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Thesis for the Degree of Master of Science

In vitro anti-diabetic and anti-diabetic complications of *Crataegus pinnatifida*

By

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Department of Food and Life Science

The Graduate School

Pukyong National University

August 2012

In vitro anti-diabetic and anti-diabetic complications of *Crataegus pinnatifida* (산사의 항당뇨 및 항당뇨 합병증 효과)

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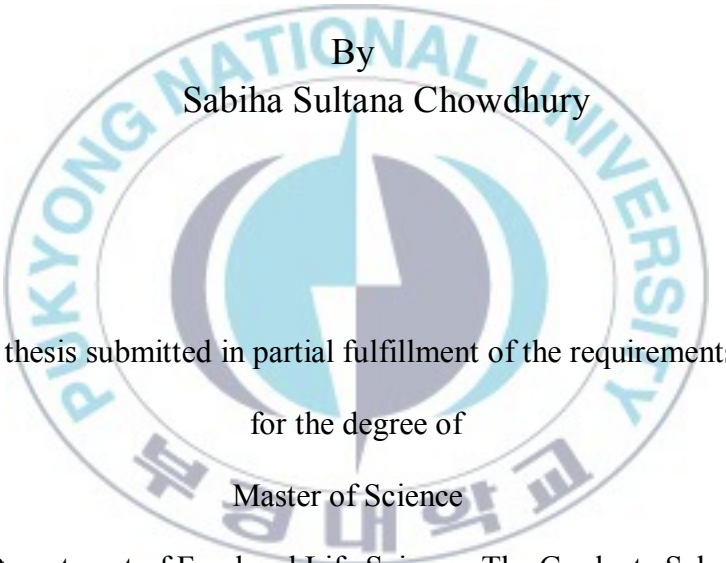

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August 2012

In vitro anti-diabetic and anti-diabetic

complications of

Crataegus pinnatifida

A Dissertation

By

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August 25, 2012

PUKYONG NATIONAL UNIVERSITY 부경대학교

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List of Abbreviations and Symbols

C. pinnatifida	: *Crataegus pinnatifida*
^{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	: herz (sec^{-1})
RP	: reverse phase
DMSO	: dimethyl sulfoxide
DMSO-d_6	: deuterium dimethyl sulfoxide
Fig	: Figure
J	: coupling constant (Hz)
δ	: chemical shift

In vitro anti-diabetic and anti-diabetic complications of *Crataegus pinnatifida*

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Abstract

Crataegus pinnatifida Bge. known as Chinese hawthorn or Shanzha belongs to the family Rosaceae, is widely distributed throughout the northern temperate regions of the world with approximately 280 species, primarily in East Asia, Europe and North America. Traditionally, preparations of leaves and fruits of *C. pinnatifida* have been used in the treatment of chronic heart failure, high blood pressure, digestive disorders, arrhythmia, and arteriosclerosis. Currently, the fruits of *C. pinnatifida* have drawn much attention in the field of food, nutraceuticals, and medicine because of its widely reported health benefits, such as lipid lowering activity, antioxidative, free radical scavenging, anti-inflammatory, vasorelaxating, and hypolipidemic effects attributed to its high contents of polyphenols, flavonoids, procyanidines as well as triterpenes. The present study investigated the anti-diabetic potential of the MeOH extract as well as its different solvent soluble fractions of the fruits of *C. pinnatifida* via α-glucosidase, protei0 tyrosine phosphatase 1B (PTP 1B), rat

lens aldose reductase (RLAR) and advanced glycation end products (AGEs) formation inhibitory assays. The MeOH extract showed potent inhibitory activity against α-glucosidase, PTP 1B, and AGEs formation with IC_{50} values of 122.11, 3.66 and 65.83 μg/mL, respectively, while it showed moderate inhibitory activity against RLAR with an IC_{50} value of 160.54 μg/mL. Among the tested fractions, the ethyl acetate (EtOAc) fraction exhibited highest α-glucosidase, PTP 1B and RLAR inhibitory activity with corresponding IC_{50} values of 22.70, 1.41 and 9.09 μg/mL while dichloromethane (CH_2Cl_2) fraction showed the highest AGEs formation inhibitory activity with an IC_{50} value of 80.53 μg/mL. Moreover, CH_2Cl_2 fraction also showed good inhibitory activity against α-glucosidase and PTP 1B inhibitory assays with IC_{50} values of 45.86 and 13.32 μg/mL, respectively. Besides, *n*-butanol (*n*-BuOH) fraction exhibited moderate PTP 1B and RLAR inhibitory activity with IC_{50} values of 18.75 and 68.60 μg/mL, respectively. In contrast, the water (H_2O) fraction showed inhibitory activity only against PTP 1B with IC_{50} value of 15.84 μg/mL. In order to isolate compounds from two active fractions, the CH_2Cl_2 fraction and EtOAc fractions were subjected to repeated column chromatography. Repeated column chromatography of the EtOAc fraction yielded hyperoside and chlorogenic acid while CH_2Cl_2 fraction yielded three triterpenic acid including ursolic acid, oleanolic acid and 3-epicorosolic acid along with *β*-sitosterol and *β*-sitosterol glucoside. Among the compounds, 3-epicorosolic acid has been isolated first time from the fruits of *C. pinnatifda*. Since all the

compounds except 3-epicorosolic acid and their bioactivities are already reported we selected 3-epicorosolic acid for further investigation. 3-Epicorosolic acid showed potent α-glucosidase inhibitory activity with IC_{50} value 30.18 μg/mL (63.84 μM) compared to the positive control acarbose with an IC_{50} value of 92.8 μg/mL (143.74 μM). On the other hand, it also exhibited potent PTP 1B inhibitory activity with an IC_{50} value of 4.08 μg/mL (8.63 μM) compared to the positive control ursolic acid with an IC_{50} value of 1.17 μg/mL (2.56 μM). Moreover, kinetic study revealed that 3-epicorosolic acid showed mixed type inhibition against PTP 1B while it showed uncompetitive inhibition against α-glucosidase. Therefore, potential anti-diabetic activity of the fruits of *C. pinnatifida* is possibly attributed to the presence of flavonoids as well as triterpenes which might be used as functional food for the treatment of diabetes and diabetes related complications. Moreover, 3-epicorosolic acid showed very strong inhibitory activity against both α-glucosidase and PTP 1B which could be explored further in order to develop therapeutic agents for the treatment of type 2 diabetes mellitus.

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I. Introduction

Natural products continued to play a highly significant role in the drug discovery and development process in health care and prevention of diseases. The ancient civilizations of the Chinese, Indians and North Africans provide written evidence for the use of natural sources for curing various diseases (Phillipson, 2001). However, it was not until the nineteenth century that scientists isolated active components from various medicinal plants. Friedrich Sertürner isolated morphine from *Papaver somniferum* in 1806, and since then natural products have been extensively screened for their medicinal purposes (Phillipson, 1995).

Fabricant and Fransworth describe the goals of using plants as sources of therapeutic agents are (a) to isolate bioactive compounds for direct use as drugs, e.g., digoxin, digitoxin, morphine, reserpine, taxol, vinblastine, vincristine; (b) to produce bioactive compounds of novel or known structures as lead compounds for semisynthesis to produce patentable entities of higher activity and/or lower toxicity, e.g., metformin, nabilone, oxycodon (and other narcotic analgesics), taxotere, teniposide, verapamil, and amiodarone, which are based, respectively, on galegine, Δ9-

tetrahydrocannabinol, morphine, taxol, podophyllotoxin, khellin, and khellin; (c) to use agents as pharmacologic tools, e.g., lysergic acid diethylamide, mescaline, yohimbine; and d) to use the whole plant or part of it as a herbal remedy, e.g., cranberry, echinacea, feverfew, garlic, ginkgo biloba, St. John's wort, saw Palmetto (Fabricant and Fransworth, 2001).

Many historical examples showed that the natural product has not just been the medicinal product but has also helped reveal a novel aspect of physiology. For example, digitalis from foxglove showed the role of sodium-potassium-ATPase; morphine pointed the way to the receptors affected by endogenous opioids; muscarine, nicotine and tubocurarine helped explore the different types of acetylcholine receptors, and so on (Sneader, 1996; Rishton, 2008). The natural products and structures derivatin from or related to natural products from all sources have played and continue to play in the development of the current therapeutic armamentarium of the physician. Most recently, Newman and Cragg reviewed the drugs introduced on the market over the last 25 years with emphasis on contributions by natural products. Despite these efforts the number of new chemical entities reaching the market has not increased. Only one drug originated from a de novo combinatorial chemistry approach.

However, the natural products remain an important source of structures contributing to mostly semi-synthetic or synthetic drugs in all disease areas. They also identified the 14 small molecule antiparasitic drugs have been approved over the years among which only four are synthetic (28.5%) and of the rest, three are artemisinin derivatives (Newman and Cragg, 2007). It is, however, arguably still true: comparisons of the information presented on sources of new drugs from 1981 to 2007 indicate that almost half of the drugs approved since 1994 are based on natural products. Thirteen natural-product- related drugs were approved from 2005 to 2007 ,and, as pointed out by Butler, five of these represented the first members of new classes of drugs: the peptides exenatide and ziconotide, and the small molecules ixabepilone, retapamulin and trabectedin (Newman and Cragg, 2007; Butler, 2008). These natural products get much attention due to their less side effects. They covered a range of therapeutic indications: anti-cancer, anti-infective, anti-diabetic, among others, and they show a great diversity of chemical structures (Ganesan, 2008).

Crataegus pinnatifida Bge. belongs to the family Rosaceae, also known as Chinese hawthorn or ShanZha , have long been used in traditional Chinese medicine and European herbal medicine (Ammon and Händel,

1981b; Chang et al., 2006). It is widely distributed throughout the northern temperate regions of the world with approximately 280 species, primarily in East Asia, Europe and North America (Zhang et al., 2002). It has been catalogued in Compendium of Materia Medica, a classic traditional Chinese medical book written by Li Shishen in Ming Dynasty, where it described as a medicinal plant to improve digestion, remove retention of food, promote blood circulation and resolve blood stasis (Chu et al., 2003).

In China and the European countries, various species of hawthorn have been widely used as medicinal and food materials, among which *Crataegus monogyna* and *Crataegus lavigata* are the major hawthorn species in Europe, and *Crataegus pinnatifida* and *Crataegus scabrifolia* in China (Fong & Bauman, 2002; Zhao & Tian, 1996). The European hawthorn species are often grown for their leaves and flowers which are used as raw materials of herbal medicines and as fencing or ornamental trees in western countries whereas the Chinese hawthorn species are commonly cultivated for their fruits, which are consumed fresh or after being processed into juice and jams (Liu et al., 2010). Although many varieties and cultivars of the genus *Crataegus* are available, *C. pinnatifida* is the most commercially important species due to its large and delicious fruits (Zhao

and Tian, 1996).

In China, hawthorn fruits have long been consumed naturally and used as raw materials in the food industry and in traditional Chinese medicine (TCM) (Chang et al., 2002). This popular fruit is not only consumed as a fresh, dried fruit but also utilized in jams, juices, and tinned foods, and also a basic ingredient for making wines and for various sweet foods (Gao et al., 1995). Traditionally, the fruits of *C. pinnatifida* have been used as medicine in the treatment of chronic heart failure, high blood pressure and various digestive ailments and as peptic agent (Chu et al., 2003). It has also been demonstrated that extracts of hawthorn fruits and leaves are safe for human consumption (Daniele et al., 2006). Pharmacological and toxicological studies have revealed that consumption of hawthorn fruits is associated with long-term medicinal benefits to cardiovascular function with little side effect (Ammon & Handel, 1981a, 1981b, 1981c). Several studies have been shown to increase myocardial contractility, reduce reperfusion arrhythmias, dilate peripheral arteries, and mildly decrease blood pressure (Ammon and Kaul, 1994). Moreover, ShanZha significantly inhibited the biosynthesis of thromboxane A2 and the platelet adhesion to result in the prevention of atheroma and/or thrombosis

(Vibes et al., 1994). ShanZha extract has also been used to treat the early stage of congestive heart failure (Weihmayr and Ernst, 1996).

Hawthorn species (Crataegus spp.) have recently attracted increasing attention in the field of food, nutraceuticals, and medicine because of their widely reported health benefits. Hawthorn fruit extract has been shown to have many health benefits including being cardiovascular protective, hypotensive and hypocholesterolemic (Zhang et al., 2001). Apart from the fruits, Crataegus leaves have been shown to possess beneficial effects on cardiovascular diseases. In certain patients Crataegus leaves with flower extract was shown to reduce the incidence of sudden cardiac death (Holubarsch et al., 2008) and be effective in treating chronic heart failure (Pittler et al., 2008). Preparations of leaves or fruits of *C. pinnatifida* is believed to improve the heart function in a situation of declining cardiac performance, deficiency in coronary blood supply, and mild forms of arrhythmia (Popping et al., 1995, Al Makdessi et al., 1996). It has documented that the hot water extract exhibited a capacity to quench free radicals and an inhibitory effect on low-density lipoprotein (LDL) oxidation in both cell and cell-free systems, decreased the serum total cholesterol, LDL-C, and triglycerides in hyperlipidemic humans (Chu et al., 2003; Chen

et al., 1995). Kao et al., demonstrated that *C. pinnatifida* exhibited antioxidant and anti-inflammatory potential and possesses potential as a cancer chemo-preventive agent against tumor promotion (Kao et al., 2005; Kao et al., 2007). Report showed that consumption of hawthorn also altered the digestive enzymes of the stomach and cholesterol metabolism of the liver. The recent studies showed that ShanZha has an ability to reduce the body weight and improve the dyslipidemia and suppress high cholesterol diet induced hypercholesterolemia in animal (Kuoa et al., 2009; Kwok et al., 2010).

Several studies showed the isolation of a series of diverse compounds from hawthorn, including flavonoids, terpenoids and organic acids. More than 170 compounds already have been isolated from Crataegus species (Chen & Song, 2005; Dauguet et al.,1993; Gao et al., 2010; Nikolov et al., 1982). The major components include flavonoids, proanthocyanidin, triterpenes, organic acids, tannin, flavane and its polymers, among which flavonoids and triterpenes were reported as the main active hypolipidemic constituents (Zhang et al., 2002; Azuma et al., 2000; Min et al., 2008; Lin et al., 2009). In addition, hawthorn contains abundant amount of anti-oxidants such as chlorogenic acid, epicatechin, hyperoside and quercetin (Liu et al.,

2010) which may be useful in alleviating the adverse effects associated with low-density lipoprotein (LDL)-cholesterol oxidation in atherosclerosis (Schwinger et al., 2000; Stocker & Keaney, 2004).

The rapidly increasing diabetes mellitus is becoming a serious threat to mankind health in all parts of the world. Diabetes mellitus (DM) is a group of metabolic diseases characterized by abnormally high plasma glucose levels resulting from the impaired action or absolute deficiency of insulin leads to imbalance of glucose metabolism (Kumar et al., 2011; Wright et al., 2006). There are mainly two types of diabetes, namely type 1 and type 2. Type 1, insulin-dependent diabetes mellitus (IDDM), in which the body does not produce any insulin, most often occurs in children and young adults. People with type 1 diabetes must take daily insulin injections to stay alive. Type 1 diabetes accounts for 5–10% of diabetes. Type 2, noninsulin-dependent diabetes mellitus (NIDDM), in which the body does not produce enough, or properly use, insulin, is the most common form of the disease, accounting for 90–95% of diabetes. Type 2 diabetes is nearing epidemic proportions, due to an increased number of elderly people, and a greater prevalence of obesity and sedentary lifestyles (Li et al., 2004).The injurious effects of DM associated with macrovascular complications

(coronary artery disease, peripheral arterial disease, and stroke) and microvascular complications (diabetic nephropathy, neuropathy, and retinopathy) becoming the major causes of morbidity and death every year (Fowler, 2008; Berger et al., 2011). It is affecting about 220 million people worldwide and projected to become one of the world's main disablers and killers within next 25 years (Ghosh et al., 2011; King et al., 1998; Zhang et al., 2010). Asia and Africa are the most affected areas where DM rates are expected to rise by two or three folds by 2030 (Shaw et al., 2010). Postprandial hyperglycemias, associated with cardiovascular complications, also play an important role in the development of type 2 DM (Baron, 1998). Thus, in the treatment of diabetes and cardiovascular complications, control of postprandial hyperglycemia has shown to be important.

The treatment of DM is considered as the main global problem and successful treatment has yet to be discovered. Varieties of new pharmacological treatments have been developed in the past 5 years to treat diabetes mellitus along with strategies along with strategies dealing to diet management and exercise. There are mainly two categories of drugs are available in the market for the treatment of diabetes mellitus, *i.e.* insulin and oral hypoglycemic agents. Even though insulin therapy and oral diabetes

mellitus they have some side effects and fail to significantly alter the course of diabetic complications (Venkatesh et al., 2010). In the management of diabetes, continuous use of synthetic agents available should be limited because of their serious side effects such as hepatotoxicity, abdominal pain, flatulence, diarrhoea, and hypoglycemia (Fujisawa et al., 2005; Singh et al., 2008). Some reports also showed an increase incidence of renal tumors, serious hepatic injury, and acute hepatitis (Diaz-Gutierrez et al., 1998; Charpentier et al., 2000). Thus, search for improved, potent and safe natural antidiabetic agent is the prime research area till now and the World Health Organization also encouraged the development of herbal medicines in this regard.

Besides the insulin therapy to reduce high glucose level and maintain normoglycemia, several therapeutic approaches have been proposed such as i) Inhibition of α-glucosidase activity, ii) inhibition of AGEs (Advanced Glycation End Products) formation, iii) inhibition of aldose reductase (AR) activity, iv) inhibition of protein tyrosine phosphatase 1B (PTP 1B) activity. α-glucosidase is the key enzyme catalyzing the final step in the digestive process of carbohydrates. α-glucosidase catalyzes the final step in the digestive process of carbohydrates and inhibition of α-glucosidase delaying

carbohydrate digestion absorption, resulting in reduced postprandial hyperglycemia (Niwa et al., 2003). PTP1B, a negative regulator of insulin signaling, has been considered as another important therapeutic strategy for the treatment of type 2 DM (Kennedy, 1999).The increased flux through the polyol pathway and the formation of advanced glycation end-products (AGEs) can cause diabetic complications (Brwonlee, 2001). Aldose reductase (AR) which is the first enzyme of the polyol pathway reduces the aldehyde form of glucose to sorbitol in the presence of NADPH as a cofactor. Sorbitol dehydrogenase oxidizes sorbitol to fructose in the polyol pathway, leading to loss of the functional integrity of the lens and subsequent cataract formation (Lee, 2002).

Currently available therapeutic options for non-insulin-dependent diabetes mellitus, such as dietary modification, oral hypoglycemics, and insulin, have limitations of their own. Searching alternative, effective and safe anti-diabetic agents is a major thrust area in the mainstream of pharmaceutical research as synthetic anti-diabetic drugs possess numerous side effects. Medicinal plants play a vital role for the development of new drugs. A vast body of literature has accumulated in the recent past linking the role of medicinal plants in the management of diabetes and their efficacy

in the amelioration of secondary complications of diabetes like cataracts (Suryanarayana et al., 2004). Therefore, much interest is being currently devoted to the physiological functions of food components relating to the prevention of diabetes and obesity. Thus our present study is concern about the antidiabetic potential of *C. pinnatifida* fruits as well as the underling mechanism of action.

II. Materials and Methods

1. Plant materials

The fruits of *Crataegus pinnatifida* were purchased from the Korea. A voucher specimen of fruits registered and deposited to the Prof. J. H. Lee, Dongguk University, Gyeongju, South Korea, for future reference.

2. Reagents and equipments

2-1. Reagents

Yeast α-glucosidase, acarbose, *p*-nitrophenyl phosphate (*p*-NPP), *p*-nitrophenyl α-D-glucopyranoside (*p*-NPG), ethylenediaminetetraacetic acid (EDTA), nicotinamide adenine dinucleotide phosphate (NADPH), DL-glyceraldehyde dimer, quercetin, bovine serum albumin (BSA), D-(-)-fructose, D-(+)-glucose, aminoguanidine hydrochloride were purchased from Sigma-Aldrich (St. Louis, MO, 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). All other chemicals and solvents used were

purchased from E. Merck, Fluka, and Sigma-Aldrich, unless otherwise stated.

2-2. Equipments

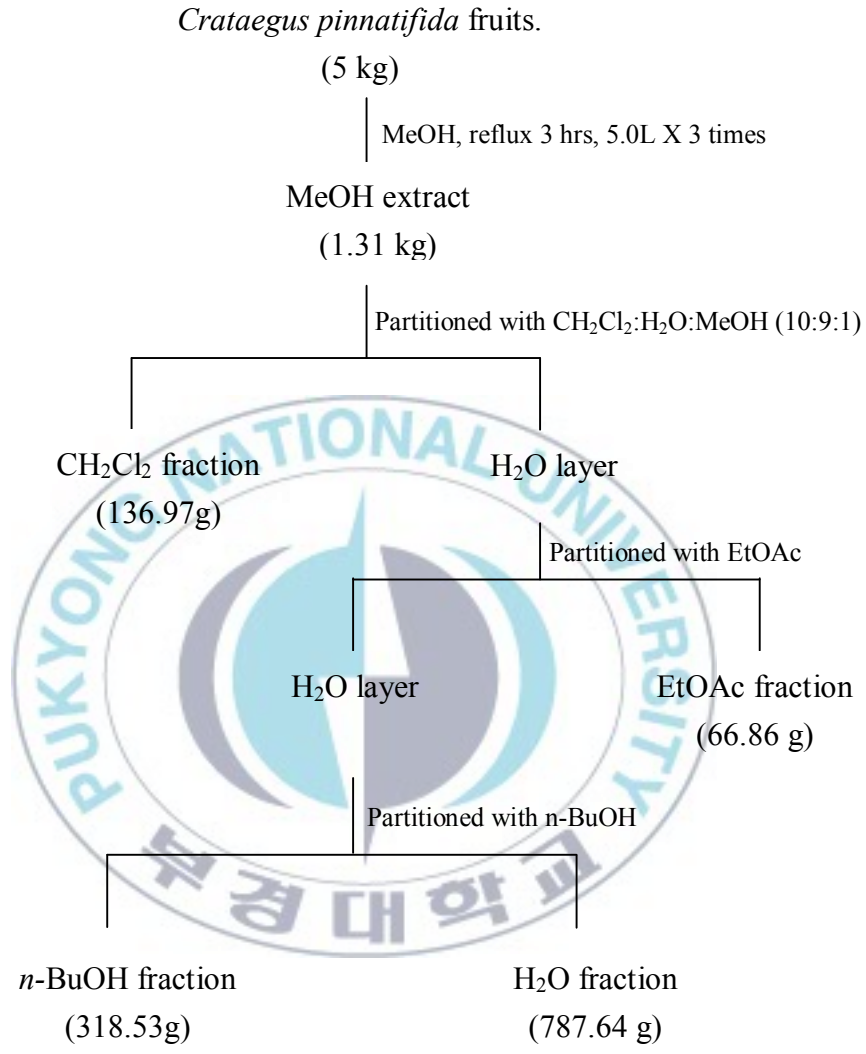
The EI-MS spectra were determined using a Hewlett-Packard 5989B spectrometer (Agilent Technologies) and a JEOL JMS-700 spectrometer. The ^{1}H- and ^{13}C-NMR spectra were measured by a JEOL JNM ECP-400 spectrometer (Tokyo, Japan) at 400 MHz for ^{1}H NMR and 100 MHz for ^{13}C NMR in deuterated dimethylsulfoxide (DMSO-d_6) and methanol (MeOH-d_4). Chemical shifts were referenced to the respective residual solvent peaks (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.

3. Methods

3-1. Extraction and fractionation

The fruits of *C. pinnatifida* (5 kg) were refluxed in MeOH for 3h (5L×3times). The total filtrate was then concentrated to dryness in vacuo at 40º C in order to render the MeOH extract (1.31kg). This extract was suspended in distilled water (H_2O) and then successively partitioned with methylene chloride (CH_2Cl_2), ethyl acetate (EtOAc), and *n*-butanol (BuOH) to yield the CH_2Cl_2 (136.97g), EtOAc (66.86g), and *n*-BuOH (318.53g) fractions, respectively, as well as a H_2O residue (787.64g) as shown in **Scheme 1-**



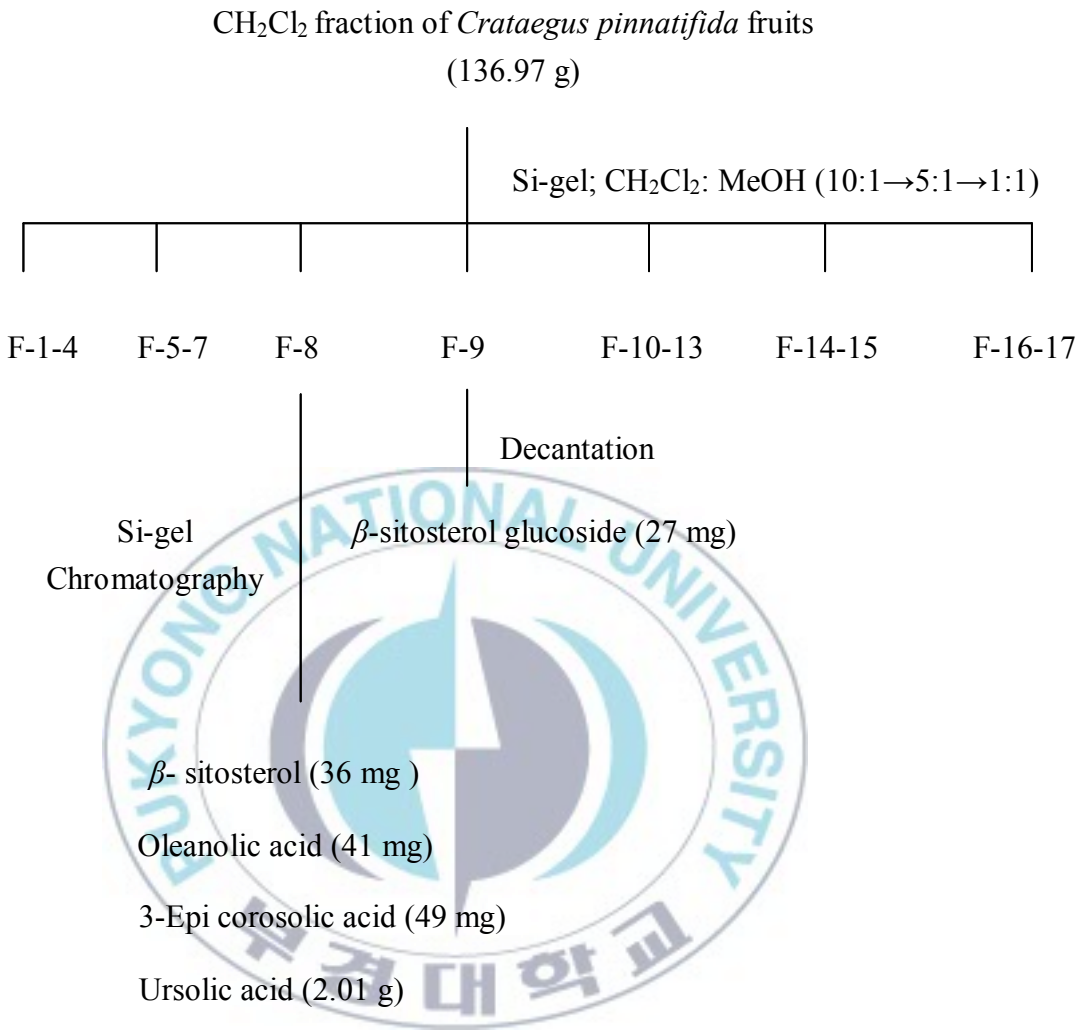
Scheme 1. Extraction and fractionation scheme of *Crataegus pinnatifida* fruits.

3-2. Isolation of the active compounds from CH_2Cl_2 and EtOAc fraction

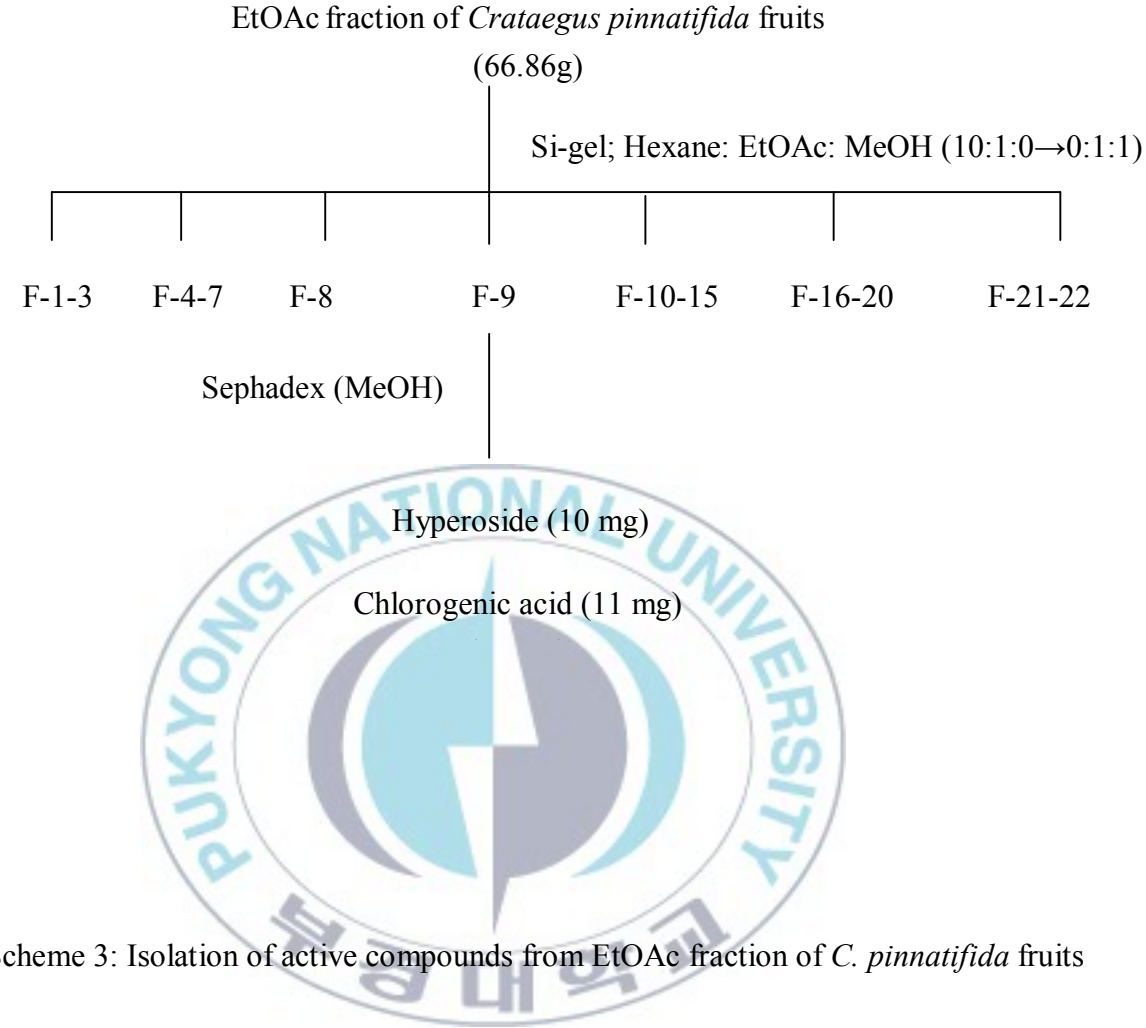
The CH_2Cl_2 fraction (136.97 g) was subjected to column chromatography on a silica gel column with a solvent mixture of CH_2Cl_2-MeOH (10:1→5:1→1:1) to get 17 subfractions (1 to 17). Among them, subfraction 9 was fitered to get precipitate as white powder as *β*-sitosterol glucoside (27 mg). From subfraction 8 *β*-sitosterol (36 mg) was found as white precipitate. Then subfraction fraction 8 was chromatographed over silica gel column using a solvent mixture of Hexane-Acetone (10:1→1:1) to obtain 10 subfractions (8-1 to 3-10). Repeated chromatography of subfractions 8-4 yielded oleanolic acid (41 mg). Subfractions 8-7 was subjected for chromatographed over a silica gel column with a solvent mixture of CH_2Cl_2: EtOAc (100:1→0:1) to get 3-epicorosolic acid (49 mg) and ursolic acid (2.01 g).

The EtOAc fraction was subjected to column chromatography on a silica gel column with a solvent mixture of Hexane-EtOAc→ EtOAc→ EtOAc-MeOH (10:1→0:1→1:1) to get 22 subfractions (1 to 22). Subfraction 9 was chromatographed repeatedly on a silica gel column with a solvent mixture of CH_2Cl_2-MeOH- H_2O (7:1:0.1→0:1:0) to obtain 4 subfractions (9-1 to 9-4). The subfraction 9-2 and 9-4 was chromatographed

on a Sephadex LH-20 column with MeOH to get compounds hyperoside (10 mg) and chlorogenic acid (11mg). Isolation of active compounds from CH_2Cl_2 and EtOAc fraction are given in Scheme 2 and Scheme 3-



Scheme 2: Isolation of active compounds from CH_2Cl_2 fraction of *C. pinnatifida* fruits



Scheme 3: Isolation of active compounds from EtOAc fraction of *C. pinnatifida* fruits

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All the compounds isolated from CH_2Cl_2 and EtOAc fractions 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. The structures are shown in Figure 1 and Figure 2. The respective spectral data of isolated compounds are as follows:

β-Sitosterol:

White needles; ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 0.68 (3H, s, H-18), 0.82 (3H, t, J=6.6 Hz, H-29), 0.83 (3H, d, *J*=6.8 Hz, H-26), 0.84 (3H, d, *J*=6.4 Hz, H-27), 0.92 (3H, d, *J*=6.4 Hz, H-21), 1.01 (3H, s, H-19), 3.52 (1H, m, H-3), 5.35 (1H, br d, *J*=5.4 Hz, H-6); ^{13}C-NMR (75 MHz, $CDCl_3$) δ: 37.2 (C-1), 31.6 (C-2), 71.8 (C-3), 42.3 (C-4), 140.7 (C-5), 121.7 (C-6), 31.9(C-7), 31.9 (C-8), 50.1 (C-9), 36.5 (C-10), 21.1 (C-11), 39.7 (C-12), 42.3 (C-13), 56.7 (C-14), 24.3 (C-15), 28.2 (C-16), 56.0 (C-17), 11.8 (C-18), 19.4 (C-19), 36.1 (C-20), 18.8 (C-21), 33.9 (C-22), 26.0 (C-23), 45.8 (C-24), 29.1 (C-25), 19.8 (C-26), !9.0 (C-27), 23.0 (C-28), 12.0 (C-29).

β-Sitosterol glucoside:

^{1}H NMR (pyridine-d_5, 400 MHz): δ 0.63 (3H, s, Me-18), 0.83–0.90 (9H, m 3× Me-26, 27, 29), 0.91 (3H, s, Me-19), 0.96 (3H, d, J = 7 Hz, Me-21), 0.99–2.73 (36H, m, methylene protons), 3.91–3.99 (2H, m, H-5 , H-3), 4.05 (1H, t, J = 8 Hz, H-2), 4.28 (2H, m, H-3 , 4), 4.39 (1H, dd, J = 12, 5 Hz, H-6 b), 4.55 (1H, dd, J = 12, 2 Hz, H-6 a), 5.04 (1H, d, J = 8, H-1), 5.32–5.33 (1H, m, H-6). ^{13}C NMR (pyridine-d_5, 100 MHz): δ 37.5 (C-1), 30.2 (C-2), 78.0 (C-3), 39.3 (C-4), 140.9 (C-5), 121.9 (C-6), 32.2 (C-7), 32.0 (C-8), 50.3 (C-9), 36.9 (C-10), 21.3 (C-11), 39.9 (C-12), 42.5 (C-13), 56.8 (C-14), 24.5 (C-15), 28.5 (C-16), 56.2 (C-17), 12.0 (C-18), 19.2 (C-19), 36.4 (C-20), 19.0 (C-21), 34.2 (C-22), 26.3 (C-23), 46.0 (C-24), 29.4 (C-25), 20.0 (C-26), 19.4 (C-27), 23.4 (C-28), 12.2 (C-29), 102.6 (C-1), 75.3 (C-2), 78.6 (C-3), 71.7 (C-4), 78.5 (C-5), 62.8 (C-6).

Oleanolic acid

White amorphous powder; ^{1}H-NMR (300 Hz, $CDCl_3$) δ 5.28 (1H, t, J = 3.5 Hz, H-12), 3.20 (1H, dd, J = 11.2, 4.2 Hz, H-3), 2.82 (1H, dd, J = 3.6, 13.2 Hz, H-18), 1.13 (3H, s, H-27), 0.77, 0.78, 0.90, 0.92 0.93, 0.99 (each 3H, s, CH_3 X 6). ^{13}C-NMR (75 MHz, $CDCl_3$) δ: 183.5 (C-28), 143.7 (C-13), 122.7 (C-12), 79.1 (C-3), 55.4 (C-5), 47.8 (C-9), 46.6 (C-17), 46.0 (C-19), 41.7

(C-14), 41.2 (C-18), 39.5 (C-8), 38.8 (C-4), 38.6 (C-1), 37.2 (C-10), 33.9 (C-21), 33.0 (C-29), 32.8 (C-7), 32.6 (C-22), 30.6 (C-20), 28.2 (C-23), 27.8 (C-15), 27.3 (C-2), 25.9 (C-27), 23.6 (C-30), 23.5 (C-16), 23.1 (C-11), 18.4 (C-6), 17.2 (C-26), 15.5 (C-24), 15.3 (C-25).

3-Epicorosolic acid

White powder; ^{1}H-NMR (pyridine-d_5, 300 MHz) δ: 5.44 (1H, t-like, H-12), 4.28 (1H, ddd, J = 11.4, 9.3, 4.5 Hz, H-2), 3.74 (1H, d, J = 9.3 Hz, H-3), 2.60 (1H, d, J = 11.1 Hz, H-18), 1.25 (3H, s, H-23), 1.15 (3H, s, H-27), 1.09 (3H, s, H-24), 1.02 (3H, s, H-25), 1.01 (3H, d, J = 6.6 Hz, H-30), 0.94 (3H, s, H-26), 0.87 (3H, d, J = 6.0 Hz, H-29); ^{13}C-NMR δ: 48.0 (C-1), 66.1 (C-2), 79.3 (C-3), 40.1 (C-4), 53.5 (C-5), 18.4 (C-6), 33.5(C-7), 40.2 (C-8), 47.8(C-9), 37.3 (C-10), 24.1(C-11), 125.6 (C-12), 139.2 (C-13), 42.9(C-14), 29.5(C-15), 26.3 (C-16), 48.7(C-17), 54.4 (C- 18), 72.4(C-19), 42.5 (C-20), 33.2 (C-21), 38.5 (C-22), 29.6 (C-23), 22.2 (C-24), 18.4 (C-25), 17.5 (C-26), 24.6 (C-27), 179.9 (C-28), 17.4 (C-29), 16.7 (C-24).

Hyperoside

Yellow amorphous powder; ^{1}H NMR (400 MHz, DMSO-d_6): δ: 12.62 (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, 2.1 Hz, H-6'), 7.53 (1H, d, J = 2.1 Hz, H-2'), 6.81 (1H, d, J = 8.5 Hz, H-5'), 6.40 (1H, d, J = 2.1 Hz, H-8), 6.20 (1H, d, J = 2.1 Hz, H-6), 5.38 (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 177.5 (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), 75.8 (C-5"), 73.2 (C-3"), 71.2 (C-2"), 67.9 (C-4"), 60.1 (C 6").

Chlorogenic acid

Slightly yellow amorphous powder; ^{1}H NMR (400 MHz, DMSO-d_6): δ 9.62 (1H, s, 40-OH), 9.19 (1H, s, 30-OH), 7.44 (1H, d, J = 16.1 Hz, H-7'), 7.06 (1H, d, J = 2.1 H-2'), 6.97 (1H, dd, J = 8.2, 2.1 Hz, H-6'), 6.75 (1H, d, J = 8.2 Hz, H-5'), 6.22 (1H, d, J = 15.7 Hz, H-8'), 5.16 (1H, ddd, J = 10.0, 6.0 Hz, H-3), 3.90 (1H, dd, 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): d 176.4 (C-7), 166.3 (C-9'), 148.6 (C-4'), 145.8 (C-7'), 144.6 (C-3'), 125.4 (C-1'), 121.2 (C-6'), 115.8 (C-5'),

114.7 (C-8'), 114.5 (C-2'), 75.2 (C-1), 73.3 (C-4), 71.6 (C-3), 71.5 (C-5), 37.2 (C-6), 36.2 (C-2).

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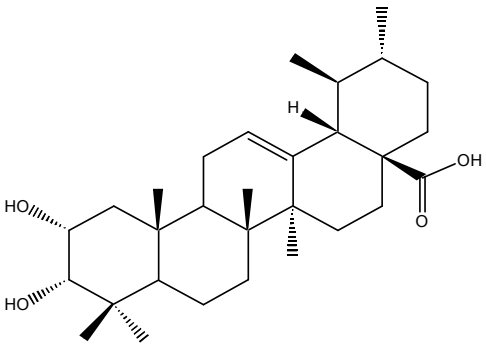
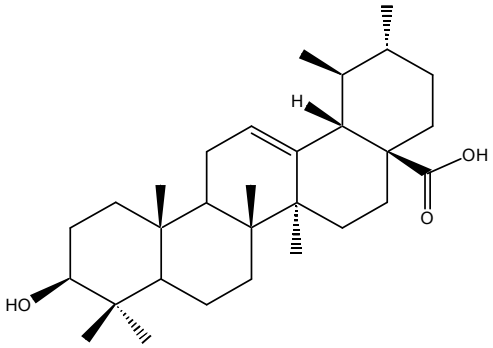
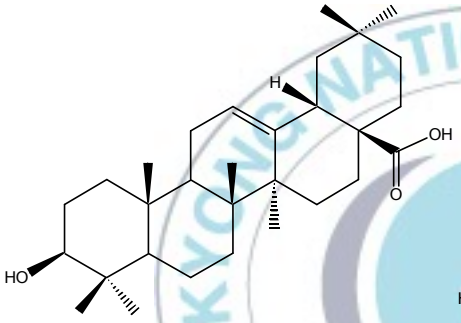


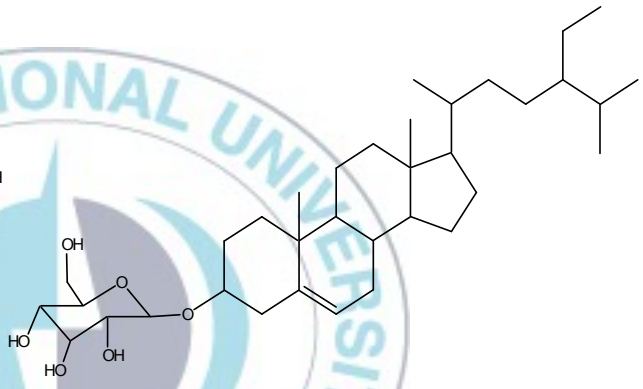
3-Epicorosolic acid



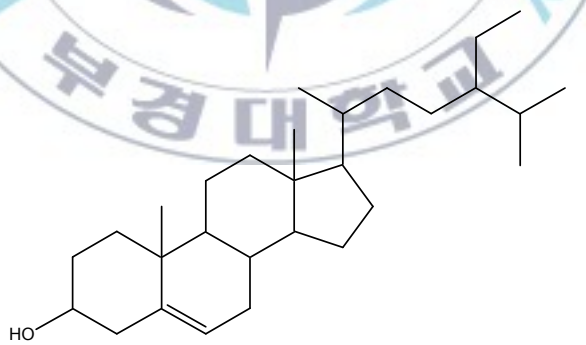
Ursolic acid



Oleanolic acid

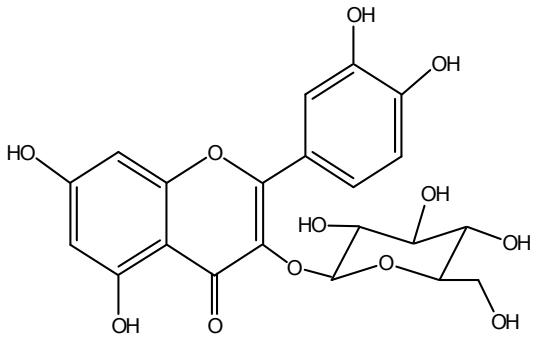


β-Sitosterol glucoside

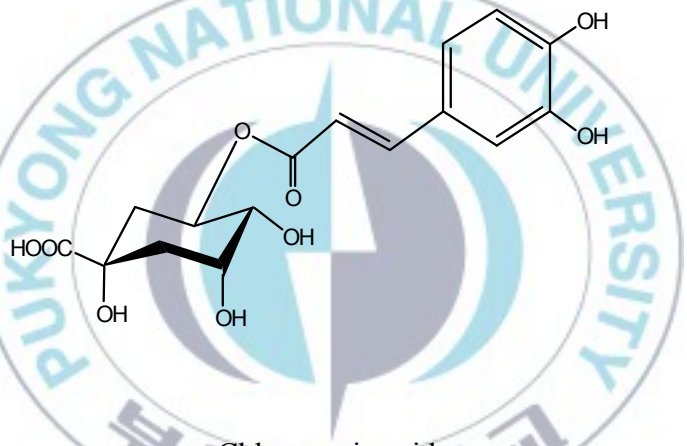


β-Sitosterol

Fig. 1: Structures of compounds isolated from CH_2Cl_2 fraction of *C. pinnatifida*.



Hyperoside



Chlorogenic acid

Fig. 2: Structures of compounds isolated from EtOAc fraction of *C. pinnatifida*.

3-3. 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 (PNP-G) (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.

3-4. Protein tyrosine phosphatase 1B 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 ℃ 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.

3-5. RLAR inhibitory assay

In these experiments we followed The Guidelines for Care and Use of Laboratory Animals as approved by Pukyong National University. According to the modified method of Hayman and Kinoshita (1965), rat lens homogenate was prepared. Briefly, the lens were removed from the eyes of Sprague-Dawley rats (Samtako BioKorea, Inc.,) weighing 250–280 g. The lens are homogenized in sodium phosphate buffer (pH 6.2), which was prepared from sodium phosphate dibasic (Na_2HPO_4 H_2O, 0.66 g) and sodium phosphate monobasic (NaH_2PO_4 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 at 4 ℃ 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 evaluations for enzyme inhibition. Reaction solution consists of 620 μl of 100 mM sodium phosphate buffer (pH 6.2), 90 μl of AR homogenate, 90 μl of 1.6 mM NADPH, 9 μl of the samples (f.c. 100 μg/ml for the extracts and fractions dissolved in 100% DMSO) or 9 μl of 100% DMSO, and 90 μl of 50 mM of dl-glyceraldehyde as the substrate. The AR activity was determined by measuring the decrease in NADPH absorption at 340 nm over a 4 min period on a Ultrospec®2100pro UV/visible spectrophotometer

with SWIFT II Applications software (Amersham Biosciences, New Jersey, USA). Quercetin well known ARIs was used as references. 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 of absorbance for 4 min with the test sample and substrate, respectively, and ΔA control/min represents the same, but with 100% DMSO instead of a sample. The IC_{50} values are expressed as means ± SEM of triplicate experiments.

3-6. AGEs 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, f.c. 200 μg/ml for the extracts and fractions) 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.

3-7. Kinetic parameters of 3-epicorosolic acid for two types of enzymatic inhibition

In order to determine the inhibition mechanism, each enzymatic inhibition at various concentrations of 3-epicorosolic acid was evaluated by the monitoring the effect of different concentration of the substrate via Dixon plots (a single reciprocal plot) Dixon plots for the inhibition of α-glucosidase by 3-epicorosolic acid obtained in the presence of different concentrations of pNPG substrate: 0.625mM (●); 1.25mM (○); and 2.5 mM (▼). The test concentrations for the 3-epicorosolic acid in the α-glucosidase kinetic analysis were 33.05 and 16.52 μM. Dixon plot for PTP 1B inhibition by the 3-epicorosolic acid was obtained in the presence of different concentrations of the pNPP substrate: 4 mM (●); 3mM (○) and 2 mM (▼). The test concentrations of the 3-epicorosolic acid used in the α- glucosidase

kinetic analysis was 10.5, 2.1 and 0.2 mM. Both enzymatic procedures applied the same aforementioned PTP1B and alpha – glucosidase assay methods. The inhibition constants (K_i) were determined by interpreting the Dixon plots, in which the value of the x-axis implies – (K_i)

Statistics

Results were expressed as respective the mean ± standard error of the mean (S.E.M) in triplicate experiments.

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II. Results

1. α-Glucosidase inhibitory activity of the extract and different fractions from *C. pinnatifida*

To evaluate the antidiabetic activity of the MeOH extract and its solvent soluble fractions of the fruits of *C. pinnatifida*, the α-glucosidase inhibitory activity was evaluated. The inhibitory activity is presented in Table 1. Among the tested fractions the EtOAc fraction has been found as the most active fraction with an IC_{50} value of 22.70 μg/mL compared to the positive control acarbose with an IC_{50} value of 81.65 μg/mL. The MeOH extract and CH_2Cl_2 fraction also exhibited potent inhibitory activity with their respective IC_{50} values of 45.86 μg/mL and 122.11 μg/mL where as the *n*-BuOH and H_2O showed no activity within the tested concentrations. The Figure 1 represents the percent inhibition of MeOH extract and solvent soluble fraction show the activity in a dose dependent manner.

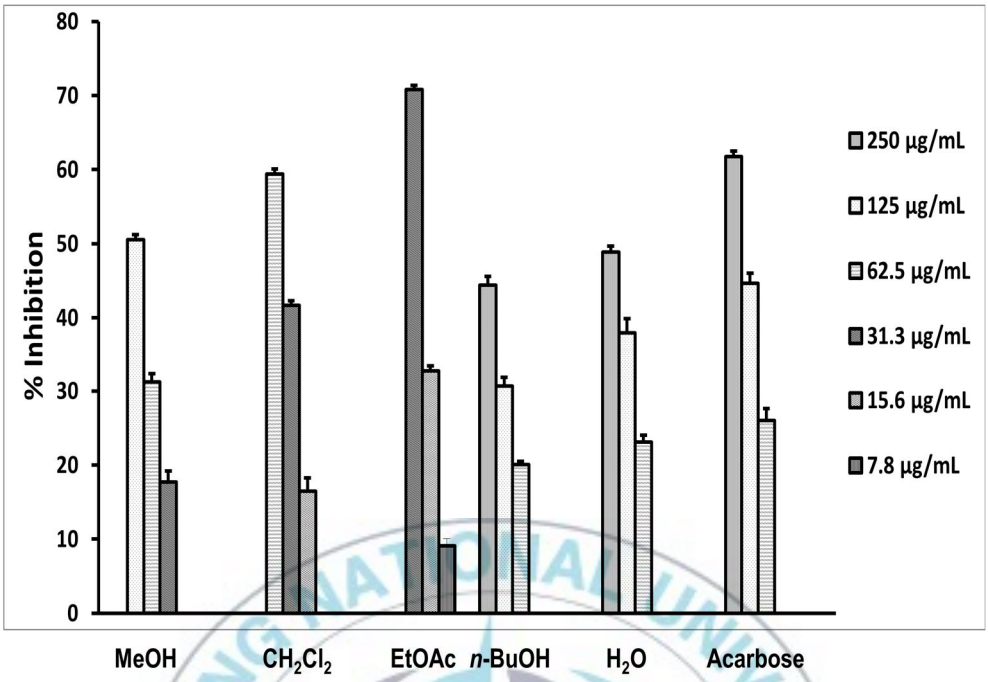

Figure 03. α- Glucosidase inhibitory activity of the extract and different fractions from *C. pinnatifida.*

MeOH = methanol extract, CH_2Cl_2 = dichloromethane fraction, EtOAc = ethyl acetate fraction, *n*-BuOH = *n*-butanol fraction, H_2O = water fraction.

Acarbose was used as the positive control in the assay.

All the values are expressed as Mean ± SEM of triplicate experiments.

2. PTP 1B inhibitory activity of the extract and different fractions from *C. pinnatifida*

The inhibitory activity of MeOH extract and its different solvent soluble fractions of *C. pinnatifida* against PTP1B which is the negative regulator of insulin receptor are presented in Table 1. Unless other assay, in PTP1B inhibitory activity the MeOH extrct and all the fractions showed inhibitory activity. The inhibitory activity ranging from the IC_{50} values from 1.41 to 18.75 μg/mL compared with the positive control ursolic acid with the IC_{50} value of 1.00 μg/mL. Among them the EtOAc fraction showed the highest activity with IC_{50} value of 1.41 μg/mL. In addition the MeOH extact also showed potent inhibitory activity with 3.66 μg/mL IC_{50} value.

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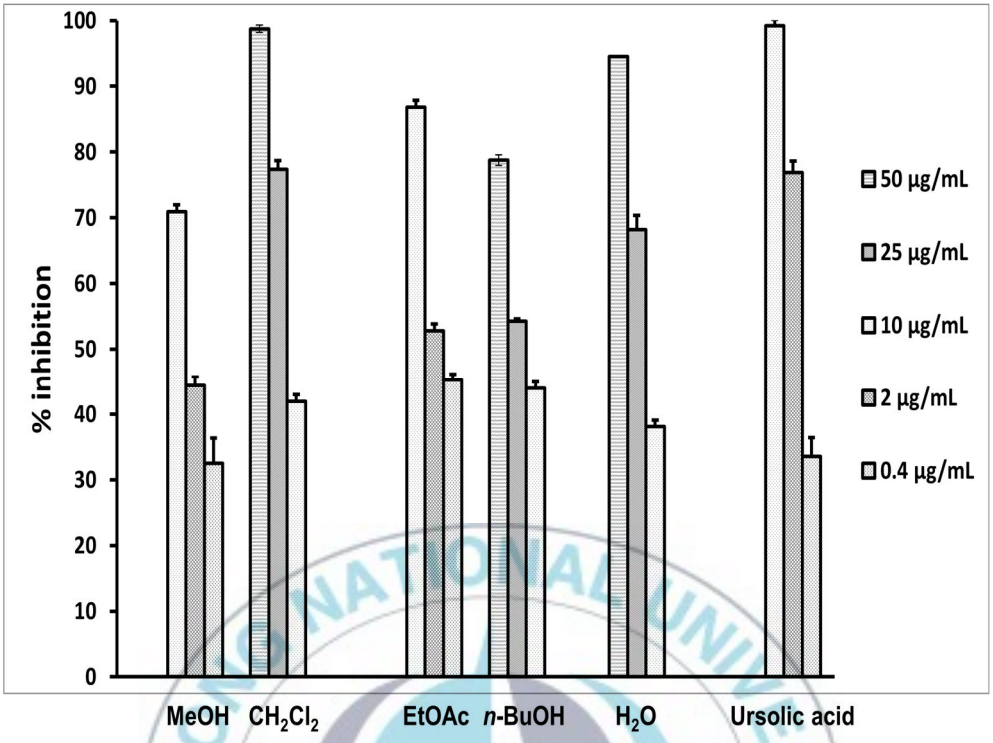

Figure 04. PTP 1B inhibitory activity of the extract and different fractions from *C. pinnatifida.*

MeOH = methanol extract, CH_2Cl_2 = dichloromethane fraction, EtOAc = ethyl acetate fraction, *n*-BuOH = *n*-butanol fraction, H_2O = water fraction.

Ursolic acid was used as the positive control in the assay.

All the values are expressed as Mean ± SEM of triplicate experiments.

Table 1: α-Glucosidase and PTP 1B Inhibitory Activity of MeOH Extracts and Different Solvent Soluble Fractions of *C. pinnatifida.*

Extract and Fractions	α- Glucosidase	PTP 1B
	IC_{50} (μg/mL) ± SEM	
MeOH extract	122.11 ± 1.19	3.66 ± 0.34
CH_2Cl_2 Fr.	45.86 ± 1.05	13.32 ± 0.84
EtOAc Fr.	22.70 ± 0.16	1.41 ± 0.20
n-BuOH Fr.	>250	18.75 ± 0.20
H_2O Fr.	>250	15.84 ± 1.20
Acarbose[a]	81.00 ± 4.07	
Ursolic acid[b]		1.00 ± 0.09

Results are expressed as the Mean ± SEM of triplicate experiments

[a, b] used as positive control in the assay.

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3. RLAR inhibitory activity of the extract and different fractions from *C. pinnatifida*

The RLAR inhibitory activity of MeOH extract and its solvent soluble fractions of the fruits of *C. pinnatifida* showed in Table 2. It is evident from the Table 2 that EtOAc fraction showed the highest RLAR inhibitory activity with an IC_{50} value of 9.09 μg/mL compared to the positive control quercetin with an IC_{50} value of 0.75 μg/mL. In addition, the MeOH extract and *n*-BuOH, CH_2Cl_2 fractions also exhibited remarkable RLAR inhibitory activities with their corresponding IC_{50} values of 160.54 μg/mL, 68.60 μg/mL and 124.50 μg/mL, respectively. At the tested concentrations H_2O fraction showed no inhibitory activity against AR.

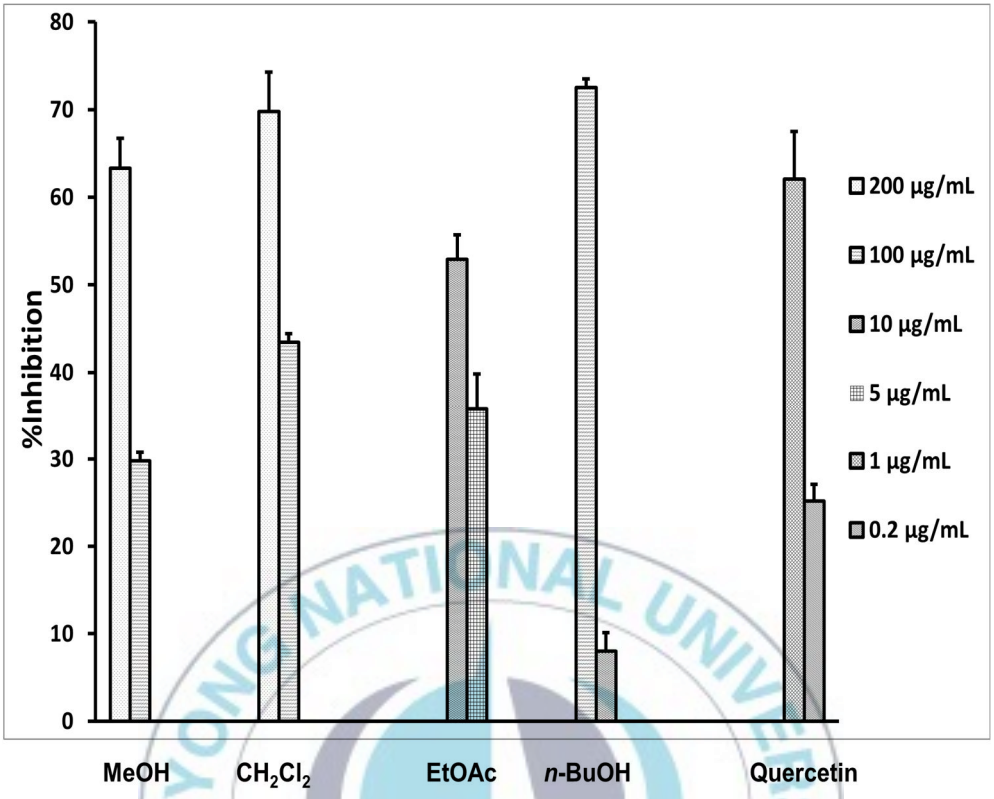

Figure 05. RLAR inhibitory activity of the extract and different fractions from *C. pinnatifida.*

MeOH = methanol extract, CH_2Cl_2 = dichloromethane fraction, EtOAc = ethyl acetate fraction, *n*-BuOH = *n*-butanol fraction.

Quercetin was used as the positive control in the assay.

All the values are expressed as Mean ± SEM of duplicate experiments.

4. AGEs formation inhibitory activity of the extract and different fractions from *C. pinnatifida*

AGEs formation inhibitory activity of the MeOH extract and its different solvent soluble fractions of *C. pinnatifida* is presented in the Table 2. As shown in the Table 2, MeOH extract exhibited highest AGEs formation inhibitory activity with the IC_{50} value of 65.83 μg/mL compared to the positive control aminoguanidine with an IC_{50} value of 127.06 μg/mL. The CH_2Cl_2 and EtOAc fractions also displayed potent AGEs formation inhibitory activity with IC_{50} values of 80.53 μg/mL and 88.90 μg/mL, respectively. Similar to the AR inhibitory assay, *n*-BuOH and H_2O fractions were showed no inhibitory activity at the concentration tested.

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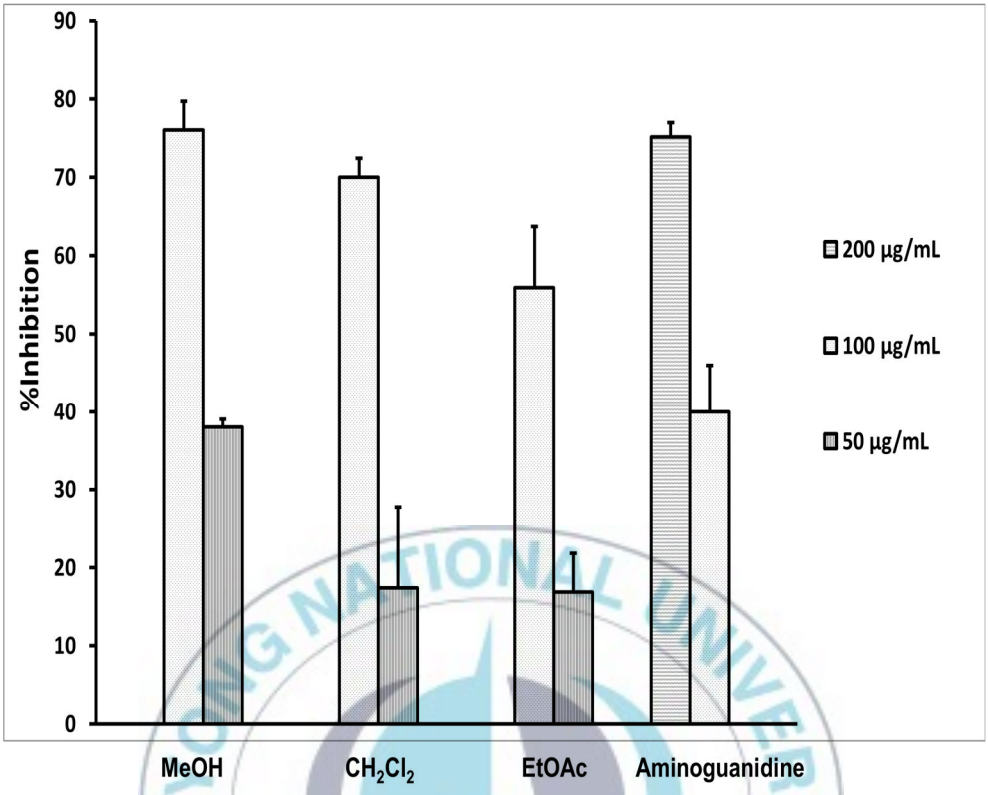

Figure 06. AGEs formation inhibitory activity of the extract and different fractions from *C. pinnatifida.*

MeOH = methanol extract, CH_2Cl_2 = dichloromethane fraction, EtOAc = ethyl acetate fraction,

Quercetin was used as the positive control in the assay.

All the values are expressed as Mean ± SEM of duplicate experiments.

Table 2: RLAR and AGEs Formation Inhibitory Activity of MeOH Extracts and Different Solvent Soluble Fractions of *C. pinnatifida*

Extract and Fractions	RLAR	AGEs
	IC_{50} (μg/mL) ± SEM	
MeOH extract	160.54 ± 0.98	65.83 ± 0.99
CH_2Cl_2 Fr.	124.50 ± 1.68	80.53 ± 1.78
EtOAc Fr.	9.09 ± 0.89	88.90 ± 4.73
n-BuOH Fr.	68.60 ± 3.18	>100
H_2O Fr.	>100	>100
Quercetin[c]	0.75 ± 0.07	
Aminoguanidine[d]		127.06 ± 7.10

Results are expressed as the Mean ± SEM of triplicate experiments.
[c, d] used as positive control in the assay.

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5. Isolation of active compounds from EtOAc fraction

The MeOH extract as well as different solvent soluble fractions of whole plants of *C. pinnatifida* displayed potential anti-diabetic activities. Different degree of anti-diabetic activities of the different solvent soluble fractions revealed their difference in chemical composition due to solvent partitioning between the CH_2Cl_2, EtOAc, *n*-BuOH, and H_2O phases. Among several solvent soluble fractions, the EtOAc fraction showed potential anti-diabetic activities via inhibition of α-glucosidase, PTP 1B, RLAR and AGEs formation. Besides EtOAc fraction, the CH_2Cl_2 fraction also showed potent indibitory activity. Therefore, CH_2Cl_2 and EtOAc fractions were selected for further works to isolate active compounds to support the activity. For these reasons, repeated column chromatography of CH_2Cl_2 fraction was accomplished to isolate three active compounds ursolic acid, oleanolic acid and 3-epi corosolic acid along with *β*-sitosterol and *β*-sitosterol glucoside (Scheme 1). The most active EtOAc fraction was subjected on column chromatography to isolate the active compounds. From this fraction we isolate two active compound including hyperoside and chlorogenic acid (Scheme 2). These compounds were characterized and identified by spectroscopic methods, including ^{1}H- and ^{13}C-NMR, and EI-MS, as well as

through comparison with published spectral data (Haque et al., 2008; Pateh et al., 2009; Hung and Yen, 2001; Wen et al., 2007; Liu et al., 2010; Nakatani et al., 2000) and TLC.

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6. α- Glucosidase and PTP 1B inhibitory activity of 3-epicorosolic acid:

The anti-diabetic activities of isolated 3-epicorosolic acid were evaluated by its α-glucosidase and PTP 1B inhibition. Against α-glucosidase enzyme 3-epicorosolic acid showed three times more potent inhibitory activity with IC_{50} value of 30.18 μg/mL (63.84 μM) compared to the positive control acarbose. In case of PTP 1B also 3-epicorosolic acid showed potent inhibitory activity with IC_{50} value of 4.08 μg/mL (8.63 μM) compared to positive control ursolic acid.

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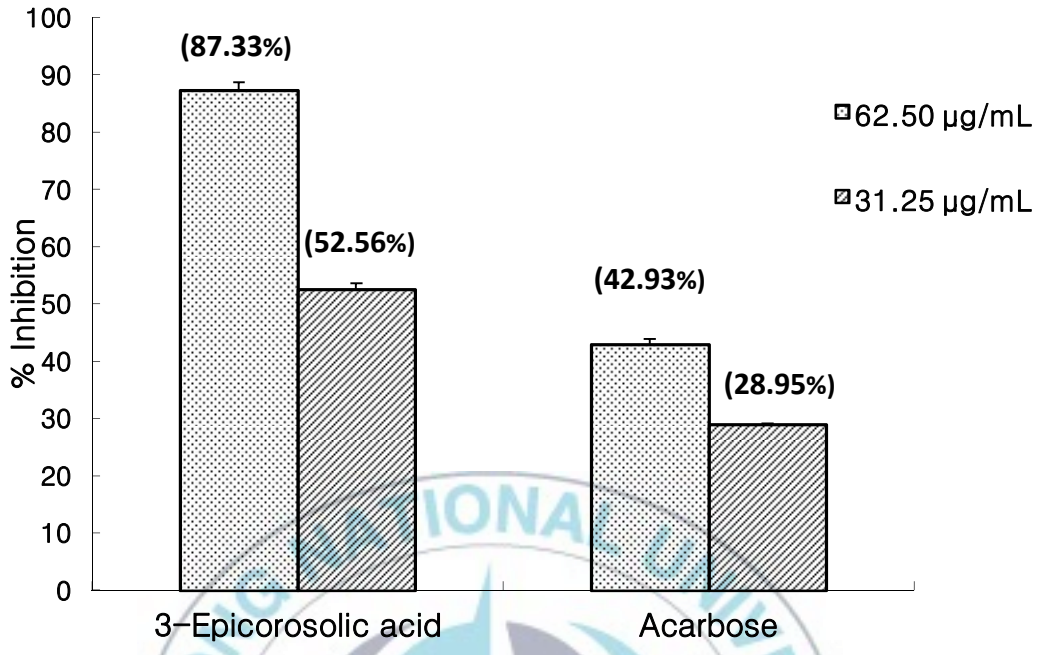

Figure 07. α- Glucosidase inhibitory activity of 3-epicorosolic acid

Acarbose was used as the positive control in the assay.

All the values are expressed as Mean ± SEM of triplicate experiments.

Figures in parenthesis indicate % inhibition.

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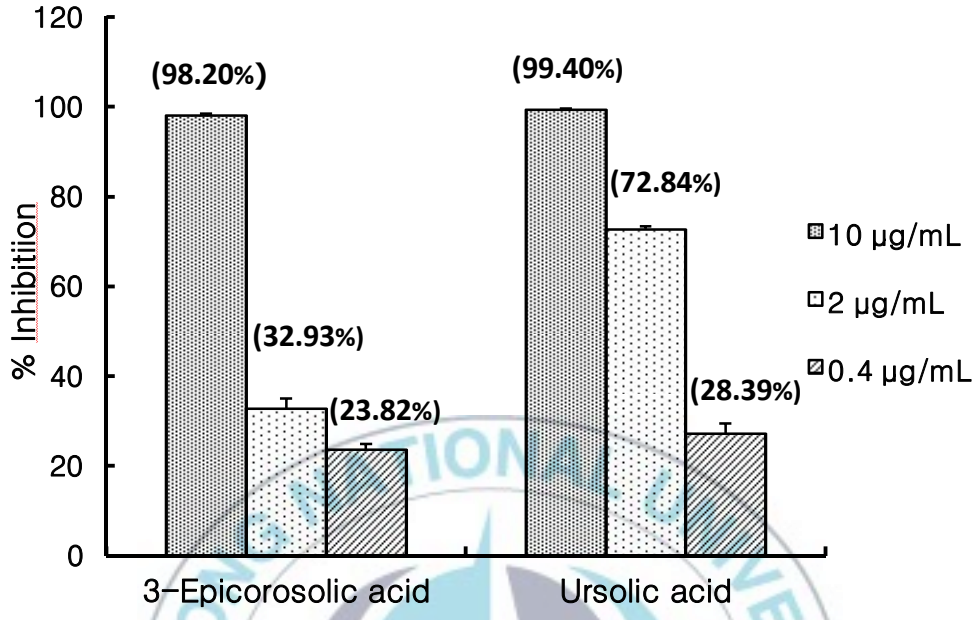

Figure 08. PTP 1B inhibitory activity of 3-epicorosolic acid.

Ursolic acid was used as the positive control in the assay.

All the values are expressed as Mean ± SEM of triplicate experiments.

Figures in parenthesis indicate % inhibition.

Table 3: α-Glucosidase and PTP 1B Inhibitory Activity of 3-epicorosolic acid

Compounds	α-Glucosidase inhibitory activity		PTP 1B inhibitory activity	
	IC_{50} (µg/mL) ± SEM			
3-Epicorosolic acid	30.18 ± 0.4	63.84µM	4.08± 0.20	8.63 µM
Acarbose[a]	92.80 ± 3.9			
Ursolic acid[b]			1.70±0.03	

Results are expressed as the Mean ± SEM of triplicate experiments

[a, b] used as positive control in the assays.

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7. Kinetics parameters of 3-epicorosolic acid:

In an attempt to explain the mode of enzymatic inhibition, kinetic analyses were performed at different concentrations of the corresponding substrate (pNPG for α-glucosidase ad pNPP for PTP 1B) and various inhibitors concentrations. Dixon plots are the graphical method [a plot of 1/enzyme velocity (1/V) against concentration (I)] for determining the type of enzyme inhibition and dissociation or inhibition constant (K_i) for an enzyme inhibitor complex, and were easily determined. Figure 9 and 10 demonstrate the enzymatic kinetic analysis of 3-epicorosolic acid on α-glucosidase and PTP 1B. 3-epicorosolic acid showed uncompetitive α-glucosidase inhibition, while against PTP 1B it showed mixed type inhibiton with K_i value of 2.45 μM. Since the K_i value represents the concentrations to form an enzyme-inhibitor complex, this value plays an important role in the development of preventive and therapeutic agents.

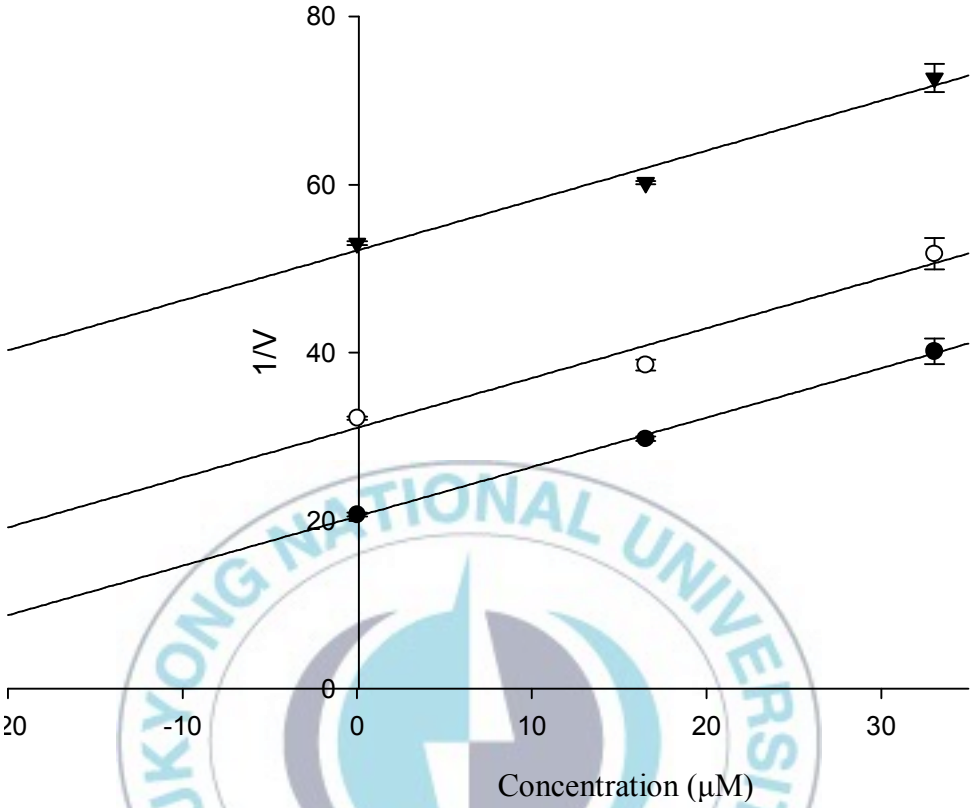

Fig. 9. Dixon plot for inhibition of 3-epicorosolic acid on α-glucosidase in the presence of different concentrations of substrate: 0.625 mM (●); 1.25 mM (○); and 2.5mM (▼) showed uncompetitive inhibition.

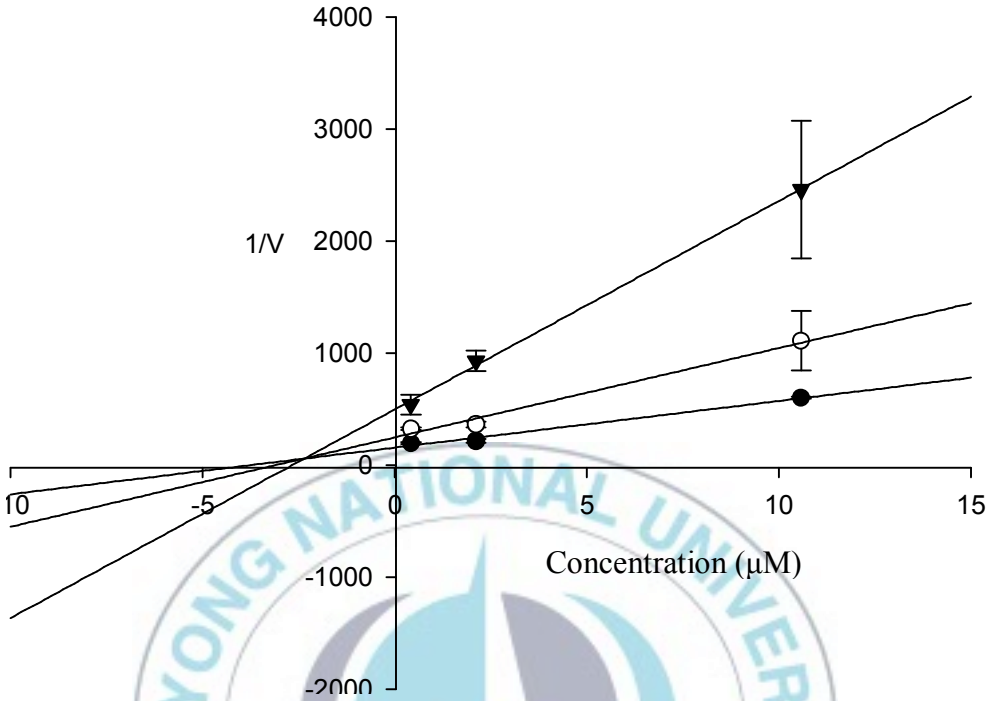

Fig. 10. Dixon plot for inhibition of 3-epicorosolic acid on PTP 1B in the presence of different concentrations of substrate: 4 mM (●); 3 mM (○); and 2 mM (▼) showed mixed type inhibition. The inhibitory constant of the compound was determined as K_i = -2.45 μM.

IV. Discussion

The prevalence of type 2 diabetes has risen at an alarming rate during recent decades, and the morbidity and mortality associated with secondary complications of the disease, such as nephropathy, retinopathy and cardiovascular diseases, have similarly increased (Stein and Colditz, 2004; Wild et al., 2004; Fonseca, 2003). It is predicted that the number of diabetes patient in the world could reach upto 366 million by the year 2030 (Patel et al., 2012). During last 5 years a varieties of new pharmacologic treatments have been developed to treat diabetes mellitus along with the strategies dealing with the diet management and exercise.

There are mainly two types of drugs available in the market for the treatment of diabetes mellitus, *i.e.* insulin and oral hypoglycemic agents (Lenzen, 2008). Sulphonylureas and biguanides, oral hypoglycemic agents, are still the major players in the management of the disease but there is growing interest in herbal remedies due to the side effects associated with the oral hypoglycemic agents. The medicinal plants, besides having natural therapeutic values against various diseases and considerable works have been done on these plants to treat diabetes mellitus, describes that the

antidiabetic activity of medicinal plants is due to the presence of phenolic compounds, flavonoids, terpenoids, coumarins and other constituents which show reduction in blood glucose levels (Rao et al., 2010).

Herbal medicines have been the highly esteemed source of medicine throughout human history. They are widely used today indicating that herbs are a growing part of modern, high-tech medicine. A variety of chemical constituents present in medicinal plants can act on variety of targets by various modes and mechanisms, which can treat various acute and chronic complications of diabetes (Bnouham et al., 2006). As a part of our continuous search of anti-diabetic agent, in the present study we investigated the anti-diabetic potential of the MeOH extract as well as different solvent soluble fractions of *C. pinnatifida* fruits via α-glucosidase, PTP 1B, RLAR and AGEs formation inhibitory assays.

Postprandial hyperglycemia is the earliest abnormality of glucose homeostasis associated with type 2 DM, may lead to β cell dysfunction. It performs a critically important function in the development of type II diabetes and complications associated with cardiovascular diseases. Thus an effective strategy to treat postprandial hyperglycemia is to reduce or slow down the dietary carbohydrate digestion and absorption (Baron, 1998). α-

glucosidase, one of the glucosidases located in the brush-border surface membranes of intestinal cells, is the key enzyme in carbohydrate digestion. α-glucosidase inhibitor is likely to retard the release of glucose from oligosaccahrides and disaccharides, thus slowing down the absorption of carbohydrate and ultimately decreases the risk of postprandial hyperglycemia in diabetic patients (Lebovitz, 1997). Acarbose, 1-deoxynojirimycin, miglitol, etc. have been found as α-glucosidase inhibitors (Hong and Jun, 2005). But it has been found that the use of acarbose may cause gastro intestinal tract disturbance and therefore, it is urgent to identify the safe and effective compounds to control the diabetes (Chougale et al., 2009). Thus in our recent study, we investigate the α-glucosidase inhibitory activity of MeOH extract and its solvent soluble fractions of *C. pinnatifida* fruits. Our study showed that the EtOAc fractions showed four times more portent α-glucosidase inhibitory activity compared to acarbose, a commercially used α-glucosidase inhibitor. CH_2Cl_2 fraction showed two times more potent inhibitory activity compare to acarbose.

PTP 1B belongs to PTPs family, a widely expressed 50kDa enzyme localized in the endoplasmic rerticulum, implicated as an important negative regulator of the insulin signaling pathway in muscle and liver (Koren and

Fantus, 2007; Frangioni et al., 1992; Egawa et al., 2001). Insulin binding to the insulin receptor (IR) results in autophosphorylation on specific tyrosine residues activates the receptor tyrosine kinase and provides binding sites for the recruitment and subsequent phosphorylation of signaling proteins such as insulin receptor substrate (IRS) 1—4. Several signaling cascades activated by phosphorylated IRS, mediate the biological effects of insulin including glucose uptake and glycogen synthesis. PTP 1B can interact with and dephosphorylate the activated IR as well as IRS proteins (Johnson et al., 2002; Asante and Kennedy, 2003). This enzyme is widely spread in insulin sensitive tissues (Goldstein, 1993).The over expression of this enzyme has been shown to inhibit the IR signaling cascade and increased expression of PTP 1B occurs in insulin resistant states (Ahmad et al., 1997). Insulin resistance is a major pathological hallmark in Type 2 DM which leads to a reduced glucose uptake and utilization, as well as increased glucose production. It is also implicated in the pathogenesis of other metabolic disorders such as atherosclerosis, hypertension and abnormal lipid profiles (Minoura et al., 2005; Kim et al., 2000; Chakrabarti et al., 2004). Thus inhibition of PTP 1B has been demonstrated to be an effective therapeutic approach for insulin-sensitive drug target for the treatment and prevention of

metabolic disorders. (Koren and Fantus, 2007; Van et al., 2002). To find out the potent PPTP 1B inhibitors, the MeOH extract of *C. pinnatifida* was evaluated against PTP 1B and showed PTP 1B inhibitory activity. Table 2 showed that among all the fractions, the EtOAc fraction showed potent PTP 1B inhibitory activity which is similar to the positive control ursolic acid. In addition, the CH_2Cl_2 fraction also showed moderate PTP 1B inhibitory activity compared to the EtOAc fraction.

In DM, persistent hyperglycemia is considered as the primary instigator for the pathogenesis of long term diabetic complications, including retinopathy, cataractogenesis, nephropathy, and nuropathy (El-Kabbani et al., 2004; Engerman and Kem, 1984**;** Beyer and Cruz, 1985). DM associated complications can arise from increased flux through the polyol pathway and the advanced glycation end-products (AGE) formation. AR, a cytosolic monomeric oxidoreductase, is the first enzyme in the polyol pathway (Brownlee, 2001). Under normoglycemic condition the AR has low affinity for glucose and a small percentage of glucose metabolisms done by this pathway whereas under hyperglycemic condition, increased intracellular glucose results in increased enzymatic conversion to sorbitol, with concomitant decreases in NADPH (Brownlee, 2001; Kinoshita et al., 1976).

The increased polyol pathway flux leads to accumulation of sorbitol in the lens fiber, which causes an influx of water, generation of osmotic stress, and cataract formation (Kubo et al., 1999; Lee and Chung, 1999). Due to the poor penetration across membranes and inefficient metabolism, sorbitol and its metabolites accumulate in the nerves, retina, and kidneys, cause development of diabetic complications, including retinopathy, neuropathy, and nephropathy (Kador et al., 1980). As it is difficult to prevent the development and progression of diabetic complications by regulating normal blood glucose levels in diabetic patient, AR inhibition has been considered as an important therapeutic approach for preventing and reducing long term diabetic complications without increase the risk of hypoglycemia (Kawamura and Hamanka, 1997; Ahmed et al., 2001; Van et al., 2004; Kim et al., 2011). In our investigation, the EtOAc fraction of *C. pinnatifida* showed potent AR inhibitory activity followed by *n*-BuOH fraction. In addition, CH_2Cl_2 fraction exhibited modest inhibitory activity against AR.

In addition to the increased polyol pathway flux, prolonged hyperglycemia also accelerates the formation of AGEs in body tissues. AGEs are a heterogeneous group of substances, involves a series of

reactions between the carbonyl group on reducing sugars and the amino group on proteins, nucleic acids, or phospholipids, forming a Schiff base followed by an Amadori rearrangement and further nonoxidative and/or oxidative modifications, which lead to the formation of advanced glycation endproducts (AGEs) (Busch et al., 2010; Reddy and Beyaz, 2006; Thorpe and Baynes, 2003). Enhanced formation and accumulation of AGEs have been implicated as a major pathogenic process leading to diabetic complications such as peripheral neuropathy, cataracts, impaired wound healing, vascular damage, arterial wall stiffening and decreased myocardial compliance and normal aging, atherosclerosis, and Alzheimer's disease (Wautier and Guillausseau, 2001; Aronson, 2003; Ahmed, 2005; Thomas et al., 2005; Wada and Yagihashi, 2005). In our present study the CH_2Cl_2 farction showed the most potent AGEs formation inhibitory activity followed by the EtOAc fraction. Moreover, the CH_2Cl_2 and EtOAc fraction showed similar type inhibition against AGEs formation which is much stronger (1.5 times) than the positive control aminoguanidine.

Based on results obtained from all assays, the EtOAc and CH_2Cl_2 fractions of the MeOH extract of *C. pinnatifida* have been shown to exhibit promising anti-diabetic as well as anti-diabetic complications potential.

Therefore, these two fractions were selected for column chromatography to isolate active compounds. Repeated column chromatography of the EtOAc fraction yielded hyperoside and chlorogenic acid while CH_2Cl_2 fraction yielded three triterpenic acid including ursolic acid, oleanolic acid and 3-epicorosolic acid along with *β*-sitosterol and *β*-sitosterol glucoside. Although 3-epicorosolic acid was isolated from other species, it is the first report of 3-epicorosolic acid isolated from the fruits of *C. pinnatifda.* Two compounds hyperoside and chlorogenic acid isolated from the EtOAc fraction have been already reported to possess potent α-glucosidase, PTP 1B, RLAR and AGEs formation inhibitory activities (Muthusamy et al, 2010; Kim et al, 2011a; Kim et al., 2011b; Lee et al., 2010; Beaulieu et al., 2010; Fan et al., 2010). On the other hand, two triterpenic acids, ursolic acid and oleanolic acid have been already reported as potent inhibitors of α-glucosidase and PTP 1B enzymes (Kang et al., 2009; Thuong et al., 2008). In addition, ursolic acid showed noncompetitive inhibition against α-glucosidase while competitive inhibition against PTP 1B enzyme (Kang et al., 2009; Na et al., 2006). Yin and Chan reported that ursolic acid and oleanolic acid inhibited the nonenzymatic formation of glyactive products such as pentosidine and carboxymethyllysine (Yin and Chan, 2007). In

addition, oleanolic acid and ursolic acid markedly suppressed renal aldose reductase activity and enhanced glyoxalase I activity,which contributed to decrease renal AGEs formation and improve renal function (Wang et al., 2010).

Since all the compounds except 3-epicorosolic acid and their anti-diabetic potential are extensively investigated we selected 3-epicorosolic acid for further investigation. In the present study we found that 3-epicorosolic acid showed 3 times potent α-glucosidase inhibitory activity compared to the positive control acarbose. On the other hand, it also exhibited potent PTP 1B inhibitory activity with an IC_{50} value of 4.08 μg/mL (8.63 μM) compared to the positive control ursolic acid with an IC_{50} value of 1.17 μg/mL (2.56 μM). Since it showed potent α-glucosidase and PTP 1B inhibitory activities we further performed kinetic study in order to find out type of enzyme inhibition. Kinetic study revealed that 3-epicorosolic acid showed uncompetitive inhibition against α-glucosidase enzyme while it showed mixed type inhibition against PTP 1B enzyme.

V. Conclusion

Considering the importance and severity of diabetic complications, mainly cardiovascular diseases, nephropathies, retinopathies and neuropathies, any new therapeutic innovation is of interest to prevent deleterious effects of hyperglycemia. In the present study, the MeOH extract as well as different solvent soluble fractions of *C. pinnatifida* showed promising inhibitory potential against α-glucosidase, PTP 1B, RLAR and AGEs formation which might be attributed to the presence of triterpinic acid (ursolic acid, oleanolic acid, 3-epicorosolic acid), hyperoside and chlorogenic acid. Taken together all these results demonstrate the potent anti-diabetic potential of the fruits of *C. pinnatifida* which could be used as functional food in the treatment of diabetes and diabetes related complications. Moreover, 3-epicorosolic acid isolated first time from *C. pinnatifida* fruits exhibited strong inhibitory activity against α-glucosidase and PTP 1B which could be further explored in order to develop therapeutic agents for the treatment of diabetes and related disease.

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VII. References

Ahmed, I., Lakhni, M. S., Gillett, M., John A., Raza, H., Hypotriglyceridemic and hypocholesterolemic effects of anti-diabetic *Momordica charantia* (karela) fruit extract in streptozotocin-induced diabetic rats. *Diabet. Res. Clin. Prac*., 51, 155-161, 2001.

Ahmed, N., Advanced glycation end products – role in pathology of diabetic complications. *Diabetes Res. Clin. Pract.,* 67, 3–21, 2005.

Ahmad, F., Azevedo, J. J., Cortright, R., Dohm, G., Goldstein, B., Alteration in skeletal muscle protein-tyrosine phosphatase activity and expression in insulin-resistant human obesity and diabetes. *J. Clin. Invest*., 100, 449-458, 1997.

Al Makdessi, S., Sweidan, H., Mullner, S., Jocob, R., Myocardial protection by pre-treatment with *Crataegus oxyacantha*. *Arzneimittel-Forschung* (Drug Research), 46, 25–27, 1996.

Ammon, H. P. T., Handel, M., Crataegus, toxicology and pharmacology. I. Toxicity. *Planta Med*., 43,105–120, 1981a.

Ammon, H. P. T., Handel, M., Crataegus, toxicology and pharmacology. II. Pharmacodynamics. *Planta Med*., 43, 209–239, 1981b.

Ammon, H. P. T., Handel, M., Crataegus, toxicology and pharmacology. III. Pharmacodynamics and pharmacokinetics. *Planta Med*., 43, 313–322, 1981c.

Ammon, H. P. T., Kaul, R., Crataegus, Herz-Kreislauf-Wirkungen von Crataegusextrakten, Flavonoiden und Procyanidinen. Teil 2: Wirkungen auf das Herz. *Deutsche Apotheker Zeitung*, 134, 2521-2535, 1994.

Aronson, D., Cross-linking of glycated collagen in the pathogenesis of arterial and myocardial stiffening of aging and diabetes. *J. Hypertens*, 21, 3–12, 2003.

Asante-Appiah, E., Kennedy, B. P., Protein tyrosine phosphatases: the quest for negative regulators of insulin action. *Am. J. Physiol. Endocrinol. Metab*., 284, E663—E670, 2000.

Azuma, K., Nakayama, M., Ito, H., Higashio, H., Terao, J., Ippoushi, K. Absorption of chlorogenic acid and caffeic acid in rats after oral administration. *J. Agric. Food Chem*., 48, 5496– 5500, 2000.

Baron, A. D., Postprandial hyperglycemia and α-glucosidase inhibitors. *Diabetes Res. Clin. Pract*., 40, S51-S55,1998.

Beaulieu, L. P., Harris, C. S., Saleem, A., Cuerrier, A., Haddad, P. S., Martineau, L. C., Bennett, S. A. L., and Arnason, J. T., Inhibitory effect of the Cree traditional medicine wiishichimanaanh (*Vaccinium vitis-idaea*) on advanced glycation endproduct formation: identification of active principles. *Phytother. Res*., 24: 741–747, 2010.

Berger, B., Stenstrom, G., and Sundkvist, G., Incidence, prevalence and mortality of diabetes in a large population: a report from the Skaraborg diabetes registry. *Diabetes Care*, 22, 773-777, 1999.

Beyer-Mears, A., Cruz, E., Reversal of diabetic cataract by sorbinil, an aldose reductase inhibitor. *Diabetes*, 34, 15—21, 1985.

Bnouham, M., Ziyyat, A., Mekhfi, H., Tahri, A., and Legssyer, A., Medicinal plants with potential antidiabetic activity-A review of ten years of herbal medicine research. *Int. J. Diabetes Metab*., 14, 1-25, 2006.

Brownlee, M., Biology and molecular cell biology if diabetic complications. *Nature*, 414, 813–820, 2001.

Butler, M. S., Natural products to drugs: natural product-derived compounds in clinical trials. *Nat. Prod. Rep*., 25, 475–516, 2008.

Chakrabarti, R., Misra, P., Vikramadithyan, R. K., Premkumar, M., Hiriyan, J., Datla, S. R., Damarla, R. K., Suresh, J., and Rajagopalan, R., Antidiabetic and hypolipidemic potential of DRF 2519--a dual activator of PPAR-alpha and PPAR-gamma. *Eur. J. Pharmacol*., 491, 195-206, 2004.

Chang, Q., Zuo, Z., Harrison, F., Chow, M. S. S., Hawthorn. *J. Clin. Pharmacol*, 42, 605−612, 2002.

Chang, Q., Zuo, Z., Chow, M. S. S., Ho, W. K. K., Effect of storage temperature on phenolics stability in hawthorn (*Crataegus pinnatifida* var. major) fruits and a hawthorn drink. *Food Chem.,* 98, 426-430, 2006.

Charpentier, G., Riveline, J. P., and Varroud-Vial, M., Management of drugs affecting blood glucose in diabetic patients with renal failure. *Diabetes Metab*., 26, 73–85, 2002.

Chen, J., and Song, S. J., Research progress in *Crataegus pinnatifida* Bge. *Research and Information on Traditional Chinese Medicine*, 7, 20-23, 2005.

Chen, J. D., Wu, Y. Z., Tao, Z. L., Chen, Z. M., Liu, X. P., Hawthorn (shanzha) drink and its lowering effect on blood lipid levels in humans and rats. *World Rev. Nutr. Diet*., 177, 147–154, 1995.

Chougale, A. D., Ghadyale, V. A., Panaskar, S. N., Arvindekar, A. U., Alpha glucosidase inhibition by stem extracts of *Tinospora cordifolia. J. Enzyme Inhib. Med. Chem.,* 24, 998–1001, 2009.

Chu, C. Y., Lee, M. J., Liao, C. L., Lin, W. L., Yin, Y. F., and Tseng, T. H., Inhibitory effect of hot-water extract from dried fruit of *Crataegus pinnatifida* on low-density lipoprotein (LDL) oxidation in cell and cell-free systems. *J. Agric. Food Chem*., 51, 7583– 7588, 2003.

Cui, L., Na, M., Oh, H. et al., Protein tyrosine phosphatase 1B inhibitors from Morus root bark. *Bioorg. Med. Chem. Lett.*, 16, 1426–1429, 2006.

Diaz-Gutierrez, F. L., Ladero, J. M., and Diaz-Rubio, M., Acarbose induced hepatitis. *American Journal of Gastroenterology*, 93, 481-481, 1998.

Daniele, C., Mazzanti, G., Pittler, M. H., and Ernst, E., Adverse-event profile of Crataegus spp.: A systematic review. *Drug Safety*, 29, 523–535, 2006.

Dauguet, J. C., Bert, M., Dolley, J., Bekaert, A., and Lewin, G., 8-Methoxykaempferol 3-neohesperidoside and other flavonoids from bee pollen of *Crataegus monogyna*. *Phytochemistry*, 33, 1503–1505, 1993.

Egawa, K., Maegawa, H., Shimizu, S., Morino, K., Nishio, Y., Bryer-Ash, M., Cheung, A. T., Kolls, J. K., Kikkawa, R., and Kashiwagi, A., Protein-tyrosine phosphatase-1B negatively regulates insulin signaling in l6 myocytes and Fao hepatoma cells. *The Journal of Biological Chemistry*, 276, 10207–10211, 2001.

Engerman, R. L., Kem, T. S., Experimental galactosemia produces diabetic-like retinopathy. *Diabetes*, 33, 97-100, 1984.

Fabricant, D. S. and Farnsworth, N. R., The value of plants used in traditional medicine for drug discovery. *Environ. Health Perspect.*, 109, 69-75, 2001.

Fan, P., Terrier, L., Hay, A. E., Marston, A., and Hostettmann, K., Antioxidant and enzyme inhibition activities and chemical profiles of *Polygonum sachalinensis* F. Schmidt ex Maxim (Polygonaceae). *Fitoterapia,* 81, 124-131, 2010.

Fong, H. H. S., and Bauman, J. L., Hawthorn. *The Journal of Cardiovascular Nursing*, 16, 1-8, 2002.

Fonseca, V., Clinical significance of targeting postprandial and fasting hyperglycemia in managing type 2 diabetes mellitus, *Curr. Med. Res. Opin.,* 19, 635–641, 2003.

Fonseca, V. A., Management of diabetes mellitus and insulin resistance in patients with cardiovascular disease. *American J. Cardiol*., 92, 50–60, 2003.

Fowler, M. J., Microvascular and macrovascular complications of diabetes, *Clinical diabetes,* 26, 77-82, 2008.

Frangioni, J. V., Beahm, P. H., Shifrin, V. et al., The nontransmembrane tyrosine phosphatase PTP-1B localizes to the endoplasmic reticulum via its 35 amino acid C-terminal sequence. *Cell*, 68, 545–560, 1992.

Fujisawa, T., Ikegami, H., Inoue, K., Kawabata, Y., and Ogihara, T., Effect of two α-glucosidase inhibitors, voglibose and acarbose, on postprandial hyperglycemia correlates with subjective abdominal symptoms. *Metabolism: Clinical and Experimental*, 54, 387–390, 2005.

Ganesan, A., The impact of natural products upon modern drug discovery. *Curr. Opin. Chem. Biol*., 12, 306–317, 2008.

Gao, G. Y., Feng, Y. X., Qin, X. Q., Analysis of the chemical constituents of hawthorn fruits and their quality evaluation. *Yaoxue Xuebao*, 30, 138-143, 1995.

Ghosh, S., Ahire, M., Patil, S., Jabgunde, A., Dusane, M.B., Joshi, B.N., Pardesi, K., Jachak, S., Dhavale, D.D., and Chopade, B.A., Antidiabetic Activity of *Gnidia glauca* and *Dioscorea bulbifera* : Potent amylase and glucosidase inhibitors. *Evidence-Based Complementary and Alternative Medicine*, doi:10.1155/2012/929051, 2012.

Goldstein, B. J., Regulation of insulin receptor signaling by protein-tyrosine dephosphorylation. *Receptor*, 3, 1-15, 1993.

Haque, M. Z., Saki, M. A. A., Ali, M. U., Ali, M. Y., and Maruf, M. A., Investigations on *Terminalia arjuna* fruits: Part 2- Isolation of compound from ethyl Acetate fraction. *Bangladesh J. Sci. Ind. Res.,* 43, 283-290, 2008.

Hayman, S., and Kinoshita, J. H., Isolation and properties of lens aldose reductase. *J. Biol. Chem*., 240, 877– 882, 1965.

Holubarsch, C. J., Colucci, W. S., Meinertz, T., Gaus, W., and Tendera, M., survival and prognosis: investigation of Crataegus extract WS 1442 in CHF (SPICE) trial study group. The efficacy and safety of Crataegus extract WS 1442 in patients with heart failure: The Spice trial. *European Journal Heart Failure*, 10, 1255–1263, 2008.

Hong, G., and Jun, K., α-Glucosidase inhibition of 6-hydroxyflavones. Part 3. Synthesis and evaluation of 2,3,4-trihydroxybenzoyl-containing flavonoid analog and 6-aminoflavones as α-glucosidse inhibitors. *Bioorg. Med. Chem.,* 13, 1661–1667, 2005.

Hung, C. Y., and Yen, G. C., Extraction and identification of antioxidative components of Hsian-tsao (*Mesona procumbens* Hemsl.). *Lebensm.-Wiss. u.-Technol*., 3, 306-311, 2001.

Johnson, T. O., Ermolieff, J., and Jirousek, M. R., Protein tyrosine phosphatase 1B inhibitors for diabetes. *Nat. Rev. Drug Discov*., 1, 696-709, 2002.

Kador, P. F., Kinoshita, J. H., Tung, W. H., and Chylack Jr., L. T., Differences in the susceptibility of various aldose reductases to inhibition. II. *Invest. Ophthalmol.Vis. Sci.,* 19, 980–982, 1980.

Kang, W., Zhang, L., Song Y., α-glucosidase inhibitors from *Luculia pinciana*. *China Journal of Chinese Materia Medica*, 34, 406-409, 2009.

Kao, E. S., Wang, C. J., Lin, W. L., Yin, Y. F., Wang, C. P., and Tseng, T. H., Anti-inflammatory potential of flavonoid content from dried fruits of *Crataegus pinnatifida in vitro* and *in vivo*. *J Agric. Food. Chem.,* 53, 430-436, 2005.

Kao, E. S., Wang, C. J., Lin, W. L., Chu, C. Y., and Tseng, T. H., Effects of polyphenols derived from fruit of *Crataegus pinnatifida* on cell transformation, dermal edema and skin tumor formation by phorbol ester application. *Food and Chemical Toxicology*, 45, 1795-1804, 2007.

Kawamura, M., and Hamanka, N., Development of Eparlestat (Kinedak), aldose reductase inhibitor. *J. Synth. Org. Chem*., 37, 787-792, 1997.

Kennedy, B. P., Role of protein tyrosine phosphatase-1B in diabetes and obesity. *Biomed. Pharmacother.,* 53, 466-70, 1999.

King, H., Aubert, R. E., and Herman, W. H., Global burden of diabetes, 1995–2025: prevalence, numerical estimates, and projections. *Diabetes Care*, 21, 1414-143, 1998.

Kim, J., Jeong, I. H., Kim, C. S., Lee, Y. M., Kim, J. M., and Kim, J. S., Chlorogenic acid inhibits the formation of advanced glycation end products and associated protein cross-linking. *Arch. Pharm. Res*., 34, 495-500, 2011a.

Kim, C. S., Kim, J., Lee, Y. M., Sohn, E., Jo, K., and Kim, J. S., Inhibitory effects of chlorogenic acid on aldose reductase activity in vitro and cataractogenesis in galactose-fed rats. *Arch. Pharm. Res*., 34, 847-852, 2011b.

Kim, J. K., Lee, Y. S., Kim, S. H., Bae, Y. S., and Lim, S. S., Inhibition of aldose reductase by phenylethanoid glycoside isolated from the seeds of *Paulownia coreana*. *Biol. Pharm. Bull*., 34, 160-163, 2011c.

Kim, J. K., Michael, M. D., Previs, S. F., Peroni, O. D., Mauvaais-Javris, F., Neschen, S., Kahn, C. R., and Shulman, G. I., Redistribution of substrates to adipose tissue promotes obesity in mice with selective insulin resistance in muscle. *J. Clin. Invest*., 105, 1791-1797, 2000.

Kinoshita, J. H., Varma, S. D., and Fukui, H. N., Aldose reductase in diabetes. *Jpn. J. Ophthalmol*., 20, 399-410, 1976.

Kubo, E., Miyoshi, N., Fukuda, M., and Akagi, Y., Cataract formation through the polyol pathway is associated with free radical production. *Exp. Eye Res*., 68, 457–464, 1999.

Kuoa, D. H., Yehb, C. H., Shieha, P. C., Cheng, K. C., Chena, F. A., and Cheng, J. T., Effect of ShanZha, a Chinese herbal product, on obesity and dyslipidemia in hamsters receiving high-fat diet. *Journal of Ethnopharmacology*, 124, 544-550, 2009.

Koren S., Fantus I. G., Inhibition of the protein tyrosine phosphatase PTP1B: potential therapy for obesity, insulin resistance and type-2 diabetes mellitus. *Best Pract. Res. Clin. Endocrinol. Metab*., 21, 621-640, 2007.

Kwok, C. Y., Wong, C. N. Y., Yau, M. Y. C., Yua, P. H. F., Au, A.L.S., Poon, C.C.W., Seto, S.W., Lam, T.Y., Kwan, Y.W., and Chan, S.W., Consumption of dried fruit of *Crataegus pinnatifida* (hawthorn) suppresses high-cholesterol diet-induced hypercholesterolemia in rats. *J Functional Foods*, 2, 179-186, 2010.

Lee, H. S., Rat lens aldose reductase inhibitory activities of *Coptis japonica* root-derived isoquinoline alkaloids. *J. Agric. Food Chem.*, 50, 7013-701, 2002.

Lee, A. Y., and Chung, S. S., Contributions of polyol pathway to oxidative stress in diabetic cataract. *FASEB J.*, 13, 23–30, 1999.

Lee, J., Jang, D. S., Yoo, N. H., Lee, Y. M., Kim, J. H., and Kim, J. S., Single-step separation of bioactive flavonol glucosides from Osteomeles schwerinae by high-speed counter-current chromatography. *J. Sep. Sci*., 33, 582–586, 2010.

Lenzen, S., The mechanism of alloxan-and streptozotocin induced diabetes. *Diabetologia*, 51, 216-226, 2008.

Lebovitz, H. E., Alpha-glucosidase inhibitors, *Endocrinol. Metab. Clin*. 26, 539–551, 1997.

Li, W. L., Zheng, H. C., Bukuru, J., and De Kimpeb, N., Natural medicines used in the traditional Chinese medical system for therapy of diabetes mellitus. *Journal of Ethnopharmacology*, 92, 1–21, 2004.

Li, T., Zhang, X. D., Song, Y. W., Liu, J. W., A microplate-based screening method for α-glucosidase inhibitors. *Nat. Prod. Res. Dev., 10*, 1128–1134, 2005.

Lin, Y., Vermeer, M. A., and Trautwein, E. A., Triterpenic acids present in hawthorn by inhibiting intestinal ACAT activity in hamsters. *eCAM*., 1-9, 2009.

Liu, P., Yang, B., and Kallio, H., Characterization of phenolic compounds in Chinese hawthorn (*Crataegus pinnatifida* Bge. var. *major*) fruit by high performance liquid chromatography–electrospray ionization mass spectrometry. *Food Chemistry*, 121, 1188-1197, 2010.

Liu, X., Wang, D., Wang, S. Y., Meng, X. S., Zhang, W. J., Ying, X. X., and Kang, T. G., LC determination and pharmacokinetic study of hyperoside in rat plasma after intravenous administration. *Yakugaku Zasshi*, 130, 873-879, 2010.

Min, S. W., Jung, S. H., Cho, K. H., and Kim, D. H., Antihyperlipidemic effects of red ginseng, *Crataegii gructus* and their main constituents ginsenoside Rg3 and ursolic acid in mice. *Biomol. Ther*., 16, 364–369, 2008.

Minoura, H., Takeshita, S., Yamamoto, T., Mabuchi, M., Takakura, J. H. S., Kawamura, I., Seki, J., Manda, T., Ita, M., and Mutoh, S., Ameliorating effect of FK614, a novel nonthiazolidinedione peroxisome proliferator-

activated receptor gamma agonist, on insulin resistance in Zucker fatty rat. *Eur. J. Pharmacol*., 519, 182-190, 2005.

Na, M., Yang, S., He, L., Oh, H., Kim, B. S., Oh, W. K., Kim, B. Y., and Ahn, J. S., Inhibition of protein tyrosine phosphatase 1B by ursane-type triterpenes isolated from *Symplocos paniculata*. *Planta Med*., 72, 261-263, 2006.

Nakatani, N., Kayano, S., Kikuzaki, H., Sumono, K., Katagiri, K., and Mitani, T., Identification, quantitative determination, and antioxidati ve activities of chlorogenic acid isomers in Prune (*Prunus domesti ca* L.). *J. Agric. Food Chem*., 48, 5512-5516, 2000.

Newa, T., Doi, U., and Osawa, T., Inhibitory activity of corn-derived bisamide compounds against α-glucosidase. *J. Agric. Food Chem.*, 51, 90-94, 2003.

Newman, D. J. and Cragg, G. M., Natural products as sources of new drugs over the last 25 years. *J. Nat. Prod*. 70, 461–477, 2007.

Nikolov, N., Seligmann, O., Wagner, H., Horowitz, R. M., and Gentili, B., New flavonoid-glycoside and *Crataegus monogyna* and *Crataegus pentagyn*. *Planta Medica*, 44, 50–53, 1982.

Pateh, U. U., Haruna, A. K., Garba, M., Iliya, I., Sule, I. M., Abubakar, M. S. and Ambi, A. A., Isolation of stigmasterol, β-sitosterol and 2-hydroxyhexadecanoic acid methyl ester from the rhizomes of *Stylochiton lancifolius* pyer and kotchy (Araceae). *Nig. Journ. Pharm. Sci*., 8, 19-25, 2009.

Patel, D. K., Kumar, R., Laloo, D., and Hemalatha, S., Diabetes mellitus: An overview on its pharmacological aspects and reported medicinal plants having antidiabetic activity. *Asia Pacific Journal of Biomedicine,* 411-420, 2012.

Phillipson, J. D., Phytochemistry and medicinal plants. *Phytochemistry,* 56, 237−243, 2001.

Phillipson, J. D., A matter of some sensitivity, *Phytochemistry,* 38, 1319-1343, 1995.

Pittler, M. H., Guo, R., and Ernst, E., Hawthorn extract for treating chronic heart failure. *Cochrane Database of Systematic Reviews*, *Cochrane Database Syst Rev,* 1, CD00531223, 2008.

Popping, S., Rose, H., Ioneseu, I., Fisher, Y., and Kammermeier, H., Effect of a hawthorn extract on contraction and energy turnover of isolated rat cardiomyocytes. *Arzneimittelforschung*, 45, 1157-1161, 1995.

Rao, M.U., Sreenivasulu, M. Chengaiah, B. Reddy, K. J., and Chetty, C.M., Herbal Medicines for Diabetes Mellitus: A Review. *International Journal of Pharm. Tech. Research*, 2, 1883-1892, 2010.

Reddy, V. P., and Beyaz, A., Inhibitors of the Maillard reaction and AGE breakers as therapeutics for multiple diseases. *Drug Discov. Today,* 11, 646-654, 2006.

Rishton, G. M., Natural products as a robust source of new drugs and drug leads: past successes and present day issues. *Am. J. Cardiol*. 101, 43D–49D, 2008.

Schwinger, R. H. G., Pietsch, M., Frank, K., and Brixius, K., Crataegus special extract WS 1442 increases force of contraction in human myocardium cAMP-independently. *Journal of Cardiovascular Pharmacology,* 35, 700-707, 2000.

Shaw, J. E., Sicree, R. A., and Zimmet, P.Z., Global estimates of the prevalence of diabetes for 2010 and 2030. *Diabetes Research and Clinical Practice*, 87, 4-14, 2010.

Singh, S. K., Rai, P. K., Jaiswal, D., and Watal, G., Evidence based critical evaluation of glycemic potential of *Cynodon dactylon*. *Evidence-Based Complementary and Alternative Medicine*, 5, 415-420, 2008.

Sneader, W., Drug prototypes and their exploitation. Wiley, UK, 1996.

Stein C. J., and Colditz G. A., The epidemic of obesity. *J. Clin. Endocrinol. Metab.,* 89, 2522–2525, 2004.

Stocker, R. M., and Keaney, J. F., Role of oxidative modifications in atherosclerosis. *Physiological Reviews*, 84, 1381–1478, 2004.

Suryanarayana, P., Kumar, A. P., Saraswat, M., Petrash, J. M., and Reddy, G. B., Inhibition of aldose reductase by tannoid principles of *Emblica officinalis*: implications for the prevention of sugar cataract. *Mol. Vis*., 10, 148–154, 2004.

Thomas, M. C., Baynes, J. W., Thorpe, S. R., and Cooper, M. E., The role of AGEs and AGE inhibitors in diabetic cardiovascular disease. *Curr. Drug Targets*, 6, 453–474, 2005.

Thorpe, S. R., and Baynes, J. W., Maillard reaction products in tissue proteins: new products and new perspectives. *Amino Acids,* 25, 275–281, 2003.

Thuong, P. T., Lee, C. H., Dao, T. T., Nguyen, P. H., Kim, W. G., Lee, S. J., and Oh, W. K., Triterpenoids from the leaves of *Diospyros kaki* (Persimmon) and their inhibitory effects on protein tyrosine phosphatase 1B. *J. Nat. Prod*, 71, 1775–1778, 2008.

Van, Huijsduijnen, R. H., Bombrun, A., and Swinnen, D., Selecting protein tyrosine phosphatases as drug targets. *Drug Discov. Today*, 7, 1013-1019, 2002.

Van Zandt, M. C., Sibely E. O., McCann, E. E., Combs, K. J., Flam, B., Sawicki D.R., Sabetta, A., Carrington, A., Sredy, J., Howard, E., Mitschler, A., and Podjarny, A. D., Design and synthesis of highly potent and selective (2-arylcarbamoyl-phenoxy)-acetic acid inhibitors of aldose reductase for treatment of chronic diabetic complications. *Bioorg. Med. Chem.,* 12, 5661-5675, 2004.

Venkatesh, S., Madhava, R. B., Dayanand, R. G., Mullangi, R., and Lakshman, M., Antihyperglycemic and hypolipidemic effects of *Helicteres isora* roots in alloxan-induced diabetic rats: A possible mechanism of action. *J. Nat. Med.,* 64, 295-304, 2010.

Vibes, J., Lasserre, B., Gleye, J., and Declume, C., Inhibition of thromboxaneA2 biosynthesis in vitro by the main components of *Crataegus oxyacantha* (Hawthorn) flower heads. *Prostaglandins, Leukotrienes, and Essential Fatty Acids* ,50, 173–175, 1994.

Vinson, J. A., and Howard III, T. B., Inhibition of protein glycation and advanced glycation endproducts by ascorbic acid and other vitamins and nutrients. *J. Nutr. Biochem*., 7, 659−663, 1996.

Wang, Z. H., Hsu, C., Huang, C. C., and Yin, M. C.,Anti-glycative effects of oleanolic acid and ursolic acid in kidney of diabetic mice. *European Journal of Pharmacology*, 628 , 255–260, 2010.

Wautier, J. L., and Guillausseau, P. J., Advanced glycation end products, their receptors and diabetic angiopathy. *Diabetes Metab*., 27, 535-542, 2001.

Weihmayr, T., and Ernst, E., Therapeutic effectiveness of Crataegus. *Fortschritte der Medizin*, 114, 27-29, 1996.

Wen, X., Xia, J.,Cheng, K., Zhang, L., Zhang, P., Liu, J., Zhang, L., Nia, P., and Suna, H., Pentacyclic triterpenes. Part 5: Synthesis and SAR study of corosolic acid derivatives as inhibitors of glycogen phosphorylases. *Bioorganic & Medicinal Chemistry Letters*, 17, 5777-5782, 2007.

Wild, S., Roglic, G., Green, A. et al., Global prevalence of diabetes: estimates for the year 2000 and projection for 2030. *Diabetes Care*, 27, 1047-1053, 2004.

Wright, E., Scism-Bacon, J. L., and Glass, L. C., Oxidative stress in type 2 diabetes: the role of fasting and post prandial glycemia. *Int. J. Clin. Pract*., 60, 308-314, 2006.

Yin, M. C., and Chan, K. C., Non-enzymatic antioxidative and antiglycative effects of oleanolic acid and ursolic acid. *J. Agric. Food Chem*., 55, 7177-7181, 2007.

Zhang, Z. S., Ho, W. K. K., Huang, Y., and Chen, Z. Y., Hypocholesterolemic activity of hawthorn fruit is mediated by

regulation of cholesterol-7a-hydroxylase and acyl CoA: cholesterol acyltransferase, *Food Res. Int*., 35, 885–891, 2002.

Zhang, P., Zhang, X., and Brown, J., Global healthcare expenditure on diabetes for 2010 and 2030. *Diabetes Research and Clinical Practice*, 87, 293–301, 2010.

Zhang, P. C., and Xu, S. X., Flavonoid ketohexosefuranosides from the leaves of *Crataegus pinnatifida* Bge. Var. major N. E. Br., *Phytochemistry*, 57, 1249–1253, 2001.

Zhang, Z., Chang, Q., Zhu, M., Huang, Y., Ho, W.K.K., and Chen, Z.Y., Characterization of antioxidants present in hawthorn fruits. *Journal of Nutritional Biochemistry* ,12 ,144–152, 2001.

Zhao, H. C., and Tian, B. F., China fruit-plant monograph, hawthorn flora. Beijing, China: Zhongguo Lin Ye Press, p.14, 1996.

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