Biological activities of three Okinawan plants, alpinia, leucaena, and pineapple

沖縄産植物ゲットウ、ギンネム及びパイナップル
の
生理活性物質

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Biological activities of three Okinawan plants, alpinia, leucaena, and pineapple

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ABSTRACT

The beneficial effects of bioactive compounds from plants have been investigated in relation to a variety of pathological diseases. Some of these identified compounds have been utilized in the agrochemicals, foods, cosmetics and pharmaceutical industries. This study is composed of investigations, screening as well as isolation and identification of compounds with specific bioactivities. Three indigenous plants from Okinawan islands were chosen for the study. These included *Alpinia zerumbet* (alpinia), *Leucaena luecocephala* (leucaena) and *Ananus comosus* (pineapple). Out of these, the alpinia and leucaena were investigated for a series of bioactivities, while in case of pineapple, the waste obtained from bromelain manufacturing process was studied for possible pharmacological importance.

The bioactive compounds from alpinia against HIV-1 integrase and neuraminidase enzymes were identified as 5,6-dehydrokawain (DK), dihydro-5,6-dehydrokawain (DDK), and 8(17),12-labdadiene-15,16-dial (labdadiene). DDK strongly inhibited integrase, an enzyme responsible for HIV-1 virus replication, with an IC_{50} of 3.6 μM. Against neuraminidase, a rational target for influenza inhibition, DDK exhibited mixed type of inhibition with IC_{50} of 24.6 μM, and K_i value of 2.8 μM. The mechanism investigative studies showed that, DDK is a slow and time-dependent reversible

inhibitor on neuraminidase, probably with a methoxy group as its functionally active site. Furthermore, on analyzing the neuraminidase and formation of atherosclerosis inhibitions by the essential oils of two varieties of alpinia leaf, it was found that the *tairin* variety, with comparatively larger number of compounds, had better bioactivities than *shima* variety.

Mimosine [β-*N*-(3-hydroxy-4-pyridone)-α-aminopropanoic acid], a non-proteinogenic amino acid, was identified as a potent neuraminidase inhibitor from the leaves of leucaena. The kinetic studies showed the mimosine inhibited neuraminidase competitively with Km and IC_{50} values of 39.1 min^{-1} and 9.8 μM, respectively. Furthermore, a series of mimosine tetrapeptides were synthesized in search for more potent neuraminidase and tyrosinase inhibitor. Mimosine-FFY (Phe-Phe-Tyr) was identified as the best synthesized tetrapeptide, and it exhibited 50% neuraminidase and tyrosinase inhibition at 1.8 and 18.3 μM, respectively.

The waste obtained during bromelain processing is often discarded and is considered as a burden while disposing them. An attempt was made to study the bioactivities of the pineapple stem waste (PSW) in regards to antimicrobial activity, advanced glycation end products formation, 15-lipoxygenase and neuraminidase inhibitions. Several phenolic acids were identified in PSW with considerable amount of

antioxidant activities mainly due to the presence of syringic, ferulic, *iso*-ferulic acids. A range of pathogenic fungi and bacteria were inhibited by the PSW, with a complete inhibition of *Colletotrichum acutatum*, a fungus with a very wide host range, but is economically most important on strawberries. The possible reasons for fungal inhibitions may be due to the presence of large amount of benzoic acid in the PSW. The other bioactivities were mainly due to the syringic and hydroxybenzoic acids present.

Furthermore, Okinawan plants are long-known for the longevity of the islanders. Most of the studies on animal models suggest that phenolic compounds present in fruits and vegetables are responsible for such an effect. In this respect, alpinia leaf, with high phenol content was investigated for longevity assays using *Caenorhabditis elegans* (*C. elegans*) as animal model. The results showed that leaves polyphenols could significantly increase the mean lifespan by 22.6%, better than the positive control, resveratrol. Moreover, both under the thermal and oxidative stressed conditions, the leaves polyphenols increase the survival rate significantly better than quercetin. It seems that the up-regulation of HSP-16.2 and SOD-3 reporters attributed to the longevity extension under normal and stressed conditions.

Our results, therefore, showed that Okinawan plants contain a wide range of phytochemicals that have a broad spectrum of activity. This study certainly aid to the

probable utilization of Okinawan plants as preventive or therapeutic agents in diseases and different functional food industries.

CHAPTER 1

GENERAL INTRODUCTION

INTRODUCTION

The therapeutic benefits of traditional medicines have been recognized for centuries. Although there is still lack of evidence for clarification of their typical mechanisms, unlike with Western medicines, it is still widely accepted by people from East Asia and beginning to be accepted by the rest of the world. Early cultures also recognized the value of using spices and herbs in preserving foods and for their medicinal value. In recent years much interest has been focused on the biologically active compounds occurring in plants and herbs for their safety and effectiveness in prevention and treatment of human diseases. Great number of plants sources is being searched for their possible antimicrobial, antitumor, antioxidant, etc activities. Most of the recent investigations have been carried out to isolate and identify the chemical nature of the substances responsible for the isolation and characterization of numerous kinds of metabolites from various plants and their structure has been elucidated.

The chemical constituents found in plants are often classified as either primary or secondary metabolites. Primary plant metabolites can be considered as those metabolites essential for the life of the plant. The plant synthesizes sugars, amino acids, and nucleotides. These simple molecules are used to produce polymers essential in the life of the plant. This aspect of the plants biochemistry can be considered as distinct

from the production of more complex molecules produced by more diverse pathways. Plants also use other metabolic pathways to produce compounds that have no readily apparent function. These are the secondary metabolites and are derived from primary metabolites but have a more limited distribution. These secondary compounds appear to play no direct role in a plant's primary metabolism, but apparently have an ecological function.

The secondary metabolites consist of phenolics, alkaloids, terpenes, etc. These are often thought to have protective roles in the plant. Researchers have utilized these compounds to investigate their bioactivity and have used them in a wide range of commercial products. They are often used as food, flavors, color dyes, poisons, perfumes, scented oils, etc. It is estimated that one-fourth of prescription drugs contain at least one chemical originally identified and extracted from a plant.

Herbal remedies have been used for centuries but more recently the compounds that are active have been identified and this has enabled them to be extracted and purified. Moreover, the ever threat of side effects imposed by the modern medicines and the awareness of consumers to the health impact of the synthetic drugs and preservatives; the demands for the natural products from herbs or plants lately increased.

CHAPTER 2

LITERATURE REVIEWS

2.1 Okinawan plants

The Okinawa area is located at the southern end of Japan and consists of more than 100 islands. The complex geographical features of many islands under the mild and rainy subtropical ocean climate created Okinawa's unique natural environment. Only Okinawa has a subtropical climate in Japan, which allows both tropical and temperate plants to grow or be cultivated. The inhabitants of Okinawa have traditionally utilized such plants as medicinal herbs or in preparing characteristic Okinawan foods. Some researchers have indicated that the intake of typical Okinawan medicinal and edible plants contributes to the longevity of the local people (Hokawa, S., 1999; Shinjo and Yamamoto, 1999). Nakatani (1992) suggested that the antioxidant compounds contained in the Okinawan plants contributed to the good health of the islanders. Most of these plants contain rich polyphenols that have antioxidant activities (Suda et al., 2005; Elzaawelly et al 2007a). The antioxidant activities of these plants were shown to protect liver from inflammation and preventing diabetes complications (Aniya et al., 2002a; Aniya et al., 2002b). Recently we have shown advanced glycation end product and atherosclerosis inhibitions by Okinawan plant, *Alpinia zerumbet* (Chompoo et al., 2011).

In this study, three plants, *Alpinia zerumbet* (alpinia), *Leucaena leucocephala*

(leucaena), and *Ananus comosus* (pineapple) were investigated for various functional properties. Alpinia (Family: Zingiberacae) is a perennial plant growing widely in subtropical and tropical regions. With fast growing and strong resistance to pests and diseases, this species grows abundantly in the Ryukyu Islands, and has been a traditionally important plant in Okinawa. The leaves are being used as a functional ingredient to make several commercial food products like noodles, tea, etc. Besides, alpinia essential oil is used in manufacturing of cosmetics, perfumes, and soaps (Tawata et al., 2008). On the other hand, leucaena is a leguminous tree that is tolerant to drought and is distributed widely in subtropical and tropical zones. In South east Asia and Africa, it 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. Pineapple is one of the most popular tropical fruits and is the leading member of the family Bromeliaceae. In Japan, most of the pineapple is grown in Okinawan islands where several products are made in pineapple canneries. In this study, we utilized the waste obtained from a pineapple industry and investigated its functional properties. To sum up, we investigated the least utilized parts of three Okinawan plants for their bioactivities.

2.2 Polyphenol

Polyphenols embrace a wide range of substances which possess an aromatic ring bearing a hydroxyl constituent, including their functional derivatives. The subdivision of polyphenols into tannins, lignins, and flavonoids is derived from secondary plant metabolism of the shikimate pathway (Dewick, 1995). Phenolic compounds are widely found in the secondary products of medicinal plants, as well as in many edible plants (Hagerman et al., 1998). The ability of phenolic compounds to serve as antioxidants has been recognized, leading to speculation about the potential benefits of ingesting phenolic rich foods. Several studies have described the antioxidant properties of medicinal plants, foods, and beverages which are rich in phenolic compounds (Brown & Rice-Evans, 1995). Phenolic compounds are the most commonly investigated constituents among potential allelochemicals (Chon et al., 2005). They occur in plant tissues as free compounds (benzoic and cinnamic acid derivatives) and in bound forms (glycosidic phenylpropanoid esters).

2.3 Antimicrobial activity

Many of the plants used today were known to the people for their preservative and medicinal values. Scientific experiments on the antimicrobial properties of plants and their components have been documented in the late 19^{th} century (Zaika, 1975). A vast majority of plant secondary metabolites have weak or narrow-spectrum antimicrobial

activities, while bacteria, yeast, and fungi produce antibiotics that both are often effective and have broad spectra of activity. The nature of this disparity is puzzling. One possibility is that plant antimicrobials actually have other functions in the plant and their low level of antimicrobial activity is accidental and largely irrelevant. Wilkins and Board (1989) reported more than 1340 plants to be potential sources of antimicrobial compounds.

2.4 Antioxidant activity

Antioxidant compounds in food play an important role as a health protecting factor. Scientific evidences suggest that antioxidants reduce the risk of chronic diseases including cancer and heart disease. Primary sources of naturally occurring antioxidants are whole grains, fruits and vegetables. The main characteristics of an antioxidant are its ability to trap free radicals. Highly reactive free radicals and oxygen species are present in the biological systems from a wide variety of sources. These free radicals may oxidize nucleic acids, proteins, or lipids. Antioxidant compounds like polyphenols, and flavonoids scavenge free radicals such as peroxide, hydroperoxide or lipid peroxyl and thus inhibit the oxidative mechanism that lead to degenerative diseases.

2.5 HIV-I integrase inhibition

HIV-I integrase catalyzes the integration of proviral DNA into the host genome, an

essential step for viral replication (Dayam et al., 2008). Inhibition of integrase catalytic activity provides an attractive strategy for antiretroviral drug design. Currently two integrase inhibitors, MK-0518 and GS-9137, are in advanced stages of human clinical trials. The integrase inhibitors in clinical evaluation demonstrate excellent antiretroviral efficacy alone or in combination regimen as compared to previously used clinical antiretroviral agents in naïve and treatment-experiences HIV-1 infected patients. Some of the previously discovered natural integrase inhibitors include equisetin, integric acid, complestatin, trichosanthin, xerocomic acid and several other compounds (Hazuda et al., 1999; Singh et al., 2000; 2001; 2003). However, the high mutation rate of HIV means that drug resistance is emerging, making it necessary to develop new drugs with different target (Deprez et al., 2004).

2.6 Neuraminidase activity

The influenza virus is a highly infective agent that causes acute pulmonary diseases. In more serious cases, influenza causes pneumonia, which can be particularly fatal in young children, the elderly, and patients with cardiopulmonary diseases. In addition, influenza spreads around the world in seasonal epidemics, killing numerous people in pandemic years (Hein et al., 2004), the impact of which is felt globally each year when the disease develops in approximately 20 percent of the world's population (Moscona,

2005). The influenza virus contains eight pieces of segmented RNA, with hemagglutinin and neuraminidase as surface antigens. Hemagglutinin is responsible for most antigenic variations and contains binding sites for sialic acid residues on the surface of target cells, mediating the binding of the virus to target cells and the subsequent entry of the viral genome (Chandrasekharan et al., 2008). Neuraminidase is involved in the release of progeny virus from infected cells, by cleaving sugars that bind the mature viral particles. Specifically, neuraminidase cleaves the α-ketosidic bond that links a terminal neuraminic-acid residue to the adjacent oligosaccharide moiety, neuraminidase is therefore essential for the movement of the virus to and from sites of infection in the respiratory tract (Itzstein, 2007; Huberman et al., 1995).

Neuraminidase is a processing hydrolase involved in removing of terminal sialic acids from a variety of glycoconjugates. It is known to play an important role in pathogenesis, bacterial nutrition, and cellular interaction. Neuraminidase is especially believed to play at least two critical roles in influenza viral life cycle: the facilitation of virion progeny release; and general mobility of the virus in the respiratory tract. Development of inhibitors of neuraminidase provides a new therapeutic approach for the treatment of the influenza virus and other diseases that arise from hydrolysis of sialic acid. With the ever-present threat of a pandemic derived from the influenza virus,

the importance of neuraminidase inhibitors escalates.

2.7 Tyrosinase inhibition

Tyrosinase (monophenol monooxygenase) (EC 1.14.18.1) is an enzyme that catalyzes the oxidation of phenols (such as tyrosine) and is widespread in plants and animals. Tyrosinase is a copper containing enzyme present in plant and animal tissues that catalyzes the production of melanin and other pigments form tyrosine by oxidation, as in the browning of a peeled or sliced potato exposed to air. In higher plants and fungi, tyrosinases occur in various isoforms such as immature, mature latent and active forms (Sanchez-Ferrer et al., 1990); however, the biochemical description regarding the kinetic characterization and relationship between these isoforms is not established yet. The biosynthetic pathway for melanin formation, operating in insects, animals, and plants, has largely been elucidated. The production of abnormal melanin pigmentation (melasma, freckles, ephelide, senile lentigines, etc.) is a serious esthetic problem in human beings. In addition, tyrosinase is responsible for the undesired enzymatic browning of fruits and vegetables (Martinez et al., 1995). A number of tyrosinase inhibitors from natural and synthetic sources that inhibited monophenolase, diphenolase, or both of these actives have been identified. Furthermore, tyrosinase inhibitors may be clinically used for the treatment of some skin disorders associated with melanin

hyperpigmentation and are also important in cosmetics for skin whitening effects (Palumbo et al., 1990).

2.8 15-Lipoxygenase inhibition

The enzyme 15-lipoxygenase has repeatedly been implicated in oxidation of low-density lipoprotein, a process that is believed to be important for the development of atherosclerosis (Cornicelli and Trivedi, 1999; Steinberg, 1999). Although the involvement of 15-lipoxygenase has been debated, recent results point toward an anti-atherosclerotic effect in vivo in rabbits for 15-lipoxygenase inhibitors without antioxidant activity (Sendobry et al., 1997; Bocan et al., 1998; Cyrus et al., 1999). Furthermore, 15-lipoxygenase has been suggested to be a mediator of oxidation of low density lipoprotein. The enzyme form hydroperoxy derivatives of linoleic acid and arachidonic acid and is induced in atherosclerotic plaques (Harats et al., 2000). Studies have shown that phenolic compounds and terpenoids have inhibited 15-lipoxygenase enzymes (Lyckander & Malterud, 1992; Amagata et al., 2003; Carroll et al., 2003)

2.9 Advanced glycation end products (AGEs) inhibitions

Advanced glycation end products (AGEs) are a complex and heterogeneous group of compounds that have been implicated in diabetes-related complications. The AGEs are closely associated with hyperglycemia and their patho-biochemistry could explain

many of the changes observed in diabetes-related complications (Singh et al., 2001). AGEs are the products of nonenzymatic glycation and oxidation of proteins and lipids. Glycation adducts of proteins are formed when proteins react with glucose reactive α-oxoaldehydes or dicarbonyls, such as glyoxal, methylglyoxal and 3-deoxyglucosone (3-DG) (Brownlee, 2001). These adducts have recently been proposed to be formed from all stages of the glycation process, by degradation of glucose or Schiff's bases in early glycation, or from Amadori products such as fructosamine in the intermediate stages of glycation. Thus, α -dicarbonyl could be considered an important focal point of how glucose can go on to form AGEs by the classical Maillard reaction, using AGEs formation pathway (Singh et al., 2001), as well as from *in vivo* factors, such as the catabolism of ketone bodies and threonine, and lipid peroxidation (Thornalley et al., 1999). Therefore, inhibition of fructosamine adducts and α -dicarbonyls could reduce AGEs formation and hence lessen the chance of diabetic complications.

2.10 Life span studies using *Caenorhabditis elegans* (*C. elegans*) as animal models

The nematode worm *Caenorhabditis elegans* (*C. elegans*) is increasingly popular as a model organism for aging studies as well as for testing antioxidants and other compounds for effects on longevity (Gruber et al., 2009). Research into the cause of aging has greatly increased in recent years. The soil-dwelling nematode (roundworm) *C.*

elegans is a small (adult size about 1 mm length), free-living, bacteriovorus vermiform with a mean lifespan of around 20 days under laboratory conditions. Under these conditions, *C. elegans* are predominantly (>99%) self-fertilizing hermaphrodites and populations can be maintained effectively isogenic, since each individual hermaphrodite produces approximately 300 clonal progeny during the first week of adult life (Klass, 1977). *C. elegans* hermaphrodite adults contain 959 somatic cells, 302 of which are neurons. The complete cell lineage is known, including those cells that undergo apoptosis during development (Sulston et al., 1983). The *C. elegans* research community has generated a library of mutant strains as well as an extensive toolkit for the genetic manipulation of this organism.

C. elegans are transparent at all developmental stages and a large number of green fluorescent protein (GFP) reporter-gene strains have been generated, making it possible to visualize anatomy, developmental processes and many signalling pathways in real time. Strains can be cryogenically preserved at -196 ^{o}C (Brenner, 1974) and many mutant strains can easily be obtained from the *C. elegans* Genetics Center. The *C. elegans* genome has been completely sequenced and contains approximately 18,000 genes, many of which have human homologs (CSC, 1998). Double-stranded RNA (dsRNA) interference can be accomplished relatively easily, i.e. by feeding worms with

bacteria expressing dsRNA for the gene of interest, thereby causing a loss of function phenotype (Timmons and Fire, 1998). When *C. elegans* was introduced as a model organism by Brenner et al. over 30 years ago, it was intended mainly as a convenient model for investigations into developmental pathways and neurobiology (Brenner, 1974). Since then it has become a widely accepted model and discoveries made in *C. elegans* have been awarded with Nobel Prizes in 2002 for work on the genetics of organ development and programmed cell death, in 2006 for RNA interference and in 2008 for work on GFP, some of it in *C. elegans*.

Plants contain a variety of polyphenolic compounds that are not involved in primary metabolism but deter herbivores and protect against UV-induced damage as well as against stress conditions. Flavonoids, a subgroup of polyphenolic plant constituents, revealed a remarkable spectrum of biochemical and pharmacological actions thought to be, at least in part, due to their antioxidative and free-radical scavenging properties (Noroozi et al., 1998; Middleton et al., 2000). Antioxidants counteract the excessive formation of reactive oxygen species (ROS) resulting in oxidative stress, a pathological situation related to various age-dependent diseases, cancer and ageing (de Grey, 1997; de Grey, 2000; Forsberg et al., 2001; Griffiths, 2005; Schroecksnadel et al., 2006). Therefore, some of the health beneficial effects of a diet

rich in herbal edibles are attributed to the consumption of these secondary metabolites.

2.11 Objectives of this study

The primary objective of this study was to investigate the functional properties of three Okinawan plants namely, *Alpinia zerumbet*, *Leucaena luecocephala*, and *Ananus comosus*. Previous studies on these plants have shown different bioactivities and have identified several secondary metabolites associated with these properties. Compounds like 5,6-dehydrokawain (DK) and dihydro-5,6-dihyrdokawain (DDK) from alpinia has been implicated with a number of bioactivities including antimicrobial, hypotensive, antiulcerogenic, antithrombic properties (Mpalantinos et al., 1998). The plant leucaena has been extensively studied for a unique non-proteinogenic amino acid, mimosine. This compound has also been studied for a wide range of activities from anticancer to DNA replication initiation prevention (DeWys & Hall, 1973; Dai et al., 1994; Andrus et al., 1998; Ju et al., 1998;). Pineapple canneries by-products, too, have been investigated widely in extracting bromelain and phenolic rich antioxidant with several bioactivities. (Ishihara et al., 2003; Correia et al., 2004).

Considering all these previous researches, this study focused on three aspects:

i) Exploring the novel bioactivities of these plants in order to develop potent neuraminidase inhibitors.

ii) Synthesis of more potent neuraminidase inhibitors form the identified ones.

iii) The anti-ageing property of alpinia leaves using animal models *C. elegans*.

Hence, this study explored the novel bioactivities of these plants in order to utilize them as preventive or therapeutic agents in diseases and in different functional food industries.

CHAPTER 3

BIOACTIVITES OF PINEAPPLE STEM WASTE

3.1 SUMMARY

Pineapple stem has been extensively used for bromelain extraction and a large amount of wastes is generated during bromelain manufacturing. So far, these wastes are not utilized and are often considered as a burden while disposing them. In this study, several bioactivities of the pineapple stem waste (PSW) were studied so as to identify its probable applications. In this regard, antioxidant, antimicrobial, and inhibitions against 15-lipoxygenase and advanced glycation end product formations by PSW were studied. The PSW had moderate bioactivities in all the performed assays. Several important phytochemicals with considerable bioactivities were found to be present in PSW. It also showed a considerable inhibition against fungal growth, probably due to high amounts of the benzoic acid present in the sample. These results indicate that PSW could be utilized as an economic source of preventive or therapeutic agent in disease and in different functional food industries.

3.2 INTRODUCTION

Pineapple (*Ananas comosus* Merr.) is one of the most popular tropical fruits and is the leading edible member of the family Bromeliaceae. In Japan, most of the pineapple is grown in Okinawa, the southern part of the archipelago. Commercially, it is mainly produced as canned fruits juices, concentrates and jam. Furthermore, bromelain, the proteolytic enzyme present in the stem of pineapple, is finding wide applications in pharmaceutical and food uses.

Search for new natural bioactive compounds has been increased dramatically over the past years and in this regard agro-industrial by-products are extensively being

explored. The low cost of these residues, which otherwise would be discarded as waste in the environment, may be one of the reasons. It is anticipated that these discarded fruits as well as the waste materials could be utilized for further industrial processes, viz. fermentation, bioactive component extraction, etc. Researchers have focused on the utilization of pineapple wastes as low-cost substrates for ethanol production (Ban-Koffi and Han, 1990; Tanaka et al., 1999; Nigam, 1999). Pineapple wastes as one of the substrates have been used to produce citric acid (Kumar et al., 2003), hydrogen gas (Wang et al., 2006) and phenolic antioxidant (Correia et al., 2004). Furthermore, dietary fiber and cellulose have been extracted from the pineapple core (Prakongpan et al., 2002) while the stem has been used as anti-dying agent (Hameed et al., 2009).

Our laboratory has carried out researches in low molecular weight bioactive compounds in pineapple juice, particularly, antihemolytic properties (Ishihara et al., 2003). Furthermore, several patented works related to antihemolytic and the bioactive compounds from pineapple juice without bromelain were carried out (Toyama et al., 1999; Ishihara et al., 2004). Besides, biological activities of compounds synthesized from cinnamic acid isolated from pineapple stem have been reported (Tawata et al., 1996; Zhu et al., 2001).

During the manufacturing of bromelain from pineapple stem, almost 98% is generated as the waste product (Morton, 1987). This huge amount of waste may cause serious environmental problems, since it accumulates in agro-industrial yards without having any significant and commercial value. Furthermore, the problem of disposing wastes is made worse by legal restrictions. Researchers have been focusing only on utilizing bromelain from the pineapple stem. However, to the best of our knowledge, no work has been done regarding the utilization of pineapple stem waste (PSW) obtained

from bromelain manufacturing process. In this light of these facts, we are interested in investigating the potentiality of using PSW as a source of phenolics for different bioactivities.

3.3 MATERIALS AND METHODS

3.3.1 Preparation of extracts

PSW is a waste of bromelain manufacturing process, obtained after removing bromelain fraction from pineapple stem. This waste was obtained from J-Dolph Pharmaceutical Company, Osaka for research purposes. Twenty grams of PSW was extracted separately by reflux in 100% methanol, 50% methanol, and water for 1 h. The cooled refluxes were filtered. Solvents were evaporated under vacuum at 40 ^{o}C and the dried extract was dissolved in water. After hydrolysis of the samples with 4M NaOH (4 h, 50 ^{o}C), the pH was adjusted to 2.25 and liquid-liquid extractions with ethyl acetate were done for all samples (Elzaawelly et al., 2007). Finally, solvents were evaporated under vacuum at 40 ^{o}C and the dried extracts were re-dissolved in methanol to make a stock solution of 1 mg/mL and refrigerated until further use. The amount of ethyl acetate fractions (E_1, E_2, E_3) (1, 2 and 3 represent samples refluxed in 100% methanol, 50% methanol, and water respectively) obtained were 0.262 g (1.3%), 0.220 g (1.1%) and 0.278 g (1.4%), respectively.

3.3.2 Total phenolic content and antioxidant assays

The amount of total phenolics of the samples was determined according to the Folin-Ciocalteu procedure (Kahkonen et al., 1999) and was expressed as gallic acid equivalents (GAE) in mg/100 g PSW. Briefly, to 200 μL of test samples (500 μg/mL), 1 mL of Folin-Ciocalteu's reagent and 0.8 mL of sodium carbonate (7.5%) were added. The mixture was allowed to stand for 30 min and the absorption was measured at 765

nm using a Shimadzu UV-160A spectrometer.

3.3.3 Free radical scavenging assay

The radical scavenging activity was evaluated as described previously with slight modifications (Elzaawelly et al., 2005). Briefly, different concentrations of 1 mL of plant extract (100-400 μg/mL) and standard phenolic compounds (1-500 μg/mL) were mixed with 0.5 mL of 0.5 mM DPPH solution and 1 mL of sodium acetate buffer (pH 5.5). The mixture was incubated in dark for 30 min at room temperature. Absorbance was measured at 517 nm using Shimadzu UV-160A spectrophotometer. BHT (butylated hydroxyl toluene) (1-50 μg/mL) was used as positive control while methanol was used as negative control. The DPPH radical scavenging activity was calculated using

% DPPH scavenging activity = $[(A_c-A_s)/A_c] \times 100$,

Where A_c is the absorbance of the control (DPPH solution without test sample) and A_s is the absorbance with test sample. The IC_{50} value was determined as the concentration required to give 50% DPPH radical scavenging activity.

3.3.4 β-Carotene bleaching assay

The antioxidant activities of various samples were evaluated as described previously (Elzaawelly et al., 2005). Briefly, 1.0 mg of β-carotene was dissolved in 10 ml chloroform. One milliliter of this solution was mixed with 20 mg linoleic acid and 200 mg Tween-40. Chloroform was evaporated and 50 ml of oxygenated water was added. Two ml of freshly prepared emulsion was added in the 24-well of microtitre plate. Sample extracts and BHT (80 μL, 1 mg/mL) was added while equal amount of methanol was used for control. The plates were incubated at 50 ^{o}C, and the absorbance was measured using Benchmark microplate reader (Bio-Rad, Hercules, CA) at 492 nm. Readings of all samples were performed immediately at zero time and every

15 min up to 180 min.

3.3.5 Reducing activity

The reducing power of the samples was determined as described previously (Elzaawelly et al., 2005). One milliliter of each extract and BHT (250, 500, and 1000 µg/mL) was mixed with 2.5 mL phosphate buffer (0.2 M, pH 6.6) and 2.5 mL potassium ferricyanide [$K_3Fe(CN)_6$] (10 mg/mL). The mixture was incubated at 50 ^oC for 30 min which was followed by addition of 2.5 mL of trichloroacetic acid (100 mg/mL). Finally, this mixture was centrifuged at 4000 rpm for 10 min, and 2.5 mL of resulting supernatant was mixed with 2.5 mL of distilled water and 0.5 ml $FeCl_3$ (1 mg/mL). Absorbance was measured at 700 nm and the increase in absorbance of the reaction mixture indicated its increased reducing power.

3.3.6 15-Lipoxygenase (15-LOX) inhibition

15-LOX from soybeans (Sigma-Aldrich, Inc. USA) was used for peroxidation of linoleic acid, and inhibition was carried out as described previously with slight modifications (Malterud & Rydland, 2000). Briefly, 12.5 µL of sample, dissolved in DMSO, was added to 487.5 µL of enzyme solution. The mixture was incubated at room temperature for 5 min. Absorbance was recorded immediately after the addition of 500 µL of substrate (250 µM linoleic acid in 0.2 M borate buffer, pH 9) every 1 min up to 5 min at 234 nm using Shimadzu UV-Vis spectrophotometer mini 1240 (Kyoto, Japan). The final enzyme concentration was 200 U/mL. The value for % inhibition of enzyme activity was calculated as $100 \times [(\Delta A_1/\Delta t) - (\Delta A_2/\Delta t)]/ (\Delta A_1/\Delta t)$, where $\Delta A_1/\Delta t$ and $\Delta A_2/\Delta t$ are values for an increase in A_{234} for sample without test substance and with test substance, respectively.

3.3.7 Advanced glycation end-products (AGEs) inhibition

The inhibition of glucose-mediated protein glycation was performed as reported previously (Matsuura et al., 2002). Briefly, the reaction mixture (500 µL) containing 10 µL of extract dissolved in DMSO (dimethyl sulfoxide), 400 µg bovine serum albumin (BSA), 200 mM glucose, 50 mM phosphate buffer (pH 7.4) were incubated at 60 ^{o}C for 30 h. Blank containing glucose and BSA, but not extract, was kept at 4 ^{o}C until measurement. After cooling, 250 µL aliquots were transferred to new tubes and 25 µL of 100% (w/v) trichloroacetic acid was added to each tube and stirred. The mixture was centrifuged at 15,000 rpm at 4 ^{o}C for 4 min and the precipitate of AGEs-BSA was dissolved in 1 mL of alkaline phosphatase buffered saline (pH 10). Fluorescence of these solutions was measured at the excitation/emission of 360/450 nm using GENIOS, Wako, Japan. Percent inhibition of AGEs formation was calculated as [1-(fluorescence sample/fluorescence control)] x 100. Experiments were performed in three replications and repeated twice. IC_{50} was calculated graphically using Microsoft Excel, 2007.

3.3.8 Antimicrobial assays

Five bacteria and three fungi responsible for food-borne and crop diseases were selected to evaluate the antimicrobial activity of PSW extracts. The antibacterial activities of all extracts, dissolved in water, were tested against *Escherichia coli*, *Micrococcus flavus*, *Brevibacterium ammoniagenese*, *Bacillus cereus* and *B. pumilus*. Antibacterial activity was determined by using the disc diffusion method (Ao et al., 2008) where 0.5 mL of 24-h test bacterial broth culture was poured evenly over the surface of 9 cm diameter Petri dishes containing 15 mL nutrient agar. Twenty microgram/disc samples were loaded on to 6 mm filter paper Whatman disc placed on the dried bacterial surface. Ampicillin (2 µg/disc) was used as positive control, while distilled water was used as negative control. The plates were incubated at 37 ^{o}C for 24 h

and the zones of inhibition were calculated by measuring diameter in mm (including the disc).

The antifungal activity was measured against three phytopathogens *Colletotrichum gloeosporiodes*, *Colletotrichum acutatum* and *Scletorinia sclerotiorum* (Deba et al., 2008) and expressed as percentage inhibition against mycelia growth diameter. Briefly, 1 mL of test samples (100 μg) was mixed with 8.0 mL of potato dextrose agar (PDA) and poured into Petri dish. After the gel was set, the spores from a 4-day old mycelia disc (6 mm) were put in the centre of the PDA plate. The plates were incubated at 27 ^{o}C for 4 days and the control contained distilled water. The antifungal activity was expressed as the percentage inhibition in the mycelia growth diameter as compared to that of control.

3.3.9 Neuraminidase inhibition assay

The enzyme assay was performed as reported with slight modifications (Jeong et al., 2009). Briefly, 4-methylumbellifery-l-α-D-*N*-acetylneuramic acid sodium salt hydrate 0.1 mM in 50 mM sodium acetate buffer (pH 5.0) was used as substrate. NA 0.1 U/mL in acetate buffer was used as enzyme source. All the inhibitors were dissolved in MeOH and diluted to appropriate concentration in acetate buffer. Fifty μL of enzyme was added to 20 μL of inhibitor mixed with 80 μL of acetate buffer in a microplate. Reaction was started by adding 50 μL substrate and fluorescence was measured using GENIOS fluorescence meter, Wako, Japan.

The inhibition was calculated using

$$\% \text{ Inhibition} = [1-(S-S_0)/(C-C_0)] \times 100$$

where, S and C represent relative fluorescence unit (RFU) for sample and control

after reaction time, and S_0 and C_0 are RFU at zero time.

3.3.10 GC-MS analysis

An aliquot of 1 µL of acetone solution of the sample was injected splitless into the GC-MS (QP-2010, Shimadzu Co., Japan). The data were obtained on a DB-5MS column, 30 m length, 0.25 mm i.d. and 0.25 µm thickness (Agilent Technologies, J&W Scientific Products, USA). Carrier gas was helium. GC oven temperature started at 50 ^{o}C and holding for 6 min to 280 ^{o}C and holding for 5 min with program rate of 5 ^{o}C/min. The injector and detector temperatures were set at 250 ^{o}C and 280 ^{o}C, respectively. The mass range was scanned from 20 to 900 amu. The control of the GC-MS system and the data peak processing were carried out by means of the Shimadzu's GC-MS solution Software, version 4. Compound identification was verified based on the relative retention time and mass fragmentation pattern spectra with those of standards and the NIST 147. LIB. database of the GC-MS system.

3.3.11 Quantification of extracted phenolic compounds

Phenolic compounds were measured at 280 nm using HPLC (SCL-10 A vp, Shimadzu Co., Japan). Separation was achieved with a Synergy 4 µm Fusion-RP (4.6 mm × 150 mm). A gradient elution was performed with solvent A (water: acetic acid, 99: 1, v/v) and B (methanol: acetonitrile: acetic acid, 75: 24: 1, v/v/v) where the concentration of B increased gradually from 5 to 100% at a flow rate of 0.8 mL/min. Identification of the phenolic compounds was carried out by comparing their retention times to those of standards. Contents of phenolic compounds were expressed in µg/g extract.

3.3.12 Statistical analyses

Data analysis was performed using the Statistical Package for Social Sciences

(SPSS, version 16.0) for Windows. Multiple comparisons between groups were performed by one-way ANOVA supplemented with Tukéys HSD post hoc test. Significance was accepted at *p* lower than 0.05.

3.4 RESULTS AND DISCUSSION

3.4.1 Antioxidant activity

The amounts of total phenolic compounds of fractions E_1, E_2, and E_3 were 118.91 ± 8.70, 155.33 ± 7.62, and 269.85 ± 12.43 GAE mg/100 g PSW, respectively (**Fig. 3.1A**). The total phenolic content of dry pineapple fruit residue has been reported as 275 ± 38.0 mg GAE/100 g (Oliveira et al., 2009). On comparing with the several other reports on total phenolic content in whole pineapple fruit (Gorinstein et al., 1999) or pulp (Alothman et al., 2009), our studies revealed that the PSW had higher phenolic content.

The IC_{50} of DPPH free radical scavenging activity of all fractions is shown in **Fig. 3.1B**. Fraction E_3 showed the highest DPPH activity with an IC_{50} of 164.21 ± 6.50 µg/mL followed by E_1 (335.20 ± 5.51 µg/mL) and E_2 (335.47 ± 8.41 µg/mL). In order to explore whether or not the individual phenolic acids present in PSW had antioxidant activity, we carried out DPPH radical scavenging activity of the standard phenolic compounds that were identified in PSW extract. It was found that the highest percentage inhibition was due to syringic acid followed by ferulic, *iso*-ferulic and vanillin (with an IC_{50} of 9.51 ± 1.41, 29.62 ± 2.54, 84.35 ± 1.27 and 249.50 ± 5.43 µg/mL, respectively) (**Fig. 3.1 B, C**). The activity of *p*-hydroxybenzoic, benzoic and cinnamic acids were very low (results not shown). BHT, a synthetic antioxidant, had the highest activity with an IC_{50} of 5.51 ± 0.80 µg/mL. Similarly, all the extracts inhibited the oxidation of β-carotene to different degrees (**Fig. 3.2A**). It was also found that the reducing power of

various extracts increased with the concentration of tested samples (**Fig. 3.2B**).

In this study, the highest DPPH activity of E_3 fraction is strongly related to the high levels of total phenolic compounds. It must be noted that benzoic acid was predominant, but seems to have no significant roles in antioxidant potentiality of the sample. The standard phenolic acids like syringic, ferulic and *iso*-ferulic, though in lesser amount, are supposedly responsible for antioxidant activity since these acids have lower IC_{50} values in DPPH scavenging assays.

3.4.2 15-LOX, AGEs and neuraminidase inhibition

Our laboratory has been working on bioactivities of pineapple products devoid of bromelain. In this regard, we wanted to examine bioactivities related to phenolic compounds of PSW. The role of phenolic compounds as antilipoperoxidants and potential lipoxygenase inhibitors have been well studied (Chen and Ho, 1997). Oxygen radicals are known to be produced during the inflammatory processes; hence we wanted to investigate the efficacy of PSW in inhibiting 15-LOX in vitro. The inhibitory activity of E_3 on 15-LOX is shown in **Table 3.1**. It was found that with an increase in the concentration, the inhibition of enzymic activity increased. At a concentration of 0.25 mg/mL, the inhibition was found to be almost 80%; IC_{50} was 0.15 mg/mL.

Furthermore, non-enzymatic glycation has been a major factor responsible for the complications in diabetes and aging (Gugliucci, 2000). Many biochemical pathways associated with hyperglycemia can increase production of free radicals (Lee and Chung, 1999). Therefore, antioxidants may play a theoretical strategy for preventing diabetic complications. In this study, since we found a number of phenolic compounds present in E_3, we were interested in investigating their effects in preventing AGEs. The effect of PSW extracts on inhibition of AGEs is shown in **Table 3.1**. A concentration of 0.5

mg/mL had inhibition of more than 80%; IC_{50} was found to be 0.28 mg/mL. The inhibition of AGEs formation by E_3 is probably due to the phenolics present in the sample.

The neuraminidase inhibition by E_3 is shown in **Table 3.1**, with IC_{50} of 0.13 mg/mL. The inhibition may be due to the presence of several phenolic acids, which are considered as a promising class of molecules against variety of diseases. Several polyphenolic compounds have been identified as neuraminidase inhibitors (Ryu et al., 2010).

Several studies have implicated the roles of phenolic acids on inhibiting 15-LOX and AGEs formation (Malterud and Rydland, 2000; Wangensteen et al., 2006). In order to study the effects of phenolic acids present in PSW, we investigated the inhibitory activities of individual phenolic acids against 15-LOX and AGEs formation (**Fig. 3.3**). The results indicated that these phenolic acids had stronger inhibitory activities against 15-LOX than AGEs formations. Syringic, ferulic, hydroxybenzoic, and vanillin had better inhibitory effects with IC_{50} ranging from 68.5 to 93.5 μg/mL. In case of AGEs inhibition, hydroxybenzoic and vanillin had better results when compared to other phenolic acids (**Fig. 3.3**). It seems that the 15-LOX inhibitory properties of PSW may be attributed to syringic, ferulic, hydroxybenzoic acids and vanillin while AGEs formation inhibition may be due to the latter two. It should also be noted that, these phenolic acids have stronger inhibitory activities against 15-LOX than against AGEs formation. These results suggest that, the presence of phenolic acids in PSW certainly had contributed to its bioactive roles. However, the effects of a mixture of these phenolic acids on various bioactivities are yet to be studied.

3.4.3 Antimicrobial assay

The present study showed that all extracts have inhibited the growth of bacteria to different degrees (**Table 3.2**). Gram positive bacteria *B. cereus* and *B. ammoniagense* were significantly inhibited by E_3. Furthermore, gram negative bacteria *E. coli* were also inhibited by E_1. Similarly, all the samples (E_1, E_2, and E_3) showed excellent results against three tested fungi (**Table 3.3**). *C. acutatum* had no growth at all when treated with ethyl acetate fractions.

The antimicrobial assays showed varied results with different extracts and organisms. These variations may reflect differences in cell surface structures between different microorganisms. At the concentrations used, samples showed better activity than the synthetic antibiotic against some bacteria. This inhibition of growth of microorganisms could be attributed to phenolic compounds present in the extracts. Phenolic compounds like ferulic acid have shown antibacterial activity against Gram-negative bacteria (Puupponen-Pimia et al., 2001). There are reports indicating that pineapple extracts have antibacterial activity against *E. coli* and *B. cereus* (Kotzekidou et al., 2008). In this study, the concentration of the standard antibiotic ampicillin used was ten times less than that of the test samples. At these concentrations, the PSW extract exhibited significant inhibitions against different bacteria indicating a moderate effect by a natural extract obtained from the waste. The antifungal activity of PSW indicated it to be a potent fungicidal at the concentrations used. All the fractions exhibited similar activity, which may be due to the presence of a high amount of benzoic acid in all samples (**Fig. 3.4**). Benzoic acids and derivatives are traditionally being used as preservatives in food products.

3.4.4 Analysis of samples by GC-MS and HPLC

The samples were subjected to further analysis by GC-MS and HPLC which

revealed that they contained a variety of phenolic compounds. The MS obtained from the GC-MS data were compared with that of standard compounds, the MS library and with our previous studies (Elzaawely et al., 2005; 2007a; 2007b). Seven compounds were quantified using HPLC, based on comparing the retention time and the peak areas of the standard compounds. The results (**Fig. 3.4A, Table 3.4**) indicated that benzoic acid was predominant followed by syringic, ferulic, *p*-hydroxybenzoic, *iso*-ferulic acids, vanillin and cinnamic acid, with 788.21 ± 13.22 , 719 ± 3.22, 680.45 ± 12.42, 202.53 ± 10.72, 192.35 ± 11.62 and 149.27 ± 11.11 µg/g extract, respectively for E_3. Interestingly, the method of extraction had a significant effect on the amount of phenolic substances present in the samples. The results showed that higher amounts of phenolic compounds could be extracted with water thereby making the extraction procedure cleaner and more economic.

Our study showed that PSW contained two groups of phenolic acids: hydroxybenzoic and hydroxycinnamic. Benzoic acid, *p*-hydroxybenzoic acid, syringic acid and vanillin fall in hydroxybenzoic acid group which have common C_6-C_1 structure, while ferulic, *iso*-ferulic, and cinnamic acids belong to aromatic hydroxycinnamic groups having three carbon side chain (C_6-C_3) (**Fig. 3.4B**). Most of these phenolic acids have been identified in pineapple juice (Wen and Wrolstad, 2002). This indicated that the waste obtained during bromelain manufacturing also contain important phenolic compounds that are present in the juice, thereby indicating its possible commercial applications.

3.5 CONCLUSIONS

The results of this study substantiate a probable role for PSW to be utilized as a natural source of antioxidant having a wide range of bioactivities. The bioactivities of

PSW are quite fair but this study certainly widens the scope of utilizing PSW which otherwise may cause serious environmental problems without having any commercial value. Since the PSW showed a good antioxidant and antimicrobial property, along with substantial 15-LOX and AGEs inhibitory activities, it could be employed as an impending nutraceutic resource, capable of offering significant low-cost, nutritional dietary supplement for low-income communities.

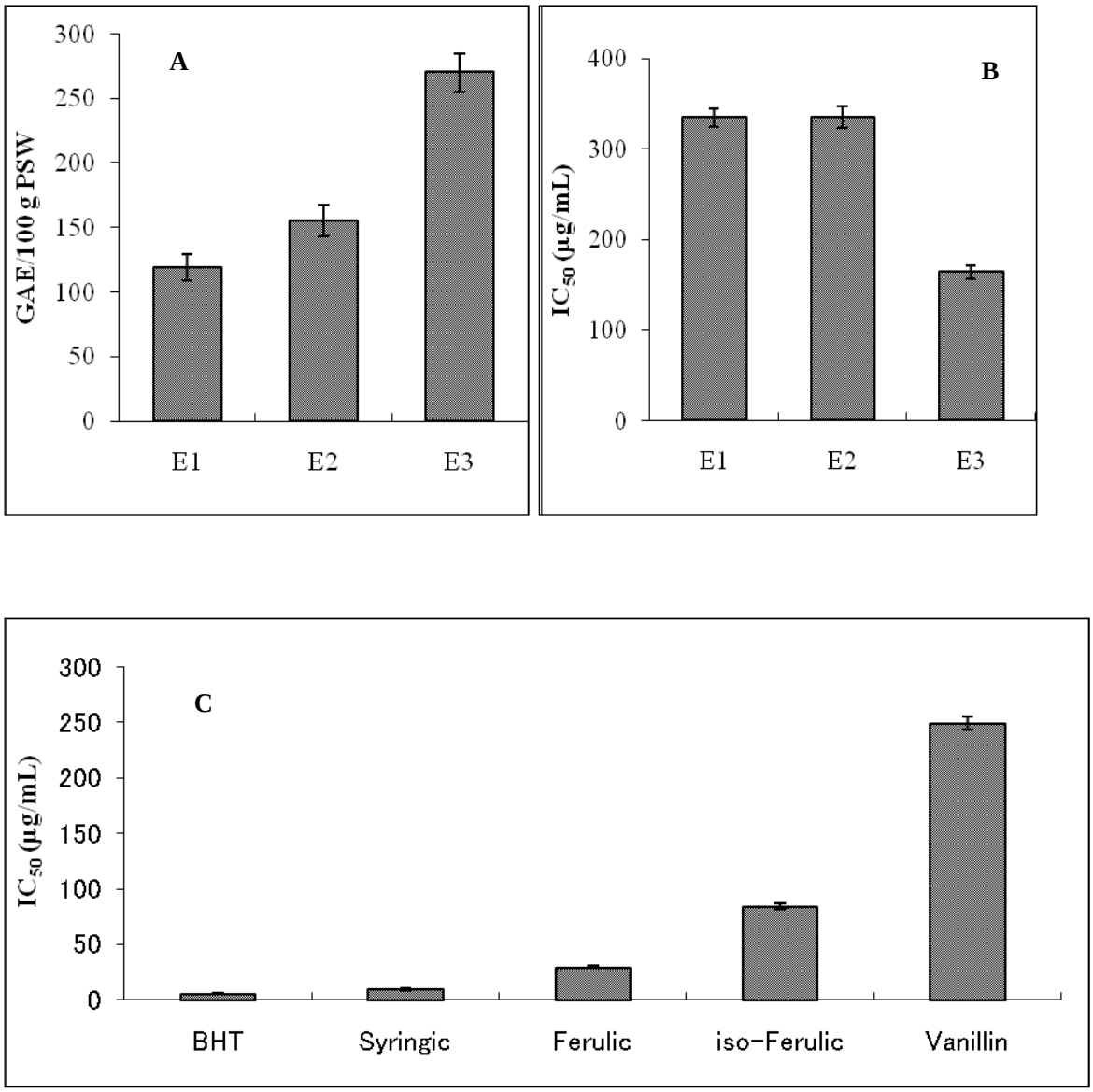
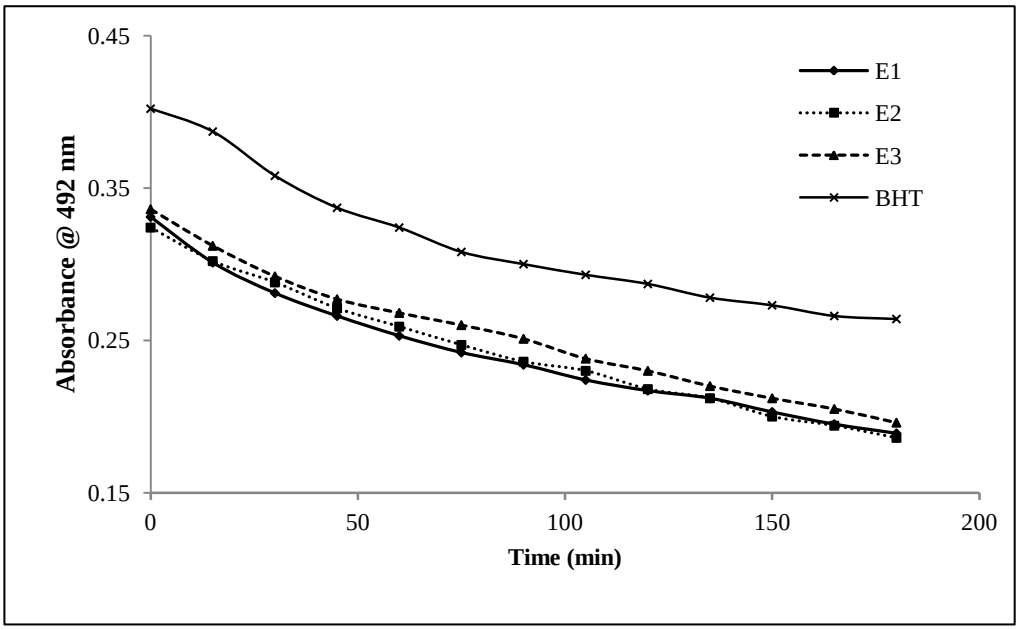
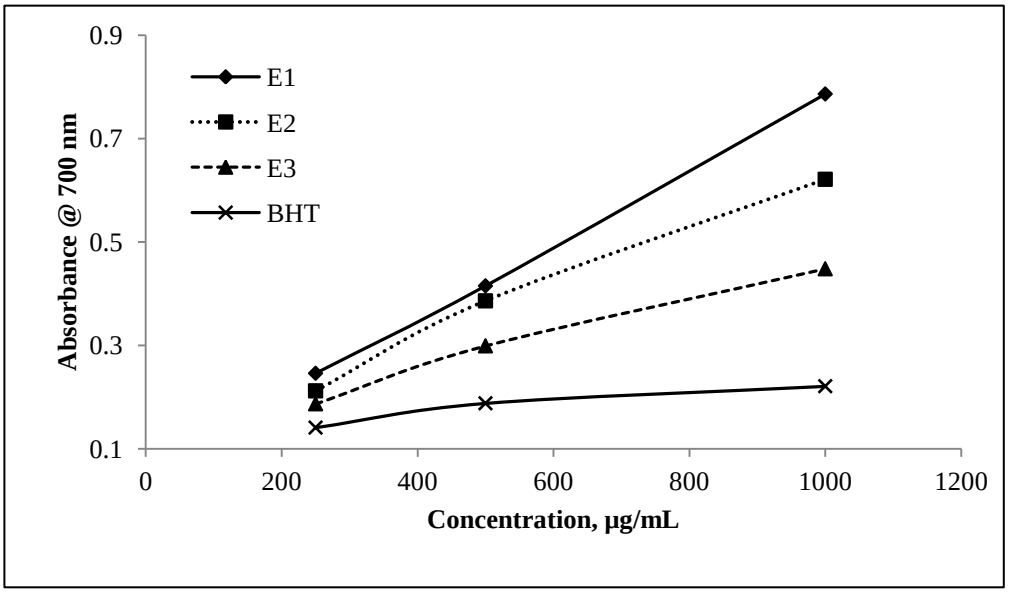


Fig. 3.1. Total phenolic content (A), and DPPH free radical scavenging activity of different fractions from PSW (B). DPPH free radical scavenging activity of standard phenolic compound (C).



A



B

Fig. 3.2. Antioxidant activity of different fractions of PSW and positive control BHT measured by β-carotene bleaching method (A) and reducing activity (B).

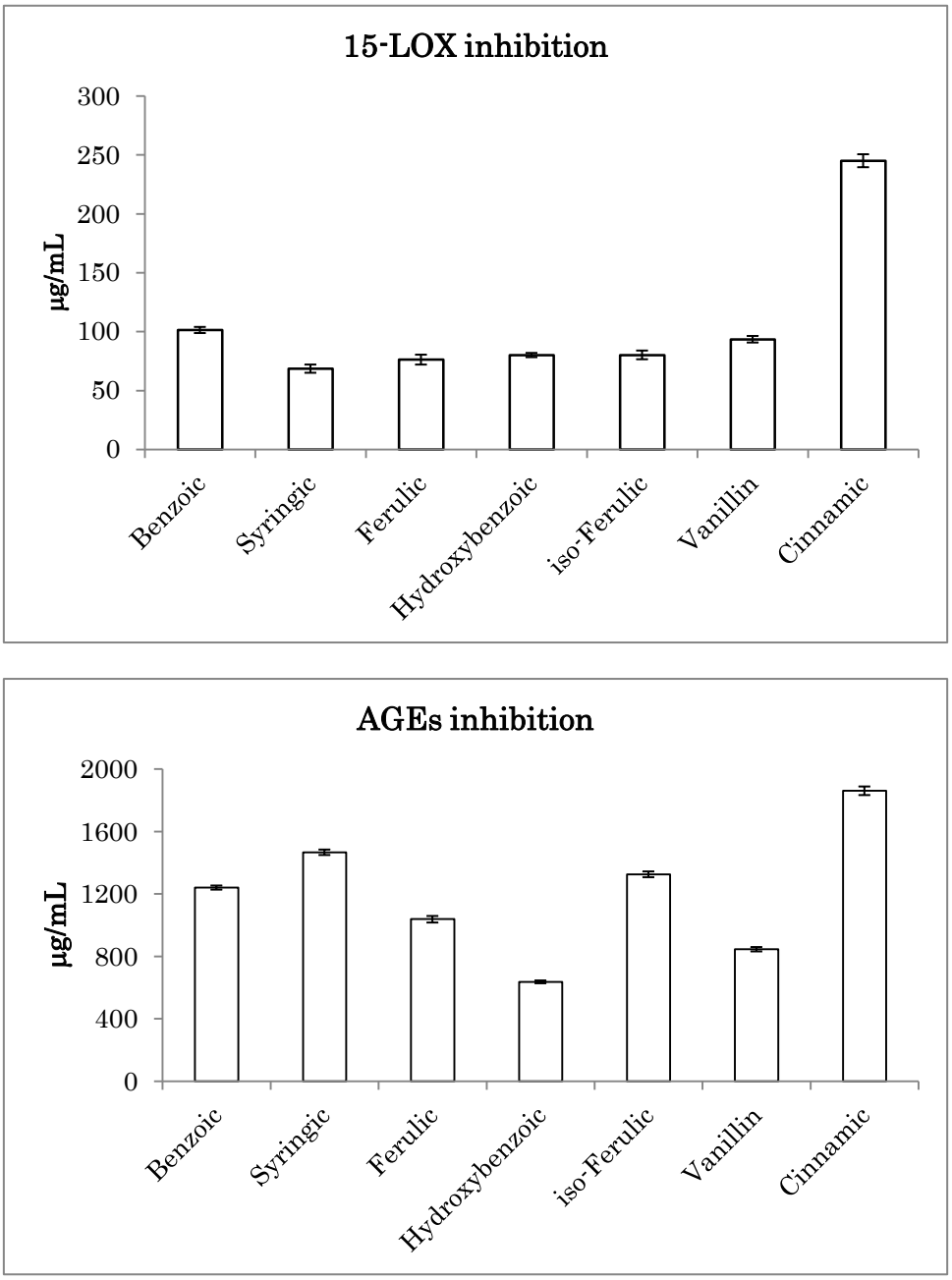
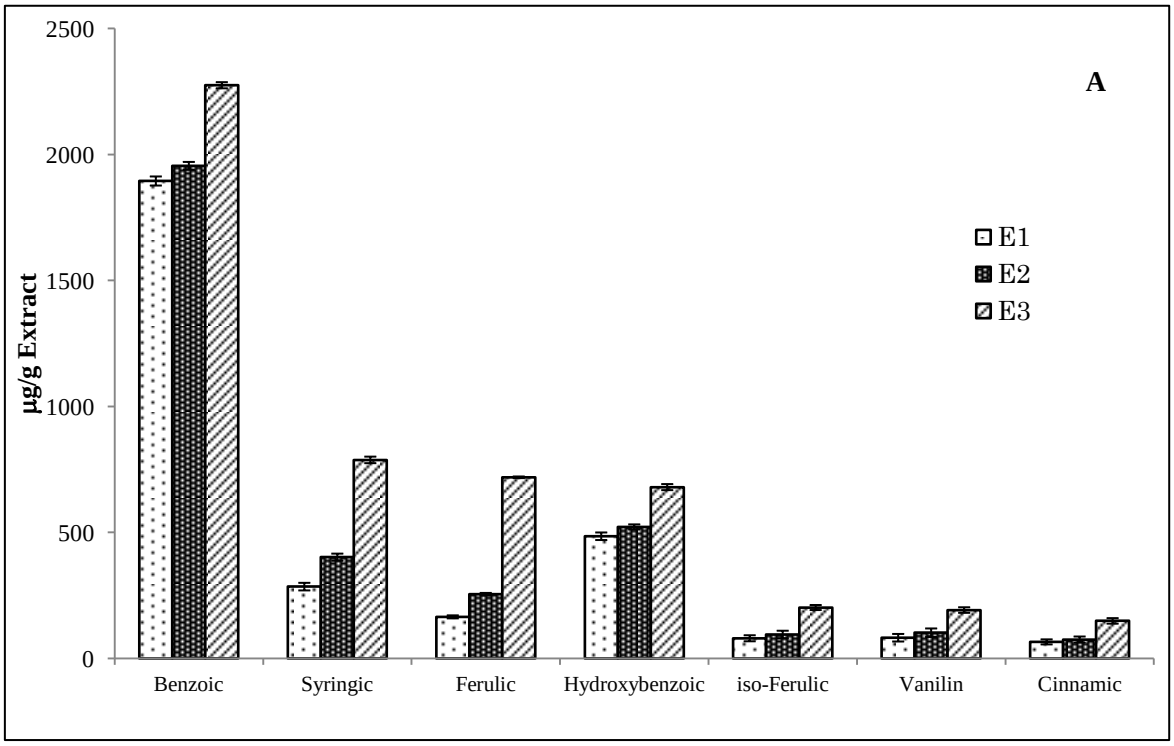
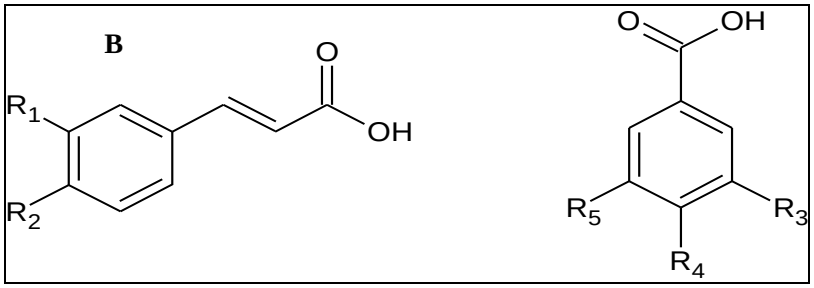


Fig. 3.3. Inhibitory effects of identified phenolic acids on 15-LOX and AGEs.





Phenolic acid	R_1	R_2	R_3	R_4	R_5
Cinnamic	H	H	-	-	-
Ferulic	OCH_3	OH	-	-	-
iso-Ferulic	OH	OCH3	-	-	-
Benzoic	-	-	H	H	H
Hydroxybenzoic	-	-	H	OH	H
Syringic	-	-	OCH_3	OH	OCH_3

Fig. 3.4. Amount of individual phenolic acid in E_3 as determined by HPLC (A).

Chemical structures of phenolic acid quantified by HPLC (B).

Table 3.1

Concentration dependent inhibition of 15-LOX, AGEs and neuraminidase by E_3 of PSW

Concentration (mg/mL)	Inhibition (%)		
	15-LOX	AGEs	Neuraminidase
0.0625	22.42 ± 1.34[a]	-	33.25 ± 1.04
0.125	41.63 ± 1.71	26.20 ± 2.11	48.64 ± 0.89
0.25	79.93 ± 2.14	49.22 ± 1.87	78.25 ± 1.10
0.5	-	80.64 ± 1.10	-

[a] Values are means of three replications ± SD.

Table 3.2

Antibacterial activities of different fractions of PSW and positive control ampicillin

Test bacteria	Inhibition zone (mm)[y]			
	E_1[x]	E_2[x]	E_3[x]	Ampicillin[z]
Escherichia coli	15.10 ± 2.10a	11.70 ± 1.90ab	9.70 ± 0.80bc	11.50 ± 1.20ab
Micrococcus flavus	11.00 ± 0.00a	14.20 ± 1.30a	11.50 ± 1.60a	10.70 ± 0.80a
Brevibacterium ammoniagenese	13.10 ± 2.30b	15.50 ± 1.00a	17.30 ± 0.80a	11.10 ± 1.50c
Bacillus cereus	11.80 ± 1.50ab	9.80 ± 0.80b	13.30 ± 2.30a	10.20 ± 1.30b
Bacillus pumilus	10.50 ± 0.60ab	9.80 ± 0.80bc	8.80 ± 1.20c	11.70 ± 1.80a

Values are means of three replications ± SD. Means with the same letter in the row are not significantly different at $p < 0.05$.

[x] 20 µg /disc.

[y] diameter of disc.

[z] 2 µg /disc.

Table 3.3

Fungicidal activity of different fractions of PSW

Test fungi	Inhibition (%)		
	E_1[a]	E_2[a]	E_3[a]
Colletotrichum gloeosporioides	73.30 ± 1.10	71.90 ± 1.70	73.00 ± 1.70
Aclerotinia sclerotiorum	81.70 ± 2.40	82.20 ± 1.10	82.20 ± 1.10
Colletotrichum acutatum	100.00	100.00	100.00

Values are means of three replications ± SD.

[a] 100 µg/mL

Table 3.4

Retention time, molecular weight and major peaks of phenolic compounds identified by GC-MS.

Compound	Retention time (min)	Chemical formula	Molecular weight	Major Peaks (*m/z*)
Benzoic acid	19.5	C_6H_5COOH	122.1	105, 122, 77
Coumaran	21.0	$C_8H_6O_2$	198.2	120, 91, 65
Vanillin	26.4	$C_8H_8O_3$	152.2	151, 109, 81
Cinnamic acid	27.3	$C_9H_8O_2$	148.2	147, 103, 77
Vanillic acid	30.6	$C_8H_8O_4$	168.2	168,153, 125
p-Hydroxybenzoic acid	30.7	$C_7H_6O_3$	138.1	168, 153, 97
4-Isopropyl-1,6-dimethyl napthalene	34.5	$C_{15}H_{18}$	198.3	183, 198, 165
Benzene-propanoic acid	34.8	$C_{10}H_{12}O_4$	196.9	137, 196,
iso-Ferulic acid	35.4	$C_{10}H_{10}O_4$	194.2	194, 133, 179
Syringic acid	36.5	$C_9H_{10}O_5$	198.2	198, 183, 127
Ferulic acid	37.8	$C_{10}H_{10}O_4$	194.2	194, 179, 133

CHAPTER 4

BIOLOGICAL ACTIVITIES OF

ALPINIA ZERUMBET

4.1 SUMMARY

AIDS and influenza are viral pandemics and remain one of the leading causes of human deaths worldwide. The increasing resistance of these diseases to synthetic drugs demands the search for novel compounds from plant based sources. In this regard, we investigated the leaves and rhizomes of *Alpinia zerumbet*, a traditionally important economic plant in Okinawa, against HIV-1 integrase and neuraminidase. The aqueous extracts of leaves and rhizomes had neruaminidase inhibitory activity with IC_{50} of 30 and 188 µg/mL, while against neruaminidase inhibition they showed 50% inhibition at concentrations of 43 and 57 µg/mL, respectively. 5,6-Dehydrokawain (DK), dihydro-5,6-dehydrokawain (DDK) and 8(17),12-labdadiene-15,16-dial (labdadiene) were isolated from the rhizomes and were tested for enzyme inhibitions. DK and DDK strongly inhibited integrase with IC_{50} of 4.4 and 3.6 µg/mL, respectively. Against neruaminidase, DK, DDK and labdadiene exhibited mixed type of inhibition with respective IC_{50} of 25.5, 24.6, and 36.6 µM and Ki values range of 0.3 to 2.8 µM. It was found that DDK is a slow and time-dependent reversible inhibitor of neruaminidase probably with methoxy group as its functionally active site. These results suggest that alpinia could be used as a source of bioactive compounds against integrase and neuraminidase, and that DK and DDK may have possibilities in the drug designing

against these viral diseases.

4.2 INTRODUCTION

Acquired immunodeficiency syndrome (AIDS) represents one of the most important modern epidemics with over 40 million people infected worldwide. The replication of HIV requires three enzymes: protease, integrase and reverses transcriptase. Insertion of viral genome inside the infected cell is mediated through the encoded enzyme integrase, which has been a rational target for treating HIV inhibition (Pommier et al., 2005). Synthetic integrase inhibitor like raltegravir (also known as isentress or MK-0518) is being used in reducing HIV load (Hanley et al., 2009). On the other hand, the impingement of influenza on human health is undeniably escalating, impact being more serious during the winter season (Moscona, 2005). Human cases of avian influenza, and more recently, the outbreak of aggressive porcine A/H1N1 strain in 2009 have heightened awareness of the threat of pandemic. The disease is associated with a RNA virus that contains hemagglutinin and neuraminidase as surface antigens. Neuraminidase is involved in the release of progeny virus from infected cells, by cleaving sugars that bind the mature viral particles (Chandrasekaran et al., 2008). Specifically, neuraminidase cleaves the α-ketosidic bond that links a terminal neuraminic-acid residue to the adjacent oligosaccharide moiety. Neuraminidase is

therefore essential for the movement of the virus to and from sites of infection in the respiratory tract (De Clercq, 2006; Von Itzstein, 2007). With the ever present threat of a pandemic derived from the HIV and influenza virus and the emergence of resistance strains against synthetic drugs, the importance of search for novel compounds from plant based source intensifies.

Alpinia [*Alpinia zerumbet* (Family Zingiberaceae)] is a perennial ginger growing widely in the subtropics and tropics. It is used in folk medicine for its anti-inflammatory, bacteriostatic and fungistatic properties (Zoghbi et al., 1999). The essential oil extracted from its leaves possessed both relaxant and antispasmodic actions in rat ileum (Bezerra, et al., 2000*).* Early reports have shown the α-pyrones, dihydro-5,6-dehydrokawain (DDK), and 5,6-dehydrokawain (DK) are major compounds in alpinia leaves and they have shown plant growth inhibition against lettuce seeds (Fujita, Nishimura, Haburagi, Mizutani, 1994), insecticidal activity against *Coptotermes formosanus* and antifungal activity against *Pythium* sp. and *Corticium rolfsii* (Tawata et al., 1996). The aqueous extract of its leaves have demonstrated hypotensive activity (Laranja et al., 1991), mainly due to flavonoids and kava pyrones (Mpalantinos et al., 1998). Furthermore, DK and DDK are reported to inhibit the aggregation of ATP release from rabbit platelets (Teng et al., 1990). DK and DDK are described to have antiulcerogenic and

antithrombotic activities (Mpalantinos et al., 1998). The inhibitory properties of DK on human platelet aggregation, anti-inflammatory and cancer chemopreventive therapeutic properties are reported (Jantan et al., 2008). Labdadiene was traditionally used as a medicine against inflammatory diseases (Kunnumakkara et al., 2008). The cardiovascular effects induced by labdadiene were evaluated in male Wistar rats (Oliveira et al., 2006). Moreover, labdadiene has also been reported to inhibit lipid peroxidation, cyclooxygenase enzymes and human tumor cell proliferation (Liu et al., 2011). Our laboratory has reported DDK and phenolic compounds and their antioxidant activities in leaves and rhizomes of the plant (Elzaawely et al., 2007).

In Okinawa, alpinia leaves have been used to prepare traditional food, *mu-chi* (**Fig. 4.1**), and there is a common folklore that it prevents from catching cold (Tawata et al., 2008). In the previous chapter, the biological activity of PSW was discussed, in this chapter, we primarily focused on investigating the integrase and neuraminidase inhibitory activities of aqueous extracts of alpinia leaf and rhizomes and three compounds isolated from rhizomes. Furthermore, in this chapter, we also exhibited the probable mechanism of neuraminidase inhibition by DDK.

4.3 MATERIALS AND METHODS

4.3.1 Preparation of plant extracts and isolation of compounds.

The leaves and rhizomes of alpinia were collected in the University of the Ryukyus campus, Okinawa. Twenty gram of fresh leaves or rhizomes were separately boiled in 1 L water for 30 min and the cooled extract was filtered and dried under vacuum at 40 ^{o}C. All the residues were weighed and dissolved in 50% DMSO to make a stock solution of 1 mg/mL of crude extracts before testing the integrase and neuraminidase inhibitory activites. In order to isolate the active compounds, we used the rhizomes of alpinia. Our laboratory has been working with two pyrone compounds (Tawata et al., 1996; Elzaawelly et al., 2007; Tawata et al., 2008; Elzaawelly et al., 2007) (**Fig. 4.2**) and thus we chose first to investigate anti-integrase and neuraminidase properties of these compounds. For the isolation of these compounds, 2 Kg of fresh alpinia rhizomes was boiled in 10 L water for 20 min. The cooled extract was filtered and reduced to 1 L under vacuum at 40 ^{o}C. Further extraction with hexane (500 mL x 3) was done and the hexane fraction was evaporated to complete dryness under vacuum. The dried extract was boiled in water and filtered hot. The residue obtained was purified by preparative HPLC to obtain DK using TSK gel ODS-100Z column (Tosoh Corporation, Japan) (15 x 0.46 cm i.d.; 5 µM particle size) monitored continuously at 280 nm. The mobile phase consisted of 0.1% acetic acid and MeOH which was increased from 50% to 100% in 20 min at a flow rate of 0.8 mL/min. For DDK, the filtrate was crystallized at 4 ^{o}C which

was further purified using preparative HPLC as above. Compounds were identified using NMR and GC-MS. The amount of the purified compounds were weighed and expressed as mg/100g fresh rhizomes. DDK and DK were determined to be 24.1 and 18.8 mg/100 g fresh rhizomes. The ^{1}H NMR (600 MHz) and ^{13}C NMR (150 MHz) spectra were recorded on a JEOL JNM-ECA600 (JEOL, Japan) in $CDCl_3$. Chemical shifts are expressed in parts per million (δ) relative to TMS. 2D NMR experiments (H, C- COSY, HMQC, HMBC) were obtained using standard pulse sequences. For DDK, electron ionization mass spectrometry (EIMS) *m/z*: 230 $[M]^+$ (30), 202 (8), 125 (30), 111 (28), 91 (100), 69 (12); ^{1}H ($CDCl_3$) δ 2.73 - 2.76 (*m*, 2H, CH_2), 2.96 - 2.97 (*m*, 2H, CH_2), 3.77 (*s*, 3H, CH_3), 5.42 (*s*, 1H, *CH*), 5.72 (*s*, 1H, *CH*), 7.18-7.29 (*m*, 5H, *aromatic*); ^{13}C NMR ($CDCl_3$) δ 32.80 (CH_2), 35.42 (CH_2), 55.80 (OCH_3), 87.68 (*CH*), 100.25 (*CH*), 126.41, 128.57, 128.57, 128.27, 128.27, 139.82 (*aromatic*), 164.32 (*CH*), 164.93 (*CH*), 170.0 (*C*) (*21*). DK was characterized by the following data: EIMS *m/*z: 228 $[M]^+$ (60), 200 (20), 157 (35), 129 (20), 69 (20), 44 (35), 40 (100); ^{1}H ($CDCl_3$) δ 3.79 (*s*, 3H, CH_3), 5.51 (*d*, 1H, *CH*), 5.97 (*s*, 1H, *CH*), 6.81 (*d*, 1H, *CH*), 7.31 (*d*, 1H, *CH*), 7.32 (*m*, 5H, *aromatic*); ^{13}C NMR ($CDCl_3$) δ 163.28 (*C*), 88.65 (*CH*), 171.21 (*C*), 100.95 (*CH*), 160.3 (*C*), 118.53 (*CH*), 135.36 (*C*), 131.88, 128.27, 129.75, 129.27, 127.98 (*aromatic*), 56.24 (OCH_3) (Dharmaratne et al., 2002). Labdadiene was isolated

by another group of our laboratory from the rhizomes of alpinia (Chompoo et al., 2011). DDK-OH was synthesized by stirring DDK in conc. HCl for 18 h followed by overnight crystallization at 8 ^{o}C (Tawata et al., 1996).

4.3.2 HIV-1 integrase assays.

The integrase inhibition was determined using multiplate integration assay (Tewtrakul et al., 2006). Fifty μL of biotinylated-LTR donor DNA was added to streptavidin coated 96 well microtitre plate and incubated for 60 min at room temperature. After discarding the solution, integrase buffer (12 μL), target DNA (5 pmol), sterilized water (32 μL), inhibitors (6 μL) and integrase (180 ng) were added to wells and incubated at 37 ^{o}C for 80 min. After the completion of reaction, unbound DNA was removed by washing with PBS containing 0.5% Tween 20 and PBS alone. The relative activity of bound DNA was determined using an alkaline phophatase labeled anti-digoxigenin antibody (50 mU) after incubation at 37 ^{o}C for 1 h. The resulting yellow color was measured using microplate reader at 405 nm. A control contained enzyme without sample, while blank did not contain enzyme.

The percent inhibition against integrase was calculated as

$$\% \text{ Inhibition} = [OD_{control} - OD_{sample}]/OD_{control} \times 100$$

where, OD = absorbance detected from each well.

4.3.3 Neuraminidase (*Clostridium perfringens*) inhibition assay.

The enzyme assay was performed as in section **3.3.9**. For kinetic studies, we used time-driven protocol with initial velocity recorded over a range of substrate concentrations for different inhibitor concentrations (0, 10, 15, and 20 μM). Dixon plots were obtained by plotting the slopes of the obtained line (Km/Vm) against substrate concentrations. For time-dependent studies, we obtained progress curves for 600 s at several pre-incubation times using 25 μM DDK and the slopes of lines obtained were plotted against pre-incubation time. To obtain the effect of enzyme concentrations, we used different enzyme concentrations over a range of inhibitor concentrations. All the data were analyzed using Microsoft Excel Office, 2007.

The inhibition was calculated using

% Inhibition = $[1-(S-S_0)/(C-C_0)] \times 100$

where, S and C represent relative fluorescence unit (RFU) for sample and control after reaction time, and S_0 and C_0 are RFU at zero time.

4.3.4 Statistical Treatment.

All the experiments were conducted in triplicates and repeated twice. The data represents mean ± s.d. of six results. IC_{50} value was determined graphically as the concentration of each sample required to give 50% inhibition activity. For kinetic

studies of neuraminidase inhibition, all calculations were performed in Excel, Microsoft Office 2007. For significant analysis, the data were analyzed by one-way ANOVA and the means were separated using Tukey HSD range test at p = 0.01. All statistical analyses were performed using SPSS version 16.0 for Windows Vista.

4.4 RESULTS AND DISCUSSION

4.4.1 Integrase inhibition assays.

The integrase inhibitory activity of different extracts and the isolated compounds are shown in **Table 4.1**. It was found that the aqueous extract of the leaves of alpinia had stronger activity than rhizomes. On carrying out the inhibition by the isolated compounds, IC_{50} of DK and DDK against integrase were 4.4 ± 0.5 and 3.6 ± 0.9 µg/mL respectively. Suramin had an IC_{50} of 2.3 ± 0.7 µg/mL under the stated conditions.

4.4.2 Neuraminidase inhibition activities.

The water extract of alpinia leaves and rhizomes showed considerable neuraminidase activity with IC_{50} of 42.7 ± 1.2 and 57.1 ± 1.1 µg/mL, respectively. In the case of neuraminidase inhibition, we also used labdadiene isolated from the rhizomes of alpinia. The IC_{50} values of the extract and the isolated compounds are shown in **Table 4.1**. When the inhibition by isolated compounds was checked, DDK (IC_{50}: 24.6 ± 0.4 µM) and DK (IC_{50}: 25.5 ± 0.7) both had more potent activity than the

positive control quercetin (*25*) with IC_{50} of 34.7 ± 0.9 μM under these conditions (**Table 4.1**). Labdadiene (IC_{50}: 36.6 ± 1.0) had activity similar to quercetin. The kinetic studies of individual compounds showed mixed type of inhibition (**Fig. 4.3**) with estimated Ki values ranging from 0.2 to 2.8 μM.

The present study examines the inhibitory properties of aqeous extract of leaves and rhizomes of alpinia against integrase and neuraminidase. DK, DDK and labdadiene were isolated from the rhizomes. In order to determine the inhibitory activity against integrase, we carried out strand transfer inhibition assay using microtitre plate method. Strand transfer is the second step of the integration reaction which corresponds to the ligation of the viral 3′-OH cDNA ends to the 5′-DNA phosphate of an acceptor DNA (Pommioer et al., 2005). In our result we found the leaves aqueous extract had higher activity than the rhizomal extract. This was also true with neuraminidase inhibition (**Table 4.1**). The leaves and rhizomes of alpinia have been a source of wide variety of bioactive constituents. The content of phenolics and flavonoids in the leaves and rhizomes of alpinia have been extensively investigated (Fujita et al., 1994; Tawata et al., 1996; Laranja et al., 1991; Mpalantinos et al., 1998; Tawata et al., 2008). Our previous work has reported the presence of phenolic compounds in the leaves and rhizomes of alpinia (Elzaawely et al., 2007). These groups of compounds have always been a

promising class of molecules against variety of diseases. Therefore, we have supposed that integrase or neuraminidase inhibitory activities of alpinia leaves and rhizomes may be attributed to the phenolic compounds. Besides, several compound present in other species of alpinia have been reported to have integrase or neuraminidase inhibitory properties (Ye et al., 2006; Grienke et al., 2010; Sawamura, et al., 2010). Furthermore, isolation and identification of specific compounds active against integrase and/or neuraminidase from alpinia are being undertaken in our laboratory. Hence, once it was confirmed that the aqueous extract of the leaves and rhizomes of alpinia had considerable activity, we explored the secondary metabolites present in the plant.

Two compounds DK and DDK were tested against integrase activity. Both of these compounds had similar activity to suramin ($p = 0.01$), which is widely used as positive control against integrase inhibition assays *in vitro* (Tewtrakul et al., 2006). Our results indicated that both DK and DDK could be used as possible candidate for integrase inhibition (**Table 4.1**). Furthermore, simple structures and low molecular weights of DK and DDK would certainly add to their merits over complex synthetic inhibitors. Besides, we had previously shown that DK could be metabolized to hispidin *in vitro* using CYP2C9 (Upadhyay et al., 2009). Since hispidin has shown to have integrase inhibitory activity (Tawata et al., 1996), DK seems to act as anti-integrase in two forms, firstly as

DK itself, and secondly after it has been catalyzed to hispidin by cytochrome P450 2C9 isozyme (CYP2C9). The mechanism of integrase inhibition by DK and DDK is still unclear while the activity of labdadiene against integrase is yet to be determined.

The IC_{50} of the investigated compounds against neuraminidase indicate that DK and DDK have significantly better inhibition properties while labdadiene had similar activity against neuraminidase when compared to quercetin (**Table 4.1**). Our results therefore suggest that DK, DDK and labdadiene could be used effectively against neuraminidase.

The role of pyrone in inhibiting integrase and neuraminidase is reported. Four naphtha-γ-pyrones belonging to the chaetochromin and ustilaginoidin family were identified as integrase inhibitors (Singh et al., 2003). On the other hand, all compounds containing pyrone rings do not show inhibitory properties. A structure-activity relationship study of 32 different flavones with γ-pyrone showed that the activity depends on the charge of γ-oxygen atom of pyrone ring (Lameira et al., 2006). However, in our study we used DK and DDK which are α-pyrone compounds. α-Pyrone compounds like tipranavir has shown to have potent HIV protease inhibition (Turner et al., 1998) and is a drug approved by Food and Drug Administration, USA (USFDA). Though yet to be confirmed, these information might suggest that the integrase

inhibition of DK and DDK may be attributed to the presence of α-pyrone group.

Furthermore, in the case of neuraminidase inhibition by α-pyrone, Grienke et al. have reported that out of four diarylheptanoids isolated from *A. katsumadai*, the most potent neuraminidase inhibitory compound had α-pyrone moiety along with an additional phenyl group (Grienke et al., 2010). However, the authors have not discussed whether the inhibition was due to pyrone moiety or some other functional groups. In order to investigate the active functional group of DDK, we examined neuraminidase inhibition by α-pyrone compound, 4-methoxy-6-methyl-2H-pyran-2-one (4MHP), a moiety present in DDK. Interestingly, the activity of 4MHP against neuraminidase was very low with an IC_{50} of 822.9 ± 7.2 μM. This made us consider that, for DDK, pyrone group may not have effective role in inhibiting neuraminidase. Our next step was to determine whether the ethyl benzene group or the methoxy group of DDK is active against neuraminidase. For this, we synthesized a compound DDK-OH (**Figure 4.2**) and examined its activity against neuraminidase. The IC_{50} of DDK-OH (525.6 ± 11.3 μM) was better than 4MHP but was more than 100-fold weaker than DDK. These two results suggest that the active functional group in DDK is probably the methoxy group present in the C-5. Hence it is likely that for DDK α-pyrone has very less functionality in inhibiting neuraminidase.

The plausible explanations of inhibition of neuraminidase by labdadiene are still under investigation. However, it is noteworthy that this compound contains double aldehyde groups in C-15 and 16 and that the commercial influenza drug Tamiflu also has an aldehyde group. Researchers have tried to develop active compounds against neuraminidase by adding various aldehyde groups to the carboxylate and acetomido moiety of Tamiflu to increase the inhibitory properties (Hochgurtel et al., 2002).

The kinetic studies of individual compounds showed mixed type of inhibition (**Fig. 4.3**). These types of inhibition are quite common with the natural compounds. The predominant inhibition mode shown by naturally occurring neuraminidase inhibitors is noncompetitive. However, the compounds isolated from alpinia exhibited mixed type of inhibition there by indicating a different class of compounds identified against neuraminidase. The estimated Ki values for DK, DDK and labdadiene are 0.2, 2.8 and 0.6 respectively (**Table 4.1**). The low Ki of DK along with its low IC_{50} value certainly makes it a potent compound against neuraminidase.

We further investigated the inhibitory mechanism of DDK at its IC_{50} concentration. We explored the effect of pre-incubation time on the inhibition of the hydrolysis of neuramic acid. Since the decrease in residual activity was observed with increasing pre-incubation time, DDK emerged to be a slow-binding inhibitor at low concentrations

(**Fig. 4.4A**). Furthermore, increasing pre-incubation time of DDK also led to a decrease in the slope thereby indicating reduction in both initial and steady state velocity (**Fig. 4.4B**). This result along with low Ki indicates that DDK is a slow binder to neuraminidase. DDK therefore is more like the drug Tamiflu which is also a slow and time-dependent inhibitor of neuraminidase (Kati et al., 1998). Moreover, when the effect of enzyme concentration on the inhibition was probed, it was found that the residual activity of neuraminidase increased with the enzyme concentration at fixed substrate amount (**Fig. 5.5A**). Plots of residual enzyme activity versus enzyme concentration at different concentrations of DDK gave a family of straight lines passing through origin indicating that DDK is a reversible inhibitor (**Fig. 5.5B**). Hence, we may say that in our study DDK acted as a time-dependent slow reversible inhibitor of neuraminidase.

4.5 CONCLUSION

The leaves and rhizomes of alpinia possess a wide range of bioactivities. We had previously reported antifungal and plant growth inhibitory activities of alpinia. In this study, we focused on integrase and neuraminidase inhibition by alpinia. We also isolated two α-pyrones from the rhizomes and for the first time showed that they have considerable integrase and significant neuraminidase inhibition activities. We also

revealed that labdane from alpinia could be used as neuraminidase inhibitor. Furthermore, we discussed the inhibitory mechanism of DDK. Since DK and DDK have significant integrase and neuraminidase inhibitory activities we may say that these compounds could be used as lead candidates for drug designing. Our results indicate that the leaves of alpinia could be utilized as preventive or therapeutic agents in diseases and in different functional food industries.

Table 4.1. Integrase and neuraminidase inhibitory activities of different extracts and isolated compounds

Alpinia/ Compound	Integrase inhibition	Neuraminidase inhibition		
	IC_{50}	IC_{50}	Type of Inhibition	Ki
Leaf	30 ± 1 µg/mL	43 ± 1 µg/mL	-	-
Rhizome	188 ± 2 µg/mL	57 ± 1 µg/mL	-	-
DK	4.4 ± 0.5 µM [a]	26 ± 1 µM [a]	Mixed	0.3 µM
DDK	3.6 ± 0.9 µM [a]	25 1 µM [a]	Mixed	2.8 µM
Labdane	-	37 ± 1 [b]	Mixed	0.6 µM
DDKOH	-	525.6 ± 11.3 µM	-	-
4MHP	-	822.9 ± 7.2 µM	-	-
Suramin	2.3 ± 0.7 µM [a]	-	-	-
Quercetin	-	35 ± 1 µM [b]	-	-

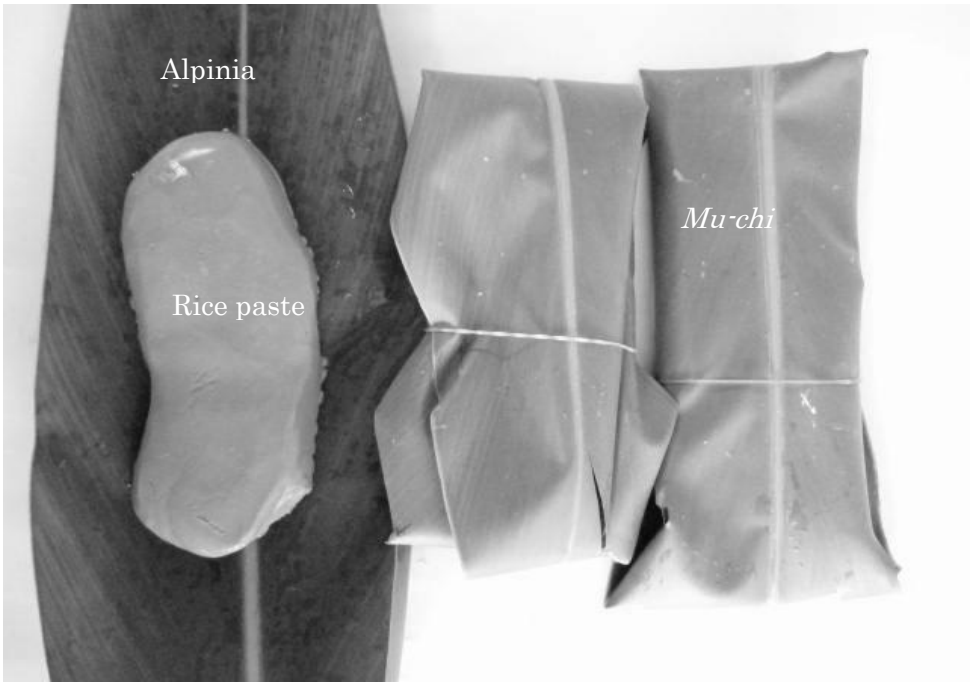


Fig. 4.1. *Mu-chi*. A traditional food of Okinawa. Rice ball or paste is wrapped in alpinia leaves and served after steaming.

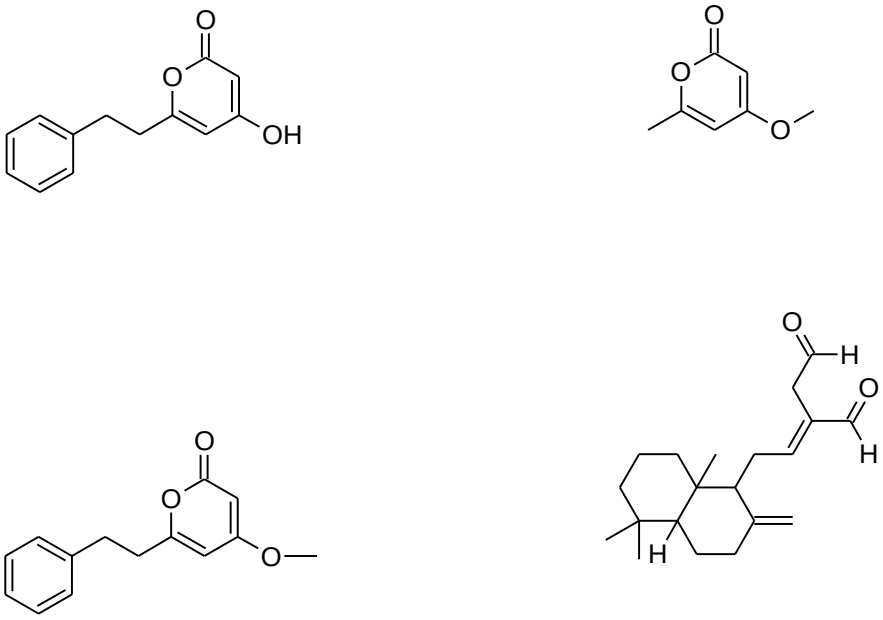


Fig. 4.2. Structures of DDK, DK, labdane and pyrone. DDK, DK and labdane were isolated from the rhizomes of alpinia.

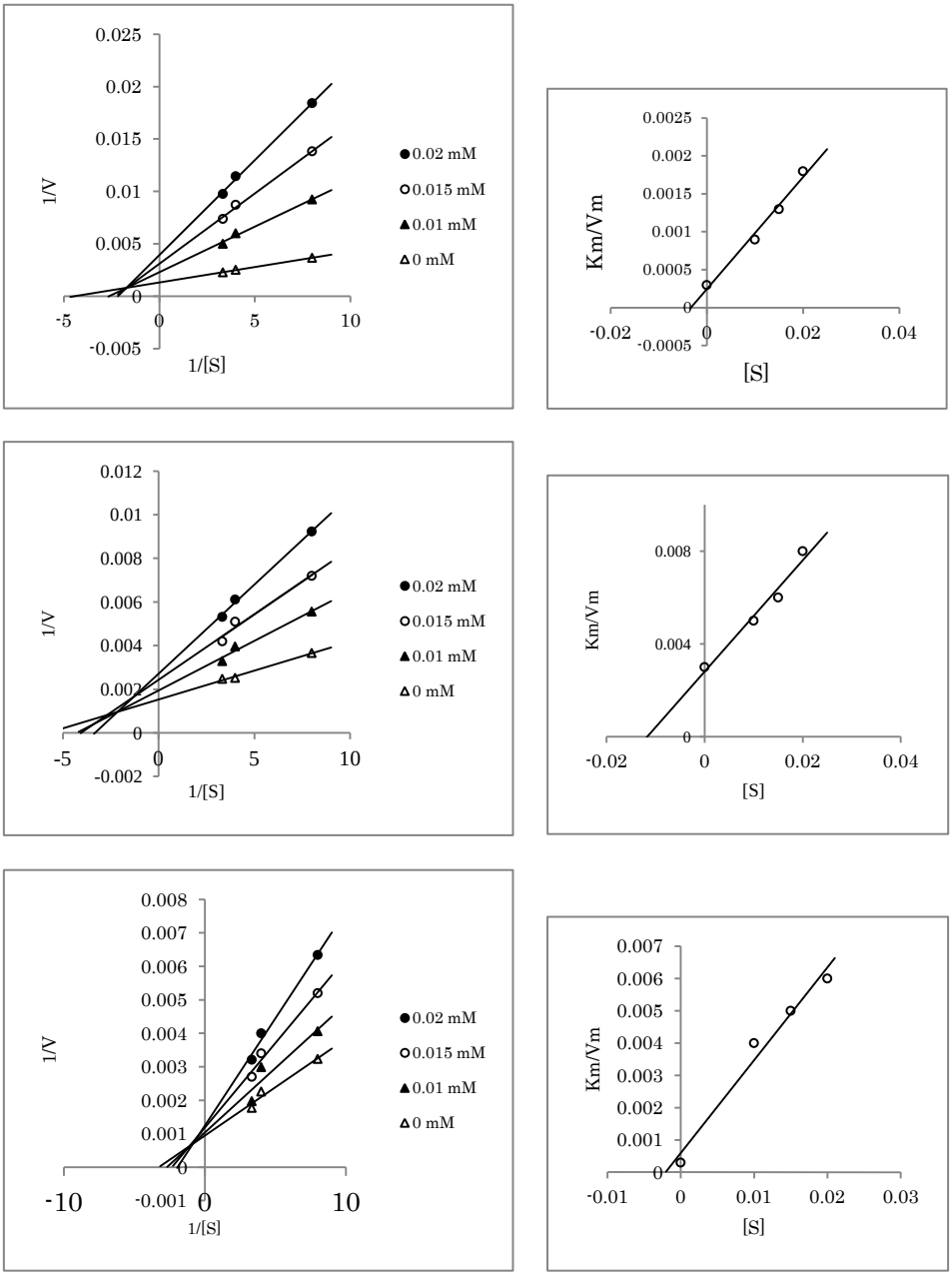
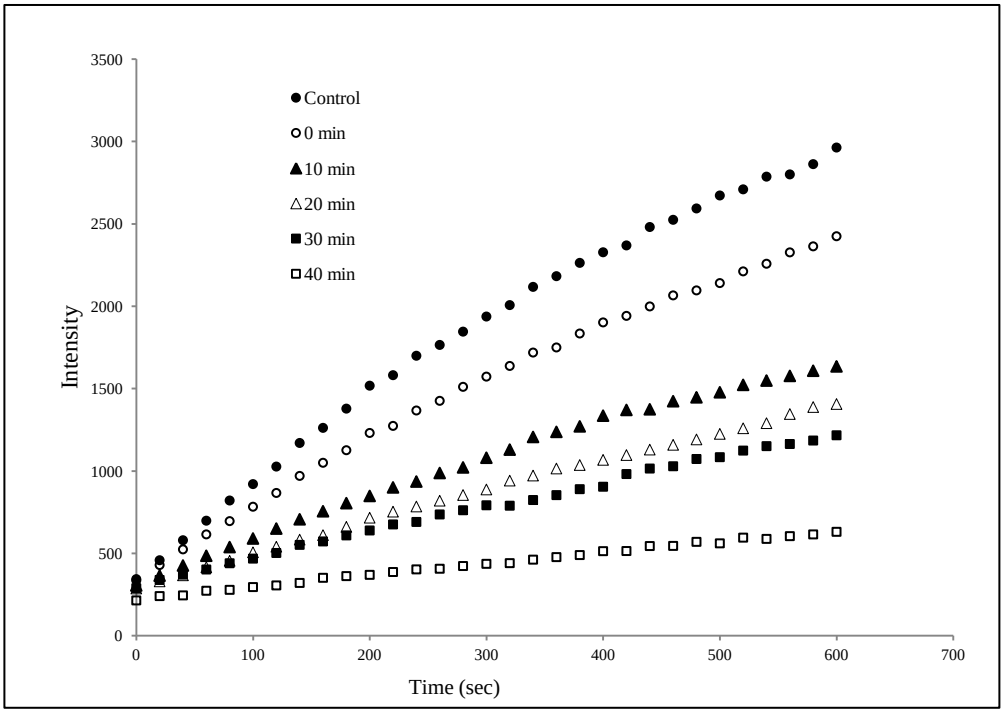
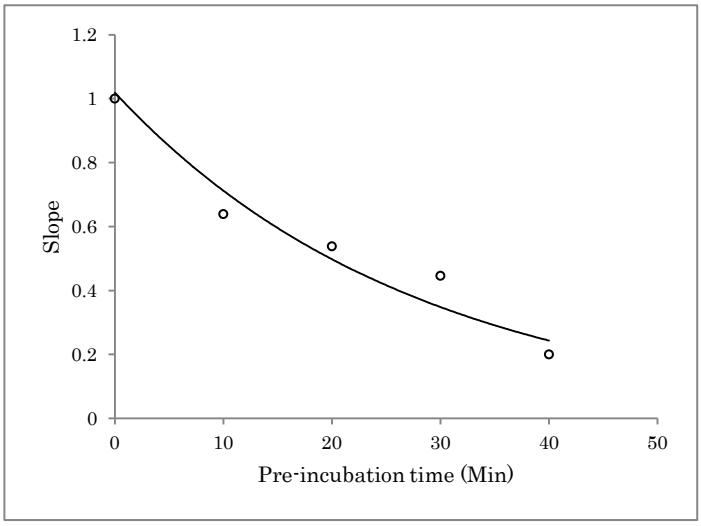


Fig. 4.3. Effect of DK (A), DDK (B) and labdadiene (C) on neuraminidase inhibition. Left: Lineweaver-Burk plot in the presence of compounds at concentrations of 0, 10, 15 and 20 µM. Neuraminidase inhibition was assayed as described in the text. Right: Secondary plot of Lineweaver-Burk plot. The slopes were plotted against respective compound concentrations. The intercept on the x-axis gives an estimate of Ki.

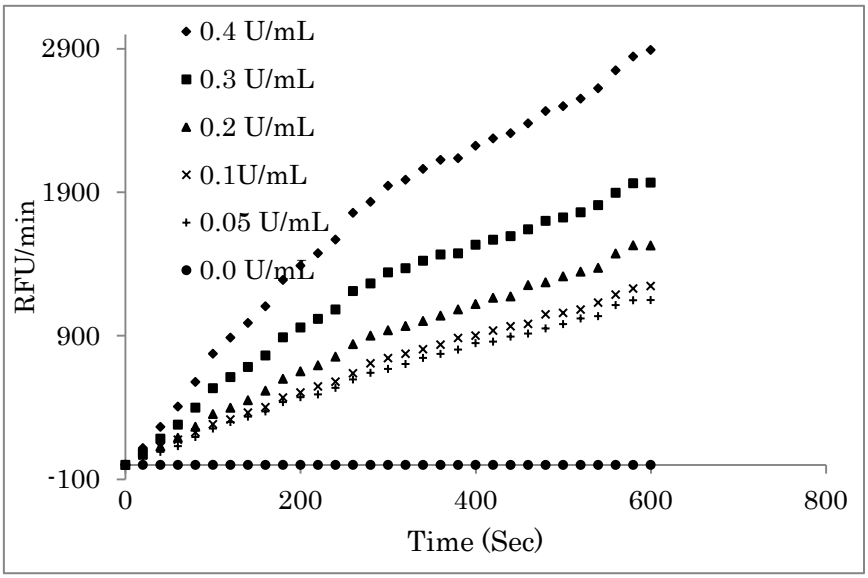


A

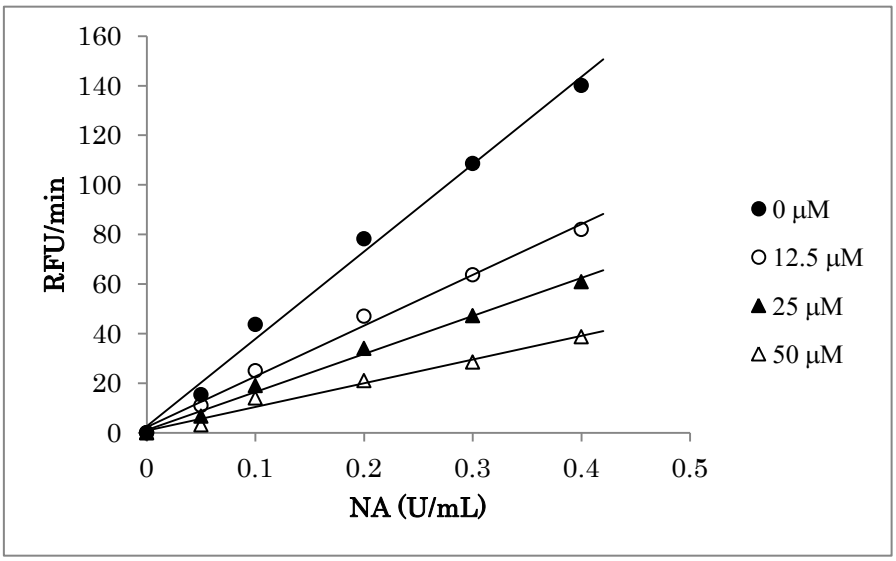


B

Figure 4.4. Effect of preincubation time on hydrolysis of substrate by neuraminidase (A) Time-dependent inhibition of neuraminidase in the presence of 25 μM DDK. (B) The decrease in slopes of the lines of panel A as a function of time.



A



B

Figure 4.5. Effect of enzyme concentration on neuraminidase inhibition (A) A typical plot of residual activity of neuraminidase at various concentrations (0-0.4 U/mL) in the presence of DDK at 25 μM. (B) The hydrolytic activity of neuraminidase as a function of enzyme concentration at different concentrations of DDK.

CHAPTER 5

NEURAMINIDASE AND TYROSIANSE INHIBTIONS BY ESSENTIAL OILS OF ALPINA LEAF

5.1 SUMMARY

The increasing toll of human casualties due to influenza and atherosclerosis has intensified the search of novel phytochemicals against these diseases. In this regard, the essential oils (EO) of two varieties of alpinia (*Alpinia zerumbet*) leaves were investigated for their inhibitory activities against neuraminidase and atherosclerosis formation. The GC-MS analysis detected more compounds in the *tairin* variety with γ-terpinene, cineole, *p*-cymene, sabinene, and 4-cravamenthenol as the major compounds. The kinetics of NA inhibition by *tairin* variety revealed competitive inhibitions at low concentrations of EO, while it exhibited a slow time-dependent inhibition at low enzyme concentrations. 15-Lipoxygenase (15-LOX) and low density lipoprotein (LDL) cholesterol oxidation inhibition assays were performed to assess the anti-atherosclerotic activities of EO. The IC_{50} values for NA, 15-LOX, and LDL oxidation inhibitions were found to be 41.5 ± 2.7 μg/mL, 0.24 ± 0.04 mg/mL, and 0.13 ± 0.01 mg/mL for *tairin*, while for *shima* varieity, the values were 62.3 ± 3.1 μg/mL, 0.41 ± 0.02 and 0.2 ± 0.02 mg/mL, respectively. These results suggest that *tairin* variety has better activity than *shima* variety and that the former could be used as a source of bioactive compounds against NA and atherosclerosis development.

5.2 INTRODUCTION

The impingement of influenza on human health is undeniably escalating, its impact being more serious during the winter season (Moscona, 2005). Human cases of avian influenza and, more recently, the outbreak of the aggressive porcine A/H1N1 strain in 2009 have heightened awareness of the threat of the pandemic. The disease is associated with a RNA virus that contains hemagglutinin and neuraminidase (NA) as surface antigens. NA is essential for the movement of the virus to and from sites of infection in the respiratory tract (De Clerq, 2006; Von Itzstien, 2007). On the other hand, atherosclerosis is an inflammatory disease due to accumulation of cholesterol and triglycerides in blood plasma. The increase in low-density lipoprotein (LDL) content is one of the principal risk factors for the development of atherosclerosis (Ross, 1999). The oxidation of LDL has been related to the enzyme 15-lipoxygenase (15-LOX) (Cornicelli and Trivedi, 1999; Steinberg, 1999) and *in vivo* experiments in rabbits against atherosclerosis using 15-LOX inhibitor (Sendobry et al., 1997; Bocan et al., 1998) also indicated the implication of 15-LOX in the development of atherosclerosis.

Alpinia [*Alpinia zerumbet* (family Zingiberaceae)] is a perennial ginger growing widely in the subtropics and tropics. It is used in folk medicine for its anti-inflammatory, bacteriostatic, and fungistatic properties (Zhogbi et al., 1999). The aqueous extract of its

leaves has demonstrated hypotensive activity (Laranja et al., 1991) mainly due to flavonoids and kava pyrones (Mpalantinos et al., 1998). In Okinawa, two varieties of alpinia are widely found: *tairin* and *shima*. *Tairin* [*A. zerumbet* (Pers.) B. L. Burtt & R. M. Sm. var. *excelsa* Funak & T. Y. Ito] has high plants with long stem while *shima* [*A. zerumbet* (Pers.) B. L. Burtt & R. M. Sm.] is short and has bushy structures. Both kinds of leaves are used to prepare a traditional food, *mu-chi*, and there is a common folklore that it prevents the common cold.

Our laboratory has reported antioxidant and antimicrobial activities (Elzaawely et 2007a, 2007b), and advanced glycation end product inhibitions (Chompoo et al., 2011) by different parts of alpinia. In the previous chapter, we showed the NA inhibitory activities of the leaf and rhizomes of alpinia and isolated active compounds. In this chapter, we report the NA inhibitions by the essential oil (EO) from the leaves of alpinia. The bioactivity of leaf EO of alpinia as a relaxant and having antispasmodic actions have been reported (Bezzera et al., 2000). However, this is the first study on the NA inhibition and atherosclerotic prevention by EO of alpinia leaves. We further discussed the kinetics of enzyme inhibition and also studied the anti-atherosclerotic properties of the EO by examining the inhibition of 15-LOX and LDL oxidation.

5.3 MATERIALS AND METHODS

5.3.1 Extraction of EO from alpinia leaf

The EO was obtained from 500 g of fresh alpinia leaf by steam distillation for 4 h. The distillate was extracted with diethyl ether and the solvent was carefully removed under vacuum at 35 ^{o}C. The obtained EO was dissolved in methanol and kept under refrigeration until use.

5.3.2 Neuraminidase inhibition assay

The enzyme assay was performed as described in sections **3.3.9** and **4.3.3.**

Anti-atherosclerotic activities

5.3.2.1 15-LOX inhibition assay

Enzyme inhibition was determined as described in section **3.3.6**.

5.3.2.2 LDL cholesterol oxidation assay

The oxidation of LDL was investigated as described by Rattan and Arad (1998). $CuSO_4$-induced oxidized-LDL generation were performed using 100 μL of 220 μg/mL LDL incubated at 37 ^{o}C in dark with 10 μL of 55 μM $CuSO_4$ and 10 μL of EO or quercetin for 24 h. The reaction was stopped by adding 50 μL of 1 M EDTA and placing the sample at -20 ^{o}C for TBA reactive substance (TBARS) assay. The generation of malonyldialdehye (MDA) equivalents during LDL oxidation was estimated by the TBARS assay using the method described elsewhere (Steinbrecher et

al., 1984). LDL oxidation was carried out as described above. After oxidation, LDL was mixed with 1.5 mL of 0.67% TBA and 1.5 mL of 20% TCA. After placing samples in boiling water (98 ^{o}C) for 30 min, the reaction product was kept for 30 min at 25 ^{o}C and centrifuged for 15 min at 4 ^{o}C. The supernatants were read on a spectrophotometer at 532 nm, using the blank containing 220 μg/mL LDL only. The yields of MDA were used as a standard and the results were expressed as nanomoles of MDA equivalents.

5.3.3 GC-MS analysis

The compounds of EO were identified using DB-5MS fused silica capillary column (30 m x 0.25 mm i.d., 0.25 μm; Agilent Technologies, J&W Scientific Products, Folsom, CA, USA). The carrier gas was helium and the GC oven temperature program was as follows: 80 ^{o}C hold for 1 min, raised at 10 ^{o}C/min to 220 ^{o}C, followed by raised to 330 ^{o}C at 20 ^{o}C/min and hold for 6 min. The injector and detector temperatures were set at 250 ^{o}C and 280 ^{o}C, respectively and the injection volume was 1.0 μL in the splitless mode. Mass spectra were scanned form *m/z* 50-600 amu and the electron impact ionization energy was 70 eV. The EO components were identified by comparing their retention times and mass fragmentation pattern with those of MS library and our previous reports (Elzawelly et al., 2007; 2007a, 2007b). Quantitative determinations of EO components were done based on the peak area measurements.

5.3.4 Statistical analysis

IC_{50} values were expressed as mean ± standard error by plotting the curve with percentage of inhibition versus concentration of the individual experiments measured (*n* = 3). Statistical analysis was performed by one-way ANOVA and upon significant difference, means were separated using Tukey's HSD range test at $P = 0.01$. All statistical analyses were performed using SPSS version 16.0 for Windows.

5.4 RESULTS AND DISCUSSION

5.4.1 Chemical composition of the EO

The GC-MS analyses identified 39 different compounds in *tairin* variety while only 14 compounds could be identified in *shima* variety. The identified compounds with their retention time and amount in percentage for two different varieties are listed in **Table 5.1**. All the compounds are arranged in the order of their retention times. The EO was a complex mixture mainly consisting of monoterpenes and sesquiterpenes. The major compounds in the EO were γ-terpinene (14.59%), cineole (13.82%), *p*-cymene (13.50%), sabinene (12.51%), and *p*-cravamenthenol (11.92%) for *tairin* variety. Other compounds with significant presence were terpinolene (4.19%), α-thugene (4.12%), caryophyllene oxide (3.02%), caryophyllene (2.4%), α-pinene (2.02%), and α-terpineol (1.28%). In the case of *shima* variety, cineole (37.8 %) was the major compound followed by

β-linalool (17.12%). The other major compounds identified were methyl cinnamate (6.34%), benzylacetone (4.21%), and α-terpineol (3.36%). The amount and number of compounds varied greatly in two different varieties with only five compounds identified in both varieties. The chemical structures of major compounds identified in EO of two varieties of alpinia leaf are shown in **Fig. 5.1A**.

5.4.2 Neuraminidase inhibitory activities of EO

With the ever present threat of a pandemic derived from influenza virus and the emergence of resistant strain to synthetic drugs, the importance of searching for novel compounds from plant-based source intensifies. In this regard, we investigated the NA inhibitory activities of EO of two varieties of alpinia leaves. The results showed a dose-dependent inhibition of NA with a sharp increase at the lower concentrations (**Fig. 5.1B**). The IC_{50} value of NA inhibition by *tairin* was found to be 41.5 ± 2.7 μg/mL, while that of *shima* variety was 62.3 ± 3.1 μg/mL (**Table 5.2**). In chapter 4 we reported that alpinia leaves and rhizomes inhibited NA with IC_{50} of 43 and 57 μg/mL, respectively. In this chapter, the activity of *tairin* was similar to the leaves while it had better results than the rhizomal extracts, however *shima* EO showed a lesser activity than our previous study.

We conducted kinetic studies of enzyme inhibition using *tairin* EO (**Fig. 5.2A**). At

EO concentrations less than its IC_{50} values (30 and 40 μg/mL), the enzyme was competitively inhibited with Km value of 0.04 min^{-1}, while at higher concentrations (50 and 60 μg/mL), EO had a mixed type of inhibition against NA. This suggests that at lower concentrations, EO binds with the free enzyme or the enzyme-substrate complex at its active site, however, at higher concentrations; it binds to a site different from the active site where the substrate binds. The estimated K_i value of 56.25 μg/mL was obtained from the secondary plot of Lineweaver-Burk plot (**Fig. 5.2B**).

We further investigated the inhibitory mechanism of *tairin* EO at its IC_{50} concentration. We began with exploring the effect of pre-incubation time on the inhibition of the hydrolysis of neuramic acid. The results did not indicate any specific relationship between the pre-incubation time and enzyme activity (**Fig. 5.3A**). Furthermore, when the slopes of the lines were plotted against the time, it was found that there is a decrease in the slope till a pre-incubation time of 20 min, making it a slow, time-dependent inhibitor (dotted line, **Fig. 5.3B**). However, when the time of pre-incubation was increased, it was found that there is a linear rise in the slope indicating that the residual activity increased with pre-incubation time (dashed lines, **Fig. 5.3B**). These results suggest that for a short pre-incubation time, it follows kinetics of a slow inhibitor, while at prolonged incubation; the EO behaves in a different manner.

The reasons for this behavior are yet to be understood, however, we assume that it may be due to the presence of a mixture of components in EO. On carrying out the statistical analysis, it was found that the inhibitory activities at pre-incubation times of 10 and 20 min are significantly higher than that of the control, thereby indicating the optimal pre-incubation times. Furthermore, when the effect of enzyme concentration on NA activity was probed, it was found that with increasing enzyme concentration, the residual activity of the enzyme also increased (**Fig. 5.4**), which is a natural phenomenon with most of the enzyme inhibitions, and agrees with our previous results in chapter 4.

There are several reports on inhibitory activities of EO of botanical families against the influenza virus (Zhai et al., 2005; Hayashi et al., 2007). In one study, compounds like terpinen-4-ol, terpinolene and α-terpineol have shown the inhibitory effect of influenza A/PR/8 virus at doses below the cytotoxic dose (Garozzo et al., 2009). However, in a recent report, it was found that although tea tree oil has anti-influenza virus activity, it did not have anti-NA activity (Garozzo et al., 2011). Therefore, while we have found NA inhibitory activities of EO of alpinia, its effectiveness against the influenza virus is yet to be confirmed.

5.4.3 Anti-atherosclerotic activities of EO

It is widely accepted that the oxidative modification of plasma lipoproteins,

particularly LDL, plays an important role in the initiation of atherosclerosis. The process of atherosclerosis begins with the accumulation of lipids within the artery wall (Shepherd et al., 1995). 15-LOX is a lipid-oxidizing enzyme that is considered to contribute to the formation of oxidized lipids in the atherosclerotic lesions (Bocan et al., 1998). Furthermore, when the levels of plasma triglycerides are low, high density lipoprotein (HDL) cholesterol levels tend to be high. HDL opposes atherosclerosis directly, by removing cholesterol from foam cells by inhibiting the oxidation of LDL (Barter, 2005). Beside LDL, the 15-LOX form hydroperoxy derivatives of linoleic acid and arachidonic acid and is induced in atherosclerotic plaques (Harats et al., 2000). Hence, if LDL cholesterol oxidation and/or 15-LOX are inhibited, the formation of atherosclerosis may be prevented.

Our results showed that EO of *tairin* had significantly better activities than *shima* EO. In both EOs, there was linear inhibition of LDL cholesterol oxidation (**Fig. 5.5A**) while 15-LOX was inhibited logarithmically (**Fig. 5.5B**). In both cases, increase in inhibitory activity was seen in a dose-dependent manner. The IC_{50} values for the 15-LOX and LDL oxidation inhibitions for *tairin* EO were found to be 0.24 ± 0.04 mg/mL and 0.13 ± 0.01 mg/mL, respectively, while for *shima* EO the values were 0.41 ± 0.02 mg/mL and 0.20 ± 0.02 mg/mL, respectively (**Table 5.2**). These results were

also similar to NA inhibitions results where *tairin* EO was found superior to *shima* EO.

A number of reports on the inhibitory activities of terpenoids against 15-LOX (Amagata et al., 2003) and LDL oxidation (Laranjinha et al., 1995; Dugas et al., 1998) are available. Our study also identified several terpenoidal compounds in EO of alpinia and we assume that these compounds might have a role in inhibiting the formation of atherosclerosis. However, the differences in the activities between the two varieties seem to be due to a large variation in the number and amount of the compounds present in the EO of two different varieties.

5.5 CONCLUSION

Essential oils have always been a subject of great interests in anti-oxidant and antimicrobial activities. However, in this study we identified novel properties of alpinia leaf EO against NA and atherosclerosis. Our results indicate that EO of alpinia leaf could be used as sources in inhibiting these diseases. We believe that further *in vivo* researches are necessary in order to use the EO of alpinia as lead materials in the drug designing against NA and atherosclerosis.

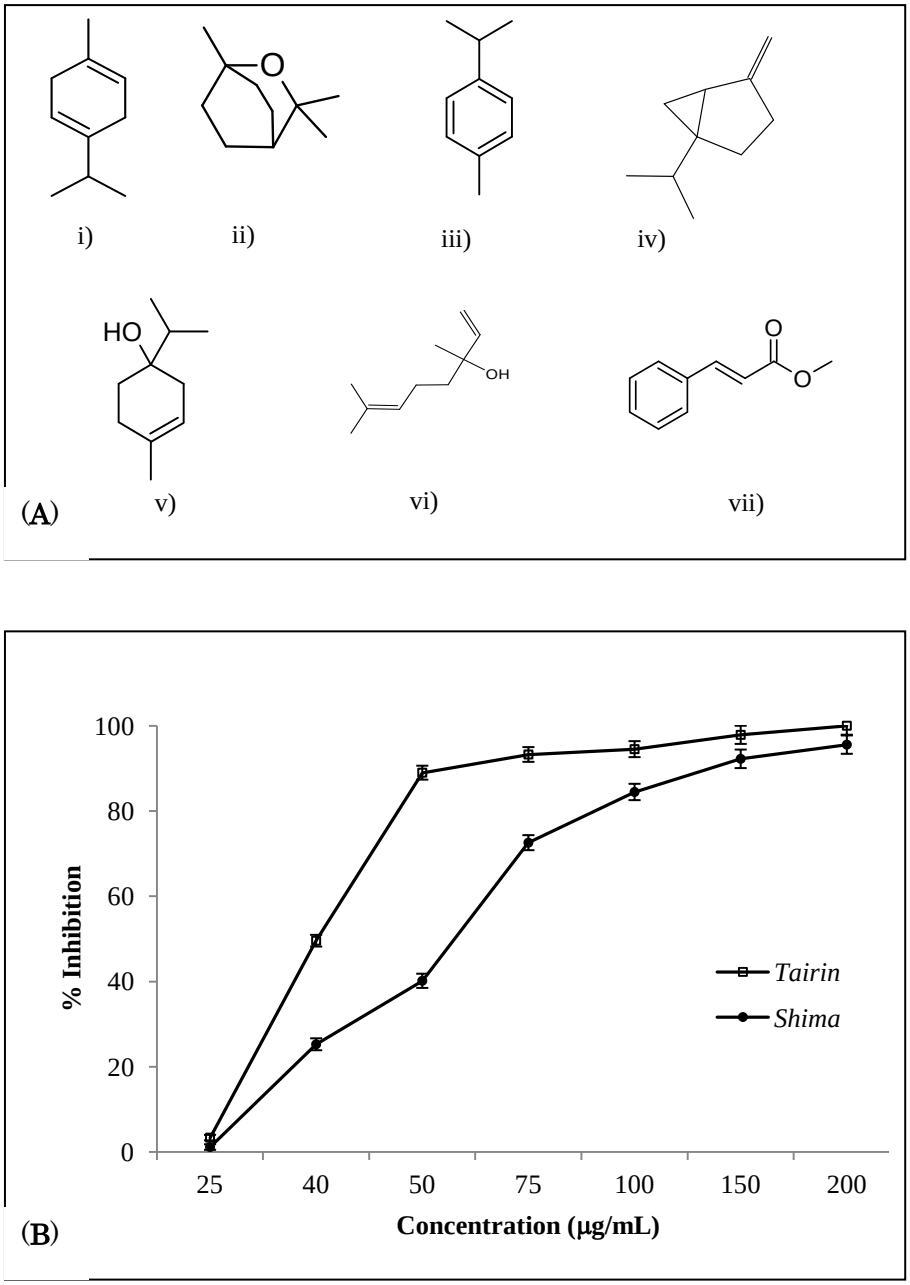
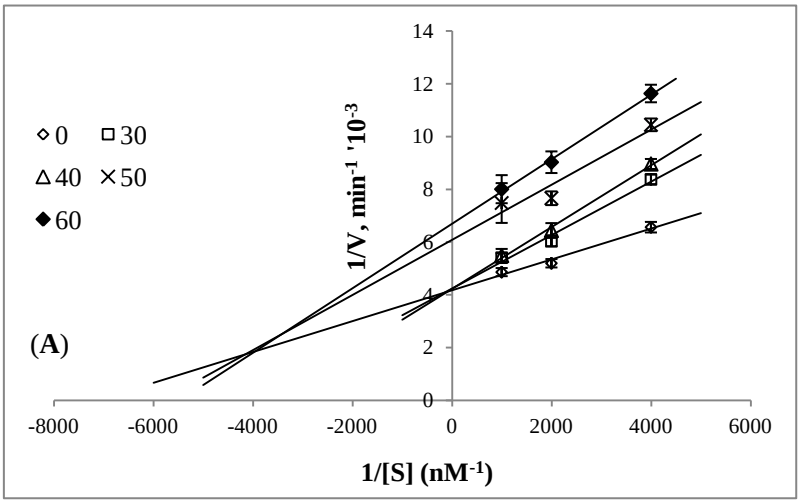


Fig. 5.1. (A) Chemical structures of major compounds present in the EO of *tairin* and *shima* leaf i) γ-terpinene, ii) cineole, iii) *p*-cymene, iv) sabinene, v) 4-cravamenthenol, vi) β-linalool, and vii) methyl cinnamate. (B). Concentration dependent inhibition of NA activity by EO of *tairin* and *shima* variety.



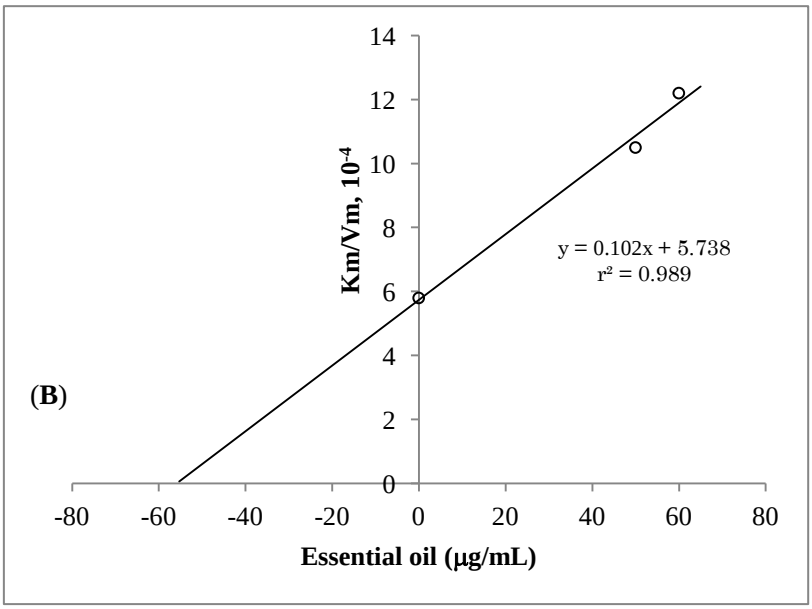


Fig. 5.2. Effect of *tairin* EO on neuraminidase inhibition. (A) Lineweaver-Burk plot in the presence of EO at concentrations of 0, 30, 40, 50, and 60 µg/mL. (B) Secondary plot of Lineweaver-Burk plot. The slopes were plotted against the respective concentrations.

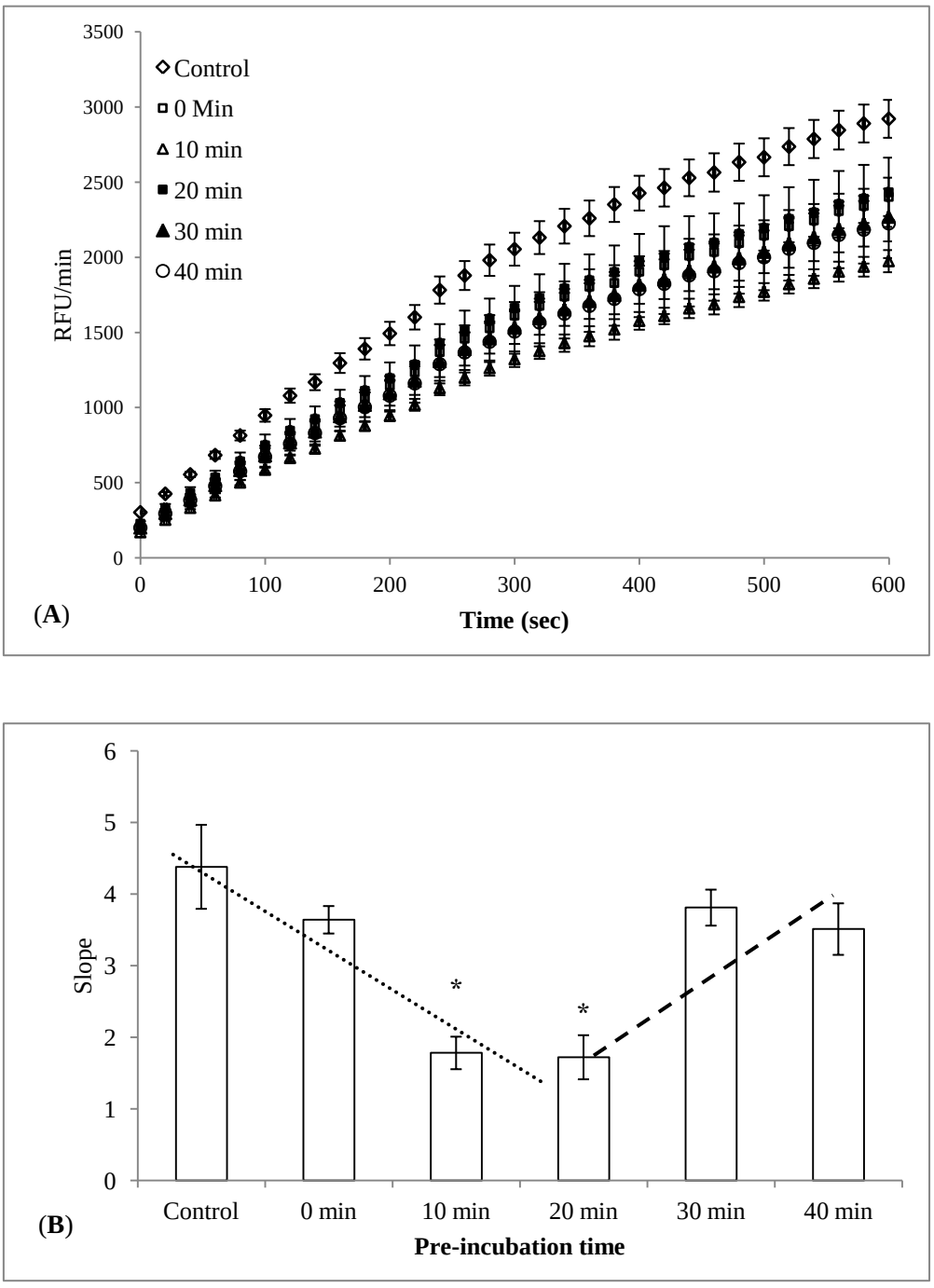


Fig. 5.3. Effect of pre-incubation time on hydrolysis of substrate by NA: (A) time-dependent inhibiton of NA in the presence of 40 μg/mL *tairin* EO; (B) slopes of the lines of panel A at different incubation time. (See text for description).

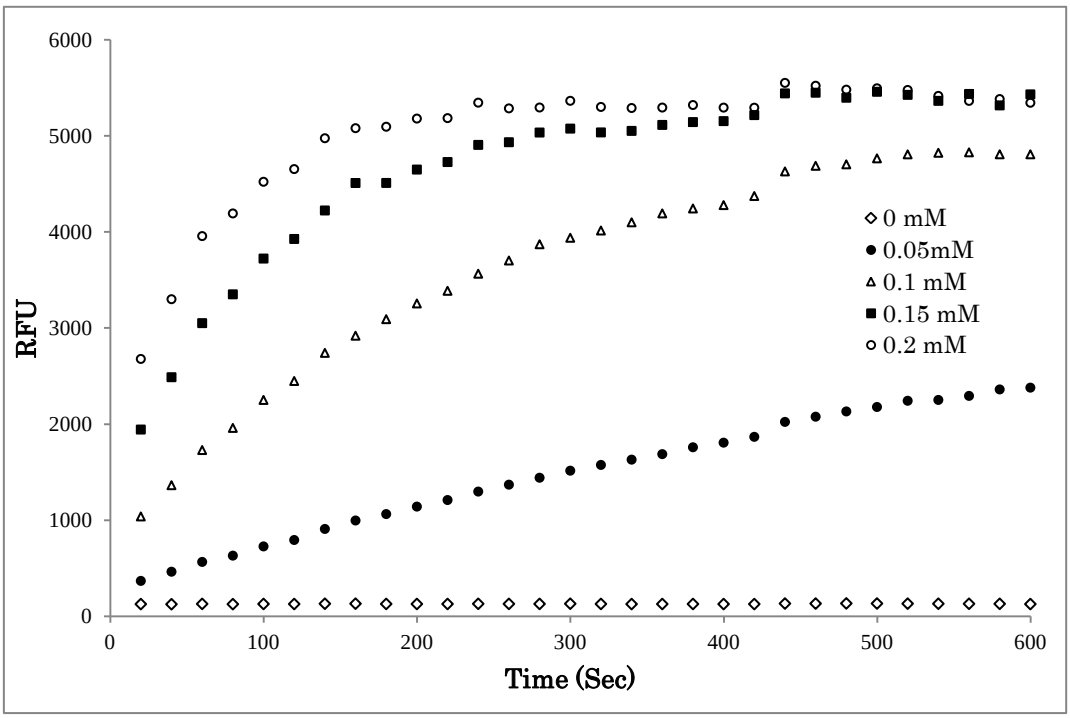
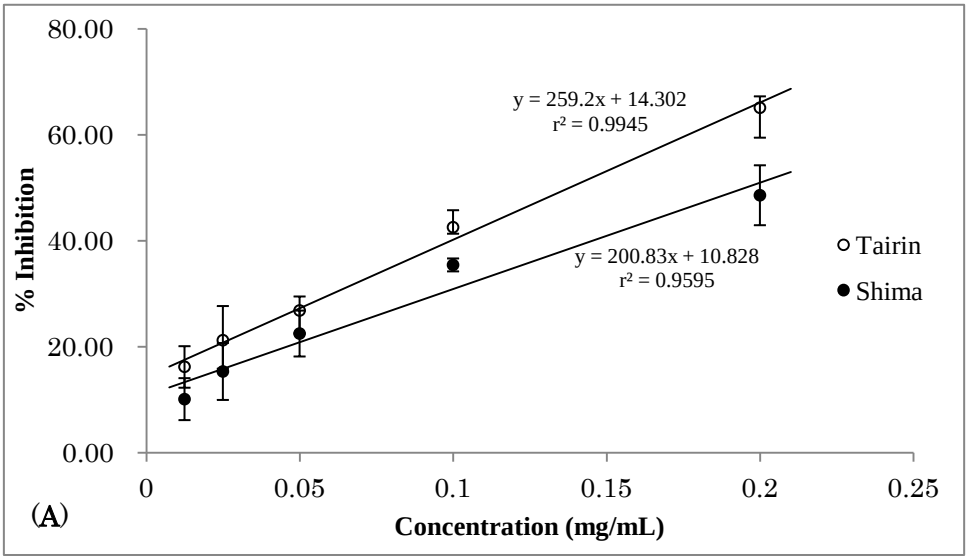


Fig. 5.4. Effect of enzyme concentration on NA inhibition. A typical plot of residual activity of NA at various enzyme concentrations (0-0.2 U/ml) in the presence of 40 µg/mL *tairin* EO.



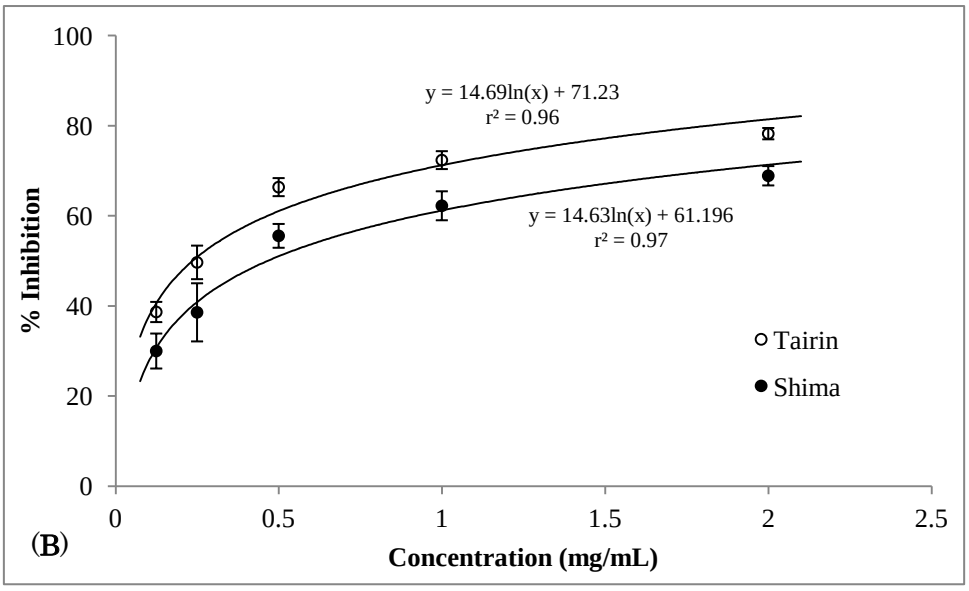


Fig. 5.5. Concentration dependent inhibition of LDL (A) and 15-LOX (B) enzymes by EO of *tairin* and *shima* varieties.

Table 5.1.

The main chemical components of the essential oil from two varieties of alpinia leaf.

S.No.	Compound	Retention time (min)	Peak area (%)	
			Tairin	*Shima*
1	2,3,4-Trimethyl-1-pentanol	3.770	0.01	-
2	α-Thugene	5.179	4.12	-
3	α-Pinene	5.322	2.02	-
4	Norborndadiene	5.607	0.08	-
5	Camphene	5.718	0.22	-
6	Benzaldehyde	6.205	-	1.59
7	Sabinene	6.517	12.51	-
8	β-Pinene	6.582	3.15	-
9	Myrcene	6.991	0.69	-
10	α-Phellandrene	7.374	0.31	-
11	Terpinolene	7.725	4.19	-
12	*p*-Cymene	8.032	13.50	-
13	Cineole	8.256	13.82	37.80
14	γ-Terpinene	9.080	14.59	-

15	cis-β-Terpineol	9.340	0.55	-
16	β-Linalool	10.316	0.50	17.12
17	2,5-Norbornadiene	10.650	-	0.71
18	cis-*p*-Menth-2-en-1-ol	10.941	0.59	-
19	4-Cravamenthenol	12.517	11.92	-
20	α-Terpineol	13.107	1.28	3.36
21	*trans*-*p*-Menth-1-en-3-ol	13.483	0.42	-
22	Benzylacetone	14.369	0.06	4.21
23	Piperitone	14.693	0.03	0.1
24	Bornyl acetate	15.611	0.37	-
25	Cumic alcohol	15.829	0.18	-
26	Carvacrol	16.089	0.13	-
27	2-*tert*-Butylphenyl pivalate	16.175	-	1.23
28	Ethyl-3-hydroxy-3-methylbutanote	16.242	-	0.09
29	Isopiperitenon	16.358	-	0.18
30	Thymol	16.668	0.05	-
31	2-Hydroxy-3,5-dimethylcyclopent-2-en-1-one	16.708	-	0.13
32	*p*-Menth-1,4-dien-7-ol	16.859	0.07	-

33	Methyl cinnamate	18.417	-	6.34
34	Caryophyllene	19.214	2.40	-
35	2,6-Diethylnitrosobenzene	19.608	-	0.69
36	α-*trans*-Bergamoene	19.646	0.09	-
37	Aristole-9-ene	19.811	0.15	-
38	α-Humulene	20.124	0.38	-
39	Butylcyclopentane	20.925	-	0.63
40	α-Selinene	20.986	0.15	-
41	γ-Cadinene	21.635	0.42	-
42	α-Bulnesene	21.866	0.25	-
43	Nerolidol	22.972	0.38	-
44	Caryophyllene oxide	23.322	3.02	-
45	Carotol	23.792	0.13	-
46	Humulene epoxide	23.949	0.29	-
47	β-Eudesmol	25.039	0.65	-
48	*iso*-Aromdendrene epoxide	25.415	0.21	-
49	α-Zingiberene	26.320	0.02	-
	Total	**-**	**93.9**	**74.18**

Table 5.2.

IC_{50} values of different inhibitory activities of EO.

Sample	*Tairin*	*Shima*
Neuraminidase (μg/mL)	41.5 ± 2.7	62.3 ± 3.1
15-LOX (mg/mL)	0.24 ± 0.04	0.41 ± 0.02
LDL oxidation (mg/mL)	0.13 ± 0.01	0.20 ± 0.02

CHAPTER 6

BIOACTIVITY OF MIMOSINE ISOLATED FROM *LEUCAENA LUECOCEPHALA* AND SYNTHESIS OF MIMOSINE TETRAPEPTIDES TO DEVELOP NEURAMINIDASE AND TYROSINASE INHIBITORS

6.1 SUMMARY

Neuraminidase is a rational target for influenza inhibition and search for the neuraminidase inhibitors has been intensified. Mimosine, a non-protein amino acid, was for the first time identified as a neuraminidase inhibitor with IC_{50} of 9.8 ± 0.2 μM. It was found that mimosine had slow, time-dependent competitive inhibition against the neuraminidase. Furthermore, a small library of mimosine tetrapeptides (M-A_1-A_2-A_3) was synthesized by solid-phase synthesis and was assayed to evaluate their neuraminidase and tyrosinase inhibitory properties. Most of the tetrapeptides showed better activities than mimosine. Mimosine-FFY was the best compound, and it exhibited 50% neuraminidase inhibition at a low micromolar range of 1.8 ± 0.2 μM, while for tyrosinase inhibition, it had IC_{50} of 18.3 ± 0.5 μM. The kinetic studies showed that all of the synthesized peptides inhibited neuraminidase noncompetitively with *Ki* values ranging from 1.9 - 7.2 μM. These results suggest that mimosine could be used as a source of bioactive compounds and may have possibilities in the design of drugs as neuraminidase and tyrosinase inhibitors.

6.2 INTRODUCTION

Neuraminidase, an enzyme present in the influenza virus, is involved in the release of progeny virus from infected cells, by cleaving sugars that bind the mature viral particles (Moscona, 2005). Specifically, neuraminidase cleaves the α-ketosidic bond that links a terminal neuraminic acid residue to the adjacent oligosaccharide moiety. Neuraminidase is therefore essential for the movement of the virus at the site of infection in the respiratory tract (De Clercq, 2006; Von itzstein, 2007). On the other hand, tyrosinase is a copper-containing enzyme that catalyzes the ortho-hydroxylation of monophenols to catechols and their subsequent oxidation to ortho-quinones. In mammals, tyrosinase is not only responsible for browning of hair and skin pigmentation (Marmol & Beermann, 1996), but also for skin anomalies such as hypo or hyper pigmentation (Piamphongsant, 1998). Furthermore, tyrosinase may play a role in cancer and neurodegenerative diseases, such as Parkinson's disease (Cavalieri et al., 2002). As both enzymes are quite a significant target in the field of medicine, the development of neuraminidase and/or tyrosinase inhibitors have received much attention.

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 pyridine ring. It is found in several tropical and sub-tropical plants of genera *Leucaena*

and *Mimosa*. Mimosine has been confirmed to be responsible for the allelopathy of the plant (Tawata et al., 1984; Tawata, 2004; Xuan et al., 2006). The therapeutic roles of mimosine include antiproliferative and antifibrotic (Ju et al., 1998), anti-tumor (DeWys & Hall, 1973), and anti-viral (Dai et al., 1994; Andrus et al., 1998). Besides the anti-tyrosinase activity of mimosine has been studied (Bubacco et al., 2000; Khan et al., 2005).

Peptides have been found to inhibit neuraminidase (Mian et al., 1979) and tyrosinase (Kim et al., 2004). In chapter 4, we isolated three compounds from *Alpinia zerumbet* rhizomes and studied the probable mechanism of neuraminidase inhibition, while in chapter 5 we showed the neuraminidase inhibitions by essential oils of two varieties of alpinia leaf. In this chapter, the neuraminidase inhibitory activity of mimosine was identified and hence mimosine peptides were synthesized to assess their neuraminidase and tyrosinase inhibitory activities. We also report the inhibition kinetics of mimosine and tetrapeptides against the neuraminidase. This is the first report on the synthesis of peptides from mimosine and investigating their therapeutic potentials.

6.3 MATERIALS AND METHODS

6.3.1 Chemicals.

The Fmoc amino acids were bought from Hipep Laboratories (Kyoto, Japan), while

Fmoc-OSu [N-(9-fluorenylmethoxycarbonyloxy)succinimide] was obtained from Merck, Germany. N,N'-Diisopropylethylamine (DIEA), 1-hydroxy-1H-benzotriazole (HOBt), and N,N'-diisopropylcarbodiimide (DIC) were bought from Wako, Japan, while HBTU was acquired from Nova Biochem, China. All other reagents used were of analytical grade and were obtained from Wako, Japan.

6.3.2 Isolation of mimosine from *Leucaena*.

One Kg of *Leucaena* leaves was boiled in 5 L water for 10 min. The cooled extract was filtered and the filtrate was added to the cation-exchange resin (2 Kg). The extract-resin mixture was shaken overnight at room temperature in a shaker (Shaking Baths SB-20, AS ONE, Osaka, Japan). The cation-exchange resin was then washed with 80% ethanol (5L) and further with distilled water several times to remove impurities other than mimosine. Finally, the resin was eluted with 4N NH_4OH (6L) to obtain mimosine. The eluate was vacuum concentrated, and the crude mimosine was further dissolved in NaOH solution. The pH of the solution was then adjusted to 4.5-5.0 by 6N HCl and kept overnight in the refrigerator for crystallization. The crystals were filtered and further dried in vacuum to obtain 5.387 g (0.54%) mimosine with a purity of more than 95%. Mimosine was identified by LC-MS (ESI-) *m/z* $[M - H]^+$; 197.2; $[M + H]^+$ 199.1.

6.3.3 Preparation of Fmoc-mimosine.

To prepare Fmoc-mimosine, 5 g of mimosine and 5.5 g of Na_2CO_3 were dissolved in 75 mL distilled water containing 75 mL dioxane. To this solution, 12.5 g of Fmoc-OSu was added and then it was incubated overnight at room temperature. Following this, 300 mL Na_2CO_3 solution (0.1M) was added and the mixture was further stirred in a magnetic stirrer (300 rpm) for 5 h at 25 ^{o}C. The obtained solution (450 mL) was filtered and washed with ethyl acetate (150 mL) to remove unreacted Fmoc-OSu and the byproducts, 9-fluorenemethanol and 9-methylenefluorene. The pH of the water fraction was lowered to 4 using 6N HCl in an ice-bath and the crystallized Fmoc-mimosine was filtered and dried under vacuum (7.108 g).

6.3.4 Solid-phase synthesis of mimosine tetrapeptides.

A solution mixture of Fmoc-amino acid (Fmoc-AA_1-OH) (1.6 mmol) in 5 mL dimethylacetoamide was prepared by adding HOBt (1.6 mmol) and DIC (1.6 mmol) and stirring for 10 min. This mixture was added to a swollen Wang resin (1 g) in DMF and the reaction mixtures were stirred for 17 h (**Fig. 6.1A**). The resins were filtered, washed with dichloromethane, isopropyl alcohol and methanol and then finally dried under vacuum. After deprotection of Fmoc with 25% piperidine (reagent a) in DMF for 30 min, the next amino acids (Fmoc-AA_2-OH) were coupled to the resin mixture solution

(Fmoc-amino acid: HOBt : HBTU : DIEA = 4 : 3 : 3.6 : 8) (reagent b). The reaction mixture was further stirred for 1 h (**Fig. 6.1B**). Coupling completeness was determined by ninhydrin test. The uncoupled Fmoc-AA_2-OH was capped with the acetyl group using a mixture of HOBt : acetic acid : DIEA : DMF (0.8 : 19 : 9 : 400) (20 mL/g resin). Similarly, Fmoc-AA_3-OH was coupled with the dipeptides to form tripeptides. After final coupling with mimosine, the resin was agitated gently with 95% trifluoroacetic acid (TFA) (reagent k) for 1 h (**Fig. 6.1C**). The resin was filtered and washed with TFA and the collected filtrate was precipitated with ice-cold diethyl ether. The obtained precipitate was filtered out, washed with diethyl ether (3 x), and dried under vacuum to obtain the desired mimosine tetrapeptide. All the crude peptides obtained were white solid and their yields are given in **Table 6.1**. However, before carrying out the enzyme inhibition assays, further purification was achieved using HPLC and the major peaks were collected using a Cadenza CD-C18 (20 x 100 mm; 3 micron) column with 0.1% trifluoroacetic acid/CH_3CN (1.5/8.5) at a flow rate of 5 mL/min. The purified compounds were identified using LC-MS (ESI-) *m/z* $[M - H]^+$ 693.2, 670.1, 654.2, 603.2, 564.1, 545.1, 554.2, and 531.1 for M-FWY , M-FYY, M-FFY, M-WGY, M-FGY, M-VGY, M-QGY, M-HGY respectively.

6.3.5 Neuraminidase inhibitory assay.

The neuraminidase inhibition was done as described in sections **3.3.9** and **4.3.3**.

6.3.6 Tyrosinase inhibition.

The inhibitory effects of mimosine and peptides on tyrosinase enzyme were assayed as described previously (Tadtong et al., 2009). Briefly, samples were dissolved in methanol to make different concentrations in μM. The 96-well plate was set up in the following order: 120 μL of phosphate buffer (20 mM, pH 6.8), 20 μL of sample and 20 μL of mushroom tyrosinase (500 units/mL in 20 mM buffer). After incubation at 25 ^{o}C for 15 min, reaction was initiated by adding 20 μL of 0.85 mM L-tyrosine solution to each well. The enzyme activity was determined by measuring the absorbance at 470 nm using the microplate reader. The percentage of tyrosinase inhibition was calculated as:

%Tyrosinase inhibition=[(A-B)-(C-D)]/(A-B) x 100

where, A and B are the absorbance of control (methanol) and test samples with enzyme, while C, and D are absorbance of control and test samples without enzyme.

6.3.7 Statistical analysis.

All the experiments were conducted in triplicates and repeated twice. The data represents the mean ± the standard deviation (SD) of six results. The IC_{50} value was determined graphically as the concentration of each sample required to give 50% inhibition activity. For kinetic studies, all calculations were performed in Excel,

Microsoft Office 2007. For significance analysis, the data were analyzed by one-way ANOVA and the means were separated using Tukey's HSD range test at p = 0.01. All the statistical analyses were performed using SPSS version 16.0 for Windows Vista.

6.4 RESULTS AND DISCUSSION

6.4.1 Neuraminidase inhibitory activity by mimosine.

In order to search for the neuraminidase inhibitors from plant based source, we first identified neuraminidase inhibition by mimosine. The IC_{50} value of mimosine against the neuraminidase was 9.8 ± 0.2 μM and the kinetic studies revealed mimosine to inhibit the enzyme competitively (Km = 39.1 ± 1.2 min^{-1}) (**Table 6.1**). The IC_{50} value indicated that mimosine is a better neuraminidase inhibitor than our previously isolated compounds viz. 5,6-dehydrokawain, dihydro-5,6-dehydorkawain, and 8(17),12-labdadiene-15,16-dial which had 50% inhibitory activities at 25.5, 24.6, and 36.6 μM, respectively, as discussed in chapter 4. The kinetic studies revealed that mimosine inhibited neuraminidase competitively (**Fig. 6.2**), which is the same mode of inhibition against tyrosinase (Cabanes et al., 1987). We further investigated the inhibitory mechanisms of mimosine at its IC_{50} concentration. We explored the effect of preincubation time on the inhibition of the hydrolysis of neuraminic acid. Because the decrease in residual activity was observed with increasing preincuabtion time, mimosine

emerged as a slow-binding inhibitor at low concentrations (**Fig. 6.3A**). Furthermore, increasing preincubation time of mimosine also led to the decrease in the slope, thereby indicating a reduction in both initial and steady-state velocity (**Fig. 6.3A**). This result indicates that mimosine is more like the drug Tamiflu, which is also a slow and time-dependent inhibitor of neuraminidase. Moreover, when the effect of enzyme concentration was probed, it was found that the residual activity of neuraminidase increased with time at fixed substrate concentration (**Fig. 6.3B**).

6.4.2 Synthesis of mimosine tetrapeptides and their neuraminidase inhibitions.

It has been reported that kojic acid (Ko) tripeptides (Ko-FWY, Ko-FFY, Ko-FYY) have inhibited tyrosinase enzyme significantly (Kim et al., 2005). This made us think to develop mimosine tetrapeptides using several amino acid combinations in order to develop a potent neuraminidase inhibitor.

In order to synthesize mimosine tetrapeptides, we applied the solid phase synthesis using the Fmoc chemistry principles. We synthesized a series of mimosine peptides, using tyrosine (Y), tryptophan (W), phenylalanine (F), glycine (G), glutamine (Q), valine (V), and histidine (H). The first coupling was done with Y and the dipeptides formed were further coupled with the next amino acid to form tripeptides. However, in order to synthesize mimosine tetrapeptides, mimosine needs to be first converted to

Fmoc-mimosine which was achieved by attaching the Fmoc group of Fmoc-OSu to mimosine. To the Fmoc-mimosine, previously formed tripeptides were coupled to obtain the desired mimosine tetrapeptides.

The synthesized compounds had significantly better inhibitions than mimosine (**Table 6.1**). The IC_{50} values of tetrapeptides were in low micromolar ranges and M-FFY had 5 times the more potency than mimosine. The low IC_{50} and Ki values of M-FFY make it a potent compound against neuraminidase. Although mimosine had competitive inhibitions, mimosine tetrapeptides exhibited noncompetitive inhibitions (**Fig. 6.4A-H**). When the neuraminidase inhibitions by individual amino acids (30 μM) were probed, the activities identified were very poor (**Table 6.2**). Our data indicated that these peptides may be more effective than the parent amino acid in case of neuraminidase inhibitions.

6.4.3 Tyrosinase inhibition by mimosine and tetrapeptides.

The inhibition of tyrosinase enzyme by mimosine is reported (Cabanes et al., 1987) and we explored the tyrosinase inhibition by mimosine tetrapeptides. The inhibition of tyrosinase enzyme by mimosine and tetrapeptides are shown in **Table 6.1**. It was found that mimosine inhibited tyrosine activity with IC_{50} of 44.7 ± 0.4 μM. When the inhibitions by the synthesized peptides were assayed, all of them showed significantly

better inhibitory properties with IC_{50} values ranging from 5.6-36.7 μM (**Table 6.1**).

In our study, we found that the most of the tetrapeptides had significantly better activities than mimosine (**Table 6.1**). Furthermore, in both the assays, we found that M-FFY had the best activity among all the synthesized peptides. The results also showed that mimosine and the most of the tetrapeptides are better inhibitors against neuraminidase enzyme. These results suggest that peptides synthesized from mimosine may have applicability in search for neuraminidase and tyrosinase inhibitors.

6.4.4 Seasonal changes of mimosine contents in *Leucaena* leaves.

In order to identify the most suitable time to extract maximum mimosine from the *Leuceana* leaves, the variation of mimosine content in the leaf was investigated from April to October. It was found that the leaves had the highest amount of mimosine during the summer period of July to September, while as the temperature reduced, the amount of mimosine also decreased (**Fig. 6.5**). An independent study on the mimosine content of the *Leuceana* leaves also have shown the similar results (Nokihara et al., 2011). It seems that the temperature of environment may have an impact on mimosine content and that the higher temperatures favor more mimosine content. Mimosine has been associated with the allelopathy of the plant (Tawata et al., 2008) and therefore, it appears that the increase in temperature may have a role in increasing the mimosine

content. However, the exact role of mimosine in relation to heat stress is yet to be studied.

6.5 CONCLUSION

We showed that mimosine could inhibit neuraminidase and for the first time synthesized mimosine tetrapeptides to investigate their inhibitory properties against neuraminidase and tyrosinase enzymes. We also discussed the kinetics of neuraminidase inhibition by mimosine and the synthesized peptides. Our results indicate that mimosine tetrapeptides could be used in the designing novel drugs against the neuraminidase. Furthermore, we are also synthesizing mimosine di- and tri-peptides in order to develop neuraminidase and/or tyrosinase inhibitors. The results of this study certainly widen the use of solid phase peptide synthesis in order to develop potent neuraminidase inhibitors.

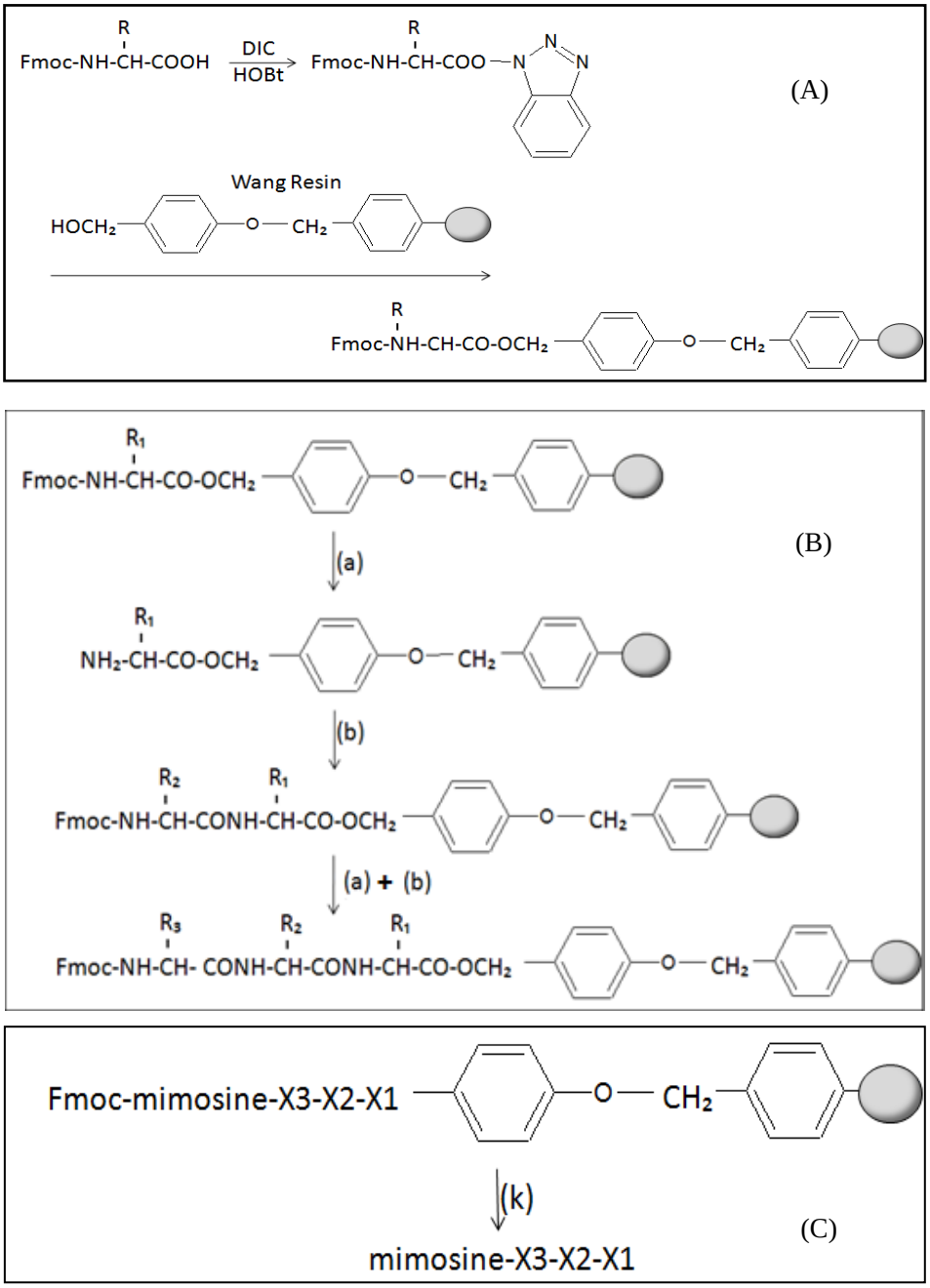


Fig. 6.1. Scheme of mimosine tetrapeptides synthesis. Attachment of Wang resin to Fmoc-amino acid (A), elongation of amino acid chain to form Fmoc-mimosine tetrapeptides (B), and removal of Fmoc-group to obtain desired mimosine tetrapeptides (C).

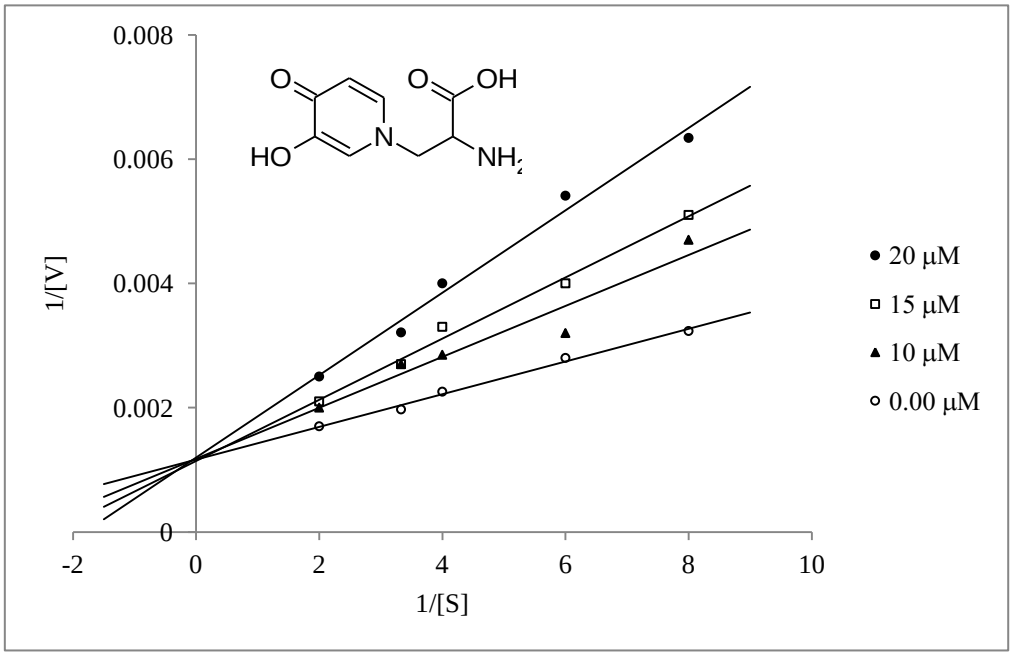
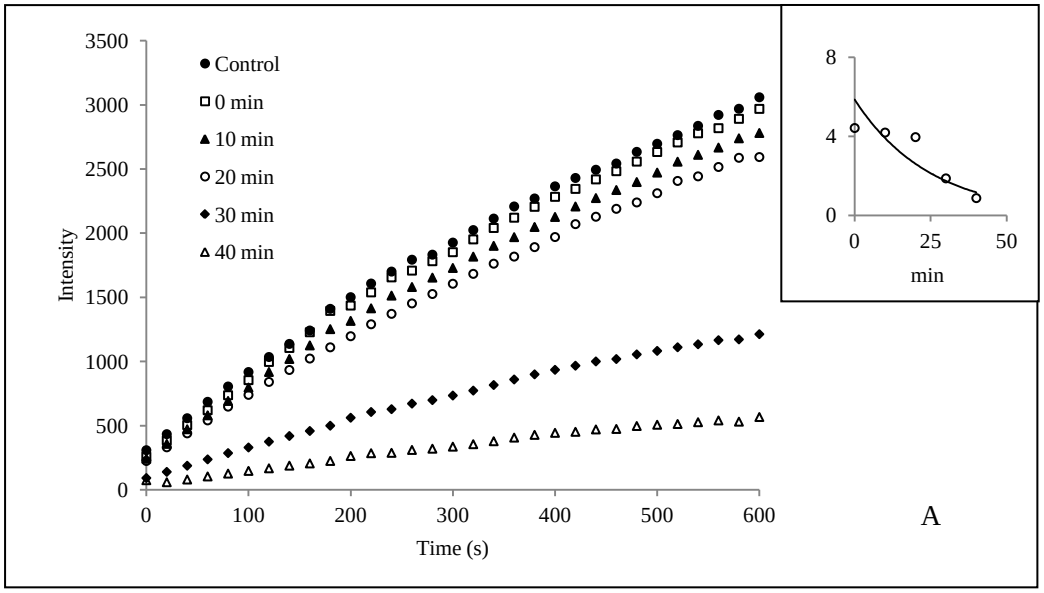


Fig. 6.2. Effect of mimosine on NA inhibition. Lineweaver-Burk plot in the presence of mimosine at concentrations of 0, 10, 15 and 20 μM. Neuraminidase inhibition was assayed as described in the text..



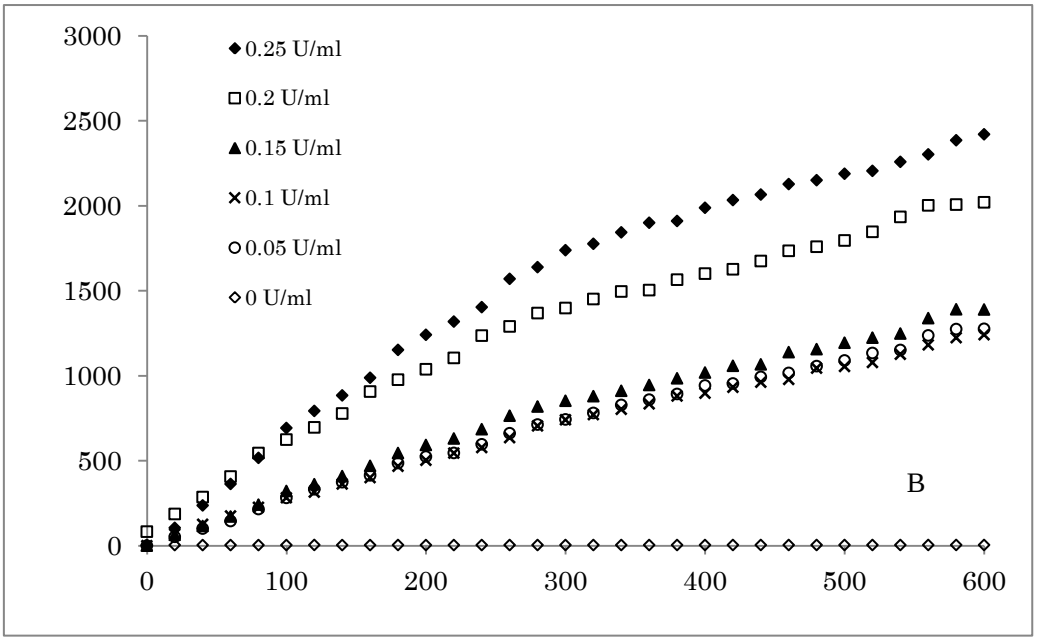


Fig. 6.3. Effect of preincubation time on hydrolysis of substrate by NA; (A) time-dependent inhibition of NA in the presence of 5 μM mimosine; (inset) decrease in slopes as a function of time; (B) effect of enzyme concentration on NA inhibition: a typical plot of residual activity of NA at various concentrations (0 – 0.4 U/mL) in the presence of mimosine at 5 μM.

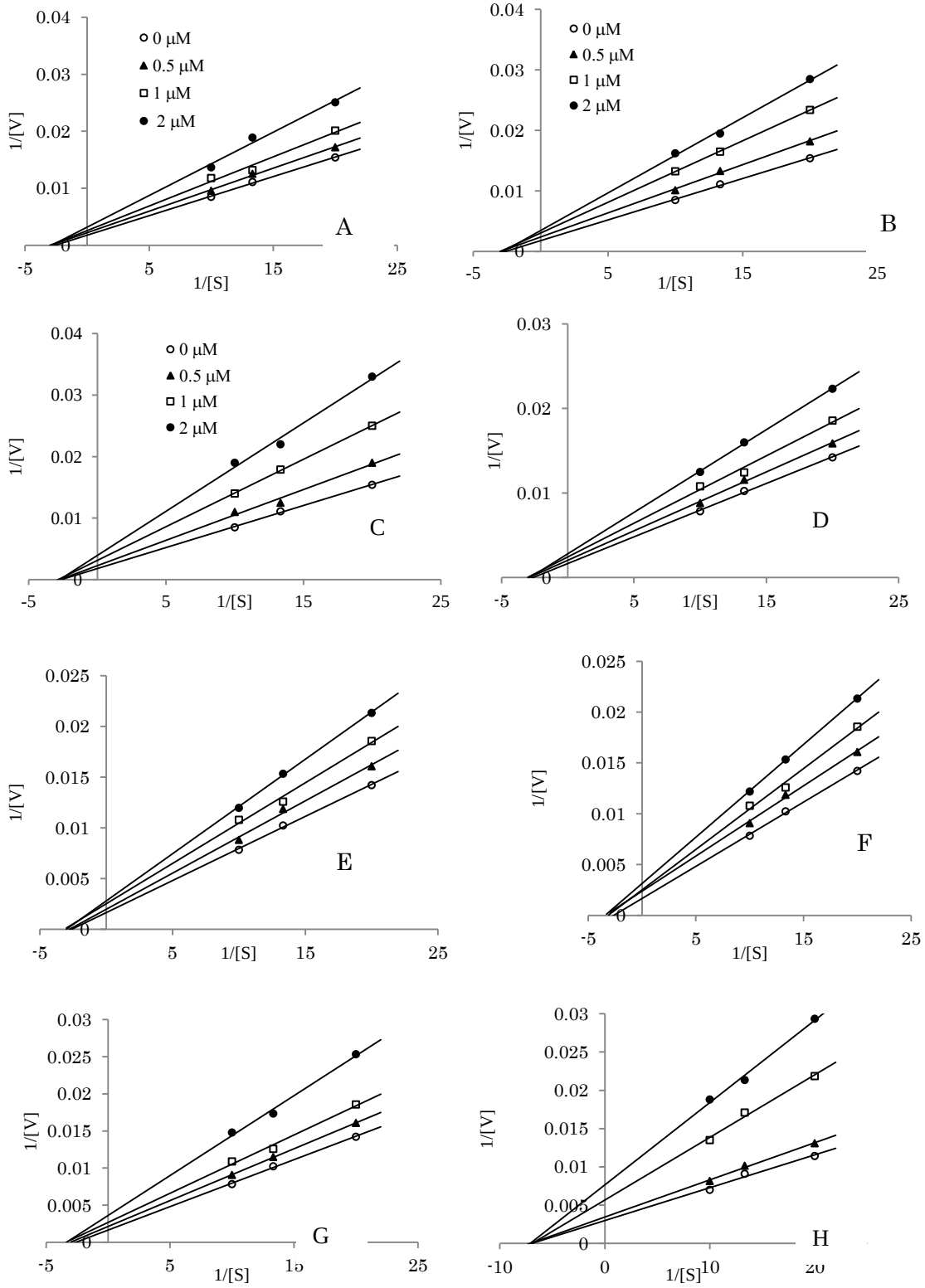


Fig. 6.4. Effect of M-FWY (A), M-FYY (B), M-FFY (C), M-VGY (D), M-QGY (E), M-WGY (F), M-FGY (G), and M-HGY (H) on NA inhibition. Lineweaver-Burk plot in the presence of 0-2 µM of mimosine tetrapeptides.

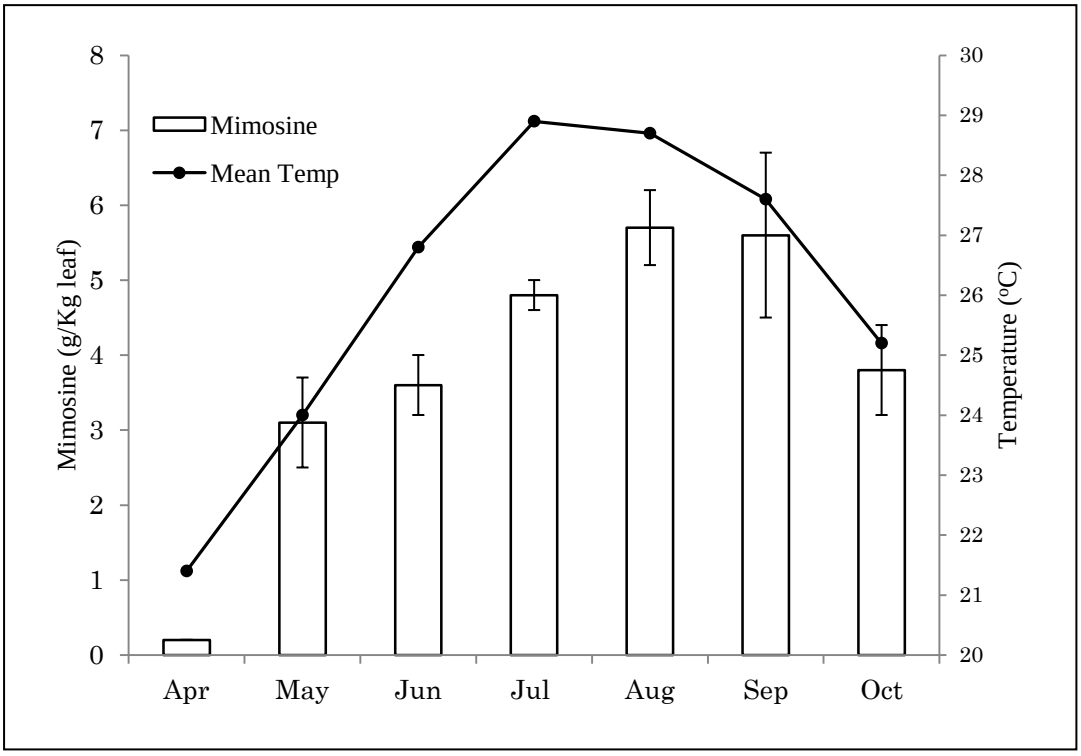


Fig. 6.5. Change in mimosine content in the leuceana leaves from April-October (2010). The line represents mean temperature (^{o}C) of Naha (Okinawa, Japan) as recorded by Japan Meteorological Agency (1990-2010).

Table 6.1. IC_{50} values of mimosine and mimosine-tetrapeptides

Inhibitor	Yield[x] (mg)	Neuraminidase IC_{50} (μM)	Neuraminidase Inhibition type (Ki, μM)	Tyrosinase IC_{50} (μM)
Mimosine	-	9.8 ± 0.2^{e}	Competitive (Km: 39.1 ± 1.2 min^{-1})	44.7 ± 0.4^{f}
M-FFY	80.2	1.8 ± 0.2^{a}	Noncompetitive (2.1 ± 0.1)	5.6 ± 0.2^{a}
M-FYY	65.7	2.8 ± 0.1^{b}	Noncompetitive (2.0 ± 0.1)	6.1 ± 0.2^{a}
M-FWY	71.5	5.9 ± 0.2^{c}	Noncompetitive (2.8 ± 0.2)	7.4 ± 0.2^{b}
M-VGY	42.5	7.0 ± 0.2^{d}	Noncompetitive (2.1 ± 0.2)	9.3 ± 0.3^{c}
M-QGY	85.6	9.2 ± 0.1^{e}	Noncompetitive (1.9 ± 0.1	9.8 ± 0.2^{c}
M-WGY	66.8	11.5 ± 0.2^{e}	Noncompetitive (2.3 ± 0.5)	9.9 ± 0.3^{c}
M-FGY	78.4	13.2 ± 0.3^{f}	Noncompetitive (2.2 ± 0.6)	16.4 ± 0.2^{d}
M-HGY	66.4	25.3 ± 0.3^{g}	Noncompetitive (7.2 ± 0.2)	36.7 ± 0.3^{e}

Different letters in the same column indicate the existence of significant difference (Tukey test, $p = 0.01$)

[x]Isolated pure crude yield

Table 6.2. Neuraminidase inhibition by individual amino acids

Amino acid (30 μM)	Neuraminidase inhibition (%)
Phenylalanine	6.1 ± 1.1
Glycine	7.0 ± 1.0
Glutamine	7.2 ± 3.2
Histidine	9.2 ± 1.2
Valine	8.7 ± 0.8
Tryptophan	9.7 ± 1.9
Tyrosine	8.5 ± 2.3

CHAPTER 7

LONGEVITY EXTENDING EFFECTS OF *ALPINIA ZERUMBET* POLYPHENOLS ON *C. ELEGANS*

7.1 SUMMARY

The beneficial effects of polyphenols compounds in fruits and vegetable are mainly extrapolated from *in vitro* studies or short-term dietary supplementation studies. Recent approaches using animal models of *C. elegans* are becoming quite popular and in this regard, the effects of *Alpinia zerumbet* leaves polyphenols on *C. elegans* lifespan in both normal and stressed conditions were investigated. The results showed that the leaves polyphenols could significantly increase the mean lifespan by 22.6%, better than the positive control resveratrol. Furthermore, both under the thermal and oxidative stressed conditions, the leaves polyphenols increased the survival rate significantly better than the quercetin. Further studies demonstrated that the significant longevity extending effects of ALP on *C. elegans* could be attributed to its *in vitro* free radical scavenging effects and its up regulation of stress resistance related proteins, including superoxide dismutase 3 (SOD-3) and heat shock protein (HSP-16.2). These results implicated that the polyphenols compounds of *A. zerumbet* have beneficial effects on the lifespan of *C. elegans* and that they may be used as a source of dietary supplement for aging or age-related diseases.

7.2 INTRODUCTION

Plants contain a variety of polyphenolic compounds that are not involved in primary metabolism but deter herbivores and protect against UV-induced damage as well as against stress conditions. Furthermore, the health beneficial effects of a diet rich in fruits and vegetables are, at least in part, attributed to polyphenols that are present in many herbal edibles. Many phenolic compounds revealed a remarkable spectrum of biochemical and pharmacological actions thought to be due to their antioxidative and free-radical scavenging properties (Noroozi et al., 1998; Middleton et al., 2000). Antioxidants counteract the excessive formation of reactive oxygen species (ROS) resulting in oxidative stress, a pathological situation related to various age-dependent diseases, cancer and aging (de Grey, 1997; de Grey, 2000; Forsberg et al., 2001; Griffiths, 2005; Schroecksnadel et al., 2006). Extensive studies suggests that reactive oxygen species (ROS), by-products of cellular respiration, play a role in normal aging by causing random deleterious oxidative damage to a variety of tissue (Harman, 1956; Muller, Lustgarten, Jang, Richardson, & Van Remmen, 2007).

The nematode worm *Caenorhabditis elegans* (*C. elegans*) is increasingly popular as a model organism for aging studies as well as for testing antioxidants and other compounds for effects on longevity (Gruber et al., 2009). The resistances to ROS due to

increasing the activity of ROS-detoxifying enzyme or low intrinsic ROS-production levels in long lived *C. elegans* mutants substantiate the free radical theory of aging (Tanja, Kai, Hannelore, & Uwe, 2010).

Alpinia zerumbet (alpinia) (Family: Zingiberaceae) is a perennial ginger growing widely in the subtropics and tropics. It is used in folk medicine for its anti-inflammatory, bacteriostatic, and fungistatic properties (Zhogbi, Andrade, & Maia, 1999). The essential oil extracted from its leaves possessed both relaxant and antispasmodic actions (Bezerra, Leal-Cardoso, Coelho-de-Souza, Criddle, Fonteles, 2000). In Okinawa, alpinia leaves are used to prepare a traditional food, *mu-chi*, which is widely believed to have preventive measures against common cold (Tawata, Fukuta, Xuan, Deba, 2008). Many researchers have focused their interests and investigation on this shrub for the purpose of finding bioactive compounds against variety of human diseases. We have reported the antioxidant activity and antimicrobial and phenolic contents in alpinia (Elzaawelly, Xuan, & Tawata, 2007a; Elzaawelly, Xuan, Koyama, & Tawata, 2007b) and have isolated active compounds from the rhizomes of *A. zerumbet* against HIV-1 integrase and neuraminidase enzymes (Chapter 4). Recently, we have reported the inhibitory effects of this plant on advanced glycation end products formation (Chompoo, Upadhyay, Kishimoto, Makise, & Tawata, 2011). In this study, we investigated the

longevity extending effects alpinia leaf polyphenols (ALP), obtained from the phenolic rich fractions of alpinia, in *C. elegans* and found that ALP could significantly improve the longevity under normal and stressed conditions.

7.3 MATERIALS AND METHODS

7.3.1 Materials

All chemicals were of analytical grade and were purchased from Wako (Japan),

7.3.2 Strains, and maintenance of C elegans

The wild type *C. elegans* strain N2 (var. Bristol), and the transgenic strains CF1553 (muls84) and the CL2070 (dvls 70) as well as the OP50 bacteria were obtained from Caenorhabditis Genetic Centre (University of Minnesota). All strains were routinely propagated at 20 ^{o}C on Nematode Growth Medium (NGM) plates with *Escherichia coli* strain OP50 as a food source. Synchronization of worm culture was achieved by treating gravid hermaphrodites with bleach (50% sodium hypochlorite; 2.5 M sodium hydroxide) and recovering the hatched L1 larvae on NGM/OP50 plates (Kampkotter, Pielarski, Rohrig, Timpel, Chovolou, Watjen, Kahl, 2005).

7.3.3 Extract preparation

Fresh alpinia leaf (50 g) was boiled in water for 20 min and the cooled extract was filtered and vacuum dried at 40 ^{o}C. The crude extract was re-dissolved in water (10

mg/mL) and kept in refrigerator until use and is denoted by ALH. For the preparation of phenol rich fraction, 50 g of fresh leaf was extracted with 50% ethanol (500 ml) for 48 h. The filtered extract was evaporated to dryness under vacuum at 40 ^{o}C and the dried extract was dispensed in water. Liquid-liquid partition with hexane and ethyl acetate was done. The stock solution (10 mg/mL) of water soluble fraction (ALP) was prepared by dissolving vacuum-dried (40 ^{o}C) extract in water and stored at 4 ^{o}C until use. To prepare plates supplemented with alpinia extract, the stock solution of extract was added to freshly autoclaved NGM plates (at 55 ^{o}C) to the desired final concentration.

7.3.4 Assessment of resistance to oxidative and thermal stress.

To the prepared NGM plates treated with alpinia extract of different concentrations and quercetin, age synchronized larva (L1) were added and maintained at 20 ^{o}C for 96 h. Around 30-40 worms were transferred to S-basal medium containing 0.5 mM H_2O_2 for 8 h (Wilson et al, 2006). After this the S-basal medium containing both living and dead nematodes were transferred to fresh NGM plates and the numbers of nematodes living and dead were counted. Survival was scored as the number of animals responsive to gentle touch as a fraction of the original number of animals on the plate. Thermotolerance assays were performed with hermaphrodites on adult day 5, after the majority of egg-laying had ceased. Animals were transferred onto 3.5 cm NGM agar

plates supplemented as indicated and then incubated at 35 ^{o}C. Scoring was done every two hours by counting the dead nematodes that were not responsive to the gentle touch by platinum tip. Animals that had died from desiccation on the sides of the plate were censored. For the transgenic strains, CL1553 and CF2070, the worms were exposed to thermal stress for 6 h and the survival rate was assessed.

7.3.5 Life span assays of C. elegans

For life span assays, the synchronized nematodes (20-30 per plate) were maintained in 3.5 cm NGM plates seeded with live OP-50 *E. coli* and supplemented with various concentrations of alpinia extract and resveratrol (100 μM). At the first day of adult hood (day 4), 400 μL 5-fluorodeoxyuridine (FUdR) was added to prevent progeny development (Fan et al, 2011). The nematodes were fed with live OP-50 and every fourth day, 80 μL of live *E. coli* were added to the NGM plates in order to prevent worms from starvation. Counting for dead animals was done every two days as mentioned above.

7.3.6 Fluorescence quantification and visualization

The GFP fluorescence of GFP-expressing populations was assayed using a MTP-880 fluorescence meter, Corona Electric, Japan. Four days old, 15 control or treated (with ALP 10μg/mL) worms were transferred in 100 μL of phosphate buffer to a

well of Nunc 96-well microtiter plate (black, clear, flat-bottom wells), and total GFP fluorescence was measured using 490 nm excitation and 530 nm emission filters (Zhang, Jie, Zhang, and Zhao, 2009). The experiment was done in triplicate with twice repetition. The results are presented as mean ± SE of six experiments. For fluorescence microscopy, the GFP pictures of transgenic worms were taken on an Olympus DP-70 microscope.

7.4 RESULTS AND DISCUSSION

7.4.1 Alpinia hot water (ALH) extract increases the tolerance for heat and oxidative stress

To investigate the protective effect of alpinia leaf hot water extract against thermal stress in *C. elegans*, young adults were pretreated with ALH 62.5, 125, and 250 μg/mL or quercetin 50 μg/mL before being exposed to 35 ^{o}C or H_2O_2. Our results showed that the pretreatment enhanced the worms' resistance to both heat shock and H_2O_2 and consequently increased the survival rates. Under heat stress, the median survival rate for the unfed nematodes was 11.5 h while a concentration-dependent increase in for the treated worms were seen (**Figure 7.1, Table 7.1**). Both ALH 125 and 250 μg/mL treated worms had significantly better performance (13.8 and 15.2 h, respectively) than the positive control quercetin (13.3 h) (**Table 7.1**). For oxidative stress, 36.4% of the total

nematodes were found surviving after 8 h or exposure in H_2O_2 in case of control group (**Table 7.2**). There was a significant increase in the median survival rate of the nematodes treated with ALH 125 and 250 μg/mL. The increase in survival rate for quercetin was found to be 65.8% while for the ALH treated worms, the rate increased by 77.2, 112.9 and 131.6% respectively for 62.5, 125, and 250 μg/mL (**Table 7.2**).

7.4.2 ALP significantly extend the lifespan of wild-type *C. elegans* N2 under normal culture conditions

Since water fractions (ALP) contained the highest amount of total phenol content **(Figure 7.2**), it was used for further assays. In order to investigate the longevity-extending effects of ALP, *C. elegans* were exposed to different concentrations of extracts (0, 25, 50, and 100 μg/mL), while resveratrol (100 μM) was used as a positive control (**Figure 7.3**). A concentration-dependent effect on longevity was observed in both the trials. There was a significant increase in the average median life of the worms fed with 50 and 100 μg/mL ALP (**Table 7.3**). When compared with the positive control (resveratrol, 100 μM), ALP 100 μg/mL had significant better lifespan extending properties. Similarly, the combined mean life span of the two trials also increased by 3.3, 15.9, and 22.9% for 25, 50, and 100 μg/mL fed ALP. Resveratrol (100 μM) had an increase of 18.9 %, when compared with that of control (**Table 7.3**).

Furthermore, when there was no worm still living in the control group, almost 40% of the worms were still alive in 100 μg/mL treatment. Similarly, the maximum lifespan of the nematodes also significantly increased by 13.0, 32.6, and 60.9% for 25, 50, and 100 μg/mL ALP concentrations. Resveratrol (100 μM) had an increase of 33.5% when compared to that of control (**Table 7.3**).

7.4.3 Effects of ALP at low concentration ranges on the thermal and oxidative stress on *C. elegans*

The effects of ALP on thermal and oxidative stress on *C. elegans* at low concentration ranges of 2.5, 5, and 10 μg/mL were investigated (**Figure 7.3**). It was found that the median survival rate for ALP 2.5, 5, and 10 μg/mL were 12.3, 13.5, and 14.0 h, with an increase in rates of 7.0, 17.4, and 21.7 %, respectively (**Table 7.1**). The ALP 10 μg/mL had significantly better performance than the positive control quercetin used. In case of oxidative stress, the median survival rates increased by 80.6, 92.5, and 112.8% for the indicated concentrations (**Table 7.2**). Our results showed that ALP 10 μg/mL had almost twice the oxidative resistive capacity than quercetin under these conditions.

7.4.4 Effects of ALP on thermal and oxidative stress on transgenic strains CL2070 and CF1553.

The effects of ALP (0, 5, and 10 μg/mL) on thermal stress on the heat sensitive transgenic strains CL2070 was investigated (**Figure 7.4**). The results showed that with increasing concentrations of ALP, the resistivity to the thermal stress also increased. The mean survival rates for the combined trials were 31.5, 54.7, and 66.5% for ALP 0, 5, and 10 μg/mL. An increase in 73.5 and 110.9% were observed for 5 and 10 μg/mL fed worms, respectively.

7.4.5 ALP up-regulates SOD-3::GFP expression in transgenic *C. elegans* CF1553

To investigate whether the increase in stress resistance was due to ALP regulating a specific stress-response gene, we examined the responsiveness of an antioxidant enzyme SOD to 10 μg/mL. ALP treatment by investigating SOD-3::GFP reporter gene expression in transgenic CF1553. The ALP treated group demonstrated significantly higher SOD-3::GFP intensity in pictures taken with confocal laser scanning microscope (**Figures 7.5A, B**). The quantification of intensity through microplate reader showed that ALP at 10 μg/mL could significantly up-regulate SOD-3::GFP expression by more than 143% (**Figure 7.5C**).

7.4.6 ALP up-regulated the expression of heat shock protein HSP-16.2 in transgenic *C. elegans* CL2070

The HSP-16.2 family of proteins is homologous to αβ-crystalline and expressed

under conditions of stress in *C. elegans* (Strayer, Wu, Christen, Link, Luo, 2003). It can serve as a stress-sensitive reporter to predict longevity in *C. elegans*. The higher levels of HSP-16.2::GFP predict the longer mean remaining longevity of *C. elegans* (Rea et al., 2005). In this study, the effect of ALP on the expression of HSP-16.2 was investigated to provide more clues to the protective effects of ALP on *C. elegans* under thermal stress. CL2070 worms containing the HSP-16.2::GFP reporter gene was treated with heat shock at 35 ^{o}C for 2 h and allowed to recover at 20 ^{o}C for 12 h. The 10 μg/mL treated sample showed higher HSP-16.2::GFP intensity, as shown by confocal laser scanning microscope (**Fig. 7.6A and B**). The intensity of the fluorescence was quantified and the results show that ALP significantly up-regulated HSP-16.2::GFP expression by almost one and half times in CL2070 (**Fig. 7.6C**, $p = 0.01$)

Researchers have 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). Nakatani (1992) suggested that the antioxidant compounds contained in the Okinawan plants contributed to the good health of the islanders. Most of these plants contain rich polyphenols that have antioxidant activities (Nakatami, 2003; Suda et al., 2005). We had previously reported the antioxidant activities and phenolic content in alpinia leaf (Elzaawelly et al., 2007a), and since the phenolic compounds have been

associated with the longevity of the nematodes, we wanted to investigate the anti-aging properties of alpinia leaf.

We began with exploring the effects of hot water extract of alpinia leaf on the thermal and oxidative stress on the wild N2 nematode. We found that there was a concentration-dependent increase in the thermo-tolerance of N2 strain. When the nematodes were exposed to the oxidative stress, similar results were obtained. In both the experiments, the worms fed with 125 and 250 μg/mL extracts had significantly better stress tolerance than the positive control quercetin (50 μg/mL).

We then studied the lifespan assays of the nematodes using the same concentration of extract. However, we found that there was no increase in the lifespan of nematodes by hot water extract of alpinia leaves (data not shown). We then investigated the lifespan and stress assays on nematodes using the phenolic rich fraction of alpinia leaf (ALP). In this study, we chose a lesser concentration of ALP (2.5, 5, 10 μg/mL) in order to assess the stress tolerance assays. There was an increase in thermo- as well as oxidative-tolerance in the *C. elegans*. For lifespan assays, we used 25, 50, and 100 μg/mL of ALP. We found that 100 μg/mL of ALP could significantly extend the life span of *C. elegans* better than resveratrol (100 μM). These results demonstrated a great longevity potential for ALP, both under normal and stressed conditions.

Phenolic compounds in plants have shown to function as free radial quenchers due to their high reactivity as electron donors (Podsedek, 2007). Researchers have shown that polyphenols from blueberry (Wilson et al., 2006), grape resveratrol (Gruber et al., 2007), *Ginkgo biloba* (Kampkotter et al., 2007), epigallocatechin gallate (EGCG) from green tea (Zhang et al., 2009) and phenolic antioxidants from spinach (Fan et al., 2011) were able to have beneficial effects on *C. elegans* aging. In this study, the phenolic rich fraction of alpinia leaves (ALP) significantly increases the lifespan of *C. elegans* under normal and stressed conditions. It has been proposed that free radical species causes deterioration of an organism (Harman, 1956). During cellular metabolism, aerobic organisms generate ROS, namely hydroxyl radical, superoxide anion, and hydrogen peroxide, which are removed by the antioxidant defense systems present in the organism (Harman, 1956). We found the radical scavenging activity of ALP *in vitro* and then investigated if it can have the same the effects *in vivo*? We found that ALP can enhance the stress resistance of *C. elegans* under environmental stress to prolong its longevity. Both SOD-3 and HSP-16.2 are the downstream effectors of DAF-16 and can serve as stress-sensitive reporters to predict longevity in *C. elegans* (Hsu et al., 2003; Rea et al., 2005). ALP could up-regulate both reporters and therefore, it seems that the longevity extension of *C. elegans* under normal and stressed conditions may be

attributed to this property **(Fig.7.7**).

7.5 CONCLUSION

Since the mortality in a population is usually closely associated with causes from environmental stress, these results demonstrated that ALP could significantly enhance the longevity of *C. elegans* under normal as well as stressed conditions. The lifespan extending ability may be attributed to its free radical scavenging activity, through up-regulating reporters such as HSP-16.2 and SOD-3 systems. These results implicated that the polyphenols compounds of *A. zerumbet* have beneficial effects on the lifespan of *C. elegans* and that they may be used as a source of dietary supplement for aging or age-related diseases.

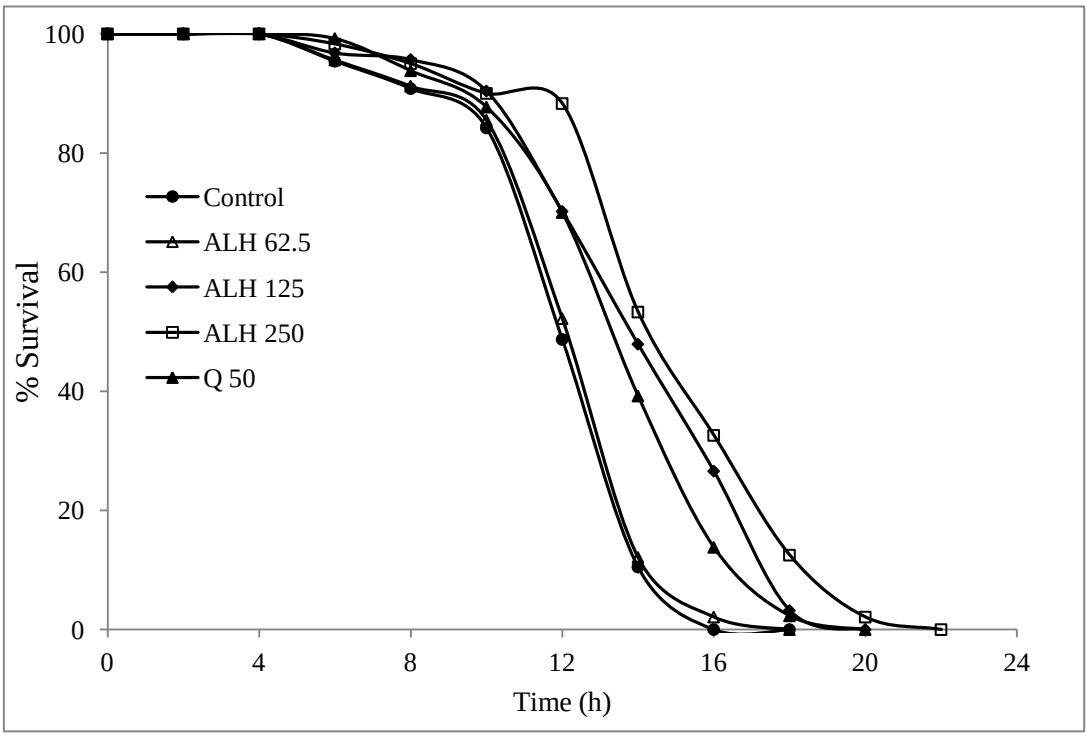


Figure 7.1. ALH improves the stress resistance of *C. elegans* under thermal stress. At 35 ^{o}C, compared with the control, ALH 250 μg/mL extended the thermal resistance by 21.1%.

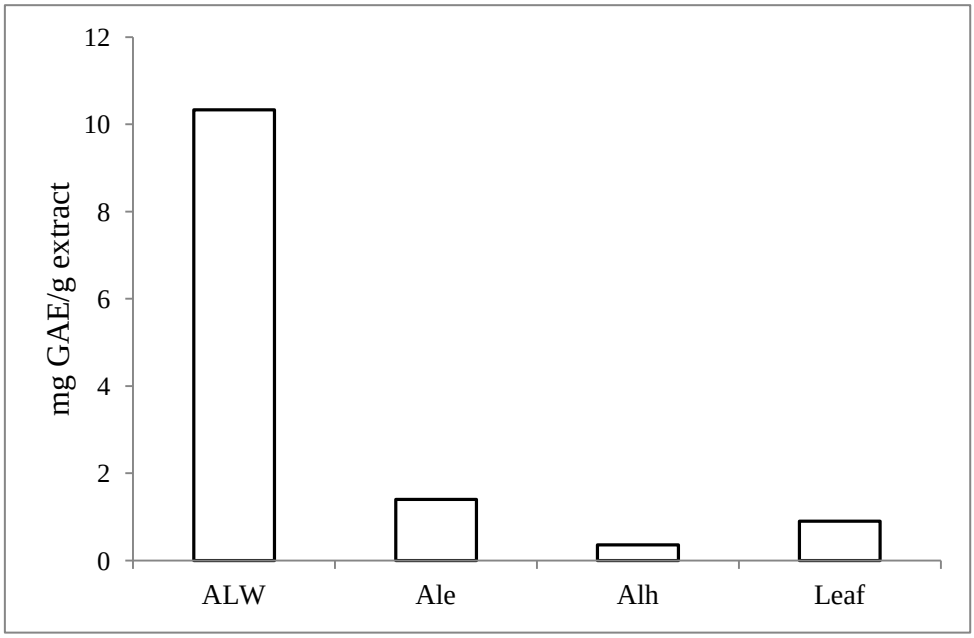


Figure 7.2. Total phenolic content of water (ALW), ethyl acetate (Ale), hexane (Alh) and leaf of alpinia

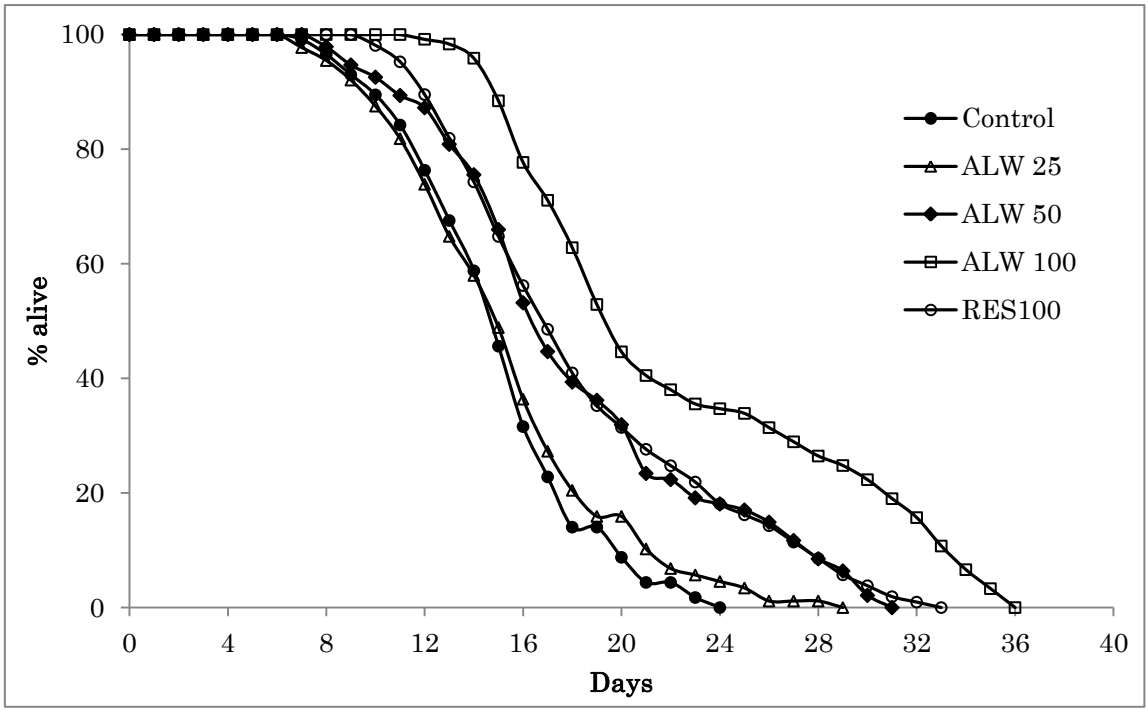


Figure 7.3. ALW extend lifespan and slow aging in *C. elegans*. Treatment with ALW (0, 25, 50, 100 μg/mL and resveratrol 100 μM.

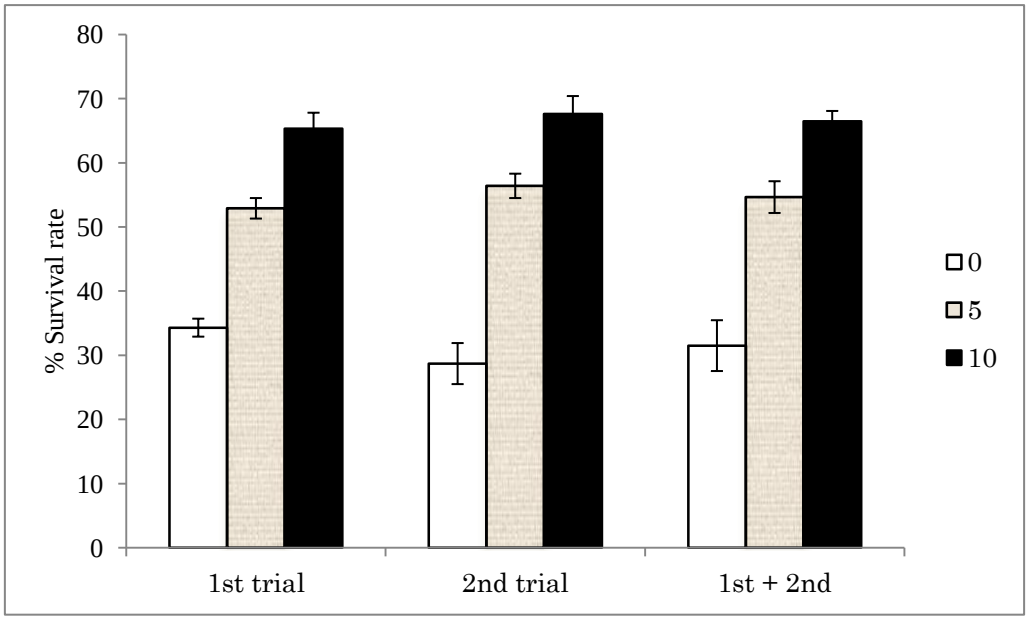
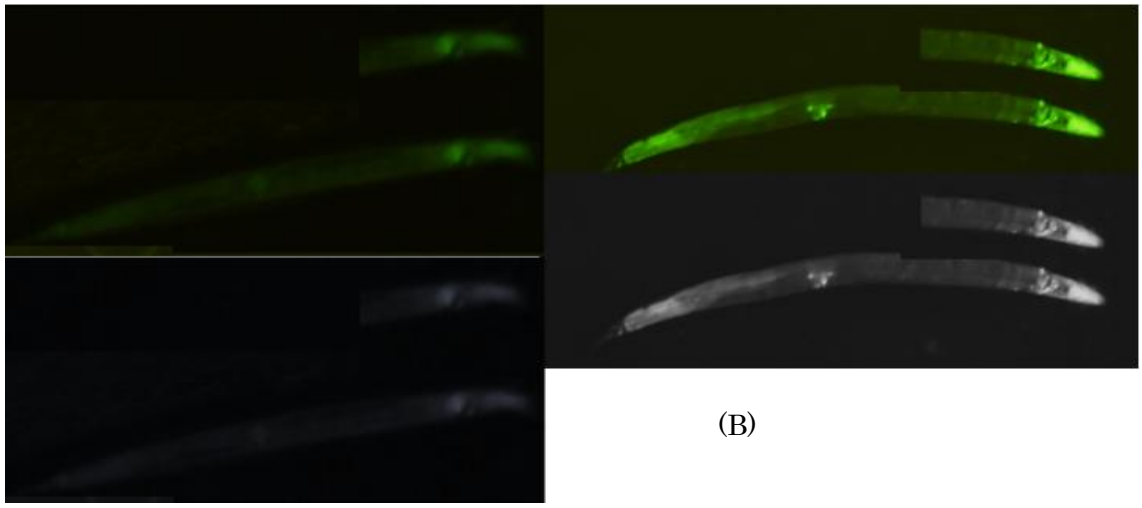
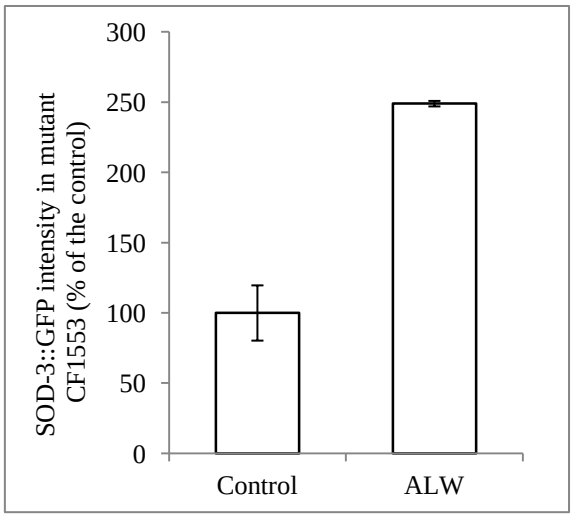


Figure 7.4. Effects of ALP on thermal stress on transgenic CL2070 *C. elegans*. The worms fed with 0, 5, and 10 μg/mL of ALP were exposed to thermal stress at 35 ^{o}C for 6 hours.



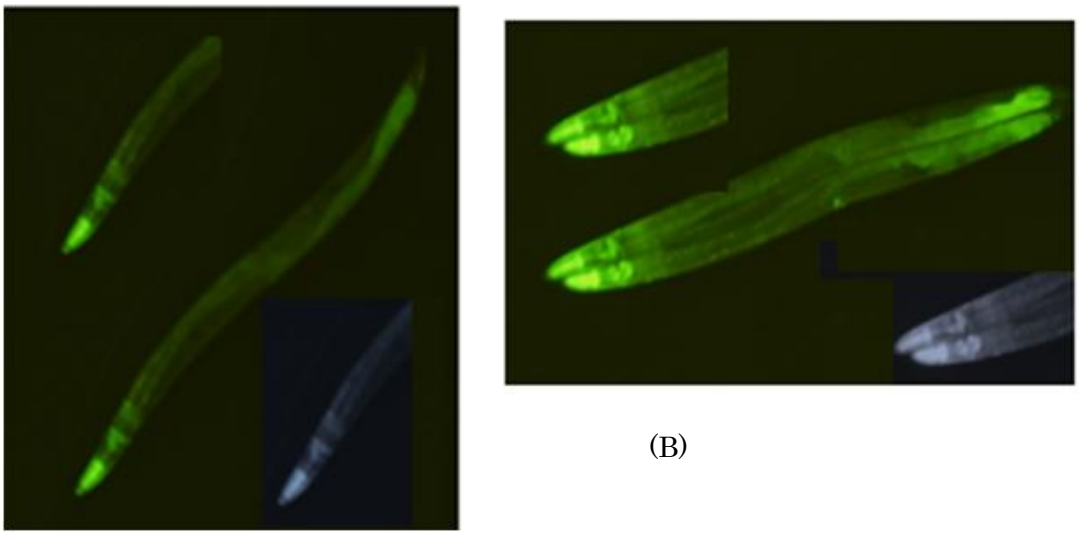
(B)

(A)

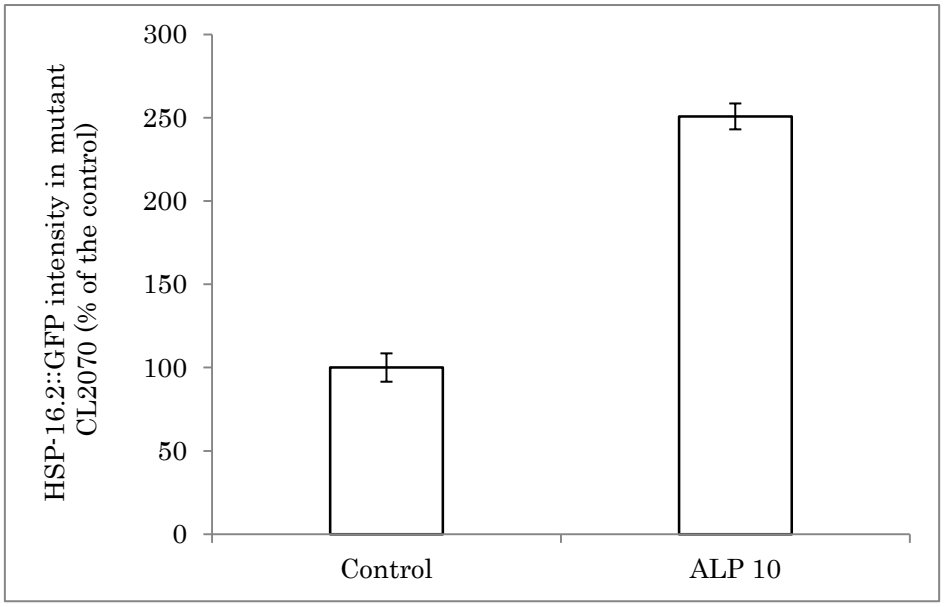


(C)

Figure 7.5. ALP up-regulates SOD-3::GFP expression in transgenic *C. elegans* CF1553. (A) Image of SOD-3::GFP expression in control worms. (B) Image of SOD-3::GFP expression in 10 μg/mL ALW-treated worms. The SOD-3::GFP expression in ALP-treated worms (B) is higher than that in control (A) both at the head and tail regions. (C) Quantified GFP intensity (SE) in CF1553 from three experiments in each group, with 20 worms in each experiment.



(A) (B)



(C)

Figure 7.6. Effects of ALP on the expression of heat shock protein HSP-16.2 in CL2070. (A) Image of HSP-16.2::GFP expression in the control worms. (B) Image of HSP-16.2::GFP expression in the 10 μg/mL ALP-treated group. The GFP pictures of the transgenic worms were taken on and Olympus DP 70 microscope. (C) Quantified GFP intensity (SE) in CL2070 from three experiments in each group with 15 worms in each experiment.

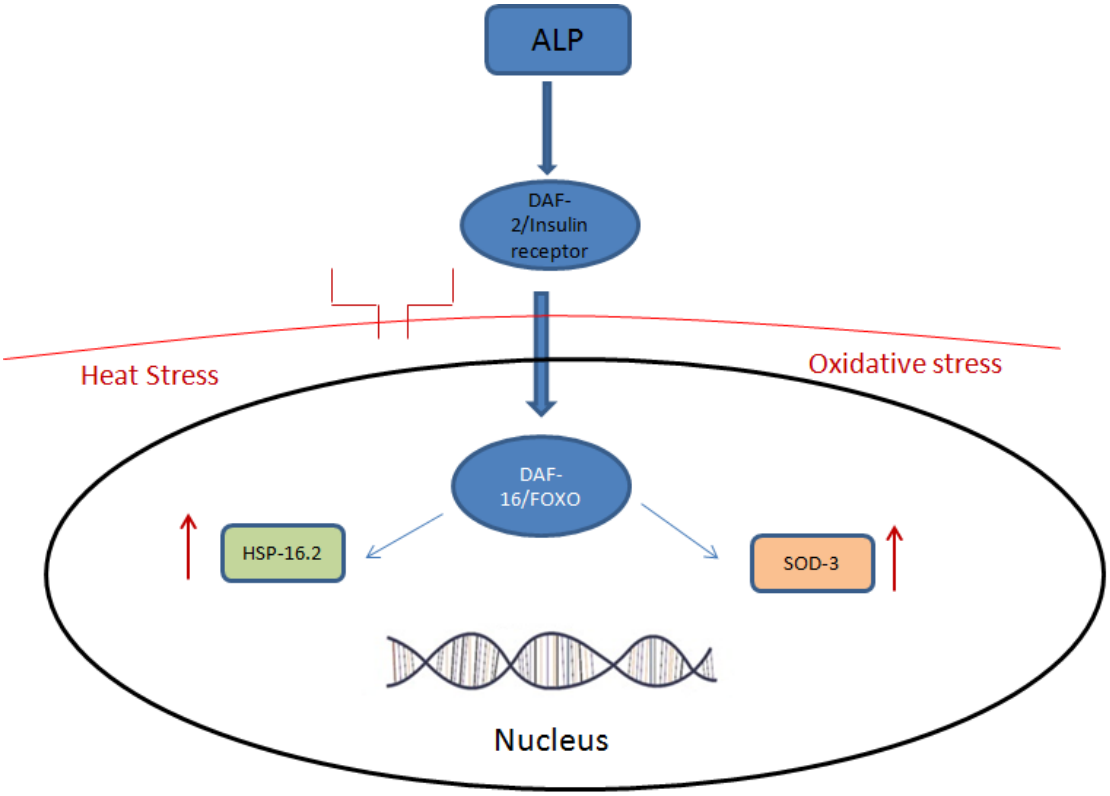


Fig. 7.7 Regulation of genes by ALP. ALP up-regulates HSP-16.2 and SOD-3 genes under the stressed conditions.

Table 7.1. Effects of ALH (62.5, 125, 250 μg/mL) and ALP (2.5, 5, 10 μg/mL) on thermal stress on wild type N2 *C. elegans* at 35 ^{o}C. Values represent the survival rate in hours.

	Trials	N2	Median	Mean	Max
Control	1st	92	11.8	12.6	14.0
	2nd	103	11.6	12.7	14.0
ALH 62.5	1st	89	12.2 (3.4)	12.8 (1.3)	16.0 (14.3)
	2nd	98	12.2 (5.2)	13.0 (2.4)	16.0 (14.3)
ALH 125	1st	86	13.8 (16.9)	14.6 (16.0)	18.0 (28.6)
	2nd	89	13.5 (16.4)	14.4 (13.4)	18.0 (28.6)
ALH 250	1st	92	14.3 (21.2)	15.4 (22.3)	20.0 (42.9)
	2nd	83	14.2 (22.4)	15.8 (24.4)	20.0 (42.9)
ALP 2.5	1st	75	12.3 (4.3)	13.5 (7.1)	20 (42.9)
	2nd	94	12.5 (7.8))	13.9 (9.5)	22.0 (57.1)
ALP 5	1st	81	13.3 (14.4)	14.2 (12.7)	20 (42.9)
	2nd	92	13.7 (18.1)	14.5 (14.2)	22.0 (57.1)
ALP 10	1st	80	14.0 (18.6)	14.5 (15.1)	20 (42.9)
	2nd	100	14.4 (24.1)	14.9 (17.3)	22.0 (57.1)
Q 50	1st	103	13.4 (16.6)	14.1 (11.9)	18 (28.6)
	2nd	88	13.9 (19.8)	14.8 (16.5)	20.0 (42.9)

Note: Numbers within parentheses indicate the percentage increase when compared with the control.

Table 7.2. Effects of ALH (62.5, 125, 250 μg/mL) and ALP (2.5, 5, 10 μg/mL) on the survival rate of nematodes exposed to oxidative stress. Q 50 is the quercetin, 50 μg/mL.

	% Survival	SE	% Increase
Control (173)	36.4	1.67	-
Q 50 (153)	60.3	0.33	65.8
ALH 62.5 (161)	64.5	13.73	77.2
ALH 125 (124)	77.5	5.87	112.9
ALH 250 (143)	84.3	2.18	131.6
ALP 2.5 (128)	65.7	4.17	80.6
ALP 5 (138)	70.5	1.55	93.7
ALP 10 (143)	77.4	1.52	112.8

Table 7.3. Effects of ALP (2.5, 5, 100 μg/mL) on the lifespan of *C. elegans* under normal conditions. Values represent the lifespan in days.

	Trial	N2	Censored	Median	Mean	Max
Control	1st	114	9	14.8	15.1	24
	2nd	108	11	14.1	15.0	22
	Combined	222	20	14.5	15.1	23
ALP 25	1st	88	7	15.0 (1.4)	15.5 (2.5)	26 (8.3)
	2nd	103	3	14.8 (4.9)	15.6 (4.0)	26 (18.2)
	Combined	183	10	14.9 (2.8)	15.6 (3.3)	26 (13.0)
ALP 50	1st	94	5	16.4 (16.4)	18.3 (21.2)	31 (29.2)
	2nd	98	-	15.8 (12.1)	16.7 (11.3)	30 (36.4)
	Combined	192	5	16.1 (11.0)	17.5 (15.9)	30.5 (32.6)
ALP 100	1st	121	13	19.4 (31.1)	22.6 (49.6)	36 (50.0)
	2nd	106	15	18.9 (34.0)	23.1 (54.0)	38 (72.7)
	Combined	227	28	19.2 (32.4)	22.9 (51.7)	37 (60.9)
Res 100	1st	105	7	16.8 (13.5)	18.7 (23.8)	33 (37.5)
	2nd	103	8	16.3 (15.6)	19.1 (27.3)	34 (54.5)
	Combined	208	15	16.6 (14.5)	18.9 (25.2)	33.5 (45.7)

Note: Numbers within the parentheses indicate the percentage increase when compared with the control.

CHAPTER 8

GENERAL CONCLUSION

8.0 GENERAL CONCLUSION

Plants are potential major natural sources of compounds with a wide range of bioactivities. Several studies show that bioactive compounds have been found to be one of the most plentiful classes of constituents in the plant kingdom, and they have been reported to have multiple biological effects.

In this study three Okinawan plants, namely, *Alpinia zerumbet*, *Leucaena luecocephala*, and *Ananus comosus* were investigated for a wide range of bioactivities. Several novel properties of these plants were identified. Pineapple stem waste was utilized for the first time in order to exploit its possible bioactive properties. This study revealed that pineapple stem waste could be utilized as an economic source of preventive or therapeutic agent in disease and in different functional food industries. The encouraging results of this waste as a fungicide certainly widen its scope in developing it as natural fungicides which may have implications in food based industries.

The plant *A. zerumbet*, on the other hand, showed a variety of bioactivities, more promising ones being the HIV-1 integrase and neuraminidase inhibitions. Both of these enzymes are necessary for the replication of two of the most atrocious virus, HIV and influenza. Three compounds, 6-dehydrokawain (DK), dihydro-5,6-dehydrokawain (DDK) and 8(17),12-labdadiene-15,16-dial (labdadiene) were isolated from the rhizomes and were shown to have very high activity. In particular, both DK and DDK showed significantly better inhibitory property than the positive control, suramin, against HIV-1 integrase. These compounds were also effective against the integrase enzyme with better performance than previously known compounds such as quercetin or ferulic acid. Compounds like DDK and DK could be used as lead compounds in

designing drugs against influenza and HIV/AIDS. Furthermore, the essential oils of two varieties of alpinia leaf were investigated against neuraminidase and atherosclerosis formations. The *tairin* variety, with higher number of compounds than shima variety, may find probable application in inhibiting influenza and/or atherosclerosis.

From the plant *Leucaena luecocephala*, mimosine, a non-proteinogenic amino acid, was identified as the most potent neuraminidase inhibitors. This amino acid had IC_{50} values at low micromolar ranges and its mechanism of inhibition was very similar to the drug Tamiflu. Furthermore, an attempt was made to synthesize a small library of tetrapeptides using mimosine as one of the amino acids. Several mimosine tetrapeptides showed much better activity towards neuraminidase and tyrosinase inhibitions, with M-FFY (mimosine-Phe-Phe-Tyr) having the most potency against both of the enzymes. The results of this study certainly expand the use of solid phase peptide synthesis in designing of potent neuraminidase inhibitor.

The final challenge of this study was to investigate the possible roles of phenolic compounds of *A. zerumbet* leaf on the lifespan assays using *C. elegans* as animal models. The results showed that polyphenols of alpinia leaves had significant longevity-extending effects on *C. elegans* both under normal and stressed conditions. The survival rate during thermal and oxidative stress increased up to 32%. A low concentration of 10 μg/mL of phenol rich fraction had a significant tolerance ranging to 21%. A 100 μg/mL fraction increased the mean life span of the nematode to almost 50%, a result significantly better than 100 μM resveratrol. These results substantiate the possible application of alpinia leaf in the field of healthy aging. It was found that the longevity extending properties of ALP may be due to the up-regulation of HSP-16.2 and SOD-3 reporters.

In conclusion, the investigated Okinawan plants showed a wide range of bioactivities. Compounds responsible for those bioactivities were identified which included, polyphenols, kawains and non-proteinogenic amino acid. On the basis of this study, it could be said that these Okinawan plants could be utilized in preparing functional foods for a series of therapeutic purposes. However, further in vivo studies are necessary in order to use these plants commercially in various industries.

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