Utilization of *Alpinia zerumbet* Components for Anti-Diabetes Medications

抗糖尿病薬開発に向けたゲットウ成分の利用

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2013

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A DISSERTATION SUBMITTED TO
THE UNITED SCHOOL OF AGRICULTURAL SCIENCES,
KAGOSHIMA UNIVERSITY,
KAGOSHIMA, JAPAN

FOR THE REQUIREMENT OF THE DEGREE OF
DOCTOR OF PHILOSOPHY
IN AGRICULTURAL SCIENCES

By

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March, 2013

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ABSTRACT

Diabetes is a disease in which a person has high blood sugar level either because the pancreas does not secret enough insulin (type 1) or the body cannot effectively use the insulin (type 2). By 2030, the WHO estimates that it is the most common endocrine disorder affecting around 2-3% of the population worldwide and its incidence is rising. These drawbacks give this research tremendous opportunity to design and develop novel drugs delivery system for overcoming the metabolism problems associated with anti-diabetes. *Alpinia zerumbet*, a common herbaceous perennial plant in Okinawa (Japan), was chosen as potential therapeutic materials because it contains numerous interesting bioactive constituents. In this study, a total of five bioactive compounds of *A. zerumbet* were isolated, two of which were previously known compounds isolated from the rhizomes namely 5,6-dehydrokawain (DK) and dihydro-5,6-dehydrokawain (DDK). In addition, three new compounds were isolated for the first time including 8(17),12-labdadiene-15,16-dial (labdadiene) and (1*E*,3*E*,5*E*)-6-methoxyhexa-1,3,5-trien-1-yl)-2,5-dihydrofuran (MTD) from rhizomes and (*E*)-2,2,3,3-tetramethyl-8-methylene-7-(oct-6-en-1-yl)octahydro-1*H*-quinolizine (TMOQ) from seeds. These compounds were examined to assess the potencies and activities in five different systems which have been associated with the development of diabetes.

In the first study, the results of the analyses revealed that labdadiene expressed antiglycation and advanced glycation end products (AGEs) formation in three different steps in the pathway. This compound suppressed the formation of Amadori's products (IC_{50} = 51.06 μg/mL), thereby reducing glycation-induce protein oxidation and dicarbonyl compounds formation.

Secondly, the antiatherosclerogenic properties of *A. zerumbet* were investigated. Cholest-4-ene-3,6-dione, a steroid present in the seed extract, showed inhibition activities against pancreatic lipase, 15-lipoxygenase and LDL oxidation (IC_{50} = 19.50, 54.79 and 16 .12 μg/mL, respectively).

Thirdly, DK could be used as a potent inhibitor and further exploited for utilization as anti-skin disease formulations. It displayed inhibitory activities for antioxidants including DPPH, ABTS and PMS-NADH scavenging (IC_{50} = 122.14, 110.08 and 127.78 μg/mL, respectively). It also showed strong inhibitory activities against skin disease-related enzymes including collagenase, elastase, hyaluronidase and tyrosinase (IC_{50} = 24.93, 19.41, 19.48 and 76.67 μg/mL, respectively).

In the fourth study involved in anti-diabetic activities of *A. zerumbet* components in gestational diabetes which is a pathologic condition related to the high blood sugar level during pregnancy. TMOQ showed high inhibitory activities on α-glucosidase, trypsin and nitric oxide. Furthermore, MTD showed dose-dependent increase in the activity of tryptophan hydroxylase. The results revealed that both compounds may have probable applications in preventing gestational diabetes formation.

In the final study, the human umbilical vein endothelial cells (HUVEC) were used to evaluate the anti-diabetic potential of *A. zerumbet* components on oxygen species release and cAMP enzyme activity. TMOQ showed high inhibitory activities on xanthine oxidase (IC_{50} = 14.70 μM), LDL oxidation (IC_{50} = 10.33 μM) and NADPH oxidase (% of inhibition = 47.84 μM), moreover, this compound had ability to increase cAMP level (% increasing = 33.50).

From the results of these experiments, it can be concluded that *A. zerumbet* is an important source of numerous phytochemical compounds which possess antidiabetic properties. Thus, this plant could be medically utilized as natural drugs against diabetes.

抗糖尿病薬開発に向けたゲットウ成分の利用

要約

糖尿病は、膵臓が十分なインシュリンを分泌しないか、インシュリンを効率的に利用できないために血糖値が高くなってしまうという疾患である。世界保健機関は、糖尿病は 2030 年までに世界人口の 20～30%の人に影響を与えている最も一般的な内分泌疾患であるとし、しかもその発生率は上昇していると見積もっている。これらのことから、抗糖尿病にまつわる代謝問題の克服へ向けた、新規薬物送達システムのデザインや開発を行う多大な機会が本研究に与えられることとなった。ゲットウは沖縄に広く分布する多年生植物であり、多くの興味深い生理活性成分を含むため、治療薬の原材料として選んだ。本研究において、計 5 種類の生理活性物質がゲットウより単離されたが、2 種については、5,6-dehydrokawain (DK) および dihydro-5,6-dehydrokawain (DDK) といった以前に根茎より単離された既知の化合物であった。さらに、以下の 3 種の新しい化合物、8(17),12-labdadiene-15,16-dial (labdadiene) と (1*E*,3*E*,5*E*)-6-methoxyhexa-1,3,5-trien-1-yl)-2,5-dihydrofuran (MTD) が根茎から、そして(*E*)-2,2,3,3-tetramethyl-8-methylene-7-(oct-6-en-1-yl)octahydro-1*H*-quinolizine (TMOQ) が種より単離された。糖尿病の発生と関連する 5 つの異なったシステムにおいて、これらの化合物の有効性と活性を調べた。

最初に行った研究において、解析の結果、labdadiene が 3 つの異なるステップにおいて糖化および AGE 生成を阻害する作用を持つことが明らかになった。この化合物により、アマドリ産物の生成が抑えられ (IC_{50} = 51.06 μg/mL)、それにより糖化によって誘導されるタンパク質の酸化およびジカルボニル化合物の

生成が減少した。

第 2 に、ゲットウの持つ抗動脈硬化作用について調べた。Cholest-4-ene-3,6-dione は種子抽出液に含まれるステロイドであるが、膵リパーゼ、1,5-リポキシゲナーゼ、LDL 酸化に対して抑制効果を示した (それぞれ IC_{50} = 19.50, 54.79, 16.12 μg/mL)。

第3に、DK の抗皮膚病薬としての利用に向けた研究を行った。DK は、DPPH、ABTS、PMS-NADH を含む抗酸化試験において阻害活性を示した(それぞれ IC_{50} =122.14, 110.08, 127.78 μg/mL)。また、コラゲナーゼ、エラスターゼ、ヒアルロニダーゼ、チロシナーゼといった皮膚病に関連した酵素に対しても強い阻害活性を示した(それぞれ IC_{50} = 24.93, 19.41, 19.48, 76.67 μg/mL)。

第 4 に、妊娠性糖尿病におけるゲットウ成分の抗糖尿病活性について調べた。TMOQ は、α-グルコシダーゼ、トリプシン、一酸化窒素に対して高い阻害活性を示した。MTD は用量依存的にトリプトファンヒドロキシラーゼを阻害した。これらの結果により、両化合物は妊娠糖尿病の予防に利用できる可能性があることが明らかになった。

最後に、ゲットウ成分が、活性酸素種の放出や cAMP 生成酵素に対して抗糖尿病作用を持つか調べるため、ヒト臍帯静脈内皮細胞 (HUVEC)を用いた。TMOQ はキサンチンオキシダーゼ (IC_{50} = 14.70 μM)、LDL 酸化 (IC_{50} = 10.33 μM)、NADPH オキシダーゼ (% of inhibition = 47.84 μM)に対して、高い阻害活性を示し、さらに cAMP のレベルも上昇させた (% increasing = 33.50)。

これらの実験の結果から、ゲットウは抗糖尿病特性を持つ多くの植物由来化合物の重要な供給源であると結論づけることができる。従って、この植物は糖尿病に対する自然薬物として医学的に利用されうるだろう。

ACKNOWLEDGEMENT

I would like to express my sincere thanks to my advisor, Prof. Shinkichi Tawata for his invaluable help and constant encouragement through the course of this research. I am most grateful for his teaching and advice, not only the research methodologies but also many other methodologies in life. I would not have achieved this far and this thesis would not have been completed without all the support that have always received from him.

I am especially thanks to my thesis committee, Assoc. Prof. Masakazu Fukuta (U. of the Ryukyus), Prof. Toshihiko Suganuma (Kagoshima U.), Prof. Kazuhiko Kinjo (U. of the Ryukyus) and Prof. Kohzo Kanda (Saga U.) for their invaluable suggestions during my Ph.D tenure.

I would like to express my appreciation to all of the past and present members in Tawata's Laboratory for good advice and collaboration. Thanks to Dr. Atul Upadhyay for his friendship and sharing knowledge. Also, I am especially grateful for the immense pleasure that we had during our research hours from great friends like Mr. Wataru Kishimoto, Mr. Nozomi Taira, Mr. Tuneaki Toyama, Mr. Masanao Chinen, Ms. Pham Thi Be Tu and many other lab-mates. In addition, I am grateful to Mr. Shinichi Gima and Mr. Tomonori Ono of the Instrumental Research Center, University of the Ryukyus (Okinawa, Japan) for skillful technique assistance.

I would like to express my gratitude to Sagawa Scholarship Foundation (Kyoto, Japan) for awarding me Sagawa scholarship in the last two years of my study course. My life in Japan would have been difficult if I did not have this support.

I am sincerely grateful to all teachers in my life for the things they have taught and guided me. And a special thanks to my boss and our staff in Kasetsart University (Thailand) for giving me a chance to carry out this Ph.D. Moreover, I am truly grateful to all the help and support that I received from the staff in Kagoshima University and University of the Ryukyus (Japan).

I would like to express my eternal appreciation towards my parents and my brother (Mr. Teerayut Chompoo) who has always been there for me no matter where I am, for all unconditional support and patience. Thank you for being ever so understanding and giving me the constant encouragement. It is a pleasure for me to thank my friends both in Thailand and in Japan (Dr. Nontivich Tandavanitj, Mr. Chanapol Kinimarn, Dr. Watchara Suyara, Dr. Khwantri Saengprachatanarug, Ms. Kamonon Khinimarn, Dr. Wanida Suebsaiprom, Dr. Kietsuda Luengwilai, Mr. Koonphol Pongmanee, Ms. Kanokwan Teingtham, Ms. Ornuma Tanadul, Ms. Cattleya Chutteang, Dr. Weeyawat Jaitrong, Dr. Wattanachai Tasen, Dr. Warachit Phayom, and Ms. Supab Poonpool) for their support throughout the period of this research and have been inspirational to me.

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March, 2013

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CHAPTER 1

GENERAL INTRODUCTION

INTRODUCTION

The role of traditional medicines as the solution of health problem is invaluable on a global level. Medicinal plants continue to provide valuable therapeutic agents, both in modern and in traditional medicine. With the associated side effects of the modern medicine, traditional medicines are gaining importance and are now being studied to find the scientific basis of their therapeutic actions. Studies of natural products stimulated development of the separation techniques, spectroscopic approaches to structure elucidation and synthetic methodologies that now constitute the foundation of contemporary organic chemistry. This research will go a long way in the scientific exploration of medicinal plants for the benefit of human and it likely to decrease the dependence on synthetic drugs.

Plants produce a vast and diverse assortment of organic compounds, primary and secondary metabolites. Primary metabolites are found in all plant cells and are essential for the survival of the plants. These include sugar (carbohydrates), amino acids (proteins), fats and oils. The research area of primary metabolites interest in growing and aspects of photosynthesis, assimilate partitioning and source-sink regulation in different types of plant-pathogen interactions. Secondary metabolites are not essential to the plant's survival, but the plant does suffer without them. Their functions, many of which remain unknown, are being elucidated with increasing frequency. Some of the roles of secondary metabolites are relatively straightforward; for instance, they play a host of general, protective role (*e.g.* as antioxidant, free radical-scavenging, UV light-absorbing and antiproliferative agents) and defend the plant against microorganisms such as bacteria, fungi and viruses.

They also manage inter-plant relationships, acting as allelopathic defenders of the plant's growing space against competitor plants. More complex roles include dictating or modifying the plant's relationship with more complex organism.

Secondary chemicals are important in plant use by humans who use some of these compounds as medicines, flavorings and recreational drugs. These chemicals can be classified on the basis of chemical structure (having rings and containing a sugar), composition (containing nitrogen or not), their solubility in various solvents, or the pathway by which they are synthesized (*e.g.* phenylpropanoid, which produces tannins). A simple classification includes three main groups: the terpenes (made from mevalonic acid, composed almost entirely of carbon and hydrogen), phenolics (made from simple sugars, containing benzene rings, hydrogen and oxygen) and nitrogen-containing compounds (extremely diverse, may also contain sulfur).

CHAPTER 2

LITERATURE REVIEWS

2.1 *Alpinia zerumbet*

Alpinia zerumbet (Pers.) B. L. Burtt. et R. M. Sm. (Family: Zingiberaceae), is a perennial herb originating in the tropical and subtropical regions of South America, Oceania and Asia (Zoghbi *et al*., 1999). In Japan, it is widespread in several southern islands, such as Okinawa Island, Amami-oshima Island and Tokunoshima Island. (Matsubara *et al*., 1994).

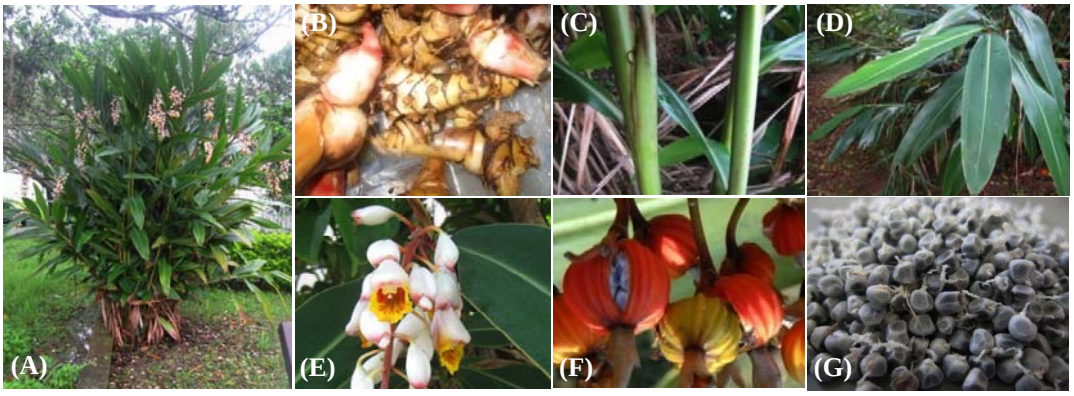

Figure 2.1 Six different structures of *A. zerumbet*; whole plant (A), rhizomes (B), stems (C), leaves (D), flowers (E), pericarps (F) and seeds (G).

From the aspect of clinical application as phytotherapy, several pharmacological effects of *A. zerumbet* have been reported. Previous studies on *A. zerumbet* have demonstrated that its extracts have many biological efficacies, including inhibition of porphyrin photooxidative reaction (Liao *et al*., 2000), as an antispasmodic agent in intestine (Bezerra *et al*., 2000), anti-hypertensive agent in vascular smooth muscle (Lahlou *et al*., 2002), vasodilatation inducer in the mesenteric vascular bed (MVB) and antihypertensive agent in rats with DOCA-salt hypertension (Lahlou *et al*., 2003). It has also been reported to have protecting effects against gastric lesion (Hsu, 1988), decoction

of diuretic properties (Laranja *et al*., 1991), antiulcerogenic properties (de Moura *et al*., 2005), and potent local anaesthetic activity (Leal-Cardoso *et al*., 2004),

Biochemical analysis of *A. zerumbet* have shown that leaves are rich in flavonoids ((+)-catechin, (-)-epicatechin, rutin, quercetin, kaempferol 3-O-rutinosideo, kaempferol 3-*O*-glucoronide, kaempferol) (Mpalantinos *et al*., 1998) and kava pyrone (dihydro-5,6-dehydrokawain (DDK) and 5,6-dehydrokawain (DK)) (Elzaawely *et al*., 2007). Methyl *trans*-cinnamic acid, DK, DDK, flavokawin-B, cardamonin and alpinetin (Itokawa *et al*., 1981), and sesquiterpenes including β-eudesmol, nerolidol, humulene epoxide II and 4α-hydroxydihydroagarofuran (Morita *et al*., 1996) have been isolated from the rhizomes. Furthermore, Hong *et al*. (1996) have isolated (E)-15,16-bisnorlabda-8(17),11-diene-13-one, coronarin E and labdane-type diterpenes, zerumin A and zerumin B from the seeds of *A. zerumbet*.

In this study, the secondary metabolites of *A. zerumbet* were isolated and studies were focused on the biological activities related to anti-diabetic properties. We investigated the inhibitory effects on various mechanisms that cause or have relationship with diabetes, including advanced glycation end products (AGEs), atherosclerosis, gestational diabetes and oxygen radical scavenging in human umbilical vein endothelial cells (HUVEC) model.

2.2 Diabetes mellitus

Diabetes is a group of metabolic diseases characterized by hyperglycemia resulting from defects in insulin secretion, insulin action, or both. The chronic hyperglycemia of diabetes is associated with long-term damage, dysfunction and

failure of different organs, especially the eyes, kidneys, nerves, heart and blood vessels. Several pathogenic processes are involved in the development of diabetes. These range from autoimmune destruction of the β-cells in the islets of Langerhans in pancreas with consequent insulin deficiency to abnormalities that result in resistance to insulin action. The basis of the abnormalities in carbohydrate, fat and protein metabolism in diabetes is deficient action of insulin on target tissues. Deficient insulin action results from inadequate insulin at one or more points in the complex pathways of hormone action (American diabetes association, 2011).

The current classification of diabetes is based upon the pathophysiology of each form of the disease. In one category, type 1 diabetes results from cellular mediated autoimmune destruction of pancreatic β-cells, usually leading to total loss of insulin secretion (American Diabetes Association, 2005). The lack of insulin production in patients with type 1 diabetes makes the use of exogenous insulin necessary to sustain life, hence the former name “insulin-dependent diabetes”. In the other, much more prevalent category, type 2 diabetes previously called non-insulin-dependent diabetes. This occurs from insulin resistance which alters the use of endogenously produced insulin at the target cells. Type 2 patients have altered insulin production as well, however, autoimmune destruction of β-cells does not occur as it does in type 1, and patients retain the capacity for insulin production because the type 2 patient still produces insulin to some extent. In both types of diabetes, the main treatment involves trying to artificially maintain “normal” or effective levels of glucose and insulin in the person’s body. This can be very difficult because so many things affect the glucose level in the blood. Different types of food from the same meal will digest at different rates, some quickly, some very slowly and

yield different amounts of glucose. The glucose consequences of a meal can last several hours, with various ways, increasing the demand for glucose in the cells (American Diabetes Association, 2005). Gestational diabetes, other type of diabetes, is a diabetic condition that some women experience in the secondary half or their pregnancy when blood sugar levels become abnormally high. The condition usually goes away after the pregnancy but in some people it does not (Mealey and Oates, 2006). Brownlee and Cerami (1981) suggested that because of high concentration of sugar in blood, the decreased uptake of glucose into muscle and adipose tissue lead to chronic extracellular hyperglycemia. This would result in tissue damage and patho-physiological complications involving heart disease, atherosclerosis, cataract formation, peripheral nerve damage, retinopathy and other diabetic complication.

2.2.1 Advanced Glycation End Products (AGEs)

AGEs are a complex and heterogeneous group of compounds that have been implicated in diabetes related complications. Structural components of the connective tissue matrix or basement membrane, such as collagen, are prime targets. The pathological cross-link formation induced by AGE lead to increased stiffness of the protein matrix, hence impeding function as well as increasing resistance to removal by proteolytic means, which in turn affects the process of tissue remodeling means, affects the pro-advancing age and accelerates in diabetes (Singh *el al*., 2001).

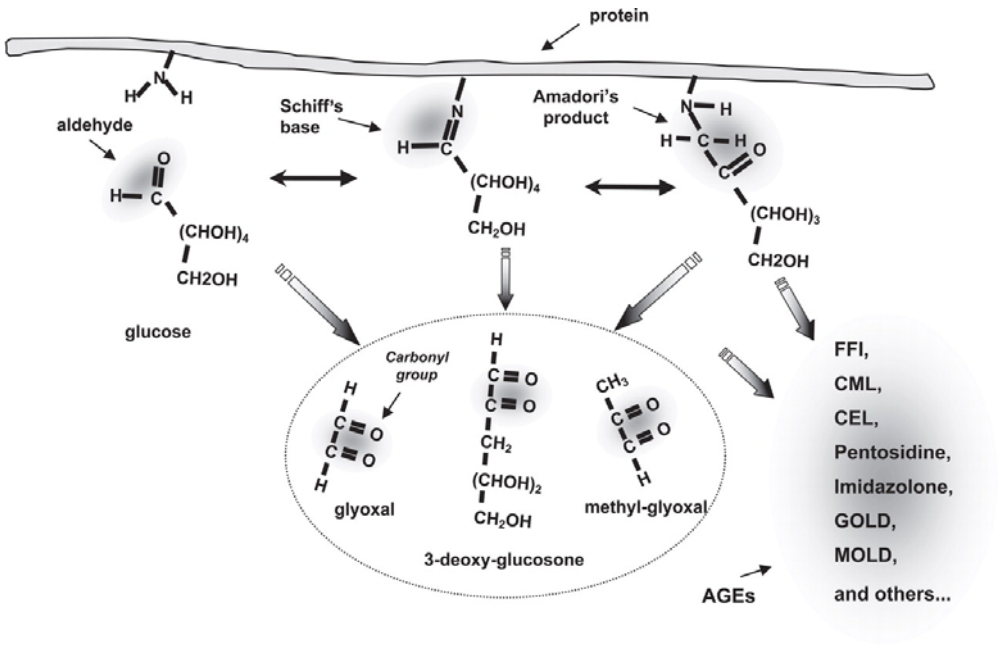

Figure 2.2 The pathway of advanced glycation end products (AGEs) formation (Basta *et al*., 2004).

The formation of AGEs through the Maillard reaction occurs in the three phases (**Figure 2.2**). First, glucose attaches to a free amino acid of a protein, lipid or DNA, in a non-enzymatic way to form a Schiff's base. A Schiff's base is a compound that has a carbon to nitrogen double bond where the nitrogen is not connected to hydrogen. The initiation of this first step depends on glucose concentration and takes place within hours. When the concentration of glucose decreases, this reaction is reversible. During the second phase, the Schiff's base undergoes chemical rearrangement over a period of days and form Amadori's products (also known as early glycation products). The Amadori's products are more stable compounds (hemoglobin A1c is the most well known), but the reaction is still reversible. When there is accumulation of Amadori's products, they will undergo complicated chemical rearrangement (oxidations, reductions and hydrations) and form cross-linked proteins.

Besides the Maillard reaction, other pathways can also lead to form AGEs. For instance, the autoxidation of glucose and the peroxidation of lipids into dicarbonyls derivatives by an increase in oxidative stress is another pathway described for the formation of AGEs. These dicarbonyl derivatives known as α-oxaldehydes (glyoxal, methylglyoxal and 3-deoxyglucosone) can interact with monoacids and form AGEs. Among the most widely studied AGEs are carboxymethyl-lysine (CML), pentosidine and pyrraline and together with methylglyoxal (an α-oxaldehyde), they have been used as biomarkers for *in vivo* formation of AGEs (Luevano-Contreras and Chapman-Novakofski, 2010). AGEs accumulate within the various organs that are damaged in diabetes, with the accumulation rate of these AGEs accelerated by hyperglycemia. The intermolecular collagen cross-linking caused by AGEs leads to diminished arterial and myocardial compliance and increased vascular stiffness, phenomena that are considered to explain partly the increase in diastolic dysfunction and systolic hypertension seen in diabetic subjects. AGEs accumulate in most sites of diabetes complications, including the kidney, retina and atherosclerotic plaques (Goh and Cooper, 2008).

Furthermore, AGEs are likely to be linked to atherosclerosis in multiple ways, including enhancing endothelial dysfunction, elevating vascular low-density lipoprotein (LDL) levels by reducing LDL uptake, promoting plaque destabilization via effects on matrix metalloproteinases, inducing neointimal proliferation and inhibiting vascular repair in response to injury. Serum levels of AGEs have increased in patients with type 2 diabetes and coronary heart disease. Futhermore, AGEs have been localized to atherosclerotic lesions, fatty streaks, lipid-containing smooth muscle cells and macrophages in individuals with diabetes (Goh and Cooper, 2008).

2.2.2 Atherosclerosis

Atherosclerosis is the most common pathological process that leads to cardiovascular diseases (CVD), a disease of large- and medium-sized arterized by a formation of atherosclerotic plaques of necrotic cores, calcified regions, accumulated modified lipids, inflamed smooth muscle cell (SMCs), endothelial cell (ECs), leukocytes and foam cells. These features of atherosclerotic plaques illustrate that atherosclerosis is a complex disease, and many components of the vascular, metabolic and immune system are involved in this process. Although low-density lipoprotein (LDL) remains the most important risk factor for atherosclerosis, immune and inflammatory mechanism of atherosclerosis that have gained tremendous interest in the past (Galkina and Ley, 2009). Atherosclerotic is accelerated in patients with diabetes and in those with impaired fasting glucose and insulin-resistant states, resulting in a 2- to 4-fold increase in cardiovascular events. Disorders of lipoprotein metabolism are thought to play a major role, as are abnormalities of the vessel wall (Mills and Grant, 2002).

Cholesterol is the major component of atherosclerotic plaque. Cholesterol accumulation within atherosclerotic plaque occurs, when cholesterol influx into the arterial wall (from apoB-containg lipoproteis) exceeds cholesterol efflux. Increased influx of cholesterol into the arterial wall is accompanied by an increased influx of monocytes/macrophages, which take up oxidized and aggregated LDL and store the cholesterol as ester (Spady, 2012).

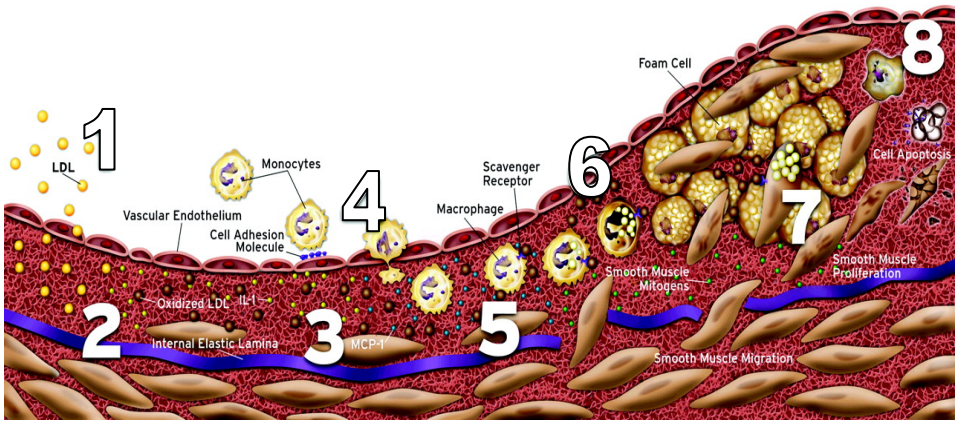

Figure 2.3 The progression of atherosclerosis. (1) Lipoprotein particles and especially LDL enter the arterial wall and undergo modification, including oxidation. (2) These oxidized lipoproteins incite a cascade of cellular responses. (3) One integral early step in atherosclerosis is the induction of adhesion molecules in the endothelium. (4) Through adhesion molecules, leukocytes attach and ultimately enter the arterial wall and attracted to these sites by chemokines. (5) Monocytes in the vessel wall take up oxidized lipoproteins, inducing their differentiation into lipid-laden foam cells (6 and 7) VSMCs migrating out of the media and into the intima. In the intima and the media, VSMCs also proliferate and elaborate extracellular matrix that contributes to the formation of the early atherosclerotic lesions. (8) Later stages of atherosclerosis involve more complicated plaques (Pltzky, 2003).

Atherosclerosis involves several highly interrelated processes that shown schematically in **Figure 2.3** and described in detail below. The earliest deposit of lipid in the intima is called a fatty streak and this appears as a yellow, slightly raised area on the luminal surface that enlarges and comes together to form irregular yellow streaks. The fatty streak contains accumulations of lipid droplets beneath the endothelium that are free and within macrophages (Ross, 1993). The endothelium is a dynamic monolayer lining the blood vessel wall and is responsible for maintaining vascular tone through the production of vasodilators (*e.g.* nitric oxide; NO) and vasoconstrictors (*e.g.* endothelin-1). As well as its vasodilation properties, NO

regulates SMC migration and growth and inhibits inflammation and platelet aggregation (Ross, 1999). The central component of the oxidation hypothesis is the oxidative modification of LDL, which acts as an immunogenic stimulus for monocyte recruitment to the vessel wall and phagocytic uptake of oxidized LDL (oxLDL) by macrophages. The process of oxidation of cellular components and particles such as lipoproteins is thought to exacerbate atherosclerosis, although this is generally a feature of late-stage lesion. (Witztum, 1997).

2.2.3 Gestational Diabetes Mellitus (GDM)

Gestational diabetes, defined as “carbohydrate intolerance of variable severity with onset or first recognition during pregnancy”, starts when the body is not able to make and use all the insulin it needs for pregnancy. Without enough insulin, glucose cannot leave the blood and be changed to energy. Gestational diabetes affects the mother in late pregnancy, after the baby’s body has been formed, but while the baby is busy growing. When the body has gestational diabetes, pancreas works overtime to produce insulin, but the insulin does not decrease blood glucose level. So extra blood glucose through the placenta and giving the baby high blood glucose levels. This causes the baby’s pancreas to make extra insulin to get rid of the blood glucose. Since the baby is getting more energy than it needs to grow and develop and then the extra energy is stored as fat (Garner, 1995).

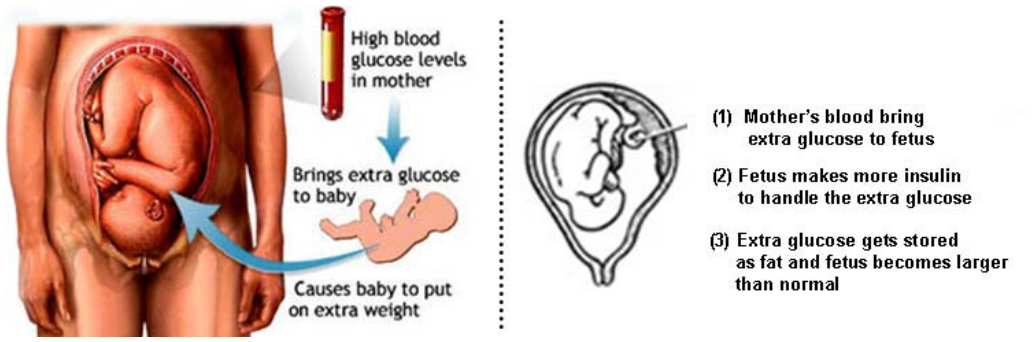

Figure 2.4 The relation between pregnancy, diabetes and gastro-mocrobiome (Available source: http://www.gestationaldiabetestest.org/).

Women with GDM have a greater severity of insulin resistance compared to the insulin resistance seen in normal pregnancies. They also have an impairment of the compensatory increase in insulin secretion; particularly first-phase insulin release may be a marker for deterioration of β-cell function. The insulin resistance is likely to play a role in ensuring that the fetus has an adequate supply of glucose by changing the maternal energy metabolism from carbohydrates to lipids (Sugiyama, 2011). Skeletal muscle is the principal site of whole-body glucose disposal and along with adipose tissue becomes severely insulin resistant during the latter half of pregnancy. In adipose tissue, the lipogenic transcription factor PPAR-γ declines in obese women during pregnancy and may shift genes in metabolic pathways to favor increased lipolysis, thus accelerating adipose tissue insulin resistance and the switch from lipid storage to lipolysis. This transition to insulin resistance contributes to greater postprandial increases in postprandial free fatty acid (FFA) and increased hepatic glucose production and results in greater fuel availability to the fetus of women with GDM. A number of risk factors have been associated with a greater likehood of developing gestational diabetes. By and large these are the same factors

that predict overt diabetes and they include advanced maternal age, a family history of diabetes in first-degree relative, obesity and glycosuria (Barbour *et al*, 2007).

2.2.4 Oxygen Radical Scavening in Human Endothelial Cells

Oxygen derived species are well known to be cytotoxic and have been implicated in the etiology of a wide array of human diseases. Hydrogen peroxide and superoxide anion are the major source of endogenous ROS (reactive oxygen species) in living cells, which are generated as products of cellular metabolism (Waris and Ahson, 2006). The endothelium is a single layer of cells that lines the inner surface of all blood vessels and the heart, forming an important interface with circulating blood. This tissue has well-established role in cardiovascular homoeostasis, including autocrine, endocrine and immunological function of the inflammatory process through increases in cell adhesiveness and permeability and the maintenance of blood fluidity (Ray and Shah, 2005). In mammalian cells, several differentially localized and expressed enzyme systems contribute to ROS formation, including endothelial NO synthases, enzymes of the respiratory chain, cytochrome P_{450} monoxygenases, xanthine oxidase and NADPH oxidases. Although all of these enzymes contribute to the oxidative burden, the evidence is accumulating that an initial generation of ROS by NADPH oxidase triggers the release of ROS by the other enzymes. Consequently, the vascular NADPH oxidases have been suggested to have a role for the development of hypertension, endothelial dysfunction, arteriosclerosis, restenosis and hypertrophy (Brandes and Kreuzer, 2005). However, following on from the seminal observation by Griendling *et al*. (1994) that vascular

smooth muscle cells also display NADPH oxidase activity, it became apparent that these enzymes are also expressed in a variety of non-phagocytic cells, including all cells present in the blood vessel wall, *i.e*. endothelial cells, smooth muscle cells and adventitial fibroblasts.

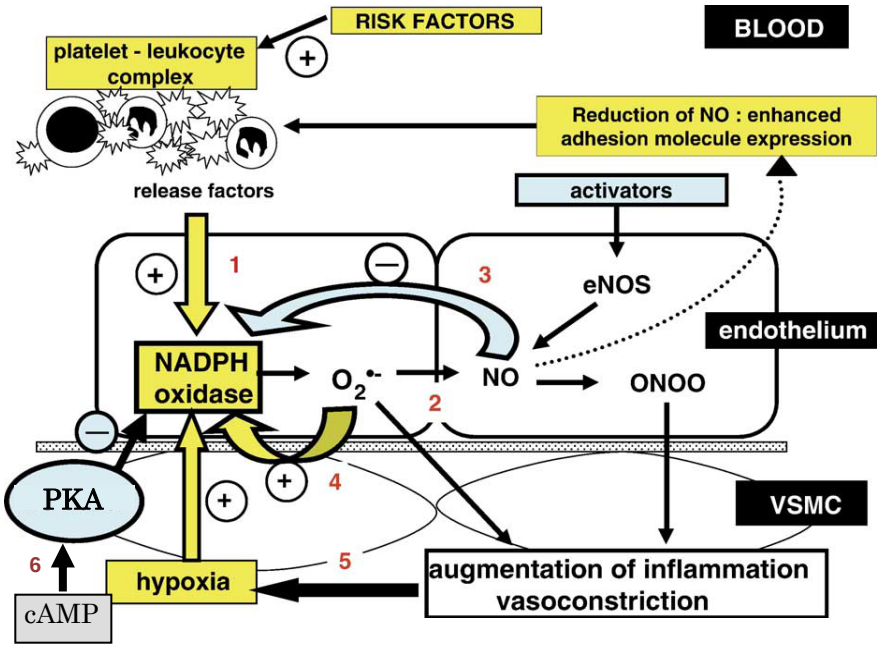

Figure 2.5 Model of the pathologic events leading to increased NADPH oxidase expression in vascular tissue. (1) Risk factors promote the adhesion of blood cells to endothelium. These release battery of factors (including cytokines and eicosanoids) that uppregulate the expression of NADPH oxidase, thereby increasing the endogenous formation of superoxide ($O_2^{\bullet-}$). (2) $O_2^{\bullet-}$ promotes inflammation and vasoconstriction in its own right, but also reduces NO derived from eNOS to form reactive nitrogen species (RNS), including peroxynitrite ($ONOO^-$). (3) NO suppresses the expression of NADPH oxidase, this reduction of NO results in further augmentation of NADPH oxidase expression and activity. (4) $O_2^{\bullet-}$ itself induces the expression of NADPH oxidase. (5) These inflammatory events may lead to tissue hypoxia, which also upregulates the expression of NADPH oxidase. (6) Cyclic adenosine monophosphate (cAMP) may inhibit the expression of NADPH oxidase via the cAMP-protein kinase A (PKA) (Muzaffar *et al*., 2005).

The cellular distribution of NADPH oxide isoform is in the vascular wall (**Figure 2.5**). NO produced by eNOS not only reacts with superoxide ($^1O_2$) to produce the reactive species peroxynitrite ($OONO^-$), it may also act to suppress NADPH oxidase activation. Beside, NADPH oxidase-derived superoxide contributes to the impairment of endothelium-dependent vasodilation by inactivating nitric oxide; the resultant endothelial dysfunction is implicated in the pathophysiology of diseases such as atherosclerosis, hypertension, diabetic vasculopathy and heart failure (Halcox *et al*., 2002).

2.2.5 The Relation of Diabetes, Atherosclerosis and Others with Skin Diseases

Hattem *et al*. (2008) reported that almost all diabetes patients eventually develop skin complications from the long-term effects of diabetes on the microcirculation and on skin collagen. Cutaneous infections are more common in type 2 diabetes, whereas autoimmune-related lesions are more common in type 1. The people with diabetes having too much glucose (sugar) in their blood for a long time can cause serious complication, including skin problem such as scleredema diabeticorum, this condition causes a thickening of the skin on the back of the neck and upper back. This condition is rare but can affect people with type 2 diabetes (Cole *et al*., 1983). Beside, vitiligo is a condition that affects skin coloration; the special cells that make pigment (the substance that controls skin color) are destroyed, resulting in patches of discolored skin. This condition is more commonly associated with type 1 diabetes (Cunliffe *et al*., 1968). Furthermore, atherosclerosis is the narrowing of blood vessels from a thickening of the vessel walls due to plaque buildup. While atherosclerosis most often is associated with blood vessels in or near

the heart, it can affect blood vessels in the body, including those that supply blood to the skin. When the blood vessels supplying the skin become narrow, changes occur to the skin due to a lack of oxygen, such as hair loss, thinning and increased shininess of skin (Tao *et al*., 1971). Reactive oxygen species (ROS) are well known to be involved in skin diseases, that are generated both from endogenous sources, such as enzyme activity or activated neutrophils and external pro-oxidant stimuli, such as ultraviolet radiation (UV). ROS-mediated oxidative damage involves a vast number of biological molecules since it cause DNA modification, lipid peroxidation and secretion of inflammatory cytokines (Briganti and Picardo, 2003). Many kinds of researches have been studied on skin diseases such as measuring enzymatic and antioxidant activities *in vivo*, defense mechanisms in animal cells *in vitro*.

2.3 The objectives of this study

Diabetes is a disease that is the most common endocrine disorder affecting around 2-3% of the population worldwide and its incidence is rising. In the year 2000, 150 million people world-wide had diabetes and is expected to double by this year, By 2030, the WHO estimates that the number of people with diabetes will almost double to 366 million (Joshi and Jasuja, 2012). These drawbacks give the aim of this study to design and develop novel drug delivery system for overcomeing the transport barriers, inherent elimination and metabolism problems associated with these anti-diabetes drugs. On the other hand, *Alpinia zerumbet* is the fast growth plant and strong resistance to pest and diseases. This species grows abundantly in the Ryukyu Island and have been a traditionally important and economic plant in

Okinawa (Tawata *et al*., 2008). This plant has been reported to contain numerous interesting bioactive constituents. Thus, *A. zerumbet* was chosen for this study.

The main objective of this study was to investigate the medicinal properties of *A. zermbet* for preventing diabetes mellitus diseases. Considering all these study focused on three aspects:

i) To investigate medicinal properties of *A. zerumbet*, specific on diabetes and other related-diseases, including AGEs inhibition, antiatherosclrosis, antigestational diabetes and anti-skin disease.

ii) To identify several secondary metabolites associated with these properties using HPLC, LC-MS, GC-MS, IR spectroscopy and NMR techniques.

iii) To study the effect of active compounds that isolated from *A. zerumbet* on oxygen radical scavenging using human umbilical vein endothelial cell (HUVEC).

CHAPTER 3

ADVANCED GLYCATION END PRODUCTS INHIBITORS FROM *A. zerumbet*

3.1 SUMMARY

Advanced glycation end products (AGEs) are major factors responsible for the complication of diabetes. The present study was carried out to investigate the inhibitory activities on fructosamine adduct and α-dicarbonyl formations by hexane extract of various parts of *A. zerumbet*. Furthermore, Two previously known compounds were isolated, namely 5,6-dehydrokawain (DK) and dihydro-5,6-dehydrokawain (DDK). For 8(17),12-Labdadiene-15,16-dial (labdadiene), this compound was isolated for the first time from the rhizome of *A. zerumbet*. The results showed that labdadiene (IC_{50} = 51.06 μg/mL) had similar activity to rutin and quercetin against fructosamine adduct. The inhibition of α-dicarbonyl compounds formation by labdadiene was significantly higher than DK and DDK. These results indicate that labdadiene is a potent antiglycation agent and that it suggests having three different steps inhibiting AGEs formation in the pathway. These data indicate that labdadiene could be used to prevent glycation-associated complications in diabetes.

3.2 INTRODUCTION

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 related to hyperglycaemia 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 dicarbonyl 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's 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* factor such as the catabolism of ketone bodies and threonine, and lipid peroxidation (Thornalley *et al.*, 1999). Therefore, the inhibitory effect on fructosamine adducts and α-dicarbonyl could be correlated to restrain AGEs formations and lessen the chance of diabetic complications.

Alpinia zerumbet (family: Zingiberaceae) is a perennial plant growing widely in the subtropical and tropical regions. It is used in traditional medicine for its anti-inflammatory, bacterostatic and fungistatic properties (Zoghbi *et al.*, 1999). DDK and DK have been detected in the rhizomes of *Alpinia speciosa* (Itokawa *et al.*, 1981). Furthermore, DK and DDK are reported to inhibit the aggregation of ATP released from rabbit platelets (Teng *et al.*, 1990). DK and DDK are described to have antiulcerogenic and antithrombotic activities (Mpalantinos *et al.*, 1998). The inhibitory effects of DK on human platelet aggregation, anti-inflammatory and cancer chemopreventive therapeutic properties are reported (Jantan *et al.*, 2008). Labdadiene, isolated from the Zingiberaceae (Sirat *et al.*, 1994), 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 (de Oliveira *et al.*, 2006). Moreover, labdadiene has also been reported to inhibit lipid

peroxidation, cyclooxygenase enzymes and human tumour cell proliferation (Liu and Nair, 2011). Our laboratory has reported DDK and phenolic compounds and their antioxidant activities in leaves and rhizomes of *A. zerumbet* (Elzaawely *et al.*, 2007). Recently, Upadhyay *et al.* (2011) have reported the HIV-1 integrase and neuraminidase inhibitory properties of *A. zerumbet* and have found that DK and DDK have significant bioactivities.

In this study, the AGEs formation inhibitory properties of hexane extracts from six different parts of *A. zerumbet* were primarily investigated. Since the bioactivities of DK and DDK were previously found against HIV-1 integrase and neuraminidase activities (Upadhyay *et al*., 2011), these compounds were focused on AGEs inhibitory properties. Furthermore, labdadiene was isolated for the first time from *A. zerumbet* and investigated its activities. The inhibition of fructosamine adducts and α-dicarbonyl formation in order to check the efficacy against AGEs. To the best of our knowledge, this is the maiden report on AGEs inhibition from *A. zerumbet* and the isolated compounds.

3.3 MATERIALS AND METHODS

3.3.1 Chemicals

Bovine serum albumin (BSA), copper (II) sulfate, D(+)-glucose, 2,4-dinitrophenyl hydrazine (DNPH), heptafluorobutylric acid (HFBA), 40% glyoxal solution, nitro blue tetrazolium chloride (NBT), sodium azide, and guanidine were obtained from Wako Pure Chemical Industries, Ltd., (Japan). Trichloroacetic acid (TCA) was purchased from Sigma-Aldrich, Inc. (Germany). Trimetylaminoacetohydrazide chloride (Girard's

reagent T) was bought from Tokyo Chemical Industry Co. Ltd. (Japan). Alkaline PBS (137 mM NaCl, 8.1 mM Na_2HPO_4, 2.68 mM KCl, 1.47 mM KH_2PO_4) was adjusted to pH 10 with NaOH.

3.3.2 Preparation of crude extracts and isolated compounds

The parts of *A. zerumbet* (including rhizomes, stems, leaves, flowers, pericarps and seeds) were collected at University of the Ryukyus, Okinawa, Japan. The samples were oven dried and ground to fine power. Fifteen grams each dried samples were soaked in 400 mL of hexane for 24 h. The extracts were filtered through filter paper and evaporated under vacuum to remove the solvent. The crude extracts were kept at 4^{o}C until using for experiments. DK and DDK were isolated from the rhizomes as the methods reported previously (Upadhyay *et al.*, 2011). The compound of labdadiene was isolated from the hexane extract of the rhizomes. Briefly, the dried rhizomes were extracted with hexane for 48 h and the crude hexane extract was filtered and dried under vacuum at 40^{o}C. The crude extract was further separated using silica gel column chromatography using hexane: acetone (9:1). The aliquots from column chromatography were separated using Diaion HP-20 resin column (Mitsubishi Chemical Co., Japan) with gradient methanol concentration from 50-100%, and further purified by HPLC to get labdadiene. Labdadiene was collected at 235 nm using a COSMOSIL HPLC (Vision Workstation Biocad Family, USA) (10 mm i.d. x 250 mm; 5 μm particle size). The mobile phase was water (solvent A) and methanol (solvent B) at flow rate of 2 ml/min. The gradient elution was performed as follows: 0-10 min, 80% B isocratic; 10-20 min, linear gradient 80-100% B, 20-40 min, 100% B isocratic. The chemical structures of isolated compounds were identified using GC-MS and NMR (**Figure 3.1**).

The ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) spectra were recorded on a Bruker Biospin (Germany) 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 on TopSpin 2.1 program Version 2.1.0. Electron ionization mass spectrometry (EIMS): *m/z* (rel. int.); 302 (20), 137 (100), 123 (50), 109 (35), 95 (73), 81 (70), 69 (55), 55 (48), 41 (50). ^{1}H ($CDCl_3$): δ 0.73, 0.82 and 0.89 (*s*, each 3H, *CH_3*, 18, 19, 20), 1.04-2.52 (*m*, 14H, *CH_2*, *CH*, 1, 2, 3, 5, 6, 7, 9, 10, 11), 3.44 (*s*, 2H, *CH_2*, 14), 4.37 (*s*, 1H, *CH_2*, 17), 4.86 (*s*, 1H, *CH_2*, 17), 6.76 (*t*, 1H, *CH*, 12), 9.40 (*s*, 1H, *CHO*, 15) and 9.63 (*s*, 1H, *CHO*, 16); ^{13}C NMR ($CDCL_3$): δ 14.4 (C-20), 19.3 (C-2), 21.7 (C-19), 24.1 (C-6), 33.6 (C-4), 37.9 (C-14), 39.3 (C-1), 39.4 (C-7), 39.6 (C-10), 42.0 (C-3), 55.4 (C-5), 56.5 (C-9), 107.8 (C-17), 134.9 (C-13), 148.0 (C-8), 159.9 (C-12), 193.5 (C-16) and 197.3 (C-15).

3.3.3 Quantification of DK, DDK and labdadiene using HPLC

The isolated compounds were measured using a Shimadzu HPLC (SCL-10A vp, Shimadzu Co., Kyoto, Japan) coupled with a UV vis detector (SPD-20A, Shimadzu). Separations were achieved on a TSKgel ODS-100Z column (Agilent Technologies, USA) (15 cm x 4.6 mm i.d.; 5 μm particle size). The mobile phase was water with 1% acetic acid (v/v) (solvent A) and methanol with 1% acetic acid (solvent B) at a flow rate of 0.8 mL/min. DK and DDK were measured at 280 nm. The gradient elution was performed as follows: 0-10 min, 50% B isocratic; 10-20 min, linear gradient 50-100% B and 20-26 min, 100-50% B. The gradient elution for labdadiene was performed as follows: 0-10 min, 80% B isocratic, 10-20 min, linear gradient 80-100% B, and 20-35 min, 100% B and monitored at 235 nm. A 5 μL methanolic solution of crude extracts at

1 mg/mL was used and the identification of the compounds (**Figure 3.1**). The quantification of each compound was determined based on peak area measurements.

3.3.4 Preparation of AGEs- BSA formation

Each sample in this experiment was dissolved in DMSO. The reaction mixtures containing 400 μg bovine serum albumin, 200 mM glucose, 10 μL of test sample and 50 mM phosphate buffer (pH 7.4) to a total volume of 500 μL, were incubated at 60^{o}C for 30 h. The blank, which contained the protein and glucose except test sample, was kept at 4^{o}C until measurement (Matsuura *et al.*, 2002).

3.3.5 Measurement of glycation of BSA

AGEs-BSA aliquots or glycated materials of 100 μL were transferred to 1.5 mL plastic tubes, and 10 μL of 100% (w/v) TCA was added to each tube. The supernatant containing glucose, inhibitor and the interfering substances was removed after centrifugation (15000 rpm, 4^{o}C, 4 min) (Wu *et al.*, 2009). Then, the precipitate of AGEs-BSA was dissolved with 100 μL of PBS to serve for screening. The comparison of fluorescence spectrum (excitation 360 nm) and the change in fluorescence intensity (excitation 360 nm, and emission 450 nm) based on AGEs were monitored using spectrofluorometer (GENios-TECAN, France). Percent inhibition of AGEs formation by each extract or compound was calculated using the following equation:

$$\text{Inhibitory activities (\%)} = [1 - (F_i / F_0)] \times 100,$$

where F_i is fluorescence of solution with inhibitors and F_0 is fluorescence of the solution without inhibitors. The concentration required for 50% inhibition (IC_{50}) of the fluorescence intensity was determined graphically.

3.3.6 Determination of fructosamine adduct

The fructosamine adduct was determined by using NBT assay as the method described by Baker *et al.* (1994). Aliquots of glycated materials 40 μL were added to reaction mixture which contained 160 μL of NBT reagent (300 μM) in sodium carbonate buffer (100 mM, pH 10.35), incubated at room temperature for 30 min, and measured absorbance at 530 nm using microplate reader (BioRad Benchmark microplate reader, UK). The percent inhibition of fructosamine produced by compounds was calculated using the following equation:

$$\text{Inhibitory activity (\%)} = [(A_c - A_t)/ A_c] \times 100,$$

where A_c is absorbance values of control and A_t is absorbance values of test samples.

3.3.7 Assay of glycation-induced protein oxidation

Sample preparation for glucose-induced protein oxidation was done as the method described by Takagi *et al.* (1995). BSA dissolved to a concentration of 10 mg/mL (150 μM) in 0.1 M sodium phosphate buffer containing 3 mM sodium azide, was incubated with 100 mM of glucose at 37^{o}C for 2 weeks. As a control, BSA was incubated in the absence of sugar. Protein carbonyl content as an indicator of protein oxidation was measured in a manner similar to that described previously (Levine *et al*., 1990). Brifely, the sample 100 μL was incubated with 500 μL of 10 mM DNPH in 2 M HCl at room temperature for 1 h. Albumin containing carbonyl residues that were chemically reacted with DNPH were precipitated with 20% TCA. The precipitate was washed three times with 1 mL of ethanol: ethylacetate (1:1) to remove unreacted DNPH and dissolved in 1 mL of 20 mM potassium phosphate buffer containing 6 M of guanidine (adjusted to pH 2.3 with trifluoroacetate). The absorbance of the DNPH-attached albumin was measured

at 374 nm using microplate reader (BioRad Benchmark microplate reader, UK). The carbonyl content was calculated based on the molar extinction coefficient of DNPH (ε = 2.2 x 10^4 cm^{-1} M^{-1}):

$$A = \varepsilon cl,$$

where *A* is the absorbance of the sample, *l* is the pathlength, and *c* is the concentration of the absorbing species in the material (moles per liter).

3.3.8 Measurement of α-dicarbonyl compounds formation

α-Dicarbonyl compounds formation was measured as reported previously with slight modifications (Mitchel and Birnbiom, 1977). Briefly, 100 μL of aliquots of glycated materials were incubated at room temperature for 1 h with reaction mixture containing 50 μL of Girard-T stock solution (500 mM) and 850 μL of sodium formate (500 mM, pH 2.9). Absorbance was measured at 290 nm using spectrophotometer (UV mini 1240, Shimadzu, Japan), and glyoxal contents were calculated using a standard curve for glyoxal. A calibration curve was prepared using 40% glyoxal solution in a similar way.

3.3.9 HPLC analyses of glyoxal content

Aliquots of glucose reaction 1 mL were initially prepared for HPLC analysis by adding 300 μL of 2 M HCl (pH 3.0) and 50 μL of Girard-T stock solution and then incubating at room temperature for 24 h to allow sufficient time for product formation from slow reacting dicarbonyl compound, such as glyoxal. Aliquots, 2 μL, were analyzed by reversed phase HPLC, using TSKgel-NH2-100 column (Tosoh Bioscience LLC, USA), 5 cm x 2.0 mm i.d., 3 μm particle size, with absorbance

detection at 280 nm. An isocratic solution of 14% acetonitrile in water containing 0.25% HFBA for 10 min was used as eluting solvent (Well-Knecht *et al.*, 1995). The amounts of glyoxal content in different samples of varying concentrations were calculated from the standard curve ($y = 2525707x + 125777$; $R^2 = 0.993$). The inhibition percent is calculated as

$$\text{Inhibition activity (\%)} = (Ac\text{-}As)/Gc \times 100,$$

where A_c is absorbance values of control and A_s is absorbance values of test samples. The IC_{50} is the concentration of sample at which glyoxal formation was reduced by 50%.

3.3.10 Statistical analysis

The data were analyzed by one-way ANOVA. Upon significant difference, mean were separated using Tukey HSD range test at $p < 0.01$ with 3 replications. In some cases, only means and standard error of the sample means were presented. All statistical analyses were performed using SPSS version 16.0 for windows Vista.

3.4 RESULTS

3.4.1 The amount of DK, DDK and labdadiene

The amount of DK, DDK and labdadiene in the crude extracts from different parts of *A. zerumbet* is shown in **Table 3.1**. DK and DDK were found in all parts of the plant. The rhizomes extract contained higher amount of DK (3.13 mg/g hexane extract), however, flowers extract contained more quantity of DDK (6.08 mg/g hexane extract). For the amount of labdadiene, the hexane extract of rhizomes

contained higher amount of ladadiene (3.97 mg/g hexane extracts) than that of seeds and pericarp (0.35 and 2.91 mg/g hexane extracts, respectively). However, the hexane extracts of leaves and flowers did not contain labdadiene.

3.4.2 Inhibitory effect of crude extracts and isolated compounds on glycation of BSA

The ability of hexane extracts from various parts of *A. zerumbet* to inhibit AGEs formation was evaluated using the BSA-glucose assay, in which bovine serum albumin served as the model protein while glucose as glycating agent. The results showed that rhizomes had the highest inhibitory activity than other parts (**Table 3.2**). On carrying out inhibition by isolated compounds, DK (IC_{50} = 15.86 μg/mL) had stronger inhibitory activity than labdadiene and DDK (IC_{50} = 108.28 and 194.47 μg/mL, respectively). On statistical analysis of the results as shown in **Figure 3.2A**, it was found that DK was not significantly different than the two positive controls rutin and quercetin (IC_{50} = 9.65 and 15.89 μg/mL, respectively) ($p < 0.01$).

3.4.3 Inhibitory effect of crude extracts and isolated compounds on the fructosamine adduct

The results of inhibitory effect of hexane extracts on fructosamine adduct showed that the IC_{50} of crude extracts from rhizomes and leaves on fructosamine adduct were stronger than stems, flowers, pericarps and seeds (**Table 3.2**). Labdadiene (IC_{50} = 51.06 μg/mL) had higher inhibitory effects than DK and DDK (IC_{50} = 360.16 and 673.41 μg/mL, respectively) (**Figure 3.2B**). Moreover, labdadeine had similar activity against fructosamine adduct formation with positive controls ($p < 0.01$).

3.4.4 Inhibitory effect of crude extracts and isolated compounds on glycation-induced protein oxidation

Oxidative modifications of BSA preparations were demonstrated using a combination of carbonyl assay; the extent of protein carbonyl formation was determined by DNPH-reagent. The inhibitory effects of crude extracts are shown in **Table 3.2**. The hexane extract of flower showed the strongest activity. The inhibitory effects of isolated compounds are shown in **Figure 3.2C**. Among the isolated compounds, labdadiene had stronger inhibition than DK and DDK.

3.4.5 Inhibitory effect of crude extracts and isolated compounds on the α-dicarbonyl compounds formation

The ability of crude extracts to inhibit α-dicarbonyl compounds formation decreased in the order flowers, rhizomes, pericarps and stems and seeds, and leaves, respectively (**Table 3.2**). The inhibitory effects of the test samples on the intensity of α-dicarbonyl compound formation were calculated using a standard curve for glyoxal (**Figure 3.3A**). The effect of isolated compounds on this process is shown in **Figure 3.3B**. The reductions of α-dicarbonyl compounds formation by labdadiene showed better inhibitory activity than DK and DDK. Moreover, labdadiene had similar activity against α-dicarbonyl compounds formation with positive controls ($p < 0.01$).

3.4.6 Identification of glyoxal content by HPLC

In order to identify the dicarbonyl sugar present in glucose incubations, a reversed phase HPLC assay was done to separate the Girard-T adduct with single peak of glyoxal as shown in **Figure 3.4**. The inhibitory effect of different parts of *A. zerumbet*

showed that rhizomes had lower glyoxal content than other parts (**Table 3.2**). The ability of isolated compounds on glyoxal content showed that labdadiene had stronger inhibition effect than DK and DDK.

3.5 DISCUSSION

The present research examines the effect of hexane extract from different parts of *A. zerumbet* and the compounds isolated from the rhizomes against AGEs formation. Researchers have focused on anti-AGEs activities of phenolic compounds of different plant extracts using high polar solvents. However, in this study hexane was chosen as the solvent for extracting bioactive phytochemicals from *A. zerumbet*. Hexane would aid in better isolation of the target compounds including DK, DDK and labdadiene.

It was found that DK and DDK were present in all six different parts of plants while labdadiene was present only in rhizomes, pericarps and seeds. Previous reports have shown that DK and DDK were found in hexane extract of rhizomes and leaves (Elzaawely *et al.* , 2007b) and in chloroform extract of flowers and seed (Elzaawely *et al.*, 2007a). Labdadiene has been reported to be present in seeds of *Alpinia galangal* (Morita and Itokawa, 1988). Another study on seeds of *A. zerumbet* has revealed the presence of labdadiene like structure (Xu *et al.*, 1996). The authors have reported the presence of carboxylic group in C-16. However, in this study isolated labdadiene with aldehyde group in C-16 as revealed from ^{1}H and ^{13}C NMR data.

The rhizomes and flowers extract had better results amongst all the tested parts. All different experiments performed, the rhizome extract inhibited the glycation of

BSA, formation of fructosamine adducts and glyoxal, while the flower exhibited better inhibitory properties against α-dicarbonyl formation and glycation-induced protein oxidation. The reasons for variations in the efficacy of different parts of *A. zerumbet* against AGEs formations are still under investigations. However, it seems that rhizome extract containing high amounts of DK and labdadiene may be the reasons for its inhibitory properties. Interestingly, though the flower extract did not contain any labdadiene, it showed superior inhibition against dicarbonyl formation and glycation-induced protein oxidation. The plausible explanation for this is unclear, however, it appears that there must be some other non-polar compounds besides DK and labdadiene in alpinia that have AGEs formation inhibition capacity. The isolation of other active compounds against AGEs formations from rhizomes and flower are being undertaken in our laboratory.

Furthermore, it is noteworthy that each inhibition of dicarbonyl compounds formation carried out with two different methods, spectrophotometric and HPLC, showed different results for crude extracts. It has been reported that only glyoxal is identified using HPLC methods, although several dicarbonyl compounds are formed during autooxidation of glucose (Wells-Knecht *et al.*, 1995). In case of spectrophotometric assays, flower extract have inhibited dicarbonyl formation more than that of rhizomes extract, while the latter has shown to inhibit glyoxal formation as indicated by the HPLC results. Furthermore, the flower extract did not contain labdadiene, which had the most activity against dicarbonyl formation in both the methods. Hence, it appears that the flower extract may contain some other compounds which are capable of inhibiting the formation of other dicarbonyl sugars such as glucosone to a greater extent than the formation of glyoxal. However, it is

also possible that the presence of more than one inhibitor in an extract may have synergistic effect, therefore further researches are necessary in order to assure that DK and/or labdadiene alone is responsible for AGEs inhibition.

The isolated compounds had different degree of activities against AGEs formation when examined using various assays. AGEs formations inhibition assay was examined by checking glycation of BSA. Glycation (nonenzymatic glycosylation) processes, also known as Maillard reaction, are a series of reactions between reducing sugars and N-terminal group of proteins in early glycation of AGEs formation (Singh *et al.*, 2001). DK had stronger reduction of reactions forming Schiff's base than labdadiene and DDK.

In order to further verify AGEs inhibition, the amount of fructosamine formed was investigated using a colorimetric assay. Fructosamine is an Amadori's product formed by the glycation of amino acid via Schiff's base (Singh *et al.*, 2001). This assay is a colorimetric test that determines the degree of glycation of serum proteins by measuring the reducing activity of serum in alkaline solution. NBT^{2+} is reduced to a stable monoformazon dye by the stepwise addition of two electrons, with the formation of the transient tetrazolinyl free radical, $NBT^{+\bullet}$ (Bielski *et al.*, 1980). Glycation-induced protein oxidation was further investigated. Reducing monosaccharides, such as glucose or fructose, can nonenzymatically react with amino groups of proteins and produce stable ketoamine products. The ketoamine can produce highly reactive hydroxyl radicals, by a process known as the Fenton reaction, which induces oxidative protein degradation (Takagi *et al.*, 1995). Finally, the amount of dicarbonyl compound (glyoxal) formed due to degradation of glucose was measured using Girard-T reagent and HPLC analysis (Wells-Knecht *et al.*, 1995).

Among the isolated compounds, labdadiene exhibited the strongest activity in four of the performed assays while DK had better results in the glycation of BSA. It is possible that the aldehyde groups of labdadiene has a significant role in inhibiting AGEs formation. These aldehyde groups may compete with sugars for Schiff's bases formation and limit the amount of amines available for glucose attachment. This study agrees with previous report which have shown the possiblitiy for aldehyde group containing compounds such as pyridoxal 5′-phosphate (an active form of vitamin B6) to inhibit AGEs formation (Nakamura *et al.,* 2007).

The fructosamine adduct assay revealed that labdadiene has strong activity when compared with the reported positive controls rutin and quercetin. However the mechanism of inhibition is likely to be different. The metal chelation by the vicinyl dihydroxyl groups of these flavonoids and/or entrapment of reactive amino group are supposed to have inhibiting roles (Morel *et al.,* 1993; Cervantes-Laurean *et al.,* 2006).

In all different forms of inhibition of AGEs by the isolated compounds, it was shown that these compounds have inhibitory activity at different steps of AGEs formation pathways. The isolated compounds inhibited glycation of amino group, the first step in this pathway. Furthermore, these compounds suppressed the formation of Amadori's products thereby reducing AGEs formation. Finally, these compounds also reduced dicarbonyl compounds formation which is the result of glucose degradation or glycation-induced protein oxidation. Therefore, our study revealed that the isolated compounds from *A. zerumbet* had three fold activities in inhibiting AGEs formation.

3.6 CONCLUSION

The rhizomes of *A. zerumbet* have several metabolites that have AGEs inhibitory properties. Two kawain were isolated, DK and DDK, and for the first time isolated labdadiene from the rhizomes of *A. zerumbet*. The results showed three-fold inhibitory properties of these compounds against AGEs formation. It was found that labdadiene showed the strongest activity and had significant results when compared with rutin and quercetin. Thus, labdadiene could be used as a preventive measure against glycation-associated complications in diabetes.

Table 3.1

Amount of DK, DDK and labdadiene in hexane extracts from different parts of *A. zerumbet.*

Sample	Amount of compound (mg/ g hexane extracts)		
	DK	DDK	Labdadiene
Rhizomes	3.13 ± 0.02 a [1]	5.41 ± 0.02 b	3.97 ± 0.02 a
Stems	2.08 ± 0.07 b	3.70 ± 0.01 c	0.00 ± 0.00 d
Leaves	1.67 ± 0.06 c	3.38 ± 0.03 d	0.00 ± 0.00 d
Flowers	2.22 ± 0.01 b	6.08 ± 0.06 a	0.00 ± 0.00 d
Pericarps	1.58 ± 0.07 c	0.13 ± 0.01 e	0.35 ± 0.01 c
Seeds	0.11 ± 0.02 d	0.22 ± 0.02 e	2.91 ± 0.01 b

[1] The data represent the means ± SD of three determinations. Values with the same letter in one column are not significantly different ($p < 0.01$) from each other.

Table 3.2

Effect of hexane extracts from various parts of *A. zerumbet* on AGEs formation.

Sample	Inhibition 50% (μg/mL)				
	Glycation of BSA	Fructosamine adducts	α-dicarbonyl formation	Glycation-induced protein oxidation	Glyoxal content by HPLC
Rhizomes	164 ± 8.11 a [1]	278 ± 2.86 a	176 ± 0.51 b	1150 ± 6.45 b	110 ± 0.66 a
Stems	275 ± 7.25 d	353 ± 4.86 c	434 ± 1.86 c	3224 ± 1.83 d	419 ± 0.99 d
Leaves	266 ± 1.38 cd	294 ± 5.06 ab	858 ± 4.22 d	1392 ± 0.73 c	784 ± 1.25 f
Flowers	231 ± 1.25 b	305 ± 3.05 b	90 ± 5.79 a	1027 ± 1.14 a	285 ± 3.98 b
Pericarps	791 ± 7.80 e	848 ± 0.83 e	459 ± 0.08 c	4213 ± 1.87 f	588 ± 8.80 e
Seeds	245 ± 5.92 bc	475 ± 6.45 d	431 ± 3.19 c	3721 ± 0.59 e	400 ± 1.59 c

[1] The data represent the means ± SD of three determinations. Values with the same letter in one column are not significantly different ($p < 0.01$) from each other.

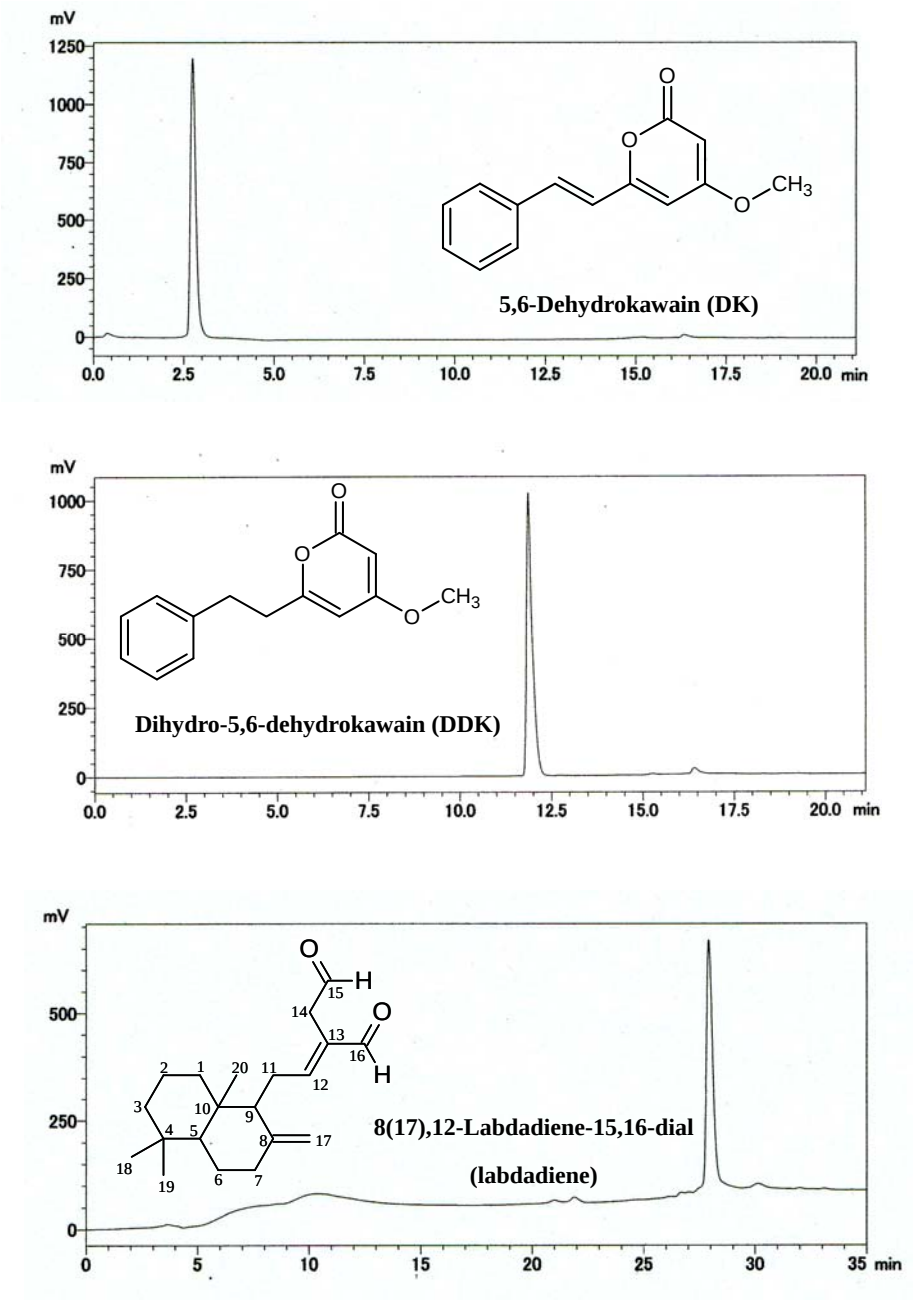

Figure 3.1 Chemical structures and chromatogram of DK, DDK and labdadiene.

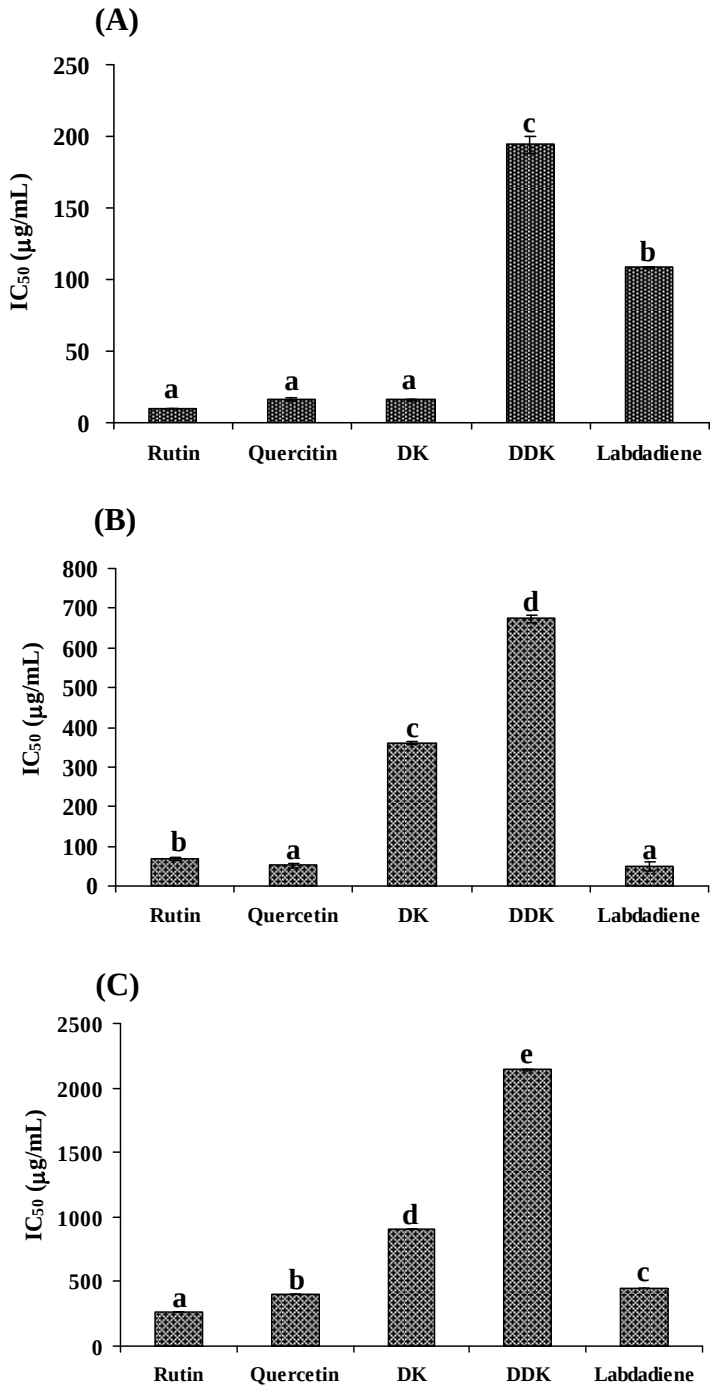

Figure 3.2 IC_{50} of DK, DDK and labdadiene on glycation of BSA (A), fructosamine adduct (B) and glycation-induced protein oxidation (C). Means with the same letter on the bars are not significantly different, $p < 0.01$ (Tukey HSD). The bars represents mean ± SD ($n = 3$).

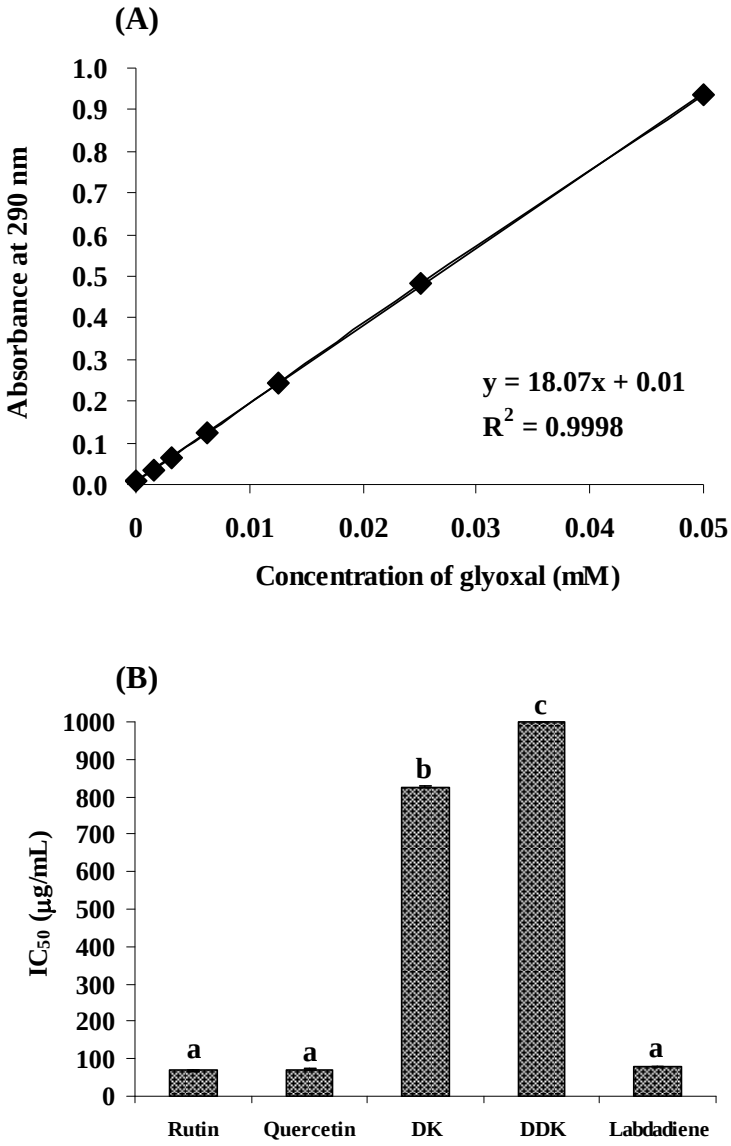

Figure 3.3 Inhibition of α-dicarbonyl compound formations by DK, DDK and labdadiene. A calibration curve of glyoxal standard (A) and effect of isolated compounds on the formation of α-dicarbonyl (B). Means with the same letter(s) on the bars are not significantly different, $p < 0.01$ (Tukey HSD). The bars represents mean ± SD ($n = 3$).

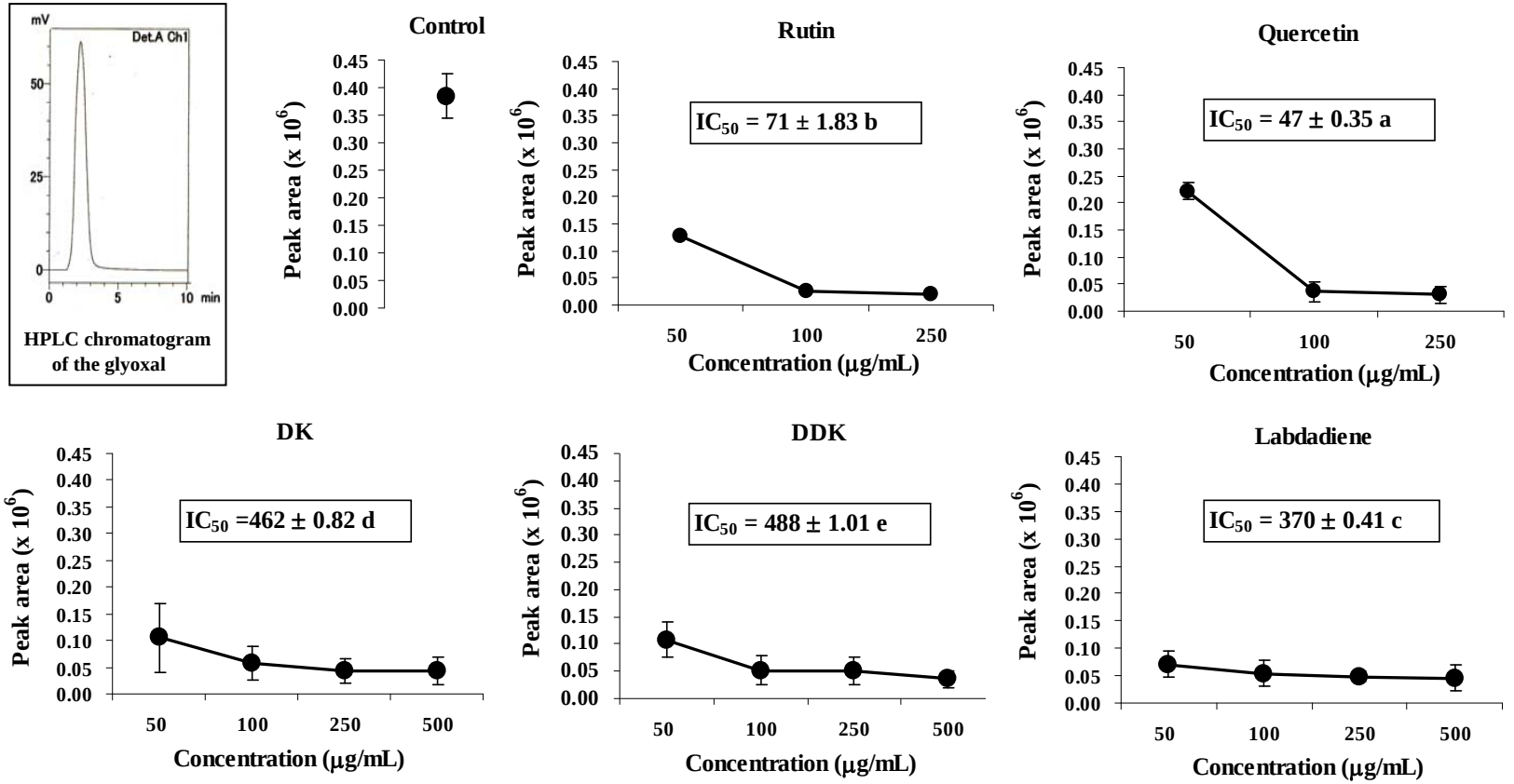

Figure 3.4 Identification of glyoxal as the dicarbonyl sugar formed during autoxidation of glucose. HPLC chromatogram of the glyoxal and peak area identification based on retention time and mass spectra of authentic standard. Means with the same letter(s) are not significantly different, $p < 0.01$ (Tukey HSD). Values represents mean ± SD ($n = 3$).

CHAPTER 4

ANTIATHEROSCLEROGENIC PROPERTIES OF *A. zerumbet*

4.1 SUMMARY

Oxidation of low-density lipoprotein (LDL) is the principal risk factor for the development of atherosclerosis. In this study, the ability of the acetone extract from rhizomes, stems, leaves, flowers, pericarps and seeds of *Alpinia zerumbet* was investigated to inhibit atherosclerosis *in vitro* using several methods. The seed extract had the strongest inhibition activity against tyrosinase, pancreatic lipase (PL), 15-lipoxygenase (15-LO) and LDL oxidation activites (IC_{50} = 2.30 ± 0.02, 5.00 ± 0.07, 1.29 ± 0.07 and 15.40 ± 0.86 μg/mL, respectively) among all different parts. It also had similar effects to the positive controls. Most of the extracts showed partial agonistic properties towards estrogenic activity. Cholest-4-ene-3,6-dione, a steroid presented only in the seed extract seems to be the compound responsible for these activities. The results showed that cholest-4-ene-3,6-dione had similar ability to curcumin and quercetin against PL and LDL oxidation (IC_{50} = 19.50 ± 1.17 and 16.12 ± 1.43 μg/mL, respectively). Furthermore, cholest-4-ene-3,6-dione (IC_{50} = 34.21 ± 1.31 μg/mL) had higher inhibition against 15-LO than quercetin (IC_{50} = 54.79 ± 1.12 μg/mL).

4.2 INTRODUCTION

Atherosclerosis is an inflammatory disease due to accumulation of cholesterol and triglycerides in blood plasma. The increasing in low-density lipoprotein (LDL) content is one of the principal risk factors for atherosclerosis (Ross, 1999). The process begins by accumulation of lipids within the artery wall (Shepherd *et al*., 1995). Besides LDL and several enzymes have been proposed to have direct or indirect effect on atherosclerosis. These include tyrosinase, pancreatic lipase (PL),

and 15-lipoxygenase (15-LO).

The copper containing protein ceruloplasmin has been found to stimulate LDL oxidation (Fox *et al*., 2000). Tyrosinase is a polyphenolase enzyme containing copper; therefore, it is believed that inhibition of tyrosinase may prevent oxidation of LDL, thereby inhibiting atherosclerosis. PL plays a key role in the efficient intestinal digestion of triglycerides, which in turn is absorbed from the small intestine to the erythrocytes resulting in hyperlipidemia (Gholamhoseinian *et al*., 2010). Therefore, atherosclerosis could be prevented when the digestion of triglycerides is blocked. Furthermore, when plasma triglycerides are low, 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). 15-LO has been suggested to be a mediator of oxidation of LDL. The 15-LO induced in atherosclerotic plaques forms hydroperoxy derivatives of linoleic acid and arachidonic acid (Harat *et al*., 2000). On the other hand, estrogens has potentially beneficial effects such as reducing LDL, increasing HDL, facilitating nitric oxide-mediated vasodilatation, and inhibiting the response of blood vessels to injury and the development of atherosclerosis. Therefore, increase in estrogenic activity prevents from atherosclerosis.

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 obsessed myorelaxant and antispasmodic actions in rat ileum (Bezerra *et al*., 2000). It has been reported the antioxidant activity and phenolic contents in flowers and seeds of the plant (Elzaawely *et al*., 2007). It has been also isolated active compounds from the rhizomes of *A. zerumbet* against HIV-1

integrase and neuraminidase enzymes (Upadhyay *et al*., 2011). In this study, acetone was chosen as the solvent for extracting the steroidal compounds from six different parts of *A. zerumbet* against atherosclerotic properties. It was observed the inhibition of LDL oxidation and determined the estrogenic activity of different extracts and steroidal compounds. Furthermore, the enzymatic activities of tyrosinase, PL and 15-LO was probed. Finally, the steroids present in different parts of the extracts were identified and their inhibition manner was investigated against these enzymes.

4.3 MATERIALS AND METHODS

4.3.1 Chemicals

Quercetin, L-tyrosine, caffeic and ferulic acids were obtained from Wako Pure Chemical Industries, Ltd. (Japan). Trichloroacetic acid (TCA), thiobarbituric acid (TBA), *p*-nitrophenylbutyrate (*p*-NPB), low-density lipoprotein cholesterol from human plasma (LDL), porcine pancreatic lipase (PL) and mushroom tyrosinase and cholest-4-ene-3,6-dione were purchased from Sigma-Aldrich, Inc. (USA). Curcumin and ethylenediaminetetraacetic acid (EDTA) were bought from Kanto Chemical Co, Inc. (Japan). Campesterol, sitosterol and stigmasterol were obtained from Tama Biochemical Co., Ltd. (Japan). 3-Morpholinopropanetetraacetic acid (MOP) was secured from Dojindo Molecular Technologies, Inc (Japan). EnBio RCAS for ERα was bought from Fujikura Kasei Co., Ltd. (Japan). 15-Lipoxygenase from soybean (15-LO) was procured from Cayman Chemical Company (USA).

4.3.2 Plant material and preparation of extracts

Rhizomes, stems, leaves, flowers, pericarps and seeds of *A. zerumbet* were collected at University of the Ryukyus, Okinawa, Japan. Twenty gram of dry samples were cut into pieces and extracted in acetone (70%) 400 mL for 24 h at room temperature. After filtration, acetone was evaporated under vacuum in a rotary evaporator at 40^{o}C. All the crude extracts were kept in refrigerator for further analysis.

4.3.3 GS-MS analysis

The steroids analytical column was HP-1MS capillary column (Agilent Technologies, J&W Scientific Products, Folsom, CA, USA). Helium was used as the carrier gas at a flow rate of 1 mL/min. The temperature was programmed at 80^{o}C for 5 min then increased to 300^{o}C at the rate of 15^{o}C per min. The temperatures of injector and EI detector (70eV) were 280^{o}C and 300^{o}C, respectively. Each plant extract of 2.0 μL was injected to the GC-MS (Phurengrat *et al*., 2006).

4.3.4 Anti-tyrosinase activity assay

Tyrosinase activity inhibition was determined by the method as described by Tadtong *et al*. (2009). In brief, sample extracts were dissolved in methanol to make the different concentrations (μg/mL). 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 phosphate 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 microplate reader (BioRad, Benchmark microplate

reader, UK). Kojic acid and quercetin were used as positive controls. The percentage of tyrosinase inhibition was calculated as follows:

Tyrosinase inhihition (%) = [(A – B) – (C – D)]/ (A – B) x 100,

where, A is absorbance of the control with enzyme, B is absorbance of the control without enzyme, C is absorbance of the test sample with enzyme and D is absorbance of the test sample without enzyme

4.3.5 Pancreatic lipase inhibition assay

The ability of the different parts of crude extracts to inhibit PL was measured as described by Kim *et al*. (2007) with slight modifications. Briefly, an enzyme buffer was prepared by the addition of 30 μL (10 units) of PL solution (in 10 mM MOPS and 1 mM EDTA, pH 6.8) to 850 μL of Tris buffer (100 mM Tris-HCl and 5 mM $CaCl_2$, pH 7.0). Then, 100 μL of the sample extracts or positive control substrances (curcumin or quercetin) was mixed with 880 μL of the enzyme buffer, and was incubated for 15 min at 37^{o}C. The reaction was allowed to proceed for 15 min at 37^{o}C after addition of 20 μL of the substrate solution (10 mM *p*-NPB in dimethyl formamide). The lipase activity was determined by measuring the hydrolysis of *p*-NPB to *p*-nitrophenol at 400 nm using microplate reader. Inhibition of the lipase activity was expressed as the decrease ratio of the OD values when PL was incubated with the test compounds.

4.3.6 15-Lipoxygenase inhibition assay

Enzyme inhibition was determined as described previously in borate buffer (0.2 M, pH 9.0) by measuring the increase in absorbance at 234 nm in 5 min after addition of 15-LO, using linoleic acid (134 μM) as substrate (Lyckander *et al*., 1996).

The final enzyme concentration was 500 units/mL and the test samples were dissolved in DMSO solutions. All measurements were carried out at least twice, in each instance using caffeic and ferulic acids, a well-known inhibitor of 15-LO, as positive controls. Calculations of enzyme activity were carried out as previously described, and IC_{50} values were determined by linear interpolation between the measuring points closest to 50% activity.

4.3.7 LDL oxidation inhibition 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 LDL (220 μg/mL) incubated at 37°C in dark with 10 μL of 55 μM $CuSO_4$ and 10 μL of test extracts or curcumin and quercetin for 24 h. The reaction was stopped by adding 50 μL of 1 M EDTA and placing the sample at -20°C for TBA reactive substance (TBARS) assay. The generation of malonyldialdehyde (MDA) equivalents during LDL oxidation was estimated by the TBARS assay using the method of 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 (100°C) for 30 min, the reaction product was kept for 30 min at 25°C and centrifuged for 15 min at 4°C. The supernatants were read on a spectrophotometer at 532 nm, 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.

4.3.8 Estrogenic activity assay

The estrogenic activity of six parts of crude extracts was determined by EnBio

RCAS for estrogen receptor α (ERα) kit (Fujikura Kasei Co., Ltd, Japan, Catalog No. 7664-93-9, UN No.2796). A peptide containing LxxLL motif of coactivator (SRC1) was immobilized on the microplate by incubating SRC1 for 1 h at room temperature. After washing by wash buffer, 5 μL of test sample and 95 μL of ERα were added to the wells. The mixtures were incubated with shaking for 1 h at room temperature. After washing, 100 μL of detection antibody solution was added and further incubated for 30 min at room temperature. 100 μL of TMB substrate solution was added after washing and further incubated without shaking for 20 min. Absorbance was measured at 450 nm after addition of 100 μL of stop solution. 17*β*-Estradiol was used as a positive control, while DMSO was used a negative control. The estrogenic activity relative to 17*β*-estradiol, B/Bmax (%), was calculated by

$$B/Bmax\ (\%) = (C - B)/ (A - B) \times 100,$$

where, A is OD_{450} value of the positive control in (SRC (+) well - SRC (-) well), B is OD_{450} value of the negative control in (SRC (+) well - SRC (-) well) and C is OD_{450} value of the test sample in (SRC (+) well - SRC (-) well)

4.3.9 Statistical analysis

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

4.4 RESULTS

4.4.1 GC-MS analysis

The steroidal compounds in different parts of *A. zerumbet* were identified using GC-MS by comparing their retention times and mass fragmentation pattern with MS library. The amount of steroidal compounds analysis in crude extracts from the different parts of *A. zerumbet* is shown in **Table 4.1**. The samples contained fourteen steroids, two in the rhizome and seed, three in the flower, four in the leaf, five in the stem and eight in the pericarp. The results showed that the pericarp extract had more number of steroids. Sitosterol was present in higher amounts in rhizome, stem and leaf while flower and pericarp were rich in stigmasterol. Campesterol was found at high amount in stem and cholest-4-ene-3,6-dione was present only in the seed extract.

4.4.2 Anti-tyrosinase activity

Anti-tyrosinase activity of different parts of *A. zerumbet* is shown in **Table 4.2**. The seed extract inhibited the tyrosinase enzyme at IC_{50} = 2.30 ± 0.02 μg/mL stronger than the other parts. On carrying out inhibition with steroidal compounds, cholest-4-ene-3,6-dione (IC_{50} = 75.11 ± 0.11 μg/mL) showed higher inhibitory effect than sitosterol, stigmasterol and campesterol (IC_{50} = 187.77 ± 0.04, 259.25 ± 4.49 and 283.78 ± 3.01 μg/mL, respectively) (**Figure 4.1A**). However, cholest-4-ene-3,6-dione showed lower inhibitory effect than quercetin and kojic acid (IC_{50} = 4.92 ± 0.21 and 4.20 ± 0.24 μg/mL, respectively).

4.4.3 Pancreatic lipase inhibition

The seed extract (IC_{50} = 5.00 ± 0.07 μg/mL) showed the highest inhibitory effect on PL activity as shown in **Table 4.2**. Cholest-4-ene-3,6-dione (IC_{50} = 19.50 ± 1.17 μg/mL) showed similar results to the positive controls, curcumin and quercetin (IC_{50} = 4.92 ± 0.21 and 18.60 ± 0.86 μg/mL, respectively) and it had stronger inhibitory effect than campesterol, stigmasterol and sitosterol (IC_{50} = 129.50 ± 7.36, 125.05 ± 4.76 and 99.99 ± 1.86 μg/mL, respectively) (**Figure 4.1B**).

4.4.4 15-Lipoxygenase inhibition

The result of IC_{50} for 15-LO inhibition of six parts of *A. zerumbet* is presented in **Table 4.2**. The highest activity towards 15-LO inhibition was observed in the seed extract (IC_{50} = 1.29 ± 0.07 μg/mL). The inhibition ability of steroidal compounds on 15-LO is shown in **Figure 4.1C**. Cholest-4-ene-3,6-dione (IC_{50} = 34.21 ± 1.31 μg/mL) had higher inhibitory effect than campesterol, sitosterol and stigmasterol (IC_{50} = 144.72 ± 3.19, 123.34 ± 1.94 and 75.26 ± 3.94 μg/mL, respectively), while it showed weaker inhibition than the positive control caffeic acid (IC_{50} = 6.43 ± 0.97 μg/mL) but stronger inhibition than ferulic acid (IC_{50} = 54.79 ± 1.12 μg/mL).

4.4.5 LDL oxidation inhibition

Table 4.2 shows 50% inhibition of various parts of *A. zerumbet* on LDL oxidation, assessed with the TBARS assay. The seed extract (IC_{50} = 15.40 ± 0.86 μg/mL) showed the highest reduction in the amount of MDA equivalents produced by copper-induced LDL oxidation compared with other parts. The inhibition ability of steroidal compounds is shown in **Figure 4.1D**. Cholest-4-ene-3,6-dione (IC_{50} =

16.12 ± 1.43 μg/mL) had higher inhibitory effect than campesterol, sitosterol and stigmasterol (IC_{50} = 149.02 ± 1.93, 97.76 ± 0.77 and 50.01 ± 0.77 μg/mL, respectively). Moreover, cholest-4-ene-3,6-dione showed significantly better activity than quercetin (IC_{50} = 22.56 ± 0.70 μg/mL), while it exhibited similar result to curcumin (IC_{50} = 16.12 ± 1.43 μg/mL).

4.4.6 Estrogenic activity

The relative activity of estrogen by various concentrations of the crude extracts from different parts of *A. zerumbet* is shown in **Figure 4.2**. The results showed that the activity increases with higher concentration. At 500 μg/mL, all samples except stem showed partial agonistic properties.

4.5 DISCUSSION

It is widely accepted that oxidative modification of plasma lipoproteins, particularly LDL, plays an important role in the initiation of atherosclerosis. It has been reported that terpenoids inhibit the oxidative modification of LDL *in vitro* (Carpenter *et al*., 1997). In addition, plant sterols and stanols (phytosterols/phytostanol) are known to reduce serum LDL-cholesterol level, and food products containing these plant compounds are widely used as a therapeutic dietary option to reduce plasma cholesterol and atherosclerotic risk (Calpe-Berdiel *et al*., 2009). In this study, the inhibitory effects of six different parts and steroidal compounds containing extracts of *A. zerumbet* against atherosclerosis were investigated. For this, inhibition of tyrosinase enzyme, which is a multifunctional, glycosylated and copper-containing oxidase was explored (Chang,

2009) that may be related with oxidation of LDL. Furthermore, as the link between PL and atherosclerosis is well established, the inhibition of PL by different extracts and steroidal compounds were checked. The inhibition of 15-LO was also investigated, since this is a lipid-oxidizing enzyme that is considered to contribute the formation of oxidized lipids in atherosclerotic lesions (Bocan *et al*., 1998). In addition, LDL oxidation inhibition was also studied because it leads to development of atherosclerosis. Finally, the effects of *A. zerumbet* parts on estrogenic activity were probed, since there is an inverse relationship between estrogenic activity and LDL oxidation (Christiansen, 1997).

The results obtained in this study indicated that seed extract had better activities amongst all of the parts. Numerous previous studies have reported similar results. For instance, Lin *et al*. (1981) had reported that *A. zerumbet* seed was effective hypolipidaemic with amazingly potent high-density lipoprotein cholesterol (HDL-C) enhancing ability as well as low-density lipoprotein cholesterol (LDL-C) suppressant in nature. It could be used as effective hepatoprotector and anti-atherosclerosis. Furthermore, several reports have been published on the other potential usages of Alpinia seed. Lee *et al*. (2003) revealed that seed extract of *A. katsumadai* inhibited H_2O_2-induced apoptosis and dose-dependently enhanced the activities of superoxide dismutase, catalase and glutathione peroxidase in Chinese hamster lung fibroblast (V79-4) cells. It had also been reported to protect neurons from ischemic damage (Li *et al*., 2011). Beside, it was found to significantly inhibit increases in Th2-type cytokine level, eosinophilia and mucus hypersecretion in the asthmatic mouse model (Lee *et al*. 2010). Moreover, Mitsui *et al*. (1976) reported that *A. galangal* seeds showed antiulcer activity in Shay rats.

In order to identify the active phytochemicals, GC-MS analysis of all different

parts was carried out. A number of steroidal compounds in six parts of *A. zerumbet* were identified. Interestingly, cholest-4-ene-3,6-dione was found to be only in the seed extract. It seems that this compound may have roles in inhibiting atherosclerosis. Furthermore, sitosterol was found in higher amounts in stem, rhizome and leaf. However, it has been reported that sitosterol and campesterol could not inhibit enzymes in the cholesterol biosynthetic pathway (Fernández *et al*., 2002) and therefore seems to have lesser effect in reducing atherosclerosis. On the other hand, stem, flower and pericarp also contained stigmasterol that has been reported to have inhibitory effects in cholesterol biosynthesis (Fernández *et al*., 2002). In order to investigate the efficacy of different steroids, which were present in relatively high amounts (>1% peak area) and pure compounds of these steroids were carried out inhibition of tyrosinase, PL, 15-LO and LDL oxidation. The results showed that cholest-4-ene-3,6-dione had the highest activity in all assays, and therefore, it seems that the activity of seed extract may be due to the presence of this steroid. The potency of numerous other steroidal compounds has been reported to possess atherosclerosis inhibition properties. The adrenal steroid dehydroepiandrosterone (DHEA, 3β-hydroxy-5-androsten-17-one) has been shown to reduce serum LDL level in normal human (Nestler *et al*., 1988) and inhibited the development of accelerated atherosclerosis in native hearts of a rabbit heart transplant model (Eich *et al*., 1993). However, cholest-4-ene-3,6-dione has been reported to show inhibitory activity on proliferation of rat hepatic stellate cells (Badria *et al*., 2005) and cytotoxic activity against human cancer cell line (Han *et al*., 2009).

Estrogens are widely regarded as beneficial to arterial wall health. Among the mechanisms of this benefit are antioxidant effects on LDL and the arterial wall (Zhu *et al*, 1999). The results on estrogenic activity reveal that all parts, except stem, had

higher induced activity with increasing concentration. At the concentrations used, the relative activity of five different parts to the positive control, 17-β-estradiol, seems to be of partial agonistic. The plausible explanation for the antagonistic effect of stem extract toward estrogenic activity is still under investigation.

Therefore, the results obtained in this study indicate that seed extracts can inhibit the formation of atherosclerosis by inhibiting tyrosinase, PL, 15-LO, and LDL oxidation and by partially inducing estrogenic activity. Furthermore, cholest-4-ene-3,6-dione, the steroidal compound, is found only in the seed of *A. zerumbet* that had high potential inhibition activities in all of performed experiments.

4.6 CONCLUSION

The acetone extract of *A. zerumbet* from seeds have several metabolites that have inhibitory effects against atherosclerosis. The present results indicate that seed extract is an efficient scavenger of free radicals and inhibition of enzymatic activities. In the preliminary studies, it was suggest that acetone extract from seeds of *A. zerumbet* may be used to produce a natural supplement or added into health food products to prevent atherosclerosis disease. Futhermore, cholest-4-ene-3,6-dione was present only in seed acetone extract that showed the strongest inhibition activities and had no significantly different results when compared with positive controls. Thus, cholest-4-ene-3,6-dione is steroidal compound that could be used to prevent LDL oxidation. This seed extract will further be studied in order to isolate and identify phytochemical compounds, involved in the ability on inhibition atherosclerotic disease.

Table 4.1

GC-MS analysis of steroidal compounds in crude extracts from six parts of *A. zerumbet*.

Compounds	RT	Peak area (%)					
		Rhizome	Stem	Leaf	Flower	Pericarp	Seed
Cholestane	13.342	-	0.33	-	-	0.20	-
3α,7β-Dihydroxy-5β,6β-epoxycholestane	17.442	-	-	-	-	0.17	0.65
Cholest-4-ene-3,6-dione	21.067	-	-	-	-	-	1.84
Cholest-5-en-3-ol	21.125	-	-	-	0.12	-	-
Ergost-5-en-3-ol	21.133	-	-	-	-	0.29	-
Sitosterol	21.333	8.25	14.49	2.86	-	0.11	-
Cholestenone	21.567	-	-	-	-	0.96	-
5α-Ergost-8(14)-ene	21.758	-	-	0.49	-	-	-
9,19-Cyclolanostan-3-ol	21.983	-	-	0.38	-	-	-
Cholest-4-en-3-ol	22.075	-	-	-	-	0.27	-
Stigmasterol	22.225	-	2.70	-	1.46	1.72	-
Cholest-8-ene-3,6-diol	22.725	0.28	0.16	0.16	-	-	-
4,22-Stigmastadiene-3-one	22.750	-	-	-	-	0.25	-
Campesterol	24.067	-	1.55	-	0.35	-	-

RT is retention time (min).

Table 4.2

Inhibitory effect of acetone extracts from various parts of *A. zerumbet* on tyrosinase, pancreatic lipase, 15-lipoxygenase and LDL oxidation activities.

Sample	50% of Inhibition (μg/mL)			
	Tyrosinase	Pancreatic lipase	15-Lipoxygenase	LDL oxidation
Rhizomes	224.53 ± 4.34 d[1]	277.75 ± 1.60 e	142.15 ± 1.44 c	164.90 ± 0.71 b
Stems	312.53 ± 1.43 e	747.09 ± 2.34 f	1866.75 ± 9.12 f	443.97 ± 3.64 d
Leaves	152.23 ± 1.66 b	58.88 ± 1.55 b	759.59 ± 3.13 d	507.59 ± 3.73 e
Flowers	210.33 ± 1.76 c	76.73 ± 0.82 c	1356.57 ± 0.52 e	423.43 ± 3.47 c
Pericarps	151.31 ± 0.26 b	256.30 ± 3.12 d	87.90 ± 1.60 b	515.48 ± 2.14 e
Seeds	2.30 ± 0.02 a	5.00 ± 0.70 a	1.26 ± 0.07 a	15.40 ± 0.86 a

[1] The data represent the means ± SD of three determinations. Values with the same letter in one column are not significantly different ($p < 0.01$) from each other.

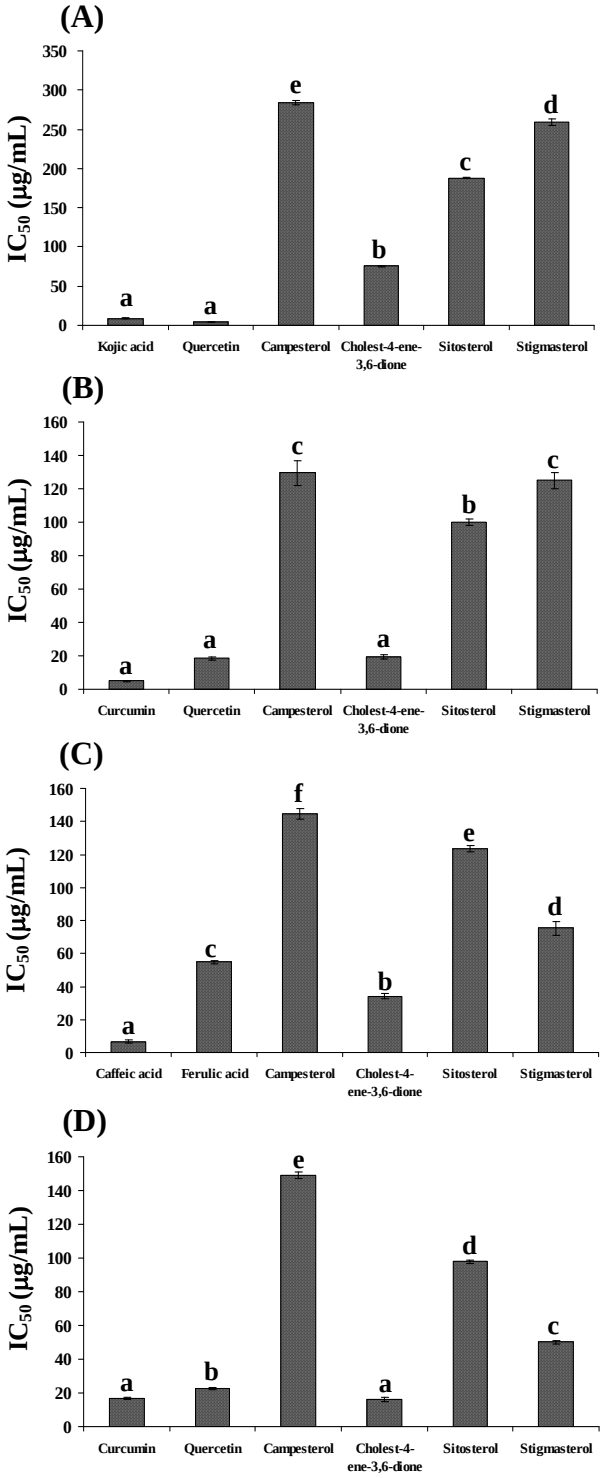

Figure 4.1 Inhibitory effect of steroidal compounds on tyrosinase (A), pancreatic lipase (B), 15-lipoxygenase (C) and LDL oxidation (D) activities. Mean with the same letter on the bars are not significantly different, $p < 0.01$ (Tukey HSD). The bars represents mean ± SD ($n = 3$).

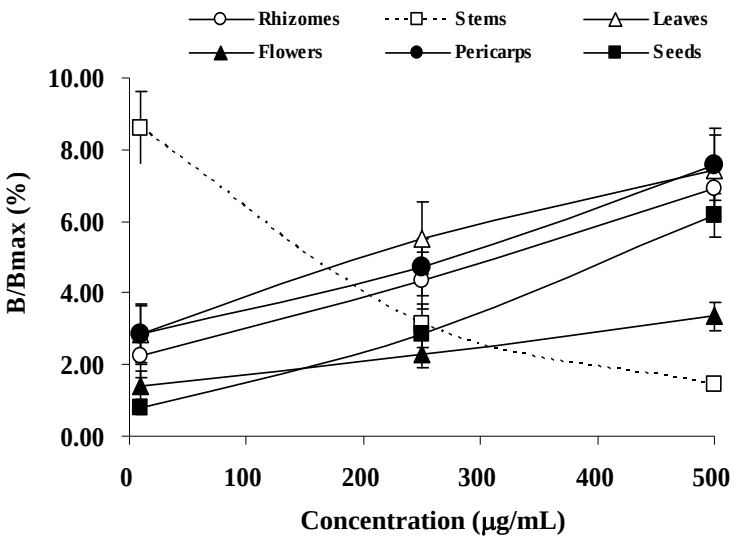

Figure 4.2 Dose response curve of different part extracts of *A. zerumbet* for estrogenic activity. Values are expressed as the mean ± SD of three replicated experiments.

CHAPTER 5

ANTIOXIDANTS AND SKIN DISEASES-RELATED ENZYMES INHIBITION ACTIVITIES OF *A. zerumbet*

5.1 SUMMARY

The skin is chronically exposed to endogenous and environmental pro-oxidant agents, leading to the harmful generation of reactive oxygen species. Antioxidant is vital substances which possess the ability to protect the body from damage cause by free radicals induce oxidative stress. *A. zerumbet*, a traditionally important economic plant in Okinawa, contains several interesting bioactive constituents and possesses health promoting properties. In this regard, it was carried out to test the inhibitory effect of aqueous and ethanol extracts and isolated compounds from *A. zerumbet* on antioxidant and skin diseases-related enzymes. The results showed that aqueous extract of the rhizome was found to have greater than the others on both inhibitory effects of oxidants and skin diseases-related enzymes. Furthermore, 5,6-dehydrokawain (DK), dihydro-5,6-dehydrokawain (DDK) and 8(17),12-labdadiene-15,16-dial (labdadiene), isolated from rhizome, were tested for antioxidant and enzyme inhibitions. It was found that DK showed higher inhibitory activities on DPPH, ABTS and PMS-NADH scavenging (IC_{50} = 122.14 ± 1.40, 110.08 ± 3.34 and 127.78 ± 4.75 μg/ml, respectively). It also had stronger inhibitory activities against collagenase, elastase, hyaluronidase and tyrosinase (IC_{50} = 24.93 ± 0.97, 19.41 ± 0.61, 19.48 ± 0.24 and 76.67 ± 0.50 μg/ml, respectively) than DDK and labdadiene.

5.2 INTRODUCTION

Hattem *et al*. (2008) reported that almost all diabetes patients eventually develop skin complications from the long-term effects of diabetes on the

microcirculation and on skin collagen. The skin is the largest organ of the human, both in terms of surface area and weight, which serves as an important environmental interface providing a protective envelope that is crucial for homeostasis. On the other hand, the skin is a major target for toxic insult by a broad spectrum of physical (UV radiation) and chemical (xenobiotic) agents that are capable of altering its structure and function (Ndiaye *et al*., 2011). Many environmental pollutants are oxidants or catalyze the production of reactive oxygen species (ROS) directly or indirectly. ROS act as cell-signaling molecules and can react with DNA, proteins, fatty acids and saccharides causing oxidative damages. Such injuries result in a number of harmful effects (disturbed cell metabolism, morphological and ultrastructural changes), attack on the regulation pathways, and alterations in the differentiation, proliferation and apoptosis of skin cells (Svobodová *et al.*, 2003). The skin possesses an array of defense mechanisms that interact with toxicants to obviate their deleterious effects. These include non-enzymatic and enzymatic molecules that function as potent antioxidant or oxidant-degrading systems (Bickers *et al*., 2006). For screening antioxidants, the methods based on a single relatively stable reagent such as $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ have most popular, because of their simple set-up and ease of control (Niederläander *et al*., 2010).

Collagenase is one of the few proteinases capable of degrading the triple-helical region of native collagen under a physiological condition. Collagen is the fibrous component of the extracellular matrix (ECM) in the skin and the collagen content in the skin decreases greatly during the aging process and due to long-term exposure to UV radiation. Inhibition of collagenase plays an important role in protecting unbalanced turn over or rapid breakdown of collagen in human inflamed

or UV-irradiated skin (Thring *et al*., 2009). Elastase is a proteolytic enzyme involved in the degradation of the ECM that includes elastin. Loss of elastin is a major part of what cause visible signs of aging (wrinkles, sagging) in the skin. Elastase secretion and activation caused by exposure to UV light or ROS, an approach that inhibits the elastase activity, could also be applied as a useful method to protect against skin diseases (Lee and Kim, 1999). Hyaluronidase is a mucopolysaccharase that hydrolyzes glycosaminoglycans, including hyaluronic acid, in the ECM during tissue remodeling. When the level of hyaluronic acid decreased under conditions in which the hyaluronidase activity is increased, the moisture and tension of the skin are reduced. Thus, hyaluronidase inhibitors are useful cosmeceutical ingredients as they have anti-wrinkle and anti-aging effects on the skin (Barla *et al*., 2009). Tyrosinase is known to be a key enzyme for the melanin biosynthesis, the compound largely responsible for hair and skin color in mammals. Melanin pigments play a crucial protective role against skin photocarcinogenesis. Tyrosinase inhibitors may be clinically helpful in dealing with skin diseases (Tief *et al*., 1996).

Alpinia zerumbet (Family Zingiberaceae) is a medicinal plant found in several islands of Japan, including Okinawa. Several pharmacological effects of essential oil from the leaves of *A. zerumbet* have been used for skin care, insect repellent and deodorant products (Murakami *et al*., 2009), antinociceptive effects on mice (de Araújo *et al*., 2005) and antihypertensive and cardiovascular effects on rat (de Moura *et al*., 2005). Phenolic compounds and their antioxidant activities in leaves and rhizomes of *A. zerumbet* have beenreported by Elzaawely *et al*. (2007) and have isolated active compounds from the rhizomes of *A. zerumbet* against HIV-1 integrase and neuraminidase enzymes (Upadhyuy *et al*., 2011). Considering all these aspects,

the present study was undertaken to evaluate antioxidant and anti-enzymatic activities of *A. zerumbet* for prevention of the skin diseases.

5.3 **MATERIAL AND METHODS**

5.3.1 Chemicals

1,1-Diphenyl-2-picrylhydrazyl (DPPH), *tert*-butyl hydroxytoluene (BHT), kojic acid, gallic acid, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)diammonium salt (ABTS), nitro blue tetrazolium (NBT), phenazine methosulfate (PMS), bovine serum albumin (BSA) and Folin-Ciocalteu reagent were purchased from Wako Pure Chemical Industries, Ltd (Japan). Nicotinamide adenine dinucleotide-reduced (NADH), N-succ-(Ala)3-nitroanilide (SANA), *N*-[3-(2-furyl)acryloyl]-Leu-Gly-Pro-Ala (FALGPA), collagenase, elastase, hyaluronidase, tyrosinase, hyaluronic acid and oleanolic acid were secured from Sigma-Aldrich, Inc. (USA).

5.3.2 Preparation of plant extracts

The six structures (rhizomes, stems, leaves, flowers, pericarps, and seeds) of *A. zerumbet* were collected at the University of the Ryukyus, Okinawa, Japan. Aqueous extract was obtained by boiling 10 g of air-dried sample for 20 min. For ethanol extract, samples were immersed in ethanol for 24 h. The aqueous and ethanol extracts were filtered, dried under vacuum and then dissolved in corresponding solvent (water and methanol, respectively) for further analysis.

5.3.3 Isolation and quantification of DK, DDK and labdadiene

The isolation of DK and DDK were isolated from rhizomes of *A. zerumbet* as reported previously (Upadhyuy *et al*., 2011). DK and DDK were purified using a TSK gel ODS-100Z column (15 x 0.46 cm i.d.; 5 μm particle size) (Tosoh Corp, Japan) and monitored continuously at 280 nm. The mobile phase consisted of water with 0.1% acetic acid (solvent A) and methanol with 0.1% acetic acid (solvent B) at a flow rate of 0.8 ml/min. The gradient elution was performed as follows: 1-10 min, 50% B isocratic, 10-20 min, linear gradient 80-100% B, and 20-30 min, 100% B.

Labdadiene was collected at 235 nm using TSK gel ODS-100Z column. The mobile phase was water with 0.1% acetic acid (solvent A) and methanol with 0.1% acetic acid (solvent B) at flow rate of 0.8 ml/min. The gradient elution was performed as follows: 0-10 min, 80% B isocratic; 10-20 min, linear gradient 80-100% B, 20-40 min, 100% B isocratic.

The quantification of each compound in six different structures of *A. zerumbet* with aqueous and ethanol extracts was determined based on peak area measurement by the same processes as described above. The structures of DK, DDK and labdadiene are shown in **Figure 5.1**. The isolated compounds were dissolved methanol for further analysis.

5.3.4 Antioxidant assays

5.3.4.1 DPPH radical scavenging activity assay

The free radical scavenging activity was evaluated as the method described by Boskou *et al*. (2006). Five hundred microlilters of the different concentration of test samples were mixed with 200 μl of 0.5 mM DPPH methanol solution and 500 μl of 0.1 M sodium acetate buffer (pH 5.5). After shaking, the mixture was incubated in

the dark at room temperature for 30 min, and then the absorbance was measured at 517 nm. BHT was used as a positive control, while water and methanol were used as controls for calculation.

5.3.4.2 Total antioxidant activity (ABTS) assay

The total antioxidant activity of test sample was measured with modified method as described previously (Hsu *et al*., 2011). $ABTS^{\bullet+}$ solution was generated by mixing 7 mM ABTS and 2.45 mM potassium persulfate ($K_2S_2O_8$) in water, which was placed in the dark at room temperature for 16 h to give the complete oxidation of ABTS. Before using, the $ABTS^{\bullet+}$ solution was diluted with water to get an absorbance of 0.700 ± 0.050 at 734 nm. Briefly, 1 ml of $ABTS^{\bullet+}$ solution was added to 30 µl of test samples and mixed thoroughly. BHT was used as positive control and the sample solutions were used as controls. The reactive mixture was incubated at room temperature for 6 min and the absorbance was immediately recorded at 734 nm.

5.3.4.3 PMS-NADH system superoxide-radical scavenging assay

The superoxide scavenging activity was assayed following the method of Lau *et al*. (2002) with minor modifications. The reaction mixture contained 0.5 ml of 105 µM NADH, 0.5 ml of 66 µM NBT dissolved in 0.1 M phosphate buffer (pH 7.4) and 0.1 ml sample in different concentrations. The reaction was initiated by adding 0.5 ml of 30 µM PMS into the reaction mixture. Methanol and water were used as a control and BHT as a positive control. After 10 min, the reaction mixture reached to stable color, measurement of absorbance was conducted at 560 nm.

The ability of the extracts to scavenge DPPH, $ABTS^{\bullet+}$, PMS-NADH radicals were calculated by following formula:

$$\text{Radical scavenging (\%)} = [(O.D_{control} - O.D_{sample})/ O.D_{control}] \times 100,$$

where O.D is the optical density in the presence or absence of the samples.

5.3.5 Determination of total phenolic contents

The amount of total phenolics was determined using the Folin-Ciocalteu reagent method (Djeridane *et al*., 2006). Briefly, 0.5 ml of Folin-Ciocalteu reagent and 0.5 ml of distilled water were added to 0.2 ml of each extract dissolved in corresponding solvent (1 mg/ml). After 1 min, 0.8 ml of sodium carbonate solution (7.5%) was added in mixture and incubated at room temperature for 30 min. Absorbance was measured at 760 nm. Total phenolic contents were expressed as gallic acid equivalents (GAE) in milligrams per gram extract.

5.3.6 Enzymatic assays

5.3.6.1 Collagenase inhibition assay

Collagenase inhibition assay was performed by the method described previously (Van Wart and Steinbrink, 1981) which based on the hydrolysis of *N*-[3-(2-furyl)acryloyl]-Leu-Gly-Pro-Ala (FALGPA) by collagenase to produce FA-Leu and Gly-Pro-Ala. The assay was performed in 50 mM Tricine buffer (400 mM NaCl and 10 mM $CaCl_2$, pH 7.5). Collagenase from *Clostridium histolyticum* (ChC) was dissolved in the buffer for use at an initial concentration of 0.8 units/ ml. The synthetic substrate, FALGPA, was dissolved in the Tricine buffer to 2 mM. Sample extracts were incubated with the enzyme in the buffer for 15 min before adding substrate to start reaction. The

final reaction mixture (150 µl total volume) contained Tricine buffer, 0.8 mM of FALGPA, 0.1 units of ChC and 25 µg of test extracts. Controls were performed with water and methanol, while oleanolic acid as a positive control. After adding substrate for 20 min, collagenase activity was measured at 340 nm.

5.3.6.2 Elastase inhibition assay

Elastase inhibition was assayed using *N*-succ-(Ala)3-nitroanilide (SANA) as the substrate, monitoring the release of *p*-nitroaniline by the method described (Kransoe *et al*., 1996) with a few modifications. The inhibitory activity determined the intensity of color released during cleavage of SANA by the action of elastase. Briefly, 1 mM SANA was prepared in 0.1 M Tris-HCl buffer (pH 8.0) and this solution (200 µl) was added to the stock sample solution (20 µl). The solutions were vortexed and preincubated for 10 min at 25^{o}C and then 20 µl of elastase from porcine pancreas (0.03 units/ml) was added. After vortexing, each solution was placed in a water bath at 25^{o}C for 10 min and the absorbance was measured at 410 nm. Controls were performed with water and methanol, while oleanolic acid as a positive control.

5.3.6.3 Hyaluronidase inhibition assay

Hyaluronidase inhibitory assay was performed by the method described previously (Kim *et al*., 2009) which depends on the fact that substance forms a precipitate with protein in an acidic solution. A test sample of 5 µl was pre-incubated with hyaluronidase from bovine test (1.50 units in 100 µl), sodium phosphate buffer 20 mM (pH 7.0) with sodium chloride 77 mM and bovine serum albumin (BSA) 0.01% for 10 min at 37^{o}C. Subsequently, the assay was initiated by adding hyaluronic acid sodium

salt from rooter comb 100 µl (0.03% in 300 mM sodium phosphate, pH 5.35) to the incubation mixture and incubated further for 45 min at 37°C. Hyaluronic acid (undigested) was precipitated with acid albumin solution (1 ml), made up of bovine serum albumin 0.1% in sodium acetate 24 mM and acetic acid 79 mM (pH 3.75). It was allowed to stand at room temperature for 10 min and then the absorbance was measured at 600 nm. Oleanolic acic was used as positive control and the sample solutions were used as controls

The percentage inhibition for collagenase, elastase and hyaluronidase assays were calculated by:

$$\text{Enzyme inhibition activity (\%)} = (1 - B/A) \times 100,$$

where A is the enzyme activity without sample and B is the activity in the presence of the sample.

5.3.6.4 Tyrosinase inhibition assay

Tyrosinase activity inhibition was determined by the method as described previously (Tadtong *et al*., 2009) by measuring the DOPA chrome formed due to the action of tyrosinase enzyme on tyrosine substrate. In brief, sample extracts were dissolved in corresponding solvent to make the different concentrations (µg/ml). 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 phosphate buffer). After incubation at 25°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. Kojic acid was used as a positive control. The percentage of tyrosinase inhibition was calculated as follows:

Tyrosinase inhihition (%) = [(A – B) – (C – D)]/ (A – B) x 100,

where A is the absorbance of the control with the enzyme, B is the absorbance of the control without the enzyme, C is the absorbance of the test sample with the enzyme and D is the absorbance of the test sample without the enzyme.

5.3.7 Statistical analysis

The data were analyzed by one-way ANOVA using SPSS version 16.0 for Windows. Upon significant difference, means were separated using Tukey HSD range test at $p < 0.01$ with three replications. In some cases, only means and standard deviation of the sample means are presented.

5.4 **RESULTS**

5.4.1 Inhibition properties of six structures of A. zerumbet

The radical scavenging activities of aqueous extracts were found superior to ethanol extracts ($p = 0.01$). The seed and rhizome had better antioxidant activities. For DPPH assay, seed extracts showed the best activity (IC_{50} = 10.33 ± 0.03 μg/ml) followed by rhizome (IC_{50} = 25.31 ± 0.67 μg/ml). In case of ABTS radical scavenging activity, the IC_{50} values for rhizome and seed extracts were 73.94 ± 1.23 and 84.29 ± 0.72 μg/ml, respectively. The scavenging of superoxide radicals also showed both seed and rhizome extract to have better activities than other parts with IC_{50} of 58.55 ± 0.31 and 64.70 ± 0.72 μg/ml, respectively. However, rhizome and seed aqueous extracts had weaker inhibitory effect than positive control BHT on DPPH, ABTS and PMS-NADH radical scavenging assays (IC_{50} = 11.74 ± 1.23, 14.26 ± 0.16 and 24.80 ± 0.98 μg/ml) ($p = 0.01$) (**Table 5.1**). It was

also found that the seed and rhizome aqueous extracts contained higher amounts of phenolic compounds (187.75 ± 0.97 and 124.51 ± 0.57 GAE mg/g extract, respectively) (**Table 5.2**).

The effect of six different structures of *A. zerumbet* on elastase, hyaluronidase and tyrosinase inhibitions showed that rhizome had stronger inhibitory activity than the other parts (IC_{50} = 57.43 ± 0.18, 35.02 ± 0.75 and 21.84 ± 0.77 μg/ml, respectively). Pericarp showed higher inhibitory activities against collagenase (IC_{50} = 45.67 ± 0.50 μg/ml). However, test samples had weaker inhibition than positive control, oleanolic acid (IC_{50} = 17.95 ± 0.23, 10.91 ± 0.03 and 5.28 ± 0.19 μg/ml, respectively) on collagenase, elastase, hyaluronidase activities and lower ihhibitory effect than kojic acid (IC_{50} = 8.90 ± 0.22 μg/ml) on tyrosinase acitivity ($p < 0.01$) (**Figure 5.2**).

5.4.2 Inhibition properties of isolated compounds from A. zerumbet

The amount of DK, DDK and labdadiene in the crude extracts of six different structures from *A. zerumbet* with aqueous and ethanol solvents extraction are presented in **Table 5.3**. DK and DDK were found in all structures of plant and in both solvent extractions. The rhizome aqueous extract contained higher amount of DK (1.23 mg/g crude extract) followed by pericarp aqueous extract and rhizome ethanol extract (1.15 and 1.14 mg/g crude extract, respectively). DDK was found in high amount in pericarp aqueous extract (0.85 mg/g crude extract) followed by rhizome aqueous extract (0.62 mg/g crude extract). Labdadiene was present in higher amount in seed ethanol and aqueous extracts (1.00 and 0.96 mg/g crude extract, respectively) followed by rhizome and pericarp aqueous extracts (0.81 and 0.75 mg/g crude extract), moreover, the crude extracts of stem, leaf and flower did not contain labdadiene.

The inhibitory properties of isolated compounds on antioxidant are shown in **Figure 5.3**. DK showed the highest inhibitory properties on DPPH, ABTS and PMS-NADH radical scavenging assays (IC_{50} = 122.14 ± 1.40, 110.08 ± 3.34and 127.78 ± 4.75 μg/ml, respectively). On statistical analysis of the result, isolated compounds were weaker inhibitors on antioxidant than BHT ($p < 0.01$).

Among the isolated compounds, DK showed stronger inhibitory activities against collagenase, elastase, hyaluronidase and tyrosinase (IC_{50} = 24.93 ± 0.97, 19.41 ± 0.61, 19.48 ± 0.24 and 76.67 ± 0.50 μg/ml, respectively) than DDK and labdadiene. However, isolated compounds had lower inhibitory effect than oleanolic acid (**Figure 5.4**) ($p < 0.01$).

5.5 DISCUSSION

Free radicals, such as ROS, are known to cause damage to cells during the process of aging thereby leading to a wide range of degenerative diseases (Duan *et al*., 2006). Furthermore, the interactions between human body and the environment make the skin chronically exposed to both endogenous and environmental pro-oxidant agents which aid to generate ROS (Briganti and Picardo, 2003). One of the important functions of skin is to protect from harmful environments. However, due to unusual disruption of connective tissues, the formation of free radicals, and the ultraviolet radiation result in skin wrinkling and pigmentation (Kim *et al*., 2005). Several studies have explored on the prevention of these skin abnormalities using plant extracts and phenolic compounds contribute in curing of skin diseases. Plant extracts such as pomegranate (Afaq *et al*., 2009), tea (Wang *et al*., 1994) and wine

(Matito *et al*., 2011) extracts have been shown to reduce the oxidative damage of UV light in skin. Purified phenolic compounds such as anthocyanins (Tsoyi *et al*., 2008), proanthocyanidin (Mantena *et al*., 2006) and EGCG (Tobi *et al*., 2002) were found to inhibit the UV-radiation-induced oxidative stress and cell damage in human keratinocytes.

In this study, the inhibitory activities of extracts obtained from six different structures of *A. zerumbet* with aqueous and ethanol extracts for ranking the efficacies were screened. The results of preliminary screening showed that the rhizome and seed aqueous extracts had high antioxidant. Likewise, it was found that the seed and rhizome aqueous extracts contained higher amounts of total phenolic contents, which may be the reasons for stronger radical scavenging activities. For skin diseases-related enzymes assays, rhizome aqueous extract exhibited the strongest activities in three of the performed assays while pericarp aqueous extract had better result in the collagenase inhibition. The amount of DK, DDK and labdadiene in all of test samples were quantified. The rhizome aqueous extract contained high amount of three compounds that may be the reason for stronger inhibitory properties. The inhibitory properties of these compounds have been published. For instance, DK had inhibitory effect on human platelet aggregation (Jantan *et al*., 2008). Beside, Upadhyay *et al.* (2011) presented that DK strongly inhibited HIV-1 integrase and neuraminidase activities.

Among the isolated compounds, DK exhibited the strongest activity in all of antioxidant assays. DK and DDK are the pyrone compounds, interestingly these compounds differ with one another by a single π-bond at C7-C8. DK contains a double bond and it seems that the presence of double bond has significant impact on

antioxidant. Singlet oxygen reacts directly with the double bond of the unsaturated fatty acid by a concerted addition, the so-called “ene” reaction. This is a concerted reaction between singlet oxygen and carbon-carbon double bond in which the oxygen molecule is inserted at either carbon atom of the C=C bond (Chan, 1977; Terao and Matsushita, 1981). Heim *et al.* (2002) have found that flavonoids with a double bond are the better antioxidant. The double bond in the heterocycle or polymerization of the nuclear structure increases activity by affording a more stable flavonoid radical through conjugation and electron delocation. Moreover, Stahl *et al.* (2003) reported that due to conjugated double bond in the polyene backbone of carotenoid structure, a major role in the protection of plants against photooxidative processes, which determines their light absorbing properties and influences the antioxidant activity. They are also part of the antioxidant defense system in animals and humans.

The skin diseases-related enzyme assays revealed that DK also has strong activity in among of isolated compounds. Several previous studies have been reported the ability of pyrone to exhibit skin diseases-related enzymes. Hosoe *et al.* (2011) reported that γ-pyrone derivative, lepidepyrone, was a major hyaluronidase inhibitory compound. Cook *et al.* (1987) presented that 3-(1-oxoalkyl)-4-hydroxy-6-alkyl-2-pyrone was found to be most effective to inhibit human sputum elastase. Huang *et al.* (2010) demonstrated that dimeric naphtha-γ-pyrone, rubrofusarin, exhibited moderate tyrosinase activity, in addition, 6-*n*-pentyl-α-pyrone have been recorded as a potent tyrosinase inhibitor (Li *et al*., 2005). Moreover, pyrone have been presented to restrain other diseases. Hong (2009) reported that tricyclic analogs and tricyclic pyrone compounds directly bind to amyloid-β (Aβ) and inhibit the formation of amyloid-β (Aβ) aggregates in cell model representing Alzheimer’s disease. Yan *et al.* (2007) mentioned that

3-hydroxy-4-pyrones and 5-amino-3-hydroxy-4-pyrone derivatives have been evaluated as inhibitor of matrix metalloproteinases. Hence it is assumed that DK is a pyrone compound and contain carbon double bond which may have a substantial role in inhibiting the skin diseases-related enzymes.

5.6 CONCLUSION

This study revealed that the rhizome aqueous extract of *A. zerumbet* indicated strong antioxidant and skin diseases-related enzymes. Since isolated compounds from rhizome of *A. zerumbet*, DK, was identified as a moderated potent inhibitor of antioxidant. Also, it was indicated that there was strong inhibition of skin diseases-related enzymes. Our results showed that DK have probable applications in preventing the skin against photo-oxidation. However further researches are necessary to use it as a lead compound in drug designing.

Table 5.1

Radical scavenging activity of six parts of *A. zerumbet* extracts on DPPH, ABTS and PMS-NADH superoxide.

Sample	Extraction	Inhibitory effect (IC_{50}; μg/mL)		
		DPPH	ABTS	PMS-NADH
BHT		11.74 ± 0.15 a [1]	14.26 ± 0.16 a	24.80 ± 0.98 a
Rhizome	Aqueous	25.31 ± 0.67 b	73.94 ± 1.23 b	64.70 ± 0.72 c
	Ethanol	145.07 ± 1.68 e	155.86 ± 4.09 f	113.37 ± 1.24 d
Stem	Aqueous	144.03 ± 0.27 e	127.97 ± 1.70 d	215.09 ± 0.28 h
	Ethanol	293.08 ± 0.17 g	223.17 ± 0.26 i	132.27 ± 1.33 e
Leaf	Aqueous	165.60 ± 0.44 f	143.77 ± 1.37 e	191.24 ± 0.20 g
	Ethanol	586.47 ± 0.77 j	206.65 ± 0.85 h	168.23 ± 4.77 f
Flower	Aqueous	132.19 ± 1.02 c	140.50 ± 1.18 e	117.32 ± 1.46 d
	Ethanol	559.01 ± 3.61 h	293.90 ± 3.64 k	210.50 ± 4.03 h
Pericarp	Aqueous	140.91 ± 0.30 de	163.41 ± 1.07 g	169.02 ± 1.01 f
	Ethanol	580.38 ± 0.26 i	246.71 ± 0.85 j	166.11 ± 0.52 f
Seed	Aqueous	10.33 ± 0.03 a	84.29 ± 0.72 c	58.55 ± 0.31 b
	Ethanol	136.63 ± 0.33 cd	137.92 ± 1.74 e	56.12 ± 1.08 b

[1] The data represent the means ± SD of three determinations. Values with the same letter are not significantly different ($p < 0.01$) from each other.

Table 5.2

Total phenolic content from six parts of *A. zerumbet* extracts.

Sample	Total phenolic content (GAE mg/g extract)	
	Aqueous extract	Ethanol extract
Rhizome	124.51 ± 0.57 b [1]	38.50 ± 0.47 h
Stem	59.41 ± 0.87 d	9.60 ± 0.04 j
Leaf	47.80 ± 0.73 f	42.14 ± 0.22 g
Flower	75.15 ± 0.23 c	12.90 ± 0.11 i
Pericarp	58.42 ± 0.18 d	10.26 ± 0.04 j
Seed	187.75 ± 0.97 a	56.31 ± 0.09 e

[1] The data represent the means ± SD of three determinations. Values with the same letter are not significantly different ($p < 0.01$) from each other.

Table 5.3

Amount of DK, DDK and labdadiene in aqueous and ethanol extracts from six different structures of *A. zerumbet.*

Sample	Extraction	Amount of compound (mg/ g crude extracts)		
		DK	DDK	Labdadiene
Rhizome	Aqueous	1.23 ± 0.03 a	0.62 ± 0.02 b	0.81 ± 0.03 b
	Ethanol	1.14 ± 0.01 b	0.08 ± 0.01 fg	0.00 ± 0.00 c
Stem	Aqueous	0.79 ± 0.03 de	0.30 ± 0.01 d	0.00 ± 0.00 c
	Ethanol	0.95 ± 0.01 c	0.17 ± 0.02 e	0.00 ± 0.00 c
Leaf	Aqueous	0.86 ± 0.02 d	0.46 ± 0.02 c	0.00 ± 0.00 c
	Ethanol	0.80 ± 0.01 de	0.07 ± 0.02 fg	0.00 ± 0.00 c
Flower	Aqueous	0.12 ± 0.02 f	0.11 ± 0.01 f	0.00 ± 0.00 c
	Ethanol	0.83 ± 0.02 de	0.06 ± 0.01 fg	0.00 ± 0.00 c
Pericarp	Aqueous	1.15 ± 0.03 b	0.85 ± 0.03 a	0.75 ± 0.04 b
	Ethanol	0.79 ± 0.03 de	0.11 ± 0.01 f	0.00 ± 0.00 c
Seed	Aqueous	0.94 ± 0.02 c	0.05 ± 0.01 g	0.96 ± 0.01 a
	Ethanol	0.78 ± 0.02 e	0.06 ± 0.02 fg	1.00 ± 0.09 a

[1] The data represent the means ± SD of three determinations. Values with the same letter in one column are not significantly different ($p < 0.01$) from each other.

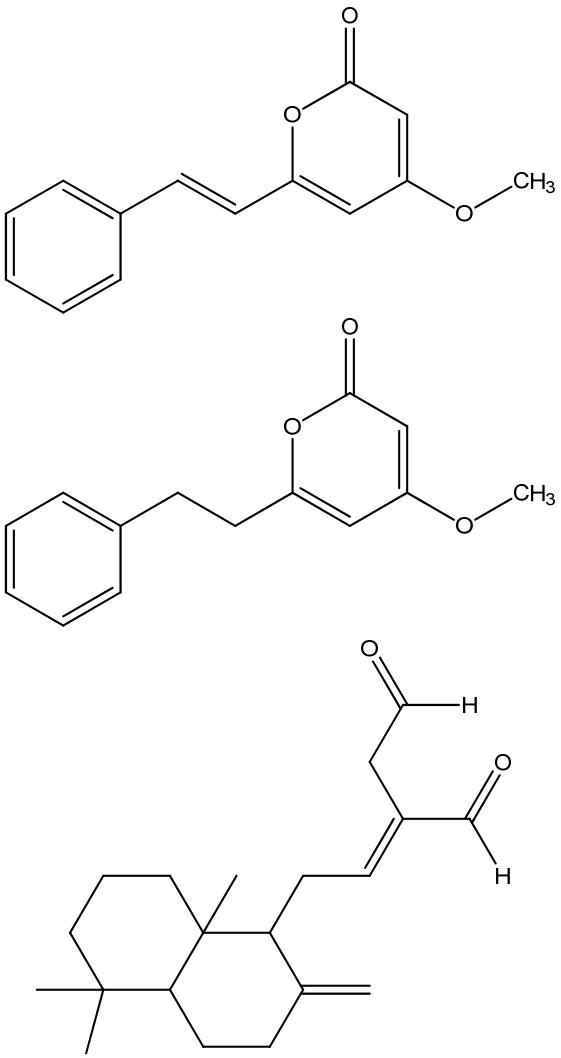

Figure 5.1 Chemical structures of 5,6-dehydrokawain; DK (A), dihydro-5,6-dehydrokawain; DDK (B) and 8(17),12-labdadiene-15,16-dial; labdadiene (C).

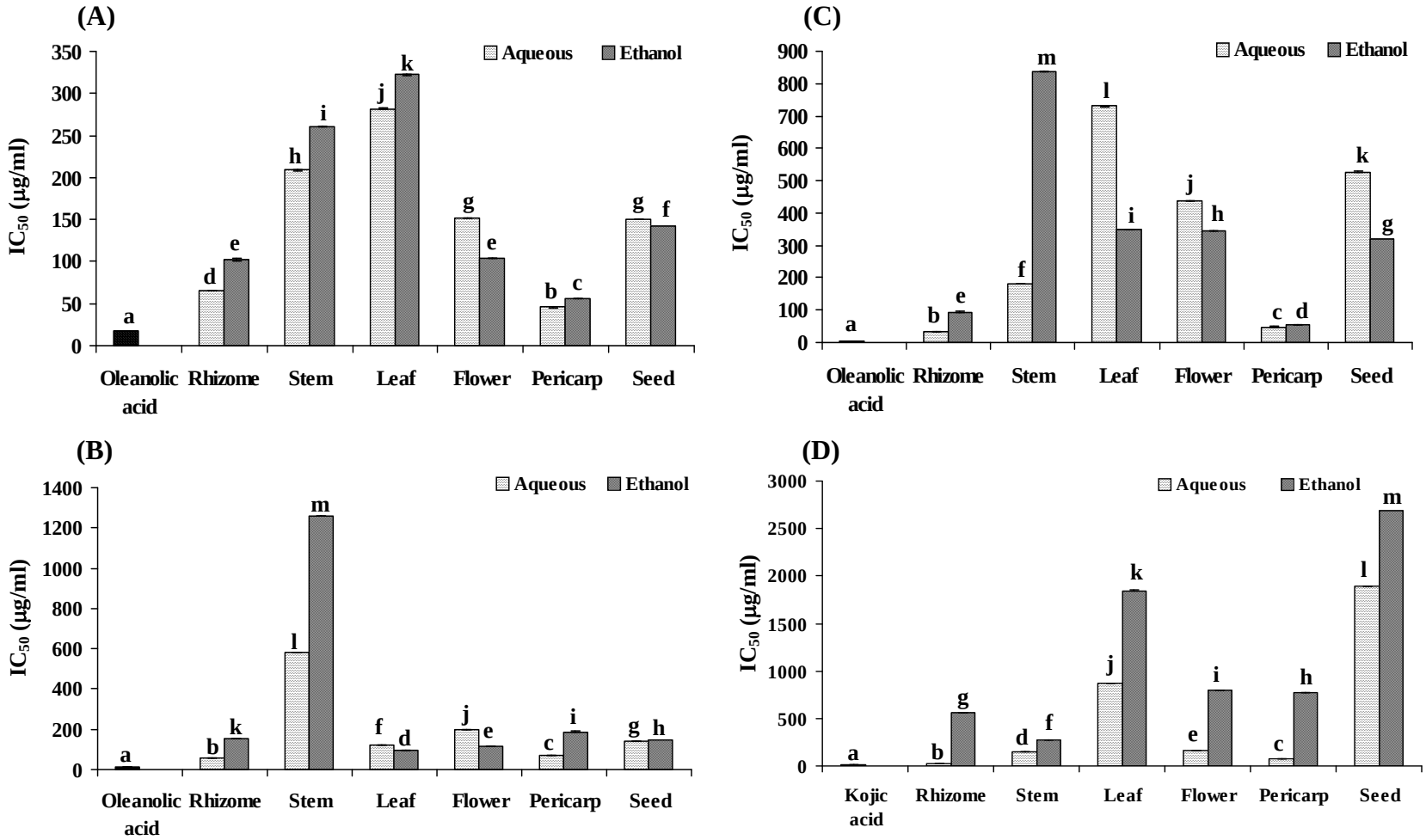

Figure 5.2 Inhibitory effects of six parts of *A. zerumbet* on collagenase (A), elastase (B), hyaluronidase (C) and tyrosinase (D) activities.

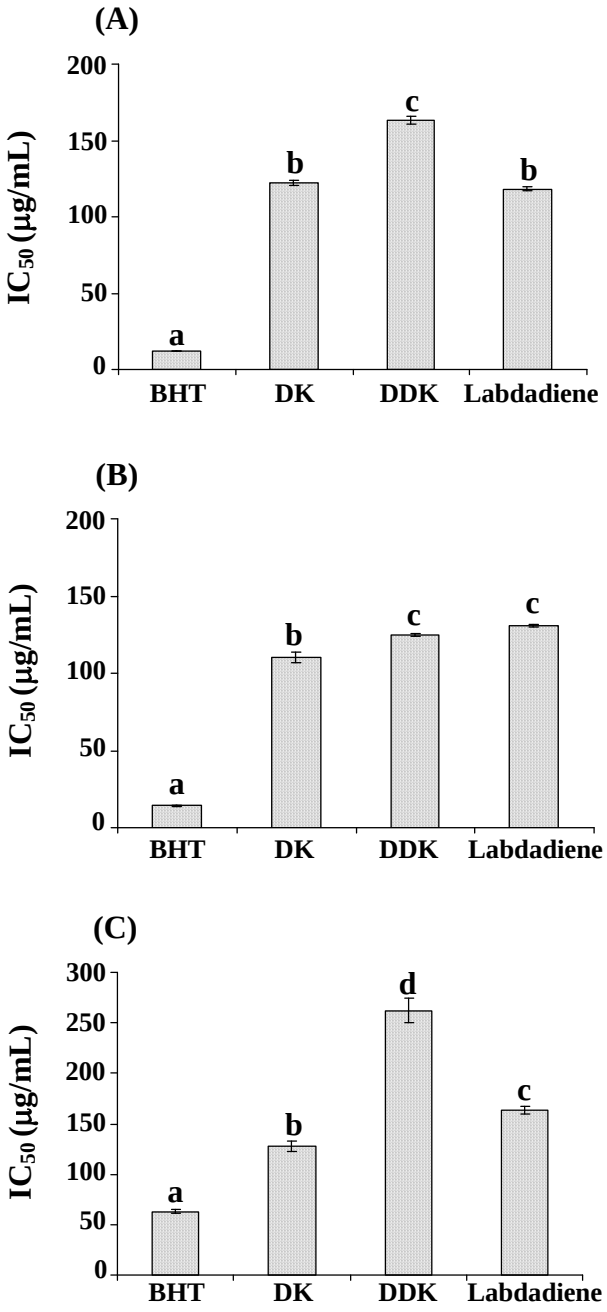

Figure 5.3 Effects of DK, DDK and labdadiene on DPPH (A), ABTS (B) and PMS-NADH (C) radical scavenging inhibitions.

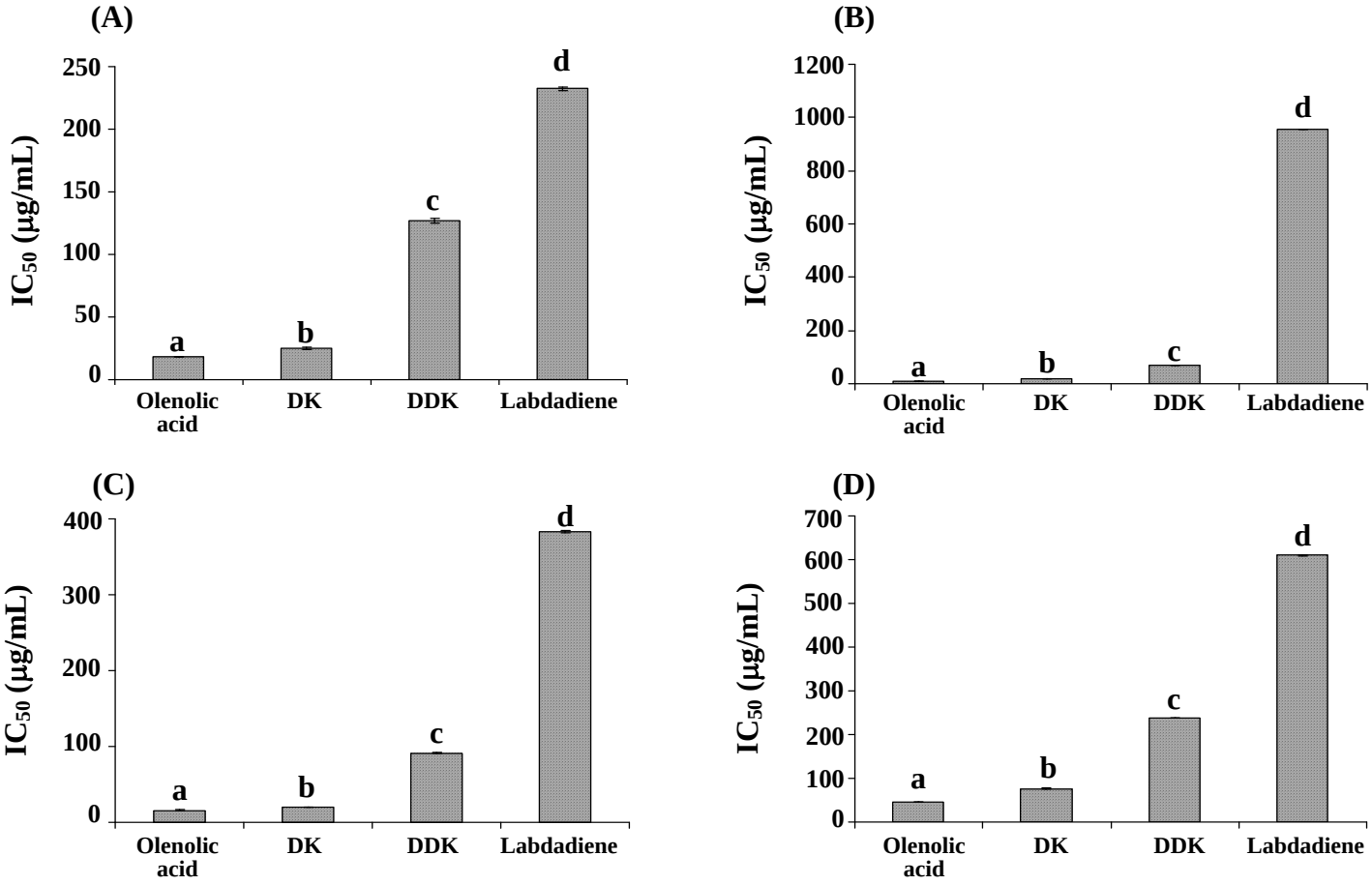

Figure 5.4 Inhibitory effects of DK, DDK and labdadiene on collagenase (A), elastase (B), hyaluronidase (C) and tyrosinase (D) activities.

CHAPTER 6

GESTATIONAL DIABETES MODULATORS INHIBITION ACTIVITY OF *A. zerumbet*

6.1 SUMMARY

Several metabolic pathways are involved in the onset of gestational diabetes, a pathologic condition related to the high blood sugar during pregnancy. In this study, the effects of isolated compounds from the rhizomes and seeds of *Alpinia zerumbet* were investigated on the activities of α-glucosidase, trypsin, nitric oxide and tryptophan hydroxylase, which have been closely associated with gestational diabetes. (*E*)-2,2,3,3-Tetramethyl-8-methylene-7-(oct-6-en-1-yl)octahydro-1*H*-quinolizine, isolated for the first time from the seeds, showed high inhibitory activities on α-glucosidase, trypsin and nitric oxide. Furthermore, the compound isolated from the rhizomes, (1*E*,3*E*,5*E*)-6-methoxyhexa-1,3,5-trien-1-yl)-2,5-dihydrofuran, showed dose-dependent increase in the activity of tryptophan hydroxylase. These studies *in vitro* reveal that both compounds may have probable applications in preventing gestational diabetes formation.

6.2 INTRODUCTION

Gestational diabetes mellitus (GDM) is defined as glucose intolerance with onset or first recognition during pregnancy (Kaaja and Rönnemaa, 2008). Several metabolic changes occur during pregnancy, including an increase in the serum concentrations of lactogenic hormones, as well as insulin resistance. To cope with insulin resistance, the pancreas increases the number of insulin-producing beta cells to augment insulin secretion. Failure to respond in this manner can lead to gestational diabetes mellitus (Georgia and Bhushan, 2010). α-Glucosidase is a membrane-bound enzyme at the epithelium of the small intestine that hydrolyses the cleavage of

glucose from disaccharides and oligosaccharide. The inhibitors of this enzyme delay carbohydrate digestion and prolong the overall carbohydrate digestion time and thus cause a reduction in the rate of glucose absorption and lower the postprandial rise in blood (Ali *et al.*, 2002). Trypsin is known to exert a cellular effect by activating protease-activated receptors (PARs) which might be up-regulated during pregnancy (Clark et al, 1991). The trypsin inhibitors were found to have decreased basal blood glucose levels and increased plasma insulin (Ihse *et al.*, 1976). Moreover, gestational diabetes is associated with increased L-arginine transport and nitric oxide (NO) synthesis (Vásquez *et al.*, 2004). NO inhibitors have been known to prevent diabetic complications (Corbett *et al.*, 1992). On the other hand, serotonin, a neurotransmitter is associated with mood changes, pain appreciation, sexual activity, appetite, endocrine and cardiac functions which are all affected during pregnancy (complementary therapies to reduce physiological stress in pregnancy). It helps to initiate the adaptive expansion of beta cell mass by lactogenic hormones during pregnancy and could be a major determinant in regulating the insulin secretion and adaptive beta cell expansion that occur during pregnancy (Georgia and Bhushan, 2010). Gestational diabetes is controlled by serotonin levels, which increase or decrease depending on how much protein pregnant mothers eat during the beginning of their pregnancies (Bole, 2010).

Plant natural products have always provided unique sources of bioactive lead compounds for drug discovery. Two α-pyrones (5,6-dehydrokawain and dihydro-5,6-dehydrokawain) were isolated from the rhizome of *A. zerumbet* and showed their neuraminidase and HIV-1 integrase inhibitory activities (Upadhyay *et al.*, 2011) and skin diseases-related enzymes (see **Chapter 5**). Besides,

8(17),12-labdadiene-15,16-dial was isolated and investigated the advanced glycation end products inhibitions (see **Chapter 3**). Cholest-4-ene-3,4-dione was identified in seed acetone extract and was found its potent antiatherogenetic properties (see **Chapter 4**).

In this study, two compounds were isolated for the first time: (1*E*,3*E*,5*E*)-6-methoxyhexa-1,3,5-trien-1-yl)-2,5-dihydrofuran from the rhizomes and (*E*)-2,2,3,3-tetramethyl-8-methylene-7-(oct-6-en-1-yl)octahydro-1*H*-quinolizine from the seeds of *A. zerumbet*. These compounds were investigated for inhibition against three enzymes, α-glucosidase, trypsin, tryptophan hydroxylase, and nitric oxide radical formation, which are known to modulate gestational diabetes. This is the first report on the isolation of these compounds from *A. zerumbet* and investigating their bioactivities against the gestational diabetes modulators.

6.3 MATERIALS AND METHODS

6.3.1 Chemicals

p-Nitrophenyl-α-D-glucopyranoside (PNG-G), PNGase F from *Elizabethkingia miricola*, *N*-α-benzoyl-DL-arginine-*p*-nitroanilide (BAPNA), trypsin from porcine pancreas, DL-dithiothreitol, ethyleneglycolbis(aminoethylether)tetra-acetate (EGTA), perchloric acid, ascorbic acid, catalase from human erythrocytes, DL-tryptophan, (6R)-5,6,7,8,-tetrahydrobiopterin dihydrochloride (BH_4), sodium nitroprusside (SNP), sulfanilic acid and *N*-(1-naphthy)ethyenediamine dihydrochloride (NEDH) were obtained from Sigma-Aldrich, Inc. (Missouri, USA). Coomassie brilliant blue G-250 (CBB G-250) and phosphoric acid were purchased from Wako Pure Chemical

Industries, Ltd. (Osaka, Japan). Rat brain was acquired from Rockland Immunochemical Inc. (Pennsylvanis, USA). Tris(hydroxymethyl)-aminomethan (Tris-HCl) was bought from Merck Chemicals Ltd. (Nottingham, UK). Sephadex G-25 was procured from GE Healthcare Bio-Science AB (Uppsala, Sweden). Ethylenediaminetetra-acetic acid (EDTA) was obtained from Kanto Chemical Co., Inc. (Tokyo, Japan). Trifluoroacetic acid (TFA) was procured from Berkin-Elmer JAPAN Co., Ltd. (Yokohama, Japan).

6.3.2 Extraction and isolation of active compounds

Rhizomes and seeds of *A. zerumbet* were collected at University of the Ryukyus, Okinawa, Japan. Air-dried rhizomes of *A. zerumbet* (400 g) were extracted with methanol (2 L) for 2 days at room temperature. After evaporation of the solvent 5.7g extract was obtained which was dissolved in distilled water (300 mL) and defatted with hexane (300 mL). The defatted aqueous extract (2.9 g) was further fractionated with $CHCl_3$ (200 mL) and then EtOAc (200 mL). The EtOAc fraction (1.6 g) was subjected to glass chromatography column containing silica gel (Silica gel 60N, particle size 63-120 μm, 70-230 mesh ASTM) and eluted with petroleum ether: $CHCl_3$ (0-100%) to afford three fractions. The fraction 2 was further purified by HPLC. The isolated compounds were collected at 280 nm using a Synergi 4u MAX-RP 80A column (150 x 4.60 mm, 4 micron; Phenomenex, Torrance, CA). The mobile phases were water with 0.1% acetic acid (v/v) (solvent A) and acetonitrile with 0.1% acetic acid (solvent B) at a flow rate of 1.00 mL/min. The gradient elution was performed as follows: 0-7 min, 40-70% B; 7-20 min, linear gradient 70-100% B; 20-30 min, 100% B. A 5 μL methanolic solution of fractions at 1 mg/mL was used

for the isolation of the compounds (**Figure 6.1A**).

The seeds (100 g) of *A. zerumbet* were extracted with 500 mL of methanol by maceration at room temperature for 2 days. After filtration, the filtrate was evaporated to dryness to give 21.6 g of dark syrupy extract. The extract was suspended in distilled water (500 mL) and partitioned with hexane (500 mL) and EtOAc (500 mL). The EtOAc extract (11.07 g) was subjected to glass chromatography column containing silica gel, eluted with MeOH in CH_2Cl_2 in a step gradient manner from 1% to 50% to obtain four fractions. Fraction 4 was further purified by the same column and condition as described above (**Fiure 6.1B**).

6.3.3 Identification of active compounds

Mass spectrometry was performed using a Shimadzu LC-20AD XR performed LC system and a Water Quattro micro API performed MS system with a 100 mm x 2.0 mmφ column of Cadenza CD-C18 (3.0 μm; Water, Milford, MA). A sample volume of 2 μL was injected. The mobile phase was composed of solvent A (0.1% formic acid in water) and solvent B (0.1% formic acid in acetonitrile) and was introduced at a constant flow rate of 0.2 mL/min. The gradient was programmed to increase the amount of B from 10-90% B; 0-30 min, linear 90% B; 30-40 min and increase to 100% B; 40-45 min. The cone and capillary voltages were 50 V and 4.0 kV, respectively. The desolation temperature was set at 350^{o}C.

The infrared (IR) spectrum was recorded in the 400-4000 cm^{-1} range using JASCO FT/IR-6100 plus spectrometer and KBr pellets.

^{1}H- and ^{13}C-NMR spectra were obtained in methanol-d_4 with a ULTRASHIELD™ PLUS 500 MHz (Bruker Biospin, Germany). Chemical shifts (δ) were obtained using

standard pulse sequences on TopSpin 2.1 program Version 2.1.0 and reported in part per million (ppm). The signals are expressed as singlet (*s*), doublet (*d*), triplet (*t*), quarter (*q*) and multiplet (*m*). Coupling constants (*J*) are reported in Hz.

6.3.4 α-Glucosidase inhibitory activity assay

The assay was performed according to Ahmad *et al.* (2011) with slight modifications. Briefly, 15 μL of various concentrations of test sample was added to 140 μL of enzyme solution (0.0073 U/mL of α-glucosidase in 0.05 M sodium phosphate buffer containing 100 mM NaCl) in 96-well plate and incubated at 37^{o}C for 15 min. After incubation, 25 μL of 0.7 mM PNP-G solution in 0.05 M sodium phosphate buffer (pH 6.8) was added to each well. The increment in absorption at 405 nm due to the hydrolysis of PNP-G by α-glucosidase was monitored continuously with a spectrophotometer (UV mini 1240, Shimadzu, Kyoto, Japan).

6.3.5 Trypsin inhibitory activity assay

Trypsin inhibitory activity was measured according to the method of Wati *et al.* (2009) using BAPNA as substrate. A solution containing 100 μL of test samples, 200 μL of trypsin (20 μg/mL) and 100 μL of distilled water were pre-incubated at 37^{o}C for 10 min. Then 500 μL of BAPNA (0.4 mg/mL, pre-warmed to 37^{o}C) was added to start reaction. After incubation at 37^{o}C for 10 min, 100 μL of acetic acid 30% (v/v) was added to terminate reaction and then subjected to centrifugation. Activity of trypsin was determined by measuring the mount of *p*-nitroaniline released at 410 nm. One unit of trypsin inhibitor was defined as decreases in absorbance at 410 nm under assay conditions compared with the control (without inhibitors).

6.3.6 Nitric oxide inhibition assay

Nitric oxide radical inhibition was estimated by using Griess Illosvoy reaction (Govindarajan *et al.*, 2003). The reaction mixture (300 μL) containing 10 mM of SNP (200 μL), phosphate buffer saline (50 μL) and test sample (50 μL) was incubated at 25^{o}C for 150 min. After incubation, 50 μL of the reaction mixture was mixed with 100 μL of sulfanilic acid reagent (0.33% in 20% glacial acetic acid) and allowed to stand for 5 min for completing diazotization. Then, 100 μL of NEDH was added, mixed and allowed to stand at 25^{o}C for 30 min. A pink color chromophore was formed in diffused light. The absorbance of these solutions was measured at 540 nm against the corresponding blank solutions. The IC_{50} value of the test compound was calculated graphically from semi-log plots of inhibitor concentration against percent inhibition. Curcumin and queretin were used as the positive controls, while methanol was used as control for calculation.

6.3.7 Tryptophan hydroxylase activity assay

The activity of tryptophan hydroxylase was determined in extracts of rat brain mesencephalic tegmentum. This brain area contains the dorsal and median raphe nuclei, the cell bodies of origin of most forebrain 5-hydroxytryptophan (5-HTP) projections and very high in TPH activity (Steinbusch, 1981). Tegmenta was frozen and stored at -80 ^{o}C until assayed. Frozen tegmenta was homogenized in 0.05 M Tris buffer, pH 7.4, containing 1 mM dithiothreitol and 1 mM ethyleneglycolbis(aminoethylether)tetra-acetate (EGTA). Homogenates were centrifuged at 40,000 *g* and the resulting supernatant was desalted on a column of Sephadex G-25 equilibrated with the same homogenizing buffer. The desalted extracts were used as the source of TPH activity without further purification. Protein was

determined by the method of Bradford (1976) at 595 nm using BSA as a standard.

The activity of TPH was determined as previously described with the following modifications for HPLC (Kuhn *et al.*, 1980). Approximately 30 μg of protein was added to the tubes containing various concentrations of samples reactions were initiated with the addition of tryptophan and BH_4. Each assay tube contained the following substituents (in the indicated final concentrations) in a volume of 100 μL: 0.05 M of Tris buffer, pH 7.4, 1 mM of EGTA, 15 μg of catalase, 250 μM of tryptophan and 100 μM of BH_4. Tubes were incubated at 37^{o}C for 10 min after which reactions were terminated by the addition of 10 μL of 6 N $HClO_4$. Precipitated protein was removed by centrifugation in a Beckman Minifuge B for 5 min and all samples were then diluted 1:10 in 0.01 N HCl, 5 mM EDTA and 0.1% (w/v) ascorbic acid. The amount of 5-HTP formed was determined by HPLC.

5-Hydroxytryptophan was identified using photodiode-array spectrophotometry as previously described. The samples were separated on RP-18 ZORBAX ODS column (150 mm x 4.6 mm i.d.; 5 μm particle size) (Agilent Technologies, USA). The mobile phase was 0.1% trifluoroacetic acid (TFA) in water as solvent A, and 0.08% TFA in 95% acetonitrile as solvent B. The concentration of solvent B was increased linearly 1% per min for 40 min at a flow rate of 1.5 mL/min at 220 nm. The percent of stimulation was calculated comparing with peak area of control follow by

$$[(P_{sample} - P_{control})/ P_{control}] \times 100,$$

where P is the peak area of HPLC.

6.3.8 Statistical analysis

The results are expressed as the mean value with its standard deviation (mean ± SD). Statistical difference among groups were estimated by one-way analysis of variance

(ANOVA), followed by Duncan's multiple range tests and analyzed by SPSS version 16.0. The *p*-value less than 0.01 were considered significant ($p < 0.01$).

6.4 RESULTS

6.4.1 Spectal details for isolated compounds

Compound R1: dihydro-5,6-dehydrokawain (DDK), HREIMS *m/z* 231.2 [M^+] (calcd for $C_{14}H_{14}O_3$, 230.25). IR *v* (KBr) cm^{-1}: 1134, 1237, 1267, 1428, 1456, 1473, 1497, 1507, 1521, 1541, 1573, 1652, 1702, 2136, 3393. ^{1}H-NMR (500 MHz, MeOD-d_4): δ 2.79 (*t*, 2H, CH_2, *J* = 5.0 Hz, 1), 2.95 (*t*, 2H, CH_2, *J* = 5.0 Hz, 2), 3.81 (*t*, 3H, OCH_3, J = 5.0 Hz, 14), 5.51 (*d*, 1H, *CH*, J = 5.0 Hz, 12), 5.92 (*d*, 1H, *CH*, J = 5.0 Hz, 10), 7.17 (*s*, 1H, *CH*, 6), 7.19 (*t*, each 1H, *CH*, *J* = 5.0 Hz, 4, 8) and 7.25 (*t*, each 1H, *CH*, *J* = 5.0 Hz, 5, 7). ^{13}C-NMR (500 MHz, MeOD-d_4): 33.80 (C-1), 36.16 (C-2), 56.85 (C-14), 88.23 (C-12), 101.92 (C-10), 127.42 (C-6), 129.42 (C-5,7), 129.56 (C-4,8), 141.34 (C-3), 166.20 (C-9), 167.65 (C-13) and 173.80 (C-11) (**Figure 6.2A**).

Compound R2: 2,5-bis (1*E*,3*E*,5*E*)-6-methoxyhexa-1,3,5-trien-1-yl)-2,5-dihydrofuran (MTD), HREIMS *m/z* 285.1 [M^+] (calcd for $C_{18}H_{22}O_3$, 286.16). IR *v* (KBr) cm^{-1}: 669, 1646, 2341, 2359. ^{1}H-NMR (500 MHz, MeOD-d_4): 3.83 (*q*, 3H, OCH_3, *J* = 5.5 Hz, 9), 5.62 (*d*, 1H, *OCH*, 2), 6.24 (*d*, 1H, *CH*, 7), 6.86 (*d*, 1H, *CH*, 6), 7.58 (*s*, 1H, *CH*, 5), 7.59 (*d*, 1H, *CH*, 4), 7.14 (*q*, 1H, *CH*, *J* = 5.0 Hz, 1), 7.41 (*t*, 1H, *CH*, *J* = 7.0 Hz, 3), 7.36 (*t*, 1H, *CH*, *J* = 4.0 Hz, 8). ^{13}C-NMR (500 MHz, MeOD-d_4): 57.01 (C-9), 89.42 (C-2), 102.78 (C-7), 120.05 (C-6), 128.63 (C-5), 129.14 (C-4), 129.99 (C-3), 130.55 (C-1), 136.66 (C-8) (**Figure 6.2B**).

Compound S1: (*E*)-2,2,3,3-tetramethyl-8-methylene-7-(oct-6-en-1-yl)octahydro-1*H*-quinolizine (TMOQ), HREIMS *m/z* 317.55 [M^+] (calcd for $C_{22}H_{39}N$, 317.2). IR *v*

(KBr) cm^{-1}: 1024, 1121, 1456, 1507, 1541, 1558, 1646, 1698, 1748, 2360, 2927, 3448. ^{1}H-NMR (500 MHz, MeOD-d_4): 0.75 (*q*, 3H, CH_3, *J* = 10.5 Hz, 20), 0.85 (*t*, 3H, CH_3, *J* = 1.5 Hz, 18), 0.89 (*q*, 3H, CH_3, *J* = 4.0 Hz, 21), 0.94 (*t*, 3H, CH_3, *J* = 2.5 Hz, 1), 1.09 (*t*, 2H, CH_2, *J* = 2.5, 8), 1.17 (*q*, 2H, CH_2, 11), 1.22 (*q*, 2H, CH_2, *J* = 1.5 Hz, 14), 1.28 (*s*, 2H, CH_2, 14), 1.39 (*q*, 1H, *NCH*, *J* = 2.5 Hz, 12), 1.43 (*q*, 2H, CH_2, *J* = 2.2 Hz, 4), 1.58 (*t*, 3H, CH_3, *J* = 2.0 Hz, 19), 1.64 (*q*, 2H, CH_2, *J* = 4.5 Hz, 7), 1.75 (*q*, 2H, CH_2, *J* = 2.0 Hz, 5), 2.00 (*d*, 2H, NCH_2, 13), 2.26 (*d*, 2H, CH_2, 6), 2.47 (*t*, 2H, NCH_2, *J* = 5.5 Hz, 17), 3.27 (*t*, 2H, CH_2, *J* = 8.0 Hz, 22), 6.02 (q, 1H, *CH*, 3) and 6.36 (*d*, 1H, *CH*, 2). ^{13}C-NMR (500 MHz, MeOD-d_4): 14.00 (C- 21), 15.48 (C-20), 20.16 (C-4), 20.39 (C-19), 22.37 (C-18), 24.53 (C-5), 31.77 (C-7), 34.02 (C-15), 34.50 (C- 1), 37.79 (C-6), 39.90 (C-16), 40.36 (C-8), 42.03 (C-11), 43.39 (C-14), 55.96 (C-9), 63.34 (C-12), 65.83 (C-17), 68.81 (C-13), 108.85 (C-22), 122.20 (C-2), 135.00 (C- 3), 150.77 (C-10) (**Figure 6.2C**).

6.4.2 α-Glucosidase inhibition

α-Glucosidase activity was determined by measuring the amount of glucose formed from PNG-G (maltose) by enzyme hydrolysis. The enzyme inhibitory activity of the isolated compounds is shown in **Figure 6.3A**. TMOQ and MTD (IC_{50} = 1.62 ± 0.04 and 1.64 ± 0.08 μg/mL, respectively) had similar effects when compared with quercetin (IC_{50} = 1.62 ± 0.04 μg/mL), where as they showed lower inhibitory activities than catechin (IC_{50} = 1.04 ± 0.07 μg/mL). Among the isolated compounds, TMOQ and MTD had stronger inhibition than DDK ($p < 0.01$).

6.4.3 Trypsin inhibition

The results showed that TMOQ (IC_{50} = 31.75 ± 1.01 μg/mL) had stronger

inhibitory activity than DDK and MTD (IC_{50} = 39.50 ± 0.35 and 41.48 ± 0.40 μg/mL, respectively). On statistical analysis, it was found that activity of TMOQ was not significantly different from activity of positive controls, catechin and quercetin (IC_{50} = 29.29 ± 0.30 and 34.68 ± 0.60 μg/mL, respectively) ($p < 0.01$) (**Figure 6.3B**).

6.4.4 Nitric oxide inhibition

The metabolic activation of SNP to nitric oxide was used to evaluate nitric oxide inhibition of isolated compounds. SNP is a rapid-acting nitrovasodilator which frequently used in pharmacological studies. It is considered to produce its vasorelaxant action by releasing NO. Moreover, NEDH is the coupling agent that is used for determining many aromatic amines by diazotization. The inhibition of NO formation by the isolated compounds is shown in **Figure 6.3C**. TMOQ (IC_{50} = 15.70 ± 0.29 μg/mL) showed better inhibitory effect than other isolated compounds. Furthermore, it had significantly better activity than quercetin (18.90 ± 0.79 μg/mL) ($p < 0.01$), where as it showed lower activity than catechin (IC_{50} = 12.03 ± 0.25 μg/mL).

6.4.5 Tryptophan hydroxylase activity

The results indicated that the amount of 5-HTP formed decreased with increasing concentrations of DDK and TMOQ when compared with control (**Figure 6.4**). Interestingly, MTD showed contradictory results implicating that MTD enhances the hydroxylase activity.

6.5 DISCUSSION

In this study, three compounds were isolated, two from the rhizomes and one from the seeds of *A. zerumbet*. Two novel compounds were isolated for the first time and all of these compounds were investigated for bioactive properties against GDM modulators.

Our laboratory had previously isolated DDK from the rhizomes of *A. zerumbet* (Upadhyay *et al.*, 2011). Beside, MTD and TMOQ were isolated from rhizomes and seeds of *A. zerumbet*, respectively. These novel compounds from *A. zerumbet* have been reported for the first time.

The activity of α-glucosidase was examined as an enzyme of therapeutic approach to decrease postprandial hyperglycemia. The effect of isolated compounds on the enzyme inhibition was investigated by measuring the hydrolysis of PNP-G. It was found that TMOQ and MTD showed stronger inhibition activity. The trypsin inhibition assay was performed by using BAPNA as the substrate of trypsin, where BAPNA was cleaved into *N*-α-benzoyl-DL-arginine and 4-nitroaniline. The result showed that TMOQ had higher inhibitory effect than DDK and MTD. Additionally, the inhibition of nitric oxide was checked using fast method for the spectrophotometric determination of sodium nitroprusside based on the application of kinetic methodology and the batch mixing technique to the coupling reaction between the diazotized nitroprusside derivative and *N*-(1-naphthyl)ethylenediamine dihydrochloride. TMOQ was the strongest inhibitor on nitric oxide formation among isolated compounds. Finally, the potency of isolated compounds was evaluated in increasing the activity of TPH extracted from rat brain. During the synthesis of

serotonin, the substrate tryptophan is hydroxylated by the enzyme TPH to form the intermediate metabolite 5-HTP. This metabolite is further decarboxylated to serotonin by L-amino acid decarboxylase. In this study, it was focused on the hydroxylation reaction only since TPH was the initial and rate-limiting enzyme in the biosynthesis of the neurotransmitter serotonin (Jéquier *et al.*, 1967). The activity of TPH was estimated by measuring the accumulation of 5-HTP using HPLC. The results showed that MTD had higher activity with increasing concentration. The plausible explanation for the effect of MTD toward TPH activity is still under investigation.

The isolated compounds exhibited different degrees of activities in various performed assays. The reasons for variations may be vast differences in the chemical structures of these compounds. DDK has been reported to have several bioactivities to inhibit such as, HIV-1 integrase and neuraminidase (Upadhyay *et al.*, 2011), aggregation and ATP release (Teng *et al.*, 1990) and singlet oxygen quenching (Liao et al., 2000).

MTD contains dihydrofuran which are important heterocyclic compounds commonly found in a wide variety of naturally occurring substances and possessing a multiplicity of biological activities. Nichols *et al.* (1986) reported that when the methoxy group was constrained into a dihydrofuran, the orientation of the ring was crucial, consistent with the notion of specific position of the oxygen unshared electron. Several researches have been reported on the potential of compounds containing dihydrofuran for pharmacological properties. Hennessy *et al.* (2000) demonstrated that 2,2-dimethyl-2,3-dihydrofuran as useful test substrate for the intermolecular asymmetric Heck reaction as it allows for easy and direct comparison of a wide range of ligands as

only one regioisomeric product can be formed. Moreover, Lin *et al.* (2007) tested the cytotoxic effects of ethyl-2-[N-p-chlorobenzyl-(2´-methyl)anikine-4-oxo-4,5-dihydrofuran-3-carboxylate in mouse leukemia and reported that this compound induced apoptosis via a microchondria-dependent pathway closely related to the level of cytoplasmic Ca^{2+} in human cervical cancer Ski cells. Zhang *et al.* (2012) presented that the 2,5-dihydrofuran derivatives could improve the anti-cancer activities effectively. Furthermore, Koike *et al.* (2011) evaluated melatonin receptor (MT_1/MT_2) ligands and reported that tricyclic dihydrofuran derivative: 8,9-dihydrofuro [3,2-*c*]pyrazolo [1,5-*a*]pyridines, was shown to penetrate the rat blood-brain barrier (BBB).

On the other hand, TMOQ contains quinolizidine ring system comprising of core structural subunits found in a large number of natural products. Unger *et al.* (1971) provided novel pharmaceutical compositions containing quinolizine derivative as active ingredients that showed effect an activity on central nervous system. The 2,9-diacettoxydibenzoquinolizine compounds with alkyl substituents bind strongly to the estrogen receptor except and with ethyl substituents strongly inhibited the growth of hormone-sensitive MCF-7 breast cancer cells (von Angerer *et al.*, 1994). Beside, new derivative of 4,5,9,10-tetrahydro-1,4-ethanobenz[*b*]quinolizine has been designed as a prodrug for its quinolizinium cation that is a potent antagonist of TCP-binding site of NMDA receptors that is a member of ion channel-type receptors for L-glutamate, an excitatory amino acid in the mammalian central nervous system which increases the influx of Ca^{2+} through the iron channel of the receptor (Sasaki *et al.*, 2001). Dugan and Nieforth (1973) synthesized (±)-*trans*-11-methylthio-1,2,3,4,6,7,12,12b-octahydro-hydroxyindolo [2,3-α]quinolizine and used as a cardiovascular agent. Moreover, the novel synthetic indoloquinolizines could serve as immunotherapeutic agents by selectively increasing the

pool of activated T lymphocytes or stimulating macrophage functions, with potential use in the treatment of infectious diseases including AIDS and cancer (Solis-Maldonado *et al.*, 2003). It seems that the functional moiety of the isolated compound, TMOQ, may have possible roles in exhibiting the bioactivities.

6.6 CONCLUSIONS

Three compounds were isolated, out of which two of them, namely, (1*E*,3*E*,5*E*)-6-methoxyhexa-1,3,5-trien-1-yl)-2,5-dihydrofuran (MTD) and (*E*)-2,2,3,3-tetramethyl-8-methylene-7-(oct-6-en-1-yl)octahydro-1*H*-quinolizine (TMOQ) were isolated for the first time from *A. zerumbet*. These results showed that these compounds have inhibitory effects against gestational diabetes modulators, particularly, α-glucosidase, trypsin, and NO, whereas, MTD increased the TPH activity. Therefore, it is assumed that these compounds may have probable applications in further researches to design gestational diabetes modulators.

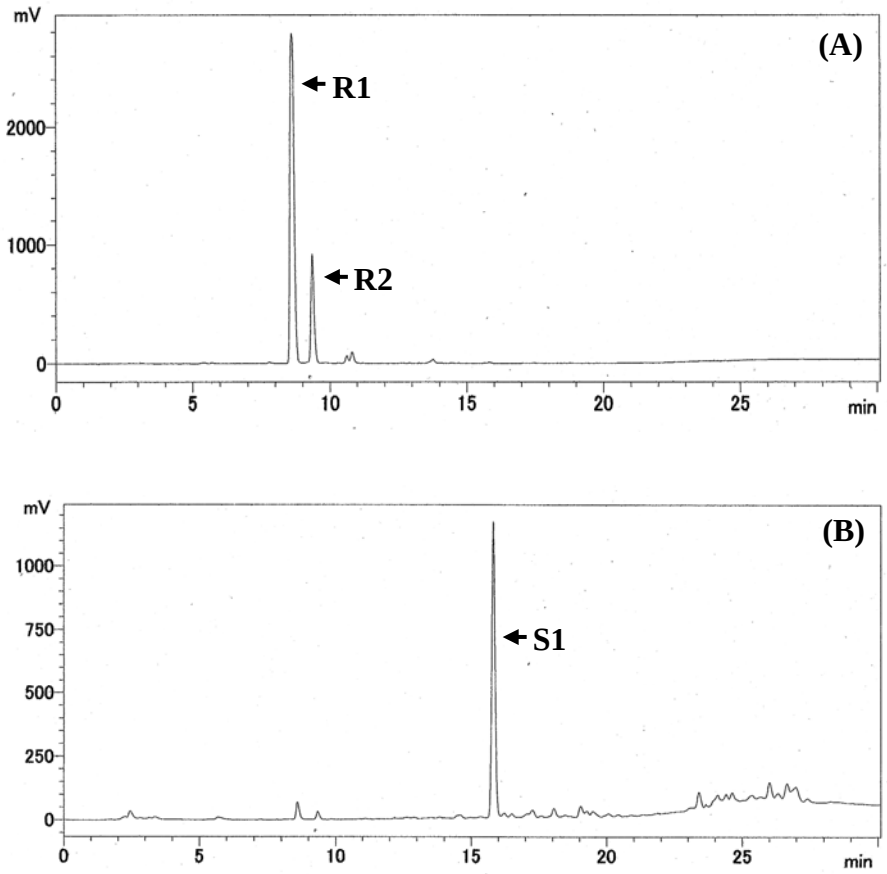

Figure 6.1 HPLC chromatograms of rhizome (A) and seed (B) fractions.

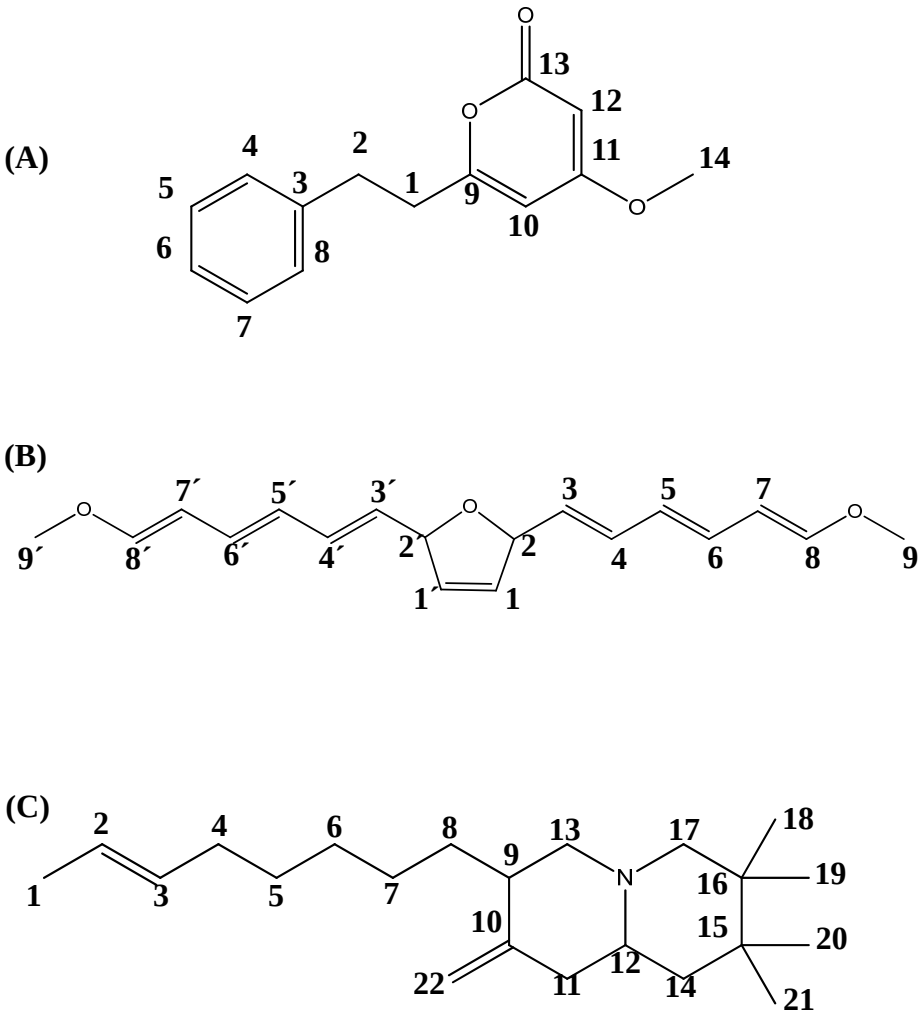

Figure 6.2 Chemical structures of isolated compounds; DDK (A), (1*E*,3*E*,5*E*)-6-methoxyhexa-1,3,5-trien-1-yl)-2,5-dihydrofuran; MTD (B) from rhizomes and (*E*)-2,2,3,3-tetramethyl-8-methylene-7-(oct-6-en-1-yl)octahydro-1*H*-quinolizine; TMOQ (C) from seeds of *A. zerumbet*.

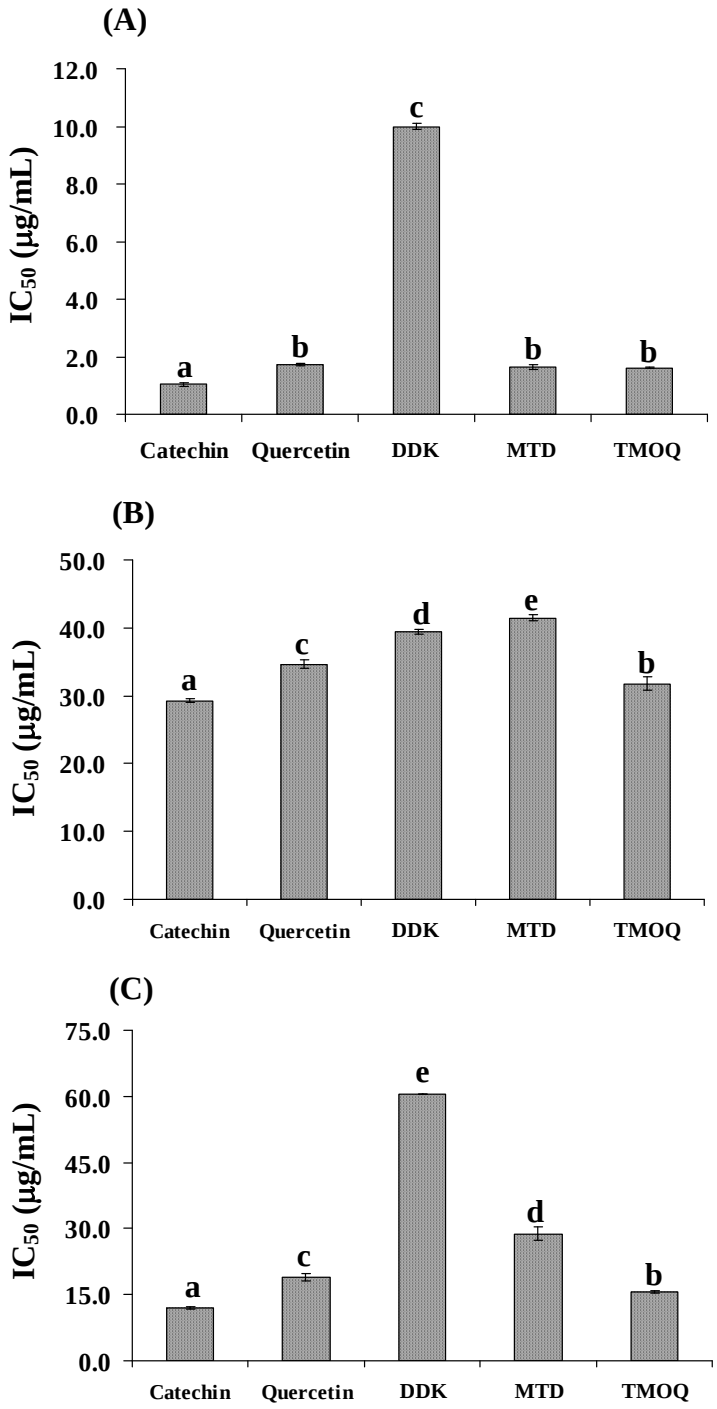

Figure 6.3 IC_{50} of isolated compounds on α-glucosidase (A), trypsin (B) and nitric oxide (C) activities. Means with the same letter on the bars are not significantly different, $p < 0.01$ (Duncan's multiple range tests). The bars represents mean ± SD ($n = 4$).

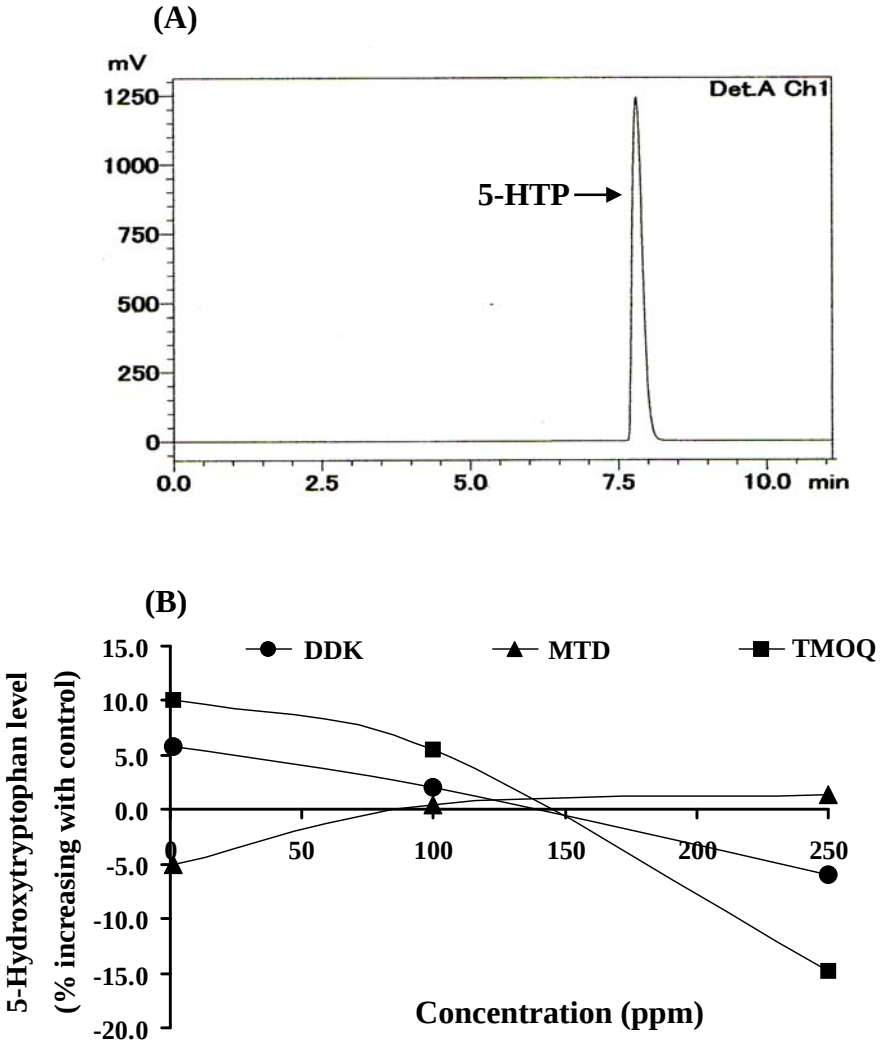

Figure 6.4 HPLC chromatogram of 5-HTP (A) and dose response curve (B) of isolated compounds for tryptophan hydroxylase activity. Values are expressed as the mean ± SD of four replicated experiments.

CHAPTER 7

A. zerumbet AS OXYGEN RADICAL SCAVENGERS IN HUVEC CELLS

7.1 SUMMARY

Endothelial dysfunction is recognized as initial step in the atherosclerotic process and is well advanced in diabetes. In current study, human umbilical vein endothelial cells (HUVEC) were used to evaluate the potential of isolated compounds from *Alpinia zerumbet* on oxygen species release and cAMP enzyme activity. it was found that (*E*)-2,2,3,3-tetramethyl-8-methylene-7-(oct-6-en-1-yl)octahydro-1*H*-quinolizine (TMOQ) showed high inhibitory activities on xanthine oxidase (IC_{50} = 14.70 ± 0.74 μM), LDL oxidation (IC_{50} = 2.10 ± 0.28 μM) and NADPH oxidase (% of inhibition = 47.84 ± 0.31, 50 μM), moreover, this compound had ability to increase cAMP level (% increasing = 33.50). These results indicate that TMOQ may have possibilities in the design of drugs against atherogenic process in endothelial cells.

7.2 INTRODUCTION

Oxygen derived species are well known to be cytotoxic and have been implicated in the etiology of a wide array of human diseases (Waris *et al.*, 2006). Hydrogen peroxide and superoxide anion are the major source of endogenous ROS in living cells, which are generated as products of cellular metabolism (Nohl *et al.*, 2003). Human umbilical vein endothelial (HUVEC) cells have played a major role as a model system for the study of the regulation of endothelial cell function and the role of the endothelium in the response of the blood vessel wall to stretch, shear forces, and the development of atherosclerotic plaques (Park *et al.*, 2006).

Low-density lipoprotein (LDL) are believed to play a major role in the

pathogenesis of atherosclerosis whereas oxidative modification of LDL (oxLDL) seems to be a key event in this process to stimulate vascular $O_2^{\bullet-}$ formation that induces apoptotic cell death in cultured HUVEC. Induction of apoptosis could be attributed to stimulation of $O_2^{\bullet-}$ formation, presumably via activation of the membrane-bound nicotinamide adenine dinucleotide phosphate (NADPH) oxidase. However, $O_2^{\bullet-}$ formation is also involved in regulation of cell proliferation, *e.g.*, it has been shown that stimulation of the membrane-bound NADPH oxidase by angiotensin II (Heinloth *et al.*, 2000). Another important source of superoxide is xanthine oxidase, an enzyme formed from xanthine dehydrogenase through either proteolytic cleavage or by oxidation of cysteines in the enzyme converting xanthine to uric acid. However, xanthine oxidase has the unique property of being able to reduce oxygen to form superoxide and hydrogen peroxide (Harrison *et al.*, 2003). Increasing of superoxide dismutase (SOD) activity reduces vascular cell-mediated oxidation of LDL suggesting that superoxide $O_2^{\bullet-}$ generated in vascular cells is involved in the oxidation of lipids/lipoproteins (Yang *et al.*, 2004). Furthermore, cyclic adenosine monophosphate (cAMP) is a ubiquitous intracellular second messenger that regulates important cellular functions. Several reports support a role of cAMP in atherogenesis by modulating the function of vascular endothelium, the production of ROS, the recruitment of circulating monocytes to the artery wall and their differentiation into macrophages- foam cells, by controlling the expression of pro- and anti-inflammatory interleukins and regulating serum levels of triglycerides and cholesterol (Fantidis, 2010). Saha *et al.* (2008) reported that increased levels of cAMP may have a protective effect in reducing oxidative stress and thereby improve vascular function.

Alpinia zerumbet (Family: Zingiberaceae) is an herbaceous perennial plant that showed various pharmacological properties. Our laboratory has suggested several possible pharmacological usages of the plant. Elzaawely *et al.* (2007) investigated that essential oil production from leaves and rhizomes of this plant may be utilized in foodstuffs as a source of natural antioxidants. 8(17),12labdadiene-15,16-dial (ladadiene) was isolated from rhizomes and reported that it could be used to prevent glycation-associated complication in diabetes (see **Chapter 3**). Upadhyay *et al.* (2011) suggested that alpinia could be used as a source of bioactive compounds against HIV-1 integrase and neuraminidase and that α-pyrones, 5,6-dehydrokawain (DK) and dihydro-5,6-dehydrokawain (DDK), isolated from rhizomes may have possibilities in the design of drug against these viral diseases. Beside, **Chapter 5** showed that DK could be used as a potent inhibitor and be further exploited to be used in anti-skin disease formulations. Furthermore, cholest-4-ene-3,6-dione contained in seeds of this plant could be used to prevent LDL oxidation which involved in the ability on inhibition atherosclerotic disease *in vitro* (see **Chapter 4**). In **Chapter 6**, (1*E*,3*E*,5*E*)-6-methoxyhexa- 1,3,5-trien-1-yl)-2,5-dihydrofuran (MTD) and (*E*)-2,2,3,3-tetramethyl-8-methylene- 7-(oct-6-en-1-yl)octahydro-1*H*-quinolizine (TMOQ) were isolated from rhizomes and seeds of *A. zerumbet* and investigated their activities against gestational diabetes modulators. The objectives of this study focused on investigating the activities of isolated compounds from *A. zerumbet* including DK, DDK, labdadiene, MTD and TMOQ as oxygen radical scavengers using *in vitro* as well as cell culture experiments. The inhibitions of activities of isolated compounds were examined for xanthine oxidase and NADPH oxidase, oxidation of LDL and generation of cyclic AMP (cAMP) in HUVEC cells.

7.3 MATERIALS AND METHODS

7.3.1 Chemicals

Medium 199, fetal bovine serum and 0.25% trypsin-EDTA were obtained from Gibco (New York, USA). Heparin sodium, L-glutamine + streptomycin + penicillin (100X), human epidermal growth factor, 0.01 mol/L phosphate buffered saline, angiotensin II, D-glucose and L-arginine were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Hydrocortisone and superoxide dismutase were bought from MP Biomedicals, LLC (Illkirch, France). Endothelial cell growth supplement, horse heart cytochrome *c*, HEPES sodium salt, malonyldialdehyde, trichloroacetic acid, thiobarbituric acid and lipoprotein from human plasma were acquired from Sigma-Aldrich, Inc. (Missouri, USA). N^G-nitro-L-arginine methyl ester (L-NAME) was procured from Dojindo (Kumamoto, Japan). Xanthine was bought from Cica (California, USA). Xanthine oxidase was purchased from Toyobo Co., Ltd. (Osaka, Japan).

7.3.2 Preparation of isolated compounds

The compounds were isolated as reported previously including DK and DDK (Upadhyay *et al.*, 2011), labdadeine (see **Chapter 3**), MTD and TMOQ (see **Chapter 6**) (**Figure 7.1**).

7.3.3 Xanthine oxidase inhibition assay

Superoxide anion radicals produced by the xanthine-xanthine oxidase system are able to reduce cytochrome *c*. Effect of isolated compounds on generation of $O_2^{\bullet-}$ were determined according to a previous report (Selloum *et al.*, 2001). The reaction

mixture contained xanthine (100 μM), horse heart cytochrome *c* (25 μM) and samples (different concentrations) in sodium phosphate buffer (50 mM, pH 7.4) supplemented with 0.1 mM of EDTA. The reaction was started by the addition of xanthine oxidase (0.07 U/mL). After 2 min, reduced cytochrome *c* was determined spectrophotmetrically at 550 nm. The amount of generated superoxide was calculated using $\varepsilon = 21{,}100\ M^{-1}cm^{-1}$.

7.3.4 LDL-oxidation inhibition assay

The generation of oxidized-LDL by $CuSO^{-}_{4}$ induced was evaluated as described by Rattan and Arad (1998). LDL was adjusted to 220 μg/mL with 10 mM PBS (pH 7.4) and then aliquots of isolated compounds (different concentration) were added to the solution. The oxidative modification of LDL was initiated by addition of 55 μM of $CuSO_4$ incubating at 37^{o}C for 24 h. The reaction was stopped by adding 50 μL of 1 M EDTA and placing the samples at -20^{o}C for TBARS assay using the method of Steinbrecher *et al*. (1984). After oxidation, LDL was mixed with 1.5 mL of 0.67% TBA and 1.5 mL of 20% TCA. After placing samples in 100^{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. The yields of MDA were used as a standard and the results were expressed as nanomoles of MDA equivalents.

7.3.5 Maintenance of HUVEC cells

Human umbilical vein endothelial cells (HUVEC) (Lot: 121210/ 255902-1R, No. 12002053) and CSC complete recombinant medium were obtained from DS

Pharma Biomedical Co., Ltd (Osaka, Japan). Cells were maintained in CSC medium containing attachment factor and cell boost at 37°C with 5% CO_2 in a humidified atmosphere.

7.3.6 The cultivation of HUVEC cells

HUVEC cells of 2-4 passages were added into gelatin-coated flasks in medium 199 supplemented with 20% of FBS, 5 U/mL of heparin, 1 mL of L-glutamine + streptomycin + penicillin (100x), 1 μg/mL of hydrocortisone, 50 μg/mL of endothelial cell growth supplement and 10 μg/mL of human epidermal growth factor. Confluent cells (10^6 cells) were incubated with 50 μM of L-NAME, DK, DDK, labdadiene, MTD and TMOQ in cell culture medium for 24 h. After incubation, the medium was removed and the cells were washed with 5 mL of PBS. Afterwards the cells were scraped off with 5 mL of 0.25% trypsin-EDTA and immediately centrifuged for 5 min at 1000g and 4°C. Cells were harvested for further analysis.

7.3.7 Determination of angiotensin II-induced $O_2^{\bullet -}$ production

Specificity of the assay for $O_2^{\bullet -}$ was ascertained by co-incubation with superoxide dismutase (SOD; 200 U/mL). The harvested cells were washed with 50 mM phosphate buffer (pH 7.4) and subsequently exposed to 1 μM angiotensin II for 6 h at 37°C in the presence of 40 μM ferricytochrome *c* in HEPES-buffered isotonic salt medium (133 mM NaCl, 6.5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 5.5 mM glucose, 50 μM l-arginine, 20 mM HEPES, pH 7.4). Reduction of ferricytochrome *c* was measured in the supernatant at 550 nm. The $O_2^{\bullet -}$ release was calculated from the difference in the setup without and with SOD (Steffen *et al*., 2008).

7.3.8 Cytochrome c reductase (NADPH) assay

The activity of NADPH was examined by Cytochrome c Reductase (NADPH) assay kit (Sigma-Aldrich, Inc., Missouri, USA, Catalog No. CY0100). In brief, the harvested cells were mechanically broken in an isotonic buffer by homogenization in a Dounce homogenizer. The samples were centrifuged at 10,000 rpm and the supernatant were removed. Finally, the microsomal pellet was obtained by further centrifuging at 12,000g for 1 h. The enzymatic assay was measured as the reduction of cytochrome *c* by NADPH-cytochrome *c* reductase in the presence of NADPH. The reduction of cytochrome *c* results in the formation of distinct bands in the absorption spectrum and the increase in absorbance was measured at 550 nm. To a 950 μL of working solution, 50 μL test samples were added. In order to prevent the cytochrome c oxidase inhibition by the interfering substances, 20 μL of inhibitor solution was also added. After mixing by inversion, the reaction was started with the addition of NADPH solution (100 μL). Blank was measured as the value given by the reagents alone without enzyme present. The enzyme activity was measured by

$$\text{Units/mL} = (\Delta A_{550}/\text{min} \times \text{dil} \times 1.1)/ (21.1 \times \text{Enzvol})$$

where, ΔA_{550}/min is ΔA_{sample} - ΔA_{blank}, dil is volume of the enzyme sample (ml), Enzvol is volume of the enzyme sample (mL), 21.1 is extinction coefficient (ε^{mM}) for reduced cytochrome *c* and 1.1 is reaction volume (mL).

7.3.9 cAMP enzyme immunoassay

To the harvested cells, 0.1 M HCL was added for cell lysis. After 10 min, the samples were centrifuged at 2000g for 10 min and the supernatants were used for assay. The cAMP activity of isolated compounds was determined by using the assay

described in cAMP Enzyme Immunoassay kit manual (Sigma-Aldrich, Inc., Missouri, USA, Catalog No. CA201). Briefly, acetylated samples (100 μL), cAMP-alkaline phosphatase (50 μL) conjugate, and cAMP antibody (50 μl) were simultaneously incubated at room temperature for 2 h in a secondary antibody coated multiwall plate. The excess reagent was washed away and the substrate was added. After a short incubation time the enzyme reaction was stopped and the yellow color produced was measured at 405 nm. The concentration of cAMP was calculated using the semi log equation of B/Bo vs cAMP concentration, where B is the average net absorbance and Bo is the percentage of absorbance of the maximum binding wells.

7.3.9 Statistical analyses

The results of cell assays are presented as mean ± SD of two experiments with three replications. Data were subjected to analysis of variance in general linear model using the SPSS version 16.0 (SPSS Ltd.). The significant treatment effects were disclosed at the probability level of $p < 0.01$. The Tukey's test was applied to determine statistical differences between means.

7.4 RESULTS

7.4.1 The Generation of $O_2^{\bullet-}$ by the xanthine-xanthine oxide activity

The results showed that TMOQ (IC_{50} = 14.51 ± 0.74 μM) had higher inhibitory effects than other isolated compounds (**Figure 7.2A**). Moreover, TMOQ had similar inhibitory activity to the positive control, L-NAME (IC_{50} = 12.88 ± 0.11 μM).

7.4.2 LDL oxidation inhibition.

The **Figure 7.2B** shows 50% inhibition of isolated compounds on LDL oxidation, assesses with the TBARS assay. TMOQ (IC_{50} = 2.10 ± 0.28 μM) showed the highest reduction in amount of MDA equivalents produced by copper-induced LDL oxidation compared with other compounds.

7.4.3 Angiotensin II-induced $O_2^{\bullet-}$ production

The effects of test samples on angiotensin II-elicited release of $O_2^{\bullet-}$ from HUVEC cells are shown in **Figure 7.3**. The result showed that all compounds had high levels of $O_2^{\bullet-}$ product. The production of $O_2^{\bullet-}$ are 96.84 ± 1.65, 95.94 ± 0.39, 93.24 ± 1.13, 84.23 ± 0.52 and 74.77 ± 1.26 % of control by DK, MTD, DDK, TMOQ and labdadiene, respectively. That showed higher $O_2^{\bullet-}$ level than L-NAME (43.00 ± 2.47 % of control).

7.4.4 Cytochrome c reductase (NADPH) activity

The inhibition of isolated compounds on NADPH activity is presented in **Figure 7.4**. Using 50 μM of test samples, the positive controls, L-NAME (% inhibition = 86.17 ± 0.66) showed higher inhibition of cytochrome *c* reductase activity than isolated compounds. Amongst isolated compounds, TMOQ (% inhibition = 47.84 ± 0.31) showed strongest inhibitory effect.

7.4.5 cAMP enzyme activity

Table 7.1 shows changes in the levels of cAMP of test samples. Increasing levels of cAMP prevent the effect of oxidative stress in vascular cells. The result

showed that TMOQ and DDK showed ability to increase cAMP in HUVEC cells comparing with non-treated (% increasing = 70.03 and 40.49, respectively). Moreover, these compounds exhibited better performances (39.99 and 15.67%, respectively) than L-NAME.

7.5 DISCUSSION

Oxygen radicals are important mediators of a variety of pathological processes including inflammation and ischemia/reperfusion. Because of its strategic position between blood and tissues, vascular endothelium is a major site for oxidative stress in postischemic periods. HUVEC have played a major role as a model system for the study of the regulation of endothelial cell function and the role of the endothelium in the response of the blood vessel wall to stretch, shear forces and the development of atherosclerotic plaques and angiogenesis. In this study HUVEC was used to investigate the effect of isolated compounds from *A. zerumbet* on $O_2^{\bullet-}$ release and cAMP enzyme activity.

The generation of $O_2^{\bullet-}$ was determined by the xanthine plus xanthine oxidase-induced (X/XO) as a model of oxidant injury. The ability of isolated compounds was tested to exhibit oxygen radicals scavenging properties (Richard *et al*., 1998). The results showed that TMOQ had strong xanthine oxidase inhibitory effects similar to that of L-NAME. For angiotensin II-induced $O_2^{\bullet-}$ production, the $O_2^{\bullet-}$ product released was investigated by Ang II using addition of SOD which a secretory copper enzyme involved in protecting Ang II-induced hypertension (Ozumi *et al*., 2012). In this process, addition of SOD to intact cells cannot prevent the Ang

II-elicited formation of reducing external ferricytochrome *c*. All of the isolated compounds showed high level of oxygen radical scavenging. Moreover, NADPH oxidase is the most important enzyme responsible for ROS formation in human vessels. The result showed that TMOQ had the highest inhibitory activity followed by MTD. However, the isolated compounds exhibited half fold inhibition than L-NAME. Because cAMP has been implicated as a mediator of endothelial permeability, it was determined whether alterations in cAMP are associated with direct oxidant injury of endothelial cells (Richard *et al*., 1998). It was found that TMOQ and DDK showed potency to increase cAMP level, furthermore, they showed higher activity than positive control, L-NAME.

As the results, TMOQ, isolated from the seeds of *A. zerumbet*, had the capacity to prevent the ROS generated by xanthine oxidase and NADPH oxidase, moreover it could be the stimulator for increasing the level of cAMP. The reason for the potency of this compound should be from the quinolizidine ring in its structure. It was suggested that TMOQ had inhibitory effect against gestational diabetes modulators *in vitro* including α-glucosidase, trypsin and nitric oxide formulation (see **Chapter 6**).

7.5 CONCLUSION

In this study, it was proven that TMOQ, the isolated compound from seeds of *A. zerumbet*, has the efficient ability to scavenge oxygen radical both *in vitro* and in HUVEC cells model. Thus, it was suggested that this compound may have plausible relevance to use it as a lead compound in drug designing for preventing the diseases related to oxygen free radicals.

Table 7.1

The effect of isolated compounds on % increasing of cAMP level.

Sample	cAMP level (pmole/mL)	% Increasing with	
		Control	L-NAME
DK	1.39	– 74.33 ± 0.79 c [l]	– 78.87 ± 1.24 d
DDK	7.59	40.49 ± 0.77 a	15.67 ± 0.45 b
Labdadiene	1.16	– 78.46 ± 0.48 c	– 82.27 ± 1.22 d
MTD	4.02	– 25.63 ± 0.34 ab	– 38.77 ± 0.61 c
TMOQ	9.19	70.02 ± 0.51 a	39.99 ± 1.94 a
L-NAME	6.57	21.46 ± 0.48 ab	–
Non-treated	5.41	–	–

[l] The data represent the mean ± SD of six determination. Values with the same letter in one column are not significantly different ($p < 0.01$) from each other.

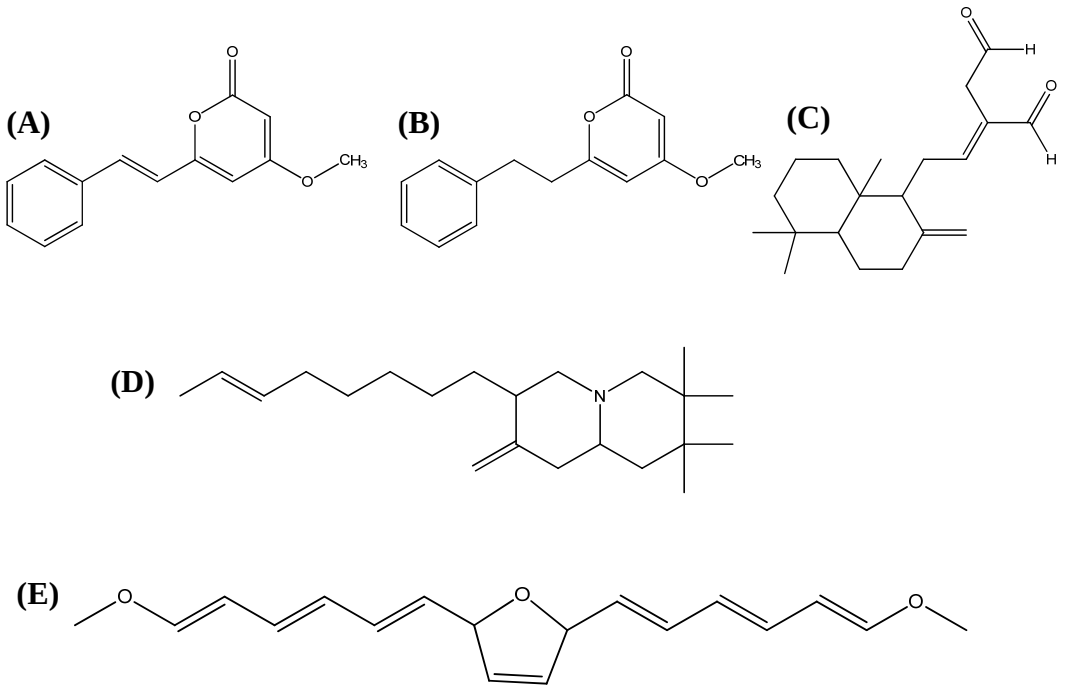

Figure 7.1 Chemical structures of DK (A), DDK (B), labdadiene (C), TMOQ (D) and MTD (E).

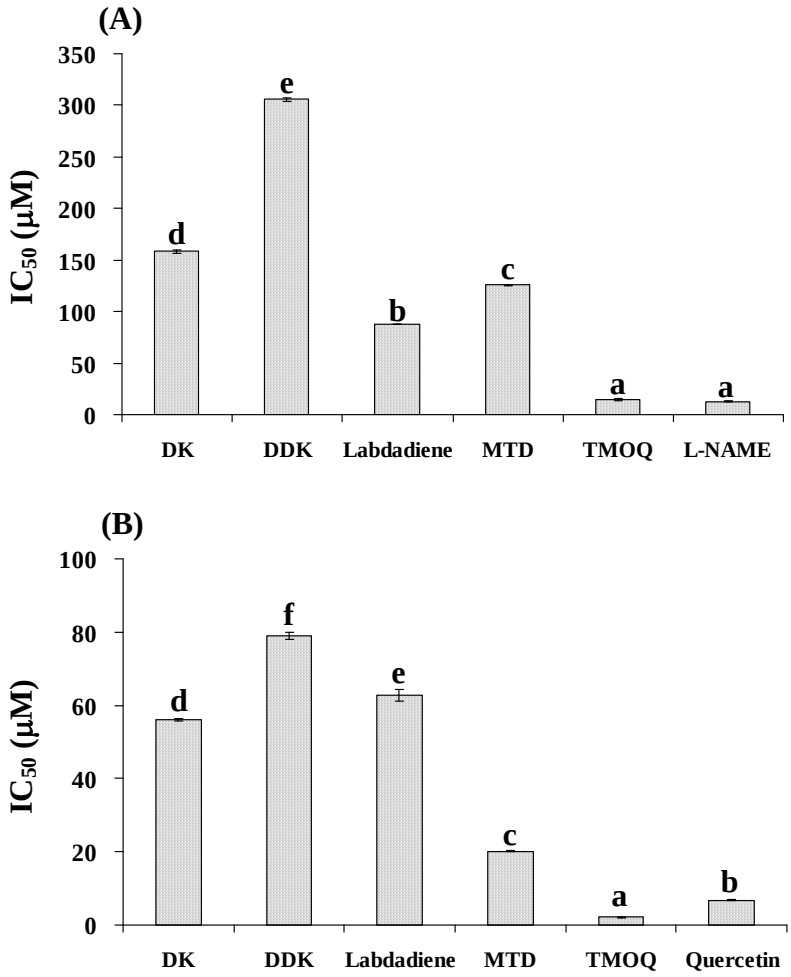

Figure 7.2 IC_{50} of isolated compounds on xanthine-xanthine oxide (A) and LDL oxidation (B) activities. Means with the same letter on the bars are not significantly different, $p < 0.01$ (Tukey's test). The bars represents mean ± SD ($n = 6$).

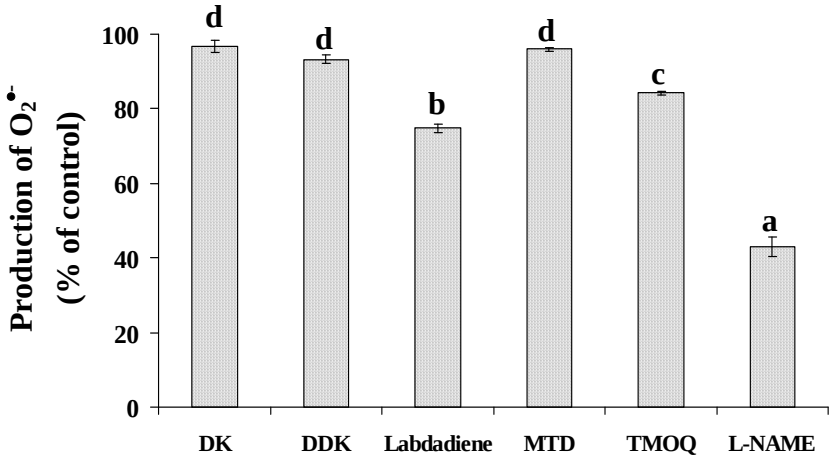

Figure 7.3 The effect of isolated compounds (at 50 μM) on angiotensin II-induced $O_2^{\bullet-}$ product. Means with the same letter on the bars are not significantly different, $p < 0.01$ (Tukey's test). The bars represents mean ± SD ($n = 6$).

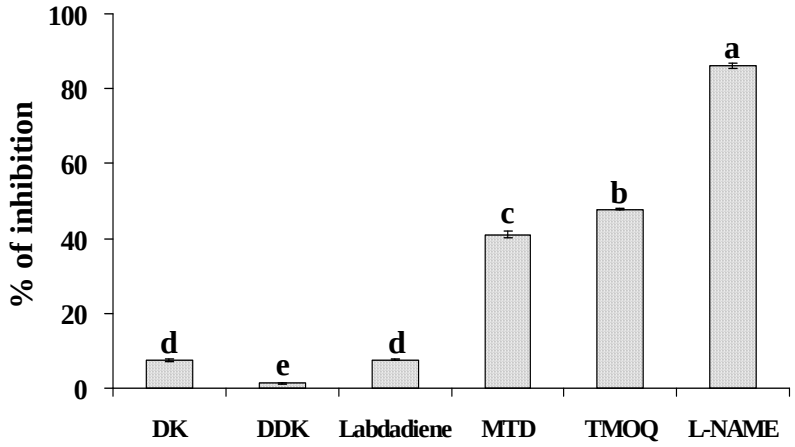

Figure 7.4 Inhibitory effects of isolated compounds (at 50 μM) on NADPH oxidase activity. Means with the same letter on the bars are not significantly different, $p < 0.01$ (Tukey's tests). The bars represents mean ± SD ($n = 6$).

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