96-well plate, and 120 μL of 20 mM sodium phosphate buffer (pH 6.8), 20 μL of 500 U/mL mushroom tyrosinase enzyme dissolved in buffer were added. The mixture was incubated at 25 °C for 15 min, followed by addition of the reaction substrate 20 μL of 0.85 mM L-tyrosine solution. The absorbance was recorded at 470 nm using a microplate reader (Benchmark plus, Biorad, Herfordshire, UK). Kojic acid was used as the positive control. For the kinetic studies of selected inhibitors, we used a range of L-tyrosine concentrations for each inhibitor concentration. Preincubation and measurement times were the same as above. Lineweaver-Burk plots were used to determine the type of enzyme inhibition, and the inhibition constant K_i was determined by plotting K_m/V_{max} versus the inhibitor concentration. Percent inhibition was calculated using the following equation:

$$\text{Inhibition } (\%) = ([C_E - C_o] - [S_E - S_o])/(C_E - C_o) \times 100 \quad (1)$$

where C_E is the absorbance of the control samples with enzyme, C_o is the absorbance of the control samples without enzyme, S_E is the absorbance of the tested sample with enzyme, and S_o is the absorbance of the tested sample without enzyme.

5.3.6 Cell culture

Mouse B16F10 cell line was obtained from American Type Culture Collection (Manassas, VA, USA). Cells were cultured in Dulbecco's modified minimum essential

medium (D-MEM) medium containing 10% fetal bovine serum (FBS), 1% penicillin/streptomycin in a humidified atmosphere with 5% CO_2 incubator at 37 °C.

5.3.7 Cell viability assay

The assay as described by Campos et al. (2013) is based upon the cleavage of the yellow tetrazolium salt MTT to give purple formazan crystals. Cells were seeded in a 96-well plate at a density 5×10^3 cells/well and cultured for 24 h before the compounds (10–500 μM) were added to the medium containing D-MEM supplemented with 10% FBS, 1% penicillin/streptomycin. Cells were incubated in a humidified atmosphere 5% CO_2 at 37 °C for 48 h. Afterward, 20 μL of MTT solution (0.5 mg/mL) were added to each well and plates were incubated for 3 h. The medium was removed and formazan was dissolved in 200 μL of DMSO. The plates were shaken for 10 min and cell viability was assessed by measuring the absorbance at 570 nm using microplate reader (Benchmark plus, Biorad, Hertfordshire, UK). Kojic acid (500 μM) was used as positive control. DMSO was used as the blank. Absorbance of sample and control subtracts the absorbance of blank. The corrected absorbance of sample divides the corrected absorbance of control, then multiplies 100% to give the percentage of cell viability.

5.3.8 Determination of melanin content in B16F10 cells

The assay was performed following previously described procedure. Cells (5×10^3 cells/well) were seeded in a 96-well plate and cultured for 24 h. Compounds (10–500 μM) were added and incubated at 37 °C for 48 h. The medium was replaced by a fresh medium containing the same concentration of compound and the cells were incubated for 48 h. The medium was removed and the cells were dissolved in 100 μL of

1 N NaOH. The reaction was heated at 90 °C for 1 h. Melanin content was estimated by the absorbance at 400 nm (Hosoya et al., 2012).

5.3.9 Intracellular tyrosinase inhibition assay

Cells were seeded in a 96-well plate at a density 5×10^3 cells/well and the compounds (10–500 μM) were added after 24 h treatment. Cells were incubated for 48 h, washed two times with 50 mM of ice-cold phosphate buffer (pH 6.8), lysed with 90 μL of 50 mM phosphate buffer (pH 6.8) containing 1% Triton-X, and frozen at −80 °C for 30 min. After thawing and mixing, 20 μL of 0.5% L-DOPA was added to each well. The mixture was incubated for 2 h at 37 °C and absorbance was measured at 490 nm (Campos et al., 2013).

5.3.10 Cyclooxygenase (COX) inhibition assay

Inhibition of COX activity was measured using a colorimetric COX (ovine) inhibitor screening assay kit (Cayman Chemical, Ann Arbor, Michigan, USA). First, 150 μL of assay buffer (0.1 M Tris-HCl, pH 8.0), 10 μL of heme, and 10 μL of enzyme (COX-1 or COX-2) were added to an anti-rabbit IgG coated 96-well microtiter plate. Immediately, 10 μL of sample was added to inhibitor wells while 10 μL of solvent or buffer was added to control and background wells, respectively. After incubation for 5 min at 25 °C, 20 μL of the colorimetric substrate solution and 20 μL of 22 mM arachidonic acid were added to all the wells. The mixture was shaken for a few seconds and incubated for an additional 5 min at 25 °C. The absorbance was determined at 590 nm using a microplate reader (Benchmark plus, Biorad, Herfordshire, UK). Indomethacin was used as the positive control. The corrected absorbance of all the samples and controls was calculated by subtracting the absorbance of the background wells. The percentage of inhibition was calculated as follows:

$$\text{Inhibition } (\%) = (A_o - A_s)/A_o \times 100 \quad (2)$$

where A_o and A_s are the absorbance of the corrected control and sample, respectively.

5.3.11 Data analysis

All statistical analyses were performed using statistical analysis system (SAS) software, version 9.1.3 (SAS Institute Inc., Cary, CA, USA). Data were analyzed by one-way analysis of variance (ANOVA) with Duncan's *post hoc* test ($p \leq 0.01$). All calculations were performed using Excel, Microsoft Office 2003. The IC_{50} values were determined graphically.

5.4 RESULTS AND DISCUSSION

5.4.1. Synthesis and tyrosinase inhibition of mimosine dipeptides

It has been reported that the attachment of amino acids to kojic acid has generated compounds that strongly inhibit tyrosinase (Noh et al., 2009). Like kojic acid, mimosine is a tyrosine analog and could potentially be used for designing more potent tyrosine inhibitors (Upadhyay et al., 2011b). Thus, I developed mimosine dipeptides and assessed their ability to inhibit tyrosinase. The general strategy for the design of these compounds was based on the conjugation of mimosine and an amino acid through solid-phase synthesis using Fmoc chemistry. Ten mimosine dipeptides were synthesized using enantiomeric amino acids, including phenylalanine (Phe), alanine (Ala), valine (Val), tryptophan (Trp), and proline (Pro). As shown in **Figure 5.1**, L- or D-amino acids were attached to Wang resin support. Mimosine was converted to Fmoc-mimosine which was then coupled to the amino acid to obtain the desired mimosine dipeptides. The chemical structures of mimosine and mimosine dipeptides are shown in **Figure 5.2**.

The in vitro assay for tyrosinase inhibition was performed using L-tyrosine as a substrate. As expected, the synthesized mimosine dipeptides inhibited tyrosinase more potently than mimosine (**Table 5.1**). In particular, conjugation of tryptophan, valine, and proline or of a D-form amino acid to mimosine led to stronger tyrosinase inhibition. Of the four most potent inhibitors, the IC_{50} values of Mi-L-Pro and Mi-D-Trp were 13 and 17 μM, respectively. The IC_{50} of Mi-L-Val and Mi-D-Val against tyrosinase was 12 and 10 μM, marginally lower than that of the positive control, kojic acid (14 μM). Because of their potent inhibition of tyrosinase, we carried out a kinetic analysis of these four compounds. Lineweaver-Burk plots of 1/[V] versus 1/[S] are shown in **Figure 5.3**. The kinetic study of tyrosinase inhibition by these selected compounds revealed that the K_m value was increased as the concentration of inhibitors was increased, while the V_{max} values remained unchanged. Therefore, like mimosine, these compounds are considered to be competitive inhibitors of tyrosinase (Cabanes et al., 1987). The K_i value of these compounds are shown in **Table 5.2**. The inhibition constant of Mi-D-Val (0.02 mM) was less than that of Mi-L-Val, Mi-L-Pro, Mi-D-Trp, suggesting that Mi-D-Val has the most potent inhibitory effect.

5.4.2. Inhibition of melanogenesis by mimosine dipeptides

As mentioned above, the results showed that a small library of mimosine dipeptides had strong tyrosinase inhibition in vitro. Thus, it is hypothesized that these compounds could exhibit melanogenesis inhibition in B16F10 melanoma cells. First, the effect of mimosine dipeptides was examined on cell survival by the thiazolyl blue tetrazolium bromide (MTT) assay, and found that none of them exhibited any significant cytotoxicity up to 100 μM. To determine the anti-melanogenic activity, their effect was measured on the melanin content in B16F10 cells. Generally, mimosine

dipeptides reduced the melanin content more effectively than mimosine. Four of them (Mi-L-Pro, Mi-D-Val, Mi-D-Trp, Mi-L-Trp) exhibited strong inhibition on melanin content, with IC_{50} values ranging 109-116 μM, compared with mimosine (IC_{50} around 226 μM) (**Table 5.3**). To examine the possible mechanism underlying their anti-melanogenic act, cellular tyrosinase activity was evaluated. The results indicated that all of them strongly inhibited intracellular tyrosinase activity with IC_{50} values ranging from 145–194 μM when compared to mimosine (IC_{50} around 388 μM) (**Table 5.3**). Mi-D-Trp and Mi-D-Val are the most potent inhibitors on melanin content and cellular tyrosinase in B16F10 melanoma cells. Taken together, their anti-melanogenic effect exerted by their cellular tyrosinase suppression in B16F10 cells. As shown in **Table 5.3**, all prepared mimosine dipeptides had more anti-melanogenic activity than mimosine. When the C-terminus of mimosine was converted to the amide form, anti-melanogenic activity became better than that of the free acid form, suggesting the importance of C-terminal amide form in mimosine-peptide conjugates. The RAC/CDC42-activated kinase 1 (PAK1) is responsible for a variety of diseases/disorders including cancers, neurofibromatosis (NF), diabetes (type 2), hypertension, short lifespan and neurodegenerative diseases such as Alzheimer's (AD) and Huntington's disease. Since PAK1 is not essential for the growth of normal cells, blocking PAK1, per se, does not cause any side effects (Maruta, 2014). Recently, it is demonstrated that PAK1 is essential for the melanogenesis in skin cells such as the melanoma B16F10 (Be Tu et al., 2015). Moreover, in the previous study, three mimosine tetrapeptides, which directly inhibit oncogenic/kinase PAK1 (Nguyen et al., 2014), are anti-melanogenic (Nguyen et al., 2015). Thus, it is most likely that mimosine dipeptides also could inhibit

melanogenesis by blocking PAK1. It is interesting to test whether mimosine dipeptides inhibit PAK1 in B16F10 cells or not.

5.4.3. Inhibition of cyclooxygenase (COX) by mimosine and their dipeptides

During screening for novel targets of mimosine, it was found that cyclooxygenase (COX) was inhibited by mimosine. As shown in **Table 5.4**, mimosine inhibited COX-1 and COX-2 with IC_{50} values of 29 and 21 μM, respectively. These results indicated that mimosine inhibits COX-2 more potently than COX-1. Mimosine was also a more potent inhibitor of COX-2 than indomethacin (IC_{50} = 28 μM). Zebardast et al. have reported that inhibition of gastroprotective prostaglandins synthesis through COX-1 pathway will lead to the gastrointestinal side effects associated with NSAIDs (Zebardast et al., 2009). Therefore, selective inhibition of COX-2 over COX-1 may be useful for treating inflammation and related disorders, as well as reducing gastrointestinal toxicities. Moreover, mimosine inhibits the transcription and translation of macrophage inflammatory protein 2 (MIP-2) and monocyte chemotactic protein 1 (MCP-1), which can induce inflammatory diseases through the recruitment of neutrophils and mononuclear cells, respectively (Conti et al., 2002). These data, in addition to previous results, suggest that mimosine may be a promising anti-inflammatory compound. Because mimosine inhibited cyclooxygenases, the effect of mimosine dipeptides on these enzymes was also explored. It was found that most of the synthesized dipeptides were more potent inhibitors of COX-1 than mimosine. The IC_{50} values of the six investigated compounds ranged as much as 18–26 μM as compared with the value of mimosine (29 μM). Mi-L-Val and Mi-L-Trp inhibited COX-2 more potently than indomethacin, with IC_{50} values of 22 and 19 μM,

respectively. In both the COX-1 and COX-2 assay, Mi-L-Trp was the most potent inhibitor among the synthesized dipeptides.

5.5 CONCLUSION

This study provides the first evidence of melanogenesis inhibition of a small library of mimosine dipeptide enantiomers. Their anti-melanogenic activity is due to intracellular tyrosinase inhibition with little effect on the growth or viability of B16F10 melanoma cells. In addition, some of these dipeptides inhibited more strongly COX-1/2 than mimosine. The results suggest that these new compounds may be the better candidates for anti-melanogenics (skin hyperpigmentation therapeutics) and COX inhibitors.

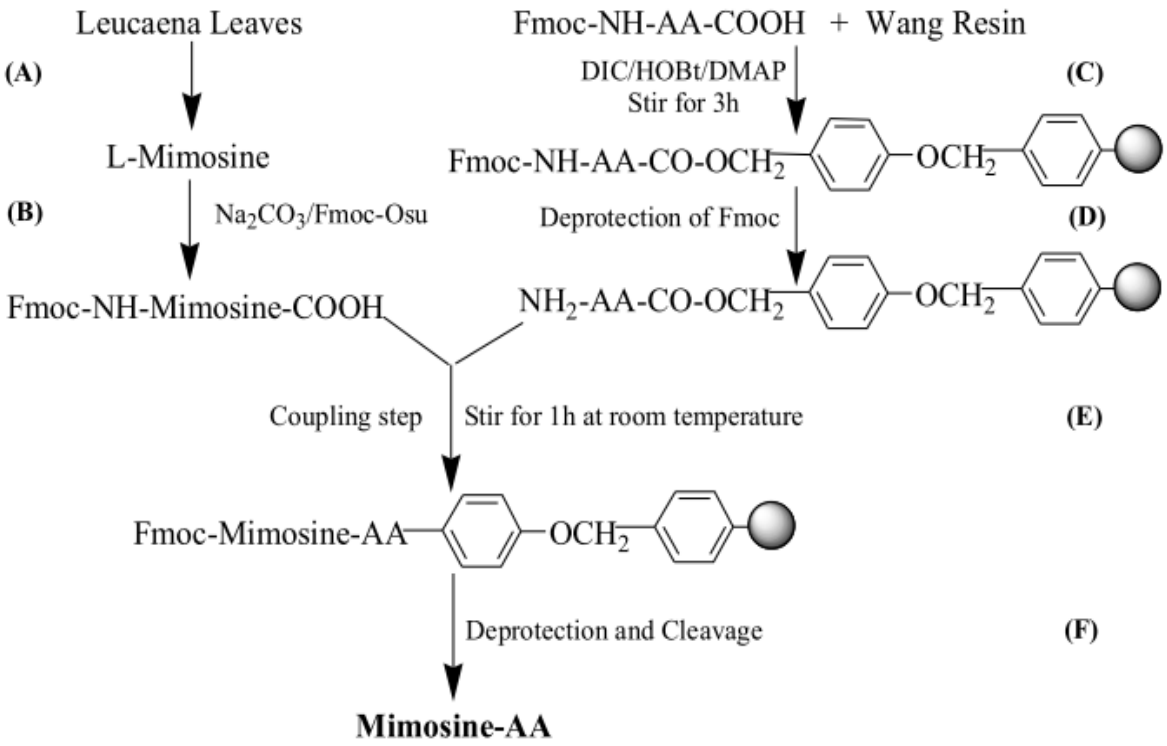

Figure 5.1. General route of mimosine dipeptide synthesis. (AA) L- or D-amino acid; (A) L-mimosine isolation from *Leucaena leucocephala* leaves using ion-exchange resin; (B) Preparation of Fmoc-mimosine; (C) Attachment of Wang resin to Fmoc-amino acid; (D) Deprotection of Fmoc using 25% piperidine; (E) Coupling of Fmoc-mimosine and amino acid-resin mixture along and the Kaiser test; (F) Deprotection and cleavage using 95% trifluoroacetic acid (TFA) to afford desired mimosine dipeptides.

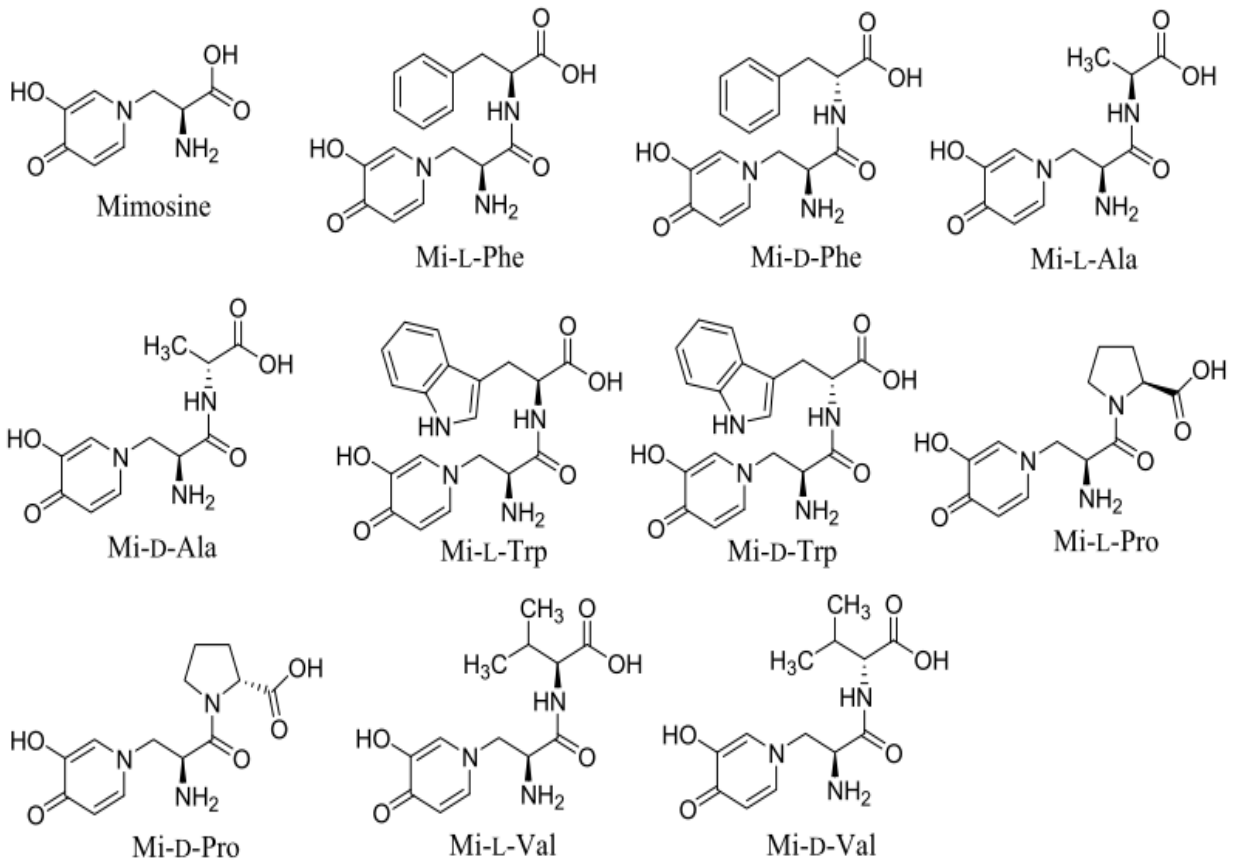

Figure 5.2. The chemical structures of mimosine and mimosine dipeptides.

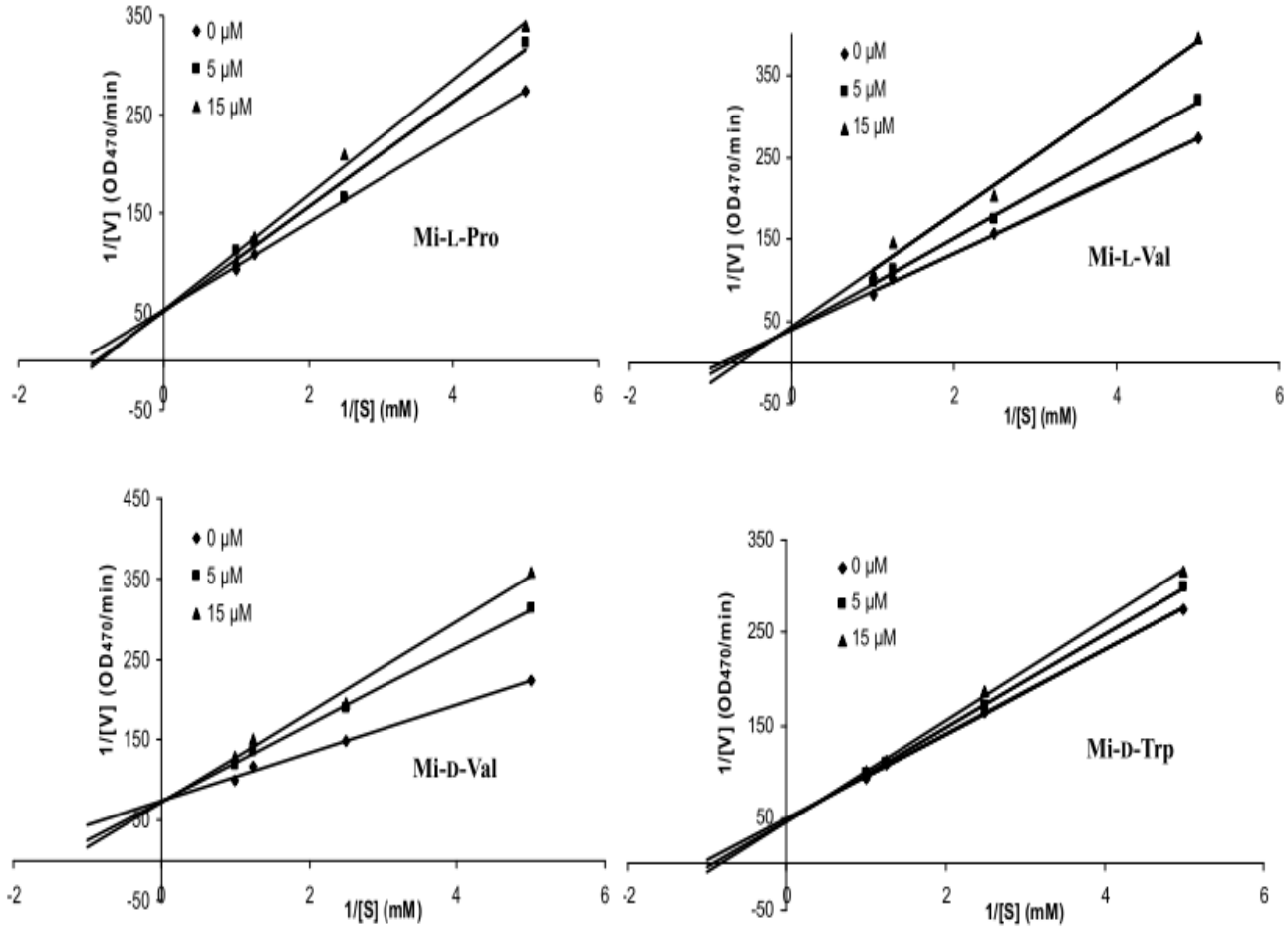

Figure 5.3. Effect of Mi-L-Pro, Mi-L-Val, Mi-D-Val, and Mi-D-Trp on tyrosinase activity. Tyrosinase activity was assayed as described in the text. Lineweaver-Burk plots were drawn in the presence of the compounds at various concentrations (0, 5, and 15 μM) with different concentrations of the substrate L-tyrosine.

Table 5.1

IC_{50} values of mimosine and their dipeptides for mushroom tyrosinase inhibition

Compound	IC_{50} (μM) mushroom tyrosinase inhibition
Mi-L-Phe	24.1 ± 3.8 ab
Mi-D-Phe	19.7 ± 0.8 bc
Mi-L-Ala	23.1 ± 2.2 b
Mi-D-Ala	17.7 ± 3.1 bc
Mi-L-Pro	13.1 ± 3.3 bc
Mi-D-Pro	17.2 ± 2.1 bc
Mi-L-Val	11.5 ± 0.5 c
Mi-D-Val	10.3 ± 0.4 c
Mi-L-Trp	19.2 ± 1.5 bc
Mi-D-Trp	16.9 ± 2.1 bc
Mimosine	32.4 ± 1.1 a
Kojic acid	13.7 ± 1.5 bc

Different letters in the same column indicate the existence of significant difference statistically. Values represented as mean ± SE.

Table 5.2

Kinetics and inhibition constant of four selected mimosine dipeptides on the activity of mushroom tyrosinase

Compound	Inhibition type	Inhibition constant (K_i) (mM)
Mi-L-Pro	competitive	0.05
Mi-L-Val	competitive	0.03
Mi-D-Val	competitive	0.02
Mi-D-Trp	competitive	0.08

Table 5.3

IC_{50} of mimosine dipeptides against intracellular tyrosinase and melanin content in B16F10 melanoma cells

Compound	IC_{50} (μM) for cellular tyrosinase inhibition	IC_{50} (μM) for melanin content inhibition
Mi-L-Phe	179.2 ± 1.3 cd	120.6 ± 8.6 de
Mi-D-Phe	194.5 ± 6.2 b	168.4 ± 1.4 c
Mi-L-Ala	172.2 ± 4.6 cd	128.0 ± 7.4 d
Mi-D-Ala	180.9 ± 6.8 bc	199.0 ± 5.2 b
Mi-L-Pro	166.8 ± 7.3 def	110.4 ± 5.3 e
Mi-D-Pro	145.9 ± 5.2 h	123.3 ± 3.0 de
Mi-L-Val	172.1 ± 6.5 de	170.5 ± 7.5 c
Mi-D-Val	162.1 ± 4.1 efg	112.2 ± 8.1 e
Mi-L-Trp	156.4 ± 8.3 fg	109.3 ± 2.7 e
Mi-D-Trp	150.0 ± 2.3 gh	116.3 ± 4.9 de
Mimosine	388.4 ± 7.2 a	225.7 ± 4.0 a

Different letters in the same column indicate the existence of significant difference statistically. Values represented as mean ± SE.

Table 5.4

IC_{50} values of mimosine and their dipeptides for cyclooxygenase (COX) isoenzymes inhibition

Compound	Yield (mg)	IC_{50} (μM) [ii]		SI [i]
		COX-1	COX-2	COX-1/COX-2
Mi-L-Phe	132.0	nt	nt	nt
Mi-D-Phe	48.5	nt	nt	nt
Mi-L-Ala	45.0	nt	nt	nt
Mi-D-Ala	79.0	19.1 ± 1.0 b	198.4 ± 3.7 a	0.10
Mi-L-Pro	105.5	17.9 ± 2.1 b	29.3 ± 3.0 c	0.75
Mi-D-Pro	43.0	21.0 ± 2.0 ab	127.5 ± 7.7 b	0.15
Mi-L-Val	17.0	24.4 ± 1.4 ab	21.7 ± 2.0 c	1.12
Mi-D-Val	25.0	nt	nt	nt
Mi-L-Trp	98.0	20.4 ± 1.6 ab	19.3 ± 1.8 c	0.95
Mi-D-Trp	71.5	26.1 ± 1.3 ab	27.5 ± 2.2 c	0.95
Mimosine		28.8 ± 2.3 a	20.9 ± 2.1 c	1.38
Indomethacin		6.1 ± 0.9 c	27.7 ± 1.9 c	0.22

[i] SI: selectivity index (COX-1 IC_{50}/COX-2 IC_{50}). [ii] Different letters in the same column indicate the existence of significant difference statistically. nt: not tested. Values represented as mean ± SE.

CHAPTER 6

MIMOSINE DERIVATIVES FOR INHIBITION OF MELANOGENESIS AND HAIR GROWTH PROMOTING ACTIVITY IN CELL CULTURE

6.1 SUMMARY

Mimosine, a non-protein amino acid found widely in subtropical and tropical plants, could be used as source of bioactive compounds for designing novel drugs. In the study, the results indicated that mimosinol and D-mimosinol, two amino alcohols of mimosine, inhibited mushroom tyrosinase with IC_{50} values around 30-40 μM, and were effective melanogenesis inhibitor in B16F10 melanoma cells without undesirable cytotoxicity. To explain possible mechanism, anti-melanogenic effect of these two compounds could be due to intracellular tyrosinase suppression. Interestingly, although mimosine is known to be responsible for hair loss, mimosinol and D-mimosinol had an outstanding of hair growth promotion effect through increasing the proliferation of human follicle dermal papilla cell, 135-140% at 200 μM. The results suggest that mimosinol and D-mimosinol could be useful compounds for the treatment of skin hyperpigmentation and hair loss.

6.2 INTRODUCTION

Melanogenesis is defined as the process leading to the formation of the dark macromolecular pigment melanin (Simon et al., 2006; Costin and Hearing, 2007) which is synthesized by melanocytes within a specialized organelle termed the melanosome (Ni-Komatsu and Orlow, 2007), and controls skin, eye, and hair color. However, the excessive melanin production in the skin has negative hyperpigmentation effects, inducing melasma, freckles, and senile lentigines (Ha et al., 2005; Min et al., 2004; Unver et al., 2006). Melanin synthesis is regulated by melanogenic enzymes such as tyrosinase, tyrosinase-related protein 1 (TRP-1) and tyrosinase-related protein 2 (TRP-

2) (Jian et al., 2011). The first enzyme is tyrosinase, which is responsible for the critical and limiting step in melanogenesis, the hydroxylation of tyrosine to form 3,4-dihydroxyphenylalanine (L-dopa) followed by oxidation of L-dopa to form dopaquinone. The second and third enzymes are TRP-1 and TRP-2 which are more concerned with the generation of different types of melanin (Hearing and Yamaguchi, 2006; Slominski et al., 2004). Since tyrosinase is the limiting step enzyme in melanogenesis, tyrosinase activity is often evaluated in investigations into the effects of depigmenting agents (Campos et al., 2013).

Hair loss (alopecia) is a common and distressing disease in human. It causes nutritional deficiency, aging, hormone imbalance, stress, and genetic disorders. To date, the number of patients suffering from hair loss has increased considerably (Zhang et al., 2013). Currently, there are several treatment options for alopecia patients such as wearing a wig, drug therapy, and human hair transplantation (Dong et al., 2014; Junlatat and Sripanidkulchai, 2014). Drug treatment provides only temporary relief, and the discontinuation of medication may results in immediate depilation (Dong et al., 2014). The development of new drugs or therapies to prevent hair loss and to enhance hair growth is still necessary (Junlatat and Sripanidkulchai, 2014). Hair growth is controlled by a cyclic process comprised of anagen, catagen and telogen phases (Kwon et al., 2007). In the hair growth cycle, the most important element of hair follicles and skin is the dermal papilla (Kawano et al., 2009), which is embedded at the bottom of the hair follicle, and is thought to be responsible for the formation of new hair follicles and the maintenance of hair growth (Rho et al., 2005). Therefore, the most useful and rapid method for evaluating hair growth promotion is by determining the dermal papilla growth rate as influenced by various samples (Kawano et al., 2009).

Mimosine [*β*-[*N*-(3-hydroxy-4-oxypyridyl)]-α-aminopropionic acid] is a non-protein amino acid containing an alanine side chain bound to the nitrogen atom of a hydroxypyridone ring. It is found in several tropical and subtropical plants and has been confirmed for a wide range of biological activities (Tawata et al., 2008). It is suggested that mimosine could be used as a source of bioactive compounds for designing novel drugs (Upadhyay et al., 2011). In the previous study, it is found that a small library of mimosine derivatives (mimosine-amino acid conjugates) have effective tyrosinase inhibitory, anti-melanogenic, and anti-alopecia activities as well (Nguyen et al., 2015bc). Therefore, in this study, two mimosine derivatives (amino alcohols) as mimosinol and D-mimosinol were used, and tested if these compounds have inhibition of melanogenesis, and hair growth promotion activity.

6.3 MATERIALS AND METHODS

6.3.1 Chemicals and reagents

Dulbecco's modified minimum essential medium (DMEM), minoxidil, and L-tyrosine were purchased from Wako Pure Chemical Industries (Osaka, Japan). Thiazolyl blue tetrazolium bromide (MTT) was obtained from Sigma-Aldrich (Japan KK, Tokyo, Japan). Fetal bovine serum (FBS) was purchased from HyClone Laboratories Inc. (Victoria, Australia). Penicillin/streptomycin was obtained from Lonza Walkersville, Inc. (Walkersville, Maryland). Papilla cell growth medium (PCGM), fetal calf serum (FCS), blueberry pituitary extract (BPE), cyproterone (Cyp), and insulin transferring triiodothyronine (ITT) were purchased from TOYOBO (Tokyo, Japan). Unless

otherwise mentioned, all reagents used were of analytical grade and were obtained from Wako Pure Chemical Industries and Kanto Chemical Co., Japan.

6.3.2 Mimosinol and D-mimosinol synthesis

Mimosinol and D-mimosinol were prepared according to procedure described previously by Nguyen et al. (2014). In brief, mimosine (0.4 g, 2 mmol) was placed in a separate round-bottom flask, and mixed with imidazole (0.15 g, 2.2 mmol) and 3.4 mL DMF:CH_2Cl_2 (1:1). The reaction flask was cooled to 0^oC, and the tris(triethylsilyl)silyl triflate was added dropwise. The reaction was stirred at room temperature for 2 h, and the mimosine ester was obtained from the filtrate by evaporation. A solution of sodium borohydride (0.28 g, 7.2 mmol) or sodium borodeuteride (0.3 g, 7.2 mmol) in 3 mL 50% ethanol was added to solution of mimosine ester in 3 mL of 50% ethanol. The resulting mixture was refluxed at room temperature for 5.5 h. The aqueous solution thus obtained was extracted with ethyl acetate; the combined extracts were washed with saturated sodium chloride, dried over anhydrous sodium sulfate, and evaporated to give mimosinol as a colorless crystal and deuterated mimosinol (D-mimosinol) as a colorless liquid. The NMR spectral data were in agreement with reported values for mimosinol and D-mimosinol.

6.3.3 In vitro tyrosinase inhibition assay

A microplate assay for tyrosinase inhibitory activity was performed following a previously described procedure (Tadtong et al., 2009). Samples (20 µL) with various concentrations were transferred into each well of a 96-well plate; 120 µL of 20 mM sodium phosphate buffer (pH 6.8) and 20 µL of 500 U/mL mushroom tyrosinase enzyme dissolved in buffer were then added to each well. The mixture was incubated at 25 ºC for 15 min, after which 20 µL of 0.85 mM L-tyrosine solution was added.

Absorbance was recorded at 470 nm using a microplate reader (Benchmark plus; Biorad, Hertfordshire, UK). Mimosine and kojic acid were used as a positive controls. The percentage of inhibition was calculated as follows:

Inhibition (%) = $[(C_E - C_o) - (S_E - S_o)]/(C_E - C_o) \times 100$

where C_E is the absorbance of the control with enzyme, C_o is the absorbance of the control without enzyme, S_E is the absorbance of the tested sample with enzyme, and S_o is the absorbance of the tested sample without enzyme.

6.3.4 Cell culture

Mouse B16F10 cell line was obtained from American Type Culture Collection (Manassas, Virginia, USA). Cells were cultured in Dulbecco's modified minimum essential medium (DMEM) medium containing 10% fetal bovine serum (FBS), 1% penicillin/streptomycin in a humidified atmosphere with 5% CO_2 incubator at 37^{o}C.

6.3.5 Cell viability assay

The assay as described by Campos et al. (2013) is based upon the cleavage of the yellow tetrazolium salt MTT to give purple formazan crystals. Cells were seeded in a 96-well plate at a density 5 x 10^3 cells/well and cultured for 24 h before the compounds (10 – 500 μM) were added to the medium containing DMEM supplemented with 10% FBS, 1% penicillin/streptomycin. Cells were incubated in a humidified atmosphere 5% CO_2 at 37^{o}C for 40 h. Afterward, 20 μL of MTT solution (0.5 mg/mL) were added to each well and plates were incubated for 3 h. The medium was removed and formazan was dissolved in 200 μL of DMSO. The plates were shaken for 10 min and cell viability was assessed by measuring the absorbance at 570 nm using microplate reader (Benchmark plus, Biorad, Hertfordshire, UK). Kojic acid (500 μM) was used as positive control. DMSO was used as the blank. Absorbance of sample and control

subtracts the absorbance of blank. The corrected absorbance of samples divide the corrected absorbance of control, then multiplies 100% to give the percentage of cell viability.

6.3.6 Determination of melanin content in B16F10 cells

The assay was performed following the previously described procedure. Cells (5 x 10^3 cells/well) were seeded in a 96-well plate and cultured for 24 h. Compounds (10 – 500 μM) were added and incubated at 37^{o}C for 48 h. The medium was replaced by a fresh medium containing the same concentration of compound and the cells were incubated for 48 h. The medium was removed and the cells were dissolved in 100 μL of 1 N NaOH. The reaction was heated at 90^{o}C for 1 h. Melanin content was estimated by the absorbance at 400 nm (Hosoya et al., 2012).

6.3.7 Intracellular tyrosinase inhibition assay

Cells were seeded in a 96-well plate at a density 5 x 10^3 cells/well and the compounds (10 – 500 μM) were added after 24 h treatment. Cells were incubated for 48 h, washed two times with 50 mM of ice-cold phosphate buffer (pH 6.8), lysed with 90 μL of 50 mM phosphate buffer (pH 6.8) containing 1 % Triton-X, and frozen at -80 ^{o}C for 30 min. After thawing and mixing, 20 μL of 0.5% L-DOPA was added to each well. The mixture was incubated for 2 h at 37^{o}C and absorbance was measured at 490 nm (Campos et al., 2013).

6.3.8 In vitro hair growth promotion assay

Human follicle dermal papilla cells (HFDPC) were purchased from TOYOBO (Tokyo, Japan). Cells were cultured in HFDPC growth medium containing 50 mL of papilla cell growth medium (PCGM), 0.5 mL of fetal calf serum (FCS), 0.5 mL of BPE, 0.25 mL of Cyp, and 0.25 mL of ITT in 51.5 mL total volume. Cell viability was

evaluated using MTT assay. HFDPCs were collected and diluted in medium containing D-MEM and 10% FBS at a cell density of 1 x 10^4 cell/mL. Then, 200 μL of cell solution (2,000 cells) was transferred into each well of collagen-coated 96 well plate, and cells were incubated for 37°C, 5% CO_2 for 3 days. The medium was removed, and 200 μL of samples prepared in DMEM only was added. After 4 days incubation, 100 μL of MTT solution in D-MEM (0.4mg/mL) was added, and the mixture was incubated for 2 h. The untransformed MTT was removed. After 100 μL of 2-propanol was added to each well, the plates were shaken for 10 min. The absorbance was read at 570 nm using a microplate reader. The cell viability rates were calculated from readings and are represented as percentages of the control value (treated cells with D-MEM only).

6.3.9 Data analysis

Statistical analyses were performed using statistical analysis system (SAS) software, version 9.1.3 (SAS Institute Inc., Cary, North Carolina). Significance was assessed by one-way ANOVA. All calculations were conducted in Microsoft Excel 2003. The IC_{50} values were determined graphically as required concentration of each compound to give 50% inhibitory activity.

6.4 RESULTS AND DISCUSSION

6.4.1 In vitro tyrosinase inhibition by mimosinol and D-mimosinol

Tyrosinase inhibitory assay was conducted with 96-well plate. As found in **Figure 6.1**, mimosinol and D-mimosinol had strong inhibitory activity against tyrosinase with IC_{50} values of 31.6 and 41.1 μM, respectively.

6.4.2 Anti-melanogenic activity of mimosinol and D-mimosinol

Mimosinol and D-mimosinol were non-cytotoxic to B16F10 cells at the concentration tested up to 500 μM (**Figure 6.2**). The suppression of mimosinol and D-mimosinol on melanin content was comparable to that of kojic acid, about 50% at 500 μM (**Figure 6.3**). D-mimosinol and mimosinol had intracellular tyrosinase inhibition 32-38% at 200 μM, 38-47% at 500 μM, respectively while kojic acid inhibited tyrosinase activity 55% at 500 μM (**Figure 6.4**).

6.4.3 Effect of mimosinol and D-mimosinol on human dermal papilla cell growth

The potential of mimosine, mimosinol and D-mimosinol to promote the growth of HFDPCs was evaluated by the MTT assay. **Figure 6.5** showed the relative growth in MTT assay with proliferative capacity of compounds compared to control. Mimosine inhibited the growth of HFDPC by 8% at the concentration of 200 μM. However, mimosinol and D-mimosinol, amimo alcohol of mimosine, promoted the proliferation of HFDPCs in vitro at about 140% for mimosinol, and 135% for D-mimosinol, relative to control at 200 μM. In this assay system, minoxidil had proliferative capacity of 115% at 10 μM (**Figure 6.5**).

6.4.4 Discussion

A desirable skin-whitening agent would inhibit melanin synthesis in the melanosomes by suppressing the synthesis or activity of tyrosinase, and would also exhibit low cytotoxicity and be non-mutagenic (Jun et al., 2008). Mimosinol and D-mimosinol were inactive in vitro mushroom tyrosinase. progressed to investigate the effects of these compounds in cultured B16F10 mouse melanoma cells. As expected, it was found that mimosinol and D-mimosinol inhibited melanin production in dose-

dependent manner in B16F10 melanoma cells. The suppression of mimosinol and D-mimosinol on melanin content was comparable to that of kojic acid (well-known melanogenesis inhibitor). This inhibitory activity could be due to reducing intracellular tyrosinase activity which is essential for the process of melanin synthesis. Based on the results, suggesting that mimosinol and D-mimosinol could be promising agents for skin brightening which had anti-melanogenic effects without association with undesirable cytotoxicity. On the other hand, in primary screening, it is also showed for the first time that mimosinol and D-mimosinol could have hair growth promotion activity through increasing proliferation of HFPDCs. The above results make mimosinol and D-mimosinol as promising agents for treating skin hyperpigmentation and hair growth promotion. It could be used as starting material for developing novel derivatives for both these characterization. However, the molecular mechanisms underlying anti-melanogenic and anti-alopecia effects of mimosinol and D-mimosinol have not been understood yet fully.

6.5 CONCLUSION

To sum up, the results indicated that amino alcohols of mimosine (mimosinol and D-mimosinol) had antimelanogenic effect in B16F10 cells which were not associated with cytotoxicity. Interestingly, mimosinol and D-mimosinol had an outstanding activity for hair growth promotion effect through increasing the proliferation of human follicle dermal papilla cell. The results suggest that mimosinol and D-mimosinol are potential compounds for treating skin hyperpigmentation and hair growth promotion.

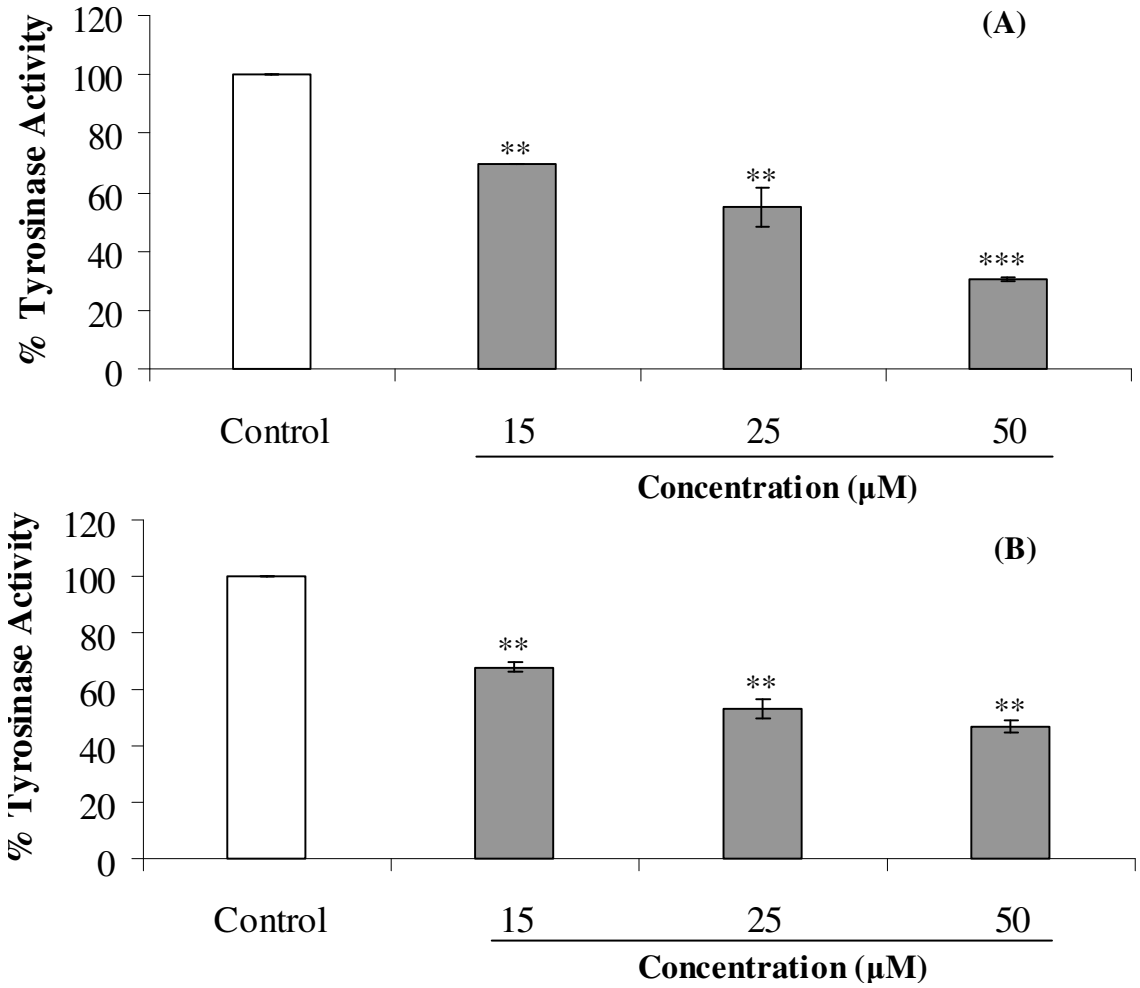

Figure 6.1. Inhibition of in vitro tyrosinase by (A) mimosinol and (B) D-mimosinol. The experiments were conducted with two repeated times, and the values are means ± SE. IC_{50} values of mimosinol and D-mimosinol are 31 and 41 µM, respectively. Values are significantly different by comparison with control. ** $p \leq 0.01$, *** $p \leq 0.001$.

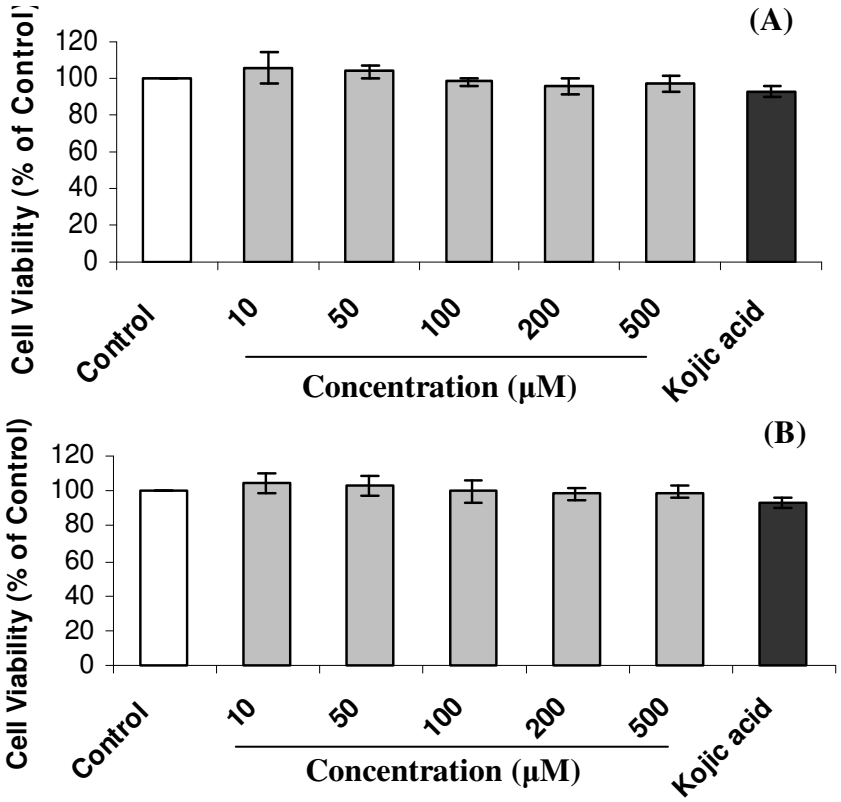

Figure 6.2. Effect of mimosinol (A) and D-mimosinol (B) on B16F10 cell viability. A: Mimosinol, B: D-mimosinol. Kojic acid (500 μM) were used as positive control. Cells were treated with various concentrations of tested compounds for 40 h, and the cell viability was measured by MTT assay. Results are expressed as percentages of cell viability relative to control. The experiments were conducted with two repeated times, and the values are means ± SE.

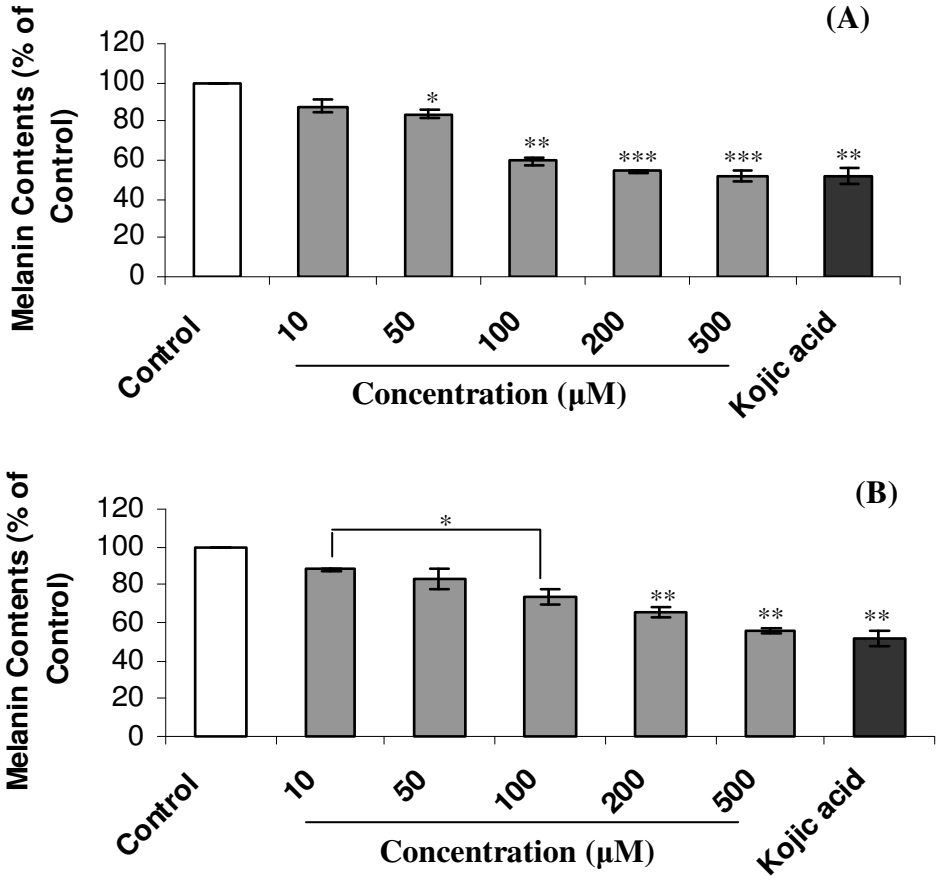

Figure 6.3. Inhibitory effect of mimosinol (A) and D-mimosinol (B) on melanin content in B16F10 cells. Kojic acid (500 μM) were used as positive control. The melanin content was measured after cells was treated with various concentrations of tested compounds for 48 h. The experiments were conducted with two repeated times, and the values are means ± SE. Results are expressed as percentages of control. The statistical analysis was conducted by one-way ANOVA at $p \leq 0.05$. Values are significantly different by comparison with control. * $0.01 \leq p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

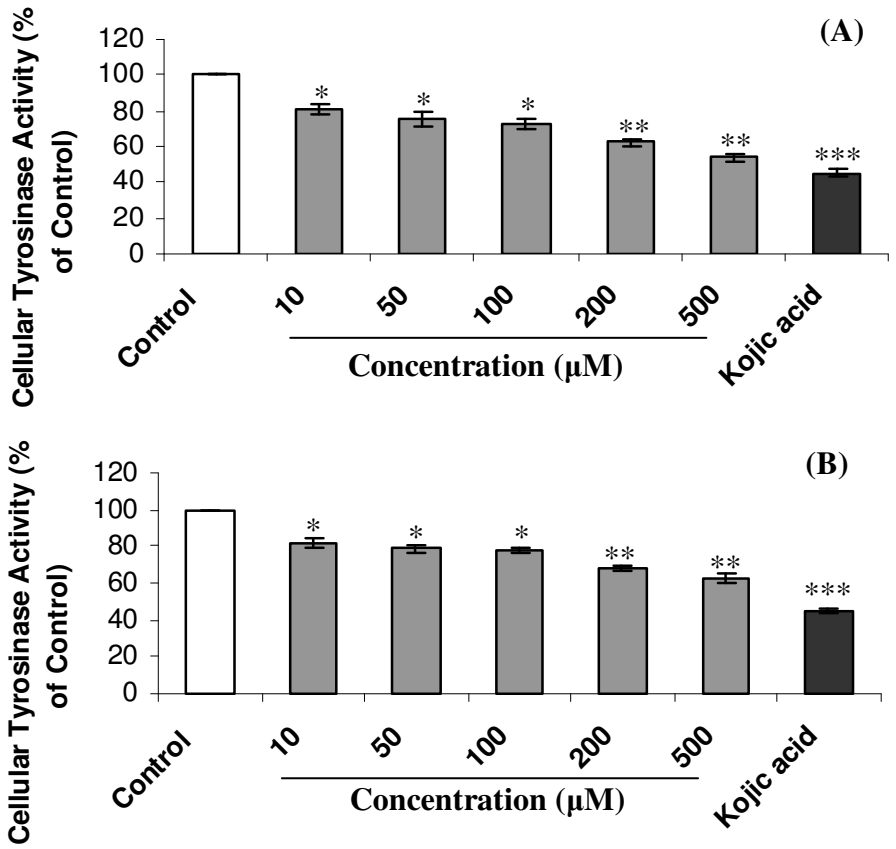

Figure 6.4. Inhibitory effect of mimosinol (A) and D-mimosinol (B) on intracellular tyrosinase activity in B16F10 cells. Kojic acid (500 μM) were used as positive control. Cells were treated with various concentrations of tested compounds for 48 h, and the tyrosinase activity was measured. Results are expressed as percentages of control. The experiments were conducted with two repeated times, and the values are means ± SE. The statistical analysis was conducted by one-way ANOVA at $p \leq 0.05$. Values are significantly different by comparison with control. * $0.01 \leq p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

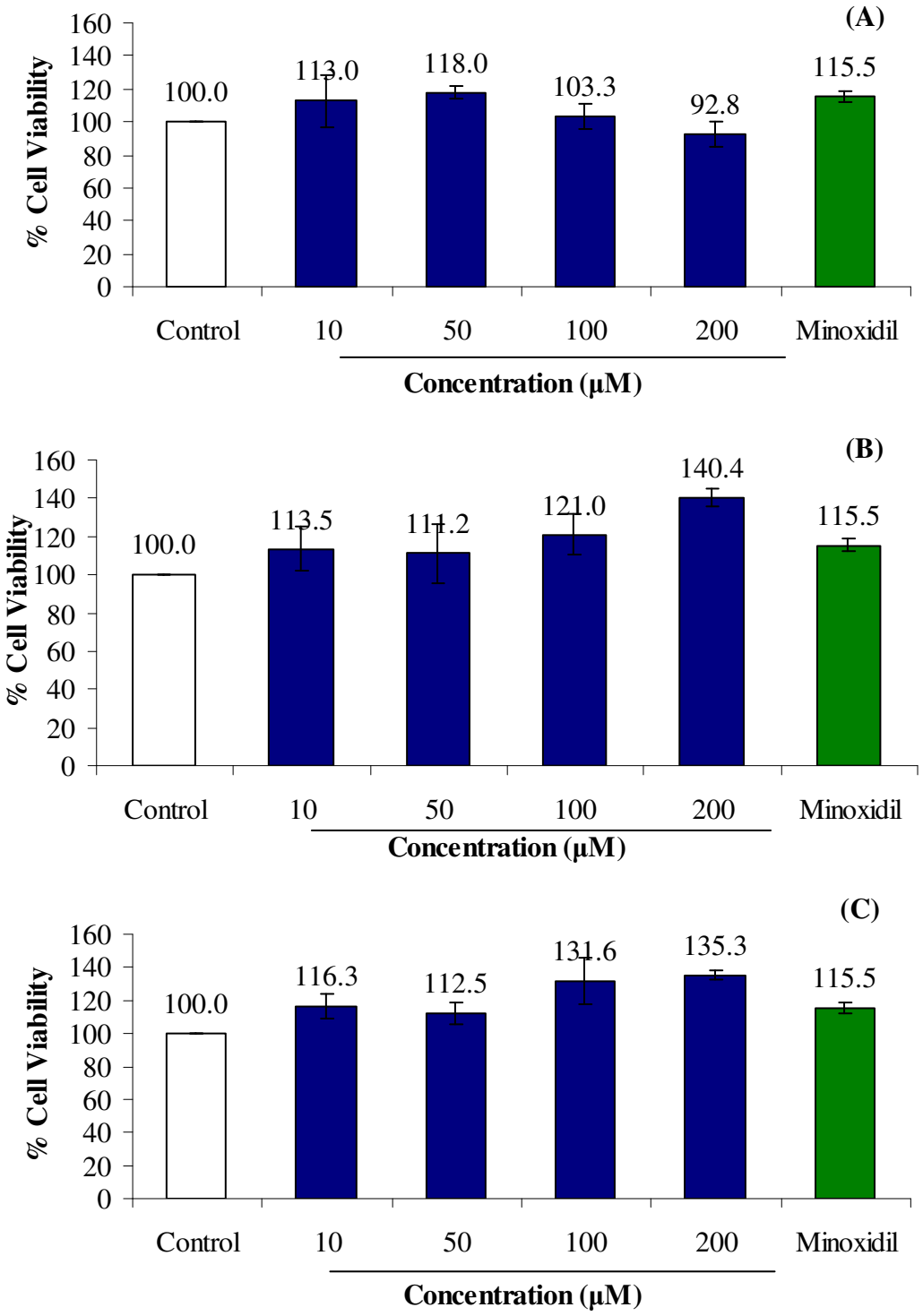

Figure 6.5. Effect of mimosine (A), mimosinol (B), and D-mimosinol (C) on proliferation of human follicle dermal papilla cells (HFDPCs). The results are mean of 20 replications/each treatment of four repeated times of two independent experiments. Minoxidil (10 µM) was used as positive control. Values are significantly different by comparison with control. * $0.01 \leq p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

CHAPTER 7

COMBINATION OF IMMUNOPRECIPITATION (IP)-ATP-GLO KINASE ASSAY FOR THE ASSESSMENT OF POTENT AND SAFE PAK1-BLOCKERS IN CELL CULTURE AND THEIR ANTI-CANCER AND ANTI-ALOPECIA ACTIVITIES

7.1 SUMMARY

Cucurbitacin I (CBI) is a triterpene from a bitter melon called Goya grown in Okinawa, Japan, and directly inhibits both the Tyr-kinase JAK2 and the G protein RAC, leading to the inactivation of PAK1 (RAC/CDC42-activated kinase 1). Bio 30, a propolis produced in New Zealand, contains CAPE (caffeic acid phenethyl ester) as the major anti-cancer ingredient which directly down-regulates RAC, leading to the inactivation of PAK1. Glaucarubinone is known as both PAK1 and PAK4 inhibitor. Since PAK1 is essential for the growth of RAS cancer cells such as A549 cell line which carry an oncogenic K-RAS mutant, and the melanogenesis in skin cells, here using these PAK1-blockers as model compounds, a new approach to the quick assessment of PAK1-blockers in cell culture was introduced. First, combining the immuno-precipitation (IP) of PAK1 from cell lysate and the *in vitro* ATP_Glo kinase assay kit (called "Macaroni-Western" assay), it was confirmed that both CBI and Bio 30 inactivated PAK1 in A549 lung cancer cells in 24 h, and inhibited their PAK1-dependent growth in 72 h. Glaucarubinone had anticancer against A549 with IC_{50} of 730 nM. Because the "Macaroni-Western" kinase assay and melanogenesis are both rather simple and quick, the combination of these two cell culture assays would be highly useful for selecting both "potent" (highly cell-permeable) and "safe" natural or synthetic PAK1-blockers. On the other hand, cucurbitacin I from bitter melon (Goya) turned out to be the most potent to promote the growth of hair cells with the effective dose around 10 nM. Bio 30 propolis and glaucarubinone also exhibited strong hair cell growth promotion at 10 μg/mL and 40 nM, respectively. Taken together, these findings suggest that CBI, Bio 30 and glaucarubinone are useful compounds for treatment of cancers or hair loss.

7.2 INTRODUCTION

RAC/CDC42-activated kinase 1 (PAK1) is essential for the growth of various solid tumors and a numerous other diseases such as Alzheimer's disease (AD), Parkinson's disease (PD), epilepsy, schizophrenia, depression, autism, diabetes (type 2), hypertension, obesity, inflammatory diseases, and infectious diseases (Maruta, 2014). PAK1 is also essential for growth and progress of many cancer cells including lung cancer cell. In other words, these cells are addicted to PAK1 for their growth and developmental progress (Maruta, 2014). More interestingly, these herbal PAK1-blockers promote hair growth, suggesting the possibility that PAK1 normally suppresses the growth of hair cells (Miyata et al., 2014). Thus, PAK1-blockers would have a potentially huge market value as therapeutics. In previous study, it is found that herbal compounds derived from three Okinawa plants as *A. zerumbet*, *L. leucocephala* and bitter melon inhibit directly the oncogenic/aging kinase PAK1 (Nguyen et al., 2014). Glaucarubinone is known as both PAK1 and PAK4 inhibitors (Blaskovich et al., 2003), while Bio 30, caffeic acid phenethyl ester (CAPE)-based propolis has been also reported as PAK1 inhibitors.

In the past decade(s), the initial screening for PAK1-blockers has been performed mainly in test tubes to select compounds that inhibit directly PAK1. Unfortunately, however, most of these PAK1-inhibitors turned out to have a poor cell-permeability. For instance, the IC_{50} for PAK1 in test tube of FRAX486 and FRAX597 are around 10 nM, but their IC_{50} for the PAK1-dependent growth of NF2-deficient tumor cells in cell culture are above 1 μM (1000 nM). Since the NF2 gene product (merlin) is a PAK1 inhibitor (Maruta, 2014), it is most likely that less than 100th of

these molecules could pass through cell membranes (Hiyashi-Takagi et al., 2014; Licciulli et al., 2013). In order to screen for highly cell-permeable PAK1-blockers more effectively, we have to conduct the assessment of test compounds in cell culture or in vivo, instead of in test tubes, in the earliest possible stage, in order to save both time and money. The current peculiar culture among pharmaceuticals that puts the first priority on the kinase-specificity of each compound in test tube over its cell-permeability needs to be reconsidered, simply because no drug would be useful for clinical application, if it hardly passes through the cell membranes.

In the past, basically two ways were used to assay the kinase activity of PAK1 in cell culture. The first (old) assay method is a "radioactive" approach in which PAK1 is immunoprecipitated (IP) with a polyclonal antibody (IgG) against PAK1 from cell lysates by means of protein A/G beads, and the resulting PAK1-IgG-bead complex is then incubated with the "radioactive" (gamma-^{32}P) ATP (adenosine triphosphate) and the substrate MBP (myelin basic protein) in vitro (test tube), and the phosphorylated (radioactive) MBP band is separated by SDS-PAGE, and the radioactivity of this band is quantitated by the auto-radiography (He et al., 2001). This method is getting less and less popular in this century, simply because the handling of radioisotopes is rather restricted in research laboratories world-wide.

The alternative (more recent) approach is the immuno-blot (also called "western-blot") approach in which a cell-lysate is separated by SDS-PAGE, and proteins including PAK1 are transferred onto nitrocellulose paper, and phosphorylated PAK1 (called "pPAK1" at Thr423, Ser199, or Ser144, case by case) band is blotted with an antibody against each pPAK1, and in the end the amount of this antibody is quantified by fluorescence technique associated with probes (biotin or horse radish peroxidase

labeling). Unfortunately, however, the "western-blot" analysis based on pPAK1 is not reliable, simply because the activation of PAK1 in cells does not always depend on the auto-phosphorylation of PAK1 at these Ser/Thr residues. The full activation of PAK1 depends on its Tyr-phosphorylation by three distinct Tyr-kinases (ETK, JAK2 and FYN) at least (He et al., 2004; Rider et al., 2007; Hirokawa et al., 2007), and dephosphorylation of PAK1 at Thr109 as well (Deguchi et al., 2010). Thus, strictly speaking, this popularized and over-simplified pPAK1-based "western-blot" analysis is rather unreliable.

Around a decade ago, an Italian group introduced a third kinase assay system which we call here "Macaroni-Western" kinase assay (Tagliati et al., 2005). This is a clever modification of the old radioactive approach by simply replacing "radioactive" ATP with a newly developed "ATP_Glo kinase assay kit" (Promega). Accordingly, PAK1 immunoprecipitated from cell lysates (after cells are treated with a given test compound) is incubated with this kit with ATP and MBP, and the remaining ATP could be quantified by the ATP-dependent "luciferin-luciferase" reaction which produces the fluorescence/luminescence. Thus, the universal "Macaroni-Western" system allows to monitor any change in the kinase activity of PAK1 in cells directly, independent of its auto-phosphorylation sites, without SDS-PAGE.

Thus, in this study, anti-cancer and anti-alopecia (hair growth promoting) activities of PAK1 blockers as cucurbitacin (CBI), glauracubinone, Bio 30 were evaluated. A new approach to the more effective assessment of PAK1-blockers in cell culture was also presented. In addition, instead of the rather unreliable pPAK1-blot analysis, combining the IP (immuno-precipitation) of PAK1 from cells treated with a given compound, and in vitro ATP _Glo kinase assay kit (Tagliati et al., 2005), the

“Macaroni-Western” PAK1 assay system was introduced to determine whether a given compound inactivates PAK1 in cell culture or not.

7.3 MATERIALS AND METHODS

7.3.1 Reagents and cell lines

Cucurbitacin I (CBI) was purified from bitter melon called “Goya” in the laboratory as previously described (Nguyen et al., 2014). Bio 30, a CAPE (caffeic acid phenethyl ester)-based propolis (tincture) from New Zealand was obtained from Manuka Health in Auckland. The content of major ingredients in Bio 30 was described in detail previously (Demestre et al., 2009). Glaucarubinone was provided by NIH. Human A549 lung cancer cell line and human follicle dermal papilla cell (HFDPC) were obtained from Japanese Collection of Research Bioresources Cell Bank (Osaka, Japan) and TOYOBO (Tokyo, Japan), respectively. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), myelin basic protein (MBP) and protein A-agarose beads were purchased from Sigma Aldrich Chemical Co. (St. Louis, MO, USA). Rabbit polyclonal anti-PAK1 IgG was obtained from Cell Signalling Technology (Danver, MA, USA). Kinase Glo reagent and ATP (ATP_Glo kinase kit) were purchased from Promega (Madison, Wisconsin, USA). Dulbecco's modified Eagle medium (DMEM), fetal bovine serum (FBS), and triton-X were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Papilla cell growth medium (PCGM), fetal calf serum (FCS), BPE, Cyp, and ITT were purchased from TOYOBO (Tokyo, Japan)All reagents were of the highest grade available.

7.3.2 Cell culture

Human A549 lung cancer cell line was cultured as described previously (Nguyen et al., 2014). Murine B16F10 melanoma cell line was cultured as described previously (Be Tu et al., 2015; Yun et al., 2015). HFDPCs were cultured the following procedure described by TOYOBO company.

7.3.3 MTT assay for cell viability or growth of A549 cell lines

Cell viability (or growth rate) was determined using an 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay, as previously described (Campos et al., 2013). Briefly, A549 cells (2×10^4 cells/well) were seeded on a 24-well plate. After preculture, cells were treated with tested compounds at various concentrations for 24 hrs and 72 h (A549) at 37^{o}C. Then cells were washed with phosphate buffer and incubated with MTT solution (0.5 mg/mL) for 3 h at 37^{o}C. After the medium was replaced by 200 µL of ethanol, the absorbance of each well was measured at 570 nm using a microplate spectrophotometer (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

7.3.4 "Macaroni-Western" (IP-ATP_Glo) kinase assay for PAK1 in A549 cells

7.3.4.1 Protocol for drug-treatment of cells followed by IPing PAK1 from cell lysates

The intracellular content of PAK1 is quite low compared with other kinases. Thus, in an attempt to optimize the selective immuno-precipitation (IP) of PAK1 alone, in particular for minimizing the non-specific binding of other kinases to beads, and maximizing the IgG-protein A beads interaction, the following protocol for monitoring the effect of test drugs on PAK1 activity in cell culture includes our substantial improvement of a decade old method (which we coined "Macaroni-Western") published by an Italian group who initially developed for monitoring in test tube the direct effect

of drugs on a variety of native abundant kinases such as CDKs (cyclin-dependent kinases) from cultured cells (Tagliati et al., 2005). The initial concentration of ATP for "test tube" kinase assay also has been optimized for monitoring both activation and inactivation of PAK1 in cells by a variety of test drugs.

Step 1: pre-culture A549 lung cancer cell line (2 x 10^5 cells/mL) on 6-well plate for 24 h

Step 2: replace by the fresh medium containing test compounds at various concentrations, and incubate for 24 h

Step 3: wash with 500 μL of ice-cold PBS (-) twice

Step 4: add 500 μL of 50 mM Tris-HCl pH 7.5 and 150 mM NaCl and 1% Triton-X (lysis buffer)

Step 5: incubate on ice for 30 min

Step 6: cell lysates were transferred into 1.5 mL tube

Step 7: spin at 1000 rpm for 5 min at 4^{o}C

Step 8: transfer the supernatant to 1.5 mL new tubes

Step 9: add 50 μL of dilution buffer alone or anti-PAK1 IgG (1:50 dilution) * to each tube

Step 10: incubate for 1 h on ice

Step 11: add 10 μL of protein A-agarose beads

Step 12: incubate for 1 h in cold room with continuous shaking by a rotary mixer (Nissin, Suginami-ku, Tokyo, Japan).

Step 13: spin at 1000 rpm for 5 min, 4^{o}C

Step 14: remove the supernatant

Step 15: resuspend the pellet with 500 μL of 50 mM Tris-HCl pH 7.5 and 150 mM NaCl (washing buffer)

Step 16: spin at 1,000 rpm for 5 min, 4^{o}C

Step 17: repeat steps 15 and 16

Step 18: resuspend the PAK1 pellet in 35 µL of 50 mM Tris-HCl pH 7.5 and 150 mM NaCl, 20 mM $MgCl_2$, 0.1 mg/mL BSA (kinase buffer)

*Note: Anti-PAK1 IgG is diluted in buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 100 µg/mL BSA) in ratio 1:50 (dilution buffer)

7.3.4.2 ATP_ Glo kinase assay

PAK1 assay was carried out in a 100 µL total volume. 35 µL of kinase buffer alone or immuno-precipitated PAK1 and 5 µL of the substrate MBP (1 mg/mL in distilled water) were incubated at 37^{o}C for 10 min. Then, 10 µL of 2 µM ATP in distilled water was added to each well. The reaction plate was incubated for 1 hr at 37^{o}C with continuous shaking. After the kinase reaction, the equal volume (50 µL) of kinase Glo reagent (Promega) was added to each well, and the plate was further incubated for 30 min to stabilize luminescent signal. The suspension was centrifuged, and the supernatant was transferred to 96-well plate for reading. Luminescence was recorded by MTP-880Lab microplate reader (Corona, Hitachinaka-ku, Ibaraki, Japan) with an integration time of 0.5 s per well.

7.3.5 Effect of CBI, Glaucarubinone, and Bio 30 on the growth of A549 cancer cells

A549 cells were seeded on 6-well plate at the density of 2 x 10^5 cells/well in D-MEM supplemented with 10% FBS, and after preculture for 24 h, cells were treated with either CBI or Bio 30 at indicated concentrations for further 72 h. Then cells were stained with trypan blue as described previously (Wu et al., 2002). Both dead cells and the total cells were counted using hemocytometer. The percentage of viable treated cells in relative to the control (non-treated) cells was calculated.

7.3.6 In vitro hair growth promotion assay

Human follicle dermal papilla cells (HFDPC) were purchased from TOYOBO (Tokyo, Japan). Cells were cultured in HFDPC growth medium containing 50 mL of papilla cell growth medium (PCGM), 0.5 mL of fetal calf serum (FSC), 0.5 mL of BPE, 0.25 mL of Cyp, and 0.25 mL of ITT in 51.5 mL total volume. Cell viability was evaluated using thiazolyl blue tetrazolium bromide (MTT) assay. HFDPCs were collected and diluted in medium containing Dulbecco's modified minimum essential medium (D-MEM) and 10% fetal bovine serum (FBS) at a cell density of 1 x 10^4 cell/mL. Then, 200 μL of cell solution (2,000 cells) was transferred into each well of collagen-coated 96 well plate, and cells were incubated for 37°C, 5% CO_2 for 3 days. The medium was removed, and 200 μL of samples prepared in DMEM only was added. After 4 days incubation, 100 μL of MTT solution in D-MEM (0.4mg/mL) was added, and the mixture was incubated for 2 h. The untransformed MTT was removed. After 100 μL of 2-propanol was added to each well, the plates were shaken for 10 min. The absorbance was read at 570 nm using a microplate reader. The cell viability rates were calculated from readings and are represented as percentages of the control value (treated cells with D-MEM only).

7.3.7 Statistical analysis

Data were expressed as mean values with their standard errors. Statistical comparisons were performed by oneway ANOVA followed by Duncan's multiple-range test. Statistical analysis was conducted using SAS (release 9.2; SAS Institute, Cary, NC, USA) and $p \leq 0.05$ was considered significant.

7.4 RESULTS AND DISCUSSION

7.4.1 "Macaroni-Western" kinase assay for the inactivation of PAK1 by CBI and Bio 30 in cell culture for 24 h

In this study, CBI, Bio 30, and glaucarubinone have chosen as potential model PAK1-blockers for the following reasons: CBI inhibits directly two activators (JAK2 and RAC) of PAK1 (Blaskovich et al., 2003; Lopez-Haber et al., 2013), while Bio 30 contains CAPE which down-regulates RAC by inhibiting directly AKR 1B10 (Aldo-Keto-Reductase 1B10) (Soda et al., 2012), and suppresses completely the PAK1-dependent growth of NF2-deficient tumor (schwannoma) in vivo (Demestre et al., 2009). Glaucarubinone is known to inhibit both PAK1 and PAK4 (Blaskovich et al., 2003).

Since the PAK1 (protein) level in melanoma cell line B16F10 is extremely low (Yun et al., 2015), as the PAK1 source for the "Macaroni-Western" kinase assay, A549 lung cancer cell line was used. This cell carries the oncogenic K-RAS mutant that highly activates PAK1, and whose growth almost solely depends on PAK1. After preculture of A549 cells for 24 h, they were treated with either CBI or Bio 30 at two indicated concentrations for 24 h, and the non-treated cells as well as treated cells were lyzed, and cell lysates were incubated with anti-PAK1 IgG and protein A-beads for immuno-precipitation (IP) of PAK1. After the PAK1-IgG-protein A-bead complex was incubated with ATP and MBP for 1 h, ATP_Glo kinase was added to measure the remaining ATP level by the released luminescence (for detail, see **Figure 7.1**). The higher the luminescence, the lower the kinase activity. As shown in **Figure 7.2**, both CBI and Bio 30 clearly inactivate PAK1 in this cancer cell line with the apparent IC_{50} around 600 nM and 60 µg/mL, respectively. The apparent down-regulation of PAK1 by

these compounds is not due to the growth inhibition of this cell line by these compounds, because for the first 24 h (just one generation of cell cycle) no growth inhibition was observed with either CBI or Bio 30 (see **Figure 7.3**).

7.4.2 Growth inhibition of A549 cancer cells by CBI, Bio 30, Glaucarubinone for 72 h

However, after treatment of A549 cancer cells with glaucarubinone, CBI or Bio 30 for 72 h (3-4 generations of cell cycle), both CBI and Bio 30 strongly inhibit the growth of this "RAS" cancer cell line with the IC_{50} around 140 nM and 8 μg/mL, respectively (see **Figure 7.4**), confirming that PAK1 is absolutely essential for the growth of this RAS cancer cell line. Glaucarubinone has anticancer activity against A549 with IC_{50} of 730 nM (**Figure 7.5**).

The major and most likely reason why the IC_{50} for the growth is several times lower than the apparent IC_{50} for the kinase activity: both IP procedure and test tube assay for PAK1 activity isolated from cells take more than 3 h in total, and in the absence of PAK1-blockers (CBI and Bio 30), the suppression of PAK1 activity could be gradually released over time. In other words, during this "time-consuming" test tube kinase assay, we cannot freeze the exact (activated or inactivated) status of PAK1 in the end of cell-treatment. That is the major demerit associated with this kinase assay, although any significant changes in the kinase activity during cell culture can be monitored regardless of the phosphorylation sites of PAK1. Thus, the apparent IC_{50} for the kinase assay obtained in test tube is only a reflection of PAK1 inhibition during cell culture, and not the true IC_{50} during cell culture. Nevertheless, the apparent IC_{50} tells clearly that CBI is far more potent than Bio 30 in suppressing PAK1 during the cell culture, as does in the cancer cell growth.

So far no herbal compound has been found to inhibit only PAK4. The majority of herbal anti-melanogenic compounds such as CAPE, curcumin, and shikonin, blocks only PAK1, and a few herbal compounds such as glaucarubinone are known to block both PAK1 and PAK4 (Blaskovich et al., 2003). In other words, if a given herbal compound inhibits the melanogenesis by only 50%, it is most likely that it is a PAK1-blocker rather than PAK4-blocker, and would be useful for improving our health and extending our lifespan. Of course, we could easily verify its anti-PAK1 activity through the quick "Macaroni-Western" kinase assay in the end.

Finally, using this opportunity, I would like to point out the followings: there are so many PAK1-blocking herbal products such as propolis available on the market world-wide which could be very useful for improving our health and even therapy of cancer and many other PAK1-dependent diseases/disorders such as Alzheimer's disease (Maruta, 2014). However, unlike FDA-approved drugs, none of them is associated with any reliable international quality control standard such as IC_{50} or ED (effective dose). For instance, the quality of propolis depends on the sources of plants where bees harvest from, and the actual content of PAK1-blocking ingredients such CAPE, apigenin, ARC (artepillin C) and propolin G in each propolis. However, since 1960s till present, the only available quality standard used for propolis is only "the total flavonoid content" for CAPE-based propolis or the ARC content in Brazilian green propolis. If these "herbal" health-promoting products are regulated by a single reliable pharmacological quality standard, it is easy to compare the quality or effectiveness from one sample to another quite objectively.

Hence, I would propose here for the first time to use a universal standard called "Anti-PAK index" which is the 100 x reciprocal of the IC_{50} in ppm (μg/mL). For instance, "Anti-PAK index" of Bio 30 is 12.5 since its IC_{50} for A549 cancer cells is around 8 ppm, whereas the "Anti-PAK index" of the "pure" compound CBI is around 1400, as the IC_{50} for A549 is around 140 nM (0.07 ppm). The higher the Anti-PAK index, the more potent a given sample. In other words, 1 mg of CBI is equivalent to 112 mg of Bio 30 for therapy of cancers and many other PAK1-dependent diseases/disorders. This rough estimation is not far from the actual in vivo data where the daily dose of cucurbitacin B (1 mg/kg), closely related to CBI, and that of Bio 30 (50 mg/kg) are their effective dose to suppress the PAK1-dependent growth of pancreatic cancers or NF tumors in mice (Demestre et al., 2009; Lopez-Haber et al., 2013), suggesting that their in vivo bioavailability is quite similar.

The only difference between these two is that Bio 30 has been available on the market world-wide for clinical uses for almost a decade, but CBI is not as yet. The only way to take CBI is to eat the edible bitter melon (Goya) grown in Okinawa (which contains around 1 g of CBI per kg) or drink Goya tea. Since roughly 90% of Goya is water, the "Anti-PAK index" of Goya extract/tea could be around 14, pretty close to that of Bio 30. Thus, the IC_{50} of Goya extract/tea on the market will be measured for calculating their "Anti-PAK index" more precisely.

7.4.3 Promotion of hair cell growth

In the past, there were a few cell culture-based evidences suggesting that herbal PAK1-blockers such as curcumin, kaempferol and apigenin from propolis promote the growth of hair cells (Huh et al., 2009). More recently ARC (artepillin C)-based green propolis from Brazil was shown to promote hair growth in mice (Miyata et al., 2014).

Interestingly, both ARC and CAPE (caffeic acid phenethyl ester) from propolis block PAK1 (Messerli et al., 2009; Demestre et al., 2009). Furthermore, propolis in general is widely known to prevent cancer patients from their hair loss caused by conventional anti-cancer drugs such as DNA/RNA/microtubule poisons. As mentioned above, Bio30, CBI, and glaucarubinone are PAK1 inhibitors, thus, here the anti-alopecia (hair growth promoting) activity of these compounds was tested in an attempt to prove or disprove the notion that PAK1 normally suppresses the hair growth. As the positive (anti-alopecia) controls, minoxidil at 10 μM as well as Bio 30, a CAPE-based propolis product at 10 μg/ml (ppm) from New Zealand that blocks PAK1 (Demestre et al., 2009) were used. Among four PAK1-blocking herbal compounds tested here, cucurbitacin I from bitter melon (Goya) extract appeared to be the most potent compound to promote the hair growth in cell culture, stimulating by around 20% even at 10 nM, 1000 times more effective than minodixil (**see Figure 7.6B**). In the past, the results indicated that Goya extract potently promotes the hair growth in cell culture (Japanese Patent, No. P2006-219407A, 2006). Thus, it is likely that CBI is the major contributer to its anti-alopecia effect. It was also found that Bio 30 and glaucarubinone also showed the anti-alopecia activity, stimulating the hair growth at 10 μg/mL and 40 nM, respectively (**Figures 7.6A and 7.6C**).

Regarding the detailed or major mechanism underlying their anti-alopecia activity, it is likely that propolis such as Bio 30 promotes the hair growth by blocking PAK1. However, it is very unlikely that CBI promotes the hair growth mainly by blocking the oncogenic JAK2-PAK1 pathway, because at 10 nM, CBI has little effect on either JAK2 or PAK1. Since the FDA approve anti-alopecia drug “Finasteride” and CBI are among triterpenes which are structurally related to DHT (dihydrotestosterone),

a hair loss hormone, it is quite possible that CBI antagonizes DHT at around 10 nM. Nevertheless, it is interesting to test whether or not ShRNA specific PAK1 alone can promote the hair growth in cell culture.

7.5 CONCLUSION

This study introduced a new approach to the quick assessment of PAK1-blockers in cell culture. First, combining the immuno-precipitation (IP) of PAK1 from cell lysate and the in vitro ATP_Glo kinase assay kit was called “Macaroni-Western” assay. CBI, Bio 30, and glaucarubinone had both anticancer and anti-alopecia activities which could be due to PAK1 blocking. Taken together, these findings suggest that CBI, Bio 30 and glaucarubinone are useful compounds for treatment of cancers or hair loss.

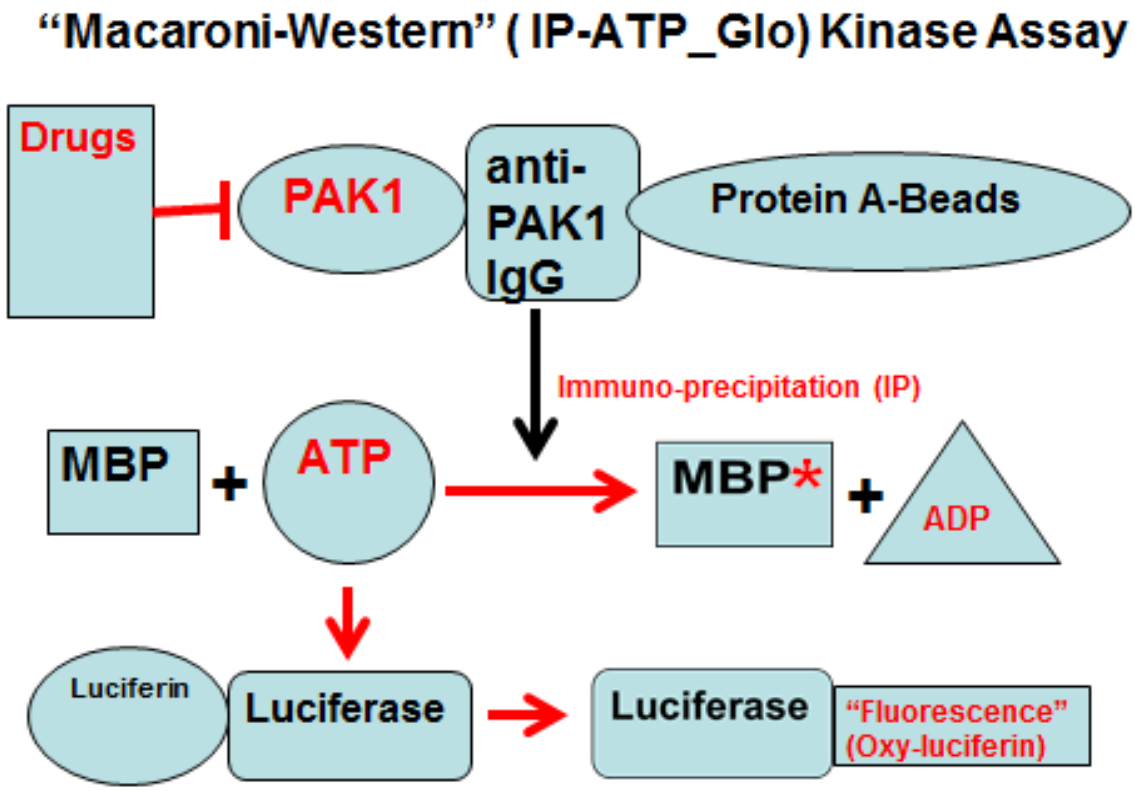

Figure 7.1. "Macaroni-Western" (IP-ATP_Glo) kinase assay. PAK1 is treated (activated or inactivated) with a given test sample in cell culture, and PAK1 is immuno-precipitated (IP) from cell lysate, and its kinase activity is measured by ATP_Glo kinase kit in test tube using ATP-dependent luciferin/luciferase reaction.

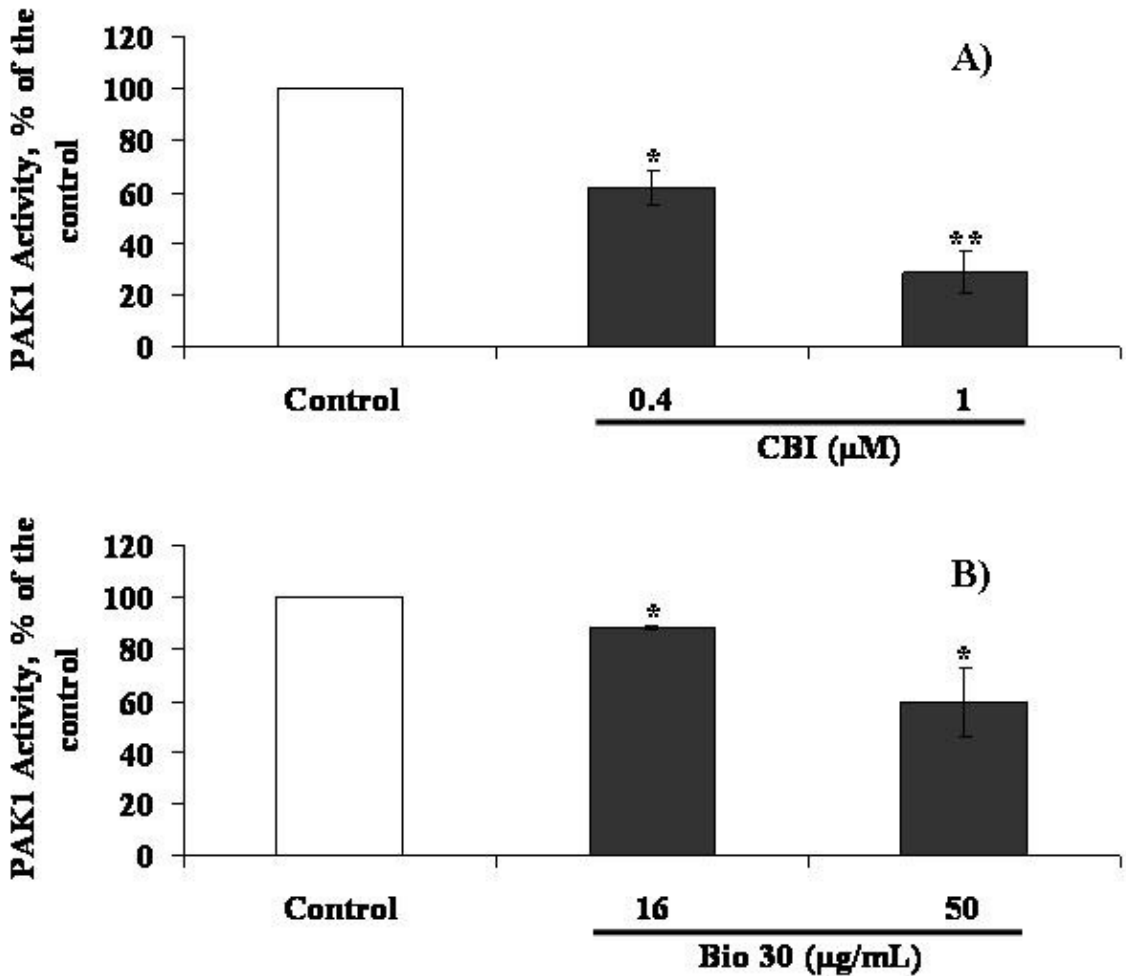

Figure 7.2. Both CBI (A) and Bio 30 (B) inactivate PAK1 in A549 cancer cells for 24 h. Inactivation of PAK1 in cells with CBI and Bio 30 was monitored by the "Macaroni-Western" kinase assay in test tube. For detail of the assay procedure, see under Materials and Methods. Data have significant difference by ANOVA analysis at $p \leq 0.05$. Statistically significant differences relative to control are indicated by asterisks. * $0.01 \leq p \leq 0.05$, ** $p < 0.01$.

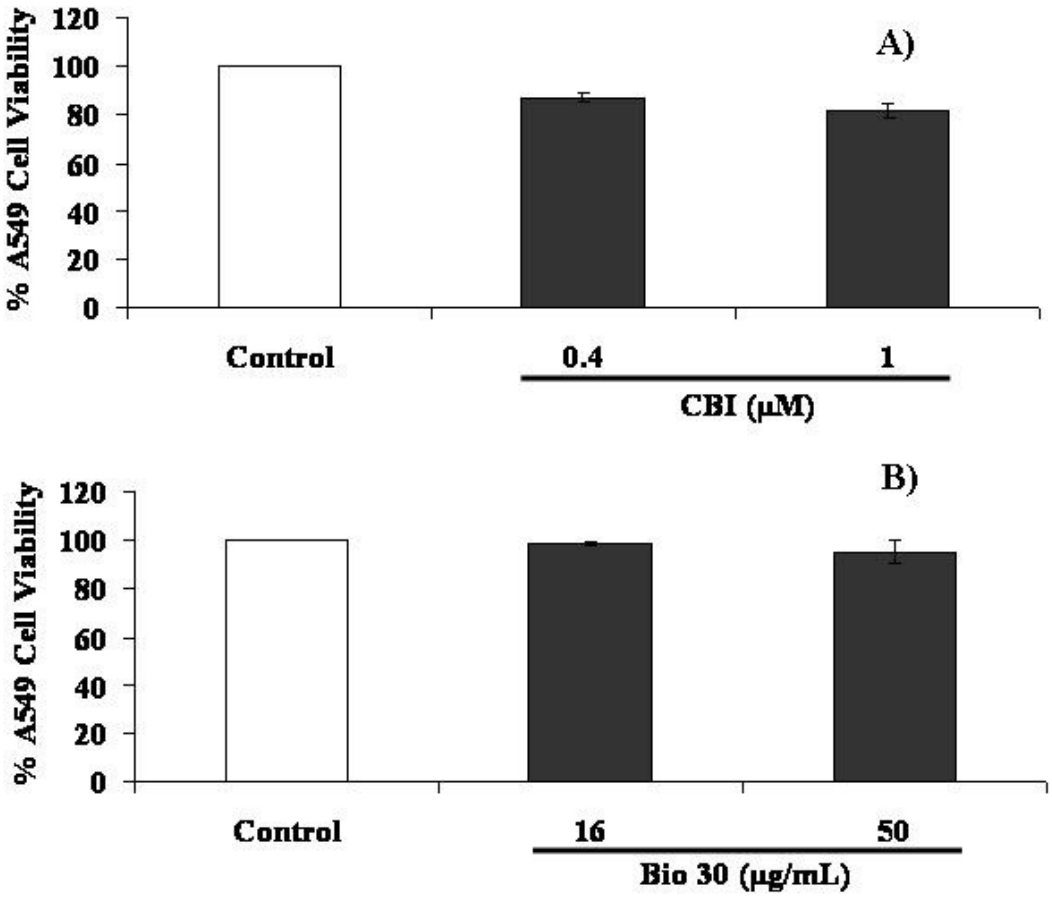

Figure 7.3. Neither CBI (A) nor Bio 30 (B) affects the viability/growth of A549 cells for the first 24 h. Data have significant difference by ANOVA analysis at $p \leq 0.05$.

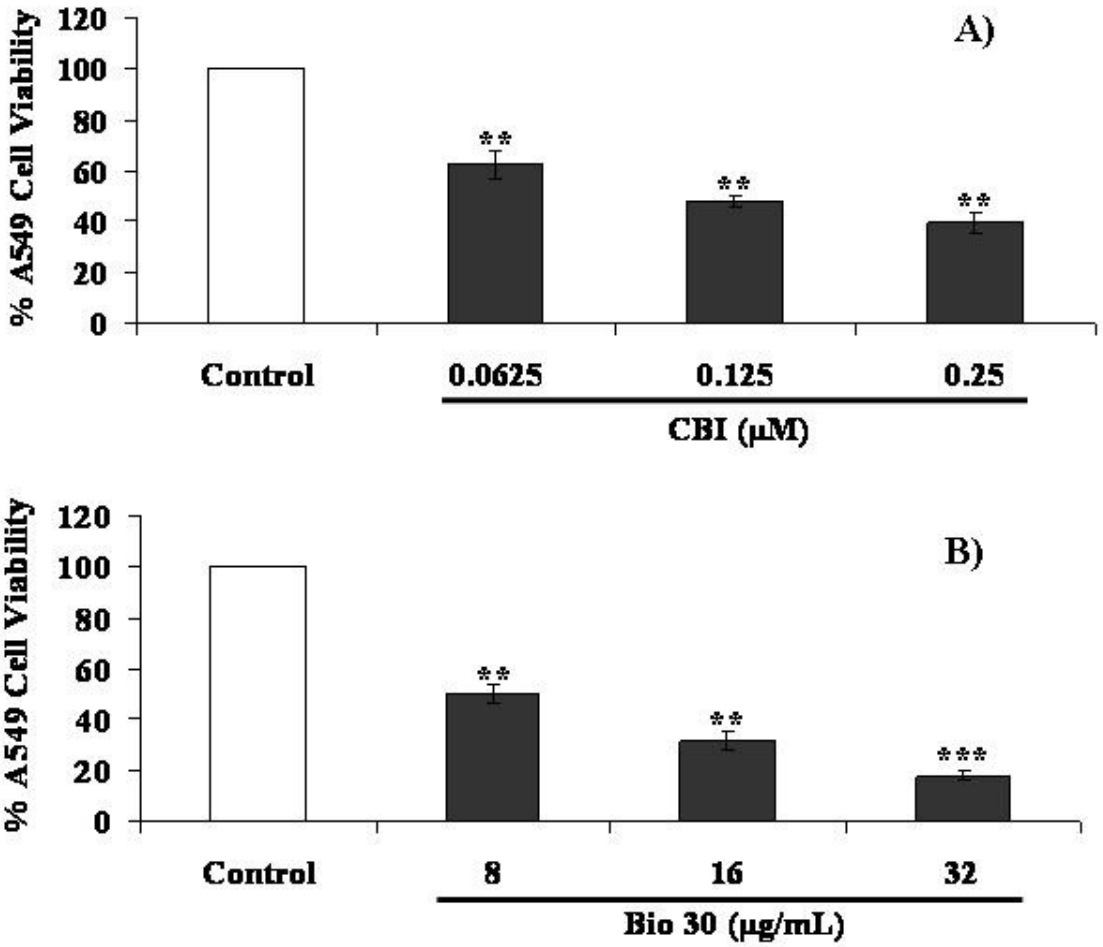

Figure 7.4. Both CBI (A) and Bio 30 (B) inhibit the growth of A549 cells for 72 h. The IC_{50} of CBI and Bio 30 are around 140 nM and 8 μg/mL (ppm), respectively. Data have significant difference by ANOVA analysis at $p \leq 0.05$. Statistically significant differences relative to control are indicated by asterisks. ** $p < 0.01$, *** $p < 0.001$.

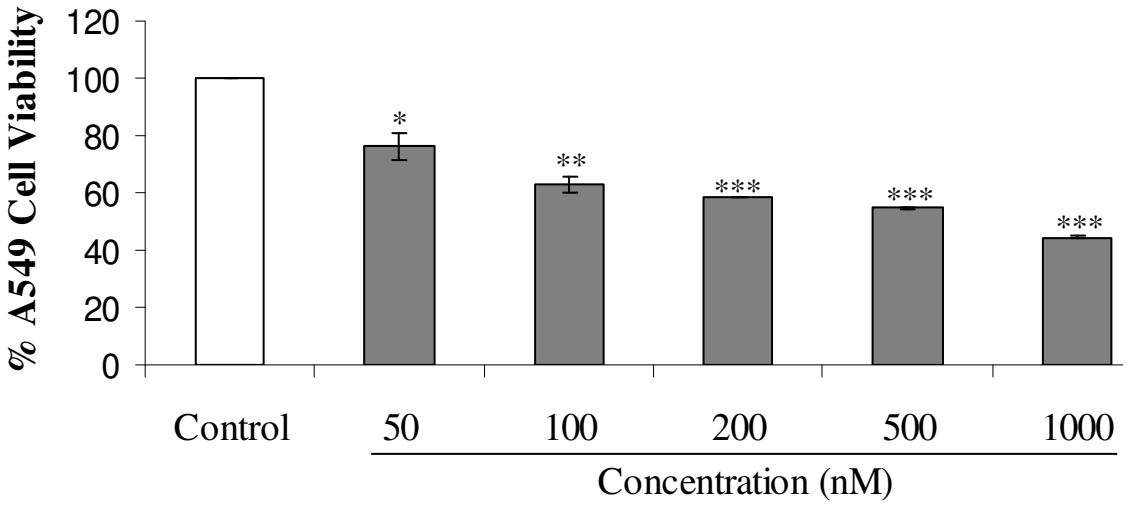

Figure 7.5. Anticancer activity of Glaucarubinone against A549 cells. Cells were treated with this compound for 72 h before the cell viability was evaluated. The results are mean ± SE of 6 replication per each treatment. Data have significant difference by ANOVA analysis at $p \leq 0.05$. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$

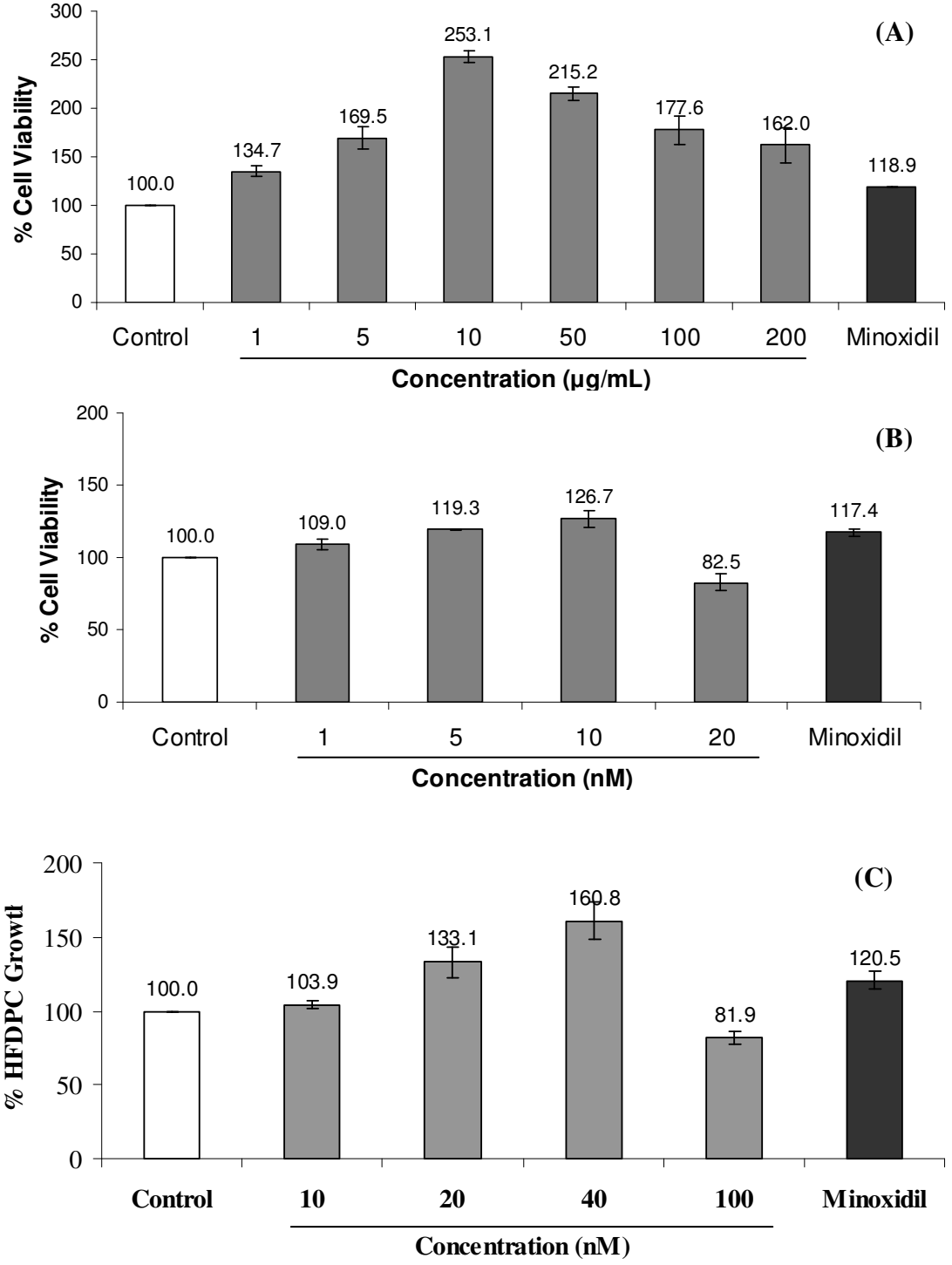

Figure 7.6. Hair cell growth promoting activity of Bio 30 (A), CBI (B) and Glaucarubinone (C). The results are mean ± SE of two repeated times. Numbers on each bar indicate the percentage of hair growth promotion.

CHAPTER 8

ARTEPILLIN C AND OTHER HERBAL PAK1-BLOCKERS: EFFECTS ON HAIR CELL PROLIFERATION AND RELATED PAK1-DEPENDENT BIOLOGICAL FUNCTION IN CELL CULTURE

8.1 SUMMARY

PAK1 (RAC/CDC42-activated kinase 1) is the major oncogenic kinase, and a number of herbal PAK1-blockers such as propolis and curcumin have been shown to be anti-oncogenic and anti-melanogenic as well as anti-alopecia (promoting hair growth). Cucurbitacin I isolated from bitter gourd/melon also exhibited a moderate anti-PAK1 activity. Hispidin, a metabolite of DK, strongly inhibited PAK1 with the IC_{50} of 5.7 μM, respectively. The IC_{50} of three hispidin derivatives (H1-3) for PAK1 inhibition ranged from 1.2 to 2.0 μM, while mimosine tetrapeptides [mimosine-Phe-Phe-Tyr (MFFY) and mimosine-Phe-Trp-Tyr (MFWY)] inhibited PAK1 at nanomolar level (IC_{50} of 0.13 and 0.60 μM, respectively). Moreover, the effects of these herbal compounds and their derivatives on the growth of cancer or normal hair cells, and melanogenesis in cell culture of A549 lung cancer, hair follicle dermal papilla cell, and B16F10 melanoma were tested. Among these herbal PAK1-inhibitors, cucurbitacin I from bitter melon (Goya) turned out to be the most potent compound to inhibit the growth of human lung cancer cells with the IC_{50} around 140 nM and to promote the growth of hair cells with the ED (effective dose) around 10 nM. Hispidin, a metabolite of 5,6-Dehydrokawain (DK) from alpinia inhibited the growth of cancer cells with the IC_{50} of 25 μM as does ARC (artepillin C), the major anti-cancer ingredient in Brazilian green propolis. Mimosine tetrapeptides (MFWY, MFYY, MFFY) and hispidin derivatives (H1-3) also exhibited a strong anti-cancer activity with the IC_{50} ranging from 16 to 30 μM. Mimosine tetrapeptides and hispidin derivatives strongly suppressed the melanogenesis in melanoma cells. Among PAK1-blockers from alpinia, hispidin was the best promoter of the hair growth in cell culture at 10 μM, far better than minoxidil. Interestingly, ARC alone promoted hair cell growth in cell culture with the ED around 20 μM. Taken together, these findings suggest that cucurbitacin I, ARC and hispidin could serve as leading herbal compounds for further

chemical modification, to produce far more cell-permeable or water-soluble derivatives which would be useful for the treatment of either cancers, hyper-pigmentation or hair loss.

8.2 INTRODUCTION

The family of p21-activated protein kinases (PAKs) belongs to RAC/CDC42-dependent Serine/Threonine kinases and in mammals consists of six distinct members (PAK1-6) (Dummler et al., 2009). Among them, PAK2 and PAK4 are absolutely essential for the development of embryos (Dummler et al., 2009; Maruta, 2014). However, apparently PAK1 is not essential for embryogenesis, and PAK1-deficient mice look perfectly healthy, and are even resistant to inflammatory diseases, and PAK1-deficient mutant of *C. elegans* lives longer than the wild-type (Maruta, 2014). PAK1 is responsible not only for a variety of inflammatory diseases such as asthma and arthritis, but also for infection of HIV and influenza virus. In addition, PAK1 is essential for the growth of majority of solid tumors as well as their metastasis and angiogenesis (blood vessel formation required for the growth of solid tumors) (Dummler et al., 2009; Maruta, 2014). In other words, hyper-activation or over-expression of PAK1 would shorten the healthy lifespan, in part by causing cancers and a variety of other diseases/disorders such as diabetes (type 2), hyper-tension and Alzheimer's disease (Maruta, 2014). Since PAK1 is not essential for the growth of normal cells (Maruta, 2014), (unlike the conventional anti-cancer drugs) blocking PAK1 *per se* does not cause any side effect. Thus, selective small molecule PAK1-blockers (natural or synthetic) would have a potentially huge market value for the treatment of a variety of PAK1-dependent diseases/disorders and the longevity as well in the future. Furthermore, considering the well-known fact that people in Okinawa have enjoyed the longest healthy lifespan among Asian population, I recently got interested in testing the possibility that foods or herbal products

derived from some of plants uniquely grown in Okinawa might contribute to the longevity among Okinawa people by blocking PAK1. Interestingly, many herbal PAK1-blockers such as propolis (CAPE/ARC-based) and curcumin have been shown to inhibit the melanogenesis by down-regulating tyrosinase that converts tyrosine to melanins through L-Dopa, as well as melanogenic/oncogenic transcription factors such as β-catenin and microphthalmia-associated transcription factor (MIFT) which are essential for activation of tyrosinase gene (Miller and Mihm, 2006; He et al., 2008; Lee et al., 2013), suggesting the possibility that PAK1 is essential for melanogenesis as well. In fact we recently have proven by silencing PAK1 or PAK4 gene with shRNAs that both PAK1 and PAK4 each contribute by 50% to melanogenesis in skin cells (Yun et al., 2015; Be Tu et al., the manuscript submitted for the publication) (**Figure 8.S1**). More interestingly, these herbal PAK1-blockers promote hair growth, suggesting the possibility that PAK1 normally suppresses the growth of hair cells (Miyata et al., 2014).

Leucaena leucocephala and *Alpinia zerumbet*, distributed widely in Okinawa in particular and in subtropical and tropical zones in general, have lent them as multi-purpose plants such as the development of novel cosmetics and medicines (Tawata et al., 2008). Alpinia is used in folk medicine for its anti-inflammatory, bacteriostatic, and fungistatic properties (Zoghbi et al., 1999). The essential oil from its leaves possesses relaxant and anti-spasmodic actions in rat ileum (Bezerra et al., 2000). Early data have indicated that mimosine, 5,6-dehydrokawain (DK) and dihydro-5,6-dehydrokawain (DDK), which are major ingredients in leucaena and alpinia, are shown to have various biological activities including anti-fungal, anti-inflammatory, anti-tumor and anti-viral (Corbi et al., 2005; Upadhyay et al., 2011). In the laboratory, several compounds have prepared from leucaena and alpinia against HIV-1 integrase and neuraminidase enzymes (Upadhyay et al., 2011a), for inhibition of advanced glycation end products and for prevention of the skin diseases (Chompoo et al.,

2011; 2012a). Bitter gourd (*Momordica charantia*), known as "goya" in Japan, has been implicated in different pharmacological activities such as anti-diabetic, anti-bacterial, anti-viral, anti-cancer, and anti-obesity (Hsu et al., 2011). In spite of given diverse biological activities, their anti-PAK1 activity still remained unknown. This study firstly focused primarily on the potential anti-PAK1 activity of compounds isolated from alpinia, leucaena and bitter gourd. Then, several derivatives were prepared from these natural compounds for further potentiation of their anti-PAK1 activity (**Figure 8.1**). To the best of my knowledge, this is the very first report showing the direct inhibition of PAK1 by compounds derived from these three Okinawa plants. On the other hand, the experiment was conducted to evaluate if these herbal compounds have any effect on the growth of normal hair and lung cancer cells or melanogenesis of melanoma in cell culture. Their anti-LIM kinase (LIMK) activity was also tested to compare with the anti-PAK1 activity in vitro for testing their kinase specificity.

8.3 MATERIALS AND METHODS

8.3.1 Chemicals and reagents

ARC (artepillin C) was kindly provided by Prof. Yoshihiro Uto at Tokushima university. Tris(triethylsilyl)silane, resveratrol were obtained from Sigma-Aldrich (Shinagawa-ku, Tokyo, Japan) whereas trifluoromethanesulfonic acid was obtained from Nacalai Tesque (Nakagyo-ku, Kyoto, Japan). Curcumin was purchased from Kanto Chemical Co. (Chuo-ku, Tokyo, Japan). Fmoc-L-amino acids were purchased from Hipep Laboratories (Kamigyo-ku, Kyoto, Japan). 2-[1H-benzotriazole-1-yl]-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) were from Novabiochem (Schuchardt, Hohenbrunn, Germany). Quercetin, wang resin (1% DVB), N,N'-diisopropylcarbodiimide (DIC), N,N'-diisopropylethylamine (DIEA), and 1-hydroxy-1*H*-benzotriazole (HOBt) were purchased

from Wako Pure Chemical Industries (Chuo-ku, Osaka, Japan). Unless otherwise mentioned, all reagents were of analytical grade and were obtained from Wako Pure Chemical Industries and Kanto Chemical Co. The ^{1}H spectra were recorded on a JEOL JNM-ECA400 (Tokyo, Japan). Chemical shifts are expressed in parts per million (δ) relative to tetramethylsilane (TMS).

8.3.2 Mimosine isolation from Leucaena leucocephala leaves

Samples of *Leucaena leucocephala* leaves were collected at the Faculty of Agriculture, University of the Ryukyus, Okinawa, Japan (lat 26ºN, long 127ºE). Fresh leaves (1.5 kg) were boiled in 5 L water for 10 min. The cooled liquid extract was sieved by suction filtration in a shaking bath (As One, Nishi-ku, Osaka, Japan) and the filtrate was mixed with ion-exchange resin (2 kg), stirred for 30 min, and left overnight. The resin was rinsed with distilled water 5–6 times and 5 L 80% ethanol was added dropwise to remove chlorophyll. Mimosine was dissolved from the resin with dropwise addition of 6 L of 2 N NH_4OH. The liquid extract was concentrated to a final volume of 300 mL at 40ºC under reduced pressure. The solution was adjusted to pH 4.5–5.0 with 6 N HCl and mimosine was precipitated at 4ºC overnight. The precipitate was recrystallized from 5 N NaOH (pH 9.0) and 6 N HCl (pH 4.5–5.0) and then allowed to stand at 4ºC to give pure mimosine. Mimosine was stored at –20ºC until further use (Tawata, 1990).

8.3.3 Preparation of mimosinol from mimosine

Trifluoromethanesulfonic acid (187 µL, 2 mmol) was added to a 25-mL round-bottom flask containing 3.4 mL dichloromethane (CH_2Cl_2). After stirring at room temperature, tris(triethylsilyl)silane solution (618 µL, 2 mmol) was added dropwise and the mixture was stirred at room temperature for 3 h until the solution become clear. Mimosine (0.4 g, 2 mmol) was placed in a round-bottom flask, to which imidazole (0.15 g, 2.2 mmol) and dimethylformamide (DMF):CH_2Cl_2 (3.4 mL, 1:1) were then added. The reaction flask was

cooled to 0ºC and tris(triethylsilyl)silyl triflate was added dropwise. After the addition was completed, the reaction was stirred at room temperature for 2 h. Mimosine ester was obtained from the filtrate by evaporation. A solution of sodium borohydride (0.28 g, 7.2 mmol) in 3 mL 50% ethanol was added to solution of mimosine ester in 3 mL of 50% ethanol. The resulting mixture was refluxed at room temperature for 5.5 h and the solvent ethanol was evaporated in vacuo. The aqueous solution thus obtained was extracted with ethyl acetate (3 × 20 mL); the combined extracts were washed with saturated sodium chloride, dried over anhydrous sodium sulfate, and evaporated to give mimosinol as a colorless crystal (352 mg, 95% yield). ^{1}H NMR (D_2O, 400 MHz) δ 7.93 (s, 1H, CH), 7.28 (s, 1H, CH), 3.02–2.86 (d, 2H, CH), 2.08–1.91 (s, 2H, CH2), 1.58–1.54 (m, 2H, CH_2), 1.22–1.11 (m, 1H, CH).

8.3.4 Synthesis of mimosine tetrapeptides

Compounds were prepared according to procedure described previously (Upadhyay et al., 2011b). In brief, Fmoc-L-amino acid (1.6 mmol) was dissolved in 5 mL of dimethylacetoamide, followed by adding HOBt (1.6 mmol) and DIC (1.6 mmol). The mixture was added to swollen Wang resin (1 g) in DMF and stirred for 17 h. After deprotection of 9-fluorenylmethoxycarbonyl (Fmoc) with 25% piperidine, the next amino acids were coupled to the resin mixture solution (Fmoc-amino acid/HOBt/HBTU/DIEA = 4:3:3.6:8) and stirred for 1 h to form tripeptides. The Kaiser's test was used to assess the completeness of the coupling reaction. After final coupling with mimosine, the final cleavage was performed by shaking the resin vigorously in 95% trifluoroacetic acid (TFA) for 1 h. The resin was filtered and washed with TFA. The obtained filtrate was precipitated with ice-cold diethyl ether. The resulting precipitate was filtered, washed with diethyl ether, and dried under a vacuum to obtain the desired mimosine tetrapeptides. The purified compounds were evaluated using LC-MS (ESI): *m/z* $[M-H]^+$ 693.2, 670.1, 654.2 for MFWY, MFYY, and MFFY, respectively.

8.3.5 Isolation of dihydro-5,6-dehydrokawain (DDK) and 5,6-dehydrokawain (DK) compounds from Alpinia zerumbet

The rhizomes of alpinia were collected from the University of the Ryukyus campus, Okinawa, Japan. The rhizomes (2 kg) were boiled in 10 L water for 20 min. The solution was cooled at room temperature and sieved by suction filtration (As One, Nishi-ku, Osaka, Japan). The filtrate was reduced to 1 L under vacuum at 40^{o}C, and extracted with hexane (3 x 500 mL). The organic layer was evaporated to complete dryness under vacuum. The dried crude extract was boiled in water and filtered hot. The residue obtained was purified by high-performance liquid chromatography (HPLC) (Shimadzu, Nakagyo-ku, Kyoto, Japan) to give DK. The filtrate was crystallized at 4^{o}C, and the crystals were purified further using HPLC to obtain DDK. For purification of DDK and DK, the major peaks were collected using mobile phase including solvent A (0.1% acetic acid in water) and solvent B (0.1% acetic acid in methanol). The gradient elution was performed as follows: 1-10 min, isocratic 50% B; 10-20 min, linear gradient 50-100% B; 20-30 min, isocratic 100% B; 30-35 min, linear gradient 100-50% B. The flow rate and absorbance wavelength were set at 0.8 mL/min and 280 nm, respectively (Upadhyay et al., 2011a).

8.3.6 Preparation of hispidin and cucurbitacin I compounds

These two compounds were prepared by other group in our laboratory. DK was converted to hispidin by cytochrome P450 2C9 enzyme (CYP2C9) in the microsomes of rabbit liver (**Figure 8.2**) (Upadhyay et al., 2009). Cucurbitacin I was isolated from Okinawa bitter gourd (*Momordica charantia*) fruits.

8.3.7 Preparation of hispidin derivatives (H1-3)

Hispidin (3 mg) was dissolved in 0.6 mL methanol:CH_2Cl_2 (1:5). The solution was cooled to 0^{o}C, and 0.5 mL of diazomethane in CH_2Cl_2 was added. The mixture was stored overnight at 4^{o}C. Solvents were evaporated, and the residue was purified by silica gel

preparative thin layer chromatography (PTLC) to obtain H1 as pale yellow powder (2 mg, 67% yield) (Singh et al., 2003). Compound H1 (3.5 mg) dissolved in 0.82 mL of MeOH:$CHCl_3$ (1:1) was stirred for 2 h in the presence of 10% of Pd/C (0.65 mg). The mixture was filtered and solvent was evaporated in vacuo. Purification was achieved by column chromatography to afford compound H2 as a white solid (3 mg, 85%) (McCracken et al., 2012). The similar procedure was used to prepare H3 from hispidin.

Data for 6-(3,4-dimethoxystyryl)-4-methoxy-2H-pyran-2-one (H1). ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.43 (d, 1H, CH), 7.07 (dd, 1H, CH), 7.00 (d, 1H, CH), 6.85 (d, 1H, CH), 6.43 (d, 1H, CH), 5.89 (d, 1H, CH), 5.46 (d, 1H, CH), 3.91 (s, 3H, OCH_3), 3.89 (s, 3H, OCH_3), 3.81 (s, 3H, OCH_3).

Data for 6-(3,4-dimethoxyphenethyl)-4-methoxy-2H-pyran-2-one (H2). ^{1}H NMR ($CDCl_3$, 400 MHz) δ 6.77 (d, 1H, CH), 6.69 (dd, 1H, CH), 6.66 (d, 1H, CH), 5.69 (d, 1H, CH), 5.40 (d, 1H, CH), 3.84 (s, 3H, OCH_3), 3.83 (s, 3H, OCH_3), 3.76 (s, 3H, OCH_3), 2.91 (m, 2H, CH2), 2.71 (m, 2H, CH2).

Data for 6-(3,4-dihydroxyphenethyl)-4-hydroxy-2H-pyran-2-one (H3). ^{1}H NMR (DMSO, 400 MHz) δ 7.29 (d, 1H, CH), 7.20 (dd, 1H, CH), 6.76 (d, 1H, CH), 6.11 (d, 1H, CH), 5.26 (d, 1H, CH), 3.34 (m, 2H, CH2), 2.99 (m, 2H, CH2).

8.3.8 In vitro assay for the kinase PAK1

Its kinase activity was measured by ADP-Glo^{TM} kinase assay kit (Promega, Madison, WI, USA) according to manufacturer's instructions. Human PAK1 (10 µL) at 25 ng/reaction concentration was incubated with 5 µL of test compounds at various concentrations for 10 min. The kinase reaction was started by the addition of 2.5 X adenosine triphosphate (ATP)/substrate mix (10 µL) which was incubated for 40 min. The reaction was terminated by 25 µL ADP-Glo^{TM} reagent, followed by 40 min incubation. To this reaction mixture was added 50 µL of the kinase detection reagent which converts adenosine diphosphate (ADP) to

ATP that eventually generates a luciferin/luciferase-based fluorescence. After 30 min incubation, luminescence was recorded by MTP-880Lab microplate reader (Corona, Hitachinaka-ku, Ibaraki Japan) with an integration time of 0.5 s per well. Blank wells lacked the test compounds and PAK1 but did include all remaining components. All procedure steps were conducted at room temperature. The percentage inhibition was calculated relative to the control kinase activity without any inhibitor.

8.3.9 Cell culture

Mouse B16F10 cell line was obtained from American Type Culture Collection (Manassas, Virginia, USA) while human A549 lung cancer cell was purchased from Japanese Collection of Research Bioresources Cell Bank (Ibaraki, Osaka, Japan). B16F10 cells were cultured in Dulbecco's modified minimum essential medium (D-MEM) medium containing 10% fetal bovine serum (FBS), 1% penicillin/streptomycin while A549 cells were cultured in D-MEM with 10% fetal bovine serum (FBS). Both cells were incubated in a humidified atmosphere with 5% CO_2 incubator at 37^{o}C.

8.3.10 Cell growth/viability assay

The assay is based upon the cleavage (reduction) of the yellow tetrazolium salt MTT to give purple formazan crystals by mitochondrial reductase in viable cells (Campos et al., 2013; Zhou et al., 2013). Cells were seeded in a 96-well plate at a density 5 x 10^3 cells/well for B16F10 and 1 x 10^4/well for A549, cultured for 24 h before the compounds were added to the medium. The cells were incubated in a humidified atmosphere 5% CO_2 at 37^{o}C for 48 or 72 h. After treatment, 20 µL of MTT solution (0.5 mg/mL) were added to each well, and plates were incubated for 3 h. The medium was removed, and formazan was dissolved in 200 µL of DMSO. After shaking the plate for 10 min, cell viability was assessed by measuring the absorbance at 490 or 570 nm using microplate reader (BioTek, Synergy HT, Winooski, Vermont, USA).

8.3.11 Cellular tyrosinase inhibition in B16F10 cells

Cells were seeded in a 96-well plate at a density 5 x 10^3 cells/well in culture medium containing D-MEM supplemented with 10% FBS, 1% penicillin/streptomycin, cultured for 24 h. After treatment with α-MSH (100 nM), the cells were treated with tested compounds for further 48 h. The cells were washed twice with 50 mM of ice-cold phosphate buffer (pH 6.8), lysed with 90 μL of 50 mM phosphate buffer (pH 6.8) containing 1% Triton-X, then frozen at -80^{o}C for 30 min. After thawing and mixing, 20 μL of 0.2% L-DOPA was added to each of 96-well plate. The mixture was incubated for 2 h at 37^{o}C, and tyrosinase activity was measured at 490 nm (Campos et al., 2013).

8.3.12 Determination of melanin content in B16F10 cells

The assay was performed following previously described protocol (Chan et al., 2014). Cells were seeded in a 96-well plate at a density 5 x 10^3 cells/well in culture medium containing D-MEM supplemented with 10% FBS, 1% penicillin/streptomycin, and cultured for 24 h. Then, the cells were treated with α-MSH (100 nM). After treatment, the cells were added tested compounds, and incubated for 48 h. The medium was removed, and the cells were dissolved in 100 μL of 1 N NaOH. The mixture was heated at 90^{o}C for 1 h. The relative melanin content was estimated by the absorbance at 405 nm.

8.3.13 Assay for hair cell growth in cell culture

Human follicle dermal papilla cells (HFDPCs) were purchased from TOYOBO (Osaka, Japan). Cells were cultured in HFDPC growth medium containing 50 mL of papilla cell growth medium (PCGM), 0.5 mL of fetal calf serum (FCS), 0.5 mL of BPE, 0.25 mL of Cyp, and 0.25 mL of ITT in 51.5 mL total volume. Cell viability was evaluated using thiazolyl blue tetrazolium bromide (MTT) assay. HFDPCs were collected and diluted in medium containing Dulbecco's modified minimum essential medium (D-MEM) and 10% fetal bovine serum (FBS) at a cell density of 1 x 10^4 cell/mL. Then, 200 μL of cell suspension

(2,000 cells/well) was transferred into each well of collagen-coated 96 well plate, and cells were incubated for 37°C, 5% CO_2 for 3 days. The medium was removed, and 200 μL of samples prepared in D-MEM only was added. After 4 days incubation, 100 μL of MTT solution in D-MEM (0.4 mg/mL) was added, and the mixture was incubated for 2 h. The un-reduced MTT was removed. After 100 μL of 2-propanol was added to dissolve the reduced MTT form (insoluble formazan) in each well with shaking, and the absorbance was read at 570 nm using a microplate reader. The cell viability rates were calculated from readings and are represented as percentages of the control value (treated cells with D-MEM only). This hair growth assay in cell culture was performed according to the procedure described by Ohto (Human follicle dermal papilla cell proliferation promoting agents and hair growth formula. Japanese Patent, No. P2006-219407A, 2006). However, the procedure is essentially very similar to that previously published (Huh et al., 2009).

8.3.14 Luciferase-based LIMK kinase assay

LIMK1 assays were carried out in 96-well white plates in a 40 μL total volume, according to the previously described procedure (Mezna et al., 2012). Five microliters of LIMK1 (7.5 ng/well), 8 μM full length cofilin in kinase buffer containing 40 mM MOPS pH 7.0, 1 mM EDTA was transferred to each well of 96-well plate, and 10 μL of test compounds diluted in appropriate solvent was added. Then, 5 μL of 20 μM adenosine triphosphate was added to each well. The reaction plate was incubated for 1 h at room temperature. After incubation, the equal volume of kinase Glo reagent (20 μL) was added to each well, and the plate was further incubated for 30 min to stabilize luminescent signal. Luminescence was recorded by MTP-880Lab microplate reader (Corona, Hitachinaka-ku, Ibaraki, Japan) with an integration time of 0.5 s per well. Control wells lacked the test compounds, but did include LIMK1 and all remaining components. Resveratrol was used as a positive control. The

percentage inhibition was calculated relative to the control LIMK1 activity without any inhibitor.

8.3.15 Data analysis

All statistical analyses were performed using statistical analysis system (SAS) software, version 9.1.3 (SAS Institute Inc., Cary, North Carolina). Data were analyzed by one-way analysis of variance (ANOVA) at $p \leq 0.05$. All calculations were performed using Excel, Microsoft Office 2003. The IC_{50} values were determined graphically.

8.4 RESULTS AND DISCUSSION

8.4.1 PAK1 inhibition

As summarized in **Table 8.1**, the anti-PAK1 activity of DK and DDK is significantly better than mimosine and mimosinol. The IC_{50} values of DK and DDK are 17 and 10 μM, respectively, while mimosine and mimosinol had the IC_{50} values of 37 and 30 μM, respectively. Furthermore, a metabolite of DK called hispidin had a strong anti-PAK1 activity (IC_{50} = 5.7 μM), almost equivalent to curcumin (IC_{50} = 7.0 μM), but clearly stronger than resveratrol (IC_{50} = 15 μM). In previous study, it is discovered that DK and DDK from alpinia rhizomes as well as mimosine from leucaena leaves are strong inhibitors of HIV-1 integrase and neuraminidase activity (Upadhyay et al., 2011a). Interestingly, PAK1 is essential for the replication of several viruses including HIV and influenza virus (Maruta, 2014). In this study it was found that DK, DDK and mimosine compounds inhibited directly the kinase activity of PAK1. Considering that one of the benzene rings in resveratrol is simply replaced by an α-pyrone ring in hispidin, it is likely that α-pyrone significantly contributes to an increase in the anti-PAK1 activity. However, Upadhyay et al. (2011a) suggested that the methoxy group at C-5 of DDK and DK could be the major contributor to their anti-HIV activity. Thus, perhaps

this methoxy group of DK and DDK could also be attributed to their anti-PAK1 activity, in addition to the α-pyrone ring. In comparison of the anti-PAK1 activity between DK and hispidin, in vivo DK could act as a PAK1 inhibitor in two ways, first as DK itself and second after it is converted to hispidin by the enzyme CYP2C9. The anti-PAK1 activity of DK is significantly weaker than its metabolite "hispidin". Thus, it is most likely that the attachment of two OH groups to the benzene ring of DK or DDK contributes to an increase in the anti-PAK1 activity. I further prepared a few hispidin derivatives in an attempt to potentiate their anti-PAK1 activity. As shown in **Table 8.1**, the two methoxy derivatives (H1-2) inhibited PAK1 more strongly than hispidin. The anti-PAK1 activity of a few mimosine tetrapeptides was also evaluated. Interestingly, at least two of these peptides inhibited PAK1 at nanomolar level.

By the simple method used in the laboratory, these pyrones (DK, DDK) as well as mimosine and mimosinol can be prepared easily from alpinia and leucaena. Moreover, the synthesis of their derivatives is not costly; thus, in theory, their further chemical modification for creating more potent PAK1-inhibitors could be economically feasible.

8.4.2. Effect on cancer cell growth/viabiltiy

As summarized in **Table 8.2**, hispidin had a strong inhibitory activity against A549 cells with the IC_{50} of 25 μM. Mimosine tetrapeptides (MFFY, MFWY, MFYY) also exhibited a strong anti-cancer activity with the IC_{50} values ranging from 27 to 30 μM. The anti-cancer activity of hispidin derivatives (H1-3) (IC_{50} of 17, 21, 17 μM) is better than that of resveratrol (IC_{50} = 23 μM). Among the compounds tested here, cucurbitacin I (CBI) showed the strongest anti-cancer activity with the IC_{50} of 140 nM (**Figure 8.3**).

It should be pointed out that the IC_{50} of CBI for inhibiting the growth of A549 lung cancer cells is far lower than the IC_{50} for inhibiting directly the kinase activity of PAK1 in vitro (around 30 μM, see **Tables 8.1 and 8.2**). Thus, it is very clear that this potent growth

inhibition is not due to the direct inhibition of PAK1. Instead, it is more likely that CBI inactivates indirectly PAK1, by a combination of direct inhibition of the oncogenic Tyr-kinase JAK2 and the oncogenic G protein RAC independently (Blaskovich et al., 2003; Lopez-Haber and Kazanietz, 2013), both of which are essential for the activation of PAK1 in cells (Maruta, 2014), with the IC_{50} ranging 100-300 nM, depending on cancer cell lines. In fact the IC_{50} of CBI against both the cancer cell growth and melanogenesis well co-related with its anti-PAK activity in cell culture measured by "Macaroni-Western" kinase assay (Nguyen et al., 2015a).

It is also worth noting that the IC_{50} of all other compounds tested here for inhibiting the cancer cell growth is far higher than their IC_{50} for direct inhibition of PAK1 in vitro (compare **Tables 8.1 and 8.2**). A549 lung cancer cell line carries an oncogenic Ki-RAS mutant and its growth strongly depends on PAK1 (Maruta, 2014). Thus, these data indicate that all compounds (except for CBI) have a very poor cell-permeability. For instance, the IC_{50} of MFFY against PAK1 in test tube is around 0.1 μM, whereas the IC_{50} against the cancer cell growth in cell culture is around 30 μM, clearly indicating that less than 1% of this molecular enters cells. In other words, these non-CBI compounds should be further chemically modified in order to potentiate their cell-permeability (and improve their water-solubility as well) for any animal experiments and clinical application (cancer therapy).

8.4.3 Anti-melanogenic activity

Anti-melanogenic activity of mimosine tetrapeptides (MFFY, MFWY, and MFYY) and hispidin derivatives (H1-3) was evaluated. None of these compounds has any significant effect on the viability of melanoma cells up to 100 μM (**Figures 8.4 and 8.5**). The mimosine tetrapeptides (MFFY, MFWY, and MFYY) inhibited the cellular tyrosinase by 40-50% at 50 μM, more potently than mimosine *per se* (only 28% inhibition at 100 μM) and kojic acid (35% inhibition at 50 μM) (see **Figure 8.6**). Hispidin derivatives (H1-3) turned out to be rather poor anti-melanogenic compounds, and their inhibition reached only 27% at 50 μM

(see **Figure 8.7**). Under α-MSH hormone stimulation (see **Table 8.3**), the anti-melanogenic activity (reducing the melanin content) of mimosine derivatives, in particular MFFY and MFWY (IC_{50} of 25 and 31 μM, respectively), was clearly stronger than that of mimosine *per se* (IC_{50} = 226 μM).

Like the anti-cancer activity of non-CBI compounds tested here, their anti-melanogenic activity turned out to be rather poor by judging their IC_{50} in cell culture, most likely due to their poor cell-permeability. On the other hands, the IC_{50} of CBI for inhibiting the melanogenesis was around 300 nM, only 1/100th of these non-CBI compounds (Nguyen et al., 2015a). Nevertheless, it is most likely that the observed anti-melanogenic activity of these compounds including CBI is mainly due to their anti-PAK1 action, because it is recently verified that PAK1 is essential for the melanogenesis in skin cells such as the melanoma B16F10 (Be Tu et al., 2015, the manuscript submitted for the publication).

8.4.4 Effect on hair cell proliferation in cell culture

In the past, there were a few cell culture-based evidences suggesting that herbal PAK1-blockers such as curcumin, kaempferol and apigenin from propolis promote the growth of hair cells (Huh et al., 2009). More recently ARC (artepillin C)-based green propolis from Brazil was shown to promote hair growth in mice (Miyata et al., 2014). Interestingly, both ARC and CAPE (caffeic acid phenethyl ester) from propolis block PAK1 (Messerli et al., 2009; Demestre et al., 2009). Furthermore, propolis in general is widely known to prevent cancer patients from their hair loss caused by conventional anti-cancer drugs such as DNA/RNA/microtubule poisons. Thus, here the effect on hair cell growth of ARC as well as cucurbitacin I and several other PAK1-blocking herbal compounds from Okinawa plants was also tested in an attempt to prove or disprove the notion that PAK1 normally suppresses the hair growth. As the positive (anti-alopecia) controls, minoxidil at 10 μM as well as Bio 30, a CAPE-based propolis product at 10 μg/mL (ppm) from New Zealand that blocks PAK1

(Demestre et al., 2009) were used. Among five PAK1-blocking herbal compounds tested here, cucurbitacin I from bitter melon (Goya) extract appeared to be the most potent compound to promote the hair growth in cell culture, stimulating by around 20% even at 10 nM, 1000 times more effective than minodixil (see **Figure 8.8**). In the past, is was found that Goya extract promotes the hair growth in cell culture by 70% with the ED around 3300 μg/mL (Japanese Patent, No. P2006-219407A, 2006). Thus, it is likely that CBI is the major contributer to its promotion of hair cell growth. Interestingly, ARC also promoted the hair cell growth with the ED (effecive dose) around 20 μM (see **Figure 8.9C**), which is in the same range of its IC_{50} against PAK1-dependent cancer cell growth and melanogenesis (Takahashi et al., unpublished observation), clearly indicating that ARC is among the major contributors to the anti-alopecia effect of Brazilian green propolis.

It was found that alpinia extract also stimulates the hair cell growth by around 2 folds at 100 μg/mL (see **Figure 8.9A**), although the ED is 10 times higher than the ED (10 μg/mL) of Bio 30 (see **Figure 8.9B**). Thus, so far Bio 30 is the most potent compound among these three herbal extracts in promoting the hair cell growth. Among PAK1-blockers from alpinia extract, hispidin appeared to be the most potent, stimulating the hair cell growth by around 2.5 folds at 10 μM (see **Figures 8.8C and 8.10**).

Regarding the detailed or major mechanism underlying their promotion of hair cell growth, it is likely that propolis such as Bio 30, ARC and hispidin from alpinia promoted the hair cell growth by blocking PAK1, because their IC_{50} for PAK1 and the ED for hair cell growth are in the same range. However, it is very unlikely that CBI promoted the hair cell growth mainly by blocking the oncogenic JAK2-PAK1 pathway, because at 10 nM, CBI has little effect on either JAK2 or PAK1. Since the FDA approve anti-alopecia drug “Finasteride” and CBI are among triterpenes which are structurally related to DHT (dihydrotestosterone), a hair loss hormone, it is quite possible that CBI antagonizes DHT at around 10 nM.

Nevertheless, I am planning to test whether or not shRNA specific PAK1 alone can promote the hair growth in cell culture.

8.4.5 LIMK1 inhibition

LIM kinase 1 (LIMK1) is an oncogenic kinase responsible for the metastasis of all solid tumors as well as a process of Alzheimer's disease (Maruta, 2014). LIMK1 is among the direct substrates of PAK1, and phosphorylates an F actin-severing protein called cofilin at Ser 3 for the inactivation. In an attempt to determine the kinase specificity of these herbal compounds, here we tested their in vitro anti-LIMK1 activity in comparison with their in vitro anti-PAK1 activity previously determined (Nguyen et al., 2014). As shown in **Table 8.1**, DK, DDK and hispidin (IC_{50} of 25, 22 and 14 μM, respectively) had LIMK1 inhibition better than resveratrol, a positive control (IC_{50} = 37 μM). Mimosine and cucurbitacin I exhibited moderately inhibitory activity with IC_{50} of 32 and 30 μM, respectively. Among tested compounds, mimosine tetrapeptides (MFFY, MFWY, MFYY) and hispidin derivatives (H1-3) had strong LIMK1 inhibitory activity. The IC_{50} values of MFFY, MFWY, MFYY range from 0.52 to 0.65 μM, while the IC_{50} values of H1, H2, H3 from 0.6 to 1.1 μM. However, in general, the inhibition (IC_{50}) of each compound appears to be quite similar (if not identical) between PAK1 and LIMK1 (see **Table 8.1**).

8.5 CONCLUSION

It is showed for the first time that several herbal compounds from Okinawa plants, which directly inhibited both PAK1 and LIMK1, inhibited both the growth of cancer cells and melanogenesis in skin cells, and promoted significantly the hair cell growth in cell culture. Cucurbitacin I from bitter melon is the most potent compound among them, inhibiting both the PAK1-dependent growth of cancer cells and melanogenesis and promoting the PAK1-

dependent growth of hair cells at nanomolar levels by inhibiting two PAK1 activators, JAK2 and RAC. Major problem with the non-CBI compounds tested here is their poor cell-permeability, which causes the relatively high IC_{50} (above 20 μM) in cell culture, more than 100 times of the IC_{50} of CBI (=140 nM).

Thus, in the future, I should focus on the far more effective screening to select only highly cell-permeable PAK1-blockers through so-called "Macaroni-Western" (IP-ATP_Glo) PAK1 assay system, that is a combination of immuno-precipitation (IP) of PAK1 from cell lysates after drug-treatment of target cells, and the (non-radioactive) luciferin/luciferase-based fluorescence detection of the remaining ATP after in vitro kinase reaction (Tagliati et al., 2005; Mezna et al., 2012). In the past, several potent synthetic PAK1-inhibitors such as FRAX486 and FRAX597 as well as PF 3758309 have been developed which inhibit PAK1 directly in vitro with the IC_{50} around 10 nM (Murray et al., 2010; Dolan et al., 2013; Licciulli et al., 2013). Unfortunately, however, none of them is highly cell-permeable (with the IC_{50} only around 1 μM in cell culture). Recently the cell-permeability of a herbal PAK1-blocker called ursolic acid from rosemary leaves was successfully boosted 200 folds by just blocking the COOH with a water-soluble triazole ring, inhibiting the cancer cell growth with IC_{50} around 100 nM (Rashid et al., 2013). Thus, currently I am taking a similar approach for a robust increase in the cell-permeability of several other herbal or synthetic PAK1-blockers (Takahashi et al., manuscript in preparation).

Mimosine tetrapeptides and hispidin derivatives are also good starting materials for developing more cell-permeable/water-soluble anti-melanogenic and anti-oncogenic compounds by a further chemical modification. Cucurbitacin I, a highly cell-permeable triterpene from bitter melon is a promising anti-cancer compound useful for the therapy of all PAK1-dependent cancers, in particular pancreatic, colon and lung cancers, and CBI-based bitter melon extract could be used as skin-whitening or anti-alopecia cosmetics.

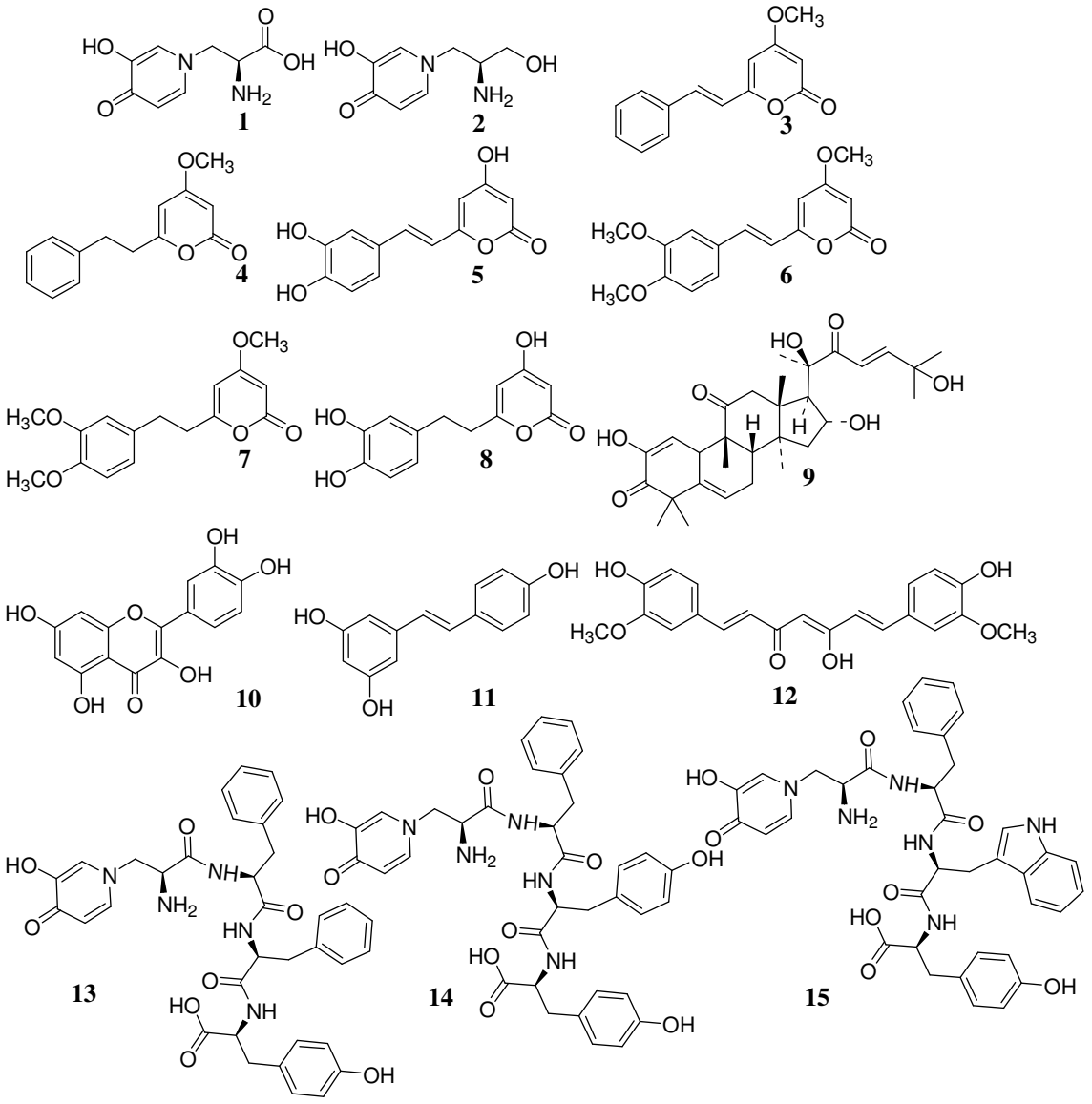

Figure 8.1. Chemical structures of compounds. Mimosine (1), mimosinol (2), DK (3), DDK (4), hispidin (5), H1 (6), H2 (7), H3 (8), cucurbitacin I (9), quercetin (10), resveratrol (11), curcumin (12), MFFY (13), MFYY (14), MFWY (15).

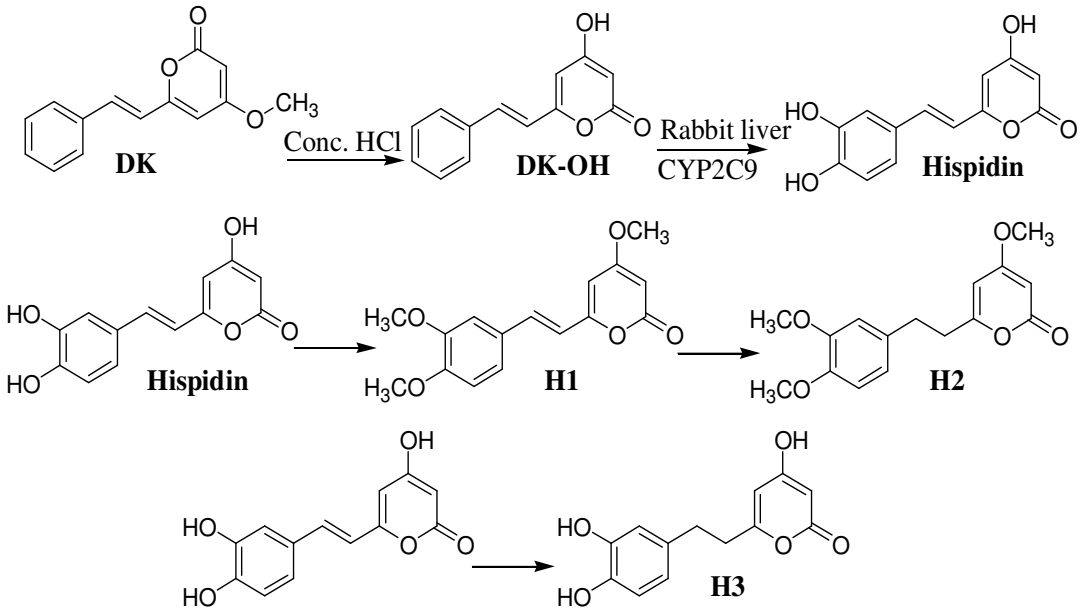

Figure 8.2. Preparation of hispidin and their derivatives. Bioconversion of 5,6-dehydrokawain (DK) to hispidin by CYP2C9 in the microsomes of rabbit liver. Hispidin derivatives (H1-3) were prepared from hispidin.

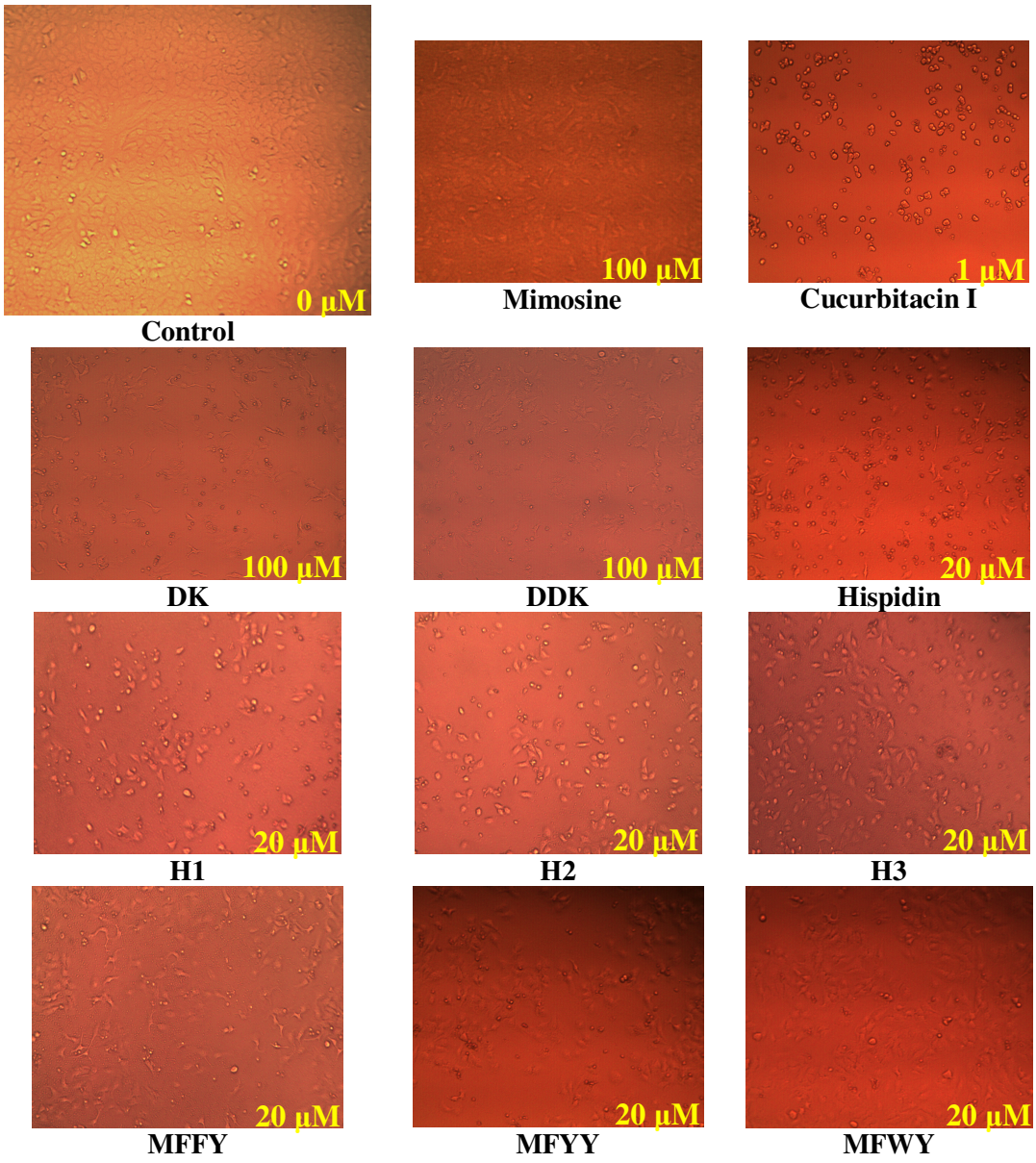

Figure 8.3. Effect of isolated compounds and their derivatives on growth of A549 human lung adenocarcinoma cells. A549 cells were treated with tested compounds for 72 h. Morphological changes of cells were detected under microscopy after incubation with mimosine, DK, DDK at 100 µM; hispidin, H1, H2, H3, MFFY, MFYY, MFWY at 20 µM and cucurbitacin I at 1 µM.

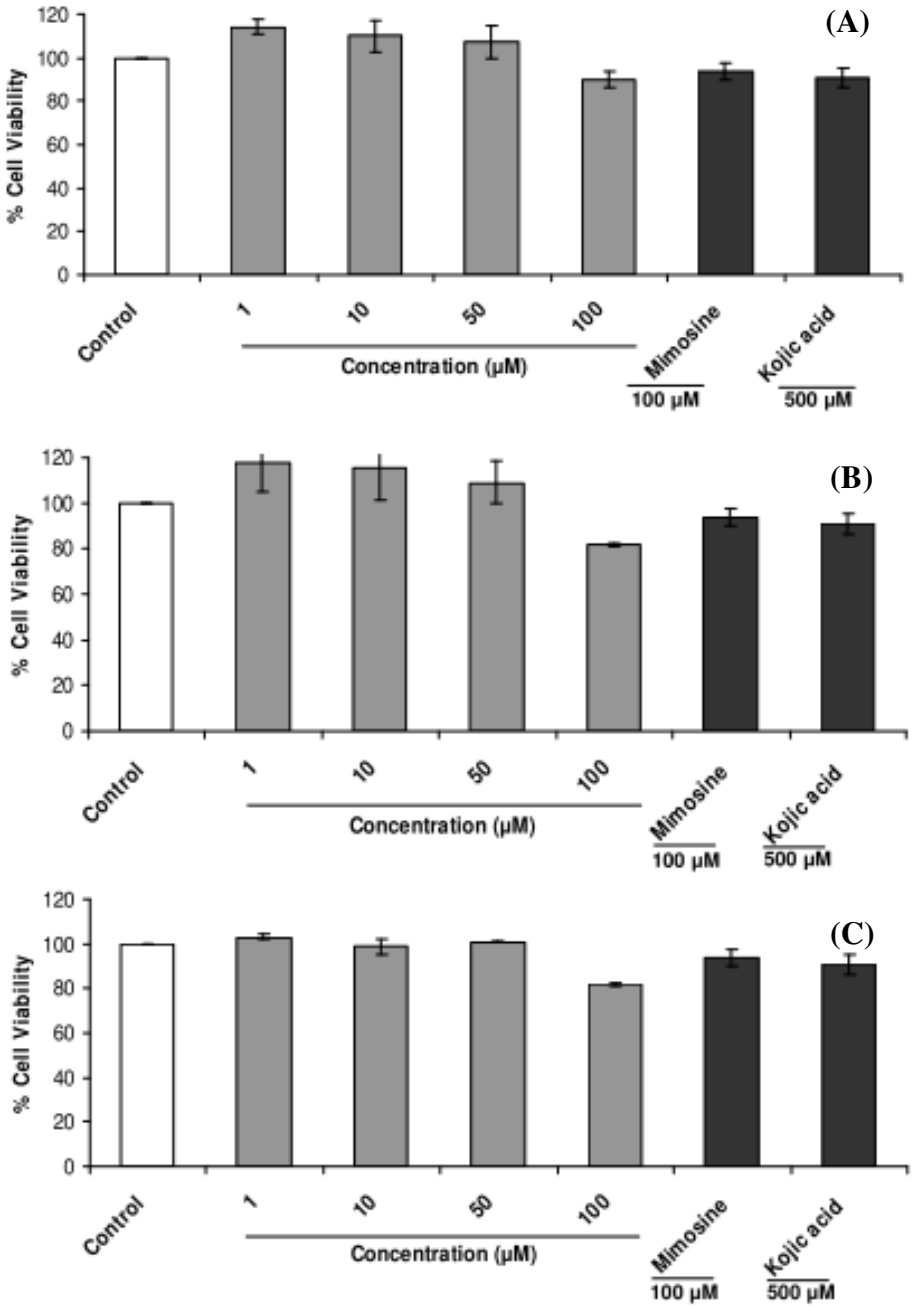

Figure 8.4. Effect of mimosine tetrapeptides on B16F10 cell viability. (A) MFFY, (B) MFWY, (C) MFYY. Mimosine (100 μM) and kojic acid (500 μM) were used as positive controls. Cells were treated with various concentrations of tested compounds for 48 h. Results are expressed as percentages of cell viability relative to control. The experiments were conducted with two repeated times, and the values are mean ± SE.

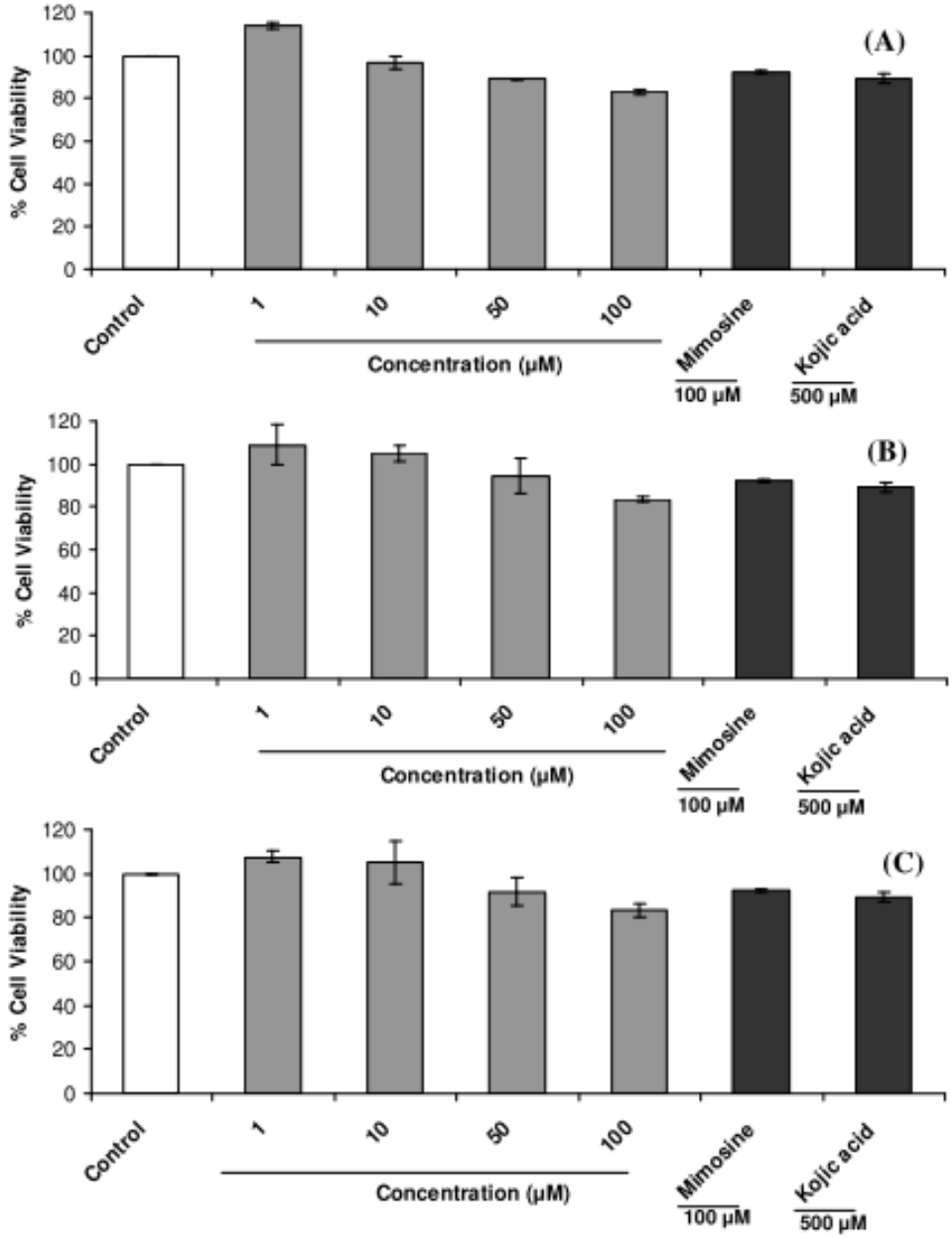

Figure 8.5. Effect of hispidin derivatives (H1-3) on B16F10 cell viability. (A) H1, (B) H2, (C) H3. Mimosine (100 μM) and kojic acid (500 μM) were used as positive controls. Cells were treated with various concentrations of tested compounds for 48 h. The experiments were conducted with two repeated times, and the values are mean ± SE.

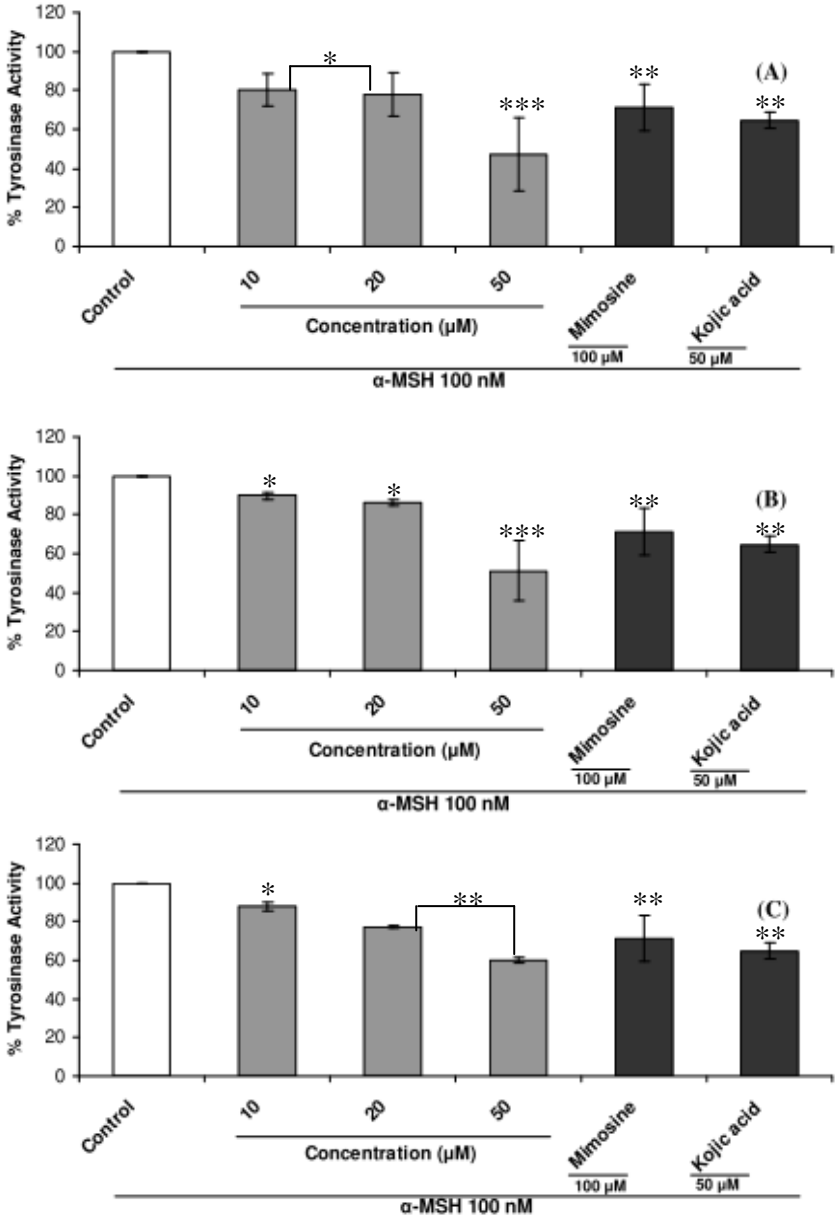

Figure 8.6. Intracellular tyrosinase inhibition of mimosine tetrapeptides in B16F10 cells. (A) MFFY, (B) MFWY, (C) MFYY. Mimosine (100 μM) and kojic acid (50 μM) were used as positive controls. Cells were treated with α-MSH and tested compounds, and tyrosinase activity was measured after 48 h treatment. The experiments were repeated two times, and the values are mean ± SE.

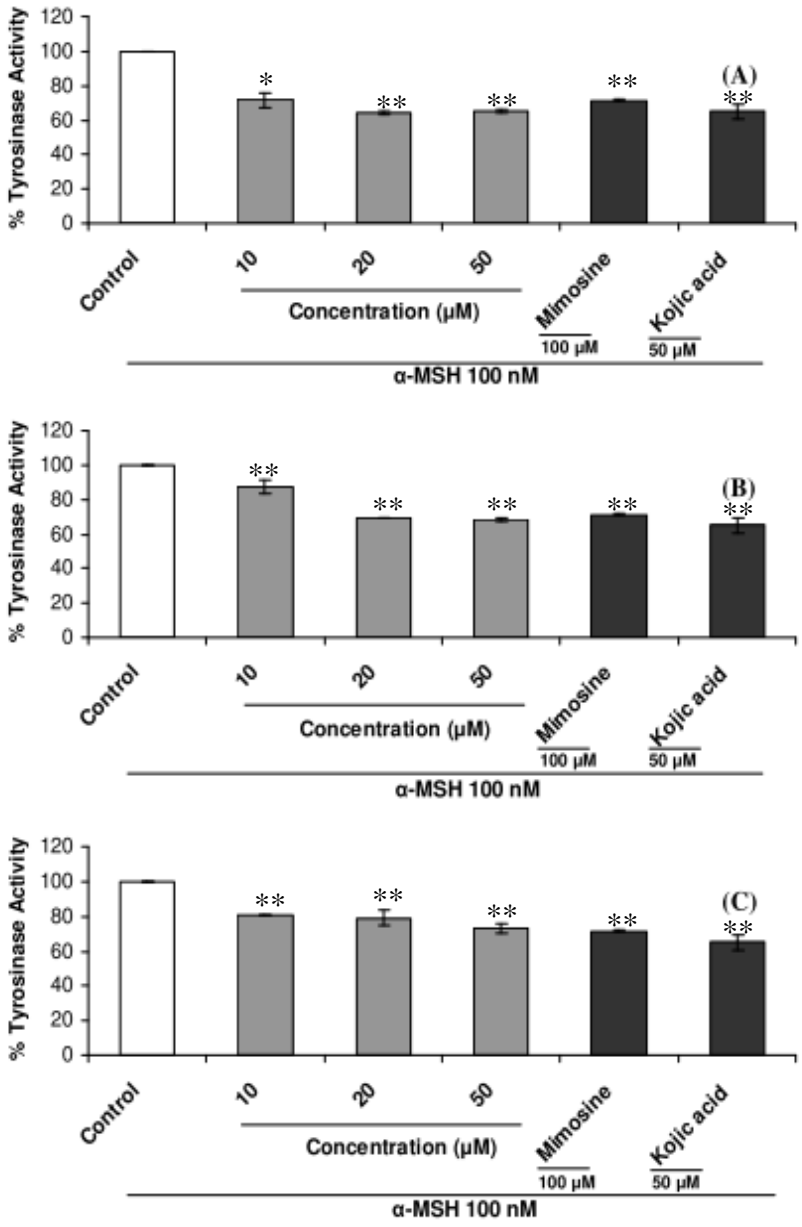

Figure 8.7. B16F10 cellular tyrosinase inhibition of hispidin derivatives (H1-3). (A) H1, (B) H2, (C) H3. Cells were treated with α-MSH and tested compounds, and tyrosinase activity was measured after 48 h treatment. Mimosine (100 μM) and kojic acid (50 μM) were used as positive controls. The experiments were repeated two times, and the values are mean ± SE.

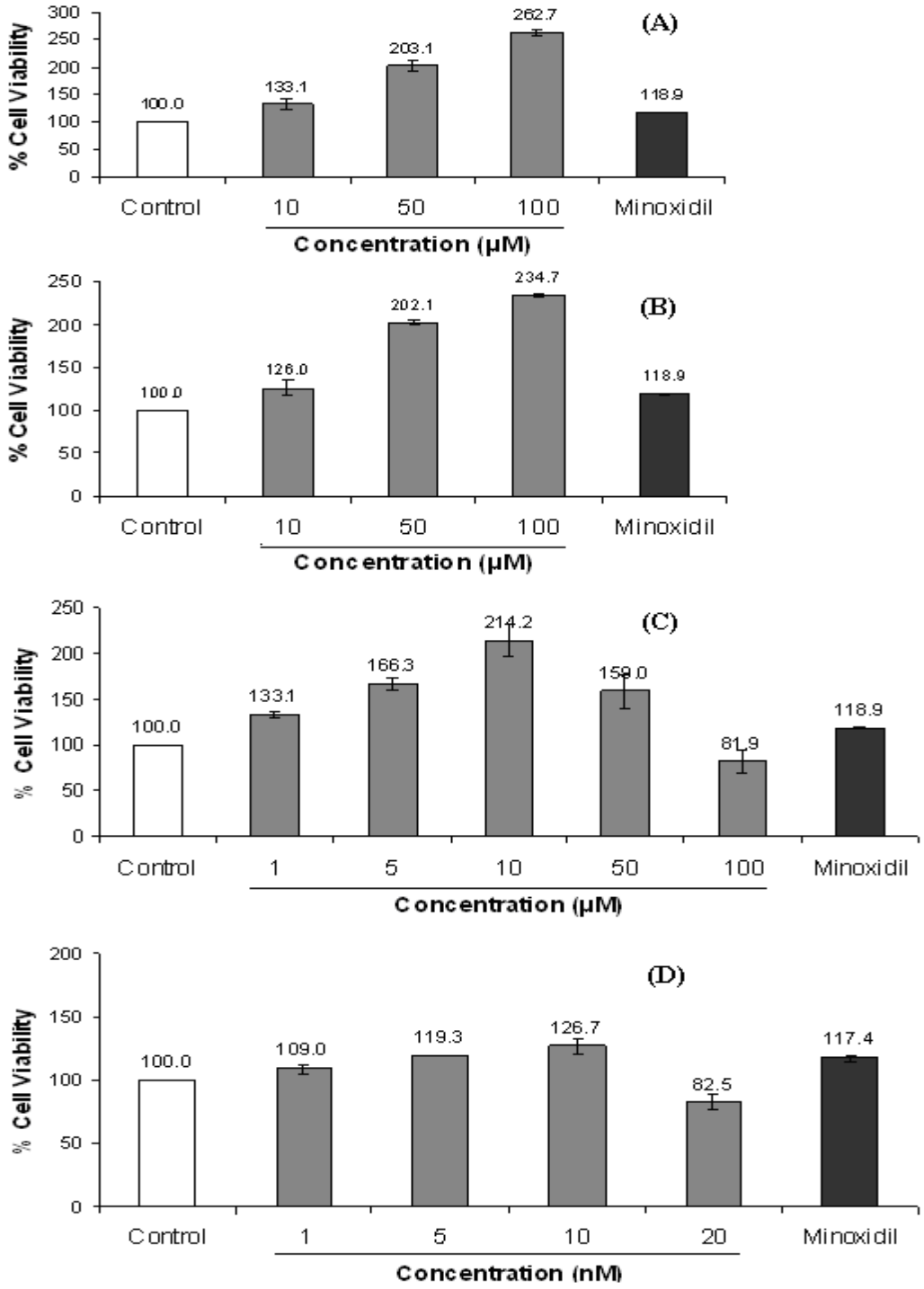

Figure 8.8. Promotion of hair growth by three alpinia compounds and Cucurbitacin I (A) DK, (B) DDK, (C) hispidin, (D) cucurbitacin I. Minoxidil (10 μM) was used as a positive control. The results are mean ± SE of two repeated times. Numbers on each bar indicate the percentage of HFDPC growth promotion.

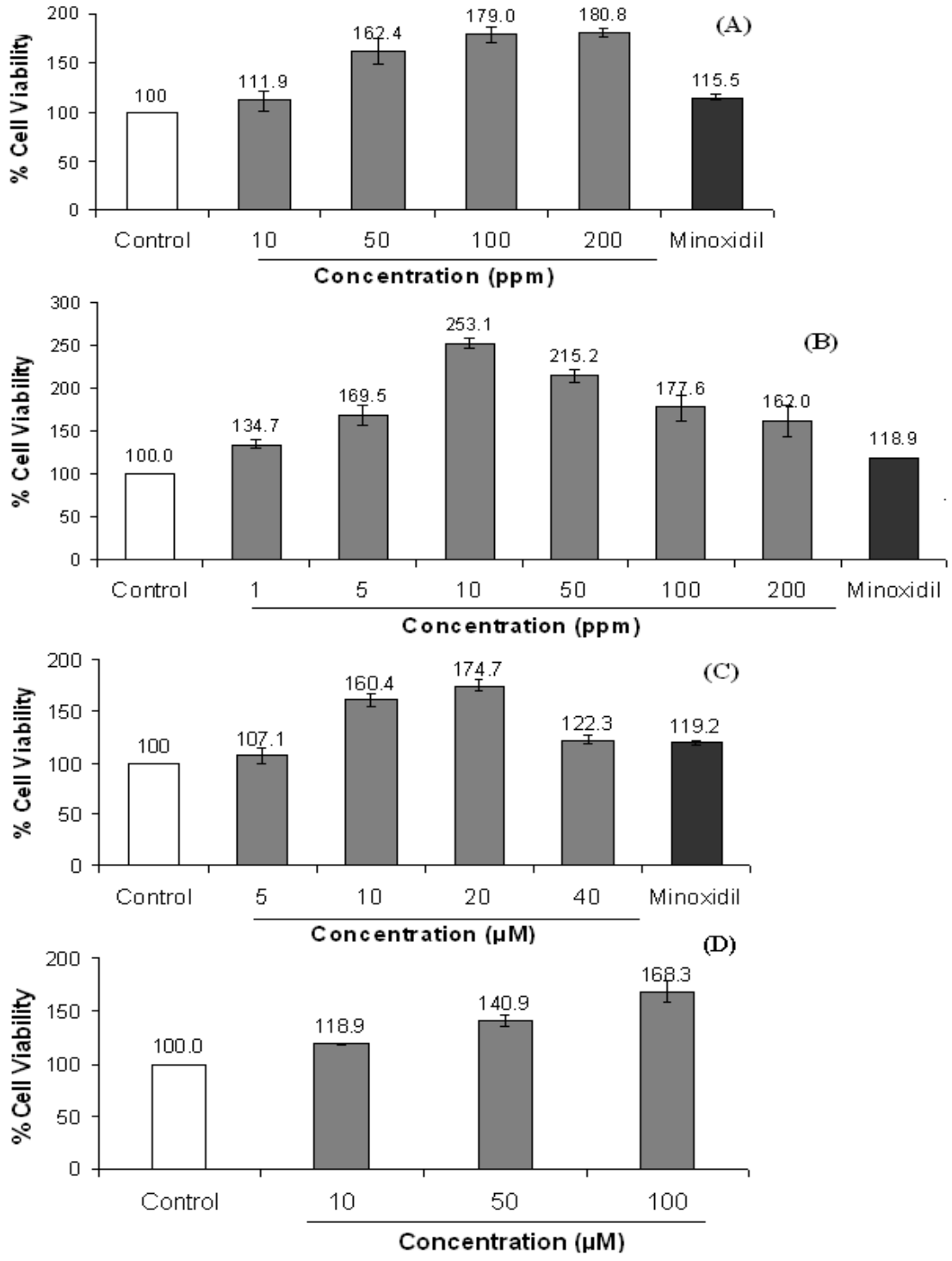

Figure 8.9. Promotion of hair growth by alpinia extract compared with a CAPE-based propolis "Bio 30", ARC from Brazilian green propolis and "Minoxidil". (A) alpinia extract, (B) Bio 30, (C) ARC and (D) minoxidil. Minoxidil (10-100 μM) was used as a positive control. The results are mean ± SE of two repeated times. Numbers on each bar indicate the percentage of HFDPC growth promotion.

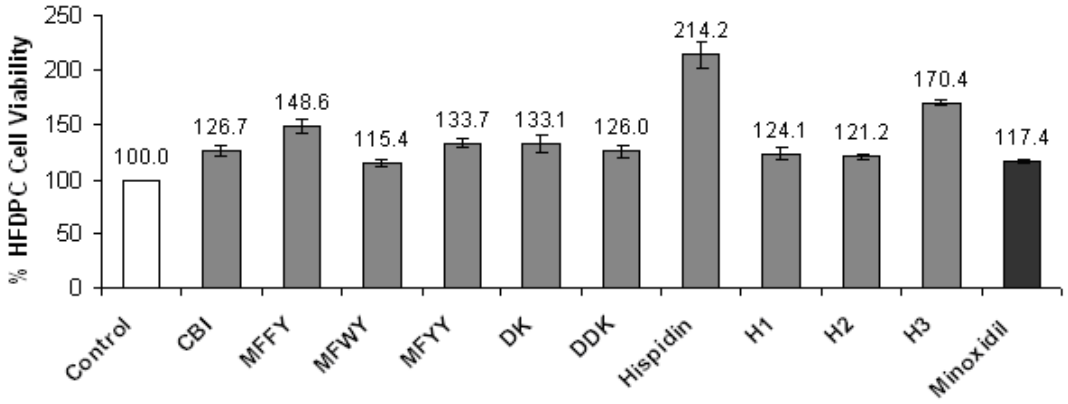

Figure 8.10. Promoting of hair cell growth by cucurbitacin I (CBI, 10 nM) and several other herbal compounds (10 μM). Minoxidil (10 μM) was used as a positive control. The results are mean ± SE of two repeated times. Numbers on each bar indicate the percentage of HFDPC growth promotion.

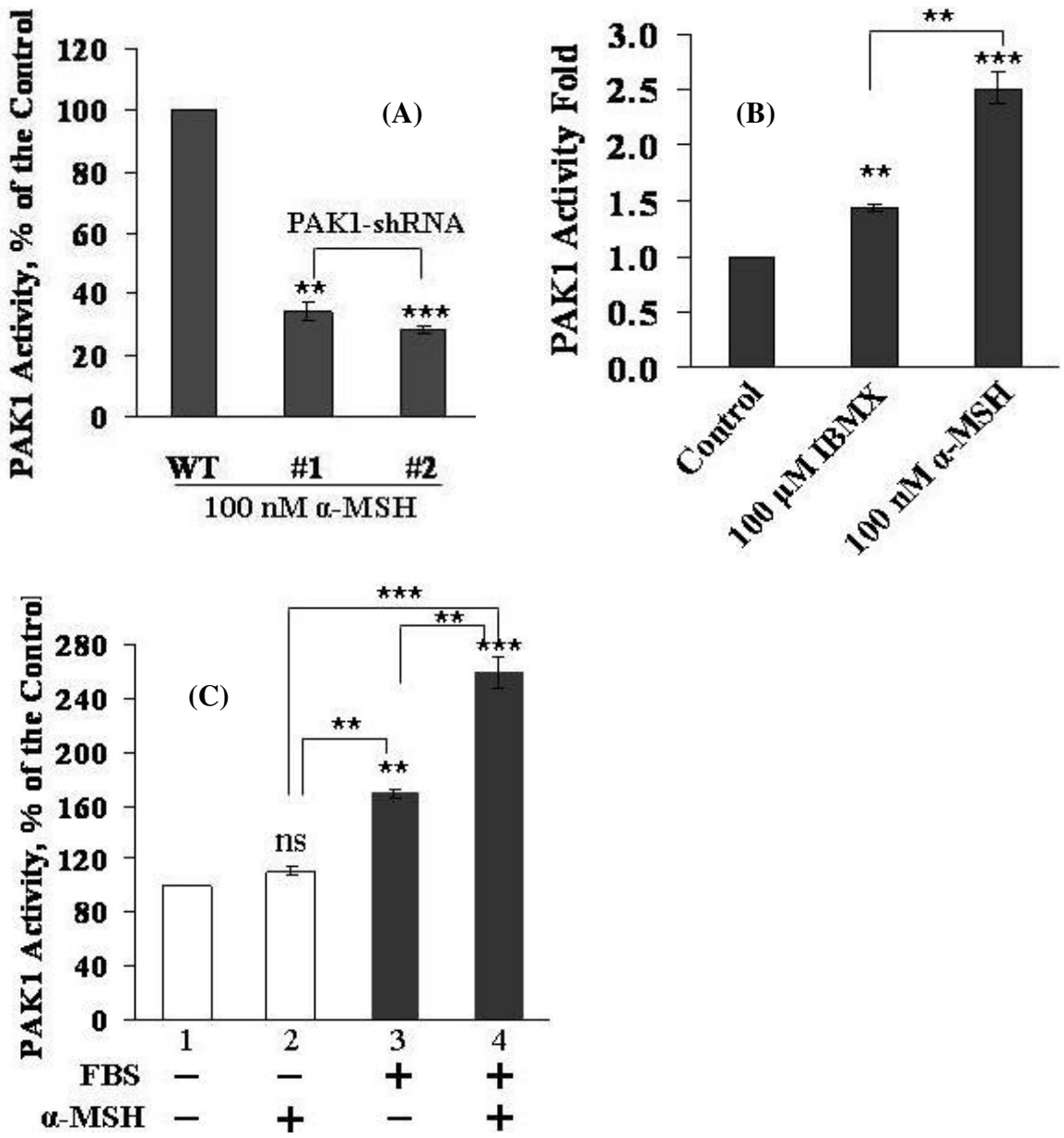

Figure 8.S1. PAK1 is involved in serum/α-MSH-induced melanogenesis. (A) Down-regulation of PAK1 by shRNAs. B16F10 cells were treated with IBMX (100 μM) and α-MSH (100 nM) for 72 h. (B) Activation of PAK1 by IBMX and α-MSH in melanocytes. Kinase activity of PAK1 in the transfectant (SH1 and SH2) compared with the WT melanocytes. Cells were treated with α-MSH (100 nM) for 48 h. (C) Serum/α-MSH-dependent PAK1 activity. PAK1 was immuno-precipitated from cell lysates, and PAK1 activity was measured by "Macaroni-Western" kinase assay. ** $p \leq 0.01$, *** $p \leq 0.001$, ns: not significant.

Table 8.1

Anticancer activity of cucurbitacin I and other herbal compounds

Compound	IC_{50} (μM)
MFFY	30.2 e
MFWY	26.7 ef
MFYY	29.5 e
DK	125.6 d
DDK	138.9 c
Hispidin	25.0 ef
H1	16.7 h
H2	21.4 fgh
H3	16.5 h
Cucurbitacin I	0.14 i
Mimosine	326.6 b
Mimosinol	506.0 a
Resveratrol	22.7 fg
Curcumin	18.2 gh

Different letters in the same column have statistically significant difference.

Table 8.2

Anti-PAK1 and anti-LIMK1 activities of cucurbitacin I and other herbal compounds

Compound	IC_{50} (μM) for LIMK1 inhibition	IC_{50} (μM) for PAK1 inhibition
Mimosine	31.6 c	37.0 a
Mimosinol	87.3 a	30.0 b
MFFY	0.6 h	0.13 j
MFYY	0.5 h	2.3 h
MFWY	0.7 h	0.60 ij
DK	25.1 e	17.1 d
DDK	22.1 f	10.3 f
Hispidin	14.1 g	5.7 g
H1	0.9 h	1.6 hi
H2	0.6 h	1.2 hij
H3	1.0 h	2.0 h
Cucurbitacin I	29.6 d	19.0 c
Resveratrol	36.9 b	15 e
Curcumin	nt	7.0 g

The IC_{50} values were determined graphically as the concentration of each compound that showed 50% inhibitory activity. M: mimosine, F: phenylalanine, W: tryptophan, Y: tyrosine. Nt: not tested. Different letters in the same column have statistically significant difference.

Table 8.3

IC_{50} values of melanin content inhibition of tested compounds

Compound	IC_{50} (μM) for melanin content inhibition
MFFY	24.9 g
MFWY	30.6 f
MFYY	93.8 e
H1	126.6 d
H2	132.2 c
H3	175.5 b
Mimosine	225.7 a

CHAPTER 9

GENERAL CONCLUSION

GENERAL CONCLUSION

Plants contain useful secondary metabolites which could be utilized as starting materials to produce economically itself new versions with improved characteristics. This semi-synthesis approach has been successfully used to generate novel drugs and pesticides in some particular cases. Therefore, it gave this research the inspiration for development of new compounds from plants with medicinal and pesticidal activities. Alpinia, leucaena, pilosa and bitter melon have many interesting biological activities which are associated with their major active components such as DDK, DK, mimosine, and CBI. These compounds were chosen and utilized for the study.

Mimosine from leucaena was identified as promising compound against insect and nematode. Since L-leucine is shown as a neuroactive substance in the blood of DDT-treated silkworm larvae, the chemical modification of the amino acid has been attempted in order to find a new lead for insecticides. In this study, novel mimosine derivatives showed strong insecticidal and nematicidal activities. Mimosinol and D-mimosinol could inhibit the development of the insects by inhibiting tyrosinase. Among the derivatives prepared from amino alcohols, two five-membered cyclic phosphorus compounds had high insecticidal (due to AChE inhibition) and nematicidal activities. Their insecticidal activity was similar to that of rotenone, commercial insecticide. These results introduced mimosine and their amino alcohols as previously unexplored scaffold of insecticide and nematicide. Moreover, mimosine was found as COX-1/2 and tyrosinase inhibitor. These enzymes are attractive targets for anti-inflammation and skin brightening therapies. Therefore, a small library of mimosine dipeptides (Mimosine-

L/D-AA) was designed and assessed their ability to inhibit enzyme activity in vitro. The results indicated that all peptides had more effective tyrosinase and COX-1/2 inhibitory activities than mimosine itself. In addition, mimosine dipeptides also exhibited suppressive effects on melanogenesis which are due to cellular tyrosinase inhibition. On the other hand, mimosinol and D-mimosinol had effective anti-melanogenesis and could be used as promising compounds in skin brightening without undesirable cytotoxicity. Furthermore, mimosinol and D-mimosinol turned out to have hair growth promoting effect through increasing the proliferation of human follicle dermal papilla cell. Taken together, mimosine derivatives prepared in this study were potential compounds as pesticides, and for the treatment of skin-whitening, anti-inflammation and hair growth promotion.

PAK1, p21(RAC/CDC42)-activated kinase 1, is the major oncogenic/aging kinase essential for a variety of diseases including cancer and Alzheimer's disease. Therefore, PAK1-blockers would be potentially useful for the treatment of various diseases/disorders in the future. Thus, in this study, a new "Macaroni-Western" PAK1 assay system was introduced for the assessment of safe and potent PAK1 blockers in cell culture. This system allowed to monitor any change in the kinase activity of PAK1 in cells directly, independent of its auto-phosphorylation sites, without SDS-PAGE.

The final challenge of this study was to investigate the anti-melanogenic, anti-oncogenic and anti-alopecia (hair growth promoting) activities of several PAK1-blocking herbal compounds. It was the first time for findings of several anti-PAK1 compounds in three distinct Okinawa plants such as leucaena, alpinia and bitter melon. 5,6-Dehydrokawain (DK) and dihydro-5,6-dehydrokawain (DDK) from alpinia inhibited directly PAK1. Cucurbitacin I from bitter melon also exhibited a moderate

anti-PAK1 activity. Hispidin and their derivatives inhibited PAK1 at low micromolar level while mimosine tetrapeptides (MFFY and MFWY) inhibited more strongly PAK1 at nanomolar level. Furthermore, these anti-PAK1 herbal compounds were found to suppress the melanogenesis in skin melanoma cells without any cytotoxicity. In addition, hispidin derivatives and mimosine tetrapeptides showed an anti-cancer activity in cell culture. Cucurbitacin I (CBI), a triterpene from bitter melon, turned out to be the most potent compound among these anti-PAK1 herbal compounds in inhibiting the growth of A549 lung cancer cells as well as to promote the hair growth in cell culture. Thus, CBI could be useful not only for cancer therapy but also for treatment of hair loss in the future.

In conclusion, from this study, DK, DDK, mimosine, and CBI could be good lead compounds for development of new substances as pesticides, and for treatment of cancer, skin-brightening, hair loss, and PAK1 inhibition. New improved "Macaroni-Western" assay system will be the most advantage technique to select the potent and safe PAK1-blockers with highly cell-permeability which are useful further clinical applications. Synthesis of new derivatives showed 100 times stronger anti-PAK1 and anticancer activities than original molecules. Because CBI and mimosine tetrapeptides exhibited strongly anti-PAK1 and anticancer activities, it is interested in testing these biological activities of new derivatives from these compounds.

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