Thesis for the Degree of Doctor of Philosophy

# Promising anti-diabetic potential of *Artemisia capillaris* and its constituents


by

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February 2014

# Promising anti-diabetic potential of *Artemisia capillaris* and its constituents (사철쑥의 잠재적 항당뇨 효과와 그 성분들)

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A thesis submitted in partial fulfillment of the requirements

for the degree of

Doctor of Philosophy

in Department of Food and Life Science, The Graduate School

Pukyong National University

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A Dissertation

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February 21, 2014

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## List of Abbreviations and Symbols

| | |
|---|---|
| *A. capillaris* | : *Artemisia capillaris* |
| $^{1}$H NMR | : Proton nuclear magnetic resonance |
| $^{13}$C NMR | : $^{13}$Carbon nuclear magnetic resonance |
| HMBC | : Heteronuclear multiple bond correlation |
| HMQC | : Heteronuclear multiple quantum coherence |
| EI-MS | : Electron impact mass spectrometry |
| TLC | : Thin layer chromatography |
| HPLC | : High performance liquid chromatography |
| $IC_{50}$ | : 50% inhibitory concentration of the test samples |
| mp | : Melting point |
| UV | : Ultraviolet |
| Hz | : Hertz ($sec^{-1}$) |
| RP | : Reversed phase |
| DMSO | : Dimethyl sulfoxide |
| DMSO-$d_6$ | : Deuterated dimethyl sulfoxide |
| $CDCl_3$ | : Deuterated chlroform |
| MeOH-$d_4$ | : Deuterated methanol |
| Pyridine-$d_5$ | : Deuterated pyridine |

| | |
|---|---|
| *J* | : Coupling constant (Hz) |
| *δ* | : Chemical shift |
| PTP1B | : Protein tyrosine phosphatase 1B |
| AGE | : Advanced glycation endproducts |
| RLAR | : Rat lens aldose reductase |

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## Abstract

*Artemisia*, a diverse genus of the family Asteraceae, has been used as anti-pyretic, anti-diabetic, anti-hypertensive, anti-hepatotoxic, anti-inflammatory, eczema, jaundice, and anti-hemorrhoid. Recently, *Artemisia* genus has emerged as a source of naturally occurring therapeutic agents for the treatment of diabetes and related complications due to the presence of wide varieties of bioactive substances including caffeoyl quinic acids, flavonoids, and coumarins. As a part of our ongoing search for anti-diabetic constituents from natural sources, we selected methanol (MeOH) extracts of 12 species of the genus *Artemisia* based on their frequent use in traditional medicines and investigated their anti-diabetic potential *via* α-glucosidase, protein tyrosine phosphatase 1B (PTP1B), and advanced glycation end products (AGE) formation inhibitory assays. The MeOH extracts of different species exhibited promising inhibitory activities ranging from 34.12 to 64.17% at a concentration of 250 μg/mL against α-glucosidase and 27.40 to 98.60% at a concentration

of 10 μg/mL against PTP1B while their AGE formation inhibitory activities ranging from 21.40 to 87.47% at a concentration of 10 μg/mL. Since the MeOH extract of *A. capillaris* exhibited the highest α-glucosidase and AGE formation inhibitory activities together with significant PTP1B inhibitory activity, it was selected for detailed investigation. Repeated column chromatography of different fractions yielded 46 compounds including 13 coumarins (esculetin, esculin, scopolin, isoscopolin, scoparone, scopoletin, isoscopoletin, artemicapin A, fraxidin, 5,6,7-trimethoxy coumarin, 6-methoxy artemicapin C, tomerin, umbelliferone) and 19 flavonoids (cirsilineol, cirsimaritin, arcapillin, linarin, quercetin, hyperoside, isorhamnetin 3-*O*-robinobioside, quercetin 5-*O*-glucoside, quercetin 3-*O*-glucoside, quercetin 3-*O*-robinobioside, chrysoeriol-7-*O*-rutinoside, quercetin 7-*O*-galatopyranosyl 7-*O*-rhamnoside, isorhamnetin 3-*O*-galactoside, isorhamnetin 3-*O*-glucoside, acacetin-7-*O*-(6″-*O*-acetyl)-β-D-glucopyranosyl-(1→2)[α-L-rhamnopyranosyl]-(1→6)-β-D-glucopyranoside, acacetin-7-*O*-β-D-glucopyranosyl-(1→2)[α-L-rhamnopyranosyl]-(1→6)-β-D-glucopyranoside, isorhamnetin-3-*O*-arabinopyranoside, vicenin 2, apigenin), 6 caffeoylquinic acid derivatives (4,5 di-*O*-caffeoyl quinic acid, 3,5 di-*O*-caffeoyl quinic acid, 3,4 di-*O*-caffeoyl quinic acid, chlorogenic acid, cryptochlorogenic acid, neochlorogenic acid), 2 chromones (capillarisin and 7-methyl capillarisin), 2 essential oils (capillin and capllinol), and 4 phenolic compounds (paramethoxy phenyl glucoside, eugenol glucoside, koaburaside and 4-hydroxy-3-*O*-glucopyranosyl benzoic acid). Among

them, acacetin-7-*O*-(6″-*O*-acetyl)-β-D-glucopyranosyl-(1→2)[α-L-rhamnopyranosyl]-(1→6)-β-D-glucopyranoside is a new acetylated flavonoid glycoside isolated for the first time. Among the isolated compounds esculetin, quercetin, scopoletin, isorhamnetin, vicenin 2, cirsilineol, 3,5-*O*-dicaffeoylquinic acid, 1,5-*O*-dicaffeoylquinic acid, 3,4-*O*-dicaffeoylquinic acid, 4,5-*O*-dicaffeoylquinic acid, exhibited potent inhibitory activities in α-glucosidase and PTP1B inhibitory assays with $IC_{50}$ values of 82.92, 58.93, 159.16, 141.17, 270.53, 351.71, 217.40, 146.06, 128.07, and 229.94 μM, respectively, compared to the positive control acarbose wth $IC_{50}$ value of 130.52 μM against α-glucosidase and 10.08, 20.35, 227.28, 43.88, 139.75, 206.78, 2.02, 16.05, 2.60, and 3.21 μM, respectively compared to the positive control ursolic acid with an $IC_{50}$ value of 4.05 μM against PTP1B. Interestingly, 3-*O*-caffeoylquinic acid, 6-methoxy artemicapin C and capillarisin exhibited potent inhibitory activity against PTP1B while hyperoside exhibited potent inhibitory activity against α-glucosidase rather than PTP1B. In addition, two essential oils, capillin and capillinol were also found to be potent inhibitors of α-glucosidase, PTP1B and RLAR. Kinetic analysis revealed that capllin noncompetitively inhibited both α-glucosidase and RLAR, while it showed mixed type inhibition against PTP1B. On the other hand, capllinol showed mixed type inhibiton against both α-glucosidase and PTP1B. In case of AGE formation inhibitory assay, esculetin, scopoletin, isoscopoletin, isoscopolin, scopolin, quercetin, hyperoside, vicenin 2, 4,5 di-*O*-caffeoyl quinic acid, and chlorogenic acid

showed remarkable inhibitory potential against AGE formation with $IC_{50}$ values of 5.02, 3.90, 18.78, 41.04, 60.10, 24.35, 48.20, 54.43, 6.48 and 21.08 μM, respectively compared to the positive control aminoguanidine with $IC_{50}$ an value of 932.66 μM. Vcenin 2 also strongly inhibited RLAR with an $IC_{50}$ value of 7.85 μM compared to the positive control quercetin with an $IC_{50}$ value of 11.64 μM. Further study with vicenin 2 revealed that it can inhibit the formation of fluorescent AGE and non-fluorescent AGE such as ($N^{\varepsilon}$-(carboxymethyl) lysine (CML). Vicenin 2 also inhibited protein oxidation indicated by decreasing protein carbonyl formation and thiol modification in BSA-glucose-fructose system. Moreover, vicenin 2 also suppressed the formation of β-cross amyloid structures of BSA. Therefore, the results of the present study clearly demonstrate the promising α-glucosidase, PTP1B, and AGE formation inhibitory potential of *A. capillaris* as well as its isolated constituents, which could be further explored to develop therapeutic or preventive agents for the treatment of diabetes and related complications.

# I. Introduction

Diabetes mellitus is a progressive metabolic disorder of glucose metabolism in which the body either does not produce enough insulin or does not respond to the produced insulin, resulting in an increase of blood glucose levels and causing serious and irreparable damage to body systems, such as blood vessels and nerves (Matsui et al., 2007). It is a global health crisis, which has been persistently affecting the humanity, irrespective of the socioeconomic profile and geographic location of the population. In recent years, nutritional balance is overlooked due to changing lifestyle as well as food habit accompanied by shifting to high energy food intake which results in sparking the incidence rate of obesity and diabetes (Alonso et al., 2004). According to an estimate, one person is detected with diabetes every 5 seconds somewhere in the world, while someone dies of it every 10 seconds (Colagiuri, 2010). Diabetes is a metabolic disorder critically afflicting the population of both developed and developing countries. According to the Diabetes Atlas, the global prevalence of diabetes is estimated to be 4.6%, representing 151 million people, and is expected to go up to 333 million people by 2025. Recent reports have estimated an increase in these figures,

with the global prevalence reaching up to 6.6%, representing 285 million people in 2010 and by 2030, it will rise up globally up to 7.8% (438 million people). Also, individual national prevalence rates from over 1% to almost 31% have been reported, severely affecting the developing countries and, more specifically, the lower socioeconomic groups. Diabetes is rapidly emerging as a major public health challenge. In 2010, diabetes will share 11.6% of the entire international healthcare expenses, much of which will comprise hospital admissions and medications (Vasim et al., 2012).

Diabetes mellitus is divided into two main types: type I (insulin -dependent diabetes mellitus or IDDM) and type II (non -insulin -dependent diabetes mellitus or NIDDM). IDDM occurs due to insulin insufficiency because the body does not generate any insulin and patients entirely depend on an exogenous supply of insulin. IDDM is more pronounced in children and young adults. It causes severe damage to the pancreatic β-cells. It is categorized as autoimmune (immune mediated) diabetes (type 1A) or idiopathic diabetes with β-cell destruction (type 1B), although the precise description of the later is still unknown (Alberti and Zimmet, 1998). Patients suffering from NIDDM are unable to respond to insulin and can be treated with exercise, diet management and medication. Mostly, its onset is in

adulthood, largely occurring in obese people over 40 years of age. NIDDM is the most widespread type. It indicates a condition with disturbed carbohydrate and fat metabolism. Hypertension, hyperlipidemia, hyperinsulinemia and atherosclerosis are often allied with diabetes. Both the types demonstrate some frequent symptoms like high blood sugar levels, unusual thirst, extreme hunger, frequent urination, extreme weakness, blurred vision etc. Although the pathophysiology of diabetes is not entirely understood, many studies indicate the participation of free radicals in the pathogenesis of diabetes (Mattecci and Giampietro, 2000) and its complications (Oberlay, 1988; Baynes, 1996; Lipinski, 2001). Free radicals are proficient enough of damaging cellular molecules, proteins, lipids and DNA, leading to alteration of cell functions. In fact, the abnormalities in lipids and proteins are one of the key reasons for the development of diabetic complications.

The long term elevated blood glucose level results in many diabetic complications such as coronary artery and peripheral vascular diseases, stroke, diabetic neuropathy, amputations, renal failure, blindness which affect quality of life, life expectancy as well as overall healthcare cost in almost every society (Amos et al., 1997). The control of hyperglycemia is

therefore of prime importance to halt the progression of the disease. Hence, a number of viable pharmacologic options such as insulin, insulin secretagogues, and insulin sensitizers constitute a base line of therapy in order to maintain normoglycemia in established diabetes mellitus (Ghadyale, 2012). With the advancement of biomedical science there are different classes of modern medicines are available for the management of diabetes, and their selection depends on the nature of the diabetes, age and situation of the person, as well as other factors. Oral antidiabetic agents exert their effects by various mechanisms: (1) stimulation of beta cells in the pancreas to produce more insulin (sulfonylureas and meglitinides), (2) increasing the sensitivity of muscles and other tissues to insulin (thiazolidinediones), (3) decreasing gluconeogenesis by the liver (biguanides), and (4) delaying the absorption of carbohydrates from the gastrointestinal tract (alpha-glucosidase inhibitors). These treatments have their own drawbacks, ranging from the developing of resistance and adverse effects to lack of responsiveness in large segment of patients population. Sulfonylureas were the first widely used oral hypoglycemic medications. They are insulin secretagogues, triggering insulin release by direct action on the $K_{ATP}$ channel of the pancreatic beta cells. The primary side effect is hypoglycemia

and weight gain. Meglitinides help the pancreas produce insulin and are often called "short-acting secretagogues. Their mode of action is original, affecting potassium channels (Rendell, 2004). By closing the potassium channels of the pancreatic beta cells, they open the calcium channels, hence enhancing insulin secretion. Adverse reactions of meglitinides include weight gain and hypoglycemia. Biguanides reduce hepatic glucose output and increase uptake of glucose by the periphery, including skeletal muscle. Although it must be used with caution in patients with impaired liver or kidney function, metformin, a biguanide, has become the most commonly used agent for type 2 diabetes in children and teenagers. Amongst common diabetic drugs, metformin is the only widely used oral drug that does not cause weight gain. However, many of such drugs withdrawn due to lactic acidosis risk (Eurich et al., 2007; Fimognari et al., 2006; Verdonck et al., 1981). Thiazolidinediones (TZDs), also known as "glitazones," bind to PPARγ, a type of nuclear regulatory proteins involved in transcription of genes regulating glucose and fat metabolism. The final result is better use of glucose by the cells. As a result of multiple retrospective studies, there is a concern about rosiglitazone's safety. The greatest concern is an increase in the number of severe cardiac events in patients taking it. Other drugs like α-

glucosidase inhibitors also called "diabetes pills" which do not have a direct effect on insulin secretion or sensitivity but these agents slow down the digestion of starch in the small intestine as well as some peptides analogues e.g. incretins which are insulin secretagogues (Briones and Bajaj, 2006; Gallwitz, 2006) but all are associated with adverse effects of weight loss and nausea. Many other potential drugs are currently in investigation by pharmaceutical companies. Some of these are simply newer members of one of the above classes, but some work by novel mechanisms. However, studies are still not completed and chances of severe side effects cannot be ruled out. Therefore, it is wise to say that to date, there exists no single drug against diabetes type 2, and discovery of a novel drug with novel mode of action would be a big breakthrough in drug development.

A great portion of the world's population still relies on the traditional medicine for the treatment of diabetes even though there are many commercially available drugs in the market. Many countries, especially in the developing world, have a long history of the use of herbal remedies in diabetes. A recent review article presents the profiles of plants with hypoglycemic properties, reported in the literature from 1990 to 2000 and states that medical plants play an important role in the management of

diabetes mellitus especially in developing countries where resources are meager (Bnouham et al., 2006). Many traditional plant treatments for diabetes exist, a hidden wealth of potentially useful natural products for diabetes control. Nonetheless, few traditional antidiabetic plants have received scientific or medical scrutiny, despite recommendations by the World Health Organization in 1980 that this should be undertaken. Presently, there is growing interest in herbal remedies due to the side effects associated with the oral hypoglycemic agents (therapeutic agent) for the treatment of diabetes mellitus. In recent years, herbal medicines have started to gain importance as a source of hypoglycemic agents. Marles and Farnsworth estimated that more than 1000 plant species are being used as folk medicine for diabetes (Marles and Farnsworth, 1995). Biological actions of the plant products used as alternative medicines to treat diabetes are related to their chemical composition. Herbal products or plant products are rich in phenolic compounds, flavonoids, terpenoids, coumarins, and other constituents which show reduction in blood glucose levels (He et al., 2005; Jung et al., 2006; Ji et al., 2009). Several species of herbal drugs have been described in the scientific and popular literature as having antidiabetic activity (Valiathan, 1998). Due to their perceived effectiveness, fewer side

effects in clinical experience and relatively low costs, herbal drugs are prescribed (Verspohl, 2002). Metformin, a less toxic biguanides and potent oral glucose-lowering agent which is only one medication approved for use in children, was developed from *Galega officianalis* and used to treat diabetes (Daniel and Norman, 2001).

*Artemisia*, a widespread and diverse genus of the family Asteraceae comprising of more than 400 species, has both high degree of therapeutic and economic importance. It is a well recognized wind pollinated cosmopolitan genus, and commonly distributed in the temperate zone of Europe, Asia and North America. It is a taxonomically complex genus since majority of its species often have different morphological forms while some species have close resemblance with each other (Mehrdad et al., 2007; Ashraf et al., 2010). Most of the species of this genus are perennial herbs or small shrubs with few exceptions of being annual or biennial (Valles et al., 2003). Different species of this genus usually have strong aroma and commonly used as medicines, food, forage, ornamentals or soil stabilizers in disturbed habitat (Pareto, 1985; Tan et al., 1998). *Artemisia* species, particularly essential oils, have been widely used as medicinal plants in folk medicine for many years and therefore, many species including *A. annua, A.*

*vulgaris, A. capillaris, A. absinthium. A. iwayomogi* incorporated into the several European and Asian Pharmacopea (Proksch, 1992). In particular, the aerial parts of *A. montana* has been used in folk medicine as an anti-pyretic, anti-diabetic, anti-hypertensive, anti-hepatotoxic, and anti-hemorrhoid (Kim, 1996). *A. iyawomogi* and *A. capillaris*, referred to as 'Haninjin' and 'Injinho', respectively, have been used in traditional medicinal system of China, Korea, and Japan for the treatment of dieresis, inflammation related diseases. Meanwhile, *A. princeps* and *A. argyi* are referred to as 'Aeyup' and are employed for the treatment of vomiting, colic pain, irregular uterine bleeding (The Korean Herbal Pharmacopoea, 2002; Kim et al., 1992; Zhao et al., 1994). *A. apiacea* is distributed at wasteland and river beaches of Korea, Japan and China, and has been used as a traditional medicine to treat eczema and jaundice (Yook, 1989). Some species of *Artemisia* including *A. santonicum, A. siebery, A. pallens, A. dracunculus,* and *A. herba-alba* have been used in traditional medicine of some Middle East and Asian countries for the treatment of diabetes. Some reports using *in vitro/in vivo* model also suggest that many of the species could be beneficial for the treatment of diabetes mellitus and related complications (Ribnickya et al., 2006; Kordali et al., 2005). Owing to high number of species, ecological and economic

importance, the genus *Artemisia* has been extensively investigated for its diverse biological activities (Valles et al., 2003). Some very important drug leads have been isolated from this genus, notably an endoperoxide sesquiterpene lactone artemisinin, a well known anti-malarial drug isolated from the aerial parts of a commonly used chinese herb *A. annua* (Koradeli et al., 2005; Wright, 2005). During last decade extensive investigation on different species of this genus has been carried out and consequently it leads to the isolation of many classes of secondary metabolites including terpenoids, flavonoids, coumarins, glycosides, sterols, and polyacetylenes which were reported to possess a wide range of biological activities, including anti-malarial, anti-viral, anti-tumour, anti-pyretic, antihemorrhagic, anti-coagulant, antifungal, anti-anginal, antioxidant, hepatoprotective, anti-ulcerogenic, anti-spasmodic, anti-complementary, and interferon inducing (Tan et al., 1998). In addition to a variety of phytochemical and biological studies, the *Artemisia* genus has recently emerged as a source of potent candidates of naturally occurring therapeutic agents for diabetes and diabetic complications due to bioactive components, including caffeoylquinic acids, flavonoids, and coumarins (Cui et al., 2009; Logendra et al., 2006; Okada et al., 1995). Despite the promising potential of the

*Artemisia* species in the treatment of diabetes and related complications, there is no systematic study on the α-glucosidase, PTP1B, and AGE formation inhibitory activities of the *Artemisia* species. Therefore, as a part of our continuous search for potent anti-diabetic compounds from the natural sources we have selected 12 species of the genus *Artemisia* based on their traditional importance and investigated *via* α-glucosidase, PTP1B, AGE formation inhibitory assays. Since the methanol extract of *A. capillaris* exhibited highest α-glucosidase inhibitory activity and AGE formation inhibitory activities together with significant PTP1B inhibitory activity, it was selected for detailed investigation to isolate active compounds as well as to establish their structure activity relationship.

*Artemisia capillaris* is commonly distributed in sandy areas along the Korean coastline and is known as halophyte. Similar to the *Artemisia* species, this plant has been frequently used in the treatment of liver disease, including hepatitis, jaundice, fatty liver and bilious disorder. Infusions of the buds, stems and leaves of *A. capillaris* have been used in TCM primarily as a choleric, anti-inflammatory, antipyretic, and diuretic agent for treating epidemic hepatitis (Tang and Eisenbrand, 1992). A wide variety of pharmacological and biological activities of *A. capillaris* have been reported

including antioxidant (Hong et al., 2007; Seo and Yun, 2008), cytoprotective (Hong et al., 2007), hepatoprotective (Lee et al., 2000; Choi et al., 2011), anti-inflammatory (Hong et al., 2004; Kwon et al., 2011), antimicrobial (Seo et al., 2010), anticancer (Cha et al., 2009), anti-obesity (Hong et al., 2009a), and choleretic effects (Okuno et al., 1981). The phytochemical properties of *A. capillaris* have been widely investigated. In particular, *A. capillaris* contains an essential oil with polyacetylene derivatives, such as capillene, capillone, and capillin, as well as mono- and sesquiterpenes (α-and β-pinene, *p*-cymene, α-terpineol, bornyl acetate, methyleugenol, β-elemene, and β-caryophyllene) (Wright, 2005). Recently, the main components of *A. capillaris* essential oil were determined to be 1,8-cineole, germacrene D, and camphor (Liu et al., 2010). Capillarisin (flavone), the major constituent of *A. capillaris*, along with capillartemisin A and B, two new stereoisomeric constituents, demonstrated choleretic effects in experimental studies (Wright, 2005). The scoparone, a coumarin derivative isolated from this species, had a preventative effect on carbon tetrachloride or galactosamine-induced hepatotoxicity in hepatocyte cell cultures (Kiso et al., 1984), as well as anti-inflammatory and analgesic effects (Yamahara et al., 1989; Jang et al., 2005). Besides these reports,

several other recent reports have proven its therapeutic value in traditional uses such as anti-inflammatory and hepatoprotective. The 70% ethanol extract of the aerial parts of *A. capillaris* showed potent inhibitory activity against 5-LOX-catalyzed leukotriene production by ionophore-induced rat basophilic leukemia-1 cells. Three compounds from the *A. capillaris* extract including quercetin, esculetin and capillarisin were found as potent 5-LOX inhibitors. In addition, esculetin, and quercetin reduced arachidonic acid-induced ear edema and delayed-type hypersensitivity response in mice (Kwon et al., 2011). In a recent report, capillarisin, isolated from *A. capillaris*, effectively inhibited the production of inflammatory mediators such as iNOS. COX-2, and TNF-α *via* the inhibition of the LPS-induced activation of NF-kB, Akt, and MAP kinase-activated inflammatory genes, which is mediated by MyD88 and TIRAP. *In vivo* experimental analysis revealed that capillarisin (20 and 80 mg/kg, i.p.) inhibited complete Freund's adjuvant (CFA)-and carrageenan-induced paw edema, nitrite production in plasma, and TNF-α, a pro-inflammatory cytokine production (Salman et al., 2013). Water extract of *A. capillaris* restored the bile duct ligation-induced depletion of glutathione content and glutathione peroxidase activity, attenuated cholestatic liver injury and collagen deposition,

suppressed expression of fibrogenic factors, including hepatic alpha-smooth muscle actin (α-SMA), platelet-derived growth factor (PDGF), and transforming growth factor beta (TGF-β). The beneficial effects of water extract of *A. capillaris* administration are associated with antifibrotic properties *via* both up-regulation of antioxidant activities and down-regulation of ECM protein production in the rat BDL model (Han et al., 2013). In addition, water extract of *A. capillaris* also showed hepatoprotective activity in alcohol-pyrazole-fed rat model via enhancement of antioxidant enzyme system and modulation of proinflammatory cytokines production (Choi et al., 2013). Hyperoside, one of the major compounds of *A. capillaris*, prevented portal inflammation, centrizonal necrosis, and Kupffer cell hyperplasia in $CCl_4$ induced liver damage in mice *via* enhancement of the antioxidant and reduction of the expression of proinflammatory cytokines (Choi et al., 2011). A water soluble polysaccharide fraction of *A. capillaris* exhibited a potent anti-proliferation activity on human nasopharyngeal carcinoma CNE-2 cells by inducing apoptosis, *via* the mitochondrial apoptotic pathway, which included the loss of mitochondrial membrane potential, release of cytochrome c and activation of caspase-3/9 (Feng et al., 2013).

# II. Materials and methods

## 2-1. Equipments and general experimental procedures

The EI-MS spectra were determined using a Hewlett-Packard 5989B spectrometer (Agilent Technologies) and a JEOL JMS-700 spectrometer. The ESI-MS was obtained on an Applied Biosystems Mariner time-of-flight mass spectrometer with an electrospray interface. The $^{1}$H- and $^{13}$C-NMR spectra were measured by a JEOL JNM ECP-400 spectrometer (Tokyo, Japan) at 400 MHz for $^{1}$H NMR and 100 MHz for $^{13}$C NMR in deuterated chloroform ($CDCl_3$), pyridine-$d_5$, dimethylsulfoxide (DMSO-$d_6$) and methanol (MeOH-$d_4$). Chemical shifts were referenced to the respective residual solvent peaks (7.25 ppm for $^{1}$H and 77.0 for $^{13}$C for $CDCl_3$, 2.50 ppm for $^{1}$H and 39.5 ppm for $^{13}$C NMR for DMSO-$d_6$; 4.80 and 3.30 for $^{1}$H and 49.0 for $^{13}$C for MeOH-$d_4$). Reversed-phase HPLC was performed on the JASCO HPLC system (Tokyo, Japan), consisting of a PU-1580 Intelligent HPLC pump, a LG-1580-04 quaternary gradient unit, a UV-1575 intelligent UV/Vis detector, a PG-1580-54 4-line degasser, and a CO-1560 intelligent column thermostat. The BORWIN chromatographic system (Le Fontanil, France) was used for HPLC data analysis. Column

chromatography was performed using silica (Si) gel 60 (70~230 mesh, Merck, Darmstadt, Germany), LiChroprep® RP-18 (40~63 μm, Merck, Darmstadt, Germany), Sephadex LH-20 (20~100 μm, Sigma, St. Louis, MO, USA), and Diaion HP-20 (250~850 μm, Sigma). Thin layer chromatography (TLC) was conducted on precoated Merck Kieselgel 60 $F_{254}$ plates (20 × 20 cm, 0.25 mm) and RP-18 $F_{254}$ plates (5 × 10 cm, Merck), using 50% $H_2SO_4$ as a spray reagent.

**2-2. Chemicals and reagents**

Yeast α-glucosidase, acarbose, *p*-nitrophenyl phosphate (*p*-NPP), *p*-nitrophenyl α-D-glucopyranoside (*p*-NPG), 1,1-diphenyl-2-picrylhydrazyl (DPPH), L-ascorbic acid, 2,2'-azinobis (3 ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), D-(−)-fructose, D-(+)-glucose, aminoguanidine hydrochloride, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox), DL-glyceraldehyde dimer, nicotinamide adenine dinucleotide phosphate (NADPH), guanidine hydrochloride, 2,4-dinitrophenylhydrazine (DNPH), nitroblue tetrazolium (NBT), 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), thioflavin T, 1-deoxy-1-demorpholino-D-fructose (DMF), L-cysteine, quercetin, coumarin, caffeic

acid, ferulic acid, dihydrocaffeic acid, *p*-coumaric acid, 3,4-dimethoxycinnamic acid, diethylenetriaminepentaacetic acid (DTPA), L-2-amino-3-mercapto-3-methylbutanoic acid (L-penicillamine), and dimethyl sulfoxide (DMSO) boviene serum albumin (BSA), and ethylenediaminetetraacetic acid (EDTA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). OxiSelect™ $N^{\varepsilon}$-(carboxymethyl) lysine (CML) ELISA kit was purchased from Cell Biolabs (San Diego, CA, USA). PTP1B (human recombinant) was purchased from Biomol® International LP (Plymouth Meeting, PA, USA), and dithiothreitol (DTT) was purchased from Bio-Rad Laboratories (Hercules, CA, USA). Sodium azide was obtained from Junsei Chemical Co. Peroxynitrite ($ONOO^-$) was purchased from Cayman Chemicals Co. Dihydrorhodamine 123 (DHR 123) was purchased from Molecular Probes. Anti-nitrotyrosine (clone 1A6, mouse-monoclonal primary antibody, IgG2b), horseradish peroxide-conjugated anti-rabbit, and antimouse antibodies were purchased from Millipore Co. Polyvinylidenefluoride (PVDF) membrane (Immobilon-P) was obtained from Millipore Co. Supersignal® West Pico Chemiluminescent Substrate was obtained from Pierce Biotechnology, Inc. All other chemicals and solvents used were purchased from Duksan Pure Chemicals co., E. Merck,

Fluka, or Sigma & Aldrch Company, unless otherwise stated.

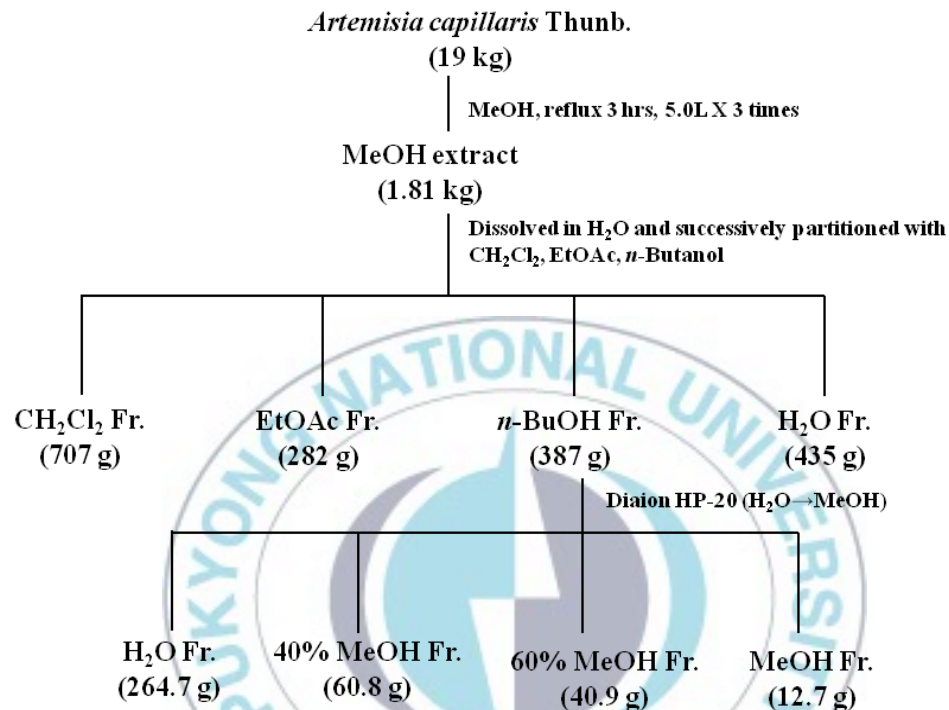
### 2-3. Plant materials

The *A. capillaris* whole plants were collected from Yeongcheon Province, Republic of Korea in August 2009, and confirmed by Prof. Je-Hyun Lee at College of Oriental Medicine, Dongguk University (Kyeongju). A voucher specimen (no. 20093008) was deposited in the author's laboratory (J. S. Choi). The MeOH extracts of 11 species (*A. apiacea* Hance, *A. rubripes* Nakai, *A. japonica* Thunb., *A. sylvatica* Max., *A. iwayomogi* Kitamura, *A. stolonifera* Max., *A. argyi* Levi. et Vaniot, *A. princeps* var. *orientalis* Pampan, *A. selengensis* Turcz, *A. capillaris* Thunb., *A. keiskeana* Miq.) were purchased from the Korean Plant Extract Bank under the Korea Research Institute of Bioscience and Biotechnology in February 2006.

### 2-4. Extraction and fractionation of *A. capillaris*

Whole plant of *A. capillaris* Thunb. was dried and grinded to powder. Then the dried powder (19.0 kg) was extracted with hot MeOH (50.0 L x 3times) for 3 hrs. After filtration, total filtrate was concentrated to dryness *in vacuo* at $40^0$C to get the MeOH extract (1.81 kg). Then the MeOH extract

was suspended in distilled water:MeOH (9:1) and successively partitioned with dichloromethane ($CH_2Cl_2$), ethyl acetate (EtOAc), *n*-butanol (*n*-BuOH) to yield $CH_2Cl_2$ (707 g), EtOAc (282 g), *n*-BuOH (387 g) fractions respectively, as well as $H_2O$ residue (435 g). The *n*-BuOH fraction was chromatographed over HP-diaion column and eluted with 100% $H_2O$, 40% MeOH, 60% MeOH, and 100% MeOH in order to get 4 subfractions $H_2O$ (264.17 g), 40% MeOH (60.82 g), 60% MeOH (40.95g) and MeOH (12.76 g), respectively (Scheme 1).




**Scheme 1.** Extraction and fractionation scheme of *A. capillaris*

## 2-5. Isolation of active compounds from the MeOH extract of *A. capillaris*

### 2-5-1. Isolation of active compounds from the *n*-BuOH fraction of *A. capillaris*

Inially, 40% MeOH subfraction was then chromatographed in a silica gel column using $CH_2Cl_2$:MeOH 10:1 with gradual increase in MeOH to get 15 subfractions (4F-1 to 4F-15). After filtration of 4F-6 to 4F-8, the PPT was recrystalized using MeOH to get quercetin-3-*O*-galactoside (3.523 g). Repeated chromatography of 4F-9 in silica gel column using $CH_2Cl_2$:MeOH (10:1→1:1) yielded 4 subfractions (4F9-1 to 4F9-4). 4F-9-4 was then chromatographed over silica gel column using EtOAc:MeOH:$H_2O$ (24:1:1) to obtain isorhamnetin-3-*O*-robinoside (267 mg). Repeated chromatography of subfraction 4F-5 over silica gel using $CH_2Cl_2$:MeOH (10:1) yielded isoscopolin (43 mg).

On the other hand, subfractions of 60% MeOH was chromatographed in a silica gel column using $CH_2Cl_2$:MeOH (20:1) with gradual increase in MeOH in order to get 17 subfractions (6F-1 to 6F-17). Decantation of subfractions 6F-3 and 6F-4 yielded two compounds isorhamnetin-3-*O*-

galactoside (510 mg) and linarin (64 mg), respectively. Subfraction 6F-2 was subjected to silica gel chromatography in a solvent system containing $CH_2Cl_2$:MeOH (15:1) which yielded 4 subfractions (6F-2-1 to 6F-2-4). 6F-2-3 was chromatographed again over silica gel column using EtOAc:MeOH:$H_2O$ (50:1:1) to get the compound isorhamnetin-3-*O*-glucoside (62 mg). All the subfractions of 6F-2 were combined and further chromatographed over silica gel column using $CH_2Cl_2$:MeOH:$H_2O$ (7:1:0.1), followed by purification of the crystal over sephadex column to get compound isorhamnetin-3-*O*-arabinopyranoside (8 mg) and eugenol glucoside (15 mg). Chromatography of 6F-1 was carried out over silica gel column using $CH_2Cl_2$:MeOH:$H_2O$ (20:1:0.1) to afford compound *p*-methoxy phenyl glucoside (1.8 mg). F-6 was chromatographed over silica gel column using solvent EtOAc:MeOH:$H_2O$ (24:1:1) to get compound acacetin-7-*O*-(6″-*O*-acetyl)-β-D-glucopyranosyl-(1→2)[α-L-rhamnopyranosyl]-(1→6)-β-D-glucopyranoside (11 mg). 6F-7 was further chromatographed over si-gel column using EtOAc:MeOH (50:1 to 0:1) in order to get acacetin-7-*O*-β-D-glucopyranosyl-(1→2)[α-L-rhamnopyranosyl]-(1→6)-β-D-glucopyranoside (2.5 mg).

On the other hand, 100% $H_2O$ fraction (65 g) was chromatographed over

si-gel column using EtOAc:MeOH:$H_2O$ (24:2:1) with gradually increasing MeOH in order to 20 subfraction (HF-1 to HF-20). HF-4 was chromatographed over si-gel column using $CH_2Cl_2$:MeOH (10:1) which yielded tomerin (9 mg). HF-17 was further chromatographed using $CH_2Cl_2$:MeOH:$H_2O$ (4:1:0.1) in order get quercetin 3-*O*-robinobioside (124 mg) and chrysoeriol 7-*O*-rutinoside (4 mg). HF-15 was chromatographed over si-gel column using $CH_2Cl_2$:MeOH:$H_2O$ (7:1:0.1) in order to get 4 subfractions (HF-15-1 to HF-15-4). Repeated chromatography of HF-15-2 yielded koaburaside (6mg), while HF-15-4 yielded quercetin 5-*O*-glucoside. HF-16 was chromatographed over si-gel in order to get quercetin 7-*O*-galactopyrnosyl-7-*O*-α-L-rhamnoside (50 mg). Repeated chromatography of HF-18 using RP, si-gel, sephadex column yielded three compounds 4,5 di-*O*-cafeoyl quinic acid (100 mg), 3,5 di-*O*-cafeoyl quinic acid (15 mg) and 3,4 di-*O*-cafeoyl quinic acid (54 mg), chlorogenic acid (67 mg), cryptochlorogenic acid (35 mg), neochlorogenic acid (15 mg), 4-hydroxy 3-*O*-β-D-glucopyranosyl benzoic acid, and vicenin 2 (30 mg).

**2-5-2. Isolation of active compounds from the EtOAc fraction of *A. capillaris***

Similarly, the EtOAc fraction was also subjected to silica gel column chromatography using *n*-hexane-EtOAc (10:1→0:1) to obtain 15 subfractions (EF01 to EF15). Fraction 3 (EF03) was further chromatographed on a silica gel column eluted with *n*-hexane-EtOAc to obtain esculetin (10 mg). Repeated chromatography of EF05 using *n*-hexane-acetone (10:1→0:1) yielded quercetin (100 mg), while chromatography of EF04 yielded apigenin.

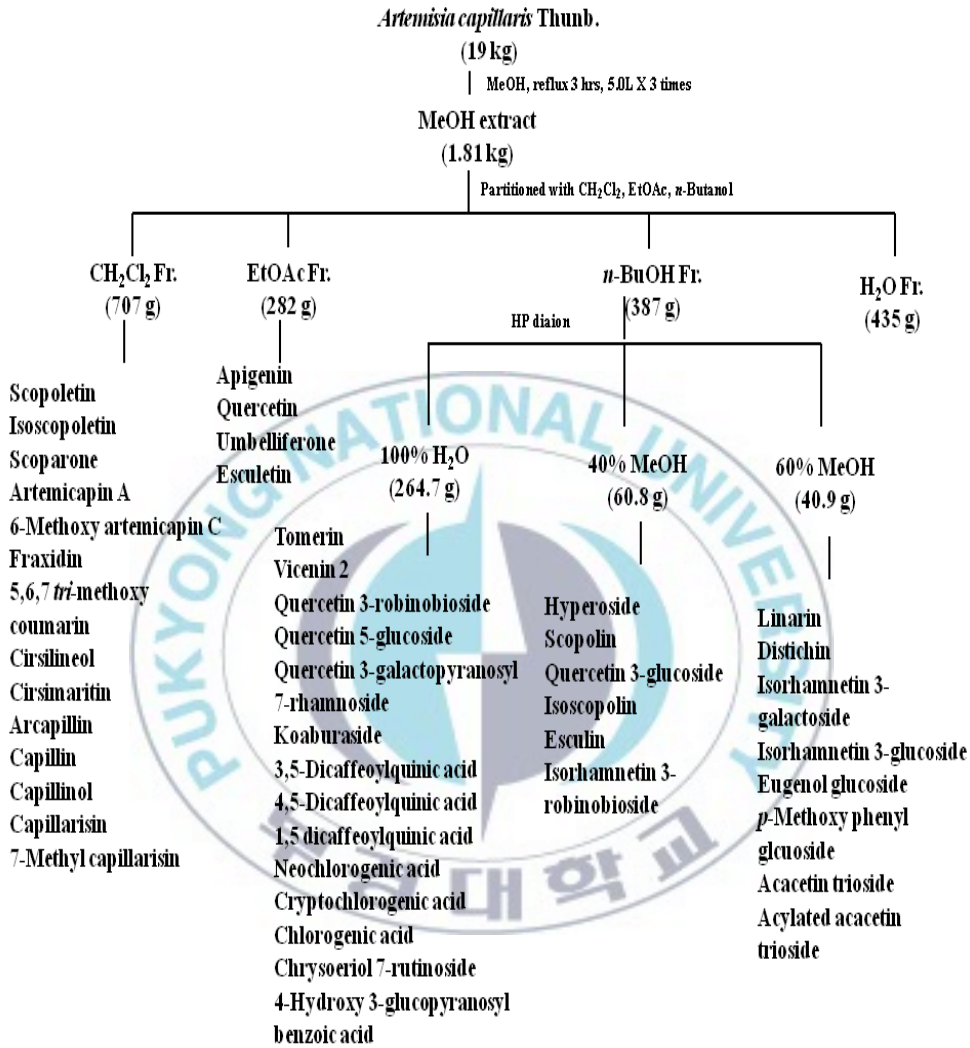
### 2-5-3. Isolation of active compounds from the $CH_2Cl_2$ Fraction of *A. capillaris*

Initially, about half of the $CH_2Cl_2$ fraction (350 g) was subjected to silica gel column chromatography eluted with $CH_2Cl_2$ followed by addition of gradual increase in MeOH amount in order to get 20 subfractions (CF-1 to CF-20). CF-1 was chromatographed over si-gel column using hexane:EtOAc (50:1→EtOAc) in order get 10 subfractions (CF-1-1 to CF-1-10). Repeated chromatogrqaphy of subfractions CF-1-5 and CF-1-6 yielded two polyacetylenes, namely capillin (340 mg) and capillinol (9 mg). CF-7 (113 g) was then chromatographed over silica gel column using Hexane:EtOAc (10:1→0:1) to afford 12 subfractions (F-7-1 to F-7-12).

Among the subfractions, CF-7-7 was filtered to separate the crystal, which was later identified as scoparone (2.70 g). Filtrate of CF-7-7 was further chromatographed over sephadex column using $CH_2Cl_2$ to get two subfractions (CF-7-7-1 to CF-7-7-2). Repeated chromatography of CF-7-7-1 in silica gel column using $CH_2Cl_2$:Hexane:MeOH (30:10:1) yielded two compounds namely 7-methyl capillarisin (54 mg) and capillarisin (330 mg). Precipitate of F-7-9 was separated after filtration and further chromatographed over silica gel column using $CH_2Cl_2$:Hexane:MeOH (30:10:1) to get cirselineol (57 mg) and cirsimaritin (11 mg). The mother liquor of CF-7-9 was chromatographed over si-gel column using Hexane:EtOAc (5:1) in order to get scopoletin (33 mg) and isoscopoletin (15 mg). Subfraction F-7-11 was filtered to get the precipitate which was identified as arcapillin (61 mg) from its spectroscopic data. CF-7-10 was chromatographed over si-gel column using $CH_2Cl_2$ (10:1) in order to get fraxidin (30 mg). CF-6 was chromatographed over si-gel column using Hexane:EtOAc (10:1) in order to get in order to get 4 subfractions (CF-6-1 to CF-6-4). Repeated chromatography of CF-6-2 using si-gel and sephadex column yielded two compounds, artemicapin A (62 mg) and 5,6,7 trimethoxy coumarin (300 mg). On the other hand, repeated

chromatography of CF-6-3 yielded 6-methoxy artemicapin C (95 mg).

All the compounds isolated from *n*-BuOH, EtOAc, and $CH_2Cl_2$ fractions of *A. capillaris* were characterized and identified by spectroscopic methods, including $^{1}H$- and $^{13}C$-NMR, as well as through comparison with published spectral data and TLC with reference compounds.




**Scheme 2.** Isolation of compounds from different fractions of *A. capillaris*

## 2-6. Experimental methods

### 2-6-1. α-Glucosidase inhibitory assay

The enzyme inhibition studies were carried out spectrophotometrically in a 96-well microplate reader using a procedure reported by Li et al., 2005. A total 60 μL reaction mixture containing 20 μL of 100 mM phosphate buffer (pH 6.8), 20 μL of 2.5 mM *p*-nitrophenyl *α-D*-glucopyranoside (pNPG) (Sigma Aldrich) in the buffer, and 20 μL of sample dissolved in 10% DMSO (fc. 125 μg/ml) were added to each well, followed by 20 μL of 10 mM phosphate buffer (pH 6.8) containing 0.2 U/mL α-glucosidase (Sigma Aldrich) to the mixture of treatment terminated wells. The plate was incubated at 37 °C for 15 min, and then adding 80 μL of 0.2 mol/L sodium carbonate solution to stop the reaction. Right after that, absorbance was recorded at 405 nm using VERSA max (Molecular Devices, Sunnyvale, CA, USA) microplate reader. Controls contained the same reaction mixture except the same volume of phosphate buffer was added instead of sample solution. Acarbose (Sigma Aldrich) was dissolved in 10% DMSO and used as a positive control. The inhibition (%) was calculated as: $(A_c-A_s)/A_c x100\%$, where $A_c$ is the absorbance of the control, and $A_s$, the

absorbance of the sample.

### 2-6-2. Protein tyrosine phosphatase 1B (PTP1B) inhibitory assay

Protein tyrosine phosphatase (PTP1B, human recombinant) was purchased from BIOMOL$^{@}$ international LP (USA) and the inhibitory activity of the plant extracts were evaluated using *p*-nitrophenyl phosphate as substrate (pNPP) (Cui et al., 2006). To each 96 well (final volume 110 μL) were added 2 mM PNPP and PTP1B in a buffer containing 50 mM citrate (pH 6.0), 0.1 M NaCl, 1 mM EDTA, and 1 mM dithiotheritol (DTT) with or without sample. The plate was preincubated at 37 ℃ for 10 mins, and then 50 μL of PNPP in buffer were added. Following incubation at 37 °C for 30 mins, the reaction was terminated with the addition of 10 M NaOH. The amount of p-nitrophenyl produced after enzymetic dephosphorylation was estimated by measuring the absorbance at 405 nm using VERSA max (Molecular Devices, Sunnyvale, CA, USA) microplate reader. The nonenzymatic hydrolysis of 2 mM PNPP was corrected by measuring the the increase in absorbance at 405 nm obtained in absence of PTP1B enzyme. The inhibition (%) was calculated as: $(A_c - A_s)/A_c x100\%$, where $A_c$ is the absorbance of the control, and $A_s$, the absorbance of the sample. Ursolic

acid was used as positive control.

### 2-6-3. Assay for rat lens aldose reductase (RLAR) inhibitory activity

This study adhered to the Guidelines for the Care and Use of Laboratory Animals approved by Pukyong National University. The rat lens homogenate was prepared according to a slightly modified method from Hayman and Kinoshita (1965). The lenses were removed from the eyes of Sprague-Dawley rats (Samtako Bio-Korea, Inc.) weighing 250-280 g. The lenses were homogenized in sodium phosphate buffer (pH 6.2), which was prepared from sodium phosphate dibasic ($Na_2HPO_4 \cdot H_2O$, 0.66 g) and sodium phosphate monobasic ($NaH_2PO_4 \cdot H_2O$, 1.27 g) in 100 mL of double distilled $H_2O$. The supernatant was obtained by centrifugation of the homogenate at 10,000 rpm and 4 $^{o}$C for 20 min and was frozen until use. A crude AR homogenate with a specific activity of 6.5 U/mg was used in the enzyme inhibition evaluations. The reaction solution consisted of 620 μL of 100 mM sodium phosphate buffer (pH 6.2), 90 μL of AR homogenate, 90 μL of 1.6 mM NADPH, and 9 μL of the samples (final concentration: 100 μg/mL for the extracts and fractions; 10 μg/mL and 100 μg/mL for the compounds dissolved in 100% DMSO) or 9 μL of 100% DMSO. The

substrate included 90 μL of 50 mM of DL-glyceraldehyde. The AR activity was determined by measuring the decrease in NADPH absorption at 340 nm over a 4 min period on an Ultrospec® 2100pro UV/Visible spectrophotometer with SWIFT II Applications software (Amersham Biosciences). Quercetin, a well-known AR inhibitor was used as a reference. The inhibition percentage (%) was calculated as [1 − (ΔA sample/min − ΔA blank/min)/(ΔA control/min − ΔA blank/min)] × 100, where ΔA sample/min represents the reduction in absorbance over 4 min for the test sample and substrate, and ΔA control/min represents the same, but with 100% DMSO instead of the test sample. The 50% inhibition concentration is expressed as the mean ± S.E.M.

**2-6-4. Kinetic study of capillin and capillinol against α-glucosidase, PTP1B, and rat lens aldose reductase**

In order to determine the kinetic mechanism, each enzymatic inhibition at various concentrations of test compounds was evaluated by monitoring the effect of different concentrations of substrates *via* Dixon plots (single reciprocal plot). We determined the enzyme kinetics of α-glcuosidase at different concentrations of pNPG (4.0 μM, 2.0 and 1.0 μM), PTP1B at

different concentrations of pNPP (4.0 μM, 2.0 μM, and 1.0 μM), and RLAR at various concentrations of DL-glyceraldehyde (0.01, 0.05, and 0.025 mM) as substrates in the absence or presence of different concentrations of the test compounds: capillin (125, 62.5 and 31.25 μg/mL for α-glcuosidse; 25, 10, and 2 μg/mL for PTP1B; and 10, 2, and 0.4 μg/mL for RLAR); capillinol (125, 62.5 and 31.25 μg/mL for α-glcuosidse; 50, 25, and 10 μg/mL for PTP1B). The enzymatic experiments consisted of the aforementioned RLAR assay method. Inhibition constants (Ki) were determined by interpreting the Dixon plots in which the value of the x-axis implies -Ki.

**2-6-5. Advanced glycation endproduct (AGE) formation inhibitory assay**

The inhibitory activity of AGE formation was examined according to the modified method of Vinson and Howard (1996). To prepare the AGE reaction solution, 10 mg/ml of bovine serum albumin in 50 mM sodium phosphate buffer (pH 7.4) was added to 0.2 M fructose, 0.2 M glucose, and 0.02% sodium azide to prevent bacterial growth. The reaction mixture (950 μl) was then mixed with various concentrations of the samples (50 μl)

dissolved in 10% DMSO. After incubating at 37 ℃ for 7 days, the fluorescence intensity of the reaction products was determined on a spectrofluorometric detector (FLx800 microplate fluorescence reader, Bio-Tek Instrument, Inc., Winooski, USA) with excitation and emission wavelengths at 350 nm and 450 nm, respectively. The $IC_{50}$ values are expressed as means ± SEM of triplicate experiments. The nucleophilic hydrazine compound, aminoguanidine hydrochloride, was used as a reference in the AGE assay. In addition, aliquots of the reaction mixtures were then assayed for fructosamine, protein carbonyl content, thiol group, amyloid cross β structure, and CML.

**2-6-6. Fructosamine measurement**

The concentration of the Amadori product fructosamine was measured by NBT assay (Ardestani and Yazdanparast, 2007). Briefly, glycated BSA (10 μL) was incubated with 100 μL of 0.5 mM NBT in 0.1 M carbonate buffer, pH 10.4 at 37 ℃. The absorbance was measured at 530 nm at 10 and 15 min time points. The concentration of fructosamine was calculated compared to DMF as the standard.

### 2-6-7. Determination of protein carbonyl content

The carbonyl group in glycated BSA, a marker for protein oxidative damage, was assayed according to the method of Levine and colleagues with minor modifications (Levine et al., 1990). Briefly, 400 μL of 10 mM DNPH in 2.5 M HCl was added to 100 μL of glycated samples. After 1 h incubation in the dark, 500 μL of 20% (w/v) TCA was used for protein precipitation (5 min on ice) and then centrifuged at 10,000 *g* for 10 min at 4 ℃. The protein pellet was washed with 500 μL of ethanol/ethyl acetate (1:1) mixture three times and resuspended in 250 μL of 6 M guanidine hydrochloride. The absorbance was measured at 370 nm. The carbonyl content of each sample was calculated based on the extinction coefficient for DNPH ($\varepsilon$ = 22,000 $M^{-1}cm^{-1}$). The results were expressed as μM carbonyl/mg protein.

### 2-6-8. Thiol group estimation

The free thiols in glycated samples were measured by Ellman's assay with minor modifications (Ellman, 1959). Briefly, 70 μL of glycated samples were incubated with 5 mM DTNB in 0.1 M PBS, pH 7.4 at 25 ℃ for 15 min. The absorbance of samples was measured at 410 nm. The

concentration of free thiols was calculated from L-cysteine standard and expressed as nmol/mg protein.

### 2-6-9. Thioflavin T assay

Thioflavin T, a marker of amyloid cross-β structure, was measured according to a previous method with minor modifications (Tupe and Agte, 2010). Briefly, 100 μL of 64 μM thioflavin T in 0.1 M PBS, pH 7.4 was added to the glycated samples (10 μL) and incubated at 25 °C for 60 min. The fluorescence intensity was measured at excitation wavelength of 435 nm and emission wavelength of 485 nm.

### 2-6-10. Assay for DPPH radical scavenging activity

The DPPH radical scavenging effect was evaluated using methods outlined in Blois (1958) with slight modifications. 160 μL of MeOH solution at various concentrations (final concentration (f.c.) 320 μg/mL for the extracts, fractions, and the compounds) were added to 40 μL DPPH methanol solution ($1.5 \times 10^{-4}$ M). After gently mixing the sample gently and letting it stand at them at room temperature for 30 min, the optical density was measured at 520 nm using a VERSAmax microplate

spectrophotometer (Molecular Devices). The antioxidant activity of the samples was expressed as IC50 values (μg/mL or μM required to inhibit DPPH radical formation by 50%), which were calculated from the log-dose inhibition curve. L-Ascorbic acid was used as the positive control. The $IC_{50}$ values are expressed as the mean ± S.E.M. of triplicate experiments.

**2-6-11. Trolox equivalent antioxidant capacity (TEAC)**

This assay was based on the ability of different substances to scavenge the ABTS radical cation ($ABTS^{\bullet+}$) as compared with the positive control trolox (Re et al., 1999). To oxidize colorless ABTS to blue-green $ABTS^{\bullet+}$, a 7 mM ABTS stock solution was mixed with 2.45 mM potassium persulfate (1:1, v/v) and left at room temperature in the dark for 12-16 h until the reaction was complete and the absorbance was stable. The blue/ green $ABTS^{\bullet+}$ solution was diluted in ethanol (EtOH) to an absorbance of 0.70 ± 0.02 at 734 nm for measurement. The photometric assay was conducted with 180 μL of the $ABTS^{\bullet+}$ solution and 20 μL of test sample dissolved in the EtOH solution (f.c. 100 μg/mL) that was stirred for 30 sec. The optical density was measured at 734 nm after 2 min using a VERSAmax microplate spectrophotometer (Molecular Devices). The antioxidant activities of the

samples were calculated by determining the decrease in absorbance at different concentrations using the following equation: E = [(Ac − At)/Ac] × 100, where At and Ac are the absorbance with and without samples, respectively. Trolox and L-ascorbic acid were used as the positive controls. The TEAC results are expressed as $IC_{50}$ values (mean ± S.E.M. of triplicate experiments).

### 2-6-12. Assay for $ONOO^-$ scavenging activity

The $ONOO^-$ scavenging activity was assessed using a modified method from Kooy et al. (1994), which involved monitoring highly fluorescent rhodamine 123 that was rapidly produced from non-fluorescent DHR 123 in the presence of $ONOO^-$. The rhodamine buffer (pH 7.4) consisted of 50 mM sodium phosphate dibasic, 50 mM sodium phosphate monobasic, 90 mM sodium chloride, 5.0 mM potassium chloride, and 100 μM DTPA. The final DHR 123 concentration was 5.0 μM. The assay buffer was prepared prior to use and placed on ice. The test sample was dissolved in 10% DMSO (f.c. 100 μM). The background and final fluorescent intensities of the samples were measured five minutes after treatment with and without the addition of authentic $ONOO^-$ (10 μM) dissolved in 0.3 N sodium hydroxide. The

fluorescence intensity of the oxidized DHR 123 was evaluated using a fluorescence microplate reader (Bio-Tek Instruments Inc., FL×800) at excitation and emission wavelengths of 480 and 530 nm, respectively. Values of $ONOO^-$ scavenging activity were calculated as the final fluorescence intensity minus the background fluorescence determined via the detection of DHR 123 oxidation. L-Penicillamine was used as the positive control.

### 2-6-13. Inhibition of $ONOO^-$-mediated tyrosine nitration

In order to examine the inhibition of $ONOO^-$ induced BSA nitration, 2.5 μL of various concentrations of each test sample dissolved in 10% EtOH (v/v) were added to 95 μL of BSA (0.5 mg protein/mL). The mixtures were incubated at room temperature (25 $^oC$) for 10 min and then mixed with 2.5 μL of $ONOO^-$ (200 μM). Following incubation at 25 $^oC$ for 10 min, the mixed sample was added to a Bio-Rad gel buffer at a ratio of 1:1 and boiled for 5 min to denature the proteins. The total protein equivalent for the reactant was separated on a 10% SDS-polyacrylamide minigel at 80 V for 30 min, followed by 100 V for 1 h, and then transferred to a PVDF membrane at 80 V for 120 min in a wet transfer system (Bio-Rad). The

membrane was immediately placed in a blocking solution (5% nonfat dry milk in Tris-buffered saline containing 0.1% Tween-20 (TBST) buffer (pH 7.4) at room temperature for 1 h. The membrane was washed three times for 10 min in TBST buffer and then incubated overnight with a monoclonal anti-nitrotyrosine antibody (5% nonfat dry milk) diluted 1:2000 in TBST buffer at 4 $^{o}$C. After another three washings in TBST buffer for 10 min, the membrane was incubated with HRP-conjugated sheep antimouse secondary antibody and diluted 1:2000 in TBST buffer at room temperature for 1 h. The membrane was then washed with TBST buffer three times for 10 min, and the antibody labeling was visualized using a Supersignal West Pico Chemiluminescent Substrate (Pierce) according to the manufacturer's instructions after exposure to X-ray film (GE Healthcare Ltd.). Prestained blue protein markers were used for molecular weight determination.

### 2-7. Statistics

Results were expressed as respective the mean ± standard deviation (STDEV) and the mean ± standard error of the mean (S.E.M) in triplicate experiments. Statistical comparisons performed using 1-way ANOVA followed by student's *t*-test.

# III. Results and discussion

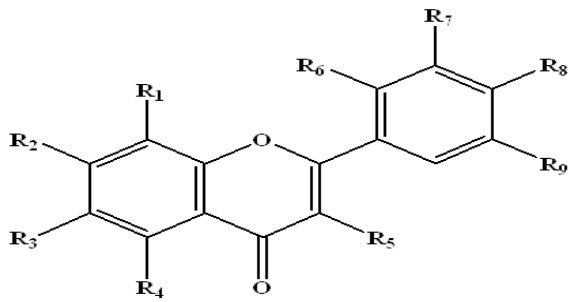
## 3-1. Isolation of the active compounds from *A. capillaris*, and their structure elucidation

### 3-1-1. Isolation of constituents from the MeOH extract of *A. capillaris*

The powder of whole plant of *A. capillaris* Thunb. was extracted with MeOH and concentrated to dryness in order get the MeOH extract. Then it was suspended in distilled water, and successively partitioned with $CH_2Cl_2$, EtOAc, *n*-BuOH to yield $CH_2Cl_2$, EtOAc, *n*-BuOH fractions respectively, as well as $H_2O$ residue. All subfractions except the $H_2O$ residue were subjected to chromatographic separation using combination of different chromatographic techniques such as normal phase, reversed phase, gel filtration and ion exchange chromatography. Extensive chromatography of different fractions yielded 46 compounds including 13 coumarins (esculetin, esculin, scopolin, isoscopolin, scoparone, scopoletin, isoscopoletin, artemicapin A, fraxidin, 5,6,7 trimethoxy coumarin, 6 methoxy artemicapin C, tomerin, and umbelliferone) and 19 flavonoids (cirsilineol, cirsimaritin, arcapillin, linarin, quercetin, hyperoside, isorhamnetin 3-*O*-robinobioside,

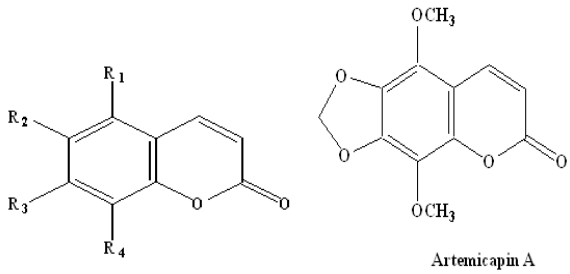
quercetin 5-*O*-glucoside, quercetin 3-*O*-glucoside, quercetin 3-*O*-robinobioside, chrysoeriol-7-*O*-rutinoside, quercetin 7-*O*-galatopyranosyl-7-*O*-rhamnoside, isorhamnetin 3-*O*-galactoside, isorhamnetin 3-*O*-glucoside, acacetin-7-*O*-(6″-*O*-acetyl)-β-D-glucopyranosyl-(1→2)[α-L-rhamnopyranosyl]-(1→6)-β-D-glucopyranoside, acacetin-7-*O*-β-D-glucopyranosyl-(1→2)[α-L-rhamnopyranosyl]-(1→6)-β-D-glucopyranoside, isorhamnetin-3-*O*-arabinopyranoside, vicenin-2, apigenin), 6 caffeoylquinic acid derivatives (4,5 di-*O*-caffeoyl quinic acid, 3,5 di-*O*-caffeoyl quinic acid, 3,4 di-*O*-caffeoyl quinic acid, chlorogenic acid, cryptochlorogenic acid, neochlorogenic acid), 2 chromones (capillarisin and 7-methyl capillarisin), 2 essential oils (capillin and caplliniol), and 4 phenolic compounds (paramethoxy phenyl glucoside, eugenol glucoside, koaburaside, 4-hydroxy-3-*O*-glucopyranosyl benzoic acid). Among them, acacetin-7-*O*-(6″-*O*-acetyl)-β-D-glucopyranosyl-(1→2)[α-L-rhamnopyranosyl]-(1→6)-β-D-glucopyranoside is a new flavonoid isolated for the first time. On the other hand, three coumarins (fraxidin, 5,6,7 trimethoxy coumarins and tomerin) and four flavnoids (linarin, chrysoeriol-7-*O*-rutinoside, quercetin 7-*O*-galatopyranosyl-7-*O*-rhamnoside, and isorhamnetin-3-*O*-arabinopyranoside) along with four phenolics (paramethoxy phenyl glucoside, eugenol

glucoside, koaburaside, 4-hydroxy-3-*O*-glucopyranosyl benzoic acid) have been isolated first time from *A. capillaris*. All the compounds isolated from *n*-BuOH, EtOAc, and $CH_2Cl_2$ fractions of *A. capillaris* were characterized and identified by spectroscopic methods, including $^{1}H$- and $^{13}C$-NMR, as well as through comparison with published spectral data and TLC with reference compound. The structures are shown in Figures 1-3.

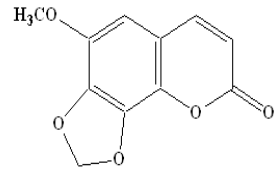



| Name | R1 | R2 | R3 | R4 | R5 | R6 | R7 | R8 | R9 |
|---|---|---|---|---|---|---|---|---|---|
| Quercetin | H | OH | H | OH | OH | H | OH | OH | H |
| Hyperoside | H | OH | H | OH | *O*-Gal | H | OH | OH | H |
| Quercetin 3-robinobioside | H | OH | H | OH | *O*-Rob | H | OH | OH | H |
| Quercetin 5-glucoside | H | OH | H | *O*-Glu | OH | H | OH | OH | H |
| Quercetin 3-galactopyranosyl-7 -rhamnoside | H | *O*-Rha | H | OH | Gal | H | OH | OH | H |
| Vicenin 2 | *C*-Glu | OH | *C*-Glu | OH | H | H | H | OH | H |
| Apigenin | H | OH | H | OH | H | H | H | OH | H |
| Isorhamnetin 3-glcucoside | H | OH | H | OH | *O*-Glu | H | $OCH_3$ | OH | H |
| Isorhamnetin 3-galactoside | H | OH | H | OH | *O*-Gal | H | $OCH_3$ | OH | H |
| Isorhamnetin 3-robinobioside | H | OH | H | OH | *O*-Rob | H | $OCH_3$ | OH | H |
| Isorhamnetin 3-arabinpyranoside | H | OH | H | OH | *O*-Rob | H | $OCH_3$ | OH | H |
| Chrysoeriol 7-rutinoside | H | *O*-Rut | H | OH | H | H | $OCH_3$ | OH | H |
| Linarin | H | O-Rut | H | OH | H | H | H | $OCH_3$ | H |
| Acacetin-7-*O*-(6''-*O*-acetyl)-β-D-glucopyranosyl-(1→2)[α-L-rhamnopyranosyl]-(1→6)-β-D-glucopyranoside | H | *O*-Glu-Rha (Ac-Glu) | H | OH | H | H | H | $OCH_3$ | H |
| Acacetin-7-O-β-D-glucopyranosyl-(1→2)[α-L-rhamnopyranosyl]-(1→6)-β-D-glucopyranoside | H | *O*-Glu-Rha (Glu) | H | OH | H | H | H | $OCH_3$ | H |
| Cirsilineol | H | $OCH_3$ | $OCH_3$ | OH | H | H | H | OH | $OCH_3$ |
| Cirsimaritin | H | $OCH_3$ | $OCH_3$ | OH | H | H | H | OH | H |
| Arcapillin | H | $OCH_3$ | $OCH_3$ | OH | H | OH | H | OH | $OCH_3$ |

**Figure 1.** Structures of flavonoid derivatives isolated from *A. capillaris*

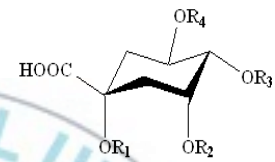


Artemicapin A



6-Methoxy artemicapin C

| Name | R1 | R2 | R3 | R4 |
|---|---|---|---|---|
| Coumarin | H | H | H | H |
| Umbelliferone | H | H | OH | H |
| Esculetin | H | OH | OH | H |
| Esculin | H | *O*-Glu | OH | H |
| Scoparone | H | $OCH_3$ | $OCH_3$ | H |
| Scopolin | H | $OCH_3$ | *O*-Glu | H |
| Scopoletin | H | $OCH_3$ | OH | |
| Isocopolin | H | *O*-Glu | $OCH_3$ | H |
| Isopoletin | H | OH | $OCH_3$ | H |
| Fraxidin | H | $OCH_3$ | $OCH_3$ | OH |
| 5,6,7 trimethoxy coumarin | $OCH_3$ | $OCH_3$ | $OCH_3$ | H |
| Tomerin | *O*-Glu | $OCH_3$ | $OCH_3$ | H |
| Daphnetin | H | H | OH | OH |
| 7-methoxy coumarin | H | H | $OCH_3$ | H |



| Name | R1 | R2 | R3 | R4 |
|---|---|---|---|---|
| Chlorogenic acid | H | Caffeoyl | H | H |
| Neochorologenic acid | H | H | H | Caffeoyl |
| Cryptochlorogenic acid | H | H | Caffeoyl | H |
| 4,5-Dicaffeoyl quinic acid | H | H | Caffeoyl | Caffeoyl |
| 3,5-Dicaffeoyl quinic acid | H | Caffeoyl | H | Caffeoyl |
| 1,5-Dicaffeoyl quinic acid | Caffeoyl | H | H | Caffeyol |
| 3,4-Dicaffeoyl quinic acid | H | Caffeoyl | Caffeoyl | H |

**Figure 2.** Structures of coumarin and caffeoylquinic acid derivatives isolated from *A. capillaris*

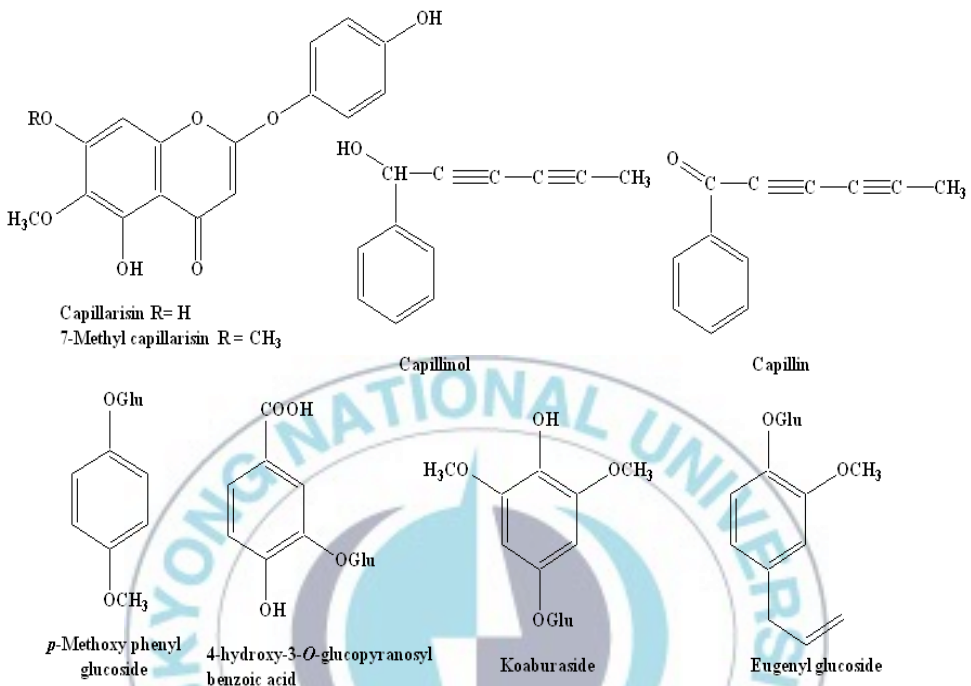



**Figure 3.** Structures of chromones, essential oils, and simple phenolic compounds isolated from *A. capillaris*

### 3-1-2. Structure elucidation of a new acetyl flavone glycoside

Acacetin-7-*O*-β-D-glucopyranosyl-(1→2)[α-L-rhamnopyranosyl]-(1→6)-β-D- glucopyranoside was identified from its respective NMR spectra and confirmed by comparison with those of published data (Veitch et al., 2010). A new acylated flavonoid glycoside was isolated as a white amorphous powder with molecular formula $C_{36}H_{44}O_{20}$ deduced from the $[M + H]^+$ and $[M - H]^-$ peaks at *m/z* 797.1 and 795.2 in combinations of positive and negative ESI-MS that were supported by the $^{13}$CNMR spectrum. Its UV spectrum exhibited characteristic absorbance bands of flavones at 268 nm and 334 nm. The $^{13}$C-NMR spectrum of the new acylated flavonoid glycoside was very similar to acacetin-7-*O*-β-D-glucopyranosyl-(1→2)[α-L-rhamnopyranosyl]-(1→6)-β-D-glucopyranoside with the exception of two additional carbon signals arising from an aliphatic carbon (20.57 ppm) and a carbonyl carbon (170.31 ppm). The aliphatic carbon was identified as a methyl carbon based on the integration of the corresponding proton resonances thus indicating the presence of an acetyl moiety. Comparison of the carbon chemical shift values of a new compound with the values of the parent glycoside showed that the C-6 carbon signal of the terminal glucopyranoside moiety had shifted downfield by about 3.3 ppm, indicating

that the acetyl group was attached to this carbon atom. This was further supported by the HMBC correlation between the acetyl carbonyl group and methylene protons (H-6'') of the terminal glucose. Therefore, the structure of the new compound was determined to be a new acylated flavonoid glycoside, acacetin-7-*O*-(6''-*O*-acetyl)-β-D-glucopyranosyl-(1→2)[α-L-rhamnopyranosyl]-(1→6)-β-D-glucopyranoside. Further confirmation of the chemical structure was determined through mild alkaline hydrolysis with 2N sodium hydroxide at ambient temperature to yield the respective deacylated flavonoid glycoside, which was then identified based on TLC or HPLC compared to an authentic sample. To the best of the authors' knowledge, this is the first report of acacetin-7-*O*-(6''-*O*-acetyl)-β-D-glucopyranosyl-(1→2)[α-L-rhamnopyranosyl]-(1→6)-β-D-glucopyranoside from natural sources. Another acacetin trioside was also isolated, and its $^{1}H$ and $^{13}C$ spectrums showed all similar peaks except the peaks for acetyl group. Therefore, the structure of another trioside is elucidated as acacetin-7-*O*-β-D-glucopyranosyl-(1→2)[α-L-rhamnopyranosyl]-(1→6)-β-D-glucopyranoside. The possibility of this compound as an artifact was excluded since many acetylated sugar derivatives of flavonoids was identified in natural products. Therefore, the new compound is truly present

in *A. capillaris.*

NMR data of other compounds are given below:

**Isorhamnetin 3-*O*-robinobioside**

Yellow amorphous powder. $^{1}$H NMR (400 MHz, DMSO-$d_6$): δ 8.01 (1H, d, *J* = 2.1 Hz, H-2'), 7.50 (1H, dd, *J* = 2.1, 8.4 Hz, H-6'), 6.97 (1H, d, *J* = 8.5 Hz, H-5'), 6.43 (1H, d, *J* = 2.3 Hz, H-8), 6.26 (1H, d, *J* = 2.1 Hz, H-6), 5.46 (1H, d, *J* = 7.8 Hz, H-1''), 4.42 (1H, d, *J* = 1.0 Hz, H-1'''), 3.84 (3H, s, -$OCH_3$). $^{13}$C (100 MHz, DMSO-$d_6$): δ 177.3 (C-4), 164.4 (C-7), 161.2 (C-5), 156.4 (C-2), 149.4 (C-3'), 147.0 (C-4'), 133.1 (C-3), 122.0 (C-6'), 121.0 (C-1'), 115.2 (C-5'), 113.4 (C-2'), 103.9 (C-10), 101.8 (C-1''), 100.0 (C-1'''), 98.8 (C-6), 93.8 (C-8), 73.5 (C-5''), 72.9 (C-3''), 71.9 (C-4'''), 71.1 (C-2''), 70.6 (C-3'''), 70.4 (C-2'''), 68.3 (C-5'''), 68.0 (C-4''), 65.2 (C-2''), 55.9 (-$OCH_3$), 17.9 (C-6''') (Park et al., 2007).

**Isorhamnetin 3-*O*-β-D-galactoside**

Yellow amorphous powder. $^{1}$H NMR (400 MHz, DMSO-$d_6$): δ 12.62 (1H, s, -OH), 10.91 (1H, brs, -OH), 9.76 (1H, brs, -OH), 8.03 (1H, d, *J* = 2.1 Hz, H-2'), 7.50 (1H, dd, *J* = 2.1 & 8.20 Hz, H-6'), 6.90 (1H, d, *J* = 8.2 Hz,

H-5'), 6.45 (1H, d, *J* = 2.1 Hz, H-8), 6.21 (1H, d, *J* = 2.1 Hz, H-6), 5.51 (1H, d, *J* = 7.8 Hz, H-1"), 3.84 (3H, s, -$OCH_3$), 3.30-3.60 (4H, m, H-2", H-3", H-4" & H-5"). $^{13}$C (100 MHz, DMSO-$d_6$): δ, 177.4 (C-4), 164.2 (C-7), 161.2 (C-5), 156.4 (C-9), 156.2 (C-2), 149.4 (C-3'), 147.0 (C-4'), 133.1 (C-3), 121.9 (C-6'), 121.1 (C-1'), 115.1 (C-5'), 113.5 (C-2'), 104.0 (C-10), 101.6 (C-1"), 98.7 (C-6), 93.8 (C-8), 75.9 (C-5"), 73.1 (C-3"), 71.2 (C-2"), 68.0 (C-4"), 60.3 (C-6"), 56.0 (-$OCH_3$) (Zuhal and Omur, 2005).

**Isorhamnetin 3-*O*-β-D-glucoside**

Yellow amorphous powder. $^1$H NMR (400 MHz, DMSO-$d_6$): δ 12.60 (1H, s, -OH), 9.87 (1H, brs, -OH), 7.94 (1H, d, *J* = 2.4 Hz, H-2'), 7.49 (1H, dd, *J* = 2.1 & 8.5 Hz, H-6'), 6.91 (1H, d, *J* = 8.5 Hz, H-5'), 6.43 (1H, d, *J* = 2.1 Hz, H-8), 6.20 (1H, d, *J* = 2.1 Hz, H-6), 5.57 (1H, d, *J* = 7.5 Hz, H-1"), 3.84 (3H, s, -$OCH_3$), 3.30-3.60 (4H, m, H-2", H-3", H-4" & H-5"). $^{13}$C (100 MHz, DMSO-$d_6$): δ, 177.4 (C-4), 164.3 (C-7), 161.2 (C-5), 156.4 (C-9), 156.2 (C-2), 149.4 (C-3'), 146.9 (C-4'), 132.9 (C-3), 122.0 (C-6'), 121.1 (C-1'), 115.2 (C-5'), 113.5 (C-2'), 103.9 (C-10), 101.8 (C-1"), 98.7 (C-6), 93.7 (C-8), 77.4 (C-5"), 76.4.1 (C-3"), 74.3 (C-2"), 69.8 (C-4"), 60.6 (C-6"), 55.7 (-$OCH_3$).

**Isorhamnetin 3-*O*-α-L-arabinofuranoside (distichin)**

Yellow amorphous powder. $^{1}$H NMR (400 MHz, DMSO-$d_6$): δ 12.61 (1H, s, -OH), 7.90 (1H, d, *J* = 2.1 Hz, H-2'), 7.57 (1H, dd, *J* = 2.1 & 8.5 Hz, H-6'), 6.91 (1H, d, *J* = 8.5 Hz, H-5'), 6.44 (1H, d, *J* = 1.7 Hz, H-8), 6.19 (1H, d, *J* = 2.1 Hz, H-6), 5.33 (1H, d, *J* = 6.15 Hz, H-1''), 3.86 (3H, s, -$OCH_3$). $^{13}$C (100 MHz, DMSO-$d_6$): δ, 177.4 (C-4), 164.6 (C-7), 161.1 (C-5), 156.3 (C-9), 156.1 (C-2), 149.5 (C-3'), 147.1 (C-4'), 133.4 (C-3), 122.2 (C-6'), 12.9 (C-1'), 115.3 (C-5'), 113.2 (C-2'), 103.8 (C-10), 101.6 (C-1''), 98.8 (C-6), 93.8 (C-8), 71.8 (C-2''), 70.9 (C-4''), 66.56 (C-3''), 65.0 (C-5''), 56.0 (-$OCH_3$).

**Quercetin 3-*O*-β-D-galactoside (hyperoside)**

Yellow amorphous powder; $^{1}$H NMR (400 MHz, DMSO-$d_6$): δ: 12.65 (1H, s, 5-OH), 10.95 (1H, brs, 7-OH), 9.82 (1H, brs, 4'-OH), 9.20 (1H, brs, 3-OH), 7.67 (2H, dd, *J* = 8.5, 2.0 Hz, H-6'), 7.53 (1H, d, *J* = 2.0 Hz, H-2'), 6.82 (1H, d, *J* = 8.5 Hz, H-5'), 6.41 (1H, d, J = 1.9 Hz, H-8), 6.21 (1H, d, *J* = 1.9 Hz, H-6), 5.38 (1H, d, *J* = 7.8 Hz, H-1''), 3.30-3.60 (4H, m, H-2'', H-3'', H-4'' & H-5''). $^{13}$C NMR (100 MHz, DMSO-$d_6$): δ 177.5 (C-4), 164.2 (C-7), 161.2 (C-5), 156.3 (C-9), 156.2 (C-2), 148.5 (C-4'), 144.8 (C-3'), 133.5 (C-

3), 122.0 (C-1'), 121.1 (C-6'), 115.9 (C-5'), 115.2 (2'), 103.9 (C-10), 101.8 (C-1"), 98.7 (C-6), 93.6 (C-8), 75.9 (C-5"), 73.2 (C-3"), 71.2 (C-2"), 67.9 (C-4"), 60.1 (C 6").

**Quercetin 3-*O*-β-D-galactopyranoside 7-*O*-α-L-rhamnoside**

$^{1}$H NMR (400 MHz, DMSO-$d_6$): δ 12.61 (1H, s, H-5), 9.79 (1H, s, H-7), 9.17 (1H, s, H-4), 7.70 (1H, dd, *J* = 2.5 & 8.5 Hz, H-6'), 7.58 (1H, d, *J* = 2.1 Hz, H-2'), 6.83 (1H, d, *J* = 8.5 Hz, H-5'), 6.79 (1H, d, *J* = 2.1 Hz, H-8), 6.43 (1H, d, *J* = 2.1 Hz, H-6), 5.55 (1H, s, H-1'"), 5.40 (1H, d, *J* = 7.9 Hz, H-1"), 5.40~3.0 (17H, m, glucose and rhamnose), 1.12 (3H, d, J = 6.1 Hz, -$CH_3$).$^{13}$C NMR (100 MHz, DMSO-$d_6$): δ 177.6 (C-4), 161.6 (C-7), 160.9 (C-5), 15.7 (C-2), 155.9 (C-9), 148.7 (C-4'), 144.9 (C-3'), 133.8 (C-3), 122.1 (C-1'), 121.0 (C-6'), 116.1 (C-5'), 115.2 (C-2'), 105.6 (C-10), 101.6 (C-1"), 99.4 (C-6), 98.43 (C-1'"), 94.3 (C-8), 75.9 (C-5"), 73.2 (C-3"), 71.6 (C-4'"), 71.2 (C-2"), 70.2 (C-3'"), 70.1 (C-2'"), 69.8 (C-5'"), 68.0 (C-4"), 60.2 (C 6"), 17.9 (C-6'") (Song et al., 2007).

**Chrysoeriol 7-*O*-rutinoside**

$^{1}$H NMR (400 MHz, DMSO-$d_6$): δ: 12.92 (1H, s, 5-OH), 7.57 (1H, dd, *J*

= 8.5 & 2.0 Hz, H-6'), 7.44 (1H, d, *J* = 2.3 Hz, H-2'), 7.13 (1H, d, *J* = 8.5 Hz, H-5'), 6.82 (1H, s, H-3), 6.76 (1H, d, *J* = 2.0 Hz, H-8), 3.87 (3H, s, 3'-$OCH_3$), 6.45 (1H, d, *J* = 2.0 Hz, H-6), 5.08 (1H, d, *J* = 7.1 Hz, H-1"), 4.55 (1H, s, H-1'"), 1.07 (3H, d, *J* = 6.5, H-6'"). $^{13}$C NMR (100 MHz, DMSO-$d_6$): δ 181.9 (C-4), 164.2 (C-2), 162.9 (C-7), 161.2 (C-5), 156.9 (C-9), 151.3 (C-3'), 146.8 (C-4'), 122.9 (C-1'), 118.9 (C-6'), 113.1 (C-2'), 112.2 (C-5'), 105.4 (10), 103.8 (C-3), 100.5 (C-1"), 99.9 (C-1"), 99.6 (C-6), 94.8 (C-8), 76.2 (C-5"), 75.6 (C-3"), 73.1 (C-4'"), 72.0 (C-2"), 70.7 (C-4"), 70.3 (C-3'"), 69.5 (C-2'"), 68.3 (C-5'"), 66.0 (C 6"), 55.8 (3'-$OCH_3$), 17.8 (C-6'") (Chen et al., 2008).

**Vicenin 2 (apigenin 6,8-*C*-glucoside)**

$^1$H NMR (400 MHz, DMSO-$d_6$): δ 10.41 (), 9.39 (), 8.03 (2H, d, *J* = 8.54 Hz, H-2' & H-6'), 6.91 (2H, d, *J* = 8.54 Hz, H-3' & H-5'), 6.81 (1H, s, H-3), 4.80 (1H, d, *J* = 9.80 Hz, H-1"), 4.75 (1H, d, *J* = 9.80 Hz, H-1'"), 3.50 ~ 3.86 (10H, m, H-2"~H6" & H-2'"~H6")'. $^{13}$C NMR (100 MHz, DMSO-$d_6$): δ 182.3 (C-4), 164.1 (C-2), 161.2 (C-7 & C-4'), 158.6 (C-5), 155.1 (C-9), 129.0 (C-2' & C-6'), 121.5 (C-1'), 115.8 (C-3' & C-5'), 107.5 (C-6), 105.3 (C-8), 103.8 (C-10), 102.6 (C-3), 81.8 (C-5"), 81.4 (C-5'"), 78.8 (C-3"), 78.8

(C-3'''), 74.1 (C-1'''), 73.8 (C-1''), 71.9 (C-2'''), 70.9 (C-2''), 70.6 (C-4''), 69.1 (C-4'''), 61.3 (C-6'''), 59.8 (C-6'') (Leosvaldo et al., 2009).

**Linarin (acacetin 7-*O*-rutinoside)**

$^{1}$H NMR (400 MHz, DMSO-$d_6$): δ: 12.91 (1H, s, 5-OH), 8.05 (2H, d, *J* = 9.22 Hz, H-2' & H-6'), 7.14 (2H, d, *J* = 8.89 Hz, H-3' & H-5'), 6.94 (1H, s, H-3), 6.79 (1H, d, *J* = 2.05 Hz, H-8), 6.45 (1H, d, *J* = 2.40 Hz, H-6), 5.07 (1H, d, *J* = 7.18 Hz, H-1''), 4.55 (1H, s, H-1'''), 4.45, 4.56, 4.70, 5.21, 5.44 (6H, s, sugar-OH), 3.86 (3H, s, 4'-$OCH_3$), 1.08 (3H, d, *J* = 6.15 Hz, H-6'''). $^{13}$C NMR (100 MHz, DMSO-$d_6$): 182.0 (C-4), 163.9 (C-2), 163.0 (C-7), 162.4 (C-4'), 161.2 (C-5), 157.0 (C-9), 128.5 (C-2' & C-6'), 122.7 (C-1'), 114.7 (C-3' & C-5'), 105.5 (C-10), 103.8 (C-3), 100.5 (C-1'''), 99.9 (C-1''), 99.7 (C-6), 94.8 (C-8), 76.3 (C-3''), 75.7 (C-5''), 73.1 (C-2''), 72.1 (C-4'''), 70.7 (C-3'''), 70.4 (C-2'''), 69.6 (C-4''), 68.3 (C-5'''), 66.1 (C-6''), 17.8 (C-6'''), 55.6 (4'-$OCH_3$) (Quintin and Lewin, 2004).

**Acacetin-7-*O*-(6''-*O*-acetyl)-β-D-glucopyranosyl-(1→2)[α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranoside**

White amorphous powder. ESI/MS (positive); 797.1, ESI/MS (negative);

795.2; $^{1}$H NMR (DMSO-$d_6$): δ 12.91 (1H, brs, 5-OH), 8.05 (2H, d, *J* = 9.0 Hz, H-2′/H-6′), 7.16 (2H, d, *J* = 9.0 Hz, H-3′/H-5′), 6.95 (1H, s, H-3), 6.75 (1H, d, *J* = 1.8 Hz, H-8), 6.48 (1H, d, *J* = 1.8 Hz, H-6), 3.87 (3H, s, 4′-$OCH_3$); β-Glc (primary): 5.23 (1H, d, *J* = 7.4 Hz, Glc H-1), 3.53 (1H, m, Glc H-2), 3.52 (1H, m, Glc H-3), 3.22 (1H, m, Glc H-4), 3.68 (1H, m, Glc H-5), 3.86 (1H, dd, *J* = 11.8, 1.9 Hz, Glc H-6a), 3.46 (1H, dd, *J* = 11.8, 6.3 Hz, Glc H-6b); α-Rha: 4.56 (1H, br d, *J* = 1.6 Hz, Rha H-1), 3.68 (1H, m, Rha H-2), 3.48 (1H, m, Rha H-3), 3.17 (1H, m, Rha H-4), 3.42 (1H, dd, *J* = 9.4, 6.3 Hz, Rha H-5), 1.08 (3H, d, *J* = 6.2 Hz, Rha H-6); β-Glc (terminal): 4.52 (1H, d, *J* = 7.8 Hz, t-Glc H-1), 3.02 (1H, br t, *J* = 8.5 Hz, t-Glc H-2), 3.22 (1H, m, t-Glc H-3), 3.08 (1H, m, t-Glc H-4), 3.42(1H, m, t-Glc H-5), 4.16 (1H, dd, *J* = 10.8, 1.9 Hz t-Glc H-6a), 4.00 (1H, dd, *J* = 10.8, 6.3 Hz, t-Glc, H-6b), 1.96 (3H, s, -$OCOCH_3$). $^{13}$C NMR (DMSO-$d_6$): δ 163.95 (C- 2), 103.84 (C-3), 182.06 (C-4), 170.31 (-$OCOCH_3$), 161.2 (C-5), 99.74 (C-6), 162.84 (C-7), 94.78 (C-8), 156.90 (C-9), 105.51 (C-10), 122.66 (C-1’), 128.40 (C-2′/C-6′), 114.73 (C-3′/C-5′), 162.50 (C-4′), 55.58 (4′-$OCH_3$), 20.57 (-$OCOCH_3$); β-Glc (primary); 98.23 (Glc C-1), 83.05 (Glc C-2), 75.58 (Glc C-3), 69.11 (Glc C-4), 75.44 (Glc C-5), 65.92 (Glc C-6); α-Rha: 100.50 (Rha C-1), 70.37 (Rha C-2), 70.75 (Rha C-3), 72.05 (Rha C-4),

68.35 (Rha C-5), 17.81 (Rha C-6); β-Glc (terminal); 104.75 (Glc C-1), 74.60 (Glc C-2), 75.89 (Glc C-3), 69.67 (Glc C-4), 73.72 (Glc C-5), 63.60 (Glc C-6).

**Acacetin-7-*O*-β-D-glucopyranosyl-(1→2)[α-L-rhamnopyranosyl]-(1→6)-β-D- glucopyranoside**

White amorphous powder. $^{1}$H NMR (DMSO-$d_6$): δ 8.05 (2H, d, $J$ = 9.0 Hz, H-2′/6′), 7.15 (2H, d, $J$ = 9.0 Hz, H-3′/5′), 6.95 (1H, s, H-3), 6.85 (1H, s, H-8), 6.51 (1H, s, H-6), 3.86 (3H, s, 4′-$OCH_3$); β-Glc (primary): 5.20 (1H, d, $J$ = 6.4 Hz, Glc H-1); α-Rha: 4.56 (1H, s, Rha H-1); β-Glc (terminal): 4.48 (1H, d, $J$ = 8.1 Hz, t-Glc H-1); $^{13}$C NMR (DMSO-$d_6$): δ 163.6 (C-2), 103.4 (C-3), 181.7 (C-4), 160.8 (C-5), 99.5 (C-6), 162.5 (C-7), 94.8 (C-8), 156.5 (C-9), 105.1 (C-10), 122.3 (C-1′), 128.1 (C-2′/C-6′), 114.4 (C-3′/C-5′), 162.1 (C-4′), 55.2 (4′-$OCH_3$); β-Glc (primary): 98.1 (Glc C-1), 82.3 (Glc C-2), 75.2 (Glc C-3), 68.9 (Glc C-4), 75.0 (Glc C-5), 65.6 (Glc C-6); α-Rha: 100.2 (Rha C-1), 70.0 (Rha C-2), 70.4 (Rha C-3), 71.7 (Rha C-4), 68.0 (Rha C-5), 17.5 (Rha C-6); β-Glc (terminal): 104.4 (t-Glc C-1), 74.4 (t-Glc C-2), 75.8 (t-Glc C-3), 69.3 (t-Glc C-4), 76.7 (t-Glc C-5), 60.3 (t-Glc C-6).

**Quercetin 3-*O*-robinibioside**

Yellow amorphous powder. $^{1}$H NMR (400 MHz, DMSO-$d_6$): δ 12.59 (1H, s, 5-OH), 10.88 (1H, brs, 7-OH), 9.82 (1brs, 4'-OH), 9.27 (1H, brs, 3-OH), 7.64 (1H, dd, *J* = 2.1, 8.4 Hz, H-6'), 7.52 (1H, d, *J* = 2.1 Hz, H-2'), 6.82 (1H, d, *J* = 8.5 Hz, H-5'), 6.39 (1H, d, *J* = 2.1 Hz, H-8), 6.19 (1H, d, *J* = 2.1 Hz, H-6), 5.32 (1H, d, *J* = 7.8 Hz, H-1"), 4.42 (1H, d, *J* = 1.3 Hz, H-1'"). $^{13}$C (100 MHz, DMSO-$d_6$): δ 177.4 (C-4), 164.2 (C-7), 161.2 (C-5), 156.4 (C-2), 148.4 (C-3'), 144.8 (C-4'), 133.5 (C-3), 121.9 (C-6'), 121.0 (C-1'), 115.9 (C-5'), 115.2 (C-2'), 103.9 (C-10), 102.0 (C-1"), 99.9 (C-1'"), 98.7 (C-6), 93.5 (C-8), 73.5 (C-5"), 73.0 (C-3"), 71.9 (C-4'"), 71.1 (C-2"), 70.6 (C-3'"), 70.4 (C-2'"), 68.3 (C-5'"), 68.0 (C-4"), 65.1 (C-6"), 17.9 (C-6'") (Rastrelli et al., 1995).

**Quercetin 3-*O*-β-D-galactoside-7-*O*-α-L-rhamnoside**

Yellow amorphous powder. $^{1}$H NMR (400 MHz, DMSO-$d_6$): δ 12.62 (1H, s, 5-OH), 9.79 (1brs, 4'-OH), 9.27 (1H, brs, 3'-OH), 7.70 (1H, dd, *J* = 2.1, 8.4 Hz, H-6'), 7.58 (1H, d, *J* = 2.1 Hz, H-2'), 6.82 (1H, d, *J* = 8.5 Hz, H-5'), 6.79 (1H, d, *J* = 2.1 Hz, H-6), 6.43 (1H, d, *J* = 2.1 Hz, H-8), 5.55 (1H, s, H-1'"), 5.40 (1H, d, *J* = 7.8 Hz, H-1"), 5.13~3.0 (17H, glucose & rhamnose),

1.12 (1H, d, *J* = 6.2 Hz, H-6'''). $^{13}$C (100 MHz, DMSO-$d_6$): δ 177.6 (C-4), 161.6 (C-7), 161.2 (C-5), 160.9 (C-5), 156.7 (C-9), 155.9 (C-2), 148.7 (C-4'), 144.9 (C-3'), 133.8 (C-3), 122.1 (C-1'), 121.1 (C-6'), 116.1 (C-5'), 15.2 (C-2'), 105.6 (C-10'), 101.6 (C-1'), 99.4 (1''), 98.4 (C-6), 94.3 (C-8), 75.9 (C-5''), 73.2 (C-3''), 71.6 (C-4'''), 71.2 (C-2''), 70.2 (C-3'''), 70.1 (C-2'''), 69.8 (C-5'''), 67.9 (C-4''), 60.2 (C 6''), 17.9 (C-6''') (Song et al., 2007).

**Quercetin 5-*O*-β-D-glucoside**

Yellow amorphous powder; $^{1}$H NMR (400 MHz, DMSO-$d_6$): δ: 10.95 (1H, brs, 7-OH), 9.42 (1H, brs, 4'-OH), 8.95 (1H, brs, 3-OH), 7.66 (1H, d, J = 2.0 Hz, H-2'),7.52 (1H, dd, *J* = 8.5 & 2.0 Hz, H-6'), 6.87 (1H, d, *J* = 8.5 Hz, H-5'), 6.76 (1H, d, *J* = 2.0 Hz, H-8), 6.64 (1H, d, *J* = 2.0 Hz, H-6), 4.78 (1H, d, *J* = 7.6 Hz, H-1''), 3.30-3.60 (4H, m, H-2'', H-3'', H-4'' & H-5''); $^{13}$C NMR (100 MHz, DMSO-$d_6$): δ 177.7 (C-4), 162.5 (C-7), 158.4 (C-5), 157.2 (C-9), 147.3 (C-4'), 145.1 (C-3'), 143.5 (C-2), 137.3 (C-3), 122.1 (C-1'), 119.5 (C-6'), 115.6 (C-5'), 114.7 (2'), 106.3 (C-10), 104.0 (C-1''), 103.2 (C-6), 97.3 (C-8), 77.5 (C-5''), 75.7 (C-3''), 73.7 (C-2''), 69.7 (C-4''), 60.8 (C 6'') (Tamuraa et al., 2002).

**Quercetin**

Yellow amorphous powder; $^{1}$H-NMR (400 MHz, DMSO-$d_6$): δ 12.48 (1H, br s, 5-OH), 7.68 (1H, d, *J* = 2.1 Hz, H-2'), 7.53 (1H, dd, *J* = 8.4, 2.1 Hz, H-6'), 6.88 (1H, d, *J* = 8.4, H-5'), 6.40 (1H, d, *J* = 2.1 Hz, H-8), 6.18 (1H, d, *J* = 2.1 Hz, H-6). $^{13}$C NMR (100 MHz, DMSO-$d_6$): δ 175.4 (C-4), 165.5 (C-7), 160.3 (C-5), 155.7 (C-9), 147.8 (C-4'), 146.8 (C-2), 145.0 (C-3'), 135.7 (C-3), 121.9 (C-1'), 119.9 (C-6'), 115.6 (C-5'), 115.0 (C-2'), 103.0 (C-10), 98.1 (C-6), 93.3 (C-8) (Lee et al., 2004).

**Arcapillin**

Yellow powder. $^{1}$H–NMR (DMSO-$d_6$, 400 MHz) δ: 12.90 (1H,br, s, -OH), 10.47 (1H, br, s, -OH), 10.06 (1H, br, s, -OH), 7.45 (1H, s, 6'-H), 7.10 (1H, s, 3-H), 6.94 (1H, s, 8-H), 6.56 (1H, s, 3'-H), 3.93 (3H, s, -$OCH_3$), 3.81 (3H, s, -$OCH_3$), 3.73 (3H, s, -$OCH_3$). $^{13}$C-NMR (DMSO-$d_6$, 100 MHz) δ: 182.2 (4-C), 161.9 (2-C), 158.4 (7-C), 153.2 (9-C), 152.6 (5-C), 152.0 (2' & 4'-C), 141.6 (5'-C), 131.6 (6-C), 111.9 (6'-C), 106.8 (3-C), 106.7 (1'-C), 104.8 (10-C), 104.4 (3'-C), 91.5 (8-C), 60.0 (-$OCH_3$), 56.8 (-$OCH_3$), 56.4 (-$OCH_3$) (Wang et al., 2007).

**Cirsilineol**

Yellow needle crystal. $^{1}$H –NMR (DMSO-$d_6$, 400 MHz) δ: 12.94 (1H,br, s, 5-OH), 10.00 (1H, br, s, 4'-OH), 7.60 (1H, dd, d, *J* = 2.4 & 8.2, 6'-H), 7.59 (1H, d, *J* = 2.4, 2'-H), 6.94 (1H, d, *J* = 8.2, 5'-H), 6.93 (1H, s, 8-H), 3.93 (3H, s, 7-$OCH_3$), 3.90 (3H, s, 5'-$OCH_3$), 3.73 (3H, s, 6-$OCH_3$). $^{13}$C-NMR (DMSO-$d_6$, 100 MHz) δ: 182.2 (4-C), 163.9 (2-C), 158.6 (7-C), 152.6 (9-C), 152.0 (5-C), 150.9 (4'-C), 148.0 (3'-C), 131.8 (6-C), 121.4 (1'-C), 120.4 (6'-C), 115.7 (5'-C), 110.2 (2'-C), 105.6 (10-C), 103.0 (3-C), 91.6, (8-C) 60.0 (6-$OCH_3$), 56.4 (5'-$OCH_3$), 56.0 (7-$OCH_3$) (Wang et al., 2007; Takahiko et al., 2006).

**Cirsimaritin**

Pale yellow fine needles $^{1}$H NMR: (DMSO-$d_6$, 400 MHz) δ: 12.93 (1H, s, 5-OH), 10.40 (1H, Br, s, 4'-OH), 7.96 (1H, d, *J* = 8.5 Hz, H-2' & H-6'), 6.93 (1H, d *J* = 8.5 Hz, H-3' & H-5'), 6.93 (1H, s, H-8), 6.85 (1H, s, H-3), 3.92 (3H, s, -$OCH_3$), 3.73 (3H, s, -$OCH_3$). $^{13}$C NMR: (DMSO-$d_6$, 100 MHz) δ: 182.2 (4-C), 164.0 (C-2), 161.3 (C-4'), 158.6 (C-7), 152.6 (C-1), 152.0 (C-5), 131.8 (C-6), 128.5 (C-2' & C-6'), 121.1 (C-1'), 115.9 (C-3' & C-5'), 105.0 (10-C), 102.6 (C-3), 91.5 (C-8), 60.0 (6-$OCH_3$), 56.4 (7-$OCH_3$)

(Takahiko et al., 2006).

**Capillarisin**

Colorless needles. EIMS *m/z*: 316 ($M^+$)$^1$H NMR (400 MHz, DMSO-$d_6$): δ 12.90 (1H, s, 5-OH), 9.76 (1H, brs, 4'-OH), 10.50 (1H, s, 7-OH), 7.17 (2H, d, *J* = 8.9 Hz, H-2' & H-6'), 6.88 (2H, d, *J* = 8.9 Hz, H-3' & H-5'), 6.45 (1H, s, H-8), 5.03 (1H, s, H-3), 3.73 (3H, s, -$OCH_3$), $^{13}$C (100 MHz, DMSO-$d_6$): δ 183.4 (C-4), 167.9 (C-2), 156.8 (C-7), 156.1 (C-4'), 152.9 (C-9), 149.8 (C-5), 143.1 (C-1'), 131.7 (C-6), 121.8 (C-2' & C-6'), 116.5 (C3' & C-5'), 102.3 (C-10), 94.1 (C-8), 86.6 (C-3), 59.9 (-$OCH_3$) (Komiya et al., 1976).

**7-Methyl capillarisin**

Colorless needles. EIMS *m/z*: 316 ($M^+$)$^1$H NMR (400 MHz, DMSO-$d_6$): δ 12.80 (1H, s, 5-OH), 9.76 (1H, brs, 4'-OH), 10.50 (1H, s, 7-OH), 7.19 (2H, d, *J* = 8.9 Hz, H-2' & H-6'), 6.87 (2H, d, *J* = 8.9 Hz, H-3' & H-5'), 6.79 (1H, s, H-8), 5.13 (1H, s, H-3), 3.89 (3H, s, -$OCH_3$), 3.71 (3H, s, -$OCH_3$). $^{13}$C (100 MHz, DMSO-$d_6$): δ 183.5 (C-4), 168.0 (C-2), 158.2 (C-7), 156.1 (C-4'), 152.2 (C-9), 150.1 (C-5), 143.1 (C-1'), 132.3 (C-6), 121.8 (C-2' & C-6'), 116.5 (C3' & C-5'), 103.3 (C-10), 91.4 (C-8), 87.2 (C-3), 60 (-$OCH_3$), 56.5 (-$OCH_3$) (Komiya et al., 1976).

**Scopoletin**

Colorless needles. $^{1}$H NMR (400 MHz, $CDCl_3$): δ 10.32 (1H, s, -OH), 7.90 (1H, d, *J* = 9.6 Hz, H-4), 7.28 (1H, s, H-5), 6.77 (1H, s, H-8), 6.21 (1H, d, *J* = 9.6 Hz, H-3), 3.81 (3H, s, $-OCH_3$). $^{13}$C (100 MHz, DMSO-$d_6$): δ 160.6 (C-2), 151.2 (C-7), 149.5 (C-9), 145.2 (C-6), 144.4 (C-4), 111.6 (C-3), 110.5 (C-10), 109.5 (C-5), 102.7 (C-8), 56.1 ($-OCH_3$) (Kim et al., 2006).

**Isoscopoletin**

Colorless needles. $^{1}$H NMR (400 MHz, DMSO-$d_6$): δ 7.61 (1H, d, *J* = 9.6 Hz, H-4), 6.97 (1H, s, H-8), 6.83 (1H, s, H-5), 6.30 (1H, d, *J* = 9.2 Hz, H-3), 3.98 (3H, s, $-OCH_3$). $^{13}$C (100 MHz, $CDCl_3$): δ 161.5 (C-2), 150.1 (C-7), 149.2 (C-9), 143.5 (C-6), 142.7 (C-4), 113.8 (C-3), 112.2 (C-5), 111.0 (C-10), 99.3 (C-8), 56.4 ($-OCH_3$) (Kim et al., 2006).

**Isoscopolin**

$^{1}$H NMR (400 MHz, DMSO-$d_6$): δ: 7.90 (1H, d, *J* = 9.5 Hz, H-4), 7.39 (1H, s, H-8), 7.09 (1H, s, H-5), 6.30 (1H, d, *J* = 9.5 Hz, H-3), 4.94 (1H, d, *J* = 7.6 Hz, H-1'), 3.86 (3H, s, $-OCH_3$), 3.15-3.60 (5H, m, H-2' ~ H-6'). $^{13}$C NMR (100 MHz, DMSO-$d_6$): 160.5 (C-2), 152.8 (C-7), 150.0 (C-9), 144.4

(C-6), 143.3 (C-4), 113.0 (C-3), 112.8 (C-5), 111.2 (C-10), 100.3 (C-1'), 77.1 (C-5'), 76.9 (C-3'), 73.1 (C-2'), 69.5 (C-4'), 60.6 (C-6'), 56.2 (-$OCH_3$).

**Scoparone**

$^1$H NMR (400 MHz, $CDCl_3$): δ 7.63 (1H, d, *J* = 9.6 Hz, H-4), 7.54 (2H, s, H-5), 7.03 (1H, s, H-8), 6.42 (1H, d, *J* = 9.6 Hz, H-3), 3.84 (3H, s, -$OCH_3$), 3.76 (3H, s, -$OCH_3$). $^{13}$C NMR (100 MHz, $CDCl_3$): δ 161.2 (C-2), 144.2 (C-6), 142.6 (C-7), 140.8 (C-4), 137.4 (C-9), 121.1 (C-10), 114.4 (C-4), 114.2 (C-8), 108.9 (C-5), 56.4 (-OCH3), 56.2 (-OCH3) (Lee et al., 2002).

**Scopolin**

Colorless prisms. $^1$H NMR (400 MHz, DMSO-$d_6$): δ 7.96 (1H, d, *J* = 9.4 Hz, H-4), 7.29 (1H, s, H-5), 7.16 (1H, s, H-8), 6.32 (1H, d, *J* = 9.4 Hz, H-3), 5.28 (1H, d, *J* = 5.2 Hz, H-1'), 3.82 (3H, s, -$OCH_3$), 3.16-3.73 (5H, m, H-2'~H-6'). $^{13}$C NMR (100 MHz, DMSO- $d_6$): δ 160.4 (C-2), 149.9 (C-7), 148.9 (C-9), 146.0 (C-6), 144.1 (C-4), 113.2 (C-3), 112.2 (C-10), 109.3 (C-5), 103.0 (C-8), 99.7 (C-1'),77.1 (C-5'), 76.7 (C-3'), 73.3 (C-2'), 69.6 (C-4'), 60.6 (C-6'), 56.0 (-$OCH_3$) (Lee et al., 2004).

**Esculetin**

$^{1}$H NMR (400 MHz, DMSO-$d_6$): δ 9.80 (1H, br s, -OH), 7.86 (1H, d, $J$ = 6.0 Hz, H-4), 6.97 (1H, s, H-5), 6.74 (1H, s, H-8), 6.16 (1H, d, $J$ = 6.0 Hz, H-3). $^{13}$C NMR (100 MHz, DMSO-$d_6$): δ 160.9 (C-2), 150.4 (C-6), 148.5 (C-7), 144.5 (C-4), 142.9 (C-9), 112.4 (C-5), 111.6 (C-3), 110.8 (C-10), 102.7 (C-8) (Wu et al., 2007).

**6-Methoxy artemicapin C**

White crystals ElMS $m/z$ [$M^+$]: 220 $^{1}$H NMR (400 MHz, DMSO-$d_6$): δ 7.93 (1H, d, $J$ = 9.6 Hz, H-4), 7.03 (1H, s, H-5), 6.32 (1H, d, $J$ = 9.6 Hz, H-3), 6.21 (2H, s, -$OCH_2O$-), 3.85 (3H, s, 6-$OCH_3$). $^{13}$C NMR (100 MHz, DMSO-$d_6$): δ 159.1 (C-2), 144.7 (C-4), 140.6 (C-7), 139.28 (C-6), 134.1 (C-8), 133.1 (C-9), 114.2 (C-10), 113.5 (C-3), 105.8 (C-5), 103.6 (-$OCH_2O$-), 56.4 (6-$OCH_3$) (Lee et al., 2003).

**Artemicapin A**

White crystals ElMS $m/z$ [$M^+$]: 220 $^{1}$H NMR (400 MHz, DMSO-$d_6$): δ 7.90 (1H, d, $J$ = 9.4 Hz, H-4), 6.22 (1H, d, $J$ = 9.8 Hz, H-3), 6.11 (2H, s, -$OCH_2O$-), 4.00 (3H, s, 8-$OCH_3$), 3.93 (3H, s, 5-$OCH_3$). $^{13}$C NMR (100 MHz,

DMSO-$d_6$): δ 159.6 (C-2), 143.5 (C-5), 142.6 (C-7), 139.1 (C-4), 134.1 (C-8), 133.0 (C-10), 130.6 (C-6), 113.2 (C-3), 108.6 (C-9), 103.6 (-$OCH_2O$-), 62.4 (5-$OCH_3$) 60.6 (8-$OCH_3$) (Wu et al., 2001).

**Fraxidin**

$^1$H NMR (400 MHz, DMSO-$d_6$): δ 7.61 (1H, d, *J* = 9.5 Hz, H-4), 6.28 (1H, d, *J* = 9.5 Hz, H-3), 6.67 (1H, s, C-5), 4.08 (3H, s, 6-$OCH_3$), 3.94 (3H, s, 7-$OCH_3$). $^{13}$C NMR (100 MHz, DMSO-$d_6$): δ 160.7 (C-2), 144.6 (C-7), 143.9 (C-4), 143.0 (C-9), 142.5 (C-8), 134.4 (C-6), 113.3 (C-3), 111.1 (C-10), 103.2 (C-5), 61.6 (6-$OCH_3$), 56.4 (7-$OCH_3$) (Olapeju and James, 2008).

**Tomerin**

$^1$H NMR (400 MHz, MeOH-$d_4$): δ 8.26 (1H, d, *J* = 9.5 Hz, H-4), 6.84 (1H, s, H-8), 6.21 (1H, d, *J* = 9.5 Hz, H-3), 5.06 (1H, d, *J* = 7.05 Hz, H-1'), 3.93 (3H, s, 7-$OCH_3$), 3.85 (3H, s, 6-$OCH_3$), 3.52-3.20 (4H, m, H-2", H-3", H-4", & H-5"). $^{13}$C NMR (100 MHz, MeOH-$d_4$): δ 163.4 (C-2), 158.8 (C-7), 152.7 (C-9), 147.4 (C-5), 142.2 (C-4), 139.9 (C-6), 112.8 (C-3), 109.8 (C-10), 105.6 (C-1), 97.8 (C-8), 78.4 (C-5'), 78.0 (C-3'), 75.5 (C-2'), 71.2 (C-4'),

62.3 (C-6'), 61.9 (6-$OCH_3$), 57.0 (7-$OCH_3$) (Masso, 1969).

**5,6,7-Trimethoxy coumarin**

Yellow oil. $^1$H NMR (400 MHz, DMSO-$d_6$): δ 7.94 (1H, d, $J$ = 9.5 Hz, H-4), 6.62 (1H, s, 8-H), 6.22 (1H, d, $J$ = 9.8 Hz, H-3), 4.04 (3H, s, 6-$OCH_3$), 3.93 (3H, s, 7-$OCH_3$), 3.87 (3H, s, 5-$OCH_3$). $^{13}$C NMR (100 MHz, DMSO-$d_6$): δ 161.2 (C-2), 157.1 (C-7), 151.4 (C-9), 149.2 (C-5), 138.0 (C-6), 112.4 (C-3), 107.1 (C-10), 95.4 (C-8), 61.7 (6-$OCH_3$), 61.1 (5-$OCH_3$), 56.3 (7-$OCH_3$) (Lee et al., 2003).

**Chlorogenic acid (3-caffeoylquinic acid)**

Slightly yellow amorphous powder, $^1$H NMR (400MHz, DMSO-$d_6$): δ 9.62 (1H, s, H-4'), 9.19 (1H, s, 3'-OH), 7.42 (1H, d, $J$ = 16.0 Hz, H-7'), 7.03 (1H, brs, H-2'), 6.98 (1H, dd, $J$ = 8.0, 2.0 Hz, H-6'), 6.76 (1H, d, $J$ = 8.0 Hz, H-5'), 6.15 (1H, d, $J$ = 16.0 Hz, H-8'), 5.06 (1H, ddd, $J$ = 10.0, 6.0 Hz, H-3), 3.92 (1H, brs, H-5), 3.42 (1H, brs, H-4), 2.03- 1.77 (4H, m, H-2/H-6). $^{13}$C NMR (100 MHz, DMSO-$d_6$): $\delta$ 175.0 (C-7), 165.8 (C-9'), 148.4 (C-4'), 145.6 (C-7'), 145.0 (C-3'), 125.6 (C-1'), 121.4 (C-6'), 115.8 (C-5'), 114.8 (C-

8'), 114.3 (C-2'), 73.5 (C-1), 70.9 (C-4), 70.3 (C-5), 68.0 (C-3), 37.2 (C-6), 36.2 (C-2) (Zhu et al., 2005).

**Cryptochlorogenic acid (4-caffeoylquinic acid)**

$^{1}$H NMR (400 MHz, $CD_3OD$): δ 7.64 (1H, d, *J* = 16.0 Hz, H-7'), 7.05 (1H, d, *J* = 2.1 Hz, H-2'), 6.97 (1H, dd, *J* = 8.0, 2.0 Hz, H-6'), 6.78 (1H, d, *J* = 8.0 Hz, H-5'), 6.38 (1H, d, *J* = 16.0 Hz, H-8'), 4.81 (1H, dd, *J* = 9.3, 3.1 Hz, H-4), 4.29 (1H, ddd, *J* = 3.5 Hz, H-5), 4.27 (1H, ddd, *J* = 3.5 Hz, H-3), 2.03-2.20 (4H, m, H-2/H-6). $^{13}$C NMR (100 MHz, $CD_3OD$): *δ* 177.4 (C-7), 169.0 (C-9'), 149.5 (C-4'), 147.1 (C-7'), 146.8 (C-3'), 127.8 (C-1'), 123.0 (C-6'), 116.5 (C-5'), 115.3 (C-8'), 115.2 (C-2'), 79.2 (C-4), 76.6 (C-1), 69.6 (C-5), 65.5 (C-3), 42.7 (C-2), 38.4 (C-6) (Zhu et al., 2005).

**Neochlorogenic acid (5-caffeoylquinic acid)**

$^{1}$H NMR (400 MHz, $CD_3OD$): δ 7.62 (1H, d, *J* = 16.0 Hz, H-7'), 7.05 (1H, d, *J* = 2.1 Hz, H-2'), 6.96 (1H, dd, *J* = 8.0, 2.0 Hz, H-6'), 6.77 (1H, d, *J* = 8.0 Hz, H-5'), 6.35 (1H, d, *J* = 16.0 Hz, H-8'), 5.34 (1H, ddd, *J* = 3.5 Hz, H-5), 4.29 (1H, ddd, *J* = 3.5 Hz, H-3), 4.13 (1H, dd, *J* = 3.5 & 7.6 Hz, H-3),

1.92-2.14 (4H, m, H-2/H-6). $^{13}$C NMR (100 MHz, $CD_3OD$): $\delta$ 181.6 (C-7), 169.0 (C-9'), 149.5 (C-4'), 147.0 (C-7'), 146.8 (C-3'), 127.9 (C-1'), 123.0 (C-6'), 116.5 (C-5'), 115.5 (C-8'), 115.1 (C-2'), 78.4 (C-1), 76.9 (C-4), 68.8 (C-5), 67.0 (C-3), 42.7 (C-2), 39.9 (C-6) (Zhu et al., 2005).

**4,5-Di-*O*-caffeoylquinic acid**

$^1$H NMR (400 MHz, $CD_3OD$): δ 7.57 and 7.50 (1H each, d, *J* = 15.9 Hz, H-7' & H-7"), 7.04 and 7.01 (1H each, d, *J* = 2.1 Hz, H-2' & H-2"), 6.92 and 6.87 (1H each, dd, *J* = 2.0 & 8.0 Hz, H-6' & H-6"),6.75 and 6.73 (1H each, d, *J* = 8.0 Hz, H-5' & H-5"), 6.29 and 6.24 (1H each, *J* = 16.0 Hz), 5.61 (1H, ddd, *J* = 4.5, 8.8, 10.8 Hz, H-5), 5.17 (1H, d, *J* = 3.2 & 8.8 Hz, H-4), 4.14 (1H, ddd, J = 3.2, 5.6, 6.4 Hz, H-3), 2.03–2.21 (4H, m, H-2 & H-6). $^{13}$C (100 MHz, $CD_3OD$): δ 181.9 (C-7), 168.4 (C-9'), 168.4 (C-9"), 149.6 (C-4'), 149.5 (C-4"), 147.4 (C-7'), 147.2 (C-7"), 146.8 (C-3'), 146.7 (C-3"), 127.8 (C-1'), 127.7 (C-1"), 123.2 (C-6'), 123.1 (6"), 116.5 (C-5'), 116.5 (C-5"),115.2 (C-2'), 115.1 (C-2"), 115.0 (C-8'), 114.9 (C-8"), 75.6 (C-1), 74.6 (C-4), 70.0 (C-3), 67.7 (C-5), 39.7 (C-6), 38.1 (C-2) (Zhu et al., 2005; Bashir, 2004).

**3,5-Di-*O*-caffeoylquinic acid**

$^{1}$H NMR (400 MHz, $CD_3OD$): δ 7.59 and 7.55 (1H each, d, *J* = 15.9 Hz, H-7' & H-7"), 7.05 and 7.04 (1H each, d, *J* = 2.1 Hz, H-2' & H-2"), 6.94 and 6.92 (1H each, d, *J* = 8.0 Hz, H-5' & H-5"), 6.77 and 6.74 (1H each, dd, *J* = 2.0 & 8.0 Hz, H-6' & H-6"), 6.39 and 6.25 (1H each, *J* = 16.0 Hz), 5.46 (1H, td, *J* = 9.0 & 7.0 Hz, H-3), 5.41 (1H, dt, *J* = 4.5 & 3.5 Hz, H-5), 3.94 (1H, dd, *J* = 9.0 & 6.4 Hz, H-4), 2.30 (1H, dd, *J* = 15.0 & 4.0 Hz, Hax-6), 2.18 (2H, m, H-2), 2.12 (1H, dd, *J* = 15.0 & 5.5, Hz, Heq-6). $^{13}$C (100 MHz, $CD_3OD$): δ 180.1 (C-7), 169.1 (C-1'), 168.8 (C-1"), 149.5 (C-7'), 149.4 (C-7"), 147.1 (C-3'), 147.0 (C-3"), 146.7 (C-6'), 146.7 (C-6"), 128.0 (C-4'), 127.8 (C-4"), 123.0 (C-9'), 123.0 (9"), 116.5 (C-5'), 116.5 (C-5"), 115.2 (C-8'), 115.8 (C-8"), 115.8 (C-2'), 115.4 (C-2"), 73.7 (C-1), 72.2 (C-4), 72.2 (C-5), 72.7 (C-5), 39.6 (C-2), 37.1 (C-6) (Zhu et al., 2005; Bashir, 2004).

**3,4-Di-*O*-caffeoylquinic acid**

$^{1}$H NMR (400 MHz, DMSO-$d_6$): δ 7.44 and 7.41 (1H each, d, *J* = 15.9 Hz, H-7' & H-7"), 7.00 and 7.00 (1H ea ch, d, *J* = 2.1 Hz, H-2' & H-2"), 6.94 and 6.92 (1H each, dd, *J* = 2.0 & 8.0 Hz, H-6' & H-6"), 6.7 and 6.69 (1H each, d, *J* = 8.0 Hz, H-5' & H-5"), 6.20 and 6.15 (1H each, *J* = 16.0 Hz),

5.52 (1H, td, *J* = 10.0 & 5.0 Hz, H-3), 4.93 (1H, dt, *J* = 4.5 & 3.5 Hz, H-4), 4.91 (1H, dd, *J* = 9.0 & 6.4 Hz, H-5), 2.03–2.21 (4H, m, H-2 & H-6).. $^{13}$C (100 MHz, $CD_3OD$): δ 176.0 (C-7), 166.2 (C-1'), 166.0 (C-1"), 148.6 (C-7'), 148.6 (C-7"), 145.6 (C-3'), 145.6 (C-3"), 145.5 (C-6'), 145.5 (C-6"), 125.3 (C-4'), 125.3 (C-4"), 121.3 (C-9'), 121.3 (9"), 115.8 (C-8'), 115.7 (C-8"), 114.8 (C-5'), 114.8 (C-5"), 113.7 (C-2'), 113.7 (C-2"), 75.8 (C-1), 68.7 (C-4), 68.1 (C-3), 67.7 (C-5), 37.8 (C-6), 37.8 (C-2) (Zhu et al., 2005; Bashir, 2004).

**Eugenyl glucoside**

$^1$H NMR (400 MHz, DMSO-$d_6$): δ 7.00 (1H, d, *J* = 8.2 Hz, H-6), 6.79 (1H, d, *J* = 2.05 Hz, H-3), 6.66 (1H, dd, *J* = 8.1 & 2.0), 5.93 (1H, ddt, *J* = 16.9, 10 & 6.8 Hz, H-8), 5.06 (1H, dd, *J* = 16.9 & 2.0 Hz, H-9), 4.82 (1H, d, *J* = 7.5 Hz, H-1'), 3.73 (3H, s, -$OCH_3$) 3.20-3.60 (4H, m, H-2", H-3", H-4" & H-5"). $^{13}$C NMR (100 MHz, DMSO-$d_6$): δ 148.9 (C-2), 144.9 (C-1), 137.9 (C-8), 133.5 (C-4), 120.3 (C-5), 115.6 (C-6), 115.5 (C-9), 112.2 (C-3), 100.2 (C-1'), 77.0 (C-3'), 76.9 (C-5'), 73.2 (C-2'), 69.7 (C-4'), 60.7 (C-6'), 55.6 (-$OCH_3$), 40.5 (C-7) (Takeda et al., 1998).

**4-Hydroxy-3-*O*-β-D-glucopyranosyl benzoic acid**

$^{1}$H NMR (400 MHz, MeOH-$d_4$): δ 7.59 (1H, d, $J$ = 3.1 Hz, H-2), 7.09 (1H, dd, $J$ = 3.1 & 8.9 Hz, H-6), 6.72 (1H, d, $J$ = 9.3 Hz, H-5), 4.77 (1H, d, $J$ = 7.22 Hz, H-1'), 3.30 ~ 3.93 (5H, m, H-2' ~ H-6'). $^{13}$C NMR (100 MHz, MeOH-$d_4$): δ 175.6 (-COOH), 158.1 (C-4), 150.9 (C-3), 123.8 (C-6), 119.6 (C-2), 117.5 (C-5), 103.8 (C-1'), 78.0 (C-5'), 77.9 (C-3'), 75.0 (C-2'), 71.4 (C-4'), 62.5 (C-6') (Rainer et al., 2000).

***p*-Methoxy phenyl glucoside**

$^{1}$H NMR (400 MHz, DMSO-$d_6$): δ 8.06 (2H, d, $J$ = 8.9 Hz, H-2 & H-6), 7.13 (2H, d, $J$ = 8.9 Hz, H-3 & H-5), 5.06 (1H, d, $J$ = 7.6 Hz, H-1'), 3.86 (3H, s, -$OCH_3$), 3.20-3.70 (5H, m, H-2' ~ H-6'). $^{13}$C NMR (100 MHz, DMSO-$d_6$): δ 163.0 (C-1), 162.4 (C-4), 128.4 (C-2 & C-6), 114.6 (C-3 & C-5), 99.9 (C-1'), 77.2 (C-5'), 76.4 (C-3'), 73.1 (C-2'), 69.5 (C-4'), 60.6 (C-6'), 55.6 (-$OCH_3$).

**Koaburaside**

$^{1}$H NMR (400 MHz, DMSO-$d_6$): δ 6.37 (2H, s, H-2 & H-6), 3.71 (6H, s, 2X-$OCH_3$). $^{13}$C NMR (100 MHz, DMSO-$d_6$): δ 150.7 (C-4), 147.8 (C-3 &

C-5), 130.3 (C-1), 101.6 (C-1'), 95.0 (C-2 & C-6), 77.2 (C-5'), 76.8 (C-3'), 73.3 (C-2'), 69.9 (C-4'), 60.6 (C-6'), 55.8 (2X-$OCH_3$) (Chen et al., 2012).

## 3-2. Potent α-glucosidase and protein tyrosine phosphatase 1B inhibitors from *Artemisia capillaris*.

### 2-2-1. α-Glucosidase inhibitory activity of the selected *Artemisia* species

In order to evaluate the a-glucosidase inhibitory activities of *Artemisia* species, the MeOH extracts of 12 different species of the Genus were selected and tested using the *in vitro* α glucosidase inhibitory assay. The α-glucosidase inhibitory activities of the selected species are summarized in the Table 1. As shown in the Table, all species showed concentration dependent a-glucosidase inhibitory activities ranging from 64.67 to 34.12 % at a concentration of 250 µg/mL. Of all these species, *A. capillaris* was found to be the most potent α-glucosidase inhibitor with inhibition percentages (%) of 64.67 ± 1.81 at a concentration of 250 µg/mL, which was significantly higher than the positive control acarbose (54.85 ± 0.76 %) at the same concentration. In addition, *A. apacea* also showed stronger inhibitory activity (57.57 ± 0.48 %) than acarbose, while *A. iyawomogi* showed similar inhibitory (54.24 ± 1.70 %) activity to acarbose. On the other hand, *A. montana*, *A. princeps*, *A. rubripes*, *A. japonica*, *A. argyi*, and *A. sylvatica* exhibited only a moderate inhibitory potential with 46.93 ± 0.88,

46.24 ± 1.96, 46.01 ± 2.00, 43.65 ± 2.29, 42.93 ± 0.31, and 41.04 ± 1.95 %, respectively.

### 3-2-2. PTP1B inhibitory activity of the selected *Artemisia* species

The inhibitory potential of the selected *Artemisia* species against PTP1B was also evaluated and the results are presented in the Table 1. The MeOH extracts of all the selected species showed concentration-dependent PTP1B inhibitory activity ranging from 98.60 to 27.40 % at a concentration of 10 µg/mL. Among the selected Artemisia species, *A. argyi, A. montana, A. stolonifera, and A. princeps* displayed significant PTP1B inhibitory activity with inhibition % of 98.60 ± 0.14, 98.58 ± 0.48, 95.23 ± 2.45, and 91.29 ± 5.42, respectively. The inhibitory activity of the positive control ursolic acid was 97.80 ± 0.74 % at the same concentration used in this assay. On the other hand, *A. sieversiana, A. apiaceae, A. rubripes, A. iwayomogi, A. capillaris, and A. japonica* showed only a moderate PTP1B inhibitory activity with inhibition % of 86.06 ± 1.80, 84.36 ± 0.51, 75.45 ± 0.93, 71.98 ± 3.14, 71.17 ± 0.49, and 66.86 ± 3.14, respectively, at a concentration of 10 µg/mL.

**Table 1.** The inhibitory activity against α-glucosidase and PTP1B by the selected *Artemisia* species

| Sample | % Inhibition ± SEM | |
|---|---|---|
| | α-Glucosidase[a] | PTP1B[b] |
| *Artemisia capillaris* | 64.67 ± 1.81 | 71.71 ± 0.49 |
| *Artemisia rubripes* | 46.01 ± 2.00 | 75.45 ± 0.93 |
| *Artemisia stolonifera* | 27.67 ± 1.41 | 95.23 ± 2.45 |
| *Artemisia iwayomogi* | 54.24 ± 1.70 | 71.98 ± 3.14 |
| *Artemisia princeps* | 46.24 ± 1.96 | 91.29 ± 5.42 |
| *Artemisia apeaceae* | 57.57 ± 0.48 | 84.36 ± 0.51 |
| *Artemisia montana* | 46.93 ± 0.88 | 98.58 ± 0.47 |
| *Artemisia sylvatica* | 41.04 ± 1.95 | 56.86 ± 3.44 |
| *Artemisia sieversiana* | 34.12 ± 0.28 | 86.06 ± 1.80 |
| *Artemisia japonica* | 43.65 ± 2.99 | 66.86 ± 3.14 |
| *Artemisia annua* | 38.47 ± 1.16 | 27.40 ± 4.43 |
| *Artemisia argyi* | 42.93 ± 0.31 | 98.60 ± 0.14 |
| Acarbose[c] | 54.85 ± 0.54 | - |
| Ursolic acid[d] | - | 97.80 ± 0.74 |

[a] Tested at a concentration of 250 μg/mL.

[b]Tested at a concentration of of 10 μg/mL.

[c,d]Used as positive controls in each assay.

### 3-2-3. α-Glucosidase and PTP1B inhibitory activity of the MeOH extract as well as its different solvent soluble fractions of *A. capillaris*

Since *A. capillaris* showed the highest α-glucosidase inhibitory activity together with significant PTP1B inhibitory activity, it was selected for further investigation. The MeOH extract of *A. capillaris* was dissolved in $H_2O$ successively partitioned with $CH_2Cl_2$, EtOAc, *n*-BuOH to obtain different solvent-soluble fractions. The α-glucosidase inhibitory activity of the individual fractions of *A. capillaris* was then evaluated. As shown in Table 2, the EtOAc fraction showed the highest α-glucosidase inhibitory activity with an $IC_{50}$ value of 63.92 ± 2.51 µg/mL followed by the $CH_2Cl_2$ fraction with an $IC_{50}$ value of 83.05 ± 1.47 µg/mL. The positive control acarbose, had an $IC_{50}$ value of 155.39 ± 2.05 µg/mL. In particular, both EtOAc fraction and $CH_2Cl_2$ fraction showed stronger inhibitory potential when compared to acarbose, which is a well-known α-glucosidase inhibitor that is in clinical use. On the other hand, the *n*-BuOH fraction showed a moderate α-glucosidase inhibitory potential with an $IC_{50}$ value of 294.35 ± 1.63 µg/mL when compared to both EtOAc and $CH_2Cl_2$ fractions. However, the $H_2O$ fraction did not exhibit any inhibitory activity at the test concentrations.

### 3-2-4. PTP1B inhibitory activity of the MeOH extract as well as its different solvent soluble fractions of *A. capillaris*

The PTP1B inhibitory activity of the extract as well as different solvent soluble fractions of *A. capillaris* was also evaluated and the results are summarized in Table 2. Similar to the α-glucosidase inhibitory assay, the EtOAc fraction exhibited the most potent PTP1B inhibitory potential with an $IC_{50}$ value of 8.66 ± 0.24 µg/mL compared to the positive control ursolic acid with an $IC_{50}$ value of 2.57 ± 0.71 µg/mL. In addition, both $CH_2Cl_2$ and *n*-BuOH fractions also showed promising PTP1B inhibitory potential with $IC_{50}$ values of 40.95 ± 1.16 and 66.24 ± 0.98 µg/mL, respectively. However, the $H_2O$ fraction was inactive as was observed in the α-glucosidase inhibitory assay.

**Table 2.** The inhibitory activities against α-glucosidase and PTP1B by the MeOH extract as well as different solvent soluble fractions of *A. capillaris*

| Sample | $IC_{50}$ values (μg/mL) ± SEM | |
|---|---|---|
| | α-Glucosidase | PTP1B |
| MeOH Extract | 109.23 ± 0.55 | 18.20 ± 0.24 |
| $CH_2Cl_2$ Fr. | 83.05 ± 1.47 | 42.24 ± 2.11 |
| EtOAc Fr. | 63.92 ± 2.51 | 8.66 ± 0.17 |
| *n*-BuOH Fr. | 294.35 ± 1.63 | 66.24 ± 0.69 |
| $H_2O$ Fr. | NA | NA |
| Acarbose[a] | 155.39 ± 2.05 | - |
| Ursolic acid[b] | - | 2.57 ± 0.50 |

NA: No activity at tested concentrations.

[a,b]Used as positive controls in each assay.

### 3-2-5. α-Glucosidase inhibitory activities of coumarins, flavonoids, and phenolic compounds isolated from *A. capillaris*

In order to determine and identify the active compounds responsible for the potent anti-diabetic activity of *A. capillaris*, the α-glucosidase inhibitory activities of the isolated compounds were evaluated. The results of the enzyme inhibitory activities of coumarins, flavonoids, and phenolic compounds are summarized in Tables 3, 4, and 5, respectively. Among the isolated compounds, quercetin and esculetin showed the highest a-glucosidase inhibitory activity with $IC_{50}$ values of 58.93 ± 6.69 and 82.92 ± 4.81 μM, respectively. The $IC_{50}$ value of the positive control acarbose was 130.52 ± 10.01 μM. In addition, hyperoside, isorhamnetin, and scopoletin exhibited moderate inhibitory activity against α-glucosidase with $IC_{50}$values of 130.77 ± 13.53, 141.17 ± 12.26, and 159.16 ± 11.71 μM, respectively. Interestingly, all isolated phenolic acids showed a relatively higher α-glucosidase inhibitory activity compared with coumarins and flavonoids. In particular, 3,5-dicaffeoylquinic acid methyl ester, 3,4-dicaffeoylquinic acid, and 1,5-dicaffeoylquinic acid exhibited significant α-glucosidase inhibitory activity with $IC_{50}$ values of 86.92 ± 4.81, 128.07 ± 1.67, and 146.06 ± 0.07 μM, respectively, whereas the $IC_{50}$ value of acarbose was 187.34 ± 3.31 lM.

In addition, 3,5-dicaffeoylquinic acid and 4,5-dicaffeoylquinic acid showed moderate α-glucosidase inhibitory activity with $IC_{50}$ values of 217.40 ± 5.45 and 229.94 ± 1.32 μM, respectively. Among the test compounds, cirsilineol, daphnetin, 6-methoxy artemicapin C, and umbelliferone displayed only a marginal inhibitory activity against a-glucosidase with $IC_{50}$ values of 351.71 ± 9.72, 560.20 ± 19.60, 563.75 ± 6.55, and 633.94 ± 23.78 μM, respectively. On the other hand, five coumarins (esculin, scopolin, 7-methoxy coumarin, isoscopolin, scoparone), four flavonoids (arcapillin, isorhamnetin 3-robinobioside, linarin, isorhamnetin 3-glucoside), one phenolic compound (3-caffeoylquinic acid), and one chromone (capillarisin) did not show inhibitory activity at the concentration tested.

### 3-2-6. PTP1B inhibitory activities of coumarins, flavonoids, and phenolic compounds isolated from *A. capillaris*

In order to determine and identify the active compounds from A. capillaris that were responsible for PTP1B inhibitory activity, the PTP1B inhibitory activity of coumarins, flavonoids, and phenolic compounds were evaluated and summarized in Tables 3, 4, and 5, respectively. Among the test compounds, all dicaffeoylquinic acid derivatives exhibited promising

PTP1B inhibitory activity. As shown in Table 5, 3,5-dicaffeoylquinic acid, 3,4-dicaffeoylquinic acid, 3,5-dicaffeoylquinic acid methyl ester, and 4,5-dicaffeoylquinic acid were found to be potent PTP1B inhibitors with $IC_{50}$ values of 2.02 ± 0.46, 2.60 ± 0.24, 2.99 ± 0.42, and 3.21 ± 0.23 μM, respectively. The $IC_{50}$ value of the positive control ursolic acid was 3.10 ± 0.05 μM. In addition, 1,5-dicaffeoylquinic acid and 3-caffeoylquinic acid also showed moderate PTP1B inhibitory activity with $IC_{50}$ values of 16.05 ± 1.45 and 17.09 ± 2.71 μM, respectively. Among the test coumarins, esculetin showed good PTP1B inhibitory potential followed by 6-methoxy artemicapin C with $IC_{50}$ values of 10.08 ± 1.93 and 27.61 ± 8.30 μM, respectively, whereas the $IC_{50}$ value of ursolic acid was 4.06 ± 0.20 μM. Among the test flavonoids, quercetin, isorhamnetin, and arcapillin also showed moderate PTP1B inhibitory activity with $IC_{50}$ values of 20.35 ± 7.56, 43.88 ± 2.26, and 86.67 ± 4.12 μM, respectively. In addition, daphnetin, scopoletin, umbelliferone, capillarisin, and cirsilineol exhibited marginal PTP1B inhibitory activity with $IC_{50}$ values ranging from 164.50 to 274.86 μM. Similar to the results observed in the α-glucosidase inhibitory assay, five inactive coumarins (esculin, scopolin, 7-methoxy coumarin, isoscopolin, scoparone) did not show PTP1B inhibitory activity at the

concentration tested. Also, four flavonoids (hyperoside, isorhamnetin 3-robinobioside, linarin, isorhamnetin 3-glucoside) showed no inhibitory activity at the concentration tested.

**Table 3.** The inhibitory activity against α-glucosidase and PTP1B by the flavonoid derivatives isolated from *A. capillaris*

| Sample | $IC_{50}$ values (μM) ± SEM | |
|---|---|---|
| | α-Glucosidase | PTP1B |
| Quercetin | 58.93 ± 6.69 | 20.35 ± 7.56 |
| Isorhamnetin | 141.17 ± 12.26 | 43.88 ± 2.26 |
| Arcapillin | NA | 86.67 ± 4.12 |
| Cirsilineol | 86.67 ± 4.12 | 206.78 ± 58.12 |
| Capillarisin | NA | 207.69 ± 2.09 |
| Hyperoside | 130.77 ± 13.53 | NA |
| Linarin | NA | NA |
| Isorhamnetin 3-*O*-robinobiside | NA | NA |
| Isorhamnetin 3-*O*-glucoside | NA | NA |
| Acarbose[a] | 130.52 ± 10.01 | - |
| Ursolic acid[b] | - | 4.06 ± 0.20 |

NA: No activity at tested concentrations.

[a,b] Used as positive controls in each assay.

**Table 4.** The inhibitory activities against α-glucosidase and PTP1B by the coumarin derivatives isolated from *A. capillaris*

| Sample | $IC_{50}$ values (μM) ± SEM | |
|---|---|---|
| | α-Glucosidase | PTP1B |
| Esculetin | 82.92 ± 4.81 | 10.08 ± 1.93 |
| 6-Methoxy artemicapin C | 563.75 ± 6.55 | 27.61 ± 8.30 |
| Daphnetin | 560.20 ± 19.60 | 164.50 ± 7.44 |
| Umbelliferone | 633.94 ± 23.78 | 274.86 ± 20.27 |
| Scopoletin | 159.16 ± 11.71 | 227.28 ± 26.71 |
| Esculin | NA | NA |
| Scopolin | NA | NA |
| Isoscopolin | NA | NA |
| Scoparone | NA | NA |
| Acarbose[a] | 130.52 ± 10.01 | - |
| Ursolic acid[b] | - | 4.06 ± 0.20 |

NA: No activity at tested concentrations.

[a,b] Used as positive controls in each assay.

**Table 5.** The inhibitory activities against α-glucosidase and PTP1B by the phenolic compounds isolated from *A. capillaris*

| Sample | $IC_{50}$ values (μM) ± SEM | |
|---|---|---|
| | α-Glucosidase | PTP1B |
| 1,5-Dicaffeoylquinic acid | 146.06 ± 0.07 | 16.05 ± 1.45 |
| 3,4-Dicaffeoylquinic acid | 128.07 ± 1.67 | 2.60 ± 0.24 |
| 3,5-Dicaffeoylquinic acid | 217.40 ± 5.45 | 2.02 ± 0.46 |
| 3,5-Dicaffeoylquinic acid methyl ester | 86.95 ± 4.10 | 2.99 ± 0.42 |
| 4,5-Dicaffeoylquinic acid | 229.94 ± 1.32 | 3.21 ± 0.23 |
| 3-Caffeoylquinic acid | NA | 17.09 ± 2.71 |
| Acarbose[a] | 187.34 ± 3.31 | - |
| Ursolic acid[b] | | 3.10 ± 0.05 |

NA: No activity at tested concentrations.

[a,b] Used as positive controls in each assay.

### 3-2-7. Discussion

Despite the advancements in biomedical science and the introduction of new treatment strategies, diabetes remains a major cause of new-onset blindness, end-stage renal disease, and cardiovascular diseases, all of which contribute to the excess morbidity and mortality in people with diabetes (WHO, 2008; Centers For Disease Control and Prevention, National Center for Health Statistics, 2010). Postprandial hyperglycemia, which results from abnormal insulin secretion by β-cells in response to eating, has been shown to impair hepatic glucose production, and defective glucose uptake by peripheral insulin sensitive tissues, particularly the skeletal muscle, is closely associated with the development of type 2 diabetes mellitus (Panunti et al., 2004). Thus, a therapeutic strategy focusing on suppressing postprandial hyperglycemia and improving insulin signaling could be a valuable treatment strategy for the not only the treatment of diabetic patients but also individuals with impaired glucose tolerance. Among the available therapeutic approaches, α-glucosidase and PTP1B inhibitors have emerged as potential therapeutic targets for the treatment of type 2 diabetes mellitus.

α-Glucosidase is a membrane-bound enzyme at the epithelium of the small intestine, which hydrolyses the cleavage of glucose from

disaccharides and oligosaccharides and thereby facilitates their absorption in the small intestine. Hence, the inhibitors of α-glucosidase delay carbohydrate digestion, prolong the overall carbohydrate digestion time, and thus cause a reduction in the rate of glucose absorption and lower the postprandial rise in blood glucose (Standl and Schnell, 2012). In particular, α-glucosidase inhibitors such as acarbose, miglitol and voglibose are most commonly used oral agents in order to ameliorate postprandial hyperglycemia. Although these drugs showed great prospect of glycemic control without the threat of hypoglycemia, hyperinsulinemia, and body weight gain, they have been reported to have side effects such as flatulence, diarrhea, abdominal distension, and meteorism (Kim et al., 2000; Standl and Schnell, 2012). On the other hand, PTP1B plays a critical role in regulating glucose homeostasis and body weight by acting as a key negative regulator of the insulin and leptin signaling pathway, respectively (Johnson et al., 2002). Insulin resistance is evident in many tissues that are important for glucose homeostasis, including muscle, liver and, more recently, in fat and at the level of the central nervous system. Metabolic insulin signal transduction occurs through activation of the insulin receptor, including autophosphorylation of tyrosine residues in the insulin receptor activation

loop (Saltiel and Pessin, 2002). It has been hypothesized that a specific protein tyrosine phosphatase (PTP) is involved in the dephosphorylation and inactivation of the insulin receptor, which attenuates insulin signaling, and disequilibrium among the insulin receptor and PTPs could be a contributing factor to the insulin resistance observed in type 2 diabetes mellitus (Tonks and Neal, 2001; Wu et al., 2003).

Apart from the currently available anti-diabetic drugs, several herbal medicines have also been recommended for the treatment of diabetes since they have fewer side effects compared to the modern therapeutic drugs. Many of them are used in traditional systems of medicines for hundreds of years in many countries (Ayodhya et al., 2010). A large number of studies have examined the anti-diabetic activity of different plants as well as a wide array of active compounds found in plants, including alkaloids, glycosides, terpenes, flavonoids, etc.; however, the main hurdle that must be overcome is the further development of such ‘leads’ into clinically useful medicines and especially phytomedicines or adequate nutritional supplements, which would be of direct benefit to the patients (Adolfo and Michael, 2005).

The genus *Artemisia*, is one of the largest and most widely distributed genera of the Asteraceae family. Some species including *A. montana*, *A.*

*santonicum*, *A. siebery*, *A. pallens*, *A. dracunculus*, and *A. herba-alba* have been used in traditional medicine for the treatment of diabetes and related complications (Ribnickya et al., 2006; Kordali et al., 2005; Jung et al., 2011). Despite the promising potential of the *Artemisia* species in the treatment of diabetes and related complications, no study has systematically evaluated the α-glucosidase and PTP1B inhibitory activity of the *Artemisia* species. Therefore, as a part of our continuous effort to identify potent anti-diabetic compounds from the natural sources, we investigated the inhibitory activity of some selected species of the genus *Artemisia* against α-glucosidase and PTP1B. Since *A. capillaris* exhibited the highest α-glucosidase inhibitory and PTP1B inhibitory activity, this plant was selected for bioactivity-guided fractionation and isolation in an attempt to isolate potent α-glucosidase and PTP1B inhibitors. Repeated chromatography yielded 45 compounds, including coumarins, flavonoids, and phenolic compounds.

In order to determine the structure–activity relationship, we further evaluated the inhibitory effects of all the compounds isolated from *A. capillaris* against α-glucosidase and PTP1B. Among the test coumarins, esculetin, scopoletin, daphnetin, 6-methoxy artemicapin C, and

umbelliferone exhibited α-glucosidase inhibitory activity. In particular, the inhibitory potentials of quercetin and esculetin against α-glucosidase were approximately 2.2-fold and 1.5-fold stronger than the clinically used drug acarbose, respectively. A comparison of the inhibitory potential of different coumarin derivatives indicated that the free hydroxyl groups at the 6 and 7 positions might play an important role in the α-glucosidase inhibitory activity (Table 4). Esculetin, which contains a free hydroxyl group at the 6 and 7 positions, showed significant inhibitory activity, while glycosylation at the 6 position drastically reduced the inhibitory activity as was observed for esculin. On the other hand, scopoletin, which is methoxylated at the 6 position and a free hydroxyl group at the 7 position, still retained inhibitory activity, although the activity was reduced compared to esculetin. Substitution at both the 6 and 7 positions with methoxyls or glycosyl groups diminished the inhibitory potential as evidenced by scoparone, scopolin, and isoscopolin. Umbelliferone, which has a free hydroxyl group at the 6 position showed marginal inhibitory potential, while methoxylation (7-methoxy coumarin) diminished the activity. Daphnetin, which has a free hydroxyl group at both the 7 and 8 positions, displayed marginal inhibitory activity but this activity was much lower compared with esculetin. By

comparing the structure and activity of 6-methoxy artemicapin C, scopoletin, and daphnetin, the free hydroxyl at the 7 position on coumarin skeleton was found to be very important to the inhibitory activity.

Among the flavonoids, only quercetin, hyperoside, isorhamnetin, and cirsilineol showed inhibitory activity against α-glucosidase. The number and positions of the hydroxyl group at the A, B, and C rings of the flavonoids plays a critical role in the inhibition of α-glucosidase (Table 3). Quercetin, which contains a free hydroxyl group at the 3 position of the C ring, exhibited the highest α-glucosidase inhibitory potential, while hyperoside with a galactosyl group at the 3 position exhibited an approximately 2.2-fold weaker activity than quercetin. Therefore, the free hydroxyl group at the 3 position of the C ring was very important for the α-glucosidase inhibitory activity. The reduced inhibitory activity of isorhamnetin clearly demonstrated that the free hydroxyl groups at the 3' and 4' positions of the B ring can play a critical role in the inhibition of this enzyme. In addition, the additional glycosylation at the 3 position abolished the inhibitory potential since isorhamnetin 3-*O*-glucoside and isorhamnetin 3-*O*-robinobioside showed no activity. Cirsilineol exhibited weaker inhibitory potential than isorhamnetin. This result implies that the methoxylation at the 7 position

may be associated with decreased activity. On the other hand, the free hydroxyl group at the 2' position also reduced the inhibitory activity as observed with arcapillin. In addition, glycosylation at the 7 position and methoxylation at the 4' position (linarin) diminished the inhibitory potential. Therefore, the presence of the hydroxyl groups at the 3, 7, 3', and 4' positions of the flavonoids largely affected the inhibitory potential against α-glucosidase. Our results are also in agreement with previously reported data (Tadera et al., 2006).

Test phenolic acids, including 3,5-dicaffeoylquinic acid methyl ester, 3,4-dicaffeoylquinic acid, and 1,5-dicaffeoylquinic acid, seemed to be potent α-glucosidase inhibitors when compared to coumarins, flavonoids, and the positive control acarbose (Table 5) (Kumar et al., 2011). Although Gao et al. reported that 3,4-dicaffeoylquinic acid, 3,5-dicaffeoylquinic acid, and 4,5- dicaffeoylquinic acid showed moderate inhibitory activity, the other dicaffeoylquinic acids, including 1,5-dicaffeoylquinic acid and 3,5-dicaffeoylquinic acid methyl ester were also shown to display α-glucosidase inhibitory activity (Gao et al., 2008). Considering the structure–activity relationship between 3,5-dicaffeoylquinic acid methyl ester and 3,5-dicaffeoylquinic acid, the presence of the methyl ester bridge at the

carboxylic acid might enhance the inhibitory activity against α-glucosidase.

Regarding the PTP1B inhibitory activity, all isolated dicaffeoylquinic acids showed significant inhibitory activity (Table 5). In particular, the activities of 3,4-dicaffeoylquinic acid, 3,5-dicaffeoylquinic acid, 3,5-dicaffeoylquinic acid methyl ester, and 4,5-dicaffeoylquinic acid were as good as the positive control ursolic acid. When considering these results, the presence of the caffeoyl group at the 3 position of the quinic acid moiety might be attributed at least in part to the PTP1B inhibitory activity and the additional caffeoyl group on quinic acid might enhance the inhibitory activity.

Among the 10 coumarins isolated from *A. capillaris* only 6 coumarins was found to be PTP1B inhibitors and the order of activity was as follows: escultein > 6-methoxy artemicapin C > daphnetin > scopoletin > umbelliferone. In particular, esculetin and 6-methoxy artemicapin C showed good PTP1B inhibitory potential. Esculetin, which has a free hydroxyl group at both the 6 and 7 positions, exhibited the highest inhibitory potential against PTP1B, while methoxylation at both positions (scoparone); methoxylation at the 6 position and glycosylation at the 7 position (scopolin); methoxylation at the 7 position and glycosylation at the 6

position (isoscopolin) drastically abolished the inhibitory activity. In addition, methoxylation at only the 6 position (scopoletin) resulted in a 20-fold decrease in the inhibitory potential. Daphnetin, which has a free hydroxyl group at both the 7 and 8 positions, displayed stronger activity than scopoletin and umbelliferone, indicating that the free hydroxyl group at the 8 position might contribute to the inhibitory potential. On the other hand, 6-methoxy artemicapin C (methoxy group at the 6 position and methylenedioxy group at both 7 and 8 positions) exhibited superior activity to daphnetin, scopoletin, umbelliferone. Based on the above structure–activity relationship, the PTP1B inhibitory activity appears to be largely dependent on the substitution of hydroxyl group at the 6, 7, and 8 positions and methoxylation or glycosylation of these positions greatly affect their potency.

Among the 10 flavonoids, only 5 flavonoids were shown to be PTP1B inhibitors. Quercetin, isorhamnetin, and arcapillin also showed moderate PTP1B inhibitory activity while capillarisin and cirsilineol exhibited marginal inhibitory activity. The structure–activity relationship strongly suggests that the position of the hydroxyl group and type of substituent correlate with the PTP1B inhibitory activity. Quercetin exhibited the highest

PTP1B inhibitory potential while its glycoside (hyperoside) exhibited very poor activity. Although isorhamnetin inhibited PTP1B, its two glycosides, namely isorhamnetin 3-*O*-glucoside and isorhamnetin 3-robinobioside, were completely inactive at the tested concentrations. Based on these observations, it is clear that the free hydroxyl group is very important for the inhibitory activity against PTP1B. In addition, the lower activity of isorhamnetin compared to quercetin implies that methoxylation of 3'-hydroxyl group diminished the inhibitory activity. Although arcapillin, cirsilineol, and capillarisin showed weaker activity than quercetin and isorhamnetin, arcapillin was more potent than cirsilineol and capillarisin. Therefore, it is expected that the free hydroxyl group at the 5, 6, and 7 positions played a critical role in the inhibition of PTP1B, while the 2'-hydroxy group increased the inhibitory activity.

In conclusion, twelve species of the genus *Artemisia* were selected and their inhibitory potential against α-glucosidase and PTP1B was evaluated in order to evaluate the anti-diabetic potential of the genus. Several *Artemisia* species were shown to be potent α-glucosidase and PTP1B inhibitors; however, *A. capillaris* as well as its isolated constituents have been shown to possess the most potent α-glucosidase and PTP1B inhibitory activities.

Esculetin, quercetin, isorhamnetin, cirsilineol, scopoletin, daphnetin and umbelliferone showed potential α-glucosidase and PTP1B inhibitory activities. In addition, 6-methoxy artemicapin C and capillrisin were determined to be more potent PTP1B inhibitors than α-glucosidase. On the other hand, hyperoside exhibited more potent inhibitory activity against α-glucosidase than PTP1B. Hence, it was hypothesized that the presence of these constituents was directly attributed to the potent α-glucosidase and PTP1B inhibitory activity of *A. capillaris*. Therefore, *A. capillaris* as well as its isolated constituents could be further explored for the development of novel therapeutic or preventive agents for the treatment of diabetes.

## 3-3. Inhibitory activity of coumarins from *A. capillaris* against advanced glycation endproduct formation

### 3-3-1. AGE formation inhibitory activity of MeOH extracts from selected *Artemisia* species

The MeOH extracts of different species were evaluated based on their inhibitory activity against AGE formation at a concentration of 10 μg/mL. The AGE inhibitory activities of the 12 selected species of the genus *Artemisia* are summarized in Table 6. Whole *A. capillaris* and *A. japonica* plants exhibited the strongest AGE inhibitory activities with inhibition percentages (%) of 87.47 ± 0.76 and 81.52 ± 0.97, respectively, as compared to aminoguanidine (67.66 ± 0.37%), a wellknown AGE inhibitor. The aerial part of *A. capillaris* and the whole *A. sylvatica* plant exhibited inhibition values of 67.34 ± 4.43% and 62.07 ± 2.09%, respectively, followed by *A. stolonifera* with 41.14 ± 2.46%. Three *Artemisia* species, including whole *A. princeps* var. *orientalis*, *A. iwayomogi*, and *A. rubripes* plants exhibited moderate AGE inhibitory activity with inhibition % of 24.41 ± 1.94, 24.06 ± 1.69, and 21.40 ± 2.88, respectively. However, *A. montana*, *A. apiacea*, *A. argyi*, *A. selengenis*, and *A. keiskeana* did not exhibit any inhibitory activity

at the test concentrations. To evaluate the potential and efficacy of the *Artemisia* species as anti-diabetic complications drugs, RLAR inhibition and AGE inhibition were used as representative mechanisms for addressing anti-diabetic complications. Interestingly, differences were noted in the anti-diabetic complication potentials of the *Artemisia* species. *A. capillaris* exhibited the most significant inhibitory activity against AGE formation and *A. montana* exhibited no inhibitory activity. On the other hand, the inhibitory activity of *A. montana* on RLAR was eight times greater than that of *A. capillaris* in our previous study (Jung et al., 2011). Although more research is needed to fully understand this difference, the slight difference in composition and concentration of bioactive compounds in the selected assays may play a role. Despite the contrasting results of the two assays, these identical species demonstrated marked inhibitory activity against RLAR activity and AGE formation, indicating *A. capillaris* has potential as a therapeutic or preventive agent for diabetic complications.

**Table 6.** AGE formation inhibitory activity of the selected *Artemisia* species

| Sample | Plant parts* | Inhibition (%)[a] ± SEM |
|---|---|---|
| *Artemisia capillaris* Thunb. | WP | 87.47 ± 0.76 |
| *Artemisia japonica* Thunb. | WP | 81.52 ± 0.97 |
| *Artemisia capillaris* Thunb. | AR | 67.34 ± 4.43 |
| *Artemisia sylvatica* Max. | WP | 62.07 ± 2.09 |
| *Artemisia stolonifera* Max. | WP | 41.14 ± 2.46 |
| *Artemisia princeps* var. *orientalis* Pampan | WP | 24.41 ± 1.94 |
| *Artemisia iwayomogi* Kitamura | WP | 24.06 ± 1.69 |
| *Artemisia rubripes* Nakai | WP | 21.40 ± 2.88 |
| *Artemisia montana* Pampan | WP | NA |
| *Artemisia apiacea* Hance | WP | NA |
| *Artemisia argyi* Levi. et Vaniot | LF | NA |
| *Artemisia selengensis* Turcz | WP | NA |
| *Artemisia keiskeana* Miq. | WP | NA |
| Aminogunaidine[b] | | 67.66 ± 0.32 |

*Abbreviations: Whole plant (WP), Leaf (LF), Aerial part (AR).

NA: No activity at the tested concentrations.

[a]Tested at a concentration of 10 μg/mL.

[b]Aminoguanidine was used as a positive control.

### 3-3-2. AGE formation inhibitory activities by the *A. capillaris* MeOH extract and its solvent soluble fractions

Since the whole *A. capillaris* plant exhibited the most potent inhibitory of AGE activity among the tested *Artemisia* species, this plant was further investigated in order to identify the active compounds responsible for the AGE inhibition. The MeOH extract of *A. capillaris* was partitioned with $CH_2Cl_2$, EtOAc, and *n*-BuOH, respectively. The AGE inhibitory effect of individual fractions was then evaluated. As shown in Table 7, the MeOH extract and *n*-BuOH fraction exhibited significant AGE inhibitory activity with $IC_{50}$ values of 2.22 ± 0.05 and 2.46 ± 0.14 μg/mL, followed by the *n*-BuOH and $CH_2Cl_2$ fractions with $IC_{50}$ values of 3.69 ± 0.16 and 5.13 ± 0.02 μg/mL, as compared to aminoguanidine with an $IC_{50}$ value of 86.84 ± 1.09 μg/mL. Thus, bioactive-guided fractionation of the MeOH extract of *A. capillaris* revealed that the EtOAc and *n*-BuOH fractions were expected to harbor a variety of potent AGE inhibitors. In addition, most flavonoids that are known AGE inhibitors were expected to be present in large amounts in the polar EtOAc and *n*-BuOH fractions (Muthenna et al., 2011; Nicolle et al., 2011).

**Table 7.** AGE formation and RLAR inhibitory activities of the MeOH extract as well as different solvent soluble fractions of *A. capillaris*

| Sample | $IC_{50}$ values (μg/mL) ± SEM | |
|---|---|---|
| | AGE | RLAR |
| MeOH Extract | 2.22 ± 0.05 | 0.46 ± 0.01 |
| $CH_2Cl_2$ Fr. | 5.13 ± 0.02 | 3.13 ± 0.28 |
| EtOAc Fr. | 2.46 ± 0.14 | 0.16 ± 0.05 |
| *n*-BuOH Fr. | 3.69 ± 0.16 | 0.20 ± 0.00 |
| $H_2O$ Fr. | 35.63 ± 0.93 | 3.98 ± 0.07 |
| Aminoguanidine[a] | 155.39 ± 2.05 | - |
| Quercetin[b] | - | 0.10 ± 0.01 |

[a,b]Used as positive controls in each assay.

### 3-3-3. *In vitro* antioxidant activities of the *A. capillaris* MeOH extract and its solvent soluble fractions

During AGE generation, highly reactive oxygen/nitrogen species and dicarbonyl intermediates are produced, which then induce the oxidation and degradation of numerous biomolecules (Rahbar, 2007). Since the oxidation process is believed to play an important role in AGEs formation, further antioxidant capacities were investigated in the DPPH, TEAC, and $ONOO^-$ assays. As demonstrated in Table 8, most fractions of three selected antioxidant assays, with the exception of the nonpolar $CH_2Cl_2$ fraction. Among the various fractions of *A. capillaris*, the EtOAc fraction exerted the most potent radical scavenging activity, with an $IC_{50}$ value of 3.16 ± 0.07 μg/mL, as compared with the positive control, L-ascorbic acid ($IC_{50}$ = 2.10 ± 0.41 μg/ mL) in the DPPH assay; an $IC_{50}$ value of 5.46 ± 0.18 μg/mL, as compared with the positive control, trolox ($IC_{50}$ = 2.10 ± 0.41 μg/mL) in the TEAC assay; and an $IC_{50}$ value of 0.55 ± 0.02 μg/mL, as compared with the positive control, L-penicillamine ($IC_{50}$ = 0.26 ± 0.03 μg/mL) in the $ONOO^-$ assay. These results correspond well with those of previous radical scavenging studies (Jung et al., 2004; Hong et al., 2007). Recently, the EtOAc fraction of *A. capillaris* has been reported to enhance the antioxidant

defense system by reducing reactive oxygen species generation and oxidative degenerative substances as well as increasing the glutathione (GSH) content and cellular antioxidant enzyme activity in high-fat diet-induced obese mice and type 2 diabetic mice (Hong and Lee, 2009b, 2009c). The *n*-BuOH fraction exhibited significant scavenging activity, with $IC_{50}$ values of 15.27 ± 0.62, 12.30 ± 0.25, and 1.46 ± 0.04 μg/mL in the DPPH, TEAC, and $ONOO^-$ assays, respectively. Since two active fractions harbored high flavonoid content and flavonoids are well-known free radical scavengers and metal ion chelators that participate in the inhibition of AGE formation (Muthenna et al., 2011), further bioactive-guided isolation of EtOAc and *n*-BuOH fractions was performed.

**Table 8.** *In vitro* antioxidant activity of the MeOH extract as well as different solvent soluble fractions of *A. capillaris*

| Extract/Fractions | $IC_{50}$ values (μg/mL) ± SEM | | |
|---|---|---|---|
| | DPPH | TEAC | $ONOO^-$ |
| MeOH Extract | 33.95 ± 2.30 | 17.96 ± 0.40 | 0.67 ± 0.08 |
| $CH_2Cl_2$ Fr. | 110.04 ± 1.42 | 45.63 ± 0.50 | 5.88 ± 0.10 |
| EtOAc Fr. | 3.16 ± 0.07 | 5.46 ± 0.18 | 0.55 ± 0.02 |
| *n*-BuOH Fr. | 15.27 ± 0.62 | 12.30 ± 0.25 | 1.46 ± 0.04 |
| $H_2O$ Fr. | 81.55 ± 1.84 | >100 | >10 |
| L-Ascorbic acid[a] | 2.10 ± 0.41 | - | - |
| Trolox[b] | - | 9.53 ± 0.02 | - |
| L-Penicillamine[c] | - | - | 0.26 ± 0.03 |

[a-c]Used as positive controls in each assay.

### 3-3-4. AGE formation inhibitory activity of coumarin derivatives

With the exception of coumarin, scoparone, and 7-methoxy coumarin, all other coumarins demonstrated significant a concentration-dependent inhibitory activity against AGE formation (Table 9). Esculin, the 6-*O*-glucoside of esculetin, demonstrated the most potent AGE inhibitory activity with an $IC_{50}$ value of 0.85 ± 0.01 μM, as compared with the positive control, aminoguanidine ($IC_{50}$ = 932.66 ± 4.94 μM), followed by umbelliferone, scopoletin, and esculetin with $IC_{50}$ values of 2.95 ± 0.02, 3.90 ± 0.05, and 5.02 ± 0.02 μM, respectively. Regarding the above results, the genin of esculin, esculetin, which has two hydroxyl groups at the C-6 and C-7 positions, exhibited approximately six-fold weaker activity than esculin. Umbelliferone, which has one hydroxyl group at the C-7 position, and scopoletin, which has an identical structure to esculetin except for an additional methoxyl group at C-6 instead of a hydroxyl group, also demonstrated significant inhibitory activity with $IC_{50}$ values of 2.95 ± 0.02 and 3.90 ± 0.05 μM, respectively. However, replacement of the C-7 hydroxyl group of umbelliferone and scopoletin with a methoxyl group (i.e., 7-methoxy coumarin and scoparone) or a glucosyl group (i.e., scopolin), or the re-moval of hydroxyl groups at the C-6 and C-7 positions (i.e.,

coumarin), significantly decreased AGE inhibition. Additionally, daphnetin with a 7,8-dihydroxyl group was found to exhibit less inhibitory activity than the 6,7-dihydroxylated coumarin (i.e., esculetin). These results suggest that the inhibitory activity of coumarins on AGE formation is largely dependent on the presence and position of a hydroxyl group in their structure. Taking into account the detailed structure-activity relationship (SAR) features of coumarin derivatives, it is clear that the hydroxyl group at the C-7 position plays a pivotal role in the observed inhibitory activity. Furthermore, the addition of a hydroxyl group in the *ortho* position to the C-7 hydroxyl group of the umbeliferone skeleton (i.e., esculetin or daphnetin) significantly decreased the inhibitory activity of these coumarins. The addition of a methoxyl group at C-6 (i.e., scopoletin) did not alter the potency, but the addition of a glucosyl moiety (i.e., esculin) greatly increased the inhibitory activity, suggesting that a free hydroxyl group at C-7 as well as a glucosyl group instead of a methoxyl group at C-6 are two important parameters in the inhibitory potential of these coumarins on AGE formation. Similarly, isoscopolin (7-methoxylated coumarin) exhibited greater inhibitory activity on AGE than scopolin (7-glucosylated coumarin). Unlike the SAR of most test coumarins, isoscopoletin (6-hydroxyl-7-

methoxyl coumarin) exhibited three times as much inhibitory activity as scopoletin (7-hydroxyl-6-methoxylated coumarin) in AGE inhibition.

Previous studies indicate scopoletin has demonstrated a remarkable inhibition of advanced glycation endproducts formation (Lee et al., 2010). Ramesh and Pugalendi (2007) revealed that umbelliferone, a natural antioxidant and benzopyrone, exerted anti-diabetic effects by controlling glycemia. Coumarin's anti-diabetic effects have been reported to include a significant protective effect on glycoprotein metabolism (Pari and Rajarajeswari, 2010; Rajarajeswari and Pari, 2011). The inhibitory activity of coumarin against carbohydrate metabolic key enzymes resulted in alterations to glucose metabolism (Pari and Rajarajeswari, 2009). Regarding the AR inhibition of coumarins, many studies indicate that simple coumarins possess a hydroxyl group at either the C-6 or C-7 position (Park et al., 2011). Additionally, substitutions in the hydroxyl group at C-7 enhance potency toward AR, while the hydroxyl group at C-7 interferes with aldehyde reductase inhibition activity (Kato et al., 2010). Umbelliferone and esculetin could also significantly inhibit the accumulation of sorbitol in human erythrocytes (Kato et al., 2008). A conjugate between coumarin and aspirin, nicousamide, was proposed to

delay diabetic nephropathy by inhibiting AGE formation and crosslinking AGEs in renal cells and a streptozotocin rat model of diabetes (Li et al., 2010, 2012). Although the inhibitory activity of coumarins and aspirin conjugate as well as several coumarins on AGE has been reported, the present study is the first to report the detailed SAR of coumarin derivatives.

**Table 9.** AGE formation inhibitory activity of the coumarin derivatives isolated from *A. capillaris*

| Sample | $IC_{50}$ values (μM) ± SEM |
|---|---|
| Coumarin | NA |
| Umbelliferone | 2.95 ± 0.02 |
| Esculetin | 5.02 ± 0.02 |
| Esculin | 0.85 ± 0.01 |
| Scopoletin | 3.90 ± 0.05 |
| Isoscopoletin | 18.78 ± 0.50 |
| Scopolin | 60.10 ± 0.11 |
| Isoscopolin | 41.04 ± 2.71 |
| Scoparone | 204.97 ± 1.34 |
| Daphnetin | 43.21 ± 1.31 |
| 7-Methoxycoumarin | 490.20 ± 9.32 |
| Aminoguanidine[a] | 932.66 ± 4.94 |

NA: No activity at the tested concentrations.

[a] Used as positive control in the assay.

### 3-3-5. *In vitro* antioxidant activities of coumarins derivatives

Recognizing the relationship between AGE inhibition and antioxidant activity, several selected coumarins were also evaluated by *in vitro* radical scavenging (Table 10) and $ONOO^-$-mediated tyrosine nitration assays (Fig. 4). Esculetin with a dihydroxyl group exhibited the most potent radical scavenging activity, with $IC_{50}$ values of 4.46 ± 0.15, 3.07 ± 0.14, and 0.42 ± 0.01 μM, in the DPPH, TEAC, and $ONOO^-$ assays, respectively. On the other hand, scopoletin with a 7-hydroxyl group demonstrated antioxidant activities in both TEAC and $ONOO^-$ assays with respective $IC_{50}$ values of 26.91 ± 0.00, 6.35 ± 0.63, and 0.42 ± 0.01 μM. Esculin and scopolin demonstrated limited and moderate antioxidant activities in the TEAC and $ONOO^-$ assays, respectively, and did not exhibit scavenging activities in other assays. Considering the antioxidant activities of scopletin, there are slight differences between these results and those reported by Lee et al. (2007), who indicated that esculetin demonstrated antioxidant activity whereas coumarin, umbelliferone, esculin, and scopoletin demonstrated weak antioxidant activity in radical scavenging assays. Since the mechanisms of AGE formation may be similar to protein nitration, additional research on the effect of coumarins on protein nitration was

performed. As demonstrated in Figure 4, esculetin, scopoletin, and scopolin exerted marked concentration-dependent inhibition, while scoparone, umbelliferone, and coumarins did not exhibit inhibition at the tested concentrations. Interestingly, the results from the $ONOO^-$-mediated tyrosine nitration assay coincided with those of the *in vitro* $ONOO^-$ scavenging assay, indicating that esculetin exerted the most potent inhibitory activity on tyrosine nitration. Although coumarin demonstrated limited activity in our assay, the oral administration of coumarin to diabetic rats resulted in significantly reduced lipid peroxides and significantly increased antioxidant enzymes as well as decreased thiobarbituric acid reactive substances (TBARS), lipid hydroperoxides, and conjugated diene concentration in the liver and kidney tissues (Rajarajeswari and Pari, 2011). Based on the current body of literature, coumarins act as effective antioxidants as well as effective inhibitors of AGE and RLAR, indicating its potential as anti-diabetic complication drugs.

**Table 10.** *In vitro* antioxidant activities of the coumarins derivatives isolated from *A. capillaris*

| Sample | $IC_{50}$ values (μM) ± SEM | | |
|---|---|---|---|
| | DPPH | TEAC | $ONOO^-$ |
| Esculetin | 4.46 ± 0.15 | 3.07 ± 0.14 | 0.42 ± 0.01 |
| Esculin | NA | 26.91 ± 0.12 | NA |
| Scopoletin | NA | 15.17 ± 0.00 | 6.35 ± 0.63 |
| Scopolin | NA | NA | 34.34 ± 2.69 |
| L-Ascorbic acid[a] | 17.00 ± 0.48 | 15.76 ± 0.23 | - |
| Trolox[b] | - | 19.21 ± 0.73 | - |
| L-Penicillamine[c] | - | - | 4.83 ± 0.24 |

NA: No activity at the tested concentrations.

[a-c]Used as positive control in the assay.

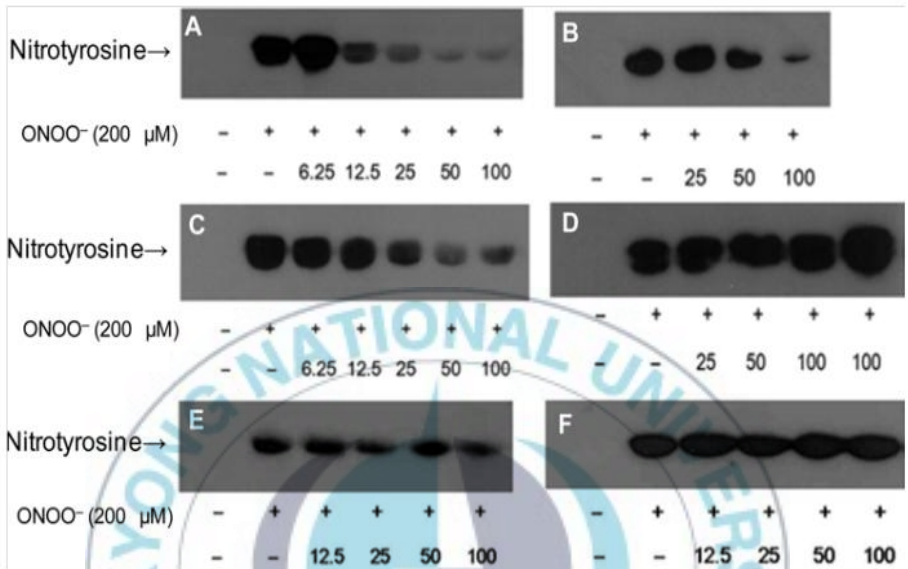



**Figure 4.** Dose-dependent inhibition of $ONOO^-$-mediated tyrosine nitration by *A. capillaris* coumarin derivatives. The mixture of test samples, BSA, and $ONOO^-$ were incubated with shaking at 37oC for 30 min. The reactant was resolved by electrophoresis in 10% polyacrylamide gel. (A) Esculetin; (B) Scopoletin; (C) Scopolin; (D) Scoparone; (E) Umbelliferone; (F) Coumarin.

### 3-3-6. AGE formation inhibitory activity of phenolic compounds

A series of phenolic compounds, including 4,5-di-*O*caffeoylquinic acid, chlorogenic acid, caffeic acid, ferulic acid, dihydrocaffeic acid, *p*-coumaric acid, and 3,4-dimethoxycinnamic acid were evaluated to assess their inhibition of AGE formation and consequently, their SAR. 4,5-Di-*O*-caffeoylquinic acid and chlorogenic acid significantly inhibited AGE formation with $IC_{50}$ values of 6.48 ± 0.04 and 21.08 ± 0.34 μM, respectively (Table 11). Supporting the SAR between caffeoylquinic acids, an additional AGE inhibition experiment was performed using a phenolic moiety (i.e., caffeic acid, ferulic acid, and dihydrocaffeic acid) and a sugar moiety (i.e., quinic acid). Much like previous research (Cui et al., 2009), results from the caffeoylquinic acids suggested that the number of caffeoyl groups and their position in the quinic acid moiety may play an important role in the inhibition of AGE formation. In addition to their AGE inhibition properties, most caffeoylquinic acids, including di-*O*-caffeoylquinic acid, tri-*O*-caffeoylquinic acid, and chlorogenic acid have been reported as potent natural AR inhibitors (de la Fuente and Manzanaro, 2003; Logendra et al., 2006). Cui et al. (2009) indicated that the number of caffeoyl groups and their position in the quinic acid moiety may play a key role in their

inhibitory activity against RLAR. 4,5-Di-*O*-caffeoylquinic acid was also reported to exhibit AR inhibitory activity (Logendra et al., 2006).

**Table 11.** AGE formation inhibitory activity of the phenolic compounds isolated from *A. capillaris*

| Sample | $IC_{50}$ values (μM) ± SEM |
|---|---|
| Caffeic acid | 47.13 ± 0.04 |
| Ferulic acid | 323.43 ± 8.10 |
| Dihydrocaffeic acid | 206.55 ± 3.06 |
| *p*-Coumaric acid | NA |
| 3,4-Dihydroxycinnamic acid | NA |
| Quinic acid | NA |
| Chlorogenic acid | 21.08 ± 0.34 |
| 4,5 Dicaffeoylquinic acid | 6.48 ± 0.04 |
| Aminoguanidine[a] | 932.66 ± 4.94 |

NA: No activity at the tested concentrations.

[a] Used as positive control in the assay.

### 3-3-7. AGE formation inhibitory activity of flavonoids

All tested flavonoids exhibited significant AGE inhibitory activities when compared with the positive control, aminoguanidine. Quercetin and its galactoside (hyperoside) exerted the most potent inhibitory activity on AGE with $IC_{50}$ values of 24.35 ± 0.95 and 48.20 ± 0.05 μM, respectively, as compared with aminoguanidine, which had an $IC_{50}$ value of 932.66 ± 4.94 μM (Table 12). There have been numerous reports on the anti-diabetic complication effects of flavonoids, especially through AR and AGE inhibition. In particular, it has been suggested that the greater the number of hydroxyl groups at the 3',4',5, and 7 position in flavonoids, the more potent it is in AGE inhibition (Matsuda et al., 2003; Stefek, 2011). Isorhamnetin and its glucosides were also identified as exhibiting anti-diabetic complication properties *in vivo* and *in vitro* (Yokozawa et al., 2002; Lee et al., 2005). However, three acacetin derivatives, including linarin, acacetin triglycoside, and its new acylated derivatives, did not demonstrate AGE inhibition at the tested concentration.

In conclusion, a variety of indigenous *Artemisia* species were selected to evaluate their AGE inhibition properties and develop therapeutic drugs that prevent diabetic complications. Although several *Artemisia* species

significantly inhibited AGE formation, *A. capillaris* exhibited the most potent AGE inhibitory activity. Further bioactivity-guided isolation led to a new acylated flavonoid glycoside, acacetin-7-*O*-(6''-*O*-acetyl)-β-D-glucopyranosyl-(1→2)[α-L-rhamnopyranosyl]-(1→6)-β-D-glucopyranoside, together with 11 known flavonoids (acacetin-7-*O*-β-D-glucopyranosyl-(1→2)[α-L-rhamnopyranosyl]-(1→6)-β-D-glucopyranoside, linarin, quercetin, hyperoside, isorhamnetin, isorhamnetin 3-galactoside, isorhamnetin 3-glucoside, isorhamnetin 3-arabinoside, isorhamnetin 3-robinobioside, arcapillin), six coumarins (umbelliferone, esculetin, scopoletin, scopolin, isoscopolin, and scoparone), and two phenolic derivatives (4,5-di- *O*-caffeoylquinic acid and chlorogenic acid). Due to limited information in recent studies, we focused on the detailed SAR between AGE inhibitory activity and coumarin derivatives. The findings of the detailed SAR between coumarins and AGE inhibition demonstrated that a free hydroxyl group at C-7 and a glucosyl group in place of a methoxyl group at C-6 are two important parameters in the inhibition potential of these coumarins on AGE formation. Since antioxidant activity may suppress AGE formation processes (i.e., glycoxidation) at the level of free radical intermediates, comprehensive and effective coumarins as AGE and RLAR

inhibitors along with antioxidants may be beneficial uses in the development of anti-diabetic complication drugs.

**Table 12.** AGE formation inhibitory activity of the flavoinoid derivatives isolated from *A. capillaris*

| Sample | $IC_{50}$ values (μM) ± SEM |
|---|---|
| Arcapillin | 77.84 ± 1.07 |
| Cirsimaritin | 138.38 ± 0.84 |
| Cirsilineol | 289.28 ± 0.94 |
| Isorhamnetin | 272.50 ± 6.40 |
| Quercetin | 24.35 ± 0.95 |
| Hyperoside | 48.20 ± 0.05 |
| Isorhamnetin 3-*O*-robinobioside | 82.90 ± 1.20 |
| Isorhamnetin 3-*O*-galactoside | 155.02 ± 8.15 |
| Linarin | NA |
| Acacetin-7-*O*-(6"-*O*-acetyl)-β-D-glucopyranosyl-(1→2)[α-L-rhamnopyranosyl]-(1→6)-β-D-glucopyranoside | NA |
| Acacetin-7-*O*-(β-D-glucopyranosyl-(1→2)[α-L-rhamnopyranosyl]-(1→6)-β-D-glucopyranoside | NA |
| Aminoguanidine[a] | 932.66 ± 4.94 |

NA: No activity at the tested concentrations.

[a]Used as positive control in the assay.

## 3-4. Promising anti-diabetic potential of two essential oils, capillin and capllinol, isolated from *A. capillaris*

### 3-4-1. α-Glucosidase inhibitory activity of capillin and capillinol

The inhibitory effect of capillin and capillinol against α-glucosidase was determined using *p*-nitrophenyl-α-D-glucopyranoside (pNPG) as a substrate. As shown in Table 13, both capillin and capillinol showed strong α-glucosidase inhibitory activity. Capillin showed the highest α-glucosidase inhibitory activity with an $IC_{50}$ value of 332.96 ± 1.14 μM compared to the positive control acarbose with an $IC_{50}$ value of 320.33 ± 4.61 μM. Capillinol also showed strong α-glucosidase inhibitory activity with an $IC_{50}$ value of 464.53 ± 2.69 μM compared to acarbose.

### 3-4-2. PTP1B inhibitory activity of capillin and capillinol

The PTP1B inhibitory effect of capillin and capllinol was determined using *p*-nitrophenyl-phosphate (pNPP) as a substrate. As can be seen in Table 13, capillin exhibited the highest PTP1B inhibitory activity with an $IC_{50}$ value of 31.90 ± 0.15 μM compared to the positive control ursolic acid with an $IC_{50}$ value of 10.42 ± 0.05 μM. Capillinol was also found as potent

inhibitor of PTP1B with an $IC_{50}$ value of 155.99 ± 0.39 μM.

### 3-4-3. RLAR inhibitory activity of capillin and capillinol

RLAR inhibitory activity of capllin and capillinol are presented in Table 13. In contrast to α-glucosidase and PTP1B, only capillin showed strong RLAR inhibitory activity with an $IC_{50}$ value of 55.34 ± 0.28 μM compared to the positive control quercetin with an $IC_{50}$ value of 11.64 ± 0.17 μM.

**Table 13.** Summary of the anti-diabetic potential of capillin and capillinol determined by α-glcuosidase, PTP1B, and RLAR inhibitory assays

| Sample | $IC_{50}$ values (μM)[a] ± SEM | | |
|---|---|---|---|
| | α-Glucosidase | PTP1B | RLAR |
| Capillin | 332.96 ± 1.44 | 31.90 ± 0.14 | 55.34 ± 0.28 |
| Capillinol | 464.53 ± 2.69 | 159.99 ± 0.73 | NA |
| Acarbose[a] | 320.33 ± 4.61 | - | - |
| Ursolic acid[b] | - | 10.42 ± 2.97 | - |
| Quercetin[c] | - | - | 11.64 ± 0.17 |

NA: No activity at the tested concentrations.

[a-c]Used as positive controls in each assay.

### 3-4-4. Inhibitory activity of capillin and capillinol on $ONOO^-$ mediated protein tyrosine nitration

Effect of capillin and capillinol on the protein tyrosine nitration was evaluated using Western blot analysis. As shown in the Figure 8, protein tyrosine nitration was largely increased when no treatment with capillin. But interestingly, treatment with capillin reduced the tyrosine nitration in a concentration-dependent manner. Notably, tyrosine nitration is almost undetectable when treatment with 100 and 200 μM of capillin. In contrast, capillinol did not inhibit $ONOO^-$ mediated protein tyrosin nitarion at the concentration tested.

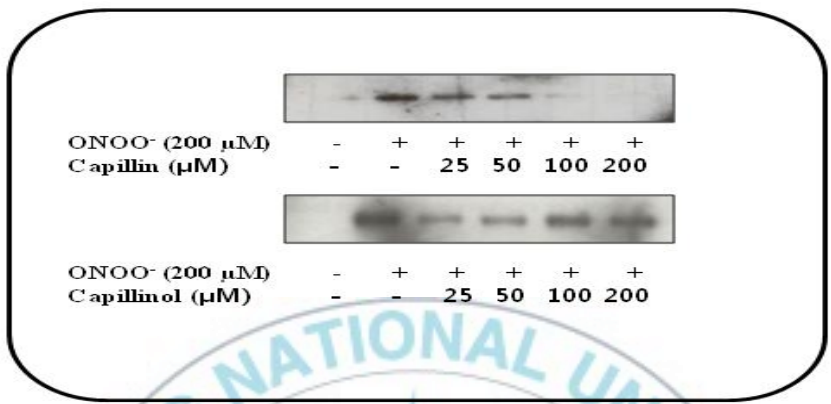



**Figure 5.** Dose-dependent inhibition of $ONOO^-$-mediated tyrosine nitration by capillin and capillinol, isolated from *A. capillaris*. The mixture of test samples, BSA, and $ONOO^-$ were incubated with shaking at 37 $^oC$ for 30 min. The reactant was resolved by electrophoresis in 10% polyacrylamide gel.

### 3-4-5. Kinetic study of capillin and capillinol against α-glucosidase, PTP1B and RLAR

In an attempt to explain the mode of enzymatic inhibition, kinetic analyses were performed at different concentration of substrate (4, 2, and 1 mM pNPG for α-glucosidase; 4, 2, and 1 mM pNPP for PTP1B; 25, 50, 100 μM DL-glyceryldehyde for RLAR) and various concentration of capillin and capillinol. Dixon plot and Lineweaver-Burk plot were drawn using the data obtained from the kinetic studies to confirm the inhibition pattern. As shown in Figure 6 A and B, capllin showed noncompetitive inhibition against α-glcucosidase with *Ki* value of 22.78 μg/mLwhile capillinol showed mixed type inhibition against α-glcucosidase (Figure 7 A and B) with Ki value of 69.57 μg/mL. In case PTP1B inhibiton, both capillin and capilllol showed mixed type inhibition with *Ki* values of 3.42 and 15.15 μg/mL, respectively (Figure 8 A and B; Figure 9 A and B). In addition, capillin also showed noncompetitive type inhibition against RLAR (Figure 10 A and B) with *Ki* value of 10.59 μg/mL.

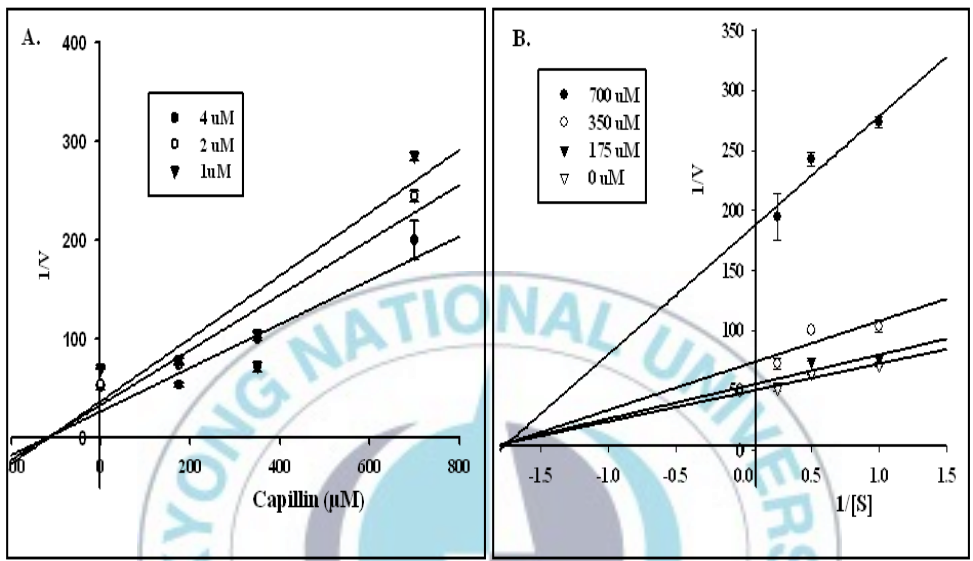



**Figure 6.** Dixon plot (A) and Lineweaver-Burk plot (B) for α-glcuosidase inhibition by capillin.

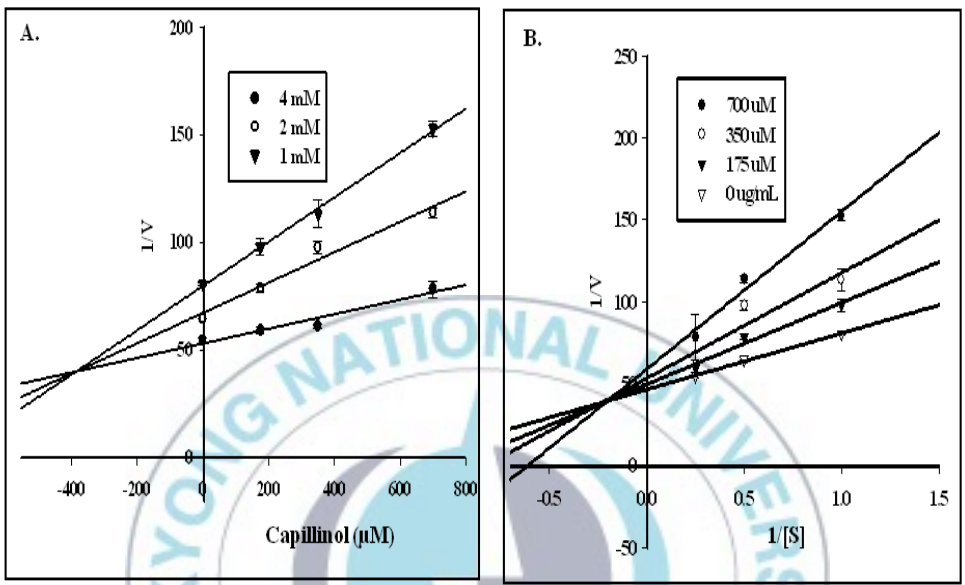



**Figure 7.** Dixon plot (A) and Lineweaver-Burk plot (B) for α-glcuosidase inhibition by capillinol.

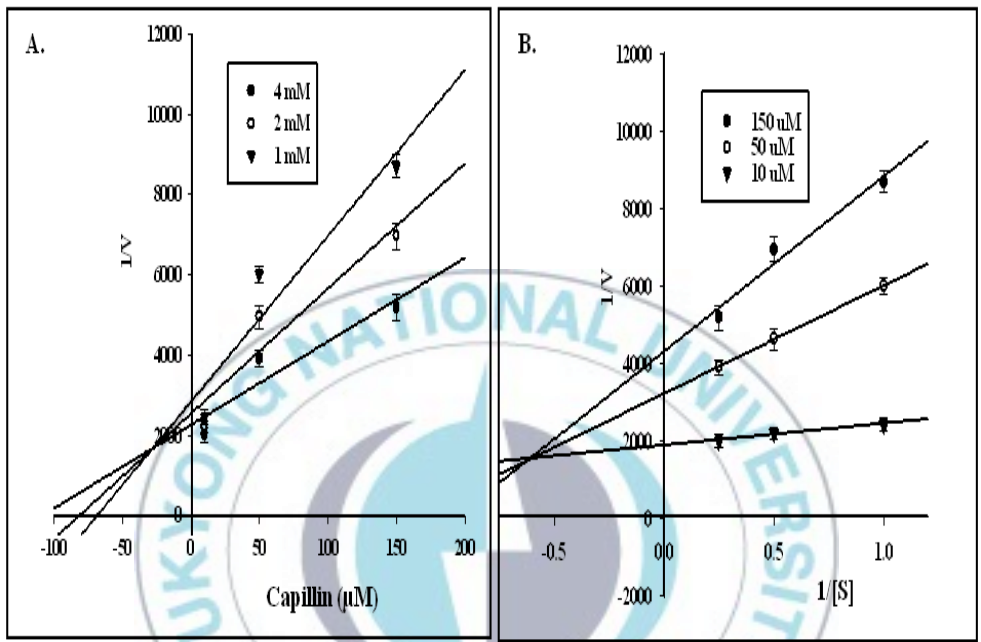



**Figure 8.** Dixon plot (A) and Lineweaver-Burk plot (B) for PTP1B inhibition by capillin.

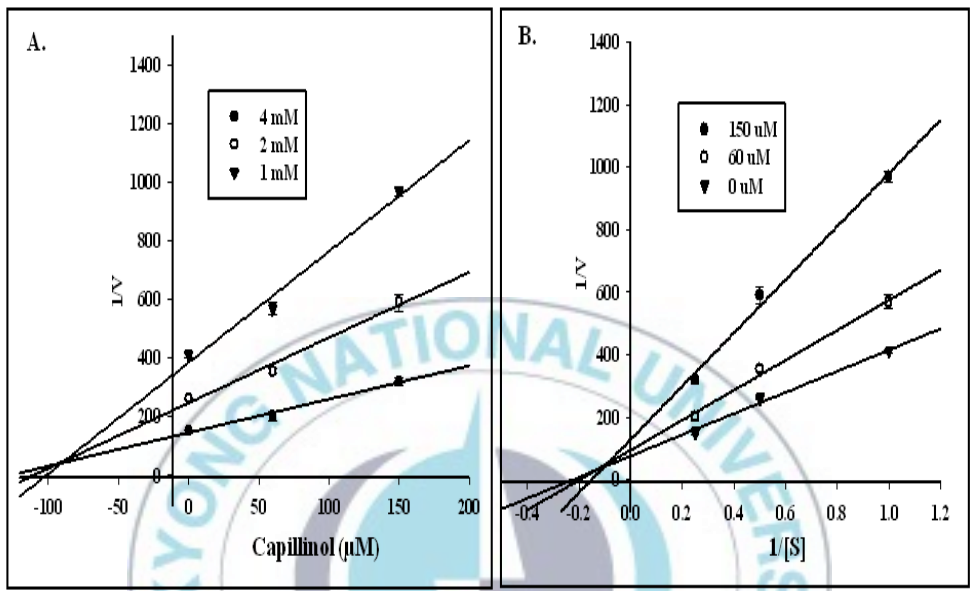



**Figure 9.** Dixon plot (A) and Lineweaver-Burk plot (B) for PTP1B inhibition by capillinol.

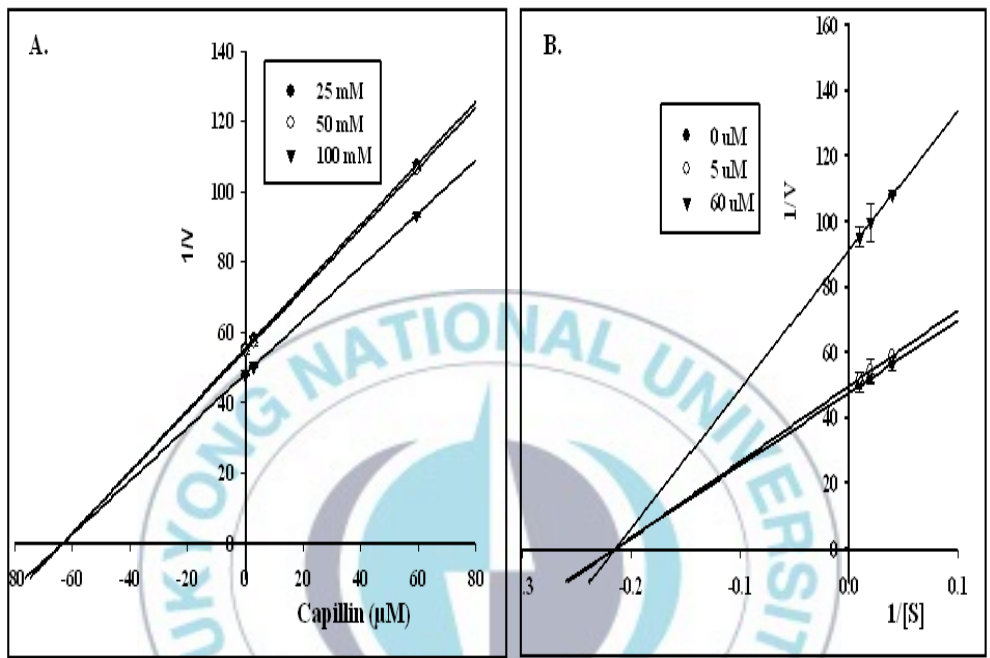



**Figure 10.** Dixon plot (A) and Lineweaver-Burk plot (B) for RLAR inhibition by capillin.

### 3-4-6. Discussion

Polyacetylenes are a group of bioactive secondary metabolites that have been considered undesirable in plant foods due to their toxicant properties such as potent skin sensitizers, allergic contact dermatitis, skin irritants, and neurotoxic at higher concentrations. They form a distinct group of chemically reactive natural products comprising more than 1400 different types and related compounds, and they are commonly found in many higher plants of the families Apiaceae, Araliaceae, and Asteraceae. Some of the most bioactive polyacetylenes are found in the common food plants such as carrot, celery, and parsley (Christensen and Brandt, 2006). In spite of their toxicity at higher concentration, many researchers have shown promising health benefits of ployacetylenes in a number of studies. Several studies have reported that polyacetylenes possess wide range of biological activities, including anti-HIV by the inhibition of sodium and potassium ATPase pump activity, immunosuppressant and antitumour activities (Fusetani et al., 1993; Hallock et al., 1995), anti-inflammatory (Shim et al., 2012), anti-diabetic (Ubillas et al., 2012), anti-platelet aggregation (Lee et al., 2012), and antibacterial activities. Although many beneficial effects of polyacetylenic compounds have been reported, there are very few reports

concerning their anti-diabetic effects. However, some recent studies support their emerging role in the area of diabetic research (Scott, 2010; Chang et al., 2004; Ubillas, 2000). Therefore, in the present study we evaluated anti-diabetic potential of two polyacetylenes, capillin and capillinol *via* α-glucosidase, PTP1B, and RLAR inhibitory assays. We also evaluated their inhibitory effect on $ONOO^-$-mediated tyrosine nitration.

In the present study we found that capillin and capillinol showed strong activity against α-glcuosidase and PTP1B enzyme. Capillin and capillinol exhibited 3.5 and 2.5 times, respectively stronger activity than acarbose, a commercially available α-glucosidase inhibitor. On the other hand, capillin showed similar inhibitory potential against PTP1B compared to the positive control ursolic acid and 6 times stronger than capillinol. In contrast, although capillin showed strong inhibitory activities against RLAR and $ONOO^-$-mediated tyrosine nitration capillinol did not exhibit inhibitory activities against RLAR and $ONOO^-$-mediated tyrosine nitration at the concentration tested.

In order to explore the mechanism of enzymatic inhibition by capillin and capillinol, kinetic analyses were performed at different concentration of substrate and various concentrations of capillin and capillinol. Dixon plot

and Lineweaver-Burk plot were drawn using the data obtained from the kinetic studies to confirm the inhibition pattern. As shown in Figure 6 A and B, all lines intersect at X-axis which implies that capllin is a noncompetitive inhibitor of α-glcucosidase. In other words, capillin can bind to the allosteric site of α-glcucosidase and inhibit the enzyme. On the other hand, capillinol showed mixed type inhibition against α-glcucosidase which inplies that capillinol can bind to both free enzyme and enzyme-substrate complex (Figure 7 A and B). In addition, lower *Ki* value (133.84 μM) of capillin compered to capillinol (413.61 μM) implies that tighter binding of capillin with the α-glcucosidase enzyme. Hence, capillin is expected to be more effective than capillinol in α-glcucosidase inhibition. On the other hand, both capillin and capilllol showed mixed type inhibition with *Ki* values of 20.09 and 90.07 μM, respectively (Figure 8 A and B; Figure 9 A and B). In addition, capillin also showed noncompetitive type inhibition against RLAR (Figure 10 A and B) with *Ki* value of 65.06 μM.

In summary, capillin and capillinol exhibited promising antidiabetic potential via inhibition of α-glcucosidase, PTP1B and RLAR. So far, this is the first report of α-glcucosidase, PTP1B and RLAR inhibitory activities of capillin and capillinol. Our results clearly demonstrate that capillin and

capillinol could be explored further for the development of anti-diabetic remedies for the treatment of diabetes and related complications. However, further extensive biological experiments should be carried out to fully elucidate their mechanism of action.

## 3-5. Vicenin 2 isolated from *A. capillaris* exhibited strong antiglycation properties in glucose and fructose mediated protein glycation

### 3-5-1. α-Glucosidase, PTP1B, and RLAR inhibitory activities of vicenin 2

α-Glucosidase, PTP1B and RLAR inhibitory activities of vicenin 2 are summarized in Table 14. Vicenin 2 exhibited strong inhibitory activity against α-glucosidase with an $IC_{50}$ value of 270.53 ± 6.23 μM compared to the positive control acarbose 320.33 ± 4.61 μM, while it showed moderate PTP1B inhibitory activity with an $IC_{50}$ value of 139.75 ± 2.97 μM compared to the positive control ursolic acid with an $IC_{50}$ value of 10.42 ± 0.05 μM. On the other hand, vicenin 2 exhibited very strong RLAR inhibitory activity with an $IC_{50}$ value of 7.85 ± 0.22 compared to the positive control quercetin with respective $IC_{50}$ value of 11.64 ± 0.17 μM.

**Table 14.** Summary of the anti-diabetic potential of vicenin 2 determined by α-glcuosidase, PTP1B, and RLAR inhibitory assays

| Sample | $IC_{50}$ values (μM)[a] ± SEM | | |
|---|---|---|---|
| | α-Glucosidase | PTP1B | RLAR |
| Vicenin 2 | 270.53 ± 6.23 | 139.75 ± 2.97 | 7.85 ± 0.22 |
| Acarbose[a] | 320.33 ± 4.61 | - | - |
| Ursolic acid[b] | - | 10.42 ± 2.97 | - |
| Quercetin[c] | - | - | 11.64 ± 0.17 |

NA: No activity at the tested concentrations.

[a-c] Used as positive controls in each assay.

### 3-5-2. Effect of Vicenin 2 on AGE formation

The formation of AGE was monitored weekly by measuring fluorescence intensity of the BSA-glucose-fructose solution. As shown in Figure 11, there was significant increased of BSA glycation with increase of the incubation time as evidenced by increasing fluorescence. When vicenin 2 was added to reaction media containing BSA-glucose-fructose system, a significant concentration-dependent reduction in the fluorescence intensity was observed throughout the study period. At week 4 of incubation, the percentage inhibition of AGE formation by vicenin 2 was found 24.4%, 34.7%, 75.0%, and 82.9% at concentrations of 42, 84, 168, and 336 μM, respectively. The positive control aminoguanidine also inhibited AGE formation 53.0% at a concentration of 1.8 mM.

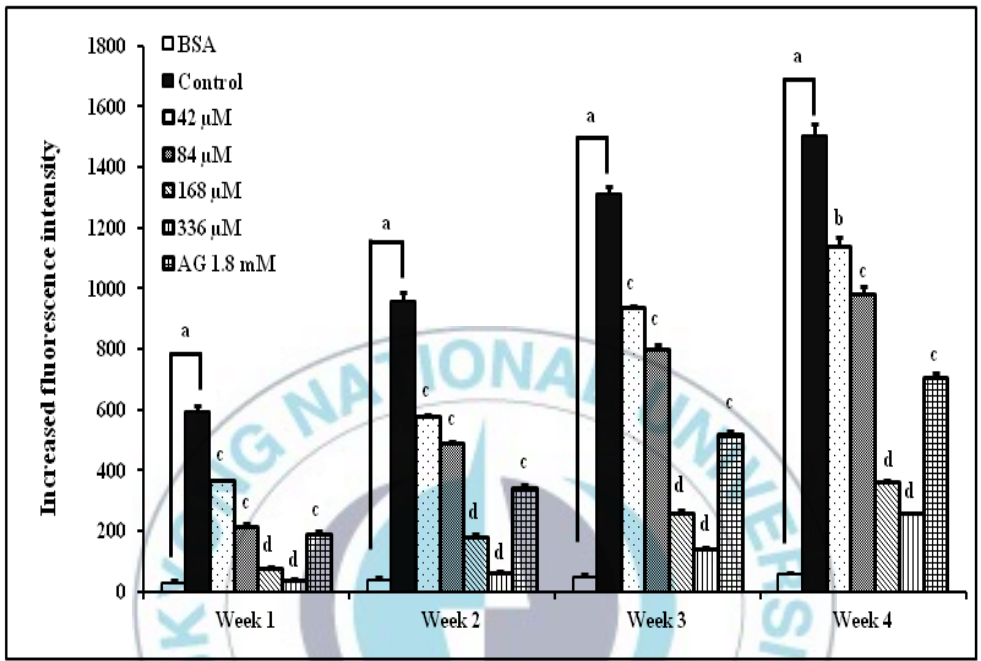



**Figure 11.** The inhibitory effect of vicenin 2 and aminoguanidine (AG) on the formation of fluorescent AGE in BSA-glcuose-fructose system. Results are expressed mean ± SEM of duplicate experiments. [a] $p < 0.001$ when compared to BSA; [b] $p < 0.05$, [c] $p < 0.01$, [d] $p < 0.001$ when compared to BSA-glucose-fructose.

### 3-5-3. Effect vicenin 2 on fructosamine formation

The amount of Amadori product, fructosamine, is shown in Figure 12. Compared with non-glycated BSA, there was significant increase in fructosamine content in BSA-glucose-fructose system after one week of study, and the formation of fructosamine continued to increase during the entire incubation period. However, treatment with vicenin 2 or aminoguanidine significantly suppressed the generation of fructosamine in a concentration-dependent manner. At the end of the study period, vicenin 2 at concentrations of 42, 84, 168, and 336 μM inhibited the formation of fructosamine in BSA-glucose-fructose systems by 2.8%, 23.8%, 29.0%, and 37.7%, respectively compared to the positive control aminoguanidine 26.3% at a concentration of 1.8 mM.

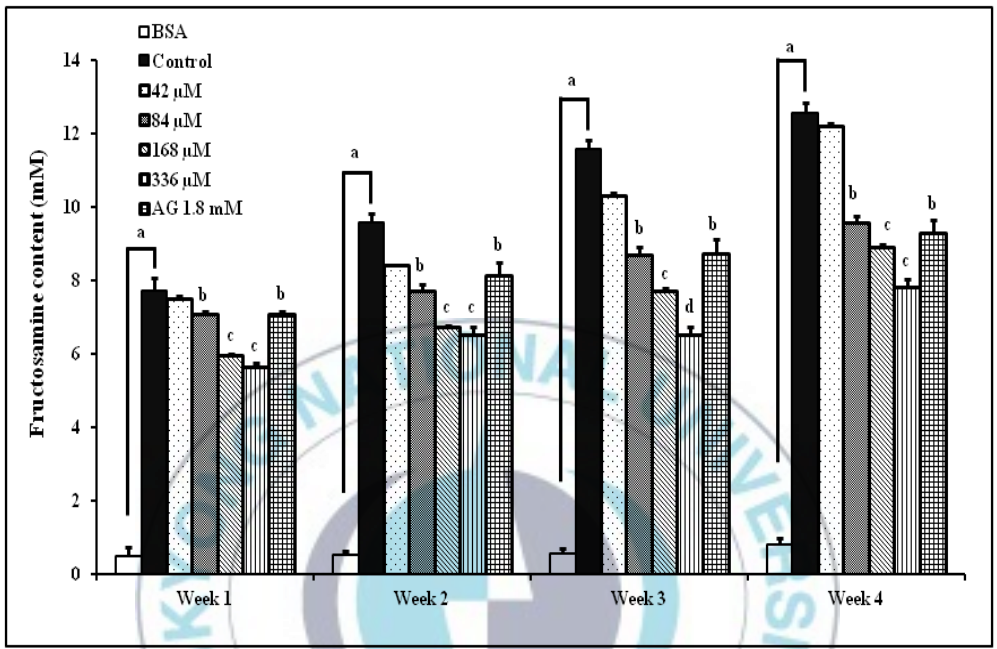



**Figure 12.** The inhibitory effect of vicenin 2 and aminoguanidine (AG) on the formation of fructosamine in BSA-glcuose-fructose system. Results are expressed mean ± SEM of duplicate experiments. [a] $p < 0.001$ when compared to BSA; [b] $p < 0.05$, [c] $p < 0.01$, [d] $p < 0.001$ when compared to BSA-glucose-fructose.

### 3-5-4. Effect of vicenin 2 on CML formation

Glucose-fructose-induced glycated BSA exhibited a 4.0 fold increase in CML formation (Figure 13), when compared to non-glycated BSA at week 4. However, treatment with vicenin 2 inhibited CML production in a concentration-dependent manner. The results showed that vicenin 2 treatment at concentrations of 42, 84, 168, and 336 μM significantly inhibited the formation of CML by 8.0%, 18.5%, 53.2%, and 61.3%, respectively at 4 week. Likewise, AG significantly reduced the level of CML by about 21.1% at a concentration of 1.8 mM.

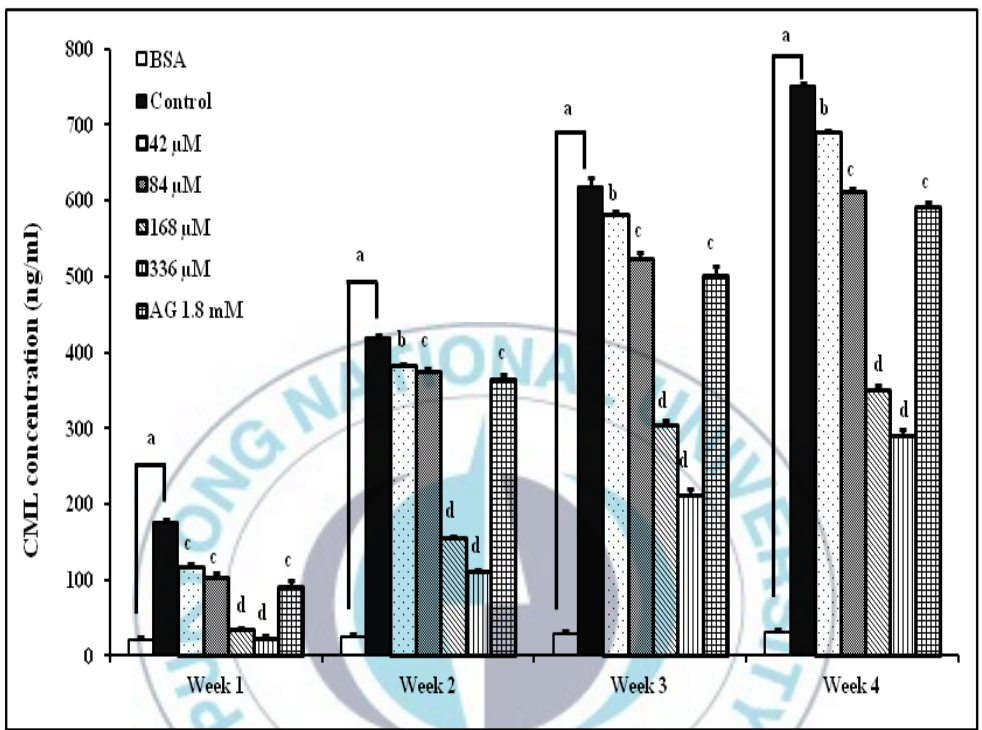



**Figure 13.** The inhibitory effect of vicenin 2 and aminoguanidine (AG) on the formation of ($N^{\varepsilon}$- carboxymethyl) lysine (CML) BSA-glcuose-fructose system. Results are expressed mean ± SEM of duplicate experiments. [a] $p <$ 0.001 when compared to BSA; [b] $p < 0.05$, [c] $p < 0.01$, [d] $p < 0.001$ when compared to BSA-glucose-fructose.

### 3-5-5. Effect of vicenin 2 on carbonyl formation

As shown in Figure 14, with the increase of incubation time the carbonyl content of glycated BSA was found to be significantly higher compared to the non-glycated BSA. The magnitude of increase in carbonyl content was approximately 7.1 fold in the BSA-glcuose-fructose system at week 4 compared to the nonglycated BSA. At week 4 of incubation, vicenin 2 significantly reduced the level of protein carbonyl by 20.9%, 28.4%, 40.0%, and 42.73% in BSA-glucose-fructose at concentrations of 42, 84, 168, and 336 μM, respectively. Aminoguanidine also decreased the protein carbonyl content by 33.1% at a concentration of 1.8 mM.

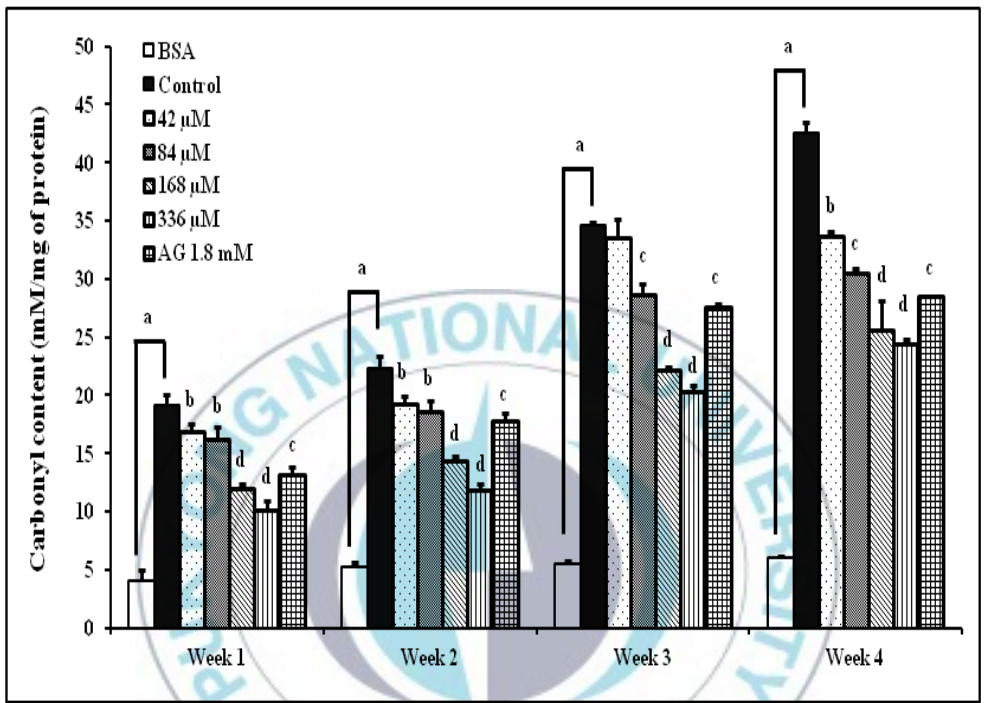



**Figure 14.** The inhibitory effect of vicenin 2 and aminoguanidine (AG) on the protein carbonyl content in BSA-glcuose-fructose system. Results are expressed mean ± SEM of duplicate experiments. [a] $p < 0.001$ when compared to BSA; [b] $p < 0.05$, [c] $p < 0.01$, [d] $p < 0.001$ when compared to BSA-glucose-fructose.

### 3-5-6. Effect of vicenin 2 on thiol oxidation

As it is shown in the Figure 15, glucose-fructose mediated glycation of BSA caused a gradual decrease in thiol groups in BSA depending on the incubation time, and at week 4 it showed about 63.9% thiol loss in BSA. However, treatment with vicenin 2 significantly prevented loss of thiol groups in a concentration–dependent manner. At week 4, vicenin 2 inhibited loss of thiol groups by 53.9%, 61.9%, 67.5%, and 83.3% at concentrations of 42, 84, 168, and 336 μM, respectively. There was also a significant improvement in the level of thiol after addition of aminoguanidine which prevented thiol loss by 59.1% at a concentration of 1.8 mM.

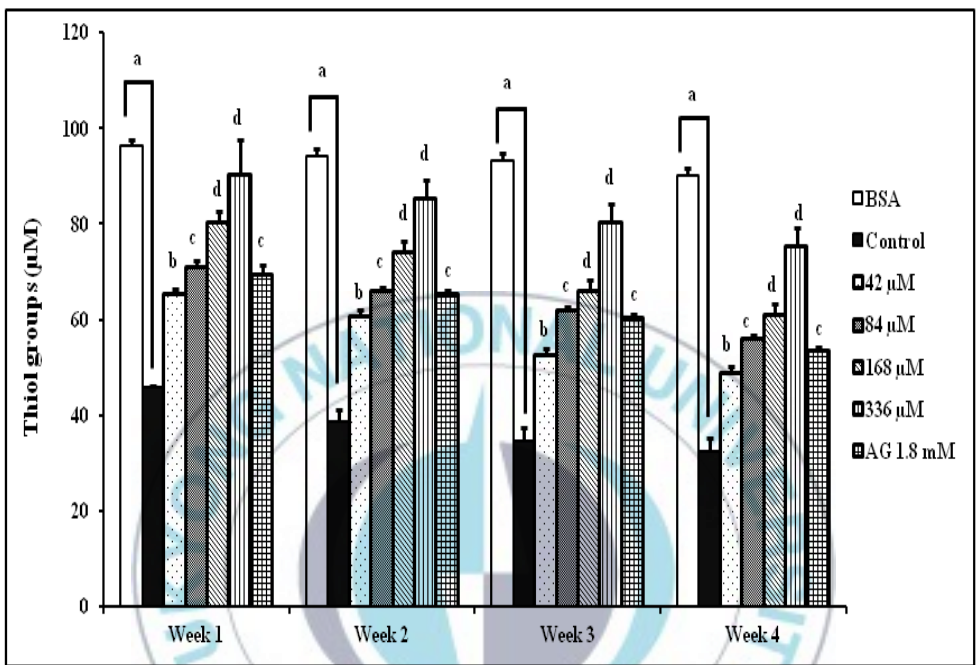



**Figure 15.** The inhibitory effect of vicenin 2 and aminoguanidine (AG) on thiol formation in BSA-glcuose-fructose system. Results are expressed mean ± SEM of duplicate experiments. [a] $p < 0.001$ when compared to BSA; [b] $p < 0.05$, [c] $p < 0.01$, [d] $p < 0.001$ when compared to BSA-glucose-fructose.

### 3-5-7. Effect of vicenin 2 on amyloid cross β-structures

As shown in Figure 16, compared with non-glycated BSA, the glycated BSA exhibited elevated amyloid cross-β conformation evidenced by gradual increase in fluorescence with increase in incubation time. At week 4, the glycated BSA exhibited 3.3 fold increase of the amyloid cross-β conformation compared to the nonglycated BSA. However, treatment with vicenin 2 reduced the level of amyloid cross-β structure by 20.8%, 33.4%, 62.5%, and 73.1% at concentrations of 42, 84, 168, and 336 μM, respectively. Similarly, aminoguanidine also decreased the level of amyloid cross-β structure by 26.7% at a concentration of 1.8 mM.

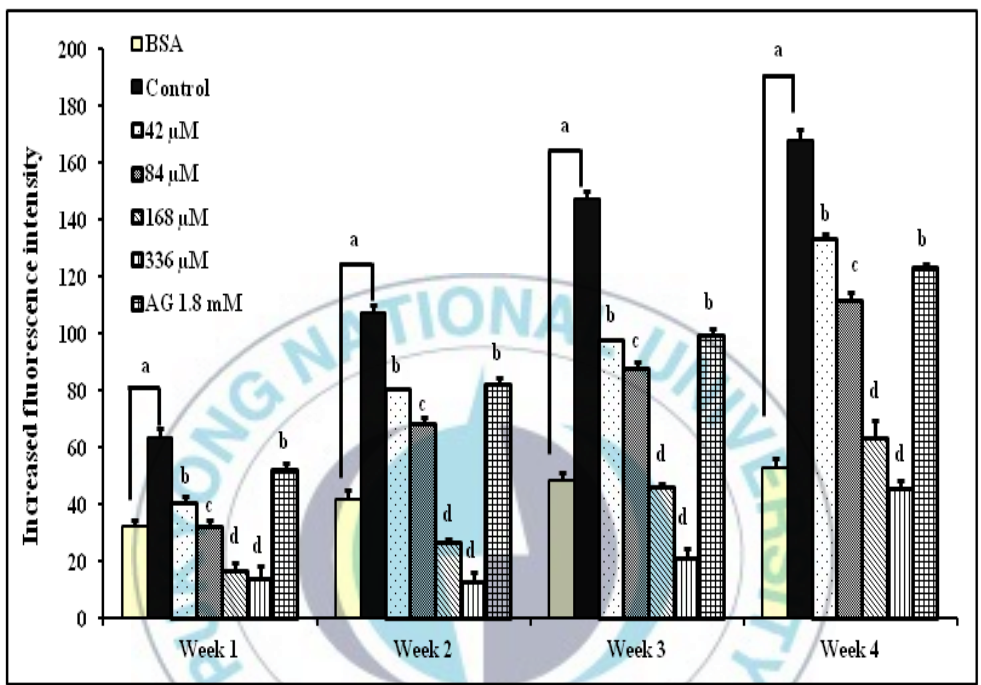



**Figure 16.** The inhibitory effect of vicenin 2 and aminoguanidine (AG) on the formation of amyloid cross β-structures in BSA-glcuose-fructose system. Results are expressed mean ± SEM of duplicate experiments. [a] $p < 0.001$ when compared to BSA; [b] $p < 0.05$, [c] $p < 0.01$, [d] $p < 0.001$ when compared to BSA-glucose-fructose.

### 3-5-8. Discussion

Vicenin 2 is a 6,8-di-*C*-glucoside of apigenin which has been isolated from *A. capillaris.* It was also previously reported in many other species including *Citrus aurantium*, *Peperomia blanda*, *Ocimum sanctum*, *Perilla frutescens, Urtica circularis, Potentilla discolor* etc. Several beneficial effects such as anti-oxidant, anti-hepatotoxic, trypanocidal, antispasmodic, improvement of functional gastrointestinal discomfort, and anti-nociceptive effects of vicenin 2 have been reported in many investigations (Kerstin et al., 1992; Grael et al., 2005; Eugen et al., 2013; Buchwald-Werner and Fujii, 2012). Recently, vicenin 2 which is a major compound of *Urtica circularis* inhibited the production of inflammatory mediators such as NO and TNF-α *via* the inhibition of NF-κB, and thereby, it showed potential anti-inflammatory effect in carrageen-induced rat paw edema model (Marrassini et al., 2011). Vicenin 2, isolated from a traditionally used medicinal herb *Ocimum sanctum*, also induced anti-proliferative, anti-angiogenic and pro-apoptopic effect on caricinoma of prostate cells. In combination with docetaxel, a drug of choice for the treatment of androgen independent carcinoma of prostate, it synergistically inhibited the growth of prostate tumors *in vivo* with a greater extent than single administration (Lokesh et al.,

2011). Although there have been many potential health benefits of vicenin 2 reported, there is no any study concerning its anti-diabetic potential till now. Therefore, in the present study we evaluated the anti-diabetic potential of vicenin 2 *via* α-glucosidase, PTP1B, RLAR and AGE formation inhibitory assays.

AGE are a complex group of compounds formed via a nonenzymatic reaction between reducing sugars and amine residues on proteins, lipids, or nucleic acids. This reaction initiates a complex cascade of condensations, rearrangements, fragmentations, and oxidative modifications that lead to poorly characterized heterogeneous products often collectively termed as AGE (Brownlee, 2001; Booth et al., 1997). Hyperglycemia-induced over production of AGE and their subsequent accumulation in tissues have been implicated in the pathogenesis of various maladies such as diabetic vascular disease (Jakus and Rietbrock, 2004), connective tissue diseases (Takahashi et al., 1994), neurodegenerative Alzheimer's disease (Munch et al., 1997) and end-stage renal disease (Ahmed, 2005; Bucala and Cerami, 1992). Several mechanisms have been proposed by which AGE lead to diabetic complications such as the accumulation of AGE in the extracellular matrix causing aberrant cross-linking, intracellular AGE formation directly alter

protein functions in cells that do not require insulin for glucose transport such as microvascular endothelial cells and neurons, and the binding of circulating AGE to the receptor of AGE (RAGE) on different cell types alters the level of gene expression *via* activation of key cell signaling pathways involved in vascular or neural pathology, by generation of reactive oxygen species and activation of transcription factor NFκB (Maya et al., 2008).

In the early stages of glycation, unstable Schiff's bases are formed and turned into Amadori products such as fructosamine, which is clinically used as an indicator for short-term control of blood sugar in diabetic patients (Ardestani and Yazdanparast, 2007). Reduction of fructosamine, therefore, is a therapeutic way to delay incident vascular complications (Shield et al., 1994). In the present study we found that vicenin 2 dose dependently inhibited fructosamine formation in BSA-glucose-fructose system. Interestingly, vicenin 2 showed much stronger inhibitory activity than the positive control aminoguanidine.

Furthermore, $N^{\varepsilon}$-(carboxymethyl) lysine (CML) can be generated either from oxidative breakdown of Amodori product (Wu et al., 2011) or polyol pathway mediated by α-oxoaldehydes such as glyoxal, methylglyoxal, and

3-deoxyglucosone (Sing et al., 2001). CML has been used as a biomarker for the formation of non-fluorescent AGE. The formation of CML was quantitatively measured by ELISA kit. It is evident from the Figure 13, that treatment with vicenin 2 strongly suppressed CML production induced by glucose-fructose mediated glycation in BSA.

Carbonyl content and thiol group formation was assessed as indicators of protein oxidation during the glycation process. Glycation is not only a major cause of AGE-mediated protein modification, but it also induces oxidation-dependent tissue damage, leading to development of complications of diseases including diabetes (Hunt and Wolff, 1991; Kalousová et al., 2002). An increase in carbonyl content and the loss of free thiol groups by modification of cysteine residues is a direct reflection of protein oxidation of BSA (Aćimović et al., 2009; Dalle-Donne et al., 2003). Reactive oxygen species generated during glycation and glyoxidation, such as superoxide, are able to oxidize side chains of amino acid residues in protein to form carbonyl derivatives and also diminish an oxidative defense of protein by decreasing thiol groups, leading to damage of cellular proteins (Balu et al., 2005). Apart from a significant role in directly resisting against these free radicals, most antioxidants also possess the ability to inhibit oxidative

damage-mediated glycation and glycoxidation by modulating abnormal levels of protein carbonyls and thiol groups (Ardestani and Yazdanparast, 2007; Balu et al., 2005). As can be seen in Figure 14 and 15, glucose-fructose-induced glycation of BSA increased carbonyl formation with concomitant loss of protein thiol groups. However, treatment with vicenin 2 or aminoguanidine suppressed carbonyl formation with simultaneous prevention against thiol loss. Interestingly, vicenin 2 has been found to be more potent than the positive control aminoguanidine.

The thioflavin T assay is commonly used to quantify the amount of a protein modification called amyloid cross-β structure in glycated BSA. Protein glycation is an important factor affecting conformational changes of polypeptides, especially the amyloid cross-β structure (Bouma et al., 2003). Amyloid cross-β is an aggregated structure of protein that accumulates as deposited fibrils in the brains of patients with glycation-related diseases such an Alzheimer's (Hardy and Selkoe, 2002; Khazaei et al., 2010). Moreover, β-amyloid deposits in the pancreas have also been found to be pathologic lesions in pancreatic β-cells of type 2 diabetic patients (Marzban et al., 2005). Notably, accumulation of protein aggregation induces pancreatic islet amyloidosis, which directly damages β-cell and impairs

insulin secretion (Tokuyama et al., 1997; Marzban and Verchere, 2004). As shown in the Figure 16, the fluorescent intensity of BSA-glucose-fructose system was gradually increased over time compared to only BSA which indicated the formation amyloid cross β-structures in the system. In the present study, we found that vicenin 2 strongly inhibited the generation of amyloid cross β-structures in glycated BSA.

Finally, vicenin 2 has been found as a potent inhibitor of α-glucosidase, PTP1B and aldose reductase. In addition, vicenin 2 inhibited the formation of both fluorescent AGE and non-fluorescent AGE e.g. CML, as well as the level of fructosamine. Vicenin 2 also decreased the formation of protein carbonyl and inhibited the modification of protein thiol groups, and thereby it suppressed glycation-induced protein oxidation. Furthermore, vicenin 2 suppressed the formation of β-cross amyloid structures of BSA. Taken together, vicenin 2 might be a new lead for the development of therapeutic modalities for the treatment of diabetes and associated complications.

# IV. Conclusion

In conclusion, twelve species of the genus *Artemisia* were selected and their inhibitory potential against α-glucosidase, PTP1B, and AGE formation was screened in order to evaluate the anti-diabetic potential of the genus. Several *Artemisia* species were found as potent α-glucosidase, PTP1B, and AGE formation inhibitors. However, *A. capillaris* as well as its isolated constituents showed the most potent α-glucosidase and AGE formation inhibitory activities along with strong PTP1B inhibitory activities. Moreover, MeOH extract as well as different solvent soluble fractions showed strong antioxidant and RLAR inhibitory activities. Chromatographic separation of *A. capillaris* extract yielded a new acetylated trioside namely acacetin-7-*O*-(6″-*O*-acetyl)-β-D-glucopyranosyl-(1→2)[α-L-rhamnopyranosyl]-(1→6)-β-D-glucopyranoside along with 45 known compounds including different types of coumarins, flavonoid, caffeoylquinic acid and phenolic derivatives. In addition, three coumarins (fraxidin, 5,6,7-trimethoxy coumarin, and tomerin) and four flavonoids (linarin, chrysoeriol-7-*O*-rutinoside, quercetin 7-*O*-galatopyranosyl-7-*O*-rhamnoside, and isorhamnetin-3-*O*-arabinopyranoside) along with six phenolics (paramethoxy phenyl glucoside,

eugenol glucoside, koaburaside, 4-hydroxy-3-*O*-glucopyranosyl benzoic acid, neochlorogenic acid, and cryptochlorogenic acid) have been isolated first time from *A. capillaris*. Among the compounds, esculetin, quercetin, isorhamnetin, cirsilineol, scopoletin, daphnetin and umbelliferone showed potential α-glucosidase and PTP1B inhibitory activities. In addition, many of the compounds such as esculetin, umbelliferone, esculin, scopoletin, isoscopoletin, cirsilineol, cirsimaritinm, arcapillin, hyperoside, and caffeoylquinic acid derivatives were found as potent inhibitors of AGE formation. Besides, anti-diabetic activity of two essential oil derivatives, capillin and capillinol, have been reported for the first time. Moreover, promising anti-diabetic potential of vicenin 2 and its mechanisms underlying inhibition of AGE formation have been reported first time. Therefore, it can be concluded that the presence of these constituents was directly attributed to the potent α-glucosidase, PTP1B, RLAR and AGE formation inhibitory activities of *A. capillaris*. Therefore, *A. capillaris* as well as its isolated constituents could be further explored for the development of novel therapeutic or preventive agents for the treatment of diabetes and diabetes associated complications.

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